The role of Glucose dependent Insulinotropic Polypeptide (GIP) and other gut hormones in glucose regulation and adipose tissue metabolism in obesity and type 2 diabetes

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Medicine by

Dr Sravan Kumar Thondam MBBS, MRCP (UK)

May 2017
I declare that this thesis entitled

**The role of Glucose dependent Insulinotropic Polypeptide (GIP) and other gut hormones in glucose regulation and adipose tissue metabolism in obesity and type 2 diabetes**

is entirely my work performed whilst registered as a candidate for the degree of Doctor of Medicine at the University of Liverpool. No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or Institute of learning.

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Thesis overview

The concept that gut derived factors can influence pancreatic secretions was known for more than 100 years. These factors later named as “incretins” were only discovered many decades later. Glucose Dependent Insulinotropic Polypeptide (GIP) previously known as gastric inhibitory peptide was the first incretin hormone to be identified in the early 1970’s followed by glucagon like peptide-1 (GLP-1) in the late 1980’s. Both these hormones are potent stimulators of insulin secretion after nutrient ingestion and regulate postprandial glucose metabolism. It was nearly 20 years later, that the first incretin based drug in the form of GLP-1 receptor agonist was used in the treatment of type 2 diabetes mellitus (T2DM). In contrast, GIP could not be used as a therapeutic agent due to its inability to secrete insulin in individuals with T2DM.

For many years, the significance of GIP was overshadowed by the expansion in physiological actions and therapeutics of GLP-1. Emerging evidence in the recent years suggest that GIP has biological actions in multiple peripheral tissues and a prominent role in promoting fat deposition in adipose tissue. GIP appears to be a hormone of evolutionary importance which facilitates energy storage by fat deposition into adipose tissue from the nutrition derived through dietary sources. However, in the current times of over-nutrition, this physiological action of GIP may have become a maladaptive response leading to undesired fat storage. Interestingly, GIP also appears to have beneficial effects in other tissues such as improving cognitive function in the brain and in prevention of bone resorption. Understanding various physiological actions of GIP would help to explore its biological significance beyond the insulinotropic action in the pancreas.

Obesity is a major risk factor for type 2 diabetes (T2DM). In the current era of the obesity pandemic and the alarming rise in the prevalence of T2DM, evidence accumulating on the pro-adipogenic properties of GIP opens a new therapeutic avenue in the treatment of obesity and obesity related diabetes. Nevertheless, it is vital to understand the effects of GIP on various tissues and its significance in humans before envisaging any therapeutic role.
The focus of my research is to understand the biological actions of GIP in adipose tissue metabolism in humans and to study if these effects differed in healthy subjects and in individuals with obesity and type 2 diabetes mellitus (T2DM). In my first study, we conducted experiments to evaluate the effects of acute GIP infusions on insulin secretion and adipose tissue metabolism in normoglycaemic men (lean and obese) and in obese individuals with impaired glucose regulation and T2DM. I was also interested in understanding the effects of commonly used hypoglycaemic agents in T2DM on the incretin system. Hence, we studied the effects of metformin treatment (most commonly used drug in T2DM) on the incretin system in my second research study described in this thesis.
Abstract

The role of Glucose dependent Insulino tropic Polypeptide (GIP) and other gut hormones in glucose regulation and adipose tissue metabolism in obesity and type 2 diabetes

Aims and hypothesis: Beyond the insulinotropic effects, glucose-dependent insulinotropic polypeptide (GIP) may regulate post-prandial lipid metabolism by promoting fat deposition and inflammation in adipose tissue after high fat diets. We hypothesised that GIP would have an anabolic action in subcutaneous adipose tissue (SAT) promoting non-esterified fatty acid (NEFA) re-esterification. We speculated these effects may be mediated by changes to the expression of key lipid metabolism enzymes and that GIP may promote inflammation by affecting the expression of key adipokines in SAT. We postulated that these effects may be different according to obesity status or glucose tolerance. Incretins and other gut hormones are affected by medications used in the treatment of T2DM. We hypothesised that metformin, a commonly used drug in T2DM, may influence the secretion of incretin and other gut hormones which may contribute to its pleotropic effects in glucose metabolism.

Subjects/Methods: We recruited 31 participants, for 2 different studies. In the first study, 23 men in four categories, normoglycaemic lean (n=6), normoglycaemic obese, (n=6), obese with impaired glucose regulation (IGR) (n=6) and obese, T2DM (n=5) participated in a double-blind, randomised, crossover study involving a hyperglycaemic clamp with a 4-hour infusion of GIP or placebo (normal saline). Serum insulin, plasma NEFA concentrations, SAT triacylglycerol (TAG) content and gene expression of key lipid metabolism enzymes, lipoprotein lipase (LPL), adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) and adipokine gene expression (TNF-α, MCP-1, osteopontin and adiponectin) in SAT were determined before and after the GIP/placebo infusions.

In the second study, eight subjects (6 male and 2 female) were studied on two occasions for 6 hours following a standard mixed meal, before and after metformin monotherapy for at least 3 months. Blood samples were taken in the fasted state and at multiple time points after the mixed meal for measuring incretin hormone, glucagon like peptide (GLP-1), ghrelin (appetite regulatory gut hormone) and dipeptidyl peptidase –IV (DPP-IV) activity.

Results: Study-1 The insulinotropic effect of GIP vs. placebo was greater in lean, obese and obese IGR groups with no significant effect in obese T2DM. In contrast, GIP lowered NEFA concentrations in obese T2DM concomitantly increasing the SAT-TAG content. Such effects were not observed in other groups. There was no change in gene expression of LPL, ATGL and HSL with GIP or placebo infusions. The gene expression of TNF-α was significantly higher in obese T2DM group and the expression of MCP-1 was higher in lean and obese subjects.

Study-2 Metformin monotherapy in obese patients with T2DM was associated with significantly increased postprandial active GLP-1 concentrations.

Conclusion: In T2DM, although the insulinotropic effect of GIP is impaired, the ability of GIP to promote fat storage seems intact lowering NEFA concentrations and increasing SAT lipid deposition which may further exacerbate obesity and insulin resistance. Oral hypoglycaemic agent metformin influences the incretin system by increasing GLP-1 concentrations and this may represent another important mechanism of its glucose-lowering effect.
Publications and presentations

Publications


Oral communications


- “Effects of chronic treatment with metformin on gut brain peptides in obese patients with Type 2 diabetes”, Mersey and Cheshire Diabetes & Endocrinology annual meeting, Liverpool Medical Institution, 13th December 2011.


Poster presentations


<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ABTF</td>
<td>Adipose tissue blood flow</td>
</tr>
<tr>
<td>Adipo-IR</td>
<td>Adipose tissue insulin resistance</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose tissue triglyceride lipase A</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BPD</td>
<td>Bilio-pancreatic diversion</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl dipeptidase -IV</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked Immuno-Sorbent Assay</td>
</tr>
<tr>
<td>NEFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>GAIT</td>
<td>Glucose incorporation into adipose tissue</td>
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<tr>
<td>GIP</td>
<td>Glucose dependent insulinoctropic polypeptide</td>
</tr>
<tr>
<td>GIP−/−</td>
<td>GIP receptor knock out</td>
</tr>
<tr>
<td>GIPR</td>
<td>Glucose dependent insulinoctropic polypeptide Receptor</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon like peptide -1</td>
</tr>
<tr>
<td>GLP-1R</td>
<td>Glucagon like peptide -1 receptor</td>
</tr>
<tr>
<td>GLP-1RA</td>
<td>Glucagon like peptide -1 receptor agonist</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated haemoglobin</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment for insulin resistance</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>IGR</td>
<td>Impaired glucose regulation</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LKB1</td>
<td>Liver kinase B1</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactant protein-1</td>
</tr>
<tr>
<td>MGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>PCOS</td>
<td>Polycystic ovarian syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activator receptor</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en-Y gastric bypass</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>SAT-TAG</td>
<td>Subcutaneous adipose tissue triacylglycerol</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TCF7</td>
<td>Transcription factor 7</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Transcription factor 7-like 2</td>
</tr>
<tr>
<td>TNF R2</td>
<td>Tumour Necrosis Factor Receptor 2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor -α</td>
</tr>
<tr>
<td>VAS</td>
<td>Visual analogue scale</td>
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<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
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<tr>
<td>VDF rats</td>
<td>Vancouver diabetic fatty rats</td>
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<tr>
<td>VLDL</td>
<td>Very Low density lipoprotein</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ΔNEFA</td>
<td>Reduction in non-esterified fatty acids</td>
</tr>
<tr>
<td>11β HSD1</td>
<td>11β Hydroxysteroid dehydrogenase</td>
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Chapter 1
Introduction and review of literature
1.1 Historical aspects of incretin hormones

Incretin hormones are gut derived peptides secreted by the intestinal mucosa in response to nutrient ingestion. These hormones stimulate insulin release from the endocrine pancreas and reduce blood glucose levels. The possible role of gut derived factors on the secretion of pancreatic juice was first described in 1902\(^1\). It was first reported in 1906 that treating patients with diabetes mellitus with the extracts of duodenal mucous membrane resolved glycosuria\(^2\). These factors were initially named “secretins”. Later in 1929, Zunz and LaBarre purified these glucose lowering factors from gut extracts and named them as “incretins,” a term coined to indicate their origin and function (INtestinal seCRETion INsulin)\(^3\). The introduction of radioimmunoassay for measurement of insulin in the 1960’s improved the understanding of communication between the intestine and the endocrine pancreas on insulin secretion\(^4\). The term “entero-insular axis” coined by Unger and Eisentraut in 1969 defines the concept of gut hormones influencing the islets of Langerhans to secrete insulin and glucagon\(^5\). An interesting observation to support this concept was that the oral administration of glucose was associated with much greater increase in plasma insulin compared to the intravenous route for the same level of plasma glucose concentration\(^6,7\). This phenomenon was later known as the “incretin effect” (Figure 1.1) which is defined as the difference in \(\beta\)-cell insulin secretory response to oral glucose load versus intravenous glucose stimuli\(^8-10\).

In search of novel gastrointestinal hormones in the late 1960s, Brown and Pederson observed a crude preparation of porcine cholecystokinin to inhibit stomach acid secretion in dogs and later named it as “Gastric Inhibitory Peptide” (GIP)\(^11,12\). Discovery of GIP relates to period between 1969 to 1971\(^13\). The amino acid sequence of GIP was first reported in 1971\(^14\). In the later years, highly purified preparations of GIP administered in physiological doses along with intravenous glucose resulted in significant increases in insulin secretion and improvement in glucose tolerance\(^15\). Inhibition of gastric acid was only seen at pharmacological doses of GIP but insulin secretory action occurred at physiological concentrations. Thus, GIP was renamed as “Glucose-dependent Insulinotropic Polypeptide”, retaining its acronym and was the first incretin hormone to be identified\(^16\).
The incretin effect however could not be fully explained by the action of GIP alone. Immuno-neutralisation of GIP in a study did not eliminate the incretin effect. Surgical resection of the ileum was associated with reduced incretin effect despite preserved GIP levels which suggested the presence of another incretin hormone secreted from the lower gastro intestinal tract\textsuperscript{17}. Another important observation was that the intravenous administration of GIP and glucose produced only 40% of the insulin response that occurred after oral glucose administration, strengthening the concept of additional insulinotropic factors being released with nutrient ingestion\textsuperscript{18}. Subsequently a second incretin hormone was identified as a potent stimulator of insulin secretion and named as glucagon like peptide -1 (GLP-1) due to its structural similarities with glucagon\textsuperscript{19,20}. GIP is secreted from the K cells of duodenum and proximal jejunum whereas GLP-1 is secreted from the L cells of the distal ileum. Both the incretin hormones are secreted in response to nutrient ingestion and potentiate glucose induced insulin secretion playing an essential role in glucose homeostasis. In addition to insulin secretory action, both these hormones are thought to play an essential role in enhancing insulin biosynthesis, beta cell proliferation and reducing beta cell apoptosis\textsuperscript{21}.

1.2 Incretin hormones and diabetes mellitus

1.2.1 Type 2 Diabetes mellitus (T2DM)

Diabetes mellitus is a metabolic disorder characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both\textsuperscript{22}. Chronic uncontrolled hyperglycaemia leads to cardiovascular complications and death. The prevalence of diabetes is rapidly rising; a recent report from the World Health Organization (WHO) indicates the current prevalence to be 8.5 % in the adult population and this has doubled since 1980 (4.7%). This is expected to rise to 9.9% by 2030 which is predominantly due to increasing number of patients with type 2 diabetes (T2DM)\textsuperscript{23}. WHO estimated that 422 million people in the world were living with diabetes in 2014 compared to 180 million people in 1980\textsuperscript{24}. The majority of these people (> 80%) have type 2 diabetes (previously known as non-insulin dependent diabetes).
Type 1 diabetes (previously known as insulin dependent diabetes) results purely from deficient insulin production. The exact causes of type 1 diabetes (T1DM) are not completely understood and this condition is not preventable. Pathogenesis of T2DM involves interplay of genetic and metabolic elements. It results from the inability of the individual to effectively use insulin and is associated with defects in insulin secretion from the endocrine pancreas. T2DM is strongly associated with risk factors such as obesity, insulin resistance and ethnicity, family history of diabetes, older age group, poor dietary habits and inactive life style. This condition is preventable and reversible by modifying the risk factors. Although insulin resistance and pancreatic beta cell dysfunction are the key pathogenic elements, significantly diminished incretin effect also contributes to the impaired postprandial glucose homeostasis in T2DM.

1.2.2 Incretin concentrations in T2DM

In healthy individuals, the incretin effect from the two incretin hormones (GLP-1 and GIP) is thought to contribute to 50- 70% of postprandial insulin response. Whereas in individuals with type 2 diabetes the incretin effect is significantly reduced (Figure 1.1) and may only contribute to < 20% of post prandial insulin secretion. Postprandial concentrations of incretin hormones are an important determinant of the incretin effect. The concentrations of GLP-1 were reported to be consistently reduced in T2DM in most studies. In contrast to GLP-1, many studies reported higher or unchanged postprandial GIP concentrations in T2DM. A meta-analysis concluded that GIP secretion in response to oral glucose and meal tests is persevered in T2DM and also suggested higher BMI to be associated with higher GIP levels but people in older age groups and those with higher glycated hemoglobin (HbA1c) had reduced GIP secretion.
Figure 1.1 Comparison of incretin effect (vertical arrow) in healthy subjects and patients with type 2 diabetes mellitus (T2DM).

*Figure re-drawn with data taken from Nauck M et al. Diabetologia 1986.*
1.2.3 Incretin based therapies in type 2 diabetes (T2DM)

T2DM is a progressive disease, intensification of glycemic control with insulin and most hypoglycemic agents causes weight gain as evident in some of the major studies like UKPDS, ACCORD and DCCT. It is also important to recognise that patients with diabetes may be on other drugs for various co-morbidities that can also induce weight gain. Physiological changes during treatment account for lower protein catabolism, decreased calories excreted in urine and reduced energy expenditure to compensate for weight loss. Obesity and progressive weight gain are therefore the major hurdles in optimising therapy and controlling cardiovascular risk factors in T2DM. Therefore, pharmacotherapy to improve glycaemic control which is either weight neutral or assists with weight reduction is more beneficial in treating an obese patient with T2DM.

Advent of incretin based therapies is a breakthrough in the pharmacotherapeutics of T2DM. The first one of this class is a GLP-1 receptor agonist. These agents enhance the incretin effect in T2DM, improve glycaemic control and have also thought to have beneficial effects on preserving β cell function. Additionally, the weight loss induced by GLP-1 receptor agonists (described later in this section) has positioned them distinctly in the treatment of obese patient with T2DM. Details of these therapeutic agents are described below.

GLP-1 receptor agonists (GLP-1RAs)

GLP-1 although reduced in T2DM, retains its insulinotropic activity when given exogenously. Pharmacological substitution of GLP-1 in the treatment of T2DM is therefore a logical therapeutic approach. Stable (GLP-1RAs) have been developed in the last decade which are now available as self-administered subcutaneous injections. The first GLP-1RA to be used is exenatide (twice daily injection) followed by commonly used once daily preparations in the UK such as liraglutide and lixisenatide. Longer acting preparations of exenatide and semaglutide are now available in the U.K that can be given once a week. All GLP-1R agonists are widely in use and proved to be effective in the treatment of T2DM associated with obesity.
In addition to the glucose lowering properties, GLP-1 receptor agonists (GLP-1RAs) induce weight loss which is thought to be due to multiple mechanisms involving reduced calorie intake, delayed gastric emptying and increased satiety levels by centrally mediated mechanisms through neuronal transmission at hypothalamic regions that regulate food intake\textsuperscript{42}. GLP-1R agonists remain at the forefront of T2DM and cardiovascular research. Previous clinical trials of GLP-1R agonists had indicated no increased cardiovascular risk in long term use but now there is clear evidence of cardiovascular benefit with liraglutide\textsuperscript{43} and semaglutide\textsuperscript{44}.

**DPP-IV inhibitors**

Both GLP-1 and GIP are rapidly degraded by the enzyme Dipeptidyl peptidase-IV (DPP-IV) expressed in multiple tissues including intestines, kidneys and liver\textsuperscript{45}. Stable inhibitors of this enzyme were developed more than 10 years ago and have been in therapeutic use around the same time as GLP-1RAs. These agents decrease serum DPP-IV activity by more than 80% with some degree of inhibition sustained for 24 hours making them suitable for once daily treatment dose\textsuperscript{46}. Several DPP-IV inhibitors are now widely used in the treatment of type 2 diabetes (sitagliptin, vildagliptin saxagliptin, linagliptin and alogliptin). These drugs are safe and effective as monotherapy or as an add on therapy to other commonly used drugs such as metformin and sulphonylureas\textsuperscript{47,48}.

**GIP agonists**

GIP, although the first incretin to be identified, could not be used as a therapeutic agent as its incretin activity is blunted in T2DM and even exogenous infusion of GIP does not have insulinotropic activity\textsuperscript{38}. In contrast to GLP-1 levels, GIP concentrations are normal or increased in patients with T2DM. The mechanisms behind the loss of insulinotropic activity of GIP in patients with T2DM remain unclear and therefore a major obstacle for its therapeutic use. Potent GIP agonists were developed by amino acid modifications to GIP molecule\textsuperscript{49-51}. However, unfavourable pharmacokinetics and rapid degradation of GIP by dipeptidyl-peptidase-IV (DPP- IV) were potential limitations for development of a stable agonist\textsuperscript{52}. Longer-acting N terminal modified GIP agonists that are DPP IV resistant have also been developed\textsuperscript{53,54}. Truncated GIP analogues D-Ala(2)-GIP(1-30) and (D-GIP(1-30)) were shown to improve glucose tolerance and insulin secretion in
diabetic Zucker fatty rats\textsuperscript{55}. Although these GIP agonists have shown glucose lowering effects in mice they have not been tested in humans. GIP antagonists have also been developed and these are discussed sections 1.4.

1.3 The link between obesity and type 2 diabetes mellitus

Obesity is a major risk factor for type 2 diabetes; the alarming increase in the global incidence of type 2 diabetes parallels the rise in obesity and strong epidemiological and pathophysiological evidence support a causal link. The national statistics for obesity in England suggest that approximately 25\% of the adult population in England are obese. The prevalence of diabetes in the UK is estimated to be 4.5\% of which T2DM constitutes about 90\% of cases. More than 80\% of patients with type 2 diabetes are either obese or overweight at the time of diagnosis\textsuperscript{56}. The risk of developing diabetes substantially increases even with modest weight gain during adulthood\textsuperscript{57}. Insulin resistance is an important precursor to T2DM, visceral and liver adiposity strongly correlate with insulin resistance independent of subcutaneous fat\textsuperscript{58,59}.

Subcutaneous and visceral fat are a major source of plasma non-esterified fatty acids (NEFA) and chronic elevations of NEFAs interfere with insulin signalling, inhibiting glucose uptake and glycogen synthesis\textsuperscript{60,61}. Recent research suggests that adipose tissue is an endocrine organ that secretes several adipokines (for example leptin, adiponectin, resistin) and pro-inflammatory cytokines (for example TNF-\textalpha and IL-6) that may modify insulin resistance. Prolonged exposure to these inflammatory agents and excess NEFAs from excess visceral and ectopic fat interferes with insulin signalling in skeletal muscle and liver\textsuperscript{62}. Management of obesity is therefore the crucial step in reducing the global impact of T2DM. Sustained weight reduction through diet and lifestyle alone is a major challenge for most patients. It is a continuous combat against strong human instinct and exceptionally efficient mechanisms of energy homeostasis to retain body weight. Treatment therefore requires a multidisciplinary approach with lifestyle intervention, behaviour modification and pharmacotherapy.
1.4 Pharmacotherapy targeting specific pathways in obesity

Although weight loss through non-pharmacological approaches is more desirable, it may not be achieved in many patients as body weight tends to reach a plateau and weight regain is common. Pharmacotherapy is therefore an important adjunct to lifestyle modification before considering bariatric surgery which is usually a last resort in many cases. The rapidly growing prevalence of obesity has led to several drugs being developed but the history of pharmacotherapy has undergone a troublesome phase with failures and withdrawals of weight loss drugs due to their side effects. An example to this is the suspension of sibutramine (a centrally acting agent) in January 2010. This was based on evidence suggestive of increased risk of non-fatal heart attacks and strokes in older patients with diabetes and pre-existing cardiovascular disease (a contraindication to the use of sibutramine) in a cardiovascular outcomes trial\textsuperscript{63}. Prior to this was the withdrawal of rimonabant in October 2008 due to increased incidence of depression. This cannabinoid receptor antagonist was shown to reduce weight and improve glucose regulation\textsuperscript{64}.

Currently there are very few potent therapeutic agents for weight loss and their use is very much limited by side-effects. Pharmacological intervention through gut hormones has opened a new avenue in the treatment of obesity with a GLP-1RA now approved as a potent weight loss medication. Several new and combination of old agents are currently being researched as anti-obesity drugs. Pharmacotherapy targeting specific pathways in obesity are described below.

1.4.1 Targeting gut hormones

**GLP-RA.** GLP-1 receptor agonists (GLP-1RAs) used predominantly for glycaemic control in T2DM have additional benefits of weight reduction as described in the previous section. Liraglutide, a (GLP-1 RA) marketed as Saxenda given at a higher dose of 3mg (compared to 1.8mg in T2DM) was approved as an anti-obesity drug by FDA and European Medicines agency (EMA) in March 2015 for the treatment of obesity. In the SCALE study (Satiety and Clinical Adiposity Liraglutide Evidence in Non-diabetic and diabetic people), a randomised control trial with 3 mg liraglutide, 63% of patients in the liraglutide group lost at least 5% of their body weight
compared to placebo group and 33% had lost more than 10% body weight compared to 10.6% with placebo\textsuperscript{65}. These results are very encouraging for future use of liraglutide in the UK as an anti-obesity drug.

**GIP antagonists** Modification to amino acids and truncations in the GIP molecule has led to development of several GIP antagonists\textsuperscript{66-69} but none of these were shown to act effectively in human physiological conditions. Recent studies showed that the most potent GIP antagonists were GIP(3–30)NH\textsubscript{2} and GIP(5–30)NH\textsubscript{2} which were developed by truncating amino acids at N-terminal of GIP and excluding C-terminal\textsuperscript{70}. Previously developed (Pro3) GIP as an antagonist in mice\textsuperscript{71} was shown to have agonist properties in human GIPR\textsuperscript{72}. The effects of GIP antagonist in prevention of diet induced obesity and improved insulin sensitivity in animal models is discussed in detail in section 1.10.

**Ghrelin antagonists** Ghrelin is a potent appetite regulatory hormone. It is synthesised and secreted by gastric oxyntic cells of the gastric fundus and plays an important role in the hypothalamic regulation of energy homeostasis\textsuperscript{73-75}. Ghrelin also previously known as ‘hunger hormone’ demonstrates circadian variation with meal times\textsuperscript{76}. Ghrelin acts by binding to the receptor known as “Growth Hormone Secretagogue Receptor–1a (GHSR-1a) making this a potential target for therapeutics. Desensitisation and down regulation of this receptor have been considered for appetite modulation therapies however, heterogeneity of GHSR-1a and blood brain barrier are a limitation to development of drugs. Synthetic ghrelin ligands and centrally penetrant GHSR-1a antagonists are currently being researched in animal models\textsuperscript{77}.

**Other gut hormones** Stable analogues of other gut hormones such as amylin, pancreatic peptide YY (PYY), cholecystokinin-I, pancreatic polypeptide, oxyntomodulin are being researched as potential therapeutic agents\textsuperscript{78}. Pramlintide (amylin analogue) is available in the United States for treatment of diabetes\textsuperscript{79}. A combination of pramlintide and metreleptin (leptin analogue) as a weight loss medication is in development\textsuperscript{80}. PYY analogue (PYY3-36) was also shown to reduce weight in animal studies\textsuperscript{81,82}.
1.4.2 Targeting fat absorption in the gut

Orlistat is a potent inhibitor of pancreatic and other intestinal lipases such as gastric lipase and carboxyl ester lipase. It acts by inhibiting the absorption of dietary of triacylglycerols (30%) Orlistat marketed as Xenical is the only drug commonly used in the UK for the treatment of obesity. Studies have shown 3 to 6% of initial body weight reduction and 37% relative risk reduction for development of T2DM in patients with impaired glucose tolerance. Compliance with this drug is poor in patients and particularly so when not adhering to a strict fat free diet due to gastrointestinal side effects. Because of fat excretion, commonly experienced side effects are abdominal bloating, flatus with discharge, steatorrhea and faecal urgency.

1.4.3 Targeting appetite regulation

Some of these drugs act by increasing satiety, reduction in food consumption and stimulating energy expenditure. Mysimba, Locaserin, Qsymia and liraglutide (Saxenda) are the new anti-obesity drugs approved by US Food and Drug Administration (FDA) but these are not currently marketed in the UK. Mysimba is a combination of Naltraxone (opioid antagonist) and bupropion (dopamine and noradrenaline re-uptake inhibitor) that targets appetite regulatory centres in the brain. Lorcaserin is a selective 5-HT2C receptor (serotonin) agonist which activates pro-opiomelanocortin production to increase satiety. Qsymia is a combination of topiramate and phentermine which acts as an appetite suppressant by stimulating synaptic noradrenaline dopamine and serotonin release. These drugs are effective in achieving at least 5% of weight loss.
1.5 Link between GIP and adiposity

The evidence to suggest a link between GIP and adiposity although existed for many years appears to have gained importance in the last decade. Animal studies have shown GIP receptor mediated effects to be the key link in the consumption of high fat energy rich diet and development of obesity and insulin resistance. Several important findings support the concept of GIP as a pro-adiposity hormone. Ingestion of food with high fat content is the most potent stimulator of GIP secretion in humans. A high fat diet has been shown to induce K cell hyperplasia and increase GIP gene expression. Raised concentrations of GIP were observed in mice with hyperinsulinaemia and metabolic abnormalities of the obesity-diabetes syndromes. At the adipocyte level GIP was shown to significantly enhance lipoprotein lipase (LPL) activity supporting its role in clearance of triglyceride rich lipo-proteins and chylomicrons from the circulation and assisting in lipid storage. The presence of functional GIP receptors on adipocytes also suggests that it has an important role in lipid metabolism. GIP could therefore play a key role in diet induced obesity. It is now well recognised that obesity is a major risk factor for insulin resistance and T2DM. Higher GIP concentrations in patients with T2DM may further worsen obesity and insulin resistance because of high fat diet leading to a vicious cycle.

It is crucial to understand the effects of GIP on various tissues in humans before envisaging its therapeutic role. Very few studies so far have investigated the in-vivo effects of GIP on human adipose tissue. The focus of our research is the effects of GIP on non-esterified fatty acid (NEFA) incorporation in the human subcutaneous adipose tissue metabolism and inflammation in adipose tissue. In the coming sections, I have discussed the structure and biological actions of GIP and elaborated on its role in lipid metabolism.
1.6 Glucose dependent Insulinotropic Polypeptide (GIP)

1.6.1 Structure and biosynthesis

Bioactive human GIP is a 42-amino acid peptide. It is derived through a post-translational cleavage of a larger 153 amino acid proGIP protein precursor. ProGIP consists of three main domains of which the middle one corresponds to active GIP with NH2 and COOH terminals on either side (Figure 1.2). Prohormone convertase 1/3 (PC1/3) is essential for cleavage of active GIP from its pro GIP precursor. Human gene for encoding GIP sequences is present in the long arm of chromosome 19. GIP in humans, mouse, rat, and porcine and bovine species exhibit more than 90% amino-acid sequence identity. The N terminus is crucial for binding to GIP receptor and GIP agonist activity. The C terminus has little impact on binding of GIP to receptor and may have some intrinsic agonistic properties. However, absence of C terminus with preserved N terminus creates a naturally occurring potent GIP antagonist.

**Figure 1.2:** Bioactive GIP with 42 amino acid structure is derived from cleavage of its precursor proGIP mediated by enzyme prohormone convertase 1/3 (PC 1/3).
1.6.2 Secretion and metabolism

GIP is synthesized and secreted from enteroendocrine K cells primarily located in the duodenum and proximal jejunum, whereas GLP-1 is secreted from L cell located predominantly in the terminal ileum (Figure 1.3A). GIP mRNA has also been detected in the α cells of human islets and both α and β cells of mice islets\(^94,95\). The role of GIP synthesis in islets is not clearly understood. GIP secretion increases after nutrient ingestion and the major stimulus for GIP secretion is fat and carbohydrate reaching the duodenum\(^88,96-98\). The rate of absorption and content of nutrition regulates GIP secretion and this appears to differ between species as fat is the most potent stimulator of GIP secretion in humans compared to carbohydrates in rodents and pigs\(^99\). The mechanisms of fat induced GIP secretion is thought to involve a transcription regulatory factor X6(Rfx6) which is solely expressed in K cells. Expression of Rfx6 was increased in K cells of high fat fed mice and knock down of Rfx6 reduced mRNA of GIP suggesting an important role for this transcription factor in GIP hypersecretion with high fat diet\(^100\).

Both the incretin hormones (GIP and GLP-1) are actively degraded by the enzyme dipeptidyl peptidase IV (DPP-IV), a serine protease enzyme that is responsible for N-terminal cleavage and inactivation of the endogenous incretin hormones. DPP-IV is expressed in multiple organs such as kidney, lung, adrenal gland, liver, intestine, spleen, pancreas and central nervous system. DPP-IV cleaves the first two N-terminal amino acids (Tyr1-Ala2) of GIP, resulting in the truncated metabolite GIP(3-42). Most radio-immunoassays measure the total GIP plasma concentrations which include the active component intact GIP(1-42) and the non-insulinotropic N-terminally truncated metabolites. Newer assays specific to the N-terminus measure only the biologically active intact GIP\(^101\). In healthy individuals, basal concentration of total GIP is ~10pmol/l reaching concentrations of 70-150 pmol/l between 30-60 minutes after meal ingestion depending on meal size. The plasma concentration of intact GIP is much lower ~30 pmol/l after a meal as shown in a study with a meal containing 250Kcal (55% carbohydrate, 30% fat and 15% protein)\(^102\) (Figure 1.3B). The half-life of GIP is approximately 7 minutes in healthy and 5 minutes in subjects with T2DM\(^101\). GIP clearance is predominantly through the kidneys and increased levels of GIP may be seen in patients with renal failure.
**Figure 1.3:** A Site of gut hormone secretion from gastro-intestinal tract and insulin and glucagon secretion from endocrine pancreas. B Graph showing concentrations of total GIP versus the active component intact GIP in a study with healthy volunteers after ingestion of a mixed meal\textsuperscript{102}.
1.6.3 GIP receptor biology

The metabolic effects of GIP are mediated by binding to its specific plasma membrane receptor. GIP receptor was first cloned in rats 1993\textsuperscript{103} and subsequently in hamsters\textsuperscript{104} and humans\textsuperscript{105}. Human GIPR gene is localised to chromosome 19\textsuperscript{106}. GIP receptor (GIPR) is a member of the secretin-vasoactive intestinal polypeptide family of G-protein-coupled receptors. GIPR has a large N terminal with sequence for N-glycosylation and C-terminal which is abundant in threonine and serine for phosphorylation sites\textsuperscript{107}.

Like all the G protein coupled receptors, GIPR shares a common molecular structure with seven transmembrane helices and a common signalling mechanisms through G- protein dependent pathways. The N terminus of GIP binds to a specific site in the transmembrane helices of GIPR\textsuperscript{108}. The specific amino acid domains involved in ligand binding site and in interaction pathways between GIPR and G-protein have been identified\textsuperscript{109}. GIP hormone binds to GIPR and triggers cyclic AMP mediated by G protein complex and subsequent signalling cascades leads to increase in intracellular calcium and insulin release which is described in detail in section 1.6.

GIPR mRNA is widely expressed in the pancreas, gut, adipose tissue, heart, pituitary, and inner layers of the adrenal cortex. It is also present in the cerebral cortex, hippocampus, and olfactory bulb, but not found in kidney, spleen, or liver. Such extensive expression suggests that GIP may have multiple unidentified actions in different organs. Importantly, the presence of functional GIP receptors in adipocytes lays emphasis on the role of GIP in adipocyte metabolism. GIPR was first identified in 1998 in rat adipocytes and differentiated mouse 3T3 cell\textsuperscript{107}. Ten years later it was demonstrated that GIPR is also expressed in well differentiated human adipocytes but not in pre-adipocytes. This indicates the GIPR expression increases as the adipocytes mature and begin to accumulate in lipid droplets favouring the concept of GIP induced lipid accumulation related to over nutrition\textsuperscript{110}. GIPR expression is reduced in mice and humans with diabetes which may explain the lack of insulinotropic activity for GIP in T2DM\textsuperscript{111,112}. 
1.7 Biological actions of GIP

1.7.1 Insulinotropic effects on β cells of pancreas
GIP as an incretin hormone has a primary physiological role in glucose dependent insulin secretion. GIP is also known to enhance β cell insulin gene transcription and insulin biosynthesis, increase β cell proliferation and reduce apoptosis\textsuperscript{21}. Both GIP and GLP-1 share the same signalling pathways. Binding of GIP to GIPR on pancreatic β cell increases in intracellular cyclic AMP mediated by G-protein complex leading to activation of protein kinase A and inactivation of voltage-dependent potassium channel (K\textsubscript{v}) which are ATP sensitive and also known as K\textsubscript{ATP} channels\textsuperscript{113}. These mechanisms are tightly linked to glucose and amino acid influx into cells that leads to ATP generation which inactivate K\textsubscript{v} channels causing potassium efflux out of the β cell. This causes membrane depolarisation on pancreatic β cell which activates the voltage gated calcium channels (VGCC) which subsequently leads to calcium influx followed by release of insulin granules\textsuperscript{25}. The cascade of events leading to insulin secretion is shown in Figure 1.4.

Complex signalling pathways of insulin secretion by GIP have been unravelled in recent years. GIP is also shown to independently regulate the voltage-dependent potassium (K\textsubscript{v}) channels on the cell surface expression which is an important step in insulin release pathway\textsuperscript{114}. Activation of protein kinase A (PKA) and exchange protein directly mediated by cyclic adenosine monophosphate (EPAC2) were shown to play a key role in GIP signalling and post-translational modification of K\textsubscript{v} channels that contributes to β cell survival\textsuperscript{115}. Inhibition of β-cell apoptosis by GIP is mediated through pathways involving Akt-dependent inhibition of apoptosis signal-regulating kinase-1, that antagonises the pro-apoptotic actions of p38 mitogen activated protein kinase (MAPK) and Jun N-terminal kinase (JNK)\textsuperscript{116}. Studies have also shown that GIP inhibits apoptosis signal-regulating kinase1 (ASK1) causing reduction in mitochondria-induced apoptosis in β-cells through protein kinase B (PKB)-mediated pathways\textsuperscript{117}. Additionally, GIP has effects on α cells by increasing glucagon secretion during fasting and hypoglycaemic conditions but not during hyperglycaemia\textsuperscript{118,119}. 
Insulin secretion pathway for GIP and GLP-1 in β cells of pancreas

Figure: 1.4 Schematic presentation on insulin secretion pathway for both incretin hormones (GIP and GLP-1) in the pancreatic β cell involving cAMP, and protein kinase A (PKA). Please see content in text for details on insulin secretion pathway. *Figure taken with permission from Meier JJ, Nauck MA, Diabetes 2010*[^25]
1.7.2 Effects on the central nervous system

GIPR is expressed in several regions of the brain and GIP was demonstrated in adult rat hippocampus\textsuperscript{120}. Distinct GIP immunoreactivity was also seen in the cerebral cortex, amygdala, substantia nigra, lateral septal nucleus and in several nuclei in the thalamus, hypothalamus and brainstem. The exact mechanism of GIP action in the brain is yet unclear however the neuronal distribution of GIP may suggest its role in neural progenitor cell proliferation and as a neuromodulator\textsuperscript{121}. There is now new evidence in animal studies that chronic treatment with stable GIP analogues may be neuroprotective. Chronic treatment with the GIP analogue, D-Ala\textsubscript{2}-GIP-glu-PAL in mice was shown to prevent dopaminergic neuronal loss in the substantia nigra pars compacta and inhibit the MTPT induced parkinsonism like motor disorders\textsuperscript{122,123}. Protease resistant long acting GIP analogues showed protective effects in animal models of Parkinson’s and Alzheimer’s disease\textsuperscript{124}. Studies using a dual GLP-1 and GIP antagonist treatments were shown to protect against neurodegeneration\textsuperscript{125,126}.

1.7.3 Effects on bone and other tissues

GIP has biological actions in multiple peripheral tissues (Figure 1.5) GIP mRNA is expressed in normal bone and GIP receptors are present in osteoblasts. GIPR is expressed in osteoblasts and osteoclasts\textsuperscript{127-129}. GIP treatment increased lysyl oxidase activities, collagen maturity and enzymatic collagen cross linking through cAMP dependent pathways leading to intra-cellular calcium influx\textsuperscript{129,130}. GIP is thought to stimulate osteoblastic activity and increase new bone formation\textsuperscript{128}. GIP was also found to inhibit osteoclast function and therefore reduced bone resorption\textsuperscript{129,131}. GIPR expression was also seen in bone marrow stromal cells (BMSCs) in mice and this expression decreased in an age-dependent manner. Stimulation of BMSCs with GIP increased osteoblastic differentiation suggesting a role of GIP in prevention of age related bone loss\textsuperscript{132}. GIP may have protective effects in bone by preventing apoptosis in human bone marrow derived mesenchymal stem cells and osteoblastic cells\textsuperscript{133}. GIPR was also shown to be expressed in adrenal cortex and vascular endothelium, where GIP induced glucocorticoid secretion in rats\textsuperscript{134} and increased intracellular calcium influx in endothelium\textsuperscript{21}. The effects of GIP in these tissues remain to be further elucidated.
Biological actions of GIP

**Figure 1.5** Biological actions of GIP on various tissues including pancreas, bone, brain, adipose tissue and blood vessels.
1.7.4 Effects of GIP on appetite and energy expenditure

The effects of GIP on energy expenditure was studied by Miyawaki et al.\textsuperscript{135} using GIPR knockout (\textit{Gipr}\textsuperscript{--/--}) mice. In addition to the lack of weight gain in \textit{Gipr}\textsuperscript{--/--} mice with high fat feed, the oxygen consumption had increased and respiratory quotient decreased compared to WT mice, suggesting increased energy expenditure in \textit{Gipr}\textsuperscript{--/--} mice\textsuperscript{135}. This occurred predominantly in the light phase when mice had low spontaneous motor activity indicating that energy expenditure was in the basal state. There was no significant difference in the dark phase (increased motor activity) in both groups. These experiments suggest inhibition or lack of GIPR prevents fat accumulation and promotes basal energy expenditure perhaps using fat as the preferred source of energy. A previous study from our research unit investigated the role of GIP in energy expenditure, appetite and energy intake in healthy subjects and those with obesity and T2DM\textsuperscript{136}. Energy expenditure (EE) was measured throughout the experiment with indirect calorimetry and subjects were given a series of visual analogue scales to rate hourly their hunger, fullness, urge to eat and prospective consumption of food. During GIP infusion (at 2 pmol/kg/min for 4 hours) there was a trend for healthy subjects to report higher hunger scores and a reduction in their EE was recorded when compared with placebo. These parameters remained unchanged in patients with T2DM.

1.7.5 Effects on adipose tissue

The insulinotropic actions of GIP have been well characterised, but its effects on lipid metabolism and adipose tissue had received far less attention previously. Identification of functional GIP receptors in animal and human adipocytes\textsuperscript{92,110} along with emerging data from studies in animal models and in cultured human adipocytes strongly support a physiological role for GIP in the adipose tissue metabolism in response to chronic exposure to nutritional excess. Several observations demonstrate that GIP exerts physiologically relevant actions critical not only for glucose but also lipid metabolism and enhance our understanding of the role of GIP receptor signalling in energy homeostasis. In the sections below, I have discussed in detail the role of GIP relevant to adipose tissue metabolism and its role in lipogenesis and lipolysis.
1.8 Adipose tissue metabolism in humans

Adipose tissue has an important role in energy balance. High calorie food intake and reduced physical activity leads to excess energy storage in the form of triacylglycerols (also referred as triglycerides) within the adipocytes. During times of energy demand (starvation, exercise and physical stress), triacylglycerols within the adipose tissue are broken down into glycerol and non-esterified fatty acids (NEFAs) to meet the energy requirements. The process of fat storage and mobilisation into the circulation is regulated by hormones such as insulin, catecholamines, growth hormone and glucocorticoids\textsuperscript{137}. The balance between energy storage and breakdown usually exists during eu-caloric states, but is dysregulated in hyper-caloric states with reduced energy expenditure which in the long term leads to adiposity.

1.8.1 Lipid storage in adipose tissue (Lipogenesis)

Human adipocytes unlike liver cells have reduced capacity for \textit{de novo} lipogenesis\textsuperscript{138,139}. Triglyceride rich lipoproteins and chylomicrons from the circulation are hydrolysed into NEFA by the enzyme lipoprotein lipase (LPL) in the adipose tissue capillary endothelium. A large proportion of these NEFAs are taken up by the adipocyte and re-esterified into glycerol 3 phosphates (triacylglycerols). Insulin enhances the activity of LPL and the process of esterification\textsuperscript{140-142}. Triacylglycerols are stored as lipid droplets coated with a protective layer of protein known as perilipin within the adipocyte (Figure 1.6). Although triacylglycerol deposition in the adipose tissue is largely mediated by LPL, the process can still occur to a certain extent in the absence of LPL and this was shown to be through upregulation of \textit{de novo} fatty acid synthesis in mice\textsuperscript{143}.

\textit{Lipoprotein lipase (LPL)}

LPL is a member of the triglyceride lipase protein family and it is the major enzyme responsible for triacylglycerol hydrolysis. It is synthesised in the adipose tissue, cardiac muscle, skeletal muscle, islets and macrophages and transported to the luminal surface of the vascular endothelium. It is bound to the endothelial wall by ion interaction with heparin sulphate proteoglycans (HSPG) and glycosyl-
phosphatidylinositol and is displaced from these sites by heparin. Several factors including the position of LPL on the endothelial lumen, apolipoprotein composition of the particles and the size of the lipoproteins influence the hydrolytic action of LPL. It is a multifunctional enzyme and in the absence of hydrolysis it anchors lipoproteins to the vessel wall and assists the uptake into the cells. Apart from hydrolysis, LPL mediates the conversion of very low density lipoproteins (VLDL) into intermediate density lipoproteins (IDL) and contributes to the transfer of surface lipid to high density lipoprotein (HDL) after lipolysis.

Adipose tissue LPL activity is enhanced by nutrient ingestion and insulin which increases fatty acid uptake and esterification by adipose tissue. This subsequently leads to fat accretion within the adipose tissue. However, in skeletal muscle insulin does not stimulate LPL activity. The LPL hydrolysis products, NEFAs and monoacylglycerols are taken up differentially in adipose tissue and muscle. They are stored as cholesteryl esters in cardiac and skeletal muscle or oxidised to meet the energy requirements. NEFAs meet >70% of the cardiac energy demands. Growth hormone, testosterone and oestrogen inhibit LPL activity in adipose tissue leading to fat mobilisation but enhance its activity in heart and skeletal muscle.

1.8.2 Adipose tissue lipolysis
Triacylglycerols in the adipose tissue are composed of 3 fatty acids bound to a glycerol molecule and these are broken down in a stepwise manner during high energy requirement states. Majority of lipolysis in adipose tissue is mediated by the enzymes hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). For the conversion of triacylglycerol (TAG) to diacylglycerol (DAG), the first step is largely mediated by ATGL and to a lesser extent by HSL which is thought to be the rate limiting step in lipolysis. The chief function of HSL is to convert diacylglycerol (DAG) to monoacylglycerol (MAG). Monoacylglycerol lipase further converts MAG into NEFAs when released into circulation are utilised during high energy demands (Figure 1.6 and 1.7).
Lipogenesis and lipolysis pathways in adipose tissue

**Figure 1.6** A schematic diagram showing lipogenesis pathway in the left half and lipolysis pathway in the right half of the figure with various enzymes and hormones involved in lipid metabolism. Please refer to text for more details on the pathways. *Figure re-drawn inspired from Frayn K et al. IJO 2003*.

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**Figure 1.6** A schematic diagram showing lipogenesis pathway in the left half and lipolysis pathway in the right half of the figure with various enzymes and hormones involved in lipid metabolism. Please refer to text for more details on the pathways. *Figure re-drawn inspired from Frayn K et al. IJO 2003*.

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**Hormone sensitive lipase (HSL)**

HSL is an intracellular neutral lipase with isoforms expressed predominantly in adipose tissue and other cholesterol storing tissues such as testes, ovaries and adrenal cortex. It plays an important role in metabolism of cholesterol esters and steroidogenesis\(^{150}\). As the name implies, HSL is regulated by hormones such as catecholamines and insulin. In adipose tissue, catecholamines stimulate lipolysis through β adrenergic pathways by activating cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) which results in phosphorylation of HSL and perilipin (protective layer of lipid droplet). Phosphorylation is a key step in lipolysis which enables translocation of HSL from adipocyte cytoplasm to the surface of lipid droplet to initiate its lipase activity\(^{137}\). Lipolysis is modulated by several hormones. Acute increases in catecholamines, natriuretic peptides, growth hormone and cortisol (stress response hormones) enhance lipolytic activity whereas insulin inhibits lipolysis. The effects of chronic exposure to glucocorticoids, although unclear may have anti-lipolytic effects and increase lipid accumulation in adipose tissue\(^{151}\).

Resistance to catecholamine-induced lipolysis in subcutaneous adipose tissue has been demonstrated in obese adults and children\(^{152,153}\) and has been attributed to alterations and impaired expression of lipolytic b2- and anti-lipolytic a2-adrenoceptors\(^{154,155}\) along with decreased expression of HSL\(^{156}\). The existence of a positive relationship between lipolytic activity in human fat cells and HSL expression\(^{157}\) and the presence of decreased expression of HSL in obese first-degree relatives of normal weight subjects\(^{158}\) underscore the importance of this HSL defect\(^{159}\). Moreover, from comparison of obese and non-obese subjects it has been demonstrated that the obese phenotype in humans is associated with a decreased HSL expression in mature fat cells and in differentiated pre-adipocytes\(^{159}\) and a decreased catecholamine-induced lipolysis, supporting a possible role of a low HSL as a primary defect in obesity.
Adipose tissue triglyceride lipase (ATGL)

Until recently, HSL was the only enzyme known to play a key role in lipolysis. However, diacylglycerols (DAGs) were shown to accumulate in the adipose tissue of HSL knock-out mice suggesting the first step in TAG hydrolysis to DAG was predominantly mediated by another enzyme identified later and named as ATGL. ATGL belongs to a gene family characterised by presence of patatin, a protein found abundantly in potato tuber. This enzyme has nearly 10-fold higher affinity for TAG than DAG and selectively hydrolyses TAGs. Unlike HSL, ATGL it is not under hormonal control and is not activated by PKA mediated phosphorylation. Its activity may be regulated by a protein known as comparative gene identification -58 (CGI-58). Phosphorylation of perilipin is thought to be associated with release of CGI-58 which increases the lipolytic activity of ATGL.

Figure 1.7. Enzymes mediating lipolysis pathway in adipose tissue.  
*Figure adapted from Peckett A.J et al 2011*
1.9 Role of GIP in adipose tissue metabolism

GIP secretion from the K-cells of the duodenum and proximal jejunum is stimulated by glucose, fat and amino acids\textsuperscript{162,163}. Amongst the various stimuli, fat ingestion was shown to be the most potent stimulator of GIP secretion\textsuperscript{97,99}. Falko et.al showed that ingestion of emulsified corn oil in healthy humans significantly increased endogenous GIP secretion from baseline fasting level by 30 minutes compared to placebo\textsuperscript{88}. Diurnal variation of endogenous GIP concentration in healthy humans was shown to parallel serum triacylglycerol concentrations for most of the day suggesting its role in metabolism of ingested fat\textsuperscript{164}. GIP is proposed to modulate other adipose tissue depots, and that excessive GIP secretion may underlie excessive visceral and liver fat deposition\textsuperscript{165,166}. A cross sectional study with large number of Danish subjects (ADDITION-PRO study) suggested that higher fasting GIP levels were associated with better low density plasma lipoprotein clearance but an unhealthy fat distribution independent of insulin. This effect on obesity varied between men and women\textsuperscript{167}.

GIP concentrations increased significantly in plasma and intestines of obese hyperglycaemic mice after eight weeks of high fat diet compared to high carbohydrate diet. The density of GIP secreting K cells although increased on both diets was higher in high fat fed mice indicating chronic fat consumption can cause hyperplasia of intestinal K cells\textsuperscript{89}. Similar findings on K cell hyperplasia were observed in another study on Wistar rats after 60 days of high fat diet\textsuperscript{168}. Although several studies demonstrated increased GIP concentrations after fat ingestion and speculated its role in lipid metabolism, it was unclear as to how GIP exerted its metabolic effects on adipose tissue. Identification of functional GIP receptors (GIPR) on rat adipocytes and mouse 3T3 cells in the late 1990s improved the understanding that GIP had a direct metabolic effect on adipose tissue through its own receptor\textsuperscript{92}. Later it was shown that functional expression of GIPR in human adipocytes was seen in differentiated adipocytes but not in pre-adipocytes. GIPR expression increased as the differentiating adipocytes start to accumulate in lipid droplets, a process of lipid storage from nutrition\textsuperscript{110} supporting the role of GIP in obesity related to over-nutrition.
1.9.1 Effects of GIP on Lipoprotein Lipase (LPL)

Insulin regulates the activity of LPL, a key enzyme in hydrolysis of triacylglycerols that facilitates lipid storage in adipocytes. A similar regulatory role of GIP on LPL was investigated by incubating cultured pre-adipocytes with GIP for 2 hours and the release of LPL and total activity were measured. GIP in pre-cultured adipocytes was shown to enhance LPL activity significantly supporting its role in clearance of triglyceride rich proteins and chylomicrons from the circulation and assisting in lipid storage. Furthermore, GIP was shown to have a direct influence on LPL activity in explants of rat epididymal adipose tissue and when combined with insulin the LPL activity is significantly greater than either hormone alone suggesting GIP compliments insulin in enhancing triacylglycerol clearance. Similar effects were not observed with the other incretin hormone GLP-1. In another study GIP was shown to enhance LPL activity in 3T3-L1 cells in a dose dependent manner. The effects of GIP on LPL and other enzymes in different studies are shown in Table 1.1.

The mechanism through which GIP stimulates LPL has been identified recently and the signal transduction pathway involves many serine/threonine protein kinases. GIP increases phosphorylation of protein kinase B (PKB) and decreases the phosphorylation of LKB1 and AMP-activated protein kinase (AMPK) that leads to activation of LPL and triacylglycerol accumulation in the adipocytes. The lipogenic effects of GIP, in the presence of insulin was shown to be partially mediated by up-regulation of adipocyte LPL gene transcription. A study in lean and obese women with a carbohydrate meal alone or combined with intravenous infusion of octreotide to supress insulin did not show any change in LPL activity in both groups whilst insulin, GIP and GLP-1 were reduced in the group who were given octreotide infusion indicating that the effects GIP are only evident when combined with insulin.

1.9.2 Effects of GIP on other enzymes involved in lipolysis

The evidence on the effects of GIP on other enzymes is very limited. GIP may increase lipolysis along with induction of inflammatory adipokines. These lipolytic effects of GIP may be enhanced with insulin deficiency. GIP induced lipolysis in the absence of insulin was shown to be through phosphorylation of HSL.
and this process was inhibited by insulin\textsuperscript{175}. In another study GIP was shown to reduce glucagon stimulated lipolysis\textsuperscript{15}. The effects of GIP on lipolytic enzymes HSL and ATGL are less well studied and therefore the role on adipose tissue lipolysis remains unclear. One study investigated the acute effects of GIP infusion on basal lipolysis and lipolytic enzymes in humans\textsuperscript{178}. In this study, healthy obese men were administered GIP (2pmol/kg/min) or normal saline in a randomised manner for 240 minutes and subcutaneous fat biopsies were taken before and after the infusion. NEFAs were significantly reduced in the circulation in a time dependent manner after GIP compared to saline infusion. GIP was shown to reduce mRNA expression and enzyme activity of 11\beta hydroxysteroid dehydrogenase type-1 (11\beta HSD-1) an enzyme that converts inactive cortisone to active cortisone. Additionally, GIP was shown to reduce the activity of both HSL and ATGL. The experiments were conducted in a euglycaemic fasting state and therefore findings may differ in hyperglycaemic or postprandial states. Hence these effects may be better studied under hyperglycaemic clamp conditions.

The above described study is the only human study to have evaluated the \textit{in-vivo} effects of GIP on lipid metabolism enzymes\textsuperscript{178}. \textit{In-vitro} experiments by the same group using 3T3-L1 differentiated cells showed that GIP reduced activity and expression of 11\beta- HSD1 and lowered the expression of ATGL and HSL. This led the authors to speculate that GIP-induced effects on lipid metabolism are due to the alteration in active cortisol content within the adipose tissue which is regulated by the enzyme 11\beta-HSD1\textsuperscript{178}. In contrary 11\beta/HSD1 gene knock out mice on high fat feed were shown to have improved glucose tolerance, reduced weight and adiposity\textsuperscript{179}. Another study showed that selective 11\beta/HSD1 inhibitors reduced insulin resistance and lowered blood glucose levels in mouse models of T2DM\textsuperscript{180,181}. It is therefore unclear at this stage weather a reduction or enhancement of 11\beta-HSD1 is likely to increase fat accumulation in the adipose tissue and the long-term changes in the metabolic parameters.
Table 1.1 Effects of GIP on enzymes involved in lipid metabolism

<table>
<thead>
<tr>
<th>Author, year</th>
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<td>Eckel RH et al. 1979&lt;sup&gt;91&lt;/sup&gt;</td>
<td>cultured pre-adipocytes</td>
<td>Cultured pre-adipocytes were incubated for 2 hours with GIP</td>
<td>↑LPL activity</td>
</tr>
<tr>
<td>Knapper JM et al. 1995&lt;sup&gt;170&lt;/sup&gt;</td>
<td>Explanted rat epididymal adipose tissue</td>
<td>Explants incubated in the presence of both insulin (0.5 nmol) and GIP (4 nmol/L)</td>
<td>↑LPL activity with each hormone, ↑↑ LPL with combination of both hormones</td>
</tr>
<tr>
<td>Ranganath LR et al. 1999&lt;sup&gt;173&lt;/sup&gt;</td>
<td>Women, lean (n=6) and obese (n=6)</td>
<td>340 kcal of carbohydrate alone or combined with IV infusion of octreotide to inhibit insulin</td>
<td>↓ Insulin, GIP and GLP-1 concentrations, No change in LPL activity in both groups</td>
</tr>
<tr>
<td>Miyawaki et al. 2002&lt;sup&gt;135&lt;/sup&gt;</td>
<td>3T3-L1 adipocytes</td>
<td>Cells incubated with GIP</td>
<td>↑LPL activity in dose dependent manner</td>
</tr>
<tr>
<td>Kim S.J et al. 2007&lt;sup&gt;182&lt;/sup&gt;</td>
<td>VDF rats Differentiated 3T3-L1 adipocytes Human adipocytes (subcutaneous)</td>
<td>GIP infusions in VDF rats and epididymal fat were harvested. Adipocytes incubated with GIP and insulin</td>
<td>↑LPL activity, ↑ TAG accumulation PKB pathway</td>
</tr>
<tr>
<td>Kim S.J et al. 2010&lt;sup&gt;172&lt;/sup&gt;</td>
<td>Cell cultures from human adipocytes</td>
<td>Overnight treatment with GIP in the presence of insulin Aimed for underlying mechanism of LPL activation by GIP</td>
<td>↑LPL gene expression, ↑LPL activity through CREB and TORC No such effects were seen with GLP-1</td>
</tr>
<tr>
<td>Szalowska E et al. 2011&lt;sup&gt;183&lt;/sup&gt;</td>
<td>Mice on high fat diet</td>
<td>2 weeks of stable GIP analogue D-Ala (2)-GIP</td>
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<td>↓ expression and enzyme activity of 11β HSD1, ↓ ATGL and HSL</td>
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<tr>
<td>Timper K et al. 2013&lt;sup&gt;175&lt;/sup&gt;</td>
<td>human adipocytes (subcutaneous)</td>
<td>Investigated GIP induced inflammation and lipolysis with and without insulin</td>
<td>↑ lipolysis with GIP in absence of insulin through phosphorylation of HSL</td>
</tr>
<tr>
<td>Kim S.J et al. 2013&lt;sup&gt;184&lt;/sup&gt;</td>
<td>Adipocytes isolated from resistin knockout mice</td>
<td>Cells treated with GIP and insulin</td>
<td>↑LPL activity Mediated by resistin</td>
</tr>
</tbody>
</table>
1.9.3 Effects of GIP on NEFA metabolism in adipose tissue

GIP secreted after fat ingestion under normoglycaemic conditions is not insulinotropic. However, under normal physiological conditions where nutrient composition is a mixture of carbohydrate, fat and protein, GIP is secreted along with insulin. As insulin is the main hormone that regulates incorporation of NEFA into adipose tissue, presence of both these hormones at the same time causes difficulty in assessing the independent action of GIP on NEFA incorporation. Earlier studies in dogs with intravenous porcine GIP infusions showed increased clearance of plasma chylomicron triacylglycerols. Beck et al. studied the influence of GIP on NEFA incorporation in rat epididymal fat pads in the presence or absence of insulin. Without insulin in the incubation medium NEFA incorporation was significantly lower compared to when both are present in the incubation medium suggesting that presence of insulin is necessary for GIP to exert its effects. In another study, exogenous GIP infusions in rats during intra-duodenal infusion of a lipid meal showed a significant decrease in plasma triacylglycerol levels. The effect of endogenous GIP was assessed by neutralising with antiserum to GIP as rats pre-treated with antiserum had significantly elevated plasma triacylglycerol levels after a fat meal suggesting an important role of GIP in clearance of triacylglycerols from the circulation by incorporating them into adipose tissue. The studies showing the effects of GIP on NEFA and triacylglycerols are listed in Table 1.2.

All in-vitro studies have shown that adipocytes incubated with combination of GIP and insulin had significantly increased NEFA incorporation. As the major physiological role of GIP was thought to be to enhance insulin secretion one could imply that GIP merely enhances NEFA incorporation through hyperinsulinaemia and does not have a direct role. However, the experiments form Miyawaki et al. with leptin gene mutant obese mice (Lep\textsuperscript{ob}/Lep\textsuperscript{ob}) has improved the understanding of this concept. Lep\textsuperscript{ob}/Lep\textsuperscript{ob} mice develop marked obesity due to hyperphagia and have gross hyperinsulinaemia. Double homozygous Lep\textsuperscript{ob}/Lep\textsuperscript{ob} and GIP receptor knockout mice (Gipr\textsuperscript{–/–}) were shown to have 23% reduced body weight compared to Lep\textsuperscript{ob}/Lep\textsuperscript{ob} alone mice at 35 weeks of age. Both these groups had similar level of hyperinsulinaemia suggesting that GIP action is not solely mediated through insulin and it has direct influence on adipose tissue deposition leading to obesity.
Most of the studies using animal models have shown that GIP along with insulin plays an important role in regulation of LPL, enhances triacylglycerol clearance from circulation and promote fatty acid incorporation into adipose tissue. However, in humans, these metabolic effects of GIP are less well studied. Asmar et al. studied the effects of GIP on plasma concentrations of TAG and NEFAs in humans. In this study, healthy human male subjects were given 20% intra-lipid (chylomicron like emulsion) through intravenous route over 1 hour and GIP (1.5 pmols/kg/min) or normal saline as a continuous infusion for a period of 300 minutes. The same experiments were repeated with glucose (25 g) infused over 30 minutes to mimic postprandial glucose excursions. There was no significant difference in TAG clearance in all the experiments with intra-lipid (IL) infusions. NEFA concentrations after IL infusion were lower with GIP compared to normal saline. In the experiments with co-infusion of IL+ glucose + GIP/saline, NEFA concentrations were significantly reduced compared to IL+GIP/saline. Insulin secretion due to glucose infusion was shown to be the most important factor in lowering NEFAs. Such reductions in NEFAs were also seen in the absence of GIP and therefore these results would suggest that GIP promotes NEFA re-esterification only through insulin stimulation and may not have a direct and independent effect.

1.9.4 Effects of GIP on human adipose tissue blood flow

Adipose tissue blood flow (ATBF) varies significantly during different physiological states (e.g. feeding, fasting and exercise). An increase in blood flow is an important marker of tissue metabolic activity. A study by Asmar et al. assessed abdominal subcutaneous adipose tissue activity in healthy lean subjects by measuring ATBF during GIP (1.5 pmoles/kg/min) or saline infusion under hyperinsulinaemic hyperglycaemic (HI-HG) clamp conditions for 5 hours. Results from this study showed that GIP in combination with hyperglycaemia and hyperinsulinaemia increased ATBF significantly compared to saline under the same HI-HG clamp conditions (Figure 1.7.). Reduction in NEFA in circulation was similar in absence of GIP under hyperglycaemic clamp conditions. Within the adipose tissue, TAG hydrolysis, glucose uptake was higher and NEFA output was lower with GIP compared to placebo under same HI-HG clamp conditions. Authors concluded that GIP increased ATBF and TAG hydrolysis resulting in reduced output of NEFAs into circulation and therefore increased re-esterification of NEFA in
adipose tissue. The insulin independent effects of GIP were difficult to tease out from these experiments but a recently published study by the same group with similar experiments and octreotide induced insulin inhibition showed that the effects of GIP may be insulin independent\textsuperscript{190}. The same research group conducted similar experiments of GIP infusion in obese and impaired glucose tolerant subjects and did not find an increase in ATBF or TAG deposition in adipose tissue compared to lean individuals\textsuperscript{191}. Subsequent experiments showed this phenomenon was reversed in the same individuals when repeated after a programmed weight loss\textsuperscript{192}. The effects of GIP on human adipose tissue metabolism do not appear to be the same in healthy, obese and diabetes states.

### 1.9.5 Effects of GIP on glucose incorporation into adipose tissue

Glucose incorporation into adipose tissue (GAIT) is the other major aspect of adipocyte metabolism influenced by insulin. The role of GIP in GAIT is controversial. Beck et al. reported that GIP enhanced insulin mediated fatty acid incorporation in to adipose tissue in dose dependent manner but had no additional effect on insulin stimulated GAIT except for very high doses\textsuperscript{193}. These results suggest that GIP mainly enhances NEFA transport but not glucose across the adipocyte membrane under physiological conditions. Miyawaki et al. studied GIP dependent response of glucose uptake (2-deoxy-D-glucose) in 3T3-L1 adipocytes in the presence or absence of insulin. GIP was shown to stimulate 2-deoxy-D-glucose uptake in a dose dependent manner in the presence of 1nM insulin but no significant changes were observed in the absence of insulin\textsuperscript{135}. Another study suggested an alternative direct action of GIP on Akt (a serine/threonine protein kinase) and glucose transporter-4 that play an important role in glucose metabolism. GIP was also shown to activate Akt in a dose dependent manner, increase glucose transporter-4 accumulation and promote glucose uptake into adipocytes\textsuperscript{194}. A recent study suggested that GIP increased sensitivity of adipocytes to insulin through a novel signalling pathway without having insulin mimetic activities\textsuperscript{195}. 
<table>
<thead>
<tr>
<th>Author, year</th>
<th>Experimental subjects</th>
<th>Methods</th>
<th>Effects of GIP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wasada T et al. 1981\textsuperscript{185}</td>
<td>Dogs</td>
<td>Infusion of porcine GIP or saline</td>
<td>↓ chylomicron triacylglycerols in circulation</td>
</tr>
<tr>
<td>Beck B et al. 1983\textsuperscript{186}</td>
<td>Rat epididymal fat pads</td>
<td>Incubation of adipocytes with GIP ± insulin</td>
<td>↓ plasma NEFA ↑ incorporation with GIP + insulin compared to GIP alone</td>
</tr>
<tr>
<td>Ebert et al. 1999\textsuperscript{187}</td>
<td>Rats</td>
<td>Intra-duodenal infusion of lipid meal + GIP infusions</td>
<td>↓ plasma triacylglycerol level</td>
</tr>
<tr>
<td>Asmar et al. 2010\textsuperscript{189}</td>
<td>Healthy men (n=10)</td>
<td>20% intra-lipid intravenously + GIP or normal saline</td>
<td>↓ NEFA with GIP compared to saline</td>
</tr>
<tr>
<td>Asmar et al. 2010\textsuperscript{189}</td>
<td>Healthy men (n=8)</td>
<td>GIP (1.5pmols/kg/min) versus saline infusion with hyperinsulinaemic hyperglycaemic clamp for 5 hours</td>
<td>↓ plasma NEFA ↑ Adipose tissue blood flow ↑ NEFA esterification with GIP infusion</td>
</tr>
<tr>
<td>Gogebakan et al. 2012\textsuperscript{178}</td>
<td>Healthy obese men (n=11) and 3T3-L1 cells</td>
<td>GIP (2pmol/kg/min) or normal saline (240 min) subcutaneous fat biopsies pre and post infusion during Euglycaemic fasting state</td>
<td>↓ plasma NEFA</td>
</tr>
<tr>
<td>Asmar et al. 2016\textsuperscript{190}</td>
<td>Obese men, before and after weight loss (n=5)</td>
<td>GIP (1.5pmols/kg/min) versus saline infusion with hyperinsulinaemic hyperglycaemic clamp</td>
<td>After weight loss: ↓ plasma NEFA and glycerol ↑ Adipose tissue blood flow</td>
</tr>
<tr>
<td>Asmar et al. 2016\textsuperscript{190}</td>
<td>Healthy men (n=6)</td>
<td>GIP (1.5pmols/kg/min) versus saline infusion with different types of euglycaemic and hyperglycaemic clamps Octreotide to inhibit insulin</td>
<td>↓ plasma NEFA and glycerol ↑ Adipose tissue blood flow Independent to insulin effect</td>
</tr>
</tbody>
</table>
1.10 Role of GIP in adipose tissue inflammation

1.10.1 Adipokines in inflammation and insulin resistance

Adipose tissue is an endocrine organ that secretes a variety of cytokines commonly referred to as adipokines or adipo-cytokines. These hormones act locally through their receptors as autocrine and paracrine factors rather than remotely acting endocrine factors\(^\text{197}\). Adipose tissue is a dynamic contributor to energy balance and glucose homeostasis\(^\text{198}\). Obesity is a state of low grade inflammation and the interaction between various adipokines and changes in balance between pro and anti-inflammatory adipokines is likely to contribute to the development of type 2 diabetes\(^\text{199}\). Leptin was the first adipocyte derived hormone to be identified. Several other adipocyte-derived factors identified in the subsequent years include interleukin-6 (IL-6), tumour necrosis factor α (TNFα), adiponectin, and resistin\(^\text{137}\). More recently identified adipokines like omentin, visfatin and osteopontin also appear to have important roles in inflammation. There are many adipokines involved in adipose tissue inflammation but I have limited my discussion to only those adipokines which may be influenced by GIP. Various studies showing the effects of GIP on adipokines are listed in Table 1.3.

1.10.2 Effects of GIP on adipokine expression

**Leptin and adiponectin**

Leptin is the best studied adipocyte hormone and established to have a regulatory role in food intake, energy balance and body weight\(^\text{200-202}\). Animals and humans with defects in leptin secretion or leptin receptors are obese. Leptin acts centrally on the hypothalamus to suppress food intake and increase energy expenditure. It has peripheral actions on glucose homeostasis through enhanced NEFA oxidation and increases insulin sensitivity in muscle and reduces intra-myocellular lipid content. Leptin inhibits hepatic triacylglycerol accumulation and improves insulin sensitivity in the liver\(^\text{198}\). Leptin therapy promotes satiety and causes weight reduction in obese children with congenital leptin deficiency and improves insulin resistance in lipodystrophy. Its therapeutic use in obesity is limited by severe leptin resistance and existing high levels of endogenous leptin in obese subjects\(^\text{203}\). Randomised controlled trials with recombinant leptin injections have shown very limited efficacy in subjects with obesity and type 2 diabetes\(^\text{204,205}\). There are no studies evaluating
the direct effects of GIP on leptin gene expression. Leptin gene mutant obese mice (Lep\textsuperscript{ob}/Lep\textsuperscript{ob}) develop marked obesity due to hyperphagia and have gross hyperinsulinaemia. Miyawaki et al showed that double homozygous Lep\textsuperscript{ob}/Lep\textsuperscript{ob} and GIP receptor knockout mice (Gipr\textsuperscript{−/−}) were shown to have 23% reduced body weight compared to Lep\textsuperscript{ob}/Lep\textsuperscript{ob} alone mice at 35 weeks of age\textsuperscript{135}. These experiments showed that GIP inhibition reduced body weight even in leptin deficient obese mice suggesting a role for factors other than leptin.

Adiponectin was identified independently by four groups and was given different names (Arcp30, AdipoQ, apM1, and GBP28)\textsuperscript{206}. Adiponectin is secreted exclusively from adipocytes and has multi-mer forms in the circulation (trimer, hexamer and a 12-18mer high molecular weight form). High concentrations of adiponectin are found in plasma and levels correlate inversely with body mass. High molecular weight adiponectin positively correlates with insulin sensitivity. Two forms of adiponectin receptors have been identified (AdipoR1 and AdipoR2). The interaction between adiponectin and its receptor are mediated through adenosine monophosphate activated protein kinase (AMPk), peroxisome proliferator-activator receptor alpha (PPAR\textsubscript{α}) and P38 mitogen activated protein kinase (P 38 MAPK)\textsuperscript{207}. High levels of adiponectin are thought to preserve β cell mass by inhibiting apoptosis and increasing β cell proliferation\textsuperscript{199}. A study in mice evaluating the effects of GIP inhibition on adiponectin levels showed that fat oxidation, adiponectin expression in adipose tissue and adiponectin levels in circulation were significantly increased after 3 weeks of high fat feed in Gipr\textsuperscript{−/−} mice\textsuperscript{208}, suggesting a role of GIP in suppressing adiponectin.

Increased adiponectin levels are considered to be protective against atherosclerosis and low levels are associated with development of coronary artery disease and type 2 diabetes\textsuperscript{209,210}. The other adipokines identified recently that have a protective role are omentin and visfatin. Similar to adiponectin, omentin levels are low in people with obesity, insulin resistance and higher levels are thought to be protective against metabolic syndrome\textsuperscript{209,211}. Visfatin is thought to enhance insulin secretion and preserve β cell function\textsuperscript{199}. 
**TNFα and Interleukin 6 (IL6)**

TNFα is a pro-inflammatory cytokine present in high levels in obesity and insulin resistant states. It is thought to induce insulin resistance in obesity through multiple mechanisms that involve altered regulation of NEFA, Glut-4 proteins and defects in insulin receptor signalling. It is also known to have an immune mediated role in β cell failure in type 1 diabetes. TNFα suppresses adiponectin and induces several pro-inflammatory cytokines (MCP-1, PAI-1) and IL-6. TNFα reduces NEFA uptake by adipocytes through inhibition of LPL expression and activity.

IL-6 is structurally related to leptin, expressed in multiple tissues and has tissue specific function. Adipose tissue has higher concentrations of IL-6 compared to TNFα. IL-6 worsens insulin resistance in adipose tissue and liver while improving insulin sensitivity in the muscle. IL-6 has similar effects to TNFα in suppressing adiponectin, visfatin and induces other pro-inflammatory cytokines. It is also thought to increase basal lipolysis in adipocytes. Additionally, IL-6 appears to have anti-inflammatory effects on islet cells and enhances glucose stimulated insulin secretion.

One study showed that human subcutaneous pre-adipocytes treated with GIP induced mRNA expression of IL-6, IL-1β, and the IL-1 receptor antagonist (IL-1Ra), but not TNFα expression. GIP induced IL-6 and IL-1Ra secretion was enhanced in the presence of lipopolysaccharides (LPS), IL-1β, and TNFα. Cytokine induction by GIP involved PKA and nuclear factor κB (NF-κB) pathways. GIP was also shown to induce lipolysis by activating hormone-sensitive lipase involving similar pathways. Lipolysis releases NEFAs that are re-esterified in the presence of GIP and this vicious cycle of lipolysis and pro-inflammatory adipokine expression may contribute to insulin resistance in adipose tissue. Currently there is insufficient evidence in humans to suggest that GIP has any effect on secretion of TNFα or IL-6. In a previous study from our research unit, acute GIP infusions in obese individuals with type 2 diabetes did not have an effect on circulating adipokine levels, however IL-6 was suppressed with acute infusion of GLP-1.
**MCP-1**

Monocyte Chemoattractant Protein-1 (MCP-1) is an extensively studied chemokine. MCP-1 is a key regulator chemokine involved in migration and infiltration of monocytes and macrophages\(^{221}\). There are a number of studies that investigated its role in the aetiologies of obesity- and diabetes-related diseases\(^{222}\). Gene expression and plasma concentrations of MCP-1 are higher in obese adults\(^{221,223,224}\). MCP-1 in circulation is higher in patients with type 2 diabetes\(^{225,226}\) and higher concentrations of MCP-1 are correlated with increased risk of cardiovascular disease and diabetic complications \(^{222,227,228}\). These correlations are stronger in obese patients with T2DM than lean patients\(^{229}\). Intraperitoneal injections in mice increase MCP-1 and IL-6 expression in adipose tissue. The only human study published recently also showed increase in MCP-1 and MCP-2 expression and increased plasma MCP-1 with GIP infusion\(^{230}\).

**Osteopontin**

Osteopontin (OPN) is another important pro-inflammatory cytokine expressed in adipocytes and other cells like osteoclasts, smooth muscle cells and hepatocytes. It is thought to increase macrophage accumulation that causes inflammation and leads to insulin resistance\(^ {231}\). OPN is classified as a T helper-1 cytokine that exacerbates numerous chronic inflammatory conditions including cardiovascular disease and atherosclerosis\(^ {232}\). Expression of OPN is higher in obesity and obesity related diabetes compared to lean individuals\(^ {233,234}\). Studies in mice have shown that inhibiting OPN action through antibody neutralisation and OPN knockout protected against insulin resistance and hepatic steatosis despite high fat diet\(^ {234,235}\).

GIP was shown to enhance OPN expression in primary rodent adipocytes\(^ {236}\). Similar effects of GIP on OPN expression was observed in human adipocytes. Experiments on adipocytes from humans with a genetic variant of GIPR with diminished function, showed reduced OPN levels and improved insulin sensitivity\(^ {237}\). Given the pro-inflammatory effects of OPN in adipocytes, an enhanced expression by GIP may exacerbate insulin resistance in adipocytes. Increased OPN expression by GIP was also observed in pancreatic β cells although this function appears to be protective in preservation of pancreatic β cell mass\(^ {238}\).
Resistin

Resistin belongs to cysteine rich resistin like molecules (RELMs). It was discovered as a product of mouse adipocytes that was suppressed by thiazolidinediones (anti-diabetic drugs that activate PPAR $\gamma$) and proposed to induce insulin resistance in adipocytes$^{239}$. In rodent models, resistin was shown to downregulate insulin receptor expression, induce insulin resistance in pancreatic islets and reduce glucose stimulated insulin secretion$^{240,241}$. Resistin in humans is predominantly expressed by non-adipocyte inflammatory cells like macrophages and mononuclear cells$^{242}$. Obese individuals have higher serum concentrations of resistin$^{243}$. Recombinant human resistin increased pro-inflammatory cytokines such as TNF$\alpha$ and IL-6 in human subcutaneous adipocytes$^{244}$. Overall resistin acts on multiple human tissues to promote inflammation, atherosclerosis and possibly insulin resistance$^{245}$. Its effects on insulin resistance in humans still remain controversial$^{246}$.

Resistin is thought to play a key role in the mechanism of GIP induced enhancement of LPL activity. GIP through its receptor activates P38 MAPK and stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) pathways leading to resistin secretion. This in turn leads to increase in protein kinase B (PKB) phosphorylation and decreases liver kinase B1 (LKB1) and AMPK phosphorylation resulting in increased LPL activity$^{247}$. Adipocytes of resistin knockout mice showed reduced GIPR expression, and GIP signalling pathways (PKB/LKB1/AMPK) that enhanced LPL activity were compromised$^{184}$. LPL responsiveness to GIP was recovered after treatment with resistin suggesting an important link with GIP mediated actions on adipogenesis.

In summary, there is evidence from studies in animals that GIP may enhance pro-inflammatory adipokines which play a significant regulatory role in adipose tissue inflammation and development of insulin resistance and type 2 diabetes. There is very little evidence in humans of the effects of GIP on adipokines which requires further exploration. Studies showing the effects of GIP on adipokines are listed in Table 1.3.
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<th>Methods</th>
<th>Effects of GIP on Adipokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khales F et al. 2016&lt;sup&gt;248&lt;/sup&gt;</td>
<td>C57BL/6j mice</td>
<td>Lipopolysaccharides (LPS) administration and injecting inflammatory cytokines</td>
<td>↑ LPS induced GIP secretion Blunted LPS induced TNF-alfa and IL6 secretion</td>
</tr>
<tr>
<td>Berglund LM et al. 2016&lt;sup&gt;49&lt;/sup&gt;</td>
<td>Mice arteries</td>
<td>GIP infusions</td>
<td>↑ Osteopontin via endothelin-1 and activation of CREB</td>
</tr>
<tr>
<td>Timper K et al. 2016&lt;sup&gt;50&lt;/sup&gt;</td>
<td>Pancreatic islets of humans &amp; mice</td>
<td>In vitro incubation with GIP</td>
<td>↑ IL-6 by α cells ↑ GLP-1 &amp; Insulin</td>
</tr>
<tr>
<td>Nakamura et al. 2016&lt;sup&gt;51&lt;/sup&gt;</td>
<td>Gastro intestinal mucosa in humans with tumours and inflammation</td>
<td>Observational study</td>
<td>↑ GIP mRNA expression in upper jejunum in group with inflammation compared to Control group</td>
</tr>
<tr>
<td>Gogebakan et al. 2015&lt;sup&gt;330&lt;/sup&gt;</td>
<td>Normoglycaemic obese men (n=17), Human and murine adipocyte /3T3 L1</td>
<td>GIP 2pmol/kg/min with Hyperglycaemic clamp In-vitro GIP experiments with co-cultures</td>
<td>↑ Gene expression of MCP-1, ↑ MCP-2, IL-6 ↑ Plasma MCP-1 ↑ mRNA expression of MCP-1</td>
</tr>
<tr>
<td>Chen S, et al. 2015&lt;sup&gt;252&lt;/sup&gt;</td>
<td>Mice</td>
<td>Intraperitoneal GIP injections</td>
<td>↑ MCP-1, IL6 expression in adipose tissue</td>
</tr>
<tr>
<td>Timper K et al. 2013&lt;sup&gt;175&lt;/sup&gt;</td>
<td>Human subcutaneous pre-adipocytes</td>
<td>In-vitro GIP cultures Chronic GIP Rx</td>
<td>↑ Gene expression of IL-6, ↑ L-1β, ↓ Glucose uptake in adipocytes</td>
</tr>
<tr>
<td>Ahlqvist E, et al. 2013&lt;sup&gt;237&lt;/sup&gt;</td>
<td>Human adipocytes</td>
<td>Genetic variant of GIPR</td>
<td>↓ Osteopontin levels and ↑ insulin sensitivity</td>
</tr>
<tr>
<td>Omar B, et al. 2012&lt;sup&gt;236&lt;/sup&gt;</td>
<td>Isolated primary rodent adipocytes</td>
<td>In-vitro incubation with GIP</td>
<td>↑ Gene expression of osteopontin</td>
</tr>
<tr>
<td>Nie Y et al. 2012&lt;sup&gt;253&lt;/sup&gt;</td>
<td>3T3 adipocytes</td>
<td>Over expression of GIPR</td>
<td>↑ IL6, TNF-alfa</td>
</tr>
<tr>
<td>Lyssenko V et al. 2011&lt;sup&gt;238&lt;/sup&gt;</td>
<td>Human and mice Pancreatic β cells</td>
<td>In-vitro GIP experiments</td>
<td>↑ Gene expression of osteopontin</td>
</tr>
<tr>
<td>Kim SJ, et al. 2013&lt;sup&gt;84&lt;/sup&gt;</td>
<td>Mouse adipocytes</td>
<td>Resistin Knockout</td>
<td>↓ GIPR expression</td>
</tr>
<tr>
<td>Naitoh R, et al. 2008&lt;sup&gt;208&lt;/sup&gt;</td>
<td>Gipr&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>GIPR knockout</td>
<td>↑ Adiponectin on high fat diet for 3 weeks. ↑ fat oxidation</td>
</tr>
</tbody>
</table>
1.11 GIP antagonism in prevention of diet induced obesity

There is substantial evidence from animal models that adipocyte GIPR activation plays a key role in GIP induced actions in adipocytes. Consumption of high fat diet leads to enhanced GIP secretion causing anabolic effects through excessive triacylglycerol accumulation in adipocytes, liver and muscle tissue. This may contribute to obesity and insulin resistance, the two key elements in the development of type 2 diabetes. Insulin resistance may lead to hyperinsulinaemia which causes further increase in fat deposition with increased nutrition leading to a vicious cycle of obesity. Such effects could be prevented in theory by inhibiting GIP signal to halt the process of diet induced obesity. Various experimental methods (Figure 1.8.) have been utilised to test this concept by GIP receptor knockout, GIPR antagonism and active immunisation of GIP and have studied the effects of blocking GIP signalling in rat models to assess the impact on their metabolic state and development of obesity.52

![Figure 1.8. Experimental methods of GIP inhibition in animal models](image-url)
1.11.1 GIP signal inhibition

**GIPR Knockout in mice**

GIP receptor knock out in transgenic mice was shown to be an exceedingly useful method in understanding the effects of GIP signal blockade. GIPR knockout mice (\textit{Gipr}⁻/⁻) were generated by replacing exons 4 and 5 of \textit{GIPR} with the PGK-neo cassette\textsuperscript{254}. Miyawaki et al. studied the effects of high fat diet and energy expenditure in \textit{Gipr}⁻/⁻ mice\textsuperscript{135}. Wild type (WT) mice and \textit{Gipr}⁻/⁻ mice were fed on a high fat diet (45% fat) or a control diet (13% fat) for 43 weeks from the age of 7 weeks. The body weight of WT and \textit{Gipr}⁻/⁻ mice remained similar on control diet. WT on high fat diet had 35% body weight gain compared to no weight gain in \textit{Gipr}⁻/⁻ mice on the same high fat diet by the end of 50 weeks (Figure 1.9). Visceral and subcutaneous fat mass and adipocyte size significantly increased in high fat fed WT mice with steatosis in liver sections but none of these were evident in high fat fed \textit{Gipr}⁻/⁻ mice other than a 10% increase in adipocyte size compared to controls. Blood glucose levels were higher in high fat fed WT mice compared to \textit{Gipr}⁻/⁻ mice on the same diet. These results showed that inhibition of GIP signal prevented insulin resistance and dietary induced obesity.

A study investigated the role of triacylglycerol accumulation into adipocytes and fat oxidation in normal dietary conditions under diminished insulin action by disrupting the insulin receptor substrate protein (IRS-1⁻/⁻). These effects were compared in mice with \textit{Gipr}⁻/⁻ and \textit{Gipr}⁺/⁺. Mice with IRS-1⁻/⁻ \textit{Gipr}⁻/⁻ exhibited lower respiratory quotient, higher fat oxidation, reduced fat accumulation in adipocytes and improved insulin sensitivity compared to IRS-1⁻/⁻ \textit{Gipr}⁺/⁺ mice\textsuperscript{255}. These results indicate an important role of GIP in triacylglycerol accumulation under diminished insulin action by switching from fat oxidation to fat accumulation and this role may otherwise be trivial in a state of normal insulin sensitivity. This study highlights the difference in GIP action in insulin sensitive and insulin deficient or resistant states. Although this was not demonstrated in humans, these findings may be of significance when evaluating the action of GIP in individuals with T2DM with some degree of β cell dysfunction and insulin resistance.
Studies published in subsequent years on GIPR knockout mice showed similar beneficial effects on prevention in development of age related insulin resistance\(^\text{256}\). Another study published recently showed that mice with adipose tissue specific receptor knockout had lower body weight, reduced insulin resistance and reduced hepatic steatosis\(^\text{257}\).

**Figure 1.9:** Picture shows that Gipr\(^{-/-}\) mice (left side) were protected from fat deposition on high fat diet compared to wild type (WT) mice (right side) with increased body weight and fat composition on same high fat diet. On control diet, there was no significant difference in fat deposition in the two groups. *Picture adapted from Miyawaki, K et al 2002\(^\text{135}\)*
**GIP receptor antagonism in mice models**

GIP receptor (GIPR) antagonists such as GIP (7-30)-NH₂ (known as ANTGIP) were developed in the past\(^{258,259}\). However, these agents were mainly used to evaluate the physiological role of GIP as a postprandial insulinoetric agent. Subsequently, Proline3 (Pro\(^3\)) GIP was developed which is a stable and specific antagonist of the GIP receptor characterised by single proline substitution at Glu\(^3\) position\(^{260}\). Initial studies by Gault V et al. showed that acute administration of (Pro\(^3\)) GIP eliminated GIP stimulated insulin release in obese diabetic mice and worsened glycaemic and insulinoetric excursions to intra-peritoneal glucose\(^{260}\). These experiments helped to improve understanding of the relative contribution of GIP action to the entero–insular axis.

Acute (Pro\(^3\)) GIP administration only abolished GIP but not GLP-1 stimulated insulin release\(^{261}\). Further experiments by the same group studied the effects of prolonged administration of GIPR antagonist that caused chemical ablation of GIPR. Daily intra-peritoneal injections of (Pro\(^3\)) GIP over a 11 day period in young adult obese mice was shown to lower plasma glucose, and significantly improved insulin sensitivity\(^{262}\). Prolonged chemical ablation with intraperitoneal daily (Pro\(^3\)) GIP injections for 8 to 16 weeks in mice fed with high-fat diet, reduced body weight significantly, enhanced loco-motor activity, improved HbA1c, glucose tolerance and insulin sensitivity\(^{263,264}\). Similar experiments with a novel long acting GIP antagonist (Pro3) GIP mini-polyethylene glycol demonstrated prolonged antagonism compared to (Pro\(^3\)) GIP\(^{265}\).

In a study done recently, Gip signalling was inhibited by using two GIP analogues GIP(3-30)Cex-K\(^{40}\) [Pal] and Pro\(^3\)GIP(3-30)Cex-K\(^{40}\) [Pal] derived from N terminal truncation of native GIP\(^{266}\). These GIP analogues were injected once daily for 21 days in normal mice and obese diabetic high fat fed mice. Both these analogues reduced the body weight and improved glucose tolerance in both groups of mice indicating beneficial effects in compromising GIP signal.
**GIP neutralisation in mice**

Active immunisation against GIP by specific neutralising antibodies is another interesting approach to inhibit GIP action. Vaccination using GIP peptides covalently attached to virus like particles (VLP-GIP) in mice induced high titres of specific antibodies and efficiently reduced body weight gain in animals fed a high fat diet\(^{267}\). Another study showed that active immunisation against GIP for 56 days showed reduced plasma glucose concentrations and showed reduced trends in insulin levels in the circulation\(^{268}\).

Another method used for prolonged disruption of GIP signalling was active immunization against (Pro3) GIP\(^{269}\). Immunisation was undertaken using GIP-ovalbumin and a mixture of Freund’s adjuvant. Mice were injected with this GIP-ovalbumin conjugate, transferred to high fat diet followed by further booster doses every 14 days until 98 days. This method of sub-chronic immunisation is intended to have a dual therapeutic effect with antibodies against (Pro\(^3\)) GIP that neutralise native GIP and albumin bound (Pro\(^3\)) GIP that serve as a long acting GIPR antagonist. Immunisation of mice against (Pro\(^3\)) GIP on high fat diet reduced plasma glucose concentrations, insulin levels, liver triacylglycerol, pancreatic insulin and circulating LDL-cholesterol levels compared to non-immunised mice. There was no significant change in body weight or eating habits between immunised and non-immunised mice.

Another study compared the effects of sub-chronic immunisation against native GIP versus (Pro\(^3\)) GIP using GIP-ovalbumin conjugates in mice. Beneficial effects on the metabolic profile very similar to other studies were observed with immunisation of GIP or (Pro\(^3\)) GIP\(^{270}\). There was no significant change in body weight, energy intake, energy expenditure and cognitive function between immunised and non-immunised mice. Lack of reduction in body weight appears to be a consistent finding in studies involving immunisation of GIP or (Pro\(^3\)) GIP compared to studies that used GIPR antagonist or GIPR knockout methods. Overall there is adequate evidence from these animal studies to suggest that metabolic profile improves with inhibition of GIP signalling independent of the method used.
1.11.2 Metabolic effects of GIP signal inhibition

GIP inhibition in animal models has shown to reduce insulin resistance and improve beta cell responsiveness and this occurs despite compromising its beneficial effects on insulin secretion. The negative effects of GIP inhibition are thought to be compensated by improved beta cell function and insulin sensitivity due to removal of triacylglycerols from pancreas, liver and muscle. Weight loss as a result of reduction in adiposity also ameliorates insulin resistance. Inhibition of GIP signalling is therefore seen as a new avenue to treat diet induced obesity and related complications such as type 2 diabetes. Potent GIP receptor antagonists have been developed in recent years to improve our understanding of the role of GIP in various tissues.

There is sufficient evidence to suggest GIP has an important role in lipid metabolism in animal models and inhibition of GIP signal prevents diet induced weight gain in mice. Although there is evidence to suggest a similar role of GIP in humans with very few studies available to date the effects of GIP antagonism have not been evaluated. Further studies in humans are required to help improve our understanding of its action on adipose tissue. GIP signal inhibition appears to be a potentially viable strategy for treatment of diet induced obesity in the future. However, the role of GIP on other tissues needs to be established before such therapeutic avenues could be ventured.
1.12 Effects of hypoglycaemic agents on incretins and gut hormones in type 2 diabetes mellitus (T2DM)

The incretin effect is known to be diminished in diabetes mellitus due to altered concentrations or reduced insulinotropic activity of incretin hormones (GLP-1 and GIP)\textsuperscript{25}. Although unclear if this is the cause or consequence of onset of diabetes mellitus, restoration of normoglycaemia in T2DM with hypoglycaemic agents may partially reverse the diminished insulinotropic activity. Drugs known to have hypoglycaemic properties but not commonly used in the treatment of diabetes have been shown to alter incretin secretion in animal studies. Resveratrol is a stilbenoid, a type of natural phenol and phytoalexin produced by several plants. Resveratrol is a potent anti-diabetic agent when used in high doses. Administering resveratrol for five weeks in mice was shown to increase portal vein concentrations of both GLP-1, insulin and intestinal content of active GLP-1\textsuperscript{273}. Berberine is an isoquinoline alkaloid that has effects on glucose and lipid metabolism. In streptozocin induced diabetes rats, 5 weeks of treatment with berberine was shown to increase pro-glucagon mRNA expression and increased L cells in ileum that produce GLP-1\textsuperscript{274}.

Hypoglycaemic agents used for treatment of T2DM in humans may also have additional benefits on secretion and efficacy of incretin and other gut hormones involved in glucose homeostasis\textsuperscript{275}. In obese patients with poorly controlled T2DM, near normalisation of blood glucose levels for 4 weeks with insulin treatment significantly improved insulin responses to both GLP-1 and GIP\textsuperscript{276}. Although insulin per se does not influence the incretin secretion, the efficacy of incretins may be increased in patients with T2DM achieving near normoglycaemia with insulin treatment. Other hypoglycaemic agents that influence incretin system are described below.

1.12.1 Effects of DPP-IV inhibitors on incretin system

The newer class of oral hypoglycaemic drugs developed more than 10 years ago to enhance the incretin axis are known as dipeptidyl peptidase-IV (DPP-IV) inhibitors. DPP-IV inhibitors enhance the incretin hormones by inhibiting the enzyme DPP-IV that rapidly degrades GLP-1 and GIP\textsuperscript{45}. These agents decrease serum DPP-IV activity by more than 80% with some degree of inhibition sustained for 24 hours.
making them suitable for once daily treatment dose. Several DPP-IV inhibitors are now widely used in treatment of type 2 diabetes (sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin) as monotherapy or as an add on therapy to metformin as a second line treatment.

1.1.2 Effects of metformin on incretins and gut hormones

In the recent years, evidence has emerged which suggests that an established older drug, metformin may also have a significant influence on incretin hormone secretion. Metformin is widely used as a first line drug in overweight and obese individuals with type 2 diabetes mellitus (T2DM). The glucose-lowering effect is mediated through reduced hepatic glucose output, increased peripheral glucose uptake and enhanced intestinal utilisation of glucose. Studies in mice and in human cell lines have shown metformin to increase glucagon-like peptide-1 (GLP-1) and its mRNA gene expression. Studies on the effects of metformin in animals and cell cultures are shown in Table 1.4. Many human studies in non-diabetic obese subjects and in individuals with T2DM, have consistently shown increases in circulating GLP-1 concentration with metformin therapy. Studies on the effects of metformin in humans are shown in Tables 1.5 and 1.6.

In contrast, little evidence exists on changes in GIP secretion after metformin treatment (Table 1.5). One study reported an increase in both GIP and GLP-1 (AUC measure) during a glucose tolerance test in women with polycystic ovarian syndrome (PCOS) after 8 months of metformin treatment. GLP-1 is rapidly degraded by the enzyme dipeptidyl peptidase-IV (DPP-IV) and administration of a single dose of metformin in patients with T2DM has been shown to inhibit DPP-IV activity which may be another important mechanism of metformin in enhancing GLP-1 concentrations. Although the effects of metformin on the incretin system may not represent the principal mechanism underlying its glucose-lowering action, it exemplifies the pleiotropic actions of this drug.

Metformin improves glycaemic control with less weight gain compared to some of the other oral anti-hyperglycaemic treatments in patients with T2DM. Although the mechanisms behind this are poorly understood, metformin has been shown to reduce food intake in obese subjects with and without diabetes mellitus. The
effects of metformin treatment on appetite regulatory hormones are less well studied. Ghrelin is a potent orexigenic hormone secreted from the gastric fundus which plays an important role in the hypothalamic regulation of energy homeostasis. The role of metformin on modulation of ghrelin levels in patients with T2DM remains unclear. Studies evaluating these effects of metformin on ghrelin have shown conflicting results.

In summary, two of the commonly used oral hypoglycaemic agents have effects on the incretin axis. Whilst DPP-IV inhibitors play a major role in enhancing both GLP-1 and GIP, there is good evidence to suggest that metformin treatment enhances total and active GLP-1 concentrations. Most the studies have shown no significant effect on GIP concentrations. Although metformin is thought to have effects on appetite regulation, currently there is little evidence available to support its effects on appetite regulatory hormones.
Table 1.4 Effect of metformin on incretins in animal studies and *in-vitro* studies

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study aims</th>
<th>Methods</th>
<th>Metformin effects on incretins and DPP-IV</th>
</tr>
</thead>
</table>
| Kappe et al. (2014) | To determine if high fat diet (HFD) and metformin in mice lowers number of entero-endocrine L cells and/or GLP-1 plasma levels | Mice received control/HFD for 12 weeks and oral metformin/ saline for last 14 days. Immunohistochemistry used to quantify GLP−1 positive cells in intestinal cells. | ↓ GLP positive cells in HFD mice  
↑ Prandial plasma GLP-1  
Upregulation of Intestinal expression of GLP-1R mRNA |
| Kappe et al. (2013) | To identify mechanisms involved in mediating lipotoxicity and metformin lipoprotection in GLP-1 secreting cells | Murine GLUT ag cell line used. DNA-fragment assay, ELISA, RT-PCR | Regulates GLP-1 receptor expression in pancreas. Protects GLP-1 cells against lipo-apoptosis.  
↑ secretion of pre-proglucagon |
| Mulherin et al. (2011) | To assess direct effects of metformin on GLP-1 secretion from intestinal L cells and assess indirect actions that increase plasma GLP-1. | In *vivo* and in *vitro* studies using murine human NCI-H716 and rat FRIC cells | ↑ GLP-1 *in vivo* only (M3 muscarinic dependent effects)  
↑ total GLP-1 over 24 hours DPP-4 activity not affected |
| Maida et al. (2011) | To assess if metformin exerts gluco-regulatory actions via modulation of the incretin axis | Used GLP-1 and GIP receptor knock-out mice and obese hyperglycaemic wild-type mice with or without GLP-1R antagonist (exendin) | ↑ GLP-1 levels in wild type mice  
No changes in GIP and PYY. Improved glucose tolerance in knock-out mice.  
↑ GLP-1R in INS-1β CELLS via PPAR-α dependent and AMPK independent pathways |
| Green et al. (2006) | To evaluate the effect of metformin on DPP-IV activity in normal and obese diabetic mice | Radioimmunoassay using blood samples taken 30 minutes post-intraperitoneal injection of glucose and GLP-1 or GLP-1+ metformin | ↑ circulating GLP-1 (7-36) amide in ↓ DPP-IV activity  
in-vivo in mice Improved glucose lowering and insulin release effects from exogenous GLP-1 |
| Hinke et al. (2002) | To Investigate whether metformin inhibits DPP-IV to increase GLP-1 in obese non-diabetic patients | In *vivo* analysis of 20% human serum, porcine kidney and recombinant human DPP-IV using mass spectrometry and surface plasma resonance | ↑ GLP-1 and glucagon secretion from pancreatic α cells and intestinal L cells  
Metformin does not act directly on DPP-IV |
| Yasuda et al. (2002) | To elucidate mechanisms behind metformin’s ability to increase GLP-1 levels | Plasma active GLP-1 changes after metformin in fasting DPP-IV positive F344/Jcl rats using valine pyrrolide a DPP-IV inhibitor | ↑ GLP-1 dose dependently  
There was no direct inhibitory effect on DPP-IV activity |
### Table 1.5: The effect of metformin on incretins in recent human studies

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study aims</th>
<th>Methods, duration of treatment and number of subjects (N)</th>
<th>Metformin effects on GLP-1, GIP DPP4 activity and gut hormones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priess D et al. (2016)</td>
<td>CAMRA study: Randomised double blind placebo controlled trial on effects of metformin in patients without cardiovascular disease in T2DM</td>
<td>CAMRA study: Fasting blood samples at 6 monthly visits after overnight fast. Duration: 18 months, n=86 (metformin), 87(lifestyle).</td>
<td>CAMERA study: ↑ Total fasting GLP-1 at 6, 12 and 18 months ↓ Leptin reduced with metformin GIP was not measured</td>
</tr>
<tr>
<td>DeFronzo R et al. (2016)</td>
<td>DIRECT study: Cross-sectional study to identify predictive biomarkers of glycaemic deterioration deep phenotyping and drug response in patients recently diagnosed with T2DM</td>
<td>DIRECT study: Fasting blood samples after a 10 hr overnight fast and metformin stopped for 24 hours. Multiple time point blood samples over 2 hours after a mixed meal. N = 270 (metformin), 505 (lifestyle).</td>
<td>DIRECT study: ↑ basal fasted GLP-1 (active and total) on metformin compared to those on lifestyle alone. There was no difference in 60-minute total GLP after mixed meal in both groups. GIP was not measured.</td>
</tr>
<tr>
<td>Otsuka Y et al. (2015)</td>
<td>Investigated the effects of delayed release metformin on glucose lowering effects and gut hormone secretion</td>
<td>Randomised, blinded cross over study using a delayed release metformin targeting the ileum Duration 5 days, n=24</td>
<td>↑ GLP-1 ↑ PYY (AUC-5 days) Similar glucose lowering effects despite reduced systemic absorption.</td>
</tr>
<tr>
<td>Wu T et al. (2014)</td>
<td>Study assessed the effects of metformin vs sitagliptin (DPP-IV inhibitor) on insulin, glucagon and incretins in Japanese patients with T2DM</td>
<td>Randomised parallel group study with hormone measurements after a meal challenge pre-and post-treatment with metformin and sitagliptin. Duration:12 weeks, n=25</td>
<td>Metformin: No significant changes in GLP-1, GIP and glucagon. C-peptide levels were slightly increased. Sitagliptin: ↑ active GLP-1 and GIP</td>
</tr>
<tr>
<td>Napolitano et al. (2014)</td>
<td>Study of Caucasian T2DM men treated with placebo or metformin to investigate effects on DPP-IV and total intact GLP-1</td>
<td>Crossover study with Intraduodenal glucose infusion on day 5 and day 8 Duration: 2 X 7 days, n=12</td>
<td>↓ Plasma fasting DPP-IV activity ↑ Plasma intact GLP-1 No significant difference in total GLP-1</td>
</tr>
<tr>
<td>Solis-Herrera et al. (2013)</td>
<td>Study of Caucasian subjects with T2DM on and off metformin to characterise gut based mechanisms</td>
<td>Post-prandial incretins, gut hormones and bile acid measurements at 4 visits on metformin treatment and after withdrawal of treatment</td>
<td>↓ GLP-1 after metformin withdrawal ↑ GLP-1after re-starting metformin Similar changes in PYY No change in GIP</td>
</tr>
<tr>
<td>Vardarli et al. (2013)</td>
<td>Study assessing glucose lowering mechanisms of sitagliptin and/or metformin in patients with T2DM</td>
<td>Cross-over study with meal tolerance testing and radioimmunoassay methods Duration: 4 X 6 weeks, n=16</td>
<td>↑ GLP-1 secretion and β cell function in metformin and sitagliptin combined (2 to 3-fold ↑ in basal plasma GLP-1 concentration) No significant ↑ with metformin alone</td>
</tr>
<tr>
<td></td>
<td>Effect of metformin, sitagliptin or both on GLP-1 responses of overweight/obese patients with T2DM</td>
<td>Cross-over study with oral glucose challenge on day 5 and IV glucose infusion on day 6 Duration: 4 X 6 days, n=20</td>
<td>Metformin: ↑ fasting total GLP-1 by ↑ insulin secretory responses. No change in fasting total or intact GIP. Sitagliptin: ↑ plasma intact GLP-1 and GIP, but ↓ total GLP-1, GIP</td>
</tr>
</tbody>
</table>
Table 1.6: The effect of metformin on incretins in previous human studies

<table>
<thead>
<tr>
<th>Author, year</th>
<th>Study aims</th>
<th>Methods, duration of treatment and number of subjects (N)</th>
<th>Metformin effects on GLP-1, GIP DPP4 activity and gut hormones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cuthbertson et al. (2011)\textsuperscript{291}</td>
<td>Acute effect of metformin and GLP-1 alone or in combination on DPP-4 activity in overweight and obese patients with T2DM</td>
<td>Blood samples after an overnight fast for DPP-4 activity, insulin, GLP-1, Duration: 1 day, n=10</td>
<td>DPP-4 only inhibited by 7% Insulin sensitizing effects important in glucose lowering by GLP-1</td>
</tr>
<tr>
<td>Migoya et al. (2010)\textsuperscript{293}</td>
<td>Effect of metformin in healthy men and women</td>
<td>Cross-over study including placebo and metformin interventions; Day 2 active and total GLP-1 and GIP and glucose plasma concentrations measured pre-meal and post-meal Duration: 4 X 2 days, n=16</td>
<td>↑ postprandial total GLP-1 ↑ postprandial active GLP-1 No effect on total or active GIP No effect on postprandial DPP-4 activity</td>
</tr>
<tr>
<td>Svendsen et al. (2009)\textsuperscript{295}</td>
<td>Effect of metformin on incretin hormones response during OGTT in women with polycystic ovarian syndrome (lean and obese PCOS)</td>
<td>Uncontrolled interventional study with 180min oral glucose tolerance tests (75 g). Incretin response over 180 mins was compared to baseline Duration: 8 months, n= 10 (lean), 12 (obese)</td>
<td>Lean: ↑ GLP-1 and GIP AUC Obese ↑ trend in GLP-1 and GIP AUC with borderline statistical significance</td>
</tr>
<tr>
<td>Mannucci et al. (2004)\textsuperscript{292}</td>
<td>Effect of metformin on 22 obese T2DM versus 12 placebo controls</td>
<td>GLP-1 measured before and after 100g glucose load after 4 weeks of 850mg metformin Duration: 4 weeks, n=34</td>
<td>Single dose didn’t modify GLP-1 Fasting GLP-1 ↑ after 4 weeks of metformin</td>
</tr>
<tr>
<td>Mannucci et al. (2001)\textsuperscript{289}</td>
<td>Effect of metformin versus placebo on GLP-1 and leptin in obese non-diabetic men before and after 14 days of treatment</td>
<td>GLP-1 measured using ELISA in fasting state and after oral glycaemic load during euglycaemic hyperinsulinaemic clamp Duration:14 days, n=10</td>
<td>Significant GLP-1 ↑ at 30 and 60 minutes after oral glucose load No changes in GLP-1 in control group</td>
</tr>
</tbody>
</table>
1.13 Effects of bariatric surgery on incretins and gut hormones in T2DM

1.13.1 Bariatric surgery in the treatment of type 2 diabetes
Bariatric surgery is currently the most effective therapeutic modality for morbid obesity. Resolution of type 2 diabetes mellitus (T2DM) has been consistently observed as an additional benefit of surgical treatment of obesity. Normoglycaemia is restored in more than 95% of patients with diabetes undergoing biliopancreatic diversion (BPD), and in 80% of patients who are treated with the Roux-en-Y gastric bypass (RYGB) procedure. RYGB is the most common gastric bypass procedure and is thought to be the gold standard procedure (Figure 1.10). Of considerable interest is the observation that normoglycaemia and normal insulin levels occur within days after surgery, long before any significant weight loss has occurred. The mechanisms underlying the dramatic effects of surgery on insulin sensitivity and β-cell function are poorly understood. Many hypotheses have been proposed to explain this phenomenon. A foregut hypothesis lays emphasis on exclusion of duodenum and jejunum from the food tract as an important mechanism. Whilst the hind gut hypothesis suggests that rapid delivery of nutrients to distal intestines may release GLP-1 and PYY which have insulinotropic and anorectic activity leading to improvement in metabolic profile.

1.13.2 Incretin hormone changes post bariatric surgery
It was speculated that eliminating GIP secretion from the foregut (duodenum and proximal jejunum) in gastric bypass surgeries may lead to rapid improvement in insulin resistance. A few studies demonstrated GIP levels to be significantly reduced along with improved insulin sensitivity within weeks after gastric bypass surgeries in obese patients with T2DM. The foregut hypothesis could therefore possibly be a parallel concept of GIP inhibition in humans. However, some of the studies have shown contrasting results of increased post-prandial levels of GIP at 4 to 6 weeks after gastric bypass surgeries in patients with T2DM. The increased post meal GIP levels were shown to persist at one year and 3 years after gastric bypass surgery. Another study in non-diabetic obese patients showed elevated GLP-1 and GIP levels at 20 years after jejuno-ileal bypass (JIB) surgery.
Overall the changes in GIP levels after bariatric surgery in various studies are inconsistent and contradictory. GLP-1 levels in contrast are consistently increased in all the studies after a glucose tolerance test or a mixed test meal\textsuperscript{126}. Increased GLP-1, peptide YY (PYY), oxyntomodulin and changes in appetite regulatory hormones such as ghrelin are thought to play an important role in T2DM remission\textsuperscript{327,328}. Based on current data, there is not enough evidence in humans to support the GIP inhibition concept through foregut exclusion in gastric bypass surgeries for resolution of diabetes. Although the exact mechanisms are unclear, favourable changes in incretin hormones, alteration in calorie intake, gastric emptying, nutrient absorption and sensing, bile acid metabolism, and microbiota may all play a significant role in remission of T2DM\textsuperscript{327}.

**Figure 1.10** Roux-en-Y Gastric Bypass (RYGB). The size of stomach is reduced to a small pouch of 30 mls. Small bowel is resected at the level of the distal jejunum and short intestinal roux limb is directly anastomosed to the small gastric pouch.
1.14 Overview of research studies in thesis

1.14.1 Study-1

The effects of acute GIP infusion on insulin secretion and adipose tissue metabolism in obesity and type 2 diabetes compared to lean controls

Hypothesis

- The effects of GIP on insulin secretion may diminish gradually from normoglycaemic to hyperglycaemic states which may explain the blunted effect of GIP in T2DM.
- GIP would have an anabolic action in human subcutaneous adipose tissue (SAT) promoting NEFA re-esterification, which we speculated may be mediated either by enhancing lipoprotein lipase (LPL) expression/activity (a lipogenic enzyme) or by reducing adipose tissue triacylglycerol lipase (ATGL) and hormone sensitive lipase (HSL) expression/activity, two key lipolytic enzymes. We postulated that this effect may be different according to obesity status or glucose tolerance.
- As demonstrated in animal models and in-vitro studies, GIP may increase the expression and secretion of some of the pro-inflammatory adipokines and reduce the expression and secretion of the anti-inflammatory cytokine, adiponectin in human SAT.

Aims and objectives

- To study the effects of acute GIP infusion versus placebo on serum insulin and plasma NEFA concentration under hyperglycaemic clamp conditions in lean, obese, obese subjects with impaired glucose regulation (IGR), and obese subjects with T2DM.
- To determine the acute, in-vivo effects of intravenous GIP versus placebo on TAG content and gene expression of LPL, ATGL, and HSL in SAT, in the four groups of individuals as described above.
- To investigate the effects of acute GIP infusions versus placebo on the gene expression and plasma concentrations of adipokines TNF-α, MCP-1, osteopontin and adiponectin in the above four groups of subjects.
1.14.2 Study-2

Effects of treatment with metformin on incretin system and gut hormones in obese patients with T2DM

**Hypothesis**
Metformin the most commonly used treatment in T2DM may affect the incretin system by altering the incretin hormone concentrations in addition to its other glucose-lowering properties. The previously reported anorectic effects of metformin that leads to improvement in glycaemic control and weight loss may be related to changes in ghrelin, an appetite regulatory hormone.

**Aims and objectives**
To study the effects of treatment with metformin (minimum 3 months) on endogenous GIP, GLP-1 concentrations, DPP-IV activity and active ghrelin levels in obese patients with T2DM.

The two research studies undertaken for this thesis work, and an overview of chapters in this thesis are shown in Figure 1.11. General methods are discussed in detail in chapter 2. The study design and protocol for each study are described in individual results chapters; study 1 in chapter 3 and study 2 in chapter 6.
Figure 1.11: Overview of research studies in this thesis.
Chapter 2

Methods
2.1 Approvals and sponsorship

Ethical approval
Ethical approval for research projects were obtained from the Northwest Research Ethics Committee, U.K (reference number: 08/H1001/20 and 05/Q1501/60).

Research Sponsorship:
Approvals were obtained from University of Liverpool to be the main sponsor and University Hospital Aintree as the co-sponsor for the research.

2.2 Participant recruitment

Research projects were advertised on the websites of University of Liverpool and University Hospital Aintree and on the notice boards in outpatient clinics. We approached various departments in University Hospital Aintree to recruit healthy volunteers. Suitable subjects attending our Hospital diabetes clinics, weight management clinics and Lipid clinics were also approached for recruitment. Some of the subjects with impaired glucose regulation and treatment naïve diabetes were recruited from a local primary health care centre. Volunteers who expressed interest in participation were given detailed information on the purpose of the study and were provided with information leaflets on study protocol and procedures involved in the study. All subjects were recruited after informed and written consent.

In study 1, we recruited 23 Caucasian male subjects into four groups: lean subjects with normoglycaemia (lean), obese subjects with normoglycaemia (obese), obese subjects with impaired glucose regulation (obese IGR) and obese subjects with type 2 diabetes mellitus (obese T2DM) who are treatment naïve and on diet control alone for diabetes. Lean were defined according to a BMI ≤25 kg/m² and obese with a BMI ≥30 kg/m² as per WHO criteria. Allocation to glucose regulation categories was based on recent medical records combined with a fasting plasma glucose concentration. Fasting plasma glucose was measured in all subjects at the start of the study and 75gram oral glucose tolerance test (OGTT) was done in those with abnormal fasting glucose (> 6 mmol/L) and no previous diagnosis of IGR and T2DM. OGTT was not repeated in those with established diagnosis of IGR and T2DM through recent OGTT and HbA1c results within 6 months.
In study 1, subjects were recruited into four groups based on BMI and glucose parameters as in table 2.1. Obese subjects allocated to the obese IGR group had one or more of the following: fasting hyperglycaemia defined as plasma glucose $\geq 6.1$ to $6.9$ mmol/l or impaired glucose tolerance OGTT with 2-hour plasma glucose $\geq 7.8$ and $<11.1$ mmol/l or HbA1c in pre-diabetes range (42-47 mmol/mol or 6 to 6.4%). Obese subjects with a diagnosis of T2DM meeting World Health Organisation (WHO) diagnostic criteria\textsuperscript{330,331} and not on pharmacological treatment for T2DM, were allocated to obese T2DM group. WHO diagnostic criteria for diabetes are the same as shown in table 2 excluding the BMI measure.

In study 2, we recruited 8 Caucasian subjects (6 male) with recent diagnosis of type 2 diabetes as per WHO diagnostic criteria who were not on any pharmacological treatment for T2DM.

<table>
<thead>
<tr>
<th></th>
<th>Lean normoglycaemia</th>
<th>Obese normoglycaemia</th>
<th>Obese (IGR)</th>
<th>Obese T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m(^2))</td>
<td>$\leq 25$</td>
<td>$\geq 30$</td>
<td>$\geq 30$</td>
<td>$\geq 30$</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>$\leq 6.0$</td>
<td>$\leq 6.0$</td>
<td>$\geq 6.1$ to $6.9$</td>
<td>$\geq 7.0$</td>
</tr>
<tr>
<td>2-hour glucose on OGTT (mmol/l)</td>
<td>$\leq 7.8$</td>
<td>$\leq 7.8$</td>
<td>$\geq 7.8$ and $&lt;11.1$</td>
<td>$\geq 11.1$</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>$\leq 42$</td>
<td>$\leq 42$</td>
<td>$\geq 42$ and $&lt;48$</td>
<td>$\geq 48^*$</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>$\leq 6%$</td>
<td>$\leq 6%$</td>
<td>6 to 6.4%</td>
<td>$\geq 6.5^*%$</td>
</tr>
</tbody>
</table>

Table 2.1 Subject allocation to categories based on BMI and glucose parameters. Allocation under T2DM is based on WHO diabetes diagnostic criteria. *Asymptomatic patients with HbA1c $\geq 48$ mmol/mol required a second result in similar range to confirm diagnosis of diabetes.
Exclusion criteria

Subjects with following conditions were excluded from the studies:

- History of severe cardiac, hepatic or renal disease
- Poor diabetes control with HbA1c > 86 mmol/mol (10%)
- Thyroid dysfunction (hyper-or hypothyroidism)
- Hypoadrenalism or excess cortisol production
- Other endocrine disturbance (acromegaly, growth hormone deficiency)
- Current malignant disease
- Known alcohol misuse
- Major psychiatric disease (including current use of antidepressants)
- History of major eating disorder (anorexia or bulimia nervosa)

2.3 Conduct of study

All experiments were performed in accordance with the principles of the Declaration of Helsinki. During each visit subjects attended the investigational unit at the Clinical Sciences Centre in University Hospital Aintree site between 8.00 to 08:30 AM, after an overnight of at least 10 hours’ duration. All subjects were asked to refrain from alcohol and strenuous physical activity for at least 24 hours. At the first visit, eligibility was confirmed and written informed consent was obtained. A detailed history on medical conditions and the list of medication were obtained.

Anthropometric assessments

Height, weight, BMI, waist circumference, and blood pressure were recorded during each visit. Height was recorded with the subject standing barefoot against a standard stadiometer to the nearest 0.1 cm and weight was recorded digitally in kilograms (kg) to the nearest 0.1 Kg at each visit using the same Tanita scales. BMI was then calculated as weight (kg) / height^2 (m). Blood pressure was recorded using an appropriate sized cuff, after at least 5 minutes of rest, in supine position. Percentage body fat estimation was determined by whole-body bioelectrical impedance analysis using a body composition analyser (Tanita Corporation, Tokyo, Japan).
Infusions and blood sampling

In study 1, after initial assessments, two intravenous cannulae (one in each arm) were inserted in antecubital veins. One cannula was used for collection of blood samples and the other for infusion of GIP/placebo and variable rate dextrose infusion for hyperglycaemic clamp (study 1). Baseline (fasting) blood samples were taken prior to the initiation of hyperglycaemic clamp. Thirty minutes after initiation of hyperglycaemic clamp an intravenous infusion of either GIP (2 pmol.kg. \(^{-1}\)min\(^{-1}\) dissolved in 0.9% saline) or placebo (0.9% saline alone) was started and maintained until 240 minutes. Further blood samples (10 ml) were taken at 15, 30, 60, 120, 180 and 240 minutes following the initiation of GIP/placebo infusion (study 1, details in chapter 3).

In study 2, blood samples were taken in the fasted state and at further 11 time points after a standard mixed meal. There were no infusions given in this study. Blood samples were collected into plastic serum separator (SST), fluoride EDTA and lithium heparin tubes for later measurement of insulin, liver function, lipid profile, glucose, NEFAs, gut hormones and adipokines. Blood samples collected into EDTA and lithium heparin tubes were pre-treated with 0.5 ml of aprotinin, to prevent degradation by dipeptidyl peptidase IV (DPP-IV) and other proteolytic enzymes. Serum samples (SST) were centrifuged (at \(-4^\circ\)C) 15 minutes after collection whereas plasma samples were centrifuged immediately. All blood samples were stored at -80°C until further analysis.

Appetite assessment

In study 2, assessment of hunger and desire to eat were done using unipolar Visual Analogue Scale (VAS) ratings. This consist of a question accompanied by a 100mm unmarked scale, with descriptors of extreme state (e.g. not hungry at all at one end to extremely hungry at other end) Participants were asked to mark on the scale, in response to a question (using paper VAS) at fasting stage and at every hour after a mixed meal. An example of VAS is attached in Appendix D.
2.4 Subcutaneous Adipose tissue biopsy (SAT biopsy)

In our first study, subcutaneous adipose tissue (SAT) biopsy was obtained at baseline (prior to initiation of hyperglycaemic clamp) and a second biopsy was obtained after 240 min of the GIP/placebo infusion from the contralateral side of abdomen on the same visit. Several adipose tissue biopsy techniques are described in the literature such as needle aspiration biopsy, Bergstrom’s technique and open incision biopsy techniques\textsuperscript{332-335}. I observed the open incision biopsy technique by Professor Tomlinson which was used in many of his research projects at the University of Birmingham, UK\textsuperscript{336}. We adopted this open incision biopsy technique as it has advantages of having large adipose tissue blocks which can be stored intact at -80°C for later analysis of lipid content and gene expression.

Volunteers were explained the procedure in detail. Subjects were placed in a supine position and the skin lateral to the umbilicus was cleaned with 3% chlorprep. The site is chosen at approximately 10 cm lateral to the umbilicus avoiding the small subcutaneous arteries that lie parallel to the midline by 2-4cm. A sterile drape is placed and the skin is anesthetized using less than 5 mls of 1% lidocaine with adrenaline of 1:200,000 concentrations, taking care not to infiltrate the subcutaneous or adipose tissue. An incision (0.75 to 1cm) was made through the skin and fascia using a sterile scalpel at this site. Skin is retracted and fat tissue tissues was incised using scalpel and Adson’s 15mm forceps. More passes were done to obtain the desired amount of tissue between 50-150 mg (Figure 2.1).

Adipose tissue samples were cleaned with normal saline and immediately snap frozen in liquid nitrogen and stored at -80°C until further analysis. All biopsies were handled under sterile conditions. Following the biopsy procedure, pressure was applied at the site of incision. The skin was cleaned with sterile saline and site was closed with steri-strips (sterile tape). Biopsy site was dressed with OPSITE waterproof dressing. Participants were provided with extra dressings and advised on dressing care. Steri-strips were removed approximately after 1 week. A new incision was made for each biopsy on both sides of abdomen.
Adipose tissue biopsy

Figure 2.1: Adipose tissue biopsy procedure on one of our study subjects (pictures taken with consent). **A** Site of incision injected with local anaesthetic (red arrow), site of old healed biopsy (blue arrow) from 1st visit, **B** Incision measuring approximately 1 cm is made using a size 11 sterile scalpel. **C** Extracting adipose tissue using Adson’s 15 mm forceps and a scalpel, **D** Intact adipose tissue removed from the site and the tissue is snap frozen immediately in liquid nitrogen.
2.5 Hyperglycaemic clamp

Rationale for hyperglycaemic clamp
Glucose homeostasis is dependent on pancreatic beta cell response to plasma glucose and sensitivity of body tissues to insulin. Hyperglycaemia stimulates insulin secretion which in turn causes cellular uptake of glucose leading to fall in plasma glucose concentrations. This sends a negative feedback signal to beta cells to prevent further insulin release. During *in-vivo* studies in normal physiological circumstances, this glucose–insulin feedback loop impedes quantification of beta cell response. The use of hyperglycaemic clamp technique allows to maintain a steady plasma glucose at a chosen hyperglycaemic plateau within narrow limits and this breaks glucose–insulin feedback loop. Maintaining a hyperglycaemic plateau controls the stimulus to beta cells and this allows to compare the beta cell sensitivity reliably in different subject populations\(^3\)\(^3\)\(^7\).

The effect of GIP on insulin secretion is dependent on plasma glucose concentrations. Studies comparing euglycaemic and hyperglycaemic clamps showed that endogenous GIP or exogenous administration of GIP enhanced insulin release only with mild to moderate hyperglycaemia but not during euglycaemic clamps\(^3\)^\(^3\)\(^8\). The effect of GIP induced insulin secretion was also not inhibited by hyperinsulinaemia\(^3\)^\(^3\)\(^9\). Although a steady state of glucose can be maintained in hyperinsulinaemic-euglycaemic clamps endogenous insulin secretion cannot be assessed accurately in different glucose tolerant states; whereas with hyperglycaemic clamp technique the insulin responses to identical glucose stimulus can be compared in glucose tolerant and intolerant states\(^3\)^\(^4\)\(^0\).

Hyperglycaemic clamp technique
All experiments in our first study with GIP/placebo infusions were conducted under hyperglycaemic clamp conditions. Several hyperglycaemic clamp protocols described in the literature\(^2\)^\(^7\)\(^6\),\(^3\)^\(^3\)\(^7\),\(^3\)^\(^3\)\(^9\),\(^3\)^\(^4\)\(^1\),\(^3\)^\(^4\)\(^2\) were reviewed and based on these principles we prepared an in-house protocol for hyperglycaemic clamp based on body weight to aim for a blood glucose \(\approx 8\) mmol/l. This was done as a two-step procedure by rising the glucose acutely in the first 5 minutes with a bolus of 20% glucose followed by variable rate of 20% glucose infusion.
Step 1- Priming dose glucose infusion
Blood glucose levels were raised acutely in the first 5 minutes with priming dose of glucose based on body weight at 120mg/kg. For example, in a subject weighing 70 kg, the priming dose of glucose required is 8400 mg (8.4 g) in the first 5 minutes. Calculations for total volume of 20% glucose in first 5 minutes and examples for priming dose based on weight is shown in in appendix A.

Step 2- Variable rate glucose infusion for maintenance
After the first 5 minutes of priming dose of glucose the infusion is switched to lower rates of variable 20% glucose infusion between 1mg/kg/min to 15mg/kg/min or higher aiming to achieve a steady state glucose of 8 mmol by 30 minutes. After 30 minutes of glucose infusion, GIP (2pmol/kg/min) or placebo was started and continued for the next 4 hours. Hyperglycaemic clamp using variable rate of glucose was a continued throughout the experiment (4 hours) aiming for glucose levels as close as possible to 8mmol. The calculations for variable rate glucose infusion for each subject is determined based on subject’s weight using excel software. An excel sheet specific to each subject was printed and used prior to starting each experiment. An example of infusion rate based on weight is shown in appendix A. Small volume blood was taken from existing cannula and blood glucose level was measured every 5 minutes from an YSI glucose analyser (YSI UK Ltd) on-site until the end of GIP/placebo infusion. The changes to variable rate infusion were determined by these blood glucose measurements aiming for glucose level of 8 mmol/l with a narrow limit of 0.5 mmol/l deviation either sides.

2.6 GIP product
GIP was sourced from Polypeptide Laboratories in Strasbourg, France and sterile-filtered by Stockport Pharmaceuticals (Stepping Hill Hospital, Stockport, UK). Certificate of sterile filtered GIP product is shown in appendix B. We used the GIP dose of 2 pmol.kg.⁻¹.min⁻¹ (dissolved in 0.9% saline) based on dose infused in previous studies. Concomitant GIP infusion at either a low (1pmol/kg/min) or a high infusion rate (4 pmol/kg/min) in healthy subjects during a hyperglycaemic clamp (7.8 mmol/l) showed no significant changes in insulin secretory responses.
2.7 Biochemical analysis

2.7.1 Glucose, insulin and metabolic profile

Glucose Blood glucose concentrations during hyperglycaemic clamp were measured using YSI 2300 STAT glucose analyser (YSI U.K Ltd, Fleet, Hampshire, U.K) on site in the research unit. Plasma glucose concentration was measured from fluoride oxalated tubes using a Cobas 8000 modular analyser (Roche diagnostics, USA) at the biochemistry department at University Hospital Aintree, Liverpool.

Insulin Serum insulin was measured by Enzyme Linked Immuno-Sorbent Assay (ELISA) using the Invitrogen Human Insulin ELISA kits (Invitrogen, Fisher Scientific Ltd Loughborough, U.K). The assay uses monoclonal antibodies directed against distinct epitopes of insulin with detection range of 0.17 to 250 uIU/ML.

Metabolic profile Glycated haemoglobin, fasting lipid profile and liver function were measured from baseline blood samples in the biochemistry department at University Hospital Aintree, Liverpool. HbA1c was determined by using high performance liquid chromatography method (Ha 8140, Menarini Diagnostics, Berkshire, UK). Fasting lipid profile (total cholesterol, HDL & triacylglycerols) was measured by homogeneous enzymatic, colorimetric method using the Cobas 8000 modular analyser (Roche diagnostics, USA).

HOMA-IR Homeostatic model assessment (HOMA-2) was used to estimate insulin resistance from fasting plasma glucose and insulin concentrations. Adipo-IR Adipose tissue insulin resistance (Adipo-IR) was calculated as a product of fasting NEFAs (mmol/L) x fasting insulin (pmol/L).
2.7.2 Non-Esterified Fatty Acids (NEFAs)

NEFAs were measured at Alder Hey Children’s Hospital, Liverpool using Randox kit on a Biostat BSD 570 analyser (Randox laboratories Ltd, London). The enzymatic reaction involved in the assay relies upon acetylation of Co-enzyme A by the NEFA in the presence of enzyme acyl CoA synthetase. The acyl- CoA produced from this reaction is oxidized by added acyl CoA oxidase with generation of hydrogen peroxide in the presence of enzyme peroxidase. This permits the condensation of 4-aminoantipyrine and N-ethyl-N-(2 hydroxy-3-sulphopropyl) m-toluidine into a purple coloured product which is measured calorimetrically.

2.7.3 Plasma gut hormones

**GIP** Intact GIP was measured at the University of Copenhagen, Denmark; this N terminal directed assay is specific for the intact N-terminus of GIP showing little cross reactivity and reduces overestimation of biologically active peptide.

**GLP-1** Active glucagon-like peptide-1 (GLP-1) was determined using commercial enzyme-linked immunoassay (ELISA) kits from Millipore, Billerica, USA. The standard curve range for GLP-1 ELISA was 2-100 pmol/L, inter and intra-assay precisions were 8 ± 4.8 % and 7.4 ± 1.1% respectively.

**Ghrelin** Active ghrelin was determined using commercial ELISA kits from Millipore, Billerica, USA. The standard curve range for ghrelin ELISA was 10-2000 pg/ml, inter and intra-assay precisions were 9.6-16.2 and 6.5-9.5% respectively.

**DPP-IV** The assay for DDP-IV was done using an in-house method adapted from methodology developed at the University of Ulster. DPP-IV activity was determined by a fluorometric method measuring 7-amino-4-methyl-coumarin (AMC) liberated from a DPP-IV substrate, Gly-Pro-AMC. The intra and inter-assay coefficients of variation of this assay were 2.1% and 6.9% respectively.
2.7.4 Plasma adipokines

**TNF R-2** Tumour Necrosis Factor Receptor 2 (TNF R2) is a marker of TNF-α activity. TNF-R2 was measured using a commercially available ELISA kit (Human sTNF RII/TNFRSF1B Quantikine ELISA Kit, DRT200, R&D Systems UK). It is a solid phase sandwich ELISA immunoassay with a sensitivity of 2.3pg/mL, with an intra-assay precision of 2.6 to 4.8 CV% and inter-assay precision of 3.5 to 5.1 CV%.

**MCP-1** Human MCP-1 was measured using commercial ELISA kit (Quantikine Human MCP-1 kit DCP00, R&D Systems UK) This is a solid phase ELISA designed to measure MCP-1 in cell culture supernates, serum, plasma, and urine. The assay range is 31 to 2000 pg/ml, with an intra-assay precision of 4.9 to 7.8 CV% and inter-assay precision of 4.6 to 6.7 CV%.

**Osteopontin** Human osteopontin was measured using commercial ELISA kit (Quantikine D0ST00, R&D Systems UK) This is a solid phase ELISA designed to measure osteopontin in in cell culture supernates, serum, plasma, urine and human milk. The assay range is 0.3 to 20ng/ml. with an intra-assay precision of 2.9 to 4 CV% and inter-assay precision of 5.4 to 6.6 CV%.

**Adiponectin** Human adiponectin was measured using a commercially available ELISA kit (Human Adiponectin ELISA, EZHADP-61K, Merck Millipore UK). The sensitivity of this assay is 1.5ng/mL, with an intra-assay precision of 1.0 to 7.4% and inter-assay precision of 2.4 to 8.4%.
2.8 Adipose Tissue analysis

2.8.1 Subcutaneous adipose tissue (SAT) biopsy lipid content
Lipid content /triacylglycerol (TAG) was quantified by measuring free glycerol output following overnight lipase treatment at 37ºC (Sigma). The values were normalized according to protein content. All samples were done in duplicate. Lysates were prepared by homogenization of fat biopsies in a buffer containing: 50mM TrisHCL pH=7.5, 150mM NaCl, 1% Triton X-100, and standard protease inhibitor cocktail (Complete Mini protease inhibitor cocktail, Roche Diagnostics, Germany). The procedure involves enzymatic hydrolysis of triglycerides in SAT to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzymatic reactions using the free glycerol reagent (Sigma-Aldrich, Saint Louis, USA) measures free glycerol.

2.8.2 SAT gene expression of lipid enzymes (LPL, ATGL and HSL)

- **RNA purification:** Total RNA was extracted from less than 100mg of adipose tissue using the RNeasy Lipid Tissue Mini Kit (Qiagen Ltd, UK). The integrity of each RNA sample was checked by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, California, USA).

- **RNA quantification:** RNA (1µl) was diluted in DEPC-treated water (199µl) and mixed by brief vortexing before quantification using a spectrophotometer (BioSpec-mini, Shimadzu Corp., Japan). A260/A280 ratio of their dilutions between 1.3 and 1.6 were deemed suitable for use.

- **Reverse transcription:** First strand complementary DNA (cDNA) synthesis was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). For each reaction, 1 µg of RNA were mixed with the Reaction Mix (4 µl), Enzyme Mix (2 µl, both provided by the kit) and H₂O, in a 20 µl reaction system, as suggested in the manufacturer’s instructions (Life Technologies).

- **qPCR:** Real-time quantitative PCR was conducted in triplicate using a BIORAD CFX-connect real time PCR instrument (BioRAD laboratories) using pre-validated TaqMan probes (Life Technologies).
2.8.3 SAT gene expression of adipokines (TNF-α, MCP-1, Osteopontin and adiponectin)

- **RNA isolation:** Total RNA isolation from all adipose tissue samples was carried out using TRIzol (Life Technologies) per manufacturer’s protocol. Adipose tissue was grounded to powder in liquid nitrogen and homogenised using 1ml of Trizol lysis method in a 2ml eppendorf tube. This was followed by additions of chloroform-isoamyl alcohol, isopropanol and centrifuged at 12,000 g for 15 minutes at 4°C. Ethanol was removed and pellets were then re-dissolved in 20μl of RNAase free water (Sigma).

- **Reserve transcription:** Complementary DNA was synthesised from 200ng of total RNA per sample using First standard buffer (Invitrogen), DTT and dNTPs (Invitrogen) followed by addition of Superscript II Reverse transcriptase (Invitrogen) RNAase free water (Sigma), RNA inhibitor (Invitrogen) This 20μl sample mixture was then incubated at 42°C for 1 hour after which the resulting cDNA product was diluted 20 times using RNAase free water. cDNA was placed at -20°C and ready to use when required for real-time quantitative PCR (qPCR).

- **Real-Time Quantitative PCR (qPCR):** Reactions were performed using the BioRad SSOAdvanced™ Universal SYBR® Green Supermix qPCR Mastermix Kit (BioRad). A reaction mixture of 12μl was made up for each sample by addition of 5μl of SYBR Supermix, 5μl of cDNA and 1μl each of forward and reverse primers respectively. Reactions were run on a BioRad CFX qPCR System (BioRad) with three technical replicates per each cDNA sample.

Further details on gene expression methods, housekeeping genes and primers are shown in appendix C.
2.9 Study protocols (in brief)

**Study 1**: A randomised double blinded cross-over study involving a hyperglycaemic clamp (glucose 8.0 mmol/l) with a 240 min GIP infusion (2pmol kg\(^{-1}\) min\(^{-1}\)) or normal saline. The details of study design, protocol and subject characteristics are discussed in chapter 3. Twenty three Caucasian men sub-divided into four groups were studied on two separate occasions with a minimum of 1 and a maximum of 3 weeks between visits. Using a computer-generated randomisation model, subjects were randomly assigned to either GIP or placebo infusion on their initial visit and received the alternate infusion on their subsequent visit. Intravenous infusion of GIP/placebo was continued from 30 minutes after initiation of hyperglycaemic clamp until 240 minutes. 10 ml blood samples were taken at baseline (prior to hyperglycaemic clamp) and at 15, 30, 60, 120, 180 and 240 minutes following the initiation of the infusion (GIP/placebo). Subcutaneous adipose tissue (SAT) biopsies were obtained at baseline and after 240 min of the GIP/placebo infusion.

**Study 2**: A prospective, observational study in patients with drug naive T2DM commencing metformin as monotherapy. Details of study design and protocol are discussed in chapter 6. Eight subjects (6 male and 2 female) with obesity and new diagnosis of T2DM were studied prior to and after at least 3 months of metformin monotherapy. Patients had a total of 3 visits to the investigation unit. Each subject was studied for 6 hours following a standard mixed meal, before and after at least 3 months of metformin monotherapy (mean dose 1.75 g daily). Blood samples were taken in the fasted state and at 11 further time points (15, 30, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes) after consumption of a standard mixed meal for measurement of gut hormones. Metformin was started after the first visit. Subjects attended for the 2\(^{nd}\) visit six weeks following initiation of treatment with metformin for a repeat HbA1c measurement, lifestyle advice and metformin dose titration as per clinical requirement. The protocol on visit 1 was repeated on visit 3 which was at least three months after metformin treatment (range 3 to 7 months).
2.10 Statistical analysis

One-way ANOVA
One-way analysis of variance (ANOVA) and Tukey’s t-tests were performed to compare participant demographics and baseline biochemical parameters between the four groups in the first study. Changes in plasma adipokines during GIP/placebo infusion studies compared to baseline values were assessed using ANOVA and Dunnett’s multiple comparison tests.

Area under the curve (AUC)
Area under the curve (AUC) was calculated by trapezoidal rule using GraphPad Prism software to give an integrated measure of responses for insulin and NEFA concentrations over 4-hour period of GIP/placebo infusion in study-1 and for gut hormones after a mixed meal in study-2. In Study-1, Paired t-tests were performed on insulin and NEFA AUC₀₋₄hr for subjects within each group (lean, obese, obese IGR and obese T2DM) to explore if AUCs differed with GIP versus placebo infusions. In study-2 paired t tests were performed on hormone AUCs pre and post metformin treatment.

Linear mixed-effects model
A linear mixed-effects model was used to model insulin secretion and NEFA concentrations using three time points (baseline, 120 minutes and 240 minutes) in study-1. A two-way interaction between treatment and group was included within the model to investigate whether the effect of GIP infusion in comparison to the placebo infusion differed between groups. Results are expressed in estimated average unit changes in insulin and NEFAs during GIP vs. placebo infusion.

Pearson correlation coefficient
A Pearson product-moment correlation coefficient was computed to assess the relationship between insulin AUC and other variables (fasting plasma glucose, HOMA-IR and Adipo-IR) during GIP and placebo infusions in study-1. The same test was used to assess the relationship between degree of NEFA reduction and other variables (fasting plasma glucose and Adipo-IR) during GIP and placebo infusions.
**Paired T tests**

Changes in SAT lipid content and gene expression are presented as a fold change values after GIP/placebo infusions compared to their respective baseline measurements on the same day. Paired t-tests were performed on these fold changes to explore whether the change over the two-time points differed between GIP and placebo. Fold changes were log transformed before analysis. P value of < 0.05 (two-tailed) was considered significant. Student’s t test was used for paired data with normal distribution and Wilcoxon signed-rank test when paired data were not normally distributed.
Chapter 3

The effects of acute GIP infusion on insulin secretion in lean, obesity and type 2 diabetes
3.1 Introduction
The two Incretin hormones GIP and GLP-1, together enhance glucose dependent insulin secretion and account for the incretin phenomenon. GLP-1 levels are reduced in patients with T2DM and therefore augmenting with GLP-1 is effective in the treatment of T2DM. In the last decade, stable GLP-1 receptor agonists (GLP-1RAs) available as self-administered subcutaneous injections are a preferred treatment choice for obese individuals with T2DM. In contrast, GIP concentrations are either normal or increased in patients with T2DM but interestingly its’ incretin activity is blunted and the reasons for this phenomenon remain unclear\textsuperscript{38}. GIP’s role in the treatment of T2DM has also been explored in recent years. Long-acting GIP receptor agonists are currently being researched as prospective therapeutic agents in T2DM. Diminished insulinotropic activity and unfavourable pharmacokinetics with rapid degradation of GIP by dipeptidyl-peptidase-IV (DPP- IV) are potential limitations for development of a stable agonist\textsuperscript{52}.

3.2 Hypothesis and aims
It is unclear if the insulinotropic activity of GIP diminishes with obesity, hyperglycaemia and insulin resistance eventually leading to a blunted effect in the diabetic state or if this is only observed as a consequence of an established diabetic state. We hypothesised that insulinotropic activity of GIP may gradually diminish from normoglycaemic to hyperglycaemic states.

The aim of this study was to explore the effects of acute GIP infusion on insulin concentration in lean, obese and in impaired glucose regulation states. To investigate these effects, we studied plasma insulin concentrations during acute GIP infusions under hyperglycaemic clamp conditions in lean, obese, obese subjects with impaired glucose regulation (IGR), and obese subjects with T2DM. This study was part of a bigger study assessing the effects of GIP infusions on adipose tissue metabolism (described in chapter 4). Our methodology did not include exploration of molecular mechanisms underlying the blunted insulinotropic effect of GIP in T2DM.
3.3 Subjects and methods
We studied 23 Caucasian men, age 49 ± 12.3 years (mean ± SD) categorised into four groups according to BMI/glucose regulation: i) lean (n=6), ii) obese (n=6), iii) obese with impaired glucose regulation [obese IGR] (n=6) and iv) obese with (treatment-naive) T2DM [obese T2DM] (n=5). Group categories based on BMI and glucose regulation are discussed in detail in chapter 2, section 2.2.

Study design (Figure 3.1)
Each subject participated in a double-blind, crossover study involving a hyperglycaemic clamp (plasma glucose 8.0 mmol/l). After overnight fasting, subjects were infused with either GIP (2 pmol.kg.\(^{-1}\).min\(^{-1}\), in 0.9% saline) or placebo (0.9% saline alone) using a computer-generated randomisation model in their first visit and received the alternate infusion on their second visit 1-3 weeks apart (Figure 3.1). Both the investigator conducting the experiments and the subject were blinded to the treatment given.

Study protocol (Figure 3.2)
After anthropometric measurements, intravenous cannulae were inserted into both antecubital fossae, for blood sampling and infusions (GIP/placebo). Plasma glucose concentration ~8.0 mmol/l was maintained during a hyperglycaemic clamp as described in detail in chapter 2. The intravenous infusion of GIP/placebo was continued from 30 minutes after initiation of hyperglycaemic clamp until 240 minutes (Figure 3.2). 10 ml blood samples were taken at baseline (prior to hyperglycaemic clamp) and at 15, 30, 60, 120, 180 and 240 minutes following the initiation of the infusion (GIP/placebo) and measured for glucose and insulin. Blood samples at baseline, 120 and 240 minutes were measured for GIP concentrations. Adipose tissue biopsies were taken at baseline and immediately after GIP/placebo infusions on contralateral sides. Samples were stored at -80 °C and later analysed for lipid content, gene expression of lipid metabolism enzymes and adipokine gene expression (discussed in chapter 4 and 5). General methods on blood sample collection, processing, storage and analysis are discussed in detail in chapter 2, section 2.3.


Figure 3.1: Study design showing four groups of subjects in a randomised cross over study undergoing GIP and placebo infusions under hyperglycaemic clamp conditions 1 to 3 weeks apart.
Study protocol

Figure 3.2: Study protocol showing duration of hyperglycaemic clamp (blue horizontal bar) aiming for glucose ~ 8mmol/l and the duration of GIP/placebo infusions and the time points for blood sampling and SAT biopsies.
3.4 Statistical Analysis

Participant demographics, baseline biochemical parameters and blood glucose concentrations during the hyperglycaemic clamp are expressed as mean ± SD; all other results are expressed as mean ± SEM. One-way analysis of variance (ANOVA) and Tukey’s t-tests were performed to compare participant demographics and baseline biochemical parameters between the four groups in this study.

Area under the curve (AUC) for insulin concentrations over 4-hour period of GIP/placebo infusion (AUC_{0-4hr}) was calculated by trapezoidal rule using GraphPad Prism software. Paired t-tests were performed on insulin-AUC_{0-4hr} for individual subjects within each group (lean, obese, obese IGR and obese T2DM) to explore if insulin-AUC differed with GIP versus placebo infusions. Tukey’s multiple comparisons test was used to compare the insulin-AUC across the four groups. Pearson product-moment correlation coefficient was computed to assess the relationship between insulin AUC and other variables (fasting plasma glucose, and HOMA-IR) during GIP and placebo infusions.

A linear mixed-effects model was used to model insulin concentrations using three time points (baseline, 120 minutes and 240 minutes). A two-way interaction between treatment and group was included within the model to investigate whether the effect of GIP infusion in comparison to the placebo infusion differed between the groups. Variables were considered to be significant at the 5% significance level. Results are expressed in estimated average unit changes in insulin during GIP vs. placebo infusion.
3.5 Results

3.5.1 Baseline characteristics and biochemical parameters

Participant demographics

Waist circumference and percentage body fat mass were significantly higher in obese, obese IGR, obese T2DM compared to the lean group. The duration of diabetes in obese T2DM group was 7 ± 5.5 months (mean ± SD), mean HbA1c of 54 ± 8.5 mmol/mol (7.1 ± 0.8 %) and all participants were naive to oral or injectable diabetes medications.

Plasma glucose and insulin concentrations

As expected, mean fasting glucose was higher in obese IGR and obese T2DM groups compared to the two other groups. Fasting insulin and HOMA-IR were significantly higher in obese, obese IGR and obese T2DM groups vs. the lean group. Adipo-IR was significantly higher in obese IGR vs. lean group and in obese T2DM group vs. lean and obese groups (Table 3.1).

Metabolic parameters

All subjects in obese IGR and obese T2DM groups had metabolic syndrome based on International Diabetes Federation 2006 criteria with most consequently treated for hypertension and dyslipidemia: angiotensin convertase enzyme (ACE) inhibitors or angiotensin receptor blockers (three subjects in obese IGR group, five subjects in obese T2DM group), beta-blockers (two obese IGR, 2 obese T2DM) and calcium channel blocker (one obese T2DM). Three subjects in each of the above two groups were on statins. Two subjects in the obese group had metabolic syndrome (one on ACE inhibitors and one a fibrate). No lean subjects had metabolic syndrome or received any regular medication. The mean fasting NEFAs were higher in obese IGR and T2DM groups vs. the normoglycaemic groups but the difference was not statistically significant (Table 3.1).
Table 3.1

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<td>6.0 ± 0.7</td>
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</tr>
<tr>
<td>(mmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting Insulin (µIU/ml)</td>
<td>11.9 ± 2.6</td>
<td>30.5 ±14.4*</td>
<td>38.3 ±12.5**</td>
<td>36.9 ± 9.1**</td>
</tr>
<tr>
<td>Fasting NEFAs(µmol/L)</td>
<td>352 ± 118</td>
<td>312 ± 123</td>
<td>421 ± 115</td>
<td>494 ± 150</td>
</tr>
<tr>
<td>HOMA-IR§</td>
<td>1.6 ± 0.3</td>
<td>3.8 ± 1.8*</td>
<td>4.8 ± 1.4**</td>
<td>4.9 ± 1.2**</td>
</tr>
<tr>
<td>Adipo-IR§ (mmol/L/pmol/L)</td>
<td>24.5 ± 8.1</td>
<td>54 ± 23.7</td>
<td>95.9 ±37.8**</td>
<td>115.7 ± 51.2**△</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>-</td>
<td>-</td>
<td>44 ± 2.3</td>
<td>54 ± 8.5</td>
</tr>
</tbody>
</table>

**Table 3.1:** Baseline demographics, anthropometric and biochemical parameters (mean ± SD) in four groups of subjects. P value for statistically significant difference vs. Lean group is indicated as * (<0.05); ** (<0.01); *** (<0.001); **** (<0.0001) and p value for significant difference vs. obese group is indicated as △ (<0.05). §Non-Esterified Fatty Acids (NEFA), ¥Homeostasis Model Assessment-Insulin resistance (HOMA-IR), §Adipose tissue insulin resistance (Adipo-IR).
3.5.2 Biochemistry changes during GIP and placebo infusions

**Blood glucose**

The blood glucose concentrations (mean ± SD) during the 4 hour hyperglycaemic clamp were similar across the four groups: lean, 8.02 ± 0.02 (GIP) vs. 8.17 ± 0.14 mmol/l (placebo); obese, 8.0 ± 0.07 (GIP) vs. 8.17 ± 0.07 mmol/l (placebo); obese IGR group, 8.08 ± 0.11 (GIP) vs. 8.11 ± 0.06 mmol/l (placebo) in and obese T2DM group, 8.35 ± 0.15 (GIP) vs. 8.46 ± 0.18 mmol/l (placebo). Blood glucose concentrations at every 15 minutes during infusions for the four groups are shown in Figure 3.3.

The volume of 20% glucose (mean ± SEM) infused to maintain the hyperglycaemic clamp during GIP vs. placebo infusions in the four groups were: lean, 1124 ± 155 mls (GIP) vs. 631 ± 152 mls (placebo); obese, 926 ± 150 (GIP) vs. 462 ± 106 mls (placebo) obese IGR group, 725 ± 139 (GIP) vs. 398 ± 34 mmol/l (placebo) in and obese T2DM group, 508 ± 72 (GIP) vs. 323 ± 14 mls (placebo).

**Plasma GIP concentrations**

Fasting plasma GIP concentrations were similar across the four groups for both visits. Higher GIP concentrations were achieved during GIP infusions and levels peaked during 2 hours and tapered towards the end of infusion (Figure 3.4).

3.5.3 Serum insulin concentrations during GIP vs placebo infusions

The insulin concentrations (mean ± SEM) at baseline (prior to hyperglycemic clamp) and at further six time points (15, 30, 60, 120, 180 and 240 minutes) during GIP and placebo infusions for all the four groups are shown in Figure 3.5 A-D. Serum insulin concentrations were higher within 15 minutes of GIP infusion compared to placebo in all four groups. This difference was sustained throughout the GIP infusion period in lean, obese and obese IGR groups. In obese T2DM group, insulin concentrations increased slightly with GIP infusion during initial 30 minutes compared to placebo which was not significant. For the rest of the duration of the infusion, there was no difference in insulin concentrations between GIP and placebo infusions.
Figure 3.3: Blood glucose concentrations (mean ± SD during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp ≈8 mmol/l) are shown in A lean individuals, B obese, individuals, C obese individuals with IGR, D obese individuals with T2DM.
Figure 3.4: Plasma GIP concentrations (mean ± SEM) during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp) are shown in A lean individuals, B obese, individuals, C obese individuals with IGR, D obese individuals with T2DM.
Figure 3.5: Serum insulin concentrations (mean ± SEM) during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp) on Y-axis are plotted against time on X-axis for each group: A lean individuals, B obese individuals, C obese individuals with IGR, D obese individuals with T2DM. Baseline blood sampling was taken at the time point -30* minutes (on X axis).
Effects of GIP vs placebo infusions on insulin concentrations

To assess the overall effect on insulin concentrations during GIP versus placebo infusion individually for different groups, statistical analysis was performed using a linear mixed-effects model with insulin concentrations at three time points (0, 120 and 240 minutes). Results are expressed in estimated average unit changes in insulin during GIP vs. placebo infusion.

The amount by which insulin concentration increases when receiving GIP differed across the groups, holding all other variables constant, these results were non-significant between obese and obese IGR groups compared to lean group. Comparing insulin secretion during GIP versus placebo within each group, there was approximately 63, 70 and 121 µIU/ml increase in insulin concentrations when receiving a GIP infusion in lean, obese and obese IGR groups respectively. In obese T2DM group, there was only a 9µIU/ml increase in insulin concentration with GIP compared to placebo infusion (Table 3.2).

<table>
<thead>
<tr>
<th>Increase in insulin concentration (µIU/ml)</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP vs. placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean</td>
<td>63</td>
<td>10 to 115</td>
</tr>
<tr>
<td>Obese</td>
<td>70</td>
<td>18 to 12</td>
</tr>
<tr>
<td>Obese IGR</td>
<td>121</td>
<td>68 to 173</td>
</tr>
<tr>
<td>Obese T2DM</td>
<td>9</td>
<td>-49 to 67</td>
</tr>
</tbody>
</table>

Table 3.2: Unit increases in insulin concentrations (linear mixed-effects model), with GIP infusion compared to placebo infusion over 240 minutes in four groups of subjects in the study, confidence intervals (CI) and p values.
Integrated insulin concentrations (AUC) during GIP vs placebo infusions

Area under the curve for insulin concentrations (Insulin AUC$_{0-4hr}$) was calculated from insulin measurements at the 7 time points as an integrated measure of insulin concentrations during the 4 hour GIP and placebo infusions. As GIP infusions enhanced the insulin secretion in the first three groups, the mean AUC$_{0-4hr}$ of insulin concentrations was higher with GIP infusion compared to placebo in lean, obese and obese IGR groups. Whereas in T2DM group, AUC$_{0-4hr}$ of insulin during GIP infusion and placebo infusions were not different (Figure 3.6). Although the mean insulin AUC$_{0-4hr}$ with GIP infusion was higher in obese group than all other groups, the difference was not statistically significant compared to insulin AUC with placebo infusion. When Insulin AUC during the GIP infusion alone is compared between the four groups but, it was not significantly different in lean, obese and obese IGR groups except for the obese T2DM group.

Insulin AUCs in subjects within the four groups

The distribution of insulin AUC$_{0-4hr}$ for individual subjects within each group during placebo and GIP infusions are shown in box plots (Figure 3.7). The insulin AUCs were significantly higher with GIP infusion compared to placebo in lean and obese IGR groups with a clear difference in the insulin AUCs for most subjects between the two infusions. Whereas in the obese group, although the insulin AUCs were higher with GIP infusion in most subjects, some of the subjects also mounted a higher insulin secretion during placebo infusion (with hyperglycaemic clamp) making this difference less significant. The insulin AUC with GIP infusion in obese T2DM group was significantly lower compared to the other 3 groups and no different compared to placebo in the same group.
Figure 3.6: A Mean insulin AUC$_{0-4hr}$ ± SEM for GIP vs placebo infusions in four groups presented as bar charts (white bar, placebo; black bar, GIP). P values presented as *<0.05, **<0.005. B AUC of insulin concentrations with placebo compared to GIP infusion over 240 minutes in all four groups, confidence intervals (CI) and p values.
Figure 3.7: Insulin AUC$_{0-4hr}$ (µIU/ml/min) in individual subjects during 4-hour infusions of GIP versus placebo in each group are presented in box plots with a median line. Whiskers represent the individuals with minimum and maximum insulin-AUC concentrations in the four groups: A Lean, B Obese, C Obese IGR and D Obese T2DM. The p values are represented by * $<0.05$ and ** $<0.005$. 
Correlation between insulin concentrations and fasting plasma glucose

There was a negative correlation between AUC of insulin and fasting plasma glucose levels during GIP infusions (Figure 3.8) for all the subjects in the study (n=23). There was no such correlation with placebo infusion. These results indicate that there is a gradual decline in insulin secretions with GIP infusions in individuals with higher fasting plasma glucose level and this has a linear relationship.

Figure 3.8: Correlation between fasting plasma glucose (mmol/l) plotted on y axis against Insulin AUC (µU/ml/min) on x axis for all subjects in the study (n=23) during A GIP infusion and B placebo infusions. C Pearson’s correlation coefficients, 95% confidence intervals and two tailed p values.
3.6 Discussion

In this study, we demonstrate that GIP infusion in a fasting state under hyperglycaemic clamp conditions stimulated insulin secretion in the lean, obese and obese subjects with IGR, whereas its insulinotropic action was significantly impaired in obese patients with T2DM. These results were consistent with findings reported by other studies previously. Additionally, our results showed that the integrated insulin concentrations (AUC) during GIP infusions correlated negatively with fasting plasma glucose for all subjects in the study. Thus, indicating a gradual decline in insulin secretion with GIP infusions in individuals with higher fasting plasma glucose levels. We also observed that the insulinotropic action of GIP did not differ significantly in normoglycaemic lean, obese and obese IGR subjects.

In the obese group, the mean insulin AUC with GIP infusion was higher than all other groups, but difference in insulin AUC was not statistically significant compared to placebo infusion within the same group. This is probably because individuals in obese group had higher fasting insulin concentrations and further increments in insulin secretion in response to hyperglycaemia during placebo infusion (with hyperglycaemic clamp) making the overall difference less significant. In the obese IGR group, who also had higher fasting insulin concentrations, the insulin secretion during placebo infusion (with hyperglycaemic clamp) was not as high as compared to obese group. This is an expected lower insulin response to hyperglycaemia in IGR group but interestingly this difference is compensated during GIP infusion achieving comparable insulin concentrations to the obese group.

Younger subjects were shown to have increased insulinotropic activity to GIP in a study\textsuperscript{350}. Subjects in our lean group were younger compared to obese and obese IGR groups but the insulin AUC during GIP infusions were comparable between these groups considering the baseline insulin concentrations are different in the three groups. There was no correlation between age and insulin AUC for all our subjects.
The incretin effect is known to be diminished in T2DM. The insulinotropic activity of both incretin hormones (GLP-1 and GIP) is impaired in diabetes states. Many studies have reported reduced concentrations of GLP-1 after a mixed meal in T2DM subjects. However increasing concentrations of GLP-1 through exogenous infusion normalises hyperglycaemia suggesting insulinotropic activity of GLP-1 is retained in T2DM. In contrast to GLP-1, GIP secretion is not impaired in T2DM. Many studies showed either normal or higher levels of GIP in individuals with T2DM. GIP secretion may vary with age obesity and metabolic status. A meta-analysis showed higher BMI (>30 kg/m²), younger age (<60 years) and low HbA1c level (<53 mmol/mol) to be associated with higher GIP secretion in response to oral glucose tolerance test (OGTT). Despite normal levels in T2DM, GIP does not enhance insulin secretion and even exogenous infusions of GIP have a blunted insulinitropic activity in individuals with T2DM. Similar effects of GIP on insulin secretion were observed even with supra physiological doses of GIP.

Both GLP-1 and GIP share similar insulin signalling pathways inside the pancreatic β cell. Although there is loss of efficacy in both incretin hormones in T2DM, GLP-1 retains insulinitropic action in T2DM indicating that the common signalling pathways remain intact in T2DM. The reasons for the diminished GIP’s action on pancreatic β cell in individuals with T2DM remains unclear. Several underlying mechanisms may contribute to this phenomenon. Decline in β-cell function in T2DM could itself play a major role in diminishing the GIP induced insulin secretion and reduce the overall incretin effect. Activation of GIPR is important for GIP’s physiological action and this is somehow glucose dependent, with GIPR expression shown to be down regulated in response to chronic hyperglycaemia. The blunted incretin effect may in part be due to reduced islet cell expression of GIP receptors (GIPR) secondary to chronic hyperglycaemia although, this has not been clearly demonstrated in humans. In a study involving 93 subjects undergoing GIP infusions under hyperglycaemic clamp conditions, the insulinitropic activity of GIP progressively decreased with near complete loss of efficacy in patients with T2DM and overt hyperglycaemia.
Studies in rats showed GIP receptor down regulation as a potential mechanism for GIP insensitivity/resistance in pancreatic beta cell. In Vancouver diabetic Zucker fatty rats GIP did not stimulate glucose-induced insulin secretion during *in-vivo* experiments or from the perfused rat pancreas and isolated perfused rat islets. The insulinotropic response of GLP-1 remained intact. In these experiments GIP failed to stimulate cAMP production in isolated and incubated rat islet cells. GIP receptor mRNA expression was also shown to be downregulated in the islets of these animals\(^{112}\). Peroxisome proliferator–activated receptor \(\gamma\) (PPAR\(\gamma\)) is thought to regulate GIPR transcription in pancreatic \(\beta\)-cells. Pancreatic PPAR \(\gamma\) knockout (PPAR\(^{-/}\)) mice demonstrated hyperglycaemia with normal \(\beta\)-cell mass but with reduced GIPR expression\(^{356}\).

Another important mechanism for reduced incretin effect in T2DM involves a transcription factor 7-like 2 (TCF7L2) protein that regulates GIPR and GLP-1 receptor (GLP-1R) and reduced levels of TCF7L2 in T2DM correlate with down regulation of GIPR and GLP-1R\(^{357}\). TCF7L2 previously known as TCF4 is a novel gene identified in the last decade and its variants are associated with susceptibility of developing T2DM\(^{358-360}\). TCF7L2 polymorphisms are considered to make a bigger contribution to the development of type 2 diabetes than any other genetic marker\(^{361}\). Common variants of TCF7L2 gene were associated with increased risk of T2DM in studies with large number of subjects of different ethnicity\(^{360,362-364}\). TCFL2 polymorphism associated T2DM may be linked to reduced incretin mediated insulin secretion\(^{365}\). Depleting TCF7L2 in isolated islets cells resulted in increased beta cell apoptosis and reduced beta cell proliferation and overexpression of TCF7L2 protected islets from glucose and cytokine induced apoptosis. These findings suggest that beta cell function and survival are regulated through interaction between TCF7L2 and GLP-1R/GIPR expression and signaling in T2DM\(^{366}\).

In contrast to TCF7L2 regulation that involves both incretin receptors, signalling from GIPR alone but not GLP-1R controls the expression of transcription factor 7 (TCF7), a member of TCF family\(^{367}\). TCF7 in turn encodes a protein known as T-cell specific transcription factor-1 (TCF1) that links GIPR signalling to control secretion of insulin and the survival of beta cells. Experiments in mice with selective ablation on GIPR in pancreatic beta cells had distinctly lower TCF-1 expression and
higher sensitivity for apoptosis. This data indicates GIPR-TCF1 axis may have a significant role in preservation of pancreatic beta cell mass. Furthermore, disruption to GIPR-TCF1 axis may be another potential mechanism for GIP resistance in T2DM which is independent of the common signalling pathways for GIPR and GLP-1R which may still be intact in T2DM. This may explain the differential response with GLP-1 and GIP on insulin secretion and diminished insulin secretion even with supra-physiological doses of GIP.

Genetic defects of GIPR were proposed as another mechanism for GIP resistance in T2DM. Reduction of b-cell mass and progressive deterioration in glycaemic control was seen in transgenic pigs expressing a dominant-negative glucose-dependent insulinotropic polypeptide receptor; such findings did not correlate in humans with T2DM. These concepts are difficult to prove in humans as it is currently not possible to quantify the effects of GIP infusions on GIPR expression in pancreatic β cell in human with in-vivo experiments.

GIP resistance in T2DM appears to be reversible with glucose normalisation. Down regulation of GIP receptor mRNA in T2DM Zucker fatty rats was almost completely reversed after 2 weeks of phlorizin treatment and normalization of hyperglycaemia. Another study showed GIP and GLP-1 receptor expression was reduced in hyperglycaemic rats after a 90% partial pancreatectomy. This was shown to be reversed on treating hyperglycaemia with phlorizin. In human subjects with T2DM and poor glycaemic control, intense insulin treatment and near normalization of glucose levels was shown to reverse the insulinotropic activity of GIP. Similarly, improved glycaemic control with 12 weeks of DPP4 inhibitor as an add on treatment to metformin showed an improved beta cell response during hyperglycaemic clamp experiments. Most subjects with T2DM in our study had stable glycaemic control with a mean HbA1c of 54 ± 8.5 mmol/mol however we still observed significantly impaired insulin secretion with GIP in this group. Nonetheless the studies described above demonstrate that reducing hyperglycaemia improves insulinotropic activity of both GIP and GLP-1.
All experiments in our study were performed under hyperglycaemic clamp conditions to achieve comparable hyperglycaemia and to mimic post-prandial increases in GIP and insulin. The peak GIP concentrations achieved in our study during GIP infusions were comparable to levels achieved in other studies\(^1\). During experiments with GIP infusions, we observed that the plasma GIP concentrations were consistently reduced by the end of experiments (on 240-minute sample) in all subjects. This is probably due to degradation of exogenous GIP by native DPP-IV or due in-homogenous mixing of GIP with normal saline in the infusate leading to reduced GIP concentrations towards the end of experiments.

Studying four distinct groups (with differing BMI and glucose tolerance) facilitates evaluation of the differential effects of GIP in insulin sensitive and resistant individuals. However, we acknowledge limitations including small group sizes and the degree of obesity: there was limited pilot data in humans prior to initiation of this study therefore a power calculation was not done for the study. BMI in our obese T2DM group was high but comparable to obese and obese IGR groups. We acknowledge that results may differ in less obese subjects.

In summary, the diminished insulinotropic effects of GIP in T2DM may be explained by multiple mechanisms involving beta cell dysfunction, down regulation of GIPR as a consequence of chronic hyperglycaemia and disruption to GIPR specific signaling in beta cells. This blunted effect appears to be partially reversed on reducing hyperglycaemia. Although our study was not aimed to identify the underlying mechanisms for GIP resistance in T2DM, we observed that overall insulin secretion as measured by AUC correlated negatively with fasting glucose levels during GIP infusions for all our subjects. This indicates chronic hyperglycaemia may influence GIP induced insulin secretion which may be a gradual effect during the transition from mild hyperglycaemia to development of T2DM with these effects more pronounced with onset of T2DM.
Chapter 4

Effects of GIP on non-esterified fatty acid (NEFA) metabolism and gene expression of lipid regulation enzymes in subcutaneous adipose tissue (SAT) in obesity and type 2 diabetes
4.1 Introduction

GIP in addition to insulinotropic activity has other important extra-pancreatic metabolic functions with receptors expressed in such tissues as bone, brain, stomach and adipose tissue, where it may modulate post-prandial lipid metabolism. In animal models of obesity-induced insulin resistance, genetic and chemical disruption of GIP signaling protects against the deleterious effects of high fat feeding by preventing lipid deposition, adipocyte hypertrophy and expansion of adipose tissue mass, and reducing triacylglycerol deposition in liver and skeletal muscle, maintaining insulin sensitivity. Thus if GIP has a potential pro-adipogenic effect, selective GIP antagonists may be beneficial in treating obesity and type 2 diabetes mellitus (T2DM).

GIP concentrations are increased with high fat diet. Given that dietary fat consumption chronically stimulates the production and secretion of GIP, inducing K cell hyperplasia, higher GIP concentrations may reflect consumption of an energy dense, high-fat diet. Early rodent studies demonstrated that a GIP infusion, during an intraduodenal lipid infusion, decreased plasma triacylglycerol levels while GIP has been shown to enhance insulin-induced fatty acid incorporation in rat adipose tissue. Thus, GIP action mediated through the adipocyte GIP receptor, is anabolic in adipose tissue promoting fat deposition.

It is important to distinguish between direct effects of GIP on fatty acid metabolism and indirect effects based on its insulinotropic action. Acute GIP infusion in lean healthy males (with hyperinsulinaemia and hyperglycaemia mimicking carbohydrate ingestion) increases adipose tissue blood flow, triacylglycerol (TAG) hydrolysis and NEFA re-esterification thus promoting triacylglycerol deposition. In healthy obese men, acute GIP infusion reduced expression and activity of 11β hydroxysteroid dehydrogenase type 1 (11β-HSD1), a fat-specific glucocorticoid metabolism enzyme that may enhance lipolysis in subcutaneous adipose tissue (SAT). Thus, from the available animal model and human data, GIP appears to have a key regulatory role in lipid metabolism and adipose tissue.
To date, very few studies have investigated the effects of GIP on human adipose tissue and none have involved subjects with T2DM although the reported presence of functional GIP receptors on adipocytes strongly suggests GIP modulates human adipose tissue metabolism\(^92,99\). However, a therapeutic benefit of inhibiting GIP action in adipose tissue in obesity-related disorders is clear due to its potential in limiting fat accretion.

### 4.2 Hypothesis and aims

Following on from the previous chapter, we hypothesised that GIP would have an anabolic action in SAT promoting NEFA re-esterification, which we speculated may be mediated either by enhancing lipoprotein lipase (LPL) expression/activity (a lipogenic enzyme)\(^91,171\), or by reducing adipose tissue triacylglycerol lipase (ATGL) and hormone sensitive lipase (HSL) expression/activity, two key lipolytic enzymes. We postulated that this effect may be different according to obesity status or glucose tolerance. Thus, we set out to determine the acute, \textit{in-vivo} effects of intravenous GIP on NEFA concentrations, and i) TAG content in SAT and ii) gene expression of the key lipid regulating genes, lipoprotein lipase (LPL), adipose tissue triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) in SAT, in obese individuals with different categories of glucose regulation (normoglycaemic, IGR and T2DM) \textit{versus} lean, normoglycaemic controls.
4.3 Subjects and methods

General methods are discussed in detail in chapter 2 (Methods). Details on subject groups, participant characteristics, study design and protocol are described in chapter 3, section 3.3. Only male subjects were studied to minimise the influence of sex steroids on lipid metabolism (e.g. considering menstrual cycle, menopause or hormone replacement therapy). Blood samples were taken at baseline (prior to initiation of hyperglycaemic clamp and at further six time points (described in chapter 3) for measurement of plasma NEFA concentrations.

**SAT biopsies** Subcutaneous adipose tissue (SAT) biopsies were obtained at baseline and after 240 min of the GIP/placebo infusion on the contralateral site. Adipose tissue samples (50-150 mg wet weight) were collected and snap frozen in liquid nitrogen and stored at -80°C. Adipose tissue was quantified for SAT-TAG content by measuring free glycerol output following overnight lipase treatment. The gene expression of the key lipid regulating genes LPL, ATGL and HSL was measured by RNA extraction and real time quantitative PCR techniques. The details of subcutaneous adipose tissue biopsy procedure and the techniques of adipose tissue analysis are discussed in detail in chapter 2 (Methods), sections 2.4 and 2.8 respectively.
4.4. Statistical analysis

Area under the curve (AUC\(_{0\text{-}4\text{hr}}\)) for NEFA concentrations over 4-hour period of GIP and placebo infusions were calculated by trapezoidal rule using GraphPad Prism software. Paired t tests were performed on NEFA-AUC\(_{0\text{-}4\text{hr}}\) for individual subjects within each group (lean, obese, obese IGR and obese T2DM) to investigate if NEFA-AUC differed with GIP versus placebo infusions. Tukey’s multiple comparisons test was used to compare the NEFA-AUC across the four groups. Pearson product-moment correlation coefficient was computed to assess the relationship between degree of NEFA reduction and other variables (fasting plasma glucose and Adipo-IR) during GIP and placebo infusions.

A linear mixed-effects model was used to model NEFA concentrations using three time points (baseline, 120 minutes and 240 minutes). Main effects for the four different groups and the treatment effect are included along with a two-way interaction between treatment and group. This allows that the overall effect of GIP infusion in comparison to the placebo infusion can be assessed individually for different groups. Results are expressed in estimated average unit changes in NEFAs during GIP vs. placebo infusion.

Change in SAT triacylglycerol (TAG) and gene expression of adipokines are presented as fold changes from baseline to the measurements from adipose tissue immediately after GIP/Placebo infusions. Paired t-tests were performed on these fold changes to explore whether the change over the two-time points differed between GIP and placebo. Fold changes were log transformed before analysis. P value of < 0.05 (two-tailed) was considered significant.
4.5 Results

4.5.1 Participant details and metabolic parameters
As described in the previous chapter, 23 individuals completed the study protocol in four sub-groups. The details of sub groups, baseline characteristics and metabolic parameters for all subjects are described in chapter 3, section 3.5 and in table 3.1. The blood glucose and GIP concentrations during hyperglycaemic clamp and the changes in insulin concentrations during GIP and placebo infusions are described in section 3.5.2 of the previous chapter. There were no significant alterations in serum triacylglycerol (TAG) concentrations with either GIP or placebo in any of the four groups (data not shown).

4.5.2 Changes in Plasma Non-Esterified Fatty Acids (NEFAs)
NEFA concentrations (mean ± SEM) at baseline (prior to hyperglycemic clamp) and at further six time points (15, 30, 60, 120, 180 and 240 minutes) during GIP and placebo infusions for all the four groups are shown in Figure 4.1 A-D. NEFA concentrations reduced from a higher baseline fasting level in all individuals during both GIP and placebo infusions with hyperglycaemic clamp. Greater reductions in NEFAs were seen during the first one hour. The degree of NEFA reduction however did not differ between GIP and placebo infusions in lean and obese groups whereas this difference was significant in obese T2DM group and a similar trend seen in obese IGR group although not statistically significant.

Effects of GIP and placebo infusions on plasma NEFA concentrations
To assess the overall effect of NEFA reduction during GIP and placebo infusions individually for different groups, statistical analysis was performed using a linear mixed-effects model with NEFA concentrations at three time points (0, 120 and 240 minutes). Results are expressed in estimated average unit changes in NEFA during GIP vs. placebo infusion.

The amount by which NEFA concentrations decreased during GIP infusions differed across the groups, holding all other variables constant, these results were non-significant between the groups. Comparing NEFA reduction during GIP versus placebo infusions within the groups, there was approximately 82.6 µmol/L reduction
in NEFAs in obese T2DM group from baseline to 240 minutes with GIP infusion compared to placebo (95% CI, -139, 26; p = 0.004) In lean, obese and obese IGR groups the decreases in NEFAs with GIP infusions were not statistically different to placebo (Table 4.1).

<table>
<thead>
<tr>
<th>Decrease in NEFA Concentrations (µmol/l)</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GIP vs. Placebo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lean</td>
<td>7.9</td>
<td>-59 to 44</td>
</tr>
<tr>
<td>Obese</td>
<td>31.2</td>
<td>-82 to 20</td>
</tr>
<tr>
<td>Obese IGR</td>
<td>11.4</td>
<td>-63 to 41</td>
</tr>
<tr>
<td>Obese T2DM</td>
<td>82.6</td>
<td>-139 to -26</td>
</tr>
</tbody>
</table>

**Table 4.1:** Unit decreases in NEFA concentrations (linear mixed-effects model) during GIP infusion compared to placebo infusion over 240 minutes in all four groups, 95 % confidence intervals (CI) and p value.
**Figure 4.1:** The plasma concentrations of NEFA (mean ± SEM) during 4 hours of GIP vs. placebo infusions (with hyperglycaemic clamp) on Y-axis are plotted against time on X axis in each group: A lean individuals, B obese individuals, C obese individuals with IGR, D obese individuals with T2DM. Baseline blood sampling was taken at the time point -30* minutes (on X axis).
Integrated NEFA concentrations in plasma (NEFA–AUC)

Area under the curve for NEFA concentrations (NEFA-AUC- _0-4hr_) was calculated from NEFA measurements at 7 time points during GIP and placebo infusions. Comparing AUCs within each group, the mean NEFA-AUC _0-4hr_ in obese T2DM group was significantly lower with GIP infusion compared to placebo (41992 ± 9843 vs 71468 ± 13605 µmol/L/min). The NEFA concentrations in obese IGR group also appeared to be lower with GIP compared to placebo infusion but difference in the mean AUC _0-4hr_ was not statistically significant. Whereas the mean AUC _0-4hr_ for NEFAs in lean and obese groups were not different during GIP and placebo infusions (Figure 4.2).

NEFA -AUCs in subjects within the four groups

The distribution of NEFA-AUC _0-4hr_ for individual subjects within each group during placebo and GIP infusions are shown in box plots (Figure 4.3) The NEFA-AUCs were very similar in all the lean subjects with both GIP and placebo infusions. Whereas in the other three groups majority of individuals had lower NEFA-AUC with GIP infusion compared to placebo but a statistically significant difference was only seen in the obese T2DM group.

4.5.3 Relation between NEFA reduction (ΔNEFA) and metabolic parameters

ΔNEFA and fasting plasma glucose

The degree of reduction in NEFA (ΔNEFA) with GIP infusion in all subjects (n=23) correlated positively with fasting plasma glucose suggesting that subjects with higher fasting plasma glucose had greater reductions in NEFA during GIP infusion. Such correlation was not seen with placebo infusion (Figure 4.4 A-B).

ΔNEFA and Adipo IR

The degree of reduction in NEFA (ΔNEFA) with GIP infusion in all subjects (n=23) correlated positively with Adipo-IR suggesting that subjects with higher Adipo-IR had greater reductions in NEFA during GIP infusion. Such correlation was not seen with placebo infusion (Figure 4.4 C-D).
Figure 4.2: A Mean NEFA AUC_{0-4hr} \pm SEM for GIP vs placebo infusions in four groups presented as bar charts (white bar - placebo; black bar - GIP). B AUC of NEFA concentrations with placebo compared to GIP infusion over 240 minutes in all four groups, confidence intervals (CI) and p values, *represents p <0.05.
Figure 4.3 Box plots showing the distribution of NEFA-AUC$_{0-4hr}$ (µmol/L/min) values for individual subjects within each group during 4-hour infusions of GIP versus placebo. The line within the box plots represents the median value and whiskers represent the minimum and maximum values of NEFA-AUC for individuals within each group: A Lean, B Obese, C Obese IGR and D Obese T2DM.
Figure 4.4: A, B Correlation between NEFA reductions (ΔNEFA) [umol/l] and fasting plasma glucose (mmol/l) on all subjects in study (n=23) during GIP vs. placebo infusion. C, D Correlation between ΔNEFA and adipo-IR(mmol/L/pmol/L) during GIP vs. placebo infusion for all subjects. Pearson correlation coefficients (r²) and two tailed p values (p) are shown in each graph.
4.5.4 Change in Subcutaneous Adipose Tissue (SAT) lipid content

The change in SAT triacylglycerol (TAG) content after 240 minutes of GIP vs. placebo infusion relative to respective baselines on each visit for the four groups are shown in Figure 4.5. In obese T2DM group, the SAT-TAG content increased by nearly 1.8-fold from baseline with GIP infusion compared to 0.86-fold with placebo (p=0.043). There was no significant difference in TAG content in lean, obese and obese IGR groups (Figure 4.5). The lipid content was measured as glycerol/protein (ug/mg). The concentrations of glycerol (normalised to protein) in the four groups before and after the infusion of placebo and GIP are shown in table 4.2.

4.5.5 Gene expression of key lipid metabolism enzymes in SAT

Lipoprotein lipase (LPL)

The changes in gene expression for the key lipid metabolism enzymes are presented as fold changes relative to baseline on the same day after GIP vs placebo infusions. In T2DM group, there was an increased trend in LPL gene expression with GIP infusion although not statistically significant (Figure 4.6A and table 4.3). In the other three groups the changes in LPL mRNA expression with GIP and placebo were comparable. In lean group, the mean LPL expression with placebo was very high as data was skewed due to an exaggerated response observed in one individual.

Adipose tissue triglyceride lipase (ATGL)

In T2DM group, ATGL expression was 1.5-fold higher with GIP infusion compared to 1.1-fold with placebo although not statistically significant (p=0.12). In the other three groups the changes in ATGL gene expression with GIP versus placebo were comparable (Figure 4.6B and table 4.3). There was an exaggerated ATGL gene expression after placebo infusion for one subject each in lean and obese groups.

Hormone sensitive lipase (HSL)

The changes in HSL gene expression were very variable across the groups with the data showing no meaningful trends. HSL expression with GIP did not differ significantly compared to placebo in all four groups (Figures 4.6C and table 4.3). There significant variations in HSL expression within each group without any meaningful trend in any of the groups.
Figure 4.5: A Fold changes (mean ± SEM) in subcutaneous adipose tissue (SAT) triacylglycerol (TAG) content after 240 min of GIP vs. placebo infusion relative to the baseline on the same day in four groups. Bars at the level of dotted line drawn from 1.0 on Y axis indicate there is no change in lipid content compared to baseline. *represents p value <0.05.
**Table 4.2:** Lipid content in SAT, presented as glycerol/protein (ug/mg), pre and post infusion of placebo and GIP. The fold change in lipid content during placebo vs GIP infusions, confidence intervals (CI) and p values are shown in the right side of the table. *represents p <0.05.
Figure 4.6: Fold changes (mean ± SEM) in SAT gene expression after 240 min of GIP vs. placebo infusion relative to baseline on the same day presented as bar charts for A LPL B ATGL and C HSL in four groups (lean, obese, obese individuals with IGR and obese individuals with T2DM.)
<table>
<thead>
<tr>
<th>Groups</th>
<th>Placebo</th>
<th>GIP</th>
<th>P value</th>
<th>Placebo</th>
<th>GIP</th>
<th>P value</th>
<th>Placebo</th>
<th>GIP</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lean</td>
<td>1.8 ± 0.4</td>
<td>1.2 ± 0.2</td>
<td>0.38</td>
<td>1.6 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>0.71</td>
<td>1.7 ± 0.6</td>
<td>1.2 ± 0.2</td>
<td>0.42</td>
</tr>
<tr>
<td>Obese</td>
<td>1.2 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>0.49</td>
<td>1.3 ± 0.2</td>
<td>1.3 ± 0.2</td>
<td>0.96</td>
<td>1.9 ± 0.6</td>
<td>1.8 ± 0.3</td>
<td>0.93</td>
</tr>
<tr>
<td>Obese IGR</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.64</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.2</td>
<td>0.90</td>
<td>0.9 ± 0.1</td>
<td>1.5 ± 0.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Obese T2DM</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>0.27</td>
<td>1.1 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>0.12</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>0.62</td>
</tr>
</tbody>
</table>

**Table 4.3** Change in SAT gene expression presented as mean fold change ± SEM for LPL, ATGL and HSL after 240 min of GIP vs. placebo infusion relative to baseline on the same day in lean, obese, obese individuals with IGR and obese individuals with T2DM.
4.6 Discussion

In this study, we demonstrate that acute GIP infusion, during fasting, under hyperglycaemic conditions, reduced serum/plasma NEFAs, concomitantly increasing SAT triacylglycerol (TAG) content in obese patients with T2DM. This anabolic effect was not observed in the lean, obese or obese patients with IGR. In contrast, while GIP stimulated insulin secretion in the lean, obese or obese patients with IGR, its insulinotropic action was not observed in obese patients with T2DM. Thus, in obese patients with T2DM, there is a dissociation of the effects on GIP on beta cells and adipocytes, with blunted insulinotropic but preserved lipogenic actions respectively.

Activation of the GIP receptor (GIPR) is somehow glucose dependent, with GIPR expression down regulated in response to hyperglycaemia\textsuperscript{111}. In patients with T2DM the blunted incretin effect (involving both incretin hormones, GLP-1 and GIP) may in part be due to reduced islet cell expression of GIP receptors (GIPR) secondary to chronic hyperglycemia\textsuperscript{25,38,343,353} although this has not been demonstrated in humans. The physiological role of GIP in adipose tissue in T2DM remains unclear although adipose GIPR expression may be similarly down regulated in insulin resistant human subjects and may represent a compensatory mechanism to reduce fat storage in insulin resistance, considering the interference of NEFAs on insulin signal transduction\textsuperscript{60,61}. However, energy dense, high fat diets in obese individuals with T2DM could result in exaggerated fat storage (through exaggerated GIP release) even in the absence of adequate insulin secretion. Although we did not measure GIPR, the lipogenic action of GIP at the adipocyte appears to be more pronounced in T2DM. Studies in patients with NAFLD suggests elevated GIP secretion is also associated with intra-hepatocellular lipid deposition\textsuperscript{372}.

Several factors may explain the differential ability of GIP to lower plasma NEFA concentrations and increase SAT lipid content in obese T2DM subjects vs. other patient groups. In lean, obese and obese individuals with IGR, where insulin secretion is potently stimulated and adipose tissue insulin sensitivity is preserved (lower Adipo-IR), insulin independently suppressed lipolysis, lowering NEFAs
perhaps leaving GIP’s effects trivial. However, in T2DM when insulin secretion is impaired and adipose tissue is insulin resistant (high Adipo-IR), the effect of GIP assumes greater importance, promoting lipid accumulation in adipocytes. This is consistent with animal data. GIP does not promote fat accumulation in adipocytes with normal insulin sensitivity, with GIPR−/− mice showing similar adiposity to wild-type on control diet135. However, under conditions of diminished insulin action, using IRS1 deficient mice, when the effects of GIP are examined (by disrupting GIP signaling, GIP−/− vs. GIPR+/+) GIP was shown to promote SAT and VAT expansion and decrease fat oxidation with greater SAT and VAT mass and lower fat oxidation in IRS-1−/−GIP−/− vs. IRS-1−/−GIPR+/+ mice 255.

A few human studies examined the metabolic effect of an acute GIP infusion in lean and obese individuals but none reported in people with T2DM. In studies to date, the effects of GIP have been examined under different experimental conditions to those here, for example during concomitant intra-lipid infusion and/or with hyperinsulinaemic-hyperglycaemic clamp conditions. These data demonstrated that in lean people, GIP in combination with hyperinsulinaemia and hyperglycemia, increased adipose tissue blood flow, glucose uptake, and NEFA re-esterification, thus resulting in increased abdominal SAT TAG deposition189,196. The same group showed that in obese and IGR subjects GIP infusion did not have the same effect on adipose tissue blood flow or TAG deposition in adipose tissue191. However, the independent contributions of insulin vs. GIP to these metabolic effects are difficult to dissect and GIP per se appeared to have little effect on human subcutaneous adipose tissue in lean insulin sensitive subjects, with an effect only apparent when GIP was co-administered with insulin during hyperglycemia. Thus, it there would appear that there are direct and indirect effects of GIP.

During nutrient excess lipogenesis is stimulated via lipoprotein lipase (LPL), hydrolysing circulating lipoprotein-derived triacylglycerols and promoting NEFA esterification into TAG and storage within lipid droplets of adipose tissue. During periods of fasting, mobilisation of NEFAs from fat depots relies on the activity of key hydrolases, including hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL). In SAT, insulin stimulates NEFA esterification and inhibits lipolytic process 137. Most of the animal studies have shown that GIP potentiates the
role of insulin in regulation of LPL, and NEFA incorporation into the adipose tissue. In cultured subcutaneous human adipocytes, LPL gene expression and activity were enhanced by GIP through activation of protein kinase B (PKB) followed by reduction in phosphorylation of Liver kinase B1 (LKB1) and AMP-activated protein kinase (AMPK). Trying to determine the molecular mechanism by which SAT-TAG content changed, we measured SAT mRNA expression of LPL, ATGL and HSL; surprisingly, we observed no significant changes in expression to account for altered serum NEFAs or SAT TAG content. This may represent a time-course phenomenon (changes in gene expression with GIP in human adipose tissue may occur over a longer interval). This speculation is consistent with the slow temporal onset of the molecular responses in adipose tissue in animal studies. GIP infusion may affect enzyme activity rather than gene expression and therefore results of ATGL and HSL may vary if phosphorylation was measured. To better appreciate the physiological effects of GIP administration on human SAT, stable isotope studies to determine dynamic changes in fat metabolism with serial tissue biopsies are required.

All studies were performed under hyperglycaemic clamp conditions to achieve comparable hyperglycaemia and to mimic post-prandial increases in GIP and insulin. The peak GIP concentrations achieved in our study during GIP infusions were comparable to levels achieved elsewhere. We believe the changes in NEFAs and SAT lipid content in our obese T2DM are more likely due to the effects of GIP, particularly in the absence of excess insulin secretion. However, the increase in lipid content (1.8-fold mean change) with GIP infusion in T2DM group appears to be excessive. It is likely that the adipose tissue sample we analysed in this group is probably not representative of the whole SAT. Individual variation in results within this group have also contributed to a higher mean change in the lipid content in this group (Table 4.2). Reductions in NEFA correlated positively with fasting glucose and Adipo-IR in all the subjects across the four groups suggesting the effects of GIP are more pronounced in hyperglycaemic and insulin resistant states. We recognise that higher ΔNEFA would be expected in subjects with higher fasting NEFA levels however correlation with Adipo-IR was only seen with GIP but not with placebo infusion (Figure 4.4). Although higher GIP peaks were noted in our T2DM group, levels were not statistically different from obese and IGR groups.
Studying four distinct groups (with differing BMI and glucose tolerance) facilitates evaluation of the differential effects of GIP in insulin sensitive and resistant individuals. However, we acknowledge limitations including small group sizes and the degree of obesity: there was limited pilot data in humans prior to initiation of this study and subsequently published human studies on GIP infusion had small number of subjects\textsuperscript{189-191}. Findings from our study may differ in less severely obese individuals. Subjects in our obese and obese T2DM groups had very high BMI but it was not significantly different between these two groups. Therefore, the effects of GIP on NEFA reductions and SAT-TAG content we observed in obese T2DM group cannot be attributed to severe obesity alone.

We invited only male subjects to participate in this study to avoid the confounding effects of sex steroid fluctuations due to menstrual cycle, menopause and hormone replacement therapy in female subjects. The relationship between sex steroids and adipocyte biology is well characterized. It was shown previously that an inverse correlation exists between serum oestradiol and LPL activity\textsuperscript{373}. LPL activity is lower under dermal patches of 17-b-oestradiol\textsuperscript{374} but increased following topical administration of progesterone patches\textsuperscript{375}. Some of our subjects were treated for hypertension and hyperlipidaemia. Unrecognised interactions between anti-hypertensive or lipid modifying medication and effects of GIP cannot be excluded.

In conclusion, we demonstrate that in obese patients with T2DM, acute GIP infusion in a fasting state, during hyperglycaemia, lowers serum NEFA and increases the SAT lipid content despite reduced insulinotropic activity. In lean, obese and obese with IGR, despite the intact insulinotropic response to GIP no lipogenic effect was observed. This anabolic effect of GIP further exacerbates obesity and insulin resistance.
Chapter 5

The influence of GIP on adipokine gene expression and secretion: effects in obesity and type 2 diabetes
5.1 Introduction

GIP has an important role in adipose tissue metabolism. In the previous chapter, we demonstrated that GIP enhanced systemic NEFA clearance, increasing its incorporation into TAGs in SAT in obese subjects with T2DM. This occurred despite diminished insulin secretion in this group. GIP may influence SAT metabolism through multiple mechanisms. In recent years, animal studies suggest that GIP may enhance the secretion of pro-inflammatory adipokines and perhaps suppress secretion of anti-inflammatory adipokines, promoting adipose tissue inflammation.

Obesity is characterised by low-grade inflammation, with the balance between secretion of pro and anti-inflammatory adipokines tipped in favour of the development of insulin resistance and type 2 diabetes\textsuperscript{199}. Amongst various pro and anti-inflammatory adipokines involved in adipose tissue inflammation, there is now evidence to suggest that the expression of some of the adipokines in adipose tissue may be influenced by GIP. Earlier studies in GIPR knock out (Gipr\textsuperscript{−/−}) mice showed increased adiponectin expression in SAT and systemic secretion after 3 weeks of high fat diet when compared to wild type mice. These findings may suggest that high fat feeds leading to increased GIP levels may suppress the expression and secretion of the anti-inflammatory adipokine, adiponectin in normal physiological circumstances\textsuperscript{208}.

GIP induced mRNA expression of pro-inflammatory adipokines IL-6, and TNF-\(\alpha\) in human subcutaneous pre-adipocytes\textsuperscript{175,253}. GIP may also influence secretion of other inflammatory adipokines such as MCP-1 and osteopontin. Intraperitoneal GIP injections in mice increased MCP-1 expression and macrophage infiltration in adipose tissue\textsuperscript{252}. GIP was shown to enhance osteopontin expression in primary rodent adipocytes\textsuperscript{236}. Human adipocytes with a genetic variant of GIPR (with diminished function) had reduced osteopontin levels and better insulin sensitivity suggesting a role for GIP in regulation of osteopontin production in adipose tissue\textsuperscript{237}. Resistin, another pro-inflammatory adipokine, is thought to play a key role in the GIP induced enhancement of lipoprotein lipase (LPL) activity. Adipocytes of resistin knockout mice showed reduced GIPR expression and compromised GIP
signalling pathways (PKB/LKB1/AMPK) that enhanced LPL activity. Currently there is no evidence to suggest changes to resistin gene expression or secretion with GIP.

There is insufficient evidence in humans to suggest that GIP has any effect on adipokine gene expression or secretion apart from one study in humans that showed an increase in MCP-1 gene expression and secretion with acute GIP infusions. In a previous study from our unit, acute GIP infusions in obese individuals with type 2 diabetes did not alter plasma IL-6, leptin, adiponectin, ghrelin and obestatin levels but IL-6 was suppressed significantly with acute GLP-1 infusions.

5.2 Hypothesis and aims

We hypothesised that GIP may increase the expression and secretion of pro-inflammatory adipokines and reduce the expression and secretion of the anti-inflammatory cytokine, adiponectin in SAT. To investigate the putative pro-inflammatory properties of GIP in human SAT, we studied the effects of acute GIP infusions versus placebo on the gene expression and plasma concentrations of adipokines TNF-α, MCP-1, osteopontin and adiponectin that were shown to be influenced with GIP treatment in animal models and in-vitro studies. We also explored whether the effects of GIP differed with obesity and with impaired glucose regulation.
5.3 Subjects and methods

General methods are discussed in detail in chapter 2 (Methods). Details on subject groups, participant characteristics, study design and protocol are described in chapter 3, section 3.3. Blood samples at baseline (taken prior to hyperglycaemic clamp), 120 minutes and 240 minutes were measured for key adipokines TNF receptor 2 (TNF R2), MCP-1, adiponectin, and osteopontin.

**SAT biopsies** Subcutaneous adipose tissue (SAT) biopsies were obtained at baseline and after 240 min of the GIP/placebo infusion on the contralateral site. Adipose tissue samples (50-150 mg wet weight) were collected and snap frozen in liquid nitrogen and stored at -80° C and later the gene expression of key adipokines TNF-α, MCP-1, osteopontin and adiponectin were determined pre and post GIP/placebo infusions by real time qPCR techniques. The details of subcutaneous adipose tissue biopsy procedure and the techniques of adipose tissue analysis are discussed in detail in Chapter 2 (Methods), sections 2.4 and 2.8 respectively.

5.4 Statistical Analysis

**Plasma Adipokine concentrations**

One-way analysis of variance (ANOVA) with Dunnett’s multiple comparison tests were performed to compare the changes in plasma adipokines at 120 minutes and 240 minutes from their baseline during GIP and placebo infusions in the four groups. Mean difference in these changes and p values were determined. P value (two tailed) <0.05 was considered to be statistically significant.

**Adipokine gene expression**

Fold change in gene expression after 240 minutes of GIP and placebo infusions compared to their respective baseline measurements on the same day were calculated (post infusion gene expression ÷ pre-expression values). Fold changes in gene expression were log transformed before analysis and paired t-tests were performed to explore whether the change over the two-time points differed between GIP and placebo. P value of < 0.05 (two-tailed) was considered significant.
5.5 Results

5.5.1 Participant details and metabolic parameters
The details of participants, sub groups, baseline characteristics and metabolic parameters for all subjects are described in chapter 3, section 3.5 and in table 3.1. The blood glucose and GIP concentrations during hyperglycaemic clamp during GIP and placebo infusions are described in chapter 3, section 3.5.2.

5.5.2 Plasma adipokine concentrations
TNF R2, MCP-1, osteopontin and adiponectin were measured at baseline and at further two time points (120 min and 240 minutes) during GIP and placebo infusion. Baseline adipokines (fasting concentrations) were compared between the four groups (lean, obese, obese IGR and obese T2DM). Changes in adipokine concentrations during GIP vs placebo infusions were compared for all subjects together (n=23) to see if there was an overall difference between GIP and placebo infusion. Adipokine concentrations at three time points during GIP versus placebo infusions in each of the four groups were also compared separately.

Fasting plasma adipokine concentrations in four groups
Adipokine concentrations measured at baseline (fasting) prior to initiation of hyperglycaemic clamp for subjects in four groups are shown in Figure 5.1. Mean fasting plasma concentrations of TNF-R2 and MCP-1 were significantly different in all the four groups based on multiple comparison tests. In comparison with the lean group (control) the fasting concentrations of TNF-R2, MCP-1 and osteopontin were significantly higher in obese T2DM. There was no significant difference seen in fasting adiponectin levels between the four groups.
Plasma adipokine concentrations during GIP vs placebo infusion

**TNF-R2**
In the lean group, there was a reduction in TNF R2 concentrations from baseline to 120-minute time point during placebo and GIP infusions (adjusted p value =0.02) but this difference was not significant at the end of infusion at 240 minutes. In other three groups reduction in TNF- R2 from baseline was observed only during placebo infusion at 120 minutes but not during GIP infusion (p= 0.01), (Figure 5.2 A-D).

**MCP-1**
There was no significant change in MCP-1 concentrations from baseline compared to both time points (120 and 240 minutes) during GIP or placebo infusion in any of the groups (Figure 5.2 E-H).

**Osteopontin**
There was no significant change in osteopontin concentrations from baseline to both time points (120 and 240 minutes) during GIP or placebo infusion in any of the groups (Figure 5.3 A-D).

**Adiponectin**
In all the groups, plasma adiponectin concentrations show a reduced trend at 120 minutes during both placebo and GIP infusions but no change at 240 minutes compared to the baseline level post infusion. Results were not statistically significant due to wide variation in individual responses. (Figure 5.3 E-H).
Figure 5.1 Baseline (fasting) plasma adipokine concentrations (Mean ± SEM) in four groups. Statistically significant difference in baseline concentrations between obese T2DM group and lean group represented are by * (p <0.05) and ** (p <0.01).
**Figure 52**: Plasma TNF-R2 and MCP-1 concentrations (mean ± SEM) during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp). **A-D** TNF-R2 concentrations in all four groups and **E-H**: MCP-1 concentration in all four groups.
Plasma osteopontin and adiponectin concentrations during GIP vs placebo infusions

Figure 5.3: Plasma osteopontin and adiponectin concentrations (mean ± SEM) during 4 hour infusions of GIP vs. placebo (with hyperglycaemic clamp) A-D osteopontin concentrations in all four groups and E-H: adiponectin concentration in all four groups.
5.5.3 Adipokine gene expression in SAT

Gene expression of key adipokines TNF-α, MCP-1, adiponectin and osteopontin are expressed as relative expression values obtained by normalisation to three housekeeping genes (ribosomal 18s, Beta 2 micro-globulin and glyceraldehyde 3-phosphate dehydrogenase). There was no significant difference in baseline (fasting) adipokine expression for any of the adipokines in all four groups (Table 5.1). For each adipokine, the gene expression results for individuals within each group (lean, obese, IGR and T2DM) are presented in a box plot distribution for both placebo and GIP infusions days. Change in gene expression from baseline to the post infusion value is presented as fold change and these values were used to compare the effects of GIP versus placebo on gene expression.

**Adipokine gene expression at baseline (fasting state)**

<table>
<thead>
<tr>
<th>Adipokine</th>
<th>Lean (N=6)</th>
<th>Obese (N=6)</th>
<th>Obese IGR (N=6)</th>
<th>Obese T2DM (N=5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>0.57 ± 0.1</td>
<td>0.82 ± 0.1</td>
<td>0.78 ± 0.2</td>
<td>0.92 ± 0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.69 ± 0.1</td>
<td>0.46 ± 0.04</td>
<td>0.59 ± 0.1</td>
<td>0.54 ± 0.1</td>
<td>0.5</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>0.87 ± 0.2</td>
<td>0.39 ± 0.1</td>
<td>0.64 ± 0.3</td>
<td>0.72 ± 0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>0.61 ± 0.2</td>
<td>1.04 ± 0.3</td>
<td>1.01 ± 0.1</td>
<td>1.01 ± 0.1</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Table 5.1** Baseline adipokine gene expression (mean ± SEM) in SAT in the four groups. P value is derived from one-way ANOVA comparing four groups indicating the overall difference in baseline concentrations between the four groups.
TNF-α gene expression in SAT
Baseline TNF-α gene expression (mean ± SEM) was lower in lean subjects but it was not statistically different from the other three groups (Table 5.1) Gene expressions pre and post infusion of placebo and GIP (2 separate days) for individuals in each group are shown in Figure 5.4 A-D. Comparing within each group, TNF-α expression in lean and obese individuals were similar with GIP or placebo infusions. Whereas in obese IGR and obese T2DM groups, the TNF-α expressions post GIP infusion are higher in most subjects compared to their respective baseline (pre) values and higher compared to the post placebo infusion values (Figure 5.4 C, D).

The changes in TNF-α gene expression (mean ± SEM fold change) from baseline to the end of infusion for placebo vs GIP visits are shown for the four groups in Figure 5.4 E. The gene expression did not change with GIP or placebo infusions in lean and obese groups. Whereas in the obese T2DM group, the change in gene expression was significantly higher with GIP infusion compared to placebo (3.5 ± 1.7-fold vs 0.8 ± 0.1-fold respectively). Similar trend was seen in obese IGR group although the results were not statistically significant.

MCP-1 gene expression in SAT
Baseline MCP-1 gene expression (mean ± SEM) was similar in all the four groups (Table 5.1). Gene expressions pre and post infusion of placebo and GIP for individuals in each group are shown in Figure 5.5. Comparing within each group, MCP-1 expression was higher in most lean and obese individuals with GIP infusion compared to their respective baseline (pre) values and higher compared to the post placebo infusion expression (Figure 5.5 A, B).

The changes in MCP-1 gene expression (mean ± SEM fold change) from baseline to the end of infusion for placebo vs GIP visits in all the four groups are shown in Figure 5.5E. The change in gene expression was significantly higher with GIP infusion compared to placebo in lean and obese groups (lean: 2.6 ± 0.5 vs. 0.9 ± 0.3-fold p=0.02; obese: 2.4 ± 0.6 vs.1.4 ± 0.4) respectively. Interestingly such difference with GIP infusion was not observed in the obese IGR and obese T2DM groups.
**TNF-α gene expression in SAT**

**Figure 5.4:** TNF-α gene expression for individual subjects pre and post infusion of placebo and GIP (2 separate days) is presented in box plots in **A** lean subjects, **B** obese subjects, **C** obese individuals with IGR and **D** obese individuals with T2DM. Horizontal line in the boxplot represents the median value and whiskers represent the minimum and maximum values of gene expression. **E** Fold changes (mean ± SEM) in TNF-α gene expression in subcutaneous adipose tissue (SAT) after 240 min of GIP vs. placebo infusion relative to the baseline on respective days are presented as bar charts in the four groups.
MCP-1 gene expression in SAT

Figure 5.5: MCP-1 gene expression for individual subjects pre and post infusion of placebo and GIP (2 separate days) is presented in boxplots in A lean subjects, B obese subjects, C obese individuals with IGR and D obese individuals with T2DM. Horizontal line within each boxplot represents the median value and whiskers represent the minimum and maximum values of gene expression, Φ represents a data point outside axis limit. E Fold changes (mean ± SEM) in MCP-1 gene expression in subcutaneous adipose tissue (SAT) after 240 min of GIP vs. placebo infusion relative to the baseline on respective days are presented as bar charts in the four groups.
Osteopontin gene expression in SAT

Baseline osteopontin gene expression (mean ± SEM) was not statistically different in the four groups (Table 5.1) Gene expressions pre and post infusion of placebo and GIP for individuals in each group are shown in Figure 5.6 A-D. Comparing within each group, there was no significant difference in gene expression from baseline (pre) to post infusion with GIP or placebo in most subjects. One subject each in obese IGR and obese T2DM groups had exaggerated gene expression response after placebo infusion that skews the data.

The change in osteopontin gene expression (mean ± SEM of fold change) from baseline to the end infusion for placebo vs GIP visits in all the four groups are shown in figure 5.6 E. There was no significant change in gene expression in all the groups. Although the fold change with placebo in obese IGR and obese T2DM groups appears to be higher, the data is skewed due to exaggerated response in individual subjects in each group and therefore results are not significant.

Adiponectin gene expression in SAT

Baseline adiponectin gene expression (mean ± SEM) was slightly lower in lean group but not significantly different from the other three groups (Table 5.1). Gene expressions pre and post infusion of placebo and GIP for individuals in each group are shown in Figure 5.7 A-D. Comparing within each group, adiponectin expression showed wide variation in lean and obese individuals with no overall change in expression from baseline (pre) to post infusion with GIP or placebo). In obese IGR and obese T2DM subjects, the adiponectin expressions were similar in both these groups and there was no change after GIP or placebo infusions.

The change in adiponectin gene expression (mean ± SEM of fold change) from baseline to the end infusion for placebo vs GIP visits in all the four groups are shown in figure 5.7 E. There was no significant change in gene expression in obese, obese IGR and obese T2DM groups. Although there appears to be a difference in the lean group between GIP and placebo, the variations in individual responses to GIP in this group skew the data and therefore this difference was not statistically significant.
Osteopontin gene expression in SAT

**Figure 5.6:** Osteopontin gene expression for individual subjects pre-and post infusion of placebo and GIP (2 separate days) is presented in boxplots in A lean subjects, B obese subjects, C obese individuals with IGR and D obese individuals with T2DM. Horizontal line within each boxplot represents the median value and whiskers represent the minimum and maximum values of gene expression for each group, Φ represents a data point outside axis limit. E Fold changes (mean ± SEM) in osteopontin gene expression in subcutaneous adipose tissue (SAT) after 240 min of GIP vs. placebo infusion relative to the baseline on respective days are presented as bar charts in the four groups.
Adiponectin gene expression in SAT

Figure 5.7: Adiponectin gene expression for individual subjects pre and post infusion of placebo and GIP (2 separate days) is presented in A lean subjects, B obese subjects, C obese individuals with IGR and D obese individuals with T2DM. Horizontal line within each boxplot represents the median value and whiskers represent the minimum and maximum values of gene expression for each group, E Fold changes (mean ± SEM) in adiponectin gene expression in subcutaneous adipose tissue (SAT) after 240 min of GIP vs. placebo infusion relative to the baseline on respective days are presented as bar charts in the four groups.
5.6 Discussion

The effects of GIP on plasma and SAT adipokine expression has not been studied before in human subjects with T2DM. In this study, as expected, the baseline plasma concentrations of TNF-α, MCP-1 and osteopontin were significantly higher in the obese T2DM group compared to the lean control group\(^{222,376-381}\) but the anticipated lower plasma adiponectin concentration in the IGR or T2DM participants was not observed with no significant differences between the four groups\(^ {382,383}\). However, in response to GIP, there were no significant changes in the concentration of any of the plasma adipokines in any of the groups. In SAT, we showed that gene expression of TNF-α in SAT of obese T2DM subjects increased significantly after GIP infusion compared to placebo and similar trends were seen in obese subjects with IGR with no change in lean and obese normoglycaemic subjects. In contrast, MCP-1 gene expression was higher with GIP infusion in lean and obese but was not different in IGR or T2DM. There was no change in osteopontin and adiponectin gene expression with GIP or placebo infusions in any of the groups.

At present, there is little literature regarding the in vivo effects of GIP on adipokines in human SAT with no evidence to support whether these effects of GIP differ according to insulin sensitivity. From earlier results, it is known that SAT retains sensitivity to GIP’s actions in T2DM subjects despite the loss of insulinotropic activity. The results we observed in gene expression of TNF-α and MCP-1 with GIP infusions are interesting as the groups which had higher gene expression of these two inflammatory adipokines were not consistent and there seems to be a difference in these gene expressions with GIP in normoglycaemic and hyperglycaemic states.

IL-6 and TNF-α are well studied adipokines in adipose tissue inflammation and their concentrations are increased in obesity and insulin resistant states. Studies in animal models and cell cultures showed consistent increases in IL-6 with GIP\(^ {175,253,384}\). TNF-α induces insulin resistance through multiple mechanisms that involve altered regulation of NEFA, Glut4 proteins and defects in insulin receptor signalling\(^ {212,213}\). Previous in-vitro studies have shown that changes in TNF-α expression with GIP treatment were inconsistent. Whilst some studies showed GIP
to increase in TNF-α expression along with IL6 and MCP-1\textsuperscript{248,253}, others showed either no change\textsuperscript{175,385} or reduced expression and inflammation with GIP treatment\textsuperscript{385,386}.

Studies exploring the molecular mechanisms suggest these actions of GIP are mediated through pathways involving Protein Kinase-A (PKA) nuclear factor κB (NF-κB) and hypoxia inducible factor-1α (HIF-1α)\textsuperscript{175,252}. GIP was also shown to induce lipolysis which increases inflammatory cytokines, a process inhibited by insulin\textsuperscript{177}. In insulin deficient states, lipolysis releases NEFAs that are re-esterified in the presence of GIP and this vicious cycle of lipolysis and pro-inflammatory adipokine expression may contribute to insulin resistance in adipose tissue. GIP was shown to increase pro-inflammatory adipokine IL-6 secretion in the presence of other cytokines such as IL-1β, TNFα and lipopolysaccharides which release inflammatory cytokines\textsuperscript{175,248} indicating GIP action may be more prominent in low grade inflammatory states.

We speculate that GIP induced TNF-α expression in obese T2DM subjects observed in our study could potentially be due to the reasons that pro-inflammatory effects of GIP may be prominent in insulin deficient subjects compared to normoglycaemic healthy individuals where anti-inflammatory effects of Insulin may override the pro-inflammatory effects of GIP\textsuperscript{387,388}. In mice models insulin was shown to inhibit TNF-α and improve endothelial function\textsuperscript{389}. Furthermore, T2DM is a low grade chronic inflammation state and GIP may have an enhanced action in the presence of other inflammatory adipokines. We are unable to explain the lack of such changes in other inflammatory adipokines to fully support this concept.

MCP-1 is a key regulator chemokine involved in migration and infiltration of monocytes and macrophages\textsuperscript{221}. A single human study in normoglycaemic obese individuals showed acute GIP infusions had increased gene expression and plasma concentrations of MCP-1 and MCP-2\textsuperscript{230}. These results are similar to the findings we observed in obese and lean individuals in our experiments. The above study used a different methodology (euglycaemic clamps and hyper-insulinemic hyperglycaemic clamps) but used similar concentrations of GIP infusions (2pmol.kg\textsuperscript{-1}.min\textsuperscript{-1}) as in our study. In the same study GIP also increased MCP-1 expression in co-cultures of
3T3L1 adipocytes. There are no studies so far evaluating these effects of GIP in individuals with T2DM.

TNF R1 and TNF R2 are the two distinct receptors that mediates the biological effects of TNF-α on adipose tissue. We measured TNF R2 in plasma which is a widely expressed receptor for TNF-α and is homologous to the archetypal TNF-α. TNF R2 expression is higher in obesity and insulin resistant states and is reliably measured in plasma. We did not observe any significant changes in adipokines secretion in plasma. It is possible that the 4-hour time frame may have not been sufficiently long for the inflammatory changes in adipose tissue to be translated into changes in the circulation.

Most studies that evaluated GIP effects on adipose tissue inflammation were done in normoglycaemic animal models and on pre-cultured adipocytes. Studying human subjects with T2DM enabled us to evaluate the differential effects of GIP in normoglycaemic and hyperglycaemic individuals with altered β cell function. All experiments were done under hyperglycaemic clamp conditions that induces hyperinsulinaemia. The effects of insulin on adipokine expression is a major factor when assessing the independent effects of GIP. We also acknowledge the limitations in accurately assessing the effects of GIP due to small group sizes in our study. We are unable to comment on the underlying mechanisms for change in TNF-α as our methodology was not aimed to explore the molecular mechanisms of GIP’s pro-inflammatory action. Unfortunately, the effects of GIP on IL-6 gene expression could not be assessed in our experiments at the time of this study due to technical difficulties with primers we used that could not be optimised to appropriate conditions to carry out qPCR.

In summary GIP appears to have pro-inflammatory effects in SAT with most of the evidence derived from animal models and in vitro studies. These effects of GIP may not be conspicuous in normoglycaemic state with normal β cell function but may be pronounced in insulin deficient states. It remains unclear if modulation of pro-inflammatory effects of GIP leading to modulation of adipokine secretion would further translate into exacerbation of insulin resistance and development of T2DM.
Chapter 6

Effects of treatment with metformin on incretin system and gut hormones in obese patients with T2DM
6.1 Introduction

The incretin effect in type 2 diabetes mellitus (T2DM) is reduced due to diminished insulinotropic activity of both incretin hormones (GLP-1 and GIP)\textsuperscript{25}. Restoration of normoglycaemia in T2DM may partially reverse the diminished insulinotropic activity\textsuperscript{276}. Hypoglycaemic agents may enhance the secretion of incretins or improve their efficacy. Whilst the newer class of dipeptidyl peptidase-IV (DPP-IV) inhibitors developed specifically for this purpose approximately 10 years ago are now widely used in T2DM, the older and well established drug, metformin also appears to have an influence on the incretin axis. The glucose-lowering effect of metformin is thought to be mediated through reduced hepatic glucose output, increased peripheral glucose uptake and enhanced intestinal utilisation of glucose\textsuperscript{278-280}.

In the last decade, there is emerging evidence that metformin treatment in animals\textsuperscript{281,283} and in humans (obese individuals without T2DM and individuals with T2DM) increases GLP-1 concentrations\textsuperscript{288-291,293,295}. There is little evidence on changes to circulating GIP concentrations with metformin treatment and no evidence so far on how metformin treatment may influence actions of GIP at the adipose tissue level. Prior to this current study, published research had evaluated the short-term effects of metformin treatment (up to four weeks) and the acute effects (after a single dose of metformin) on GLP-1 and DPP-IV activity in patients with T2DM\textsuperscript{289,291-293,295}. At the time of this study, there was limited data to support that these effects are sustained with longer term treatment.

Metformin improves glycaemic control with less weight gain compared to some of the other oral anti-hyperglycaemic treatments in patients with T2DM\textsuperscript{303}. Although the mechanisms behind this are poorly understood, metformin has been shown to reduce food intake in obese subjects with and without diabetes mellitus\textsuperscript{304,305}. The effects of metformin treatment on appetite regulatory hormones are less well studied. Ghrelin is a potent orexigenic (appetite stimulant) hormone secreted from the gastric fundus which plays an important role in the hypothalamic regulation of energy homeostasis \textsuperscript{73}. The role of metformin on modulation of ghrelin levels in patients with T2DM remains unclear. Studies in this area so far have shown conflicting results\textsuperscript{306-309}. 
6.2 Hypothesis and aims
We hypothesised that metformin treatment may affect the incretin system by altering the incretin hormone concentrations which may contribute to its additional glucose-lowering properties. The previously reported anorectic effects of metformin that leads to improvement in glycaemic control and weight loss may be related to changes in ghrelin levels.

Our aim was to study the effects of treatment with metformin (minimum 3 months) on endogenous GIP, GLP-1 concentrations, DPP-IV activity and active ghrelin concentrations in obese patients with T2DM.

6.3 Subjects and Methods

Study design
This was a prospective observational study in patients with drug naive T2DM commencing metformin as monotherapy. We studied eight subjects (6 males and 2 female) age 58.7 ± 2.6 years with obesity and new diagnosis of T2DM. None of these subjects were on treatment for T2DM at the start of this study. Each subject was studied for 6 hours with blood sampling at multiple time points following a standard mixed meal, before and after at least 3 months of metformin monotherapy.

Study protocol (Figure 6.1)
All participants had 3 visits (Figure 6.1) to the investigations unit in Clinical Sciences Centre, University Hospital Aintree, Liverpool. A detailed history was taken on the first visit and anthropometric assessments were done on all three visits.

Visit 1
On their 1st visit, an intravenous cannula was inserted and fasting blood samples were collected into EDTA, lithium heparin and serum separator tubes for later measurement of DPP-IV activity, GLP-1 and ghrelin. Blood collection tubes had 50 microliters aprotinin, to prevent degradation by DPP-IV and other proteolytic enzymes. Details on sample collection processing and storage are described in chapter 2 (Methods).
After collection of blood samples in the fasted state (time = 0 minutes), patients were asked to consume a mixed meal calculated to provide approximately 600 kcal (cereals, toast, flora, jam and orange juice). The energy load of the meal was calculated to be 23% fat, 13% protein and 64% carbohydrate. Blood samples were taken for later measurement of DPP-IV activity, GLP-1 and ghrelin at 15, 30, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes after consumption of the test meal. The postprandial peak of GLP-1 concentrations is usually seen between 60 to 120 minutes, we sampled blood for a longer duration to see if the observed effects were sustained and to detect any changes in the other variables (DPP-IV and ghrelin). Participants marked their hunger score and desire to eat on a visual analogue scale (0-100) in the fasted state and at every hour after a mixed meal. A buffet lunch was provided at the end of the experiments and total calorie intake during this lunch was recorded.

Following visit 1, patients were commenced on metformin tablets, starting initially at 500 mg daily, and the dose titrated upwards over a period of six to eight weeks to the dose that produced satisfactory metabolic control or titrated to the maximum dose tolerated by the patient. The maximum dose for metformin given was 1000 mg three times daily. Subjects with gastrointestinal side effects during the period of upward titration of the dose were instructed to return to the dose previously tolerated for a further two weeks, and then an increase in the dosage was re-attempted if tolerated. During this time, all patients were instructed to continue with their diet as previously recommended.

**Visit 2**

Six weeks following initiation of treatment with metformin, participants were asked to attend the investigational unit for a 2nd visit. At this stage blood samples were taken to measure HbA1c. Lifestyle advice was offered (as in routine clinical practice), compliance with medication was assessed and further dose titration was recommended as appropriate.
Visit 3
All subjects attended for their 3rd visit at least three months (range 3 to 7 months) after the initiation of metformin monotherapy, and the procedures of visit 1 were repeated in an identical manner. Telephone contact was maintained with all subjects between visits 1 and 3 to monitor compliance with medication and to offer advice on problems with medication tolerance.

Figure 6.1 Study design showing 3 visits for each participant and the study protocol during each of these visits.
6.4 Statistical Analysis

Hormone profiles at multiple time points after a standard mixed meal were compared with baseline fasting concentrations using One-ANOVA and Dunnett’s multiple comparison tests.

Area under the curve (AUC) was calculated for all the variables measured at 12 time points over a period of 6 hours to give an integrated measure of responses. AUC was calculated by the trapezoidal rule using GraphPad Prism software. AUCs pre and post metformin treatment (visit 1 and visit 3) were compared using Student’s t test for paired data and Wilcoxon signed-rank test when paired data were not normally distributed. Mean observations at each time point pre and post metformin were also compared for all variables. P value of < 0.05 (two-tailed) was considered to be significant.
6.5 Results

6.5.1 Participant demographics and metabolic parameters

Eight subjects (6 male and 2 female) with a mean age of 58.7 years (range 47 to 69 years) were recruited. Mean dose of metformin at visit 3 was 1.75 g daily (range 1 to 3 g daily). As expected glycaemic control improved in all patients after at least 3 months of metformin therapy (range 3 to 7 months). The mean HbA1c reduction was $13.5 \pm 4$ mmol/mol ($p = 0.017$) with metformin treatment but there was no significant change in body weight (Figure 6.2 and Table 6.1).

![Figure 6.2](image)

**Figure 6.2:** A HbA1c (mmol/mol) and B body weight (kg) before and after 3 months of metformin treatment for all subjects presented in box plots with median line. Whiskers represent minimum and maximum measures of Hba1c and weight. *p=<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pre-metformin</th>
<th>On metformin (≈ 3 months)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>69 ± 6</td>
<td>56 ± 4</td>
<td>0.017*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.5 ± 0.5 %</td>
<td>7.3 ± 0.3 %</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>114.5 ± 9.5</td>
<td>114 ± 9.1</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>41.1 ± 2.9</td>
<td>40.9 ± 2.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table 6.1:** Changes in glycaemic control and body weight after 3 months of metformin treatment. Variables are shown as mean ± SEM, NS denotes not significant.
6.5.2 Fasting hormone profiles pre and post metformin

**GLP-1** Following 3 months of metformin monotherapy, the mean fasting GLP-1 levels were higher compared to the baseline but there was significant variation in GLP-1 levels in individual subjects and the results were not statistically significant (Figure 6.3A). [pre-metformin: 5.3 ± 2 vs. post metformin: 7.3 ± 3.4 pmoles/litre, (Mean ± SEM); \( P = 0.2 \)].

**DPP-IV activity:** The mean fasting DPP-IV activity was significantly lower after 3 months of metformin treatment compared to the baseline (pre-metformin: 43.8 ± 3.6 vs. post metformin: 36.7 ± 1.4 pmoles/min/ng; \( P = 0.012 \)). Most individuals had lower fasting DPP-IV activity after metformin treatment (Figure 6.3 B).

**Ghrelin:** The mean fasting ghrelin levels were not different before and after metformin treatment (pre-metformin: 42.4 ± 8.8 vs. post metformin: 47.1 ± 10.4 pg/ml; \( P = 0.6 \)). (Figure 6.3C).

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**Figure 6.3** Fasting concentrations of plasma GLP-1, DPP-IV activity and ghrelin before and after 3 months of metformin treatment for all subjects presented as box plots with median line. Whiskers represent minimum and maximum measures. *\( p<0.05 \).
6.5.3 Postprandial hormonal profile after a standard test meal

**GLP-1:** The mean GLP-1 concentrations measured at 12 time points after the test meal were consistently higher after 3 months of metformin monotherapy compared with the pre-metformin visit (Figure 6.4 A). The GLP-1 concentrations increased within 30 minutes after the standard mixed meal (post metformin) but concentrations at all time points were not significantly different from baseline/fasting state. Mean AUC$_{0-6h}$ of GLP-1 was significantly higher on metformin (pre: 1750.8 ± 640 vs. post: 2718.8 ± 1182.3 pmoles/litre/min; \( P = 0.017 \)) [Figure 6.4 B].

**DPP-IV activity:** The mean DPP-IV activity measured at all time points after the test meal were consistently lower after 3 months of metformin monotherapy compared with the pre-metformin visit (Figure 6.5 A). The DPP-IV concentrations at any of the time points were not significantly different from baseline/fasting state. Mean AUC$_{0-6h}$ of DPP-IV activity was lower post metformin but this did not reach statistical significance (pre: 14099.4 ± 989 vs. post metformin 11988.2 ± 737.8 pmoles/min/ng; \( P = 0.1 \)).

**Ghrelin:** Ghrelin concentrations measured at 12 time points after the test meal before and after 3 months of metformin monotherapy were very variable between the two visits and did not show any meaningful trend. At all-time points concentrations of ghrelin were not significantly different from baseline/fasting state. (Figure 6.6 A). There was no significant difference in mean AUC$_{0-6h}$ of ghrelin following treatment with metformin compared with baseline (pre: 18425.9 ± 2436 vs post: 18787.6 ± 2485 pg/ml/min, \( P = 0.9 \)).
Figure 6.4: A Mean GLP-1 ± SEM (pmol/L) for all subjects at 12 time points after a standard mixed meal pre and post metformin treatment. B AUC of GLP-1 for all subjects before and after 3 months of metformin treatment are presented in box plots with median line. Whiskers represent minimum and maximum AUC measures of GLP-1.
Figure 6.5: A Mean DPP-IV activity ± SEM (pmol/min/ng) for all subjects at 12 time points after a standard mixed meal pre and post metformin treatment. B AUC of DPP-IV activity for all subjects before and after 3 months of metformin treatment are presented in box plots with median line. Whiskers represent minimum and maximum AUC measures of DPP-IV activity.
Figure 6.6: A Mean ghrelin ± SEM (pg/ml) of all subjects at 12 time points after a standard mixed meal pre and post metformin treatment. B AUC of DPP-IV activity for all subjects before and after 3 months of metformin treatment are presented in box plots with median line. Whiskers represent minimum and maximum AUC measures of ghrelin.
6.5.4 Scores on visual analogue scales and total calorie intake

All subjects marked their scores of hunger and their desire to eat on a visual analogue scale (0-100) on the two visits (pre and post metformin treatment). There was no difference in the scores on visual analogue scales pre and post metformin (Figure 6.7 A, B). Total calorie intake during a buffet lunch at the end of the experiments did not differ between pre and post metformin visits (Figure 6.7 C).

Figure 6.7 Visual analogue scores (Mean ± SEM) at fasting and at hourly time points after a standard mixed meal A Hunger scores, B Desire to eat, C Total calorie (Kcal) intake during buffet lunch pre and post metformin treatment
6.6 Discussion

In this study, we have shown that three or more months of metformin monotherapy in obese patients with T2DM is associated with a significant increase in postprandial active GLP-1 concentration, which is maintained for up to six hours after a standard meal. We also observed an increased trend in fasting GLP-1 levels although the difference was not statistically significant. DPP-IV activity was significantly lower in the fasting state after 3 months of metformin therapy. Postprandial DPP-IV activity seemed to be lower after metformin treatment but this difference was not statistically significant. Reduced fasting DPP-IV may have contributed to the enhanced post prandial GLP-1 secretion in our study. However, our methodology was not aimed at exploring the underlying mechanisms of increased GLP-1 secretion. Glycaemic control improved as expected but no significant weight change occurred during this period despite the observed increased postprandial GLP-1 responses. There was no difference in participants’ visual analogue score of hunger or their desire to eat on pre and post metformin visits. Furthermore, there was no difference in calorie intake during a buffet lunch before and after metformin treatment.

The results from our study are consistent with the other human studies in the past that showed increased post prandial GLP-1 levels with metformin treatment in non-diabetic lean and obese subjects and in obese subjects with T2DM. Metformin treatment in these studies was for a shorter duration ranging from 4 days to 4 weeks. At the time of our study there was dearth of evidence on the effects of longer term metformin treatment on incretins and gut hormones in T2DM. One study undertaken in non-diabetic women with polycystic ovarian syndrome (PCOS) showed increased AUC of both GIP and GLP-1 after a 75g oral glucose tolerance test in lean women (BMI < 25 kg/m²). A similar trend was seen in obese women (BMI≥ 32 kg/m²) with PCOS but the results were not statistically significant.

Larger studies published recently strengthen the concept of metformin enhanced GLP-1 concentrations. Data taken from a large cross sectional study of 836 people with recent diagnosis of T2DM aimed at investigating predictive bio markers of
glycaemic deterioration and phenotyping (DIRECT study) showed fasting GLP-1 levels to be significantly higher in patients treated with metformin compared to those on life style modification. A randomised double blind placebo controlled trial (CAMERA study) on effects of metformin on cardiovascular disease in patients without diabetes (n=86) showed that metformin treated subjects consistently had higher fasting GLP-1 levels at 6, 12 and 18 month intervals compared to those on life style measures alone (n=87). A randomised cross over trial using delayed release metformin showed that 5-day treatment period resulted in significant increases in GLP-1 and PYY concentrations. Other smaller studies in subjects with T2DM published in recent years after our study, have shown varied results with some studies suggesting metformin enhanced GLP-1 concentrations but had no effects on GIP concentrations. Whereas studies of patients with T2DM on combination treatment with metformin and sitagliptin (DPP-IV inhibitor) showed higher GIP and GLP-1 concentrations with the combination or sitagliptin alone but not with metformin alone.

The precise mechanism(s) through which metformin increases GLP-1 concentrations in the plasma remain unclear. Although improved glycaemic control and reduced metabolic risk factors may partially restore the diminished incretin effect in T2DM, data from the DIRECT study suggested that GLP-1 concentrations were higher in metformin treated patients despite adjusting for HbA1c, fasting glucose and lifestyle factors, suggesting an independent effect of metformin in addition to the glucose lowering properties. Treatment with delayed release metformin showed similar levels of glucose reduction and enhanced GLP-1 and PYY secretion compared to immediate release metformin despite 60% reduction in systemic exposure to metformin with the delayed release preparation. This suggests a local action of metformin in the distal ileum mediated by gut hormone secretion which may contribute significantly to its glucose lowering effects. DPP-IV inhibition by metformin has been proposed as another mechanism that enhances GLP-1 levels. We also observed in our study a reduction in fasting/baseline DPP-IV activity although a causal relationship with the raised post-prandial GLP-1 levels cannot be established. Cuthbertson et al demonstrated that in patients with T2DM under fasting conditions, a single dose of subcutaneous GLP-1 (7-36) amide, in combination with 1 gram of metformin, resulted in reduced DPP-IV activity and
increased plasma active concentrations of the injected GLP-1 (7-36) amide than when GLP-1 was injected alone, suggesting metformin increases GLP-1 via inhibition of DPP-IV.

Results from other studies contradict the DPP-IV inhibition concept and favour the notion that metformin directly enhances the synthesis of GLP-1. One previous study showed a dose dependent increase in active GLP-1 levels even in DPP-IV-deficient F344/DuCrj rats after treatment with metformin, suggesting a mechanism independent of DPP-IV inhibition. Another study that employed in vitro methods demonstrated that metformin did not alter GLP-1 degradation suggesting that it did not act through DPP-IV inhibition. Migoya et al showed that in healthy individuals without T2DM, 2 days of treatment with metformin (1 gram per day orally) increased the total and active GLP-1 concentrations but had no effect on the other incretin hormone, glucose dependent insulinotropic polypeptide (GIP). Furthermore, it has been demonstrated that metformin treatment increases pre-proglucagon gene (Gcg) expression in the large intestine of mice leading to increased total GLP-1 concentration. Another recent study in mice has shown that metformin enhances expression of the GLP-1 receptor through peroxisome proliferator-activated receptor-α (PPAR-α).

GLP-1 is rapidly degraded by the enzyme DPP-IV secreted from endothelial capillaries of the intestinal mucosa. Nearly 75% of GLP-1 is inactivated before it enters the systemic circulation. There are arguments for and against GLP-1 mediation as the sole or major therapeutic action of DPP-IV inhibition. It has been proposed that endogenous GLP-1 exerts immediate local effects in the gut and hepatic portal bed to stimulate hypothalamic neural pathways for insulin secretion, which occurs before degradation by DPP-IV. Therefore DPP-IV inhibition only protects the endocrine route but not the important neural pathways for insulin secretion. The synergistic action of metformin and DPP-IV inhibitor combination therapy in T2DM is recognised to result in superior glycaemic control compared with the cumulative individual maximal monotherapies. Given the potency of DPP-IV inhibitors in reducing the serum DPP-IV activity by more than 80%, a small degree of further inhibition by metformin may not completely explain the superior glycaemic control. Moreover, lack of postprandial rise in active GLP-1 is a
recognised problem in patients with T2DM. Therefore, the ability of metformin to enhance endogenous GLP-1 secretion and the unidentified benefits of DPP-IV inhibition in addition to enhancing GLP-1 in the circulation would seem as a favourable explanation for this synergism.

There were no significant changes in ghrelin levels before and after metformin treatment in our study. These results are in contrast to a previous study from our research unit which demonstrated that patients with T2DM on metformin monotherapy exhibit a prolonged fall in ghrelin concentrations after a mixed meal compared with matched controls on diet alone. The study described above was a cross-sectional study and ghrelin levels were not studied pre and post metformin treatment in the same group of patients which may account for some of this variation. Similarly, another study has shown that four months of treatment with metformin but not pioglitazone reduced ghrelin levels after a glucose load despite similar reductions in HbA1c in both groups. However, subjects in that study had longer duration of diabetes and the majority were already on sulphonylureas. Other studies have shown contradictory results suggesting an increase in ghrelin levels after metformin treatment. Douge et al. showed that ghrelin levels increased after 6 weeks of metformin treatment in obese patients with T2DM but this was not accompanied by changes in hunger and fullness scores. There were variations in the individual responses to ghrelin in our study; we are therefore unable to comment on any trends in ghrelin levels in individuals on metformin therapy.

The strengths of our study include testing the effects of long term metformin treatment on a clinically appropriate patient group (obese patients with drug naive T2DM) and increased frequency of blood sampling after a standard meal. Although the postprandial peak of GLP-1 concentration is usually seen between 60 to 120 minutes, we sampled blood for a longer duration to see if the observed effects were sustained and to detect any changes in the other variables (DPP-IV and ghrelin). We did not measure GIP due to lack of reliable GIP assay locally at the time of this study and there was insufficient evidence from many studies to suggest any changes in GIP concentrations with metformin treatment in T2DM. The small sample size and the significant variation among individuals in the postprandial responses of GLP-1, ghrelin and DPP-IV activity are possible limitations. However, a previous
study with a sample size of 8 was shown to have 90% power to detect a 25% difference in DPP-IV activity at the 5% significance level. Having a placebo arm may have strengthened our study further, however we felt it would be unethical to withhold treatment in any of our subjects given their HbA1c result.

In summary, three months or more of metformin monotherapy in obese patients with T2DM is associated with significantly increased postprandial GLP-1 levels and reduced fasting DPP-IV activity. Previous studies have suggested no significant changes in GIP concentrations. The pleotropic effects of metformin on the entero-insular axis may represent yet another important mechanism underlying its glucose lowering properties.
Chapter 7

Final discussion
7.1 Summary of results

Study 1

We demonstrate that acute GIP infusion in a fasting state under hyperglycaemic clamp conditions stimulated insulin secretion in the lean, obese and obese subjects with IGR, whereas its insulinotrophic action was significantly impaired in obese patients with T2DM, consistent with results from previous studies. Additionally, the integrated insulin concentrations (AUC) during GIP infusions correlated negatively with fasting plasma glucose for all subjects in the study, indicating a gradual decline in insulin secretion with GIP infusions in individuals with higher fasting plasma glucose levels.

GIP infusion reduced plasma NEFAs, concomitantly increasing SAT triacylglycerol (TAG) content in obese patients with T2DM. This anabolic effect was not observed in the lean, obese or obese patients with IGR. In contrast, while GIP stimulated insulin secretion in the lean, obese and obese patients with IGR, its insulinotrophic action was not observed in obese patients with T2DM. The reduction in NEFA concentration with GIP correlated with adipose tissue insulin resistance for all subjects. There were no significant gene expression changes in key SAT lipid metabolism enzymes. Thus, in obese patients with T2DM, there is a dissociation of the effects on GIP on beta cells and adipocytes, with blunted insulinotrophic but preserved lipogenic actions respectively.

Baseline plasma concentrations of TNF-α, MCP-1 and osteopontin were significantly higher in the obese T2DM group compared to the lean control group. The gene expression of TNF-α in SAT of obese T2DM subjects increased significantly after GIP infusion compared to placebo and similar trends were seen in obese subjects with IGR with no change in lean and obese normoglycaemic subjects. In contrast, MCP-1 gene expression was higher with GIP infusion in lean and obese but was not different in IGR or T2DM. There was no change in osteopontin and adiponectin gene expression with GIP or placebo infusions in any of the groups. Summary of results for study 1 are shown in Table 7.1.
Study 2
Three or more months of metformin monotherapy in obese patients with T2DM is associated with a significant increase in postprandial active GLP-1 concentration, which is maintained for up to six hours after a standard meal. We also observed an increased trend in fasting GLP-1 levels although the difference was not statistically significant. DPP-IV activity was significantly lower in the fasting state after 3 months of metformin therapy. Post prandial DPP-IV activity seemed to be lower after metformin treatment but this difference was not statistically significant. There was no change in ghrelin levels post metformin treatment.

7.2 Limitations of studies
We acknowledge the small number of subjects in each group. There was limited pilot data in humans prior to initiation of this study and subsequently published human studies on GIP infusion had similar number of subjects between 6-8 participants per group\textsuperscript{189-191}. Subjects other than controls had very high BMI therefore the findings may differ in less severely obese individuals with BMI < 40 kg/m\textsuperscript{2}. Only male subjects were recruited for GIP infusion studies to avoid the confounding effects of sex steroid fluctuations due to menstrual cycle, menopause and hormone replacement therapy in female subjects. Results may therefore vary in female subjects. Some subjects in obese IGR and T2DM group were on medication for hypertension and dyslipidaemia. Unrecognised interactions between anti-hypertensive or lipid modifying medication and effects of GIP cannot be excluded.

We acknowledge assessing the effects of GIP on glucagon secretion would have been a useful measure but we did not measure this as our experiments were conducted under hyperglycaemic clamps where glucagon is usually supressed. GIP infusion may affect enzyme activity rather than gene expression and therefore results of LPL, ATGL and HSL may vary if activity was measured. Unfortunately, the effects of GIP on IL-6 gene expression could not be assessed in our experiments at the time of this study due to technical difficulties with primers that could not be optimised to appropriate conditions to carry out qPCR. In study 2, we did not measure GIP due to lack of reliable GIP assay locally at the time of this study and there is insufficient evidence from several studies on any changes in GIP concentrations with metformin treatment in T2DM.
Table 7.1 Summary of results for study-1

<table>
<thead>
<tr>
<th>Effects of GIP vs placebo</th>
<th>Lean</th>
<th>Obese</th>
<th>Obese IGR</th>
<th>Obese T2DM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↔</td>
</tr>
<tr>
<td>NEFA</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Adipokines</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td><strong>SAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triacylglycerol content</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
</tr>
<tr>
<td>Gene expression of SAT enzymes</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
</tr>
<tr>
<td>Gene expression of SAT adipokines</td>
<td>↑ MCP-1</td>
<td>↑ MCP-1</td>
<td>↔</td>
<td>↑ TNF-α</td>
</tr>
</tbody>
</table>

Table 7.1: Summary of results on the effects of GIP vs placebo in four groups. ↔ indicates no difference in effects compared to placebo.
7.3 Physiological role of GIP: an overview

GIP is likely to have many different actions in multiple peripheral tissues. Although its’ principal action was thought to be in postprandial glucose regulation through insulin secretion, blocking GIP signalling surprisingly caused only a minor disturbance in glucose regulation with benefits on other metabolic profiles questioning its primary physiological role in the pancreas. Its role in adipose tissue metabolism has gained more understanding in the last two decades but most of the evidence is from animal studies. Although there is some evidence to suggest it is pro-adipogenic in humans, the results are not consistent in all subject groups and there are very few studies in humans to date. New evidence from animal studies suggests GIP may have beneficial effects in bone and brain but this concept is at a very early stage. In this section, I have discussed the role of GIP in specific tissues and the implications in healthy individuals compared to those with T2DM.

Effects in pancreas: is this the primary physiological role of GIP?

In healthy individuals, GIP mediates the bulk of incretin effect thus, playing an important role in post prandial glucose metabolism. Both incretin hormones enhance insulin biosynthesis and β cell survival. Whilst GLP-1 retains its insulino-tropic activity in people with T2DM, GIP has a blunted effect despite preserved secretion of GIP in these individuals. Interestingly, the effects of GIP differ from GLP-1 on glucagon secretion. We are now aware that GIP increases glucagon secretion in healthy individuals during fasting and hypoglycaemic conditions but not during hyperglycaemia. Similar effects of GIP on glucagon were also observed in patients with type 1 diabetes. Although glucagon secretion by GIP appears to be a protective mechanism to stabilise glucose in healthy individuals, it may worsen glucose intolerance in people with T2DM. Additionally, GIP was shown to enhance another islet cell hormone known as pancreatic peptide (PP) secreted from PP cells which is also elevated along with glucagon in T2DM. GIP therefore seems to have effects on multiple cells in pancreatic islets that can influence glucose metabolism. Whilst GIPR is down regulated in hyperglycaemic states with loss of GIP sensitivity in β cells for insulin secretion, the action of GIP in α cells and PP cells may be retained leading to increased glucagon and PP secretion and worsening of glucose intolerance in T2DM.
Studying the metabolic effects through GIP signal blockade helps to understand the overall contribution of GIP in glucose regulation. Most studies in GIPR knockout mice showed that glucose tolerance and insulin sensitivity had improved on high fat diet in contrast to what would have been expected with the loss of insulinotropic activity from GIP\textsuperscript{135,256,257,407}. As the metabolic profile improves with inhibition of GIP signal, it is possible that the negative effects of GIP inhibition at pancreas are compensated by beneficial effects in other tissues leading to improved beta cell function and insulin sensitivity due to removal of triacylglycerols from pancreas, liver and muscle. Weight loss as a result of reduction in adiposity may also ameliorate insulin resistance. Data from these studies indicate that effects of GIP in pancreas may not be as vital as it was thought to be in the past. Whilst other glucose regulatory mechanisms and GLP-1 may compensate for glucose metabolism in healthy individuals, preventing GIP action in α and PP cells of pancreas would seem beneficial in T2DM with reduction in counter regulatory hormones.

The effects of GIP in adipose tissue: Is it harmful or beneficial?

Storage of energy deposits in adipose tissue is an adaptive mechanism of evolutionary importance. Perhaps the effects of GIP in adipose tissue was a protective mechanism during the times of under-nutrition and famine which may have become maladaptive with surplus dietary intake. The excess fat deposition by GIP seen in animal studies was only with high fat diets and not observed with normal diet. Demonstration of fat ingestion as the most potent stimulator of GIP secretion in animals\textsuperscript{89,98,99} and humans\textsuperscript{88,97} indicates a significant role for GIP in post prandial lipid metabolism. The effects of GIP on enhancing LPL activity seen in animal studies were not observed in the limited human studies. The most convincing evidence in humans is the effect of GIP on NEFA metabolism. Most studies (including our experiments) show a consistent reduction in plasma NEFA with GIP suggesting a re-esterification process in adipose tissue\textsuperscript{178,188-192}. If the source of NEFA reaching adipocyte endothelium for re-esterification is derived from diet, one would expect a reduction in plasma triacylglycerols in the post prandial state. However, in studies using meal experiments, surprisingly there was no reduction in plasma triacylglycerols levels with GIP infusions\textsuperscript{196,408}. GIP mediated effects on lipid metabolism via peripheral lipid clearance in humans warrants further examination.
GIP may modulate other fat deposits that leads to excess liver and visceral fat deposits\textsuperscript{165,166}. In support of this, results from a large cross-sectional study of Danish men demonstrated an association between higher stimulated levels of GIP (during a glucose tolerance test) and a metabolically unfavorable phenotype (higher visceral: subcutaneous fat distribution and a higher waist-hip ratio). This study also demonstrated that higher fasting GIP levels were associated with lower LDL cholesterol (men and women) and higher HDL in women.\textsuperscript{167} Whilst clearance of LDL by GIP from circulation is beneficial in reducing the lipotoxicity, unhealthy distribution of fat in in visceral adipose tissue potentially mediated by GIP would have long term deleterious effects.

It is possible that GIP acts as a regulator of fat deposits. In addition to its lipogenic action, it is thought to increase lipolysis and induce inflammatory adipokines\textsuperscript{174-176}. These lipolytic effects of GIP are enhanced with insulin deficiency\textsuperscript{177}. In healthy individuals under normal diet, these counter regulatory effects of GIP in adipose tissue may be balanced without excess fat deposition. In individuals with chronic excess high fat consumption and obesity the balance may tilt more towards increased fat deposition. In people with T2DM with lack of insulin secretion on high fat diets, GIP may lead to exaggerated fat deposition and inflammation (Figure 7.1).

We know that visceral fat is a major source of NEFA and chronic elevations of NEFAs interfere with insulin signalling, inhibiting glucose uptake and glycogen synthesis\textsuperscript{60,61}. Free fatty acid (NEFA) uptake is higher in visceral fat compared to subcutaneous fat\textsuperscript{409} and visceral fat deposits are more susceptible to lipolytic stimuli such as catecholamines\textsuperscript{410,411} and this lipolytic process is enhanced in the absence of insulin. Whilst clearance of NEFA from circulation by GIP may be beneficial, increasing subcutaneous and potentially visceral fat deposits with over-nutrition would result in fat storage in metabolically unfavourable sites. These fat deposits can be a constant source of excess NEFA in circulation. We speculate that with consumption of energy dense high fat diet, the effects of GIP in adipose tissue may lead to a vicious cycle of fat storage and lipolysis releasing excess NEFA which further exacerbates insulin resistance and obesity (Figure 7.2).
Figure 7.1 In healthy people, GIP acts on its receptors on beta cells and adipocytes to promote insulin secretion (insulinotropic action) and lipid deposition (adipogenic action) (left figure). In obesity, with consumption of an energy-dense, higher fat diet, there is enhanced insulin secretion (which may help overcome peripheral insulin resistance) and increased lipid deposition (which will further enhance fat storage) (middle figure). In T2DM, the effects of GIP on beta cell are impaired with reduced insulin secretion; the effects on the adipocyte seem to be preserved further promoting lipid deposition (right figure).
Figure 7.2 Pro-adipogenic effects of GIP in subcutaneous and visceral adipose tissue (SAT/VAT) with high fat diet consumption leading to a vicious cycle of fat deposition and lipolysis exacerbating adiposity and insulin resistance.
Does GIP have beneficial effects in other tissues?

The role of incretin hormones in bone physiology is a fairly new concept\textsuperscript{412}. GIP appears to have direct beneficial effects in bone with GIPR expressed in osteoblasts and osteoclasts\textsuperscript{127-129}. To date there are very few studies that have evaluated the effects of GIP on human bone metabolism. Experiments in healthy male subjects showed that glucose when given orally leading to incretin secretion, the peaks in GIP were associated with turnover markers that indicate reduced bone resorption\textsuperscript{413}. Similarly, even exogenous GIP infusions in healthy males reduced circulating markers of bone resorption\textsuperscript{414}. Polymorphisms in GIPR were associated with reduction in bone mineral density and increased fracture risk in post-menopausal women\textsuperscript{415}. Treatment of mice with stable GIP analogues have shown to reduce osteoclast formation and bone resorption suggesting there may be potential benefits on bone with a GIP agonist treatment. Based on available data the beneficial action of GIP appears to be is mainly through reduction bone resorption. There may be some favourable effects of GIP on the brain but all the evidence is derived from animal models suggesting a role in neural progenitor cell proliferation\textsuperscript{121}.

GIP: A friend or foe?

GIP appears to be multi-dimensional in its action with positive and negative effects in many tissues. Whilst the insulinotropic action of GIP remains important in regulating glucose metabolism, the effects of glucagon stimulation in healthy individuals and people with T2DM needs further evaluation. The role of GIP in adipose tissue metabolism seem to differ in healthy and diabetes states as we have observed in our study. The pro-adipogenic effect of GIP is mostly by enhancing NEFA incorporation into adipose tissue. It is not completely clear if these effects of GIP are deleterious or may even have some benefits in reducing lipotoxicity. GIP also has lipolytic properties and may increase pro-inflammatory adipokines. These effects appear to be more pronounced with insulin deficiency. Recent studies suggest beneficial effects of GIP in bone and brain although the evidence in this area is still premature. In view of both beneficial and undesirable effects, targeting GIP either by blocking or enhancing its action is likely to compromise some of its important actions or cause deleterious effects making it difficult to manipulate this hormone in therapeutics.
7.4 Therapeutic potential of GIP

Therapeutics of GIP is an interesting area as both GIP agonists and antagonists have been developed in the recent years and were used in animal models. These are discussed in detail in Chapter 1, section 1.3 and 1.4. Amongst the GIP agonists, the truncated analogue, GIP 1-30 appears to be effective in insulin secretion and was also shown to have longer term benefits in β cell preservation. Single molecule drugs which are dual activators of GIP and GLP-1 receptors are also currently in development and have been shown to be beneficial and both glucose metabolism and weight reduction in animal models.

Contrary to the above, several observations in animal models support the concept of inhibition of GIP action to reduce dietary induced obesity along with improvement in metabolic parameters (discussed in Chapter 1 section 1.10). GIP antagonism could offer a potentially novel approach in the future for treatment of dietary induced obesity and its complications such as T2DM. However, it is important to understand the actions of GIP on other vital organs like brain and bone in humans. Recent evidence from animal studies show that long term inhibition of GIP may have some adverse effects on cognitive function and bone remodelling.

Studies evaluating the effects of GIPR knockout in other tissues showed that Gipr−/− mice had impaired learning, reduced synaptic activity and neurogenesis. Prolonged GIP receptor activation with GIP agonists had improved cognitive function and hippocampal synaptic plasticity in high fat fed mice. GIPR knockout mice were observed to have earlier age related changes and altered bone turnover. Alteration in trabecular bone volume and reduction in bone strength was also seen with GIP inhibition. A study evaluated the effects of GIPR knockout in mice in multiple tissues and suggested that partial receptor knockout did not change glucose tolerance but reduced obesity with preservation of bone volume as opposed to reduction in bone volume with complete GIPR knockout. Therefore, the intended improvements in metabolic profile by GIP antagonism may compromise the benefits in bone and brain tissues which needs further careful evaluation.
7.5 Future direction of GIP research

Results presented in this thesis and recent evidence from other studies on the effects of GIP in humans raise additional questions that need to be addressed in future studies. It is now evident that the effects of GIP in human adipose tissue may vary with obesity and in T2DM. It is important to evaluate the effects of GIP in a larger group of patients with T2DM to establish whether the effects we observed on lipid deposition can be replicated. It is difficult to dissect the insulin independent effects of GIP in normal physiological circumstances therefore testing this concept in insulin deficient states would be of significant value. The effects of GIP on glucagon secretion in T2DM which may alter glucose tolerance needs further evaluation.

The pro-adipogenic effects of GIP were only seen in animals with high fat diet indicating GIP mainly alters post prandial lipid metabolism. The source of NEFA that undergo re-esterification in human studies is unclear. Studies using stable isotopes help to study the dynamic changes in lipid metabolism\textsuperscript{424}. Tracers [U-\textsuperscript{13}C] palmitate and \textsuperscript{13}C-carbon labeled substrates have been used to assess NEFA metabolism\textsuperscript{425,426}. Future GIP infusion studies using tracer labelled test meals and subsequent mass spectrometry analysis of NEFA would give better understanding into the synthesis and uptake of NEFA in fasting and postprandial states. GIP may promote fat deposition in visceral adipose tissue and liver but this is not proven and therefore the use of isotope traces methods would also help to evaluate this aspect.

In the light of GIP agonists and antagonists being considered as therapeutic agents for diabetes and obesity, the effects of GIP in central nervous system and bone metabolism in humans needs further evaluation.
Appendices
Appendix A

Hyperglycaemic clamp protocol

Equipments required
YSI Glucose analyser
Pump for glucose infusion
Pump for GIP/placebo infusion

Consumables
Green Veflons, Venflon dressing packs Chlorprep, BD –Q–syte, 3 way taps
Syringes 1 ml, 2ml, 5ml, 10 ml, 20 ml, infusion sets, 20% glucose (500 ml bags),
0.9% normal saline (1000 ml bags) and GIP product in ampules
Blood collection tubes (serum separator, EDTA, lithium heparin and fluoride oxalate).

Aim: To maintain blood glucose concentration as close as possible to 8mmol with a narrow limit of 0.5 mmol/l on either side.

Step -1
Priming dose of glucose 120mg/kg to 130mg/kg in 5 minutes

Example 1
If 120mg/kg body weight is used for 70 kg man: 120x 70 /1000 = 8.4 g to be given in the first 5 minutes which is to be infused as 20% glucose.

The calculations for the volume of 20% glucose to be given is as follows:
Concentrated glucose is available as 20% glucose infusion in 500 ml volume bags
1ml of 20% glucose = 0.2 gram of glucose
5 ml of 20% glucose = 1gram of glucose
8.4 g of glucose would require 8.4 x 5ml of 20% glucose = total 42 ml
To give 42 ml of 20% glucose in 5 minutes the rate of infusion (ml/hr) to be set on the infusion pump is 42 x 12 ml/ hour = 504 ml/hour

Final formula
Rate 20% glucose of infusion (ml/hr) = (120 x wt (kg) /1000) x 5 x 12 (ml/hr)
Examples of infusion rates in first 5 minutes for glucose load of 120mg/kg weight

<table>
<thead>
<tr>
<th>Participant’s weight in kg</th>
<th>Glucose required in grams</th>
<th>Millilitres of 20% glucose in 5 minutes</th>
<th>Rate of 20% glucose infusion in mls/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>7.2</td>
<td>36</td>
<td>432</td>
</tr>
<tr>
<td>65</td>
<td>7.8</td>
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<td>468</td>
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<td>70</td>
<td>8.4</td>
<td>42</td>
<td>504</td>
</tr>
<tr>
<td>75</td>
<td>9.0</td>
<td>45</td>
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</tr>
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<td>80</td>
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<td>576</td>
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<td>60</td>
<td>720</td>
</tr>
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</tr>
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<td>200</td>
<td>24</td>
<td>120</td>
<td>1440</td>
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</table>

Examples of infusion rates for first 5 minutes with higher glucose load of 130mg/kg is are shown below:

Final formula for this would be \((130 \times \text{wt (kg)}/1000) \times 5 \times 12\) (ml/hr)

<table>
<thead>
<tr>
<th>Participant’s weight in kg</th>
<th>Glucose required in grams</th>
<th>Millilitres of 20% glucose in 5 minutes</th>
<th>Rate of 20% glucose infusion in ml/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>7.8</td>
<td>39</td>
<td>468</td>
</tr>
<tr>
<td>65</td>
<td>8.45</td>
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<td>504</td>
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<td>13.0</td>
<td>65</td>
<td>780</td>
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</tbody>
</table>
Step 2
Variable 20% glucose infusion

After 1st five minutes of high dose priming infusion, the rate of infusion is lowered to variable 20% glucose infusion from 1mg/kg/min to 15mg/kg/min or higher aiming to achieve a steady state glucose of 8 mmol/l by 30 minutes. At the end of 30 minutes, GIP at 2pmol/kg/min or 0.9% saline (placebo) is started and continued for 4 hours Simultaneously, the variable rate 20% glucose infusion is continued for the whole experiment aiming for a blood glucose close to 8mmol.

Example
For a 70-kg person at 1mg/kg/min will be 70mg/min at the rate of 21ml/hr and 15 mg/kg/min will be 1050mg/min, at the rate of 315mls/hr.

Calculation on an excel sheet for variable glucose infusion based on weight are shown below for a subject weighing 70kg.

<table>
<thead>
<tr>
<th>Glucose Infusion Rate (mg/kg/min)</th>
<th>Infusion of 20% glucose (ml/hr)</th>
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</thead>
<tbody>
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<td>mg/min</td>
<td>mg/min</td>
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<td>14.5</td>
<td>1015</td>
</tr>
<tr>
<td>15</td>
<td>1050</td>
</tr>
</tbody>
</table>
Appendix B

GIP product - Certificate of analysis

---

**SEQUENCE**  (TFA salt)

**QUANTITY**
Net Peptide Weight : mg
Net peptide content (AAA) : 74.9 %
HPLC ANALYSIS : 95.4 %

**MASS SPECTROMETRY**
Theoretical molecular weight : 4983.7
Experimental molecular weight : 4983.3 ES+

**AMINO ACID ANALYSIS**
Asx : 6.34 / 7
Glx : 5.29 / 5
Gli : 1.92 / 2
His : 1.89 / 2
Gly : 2.14 / 2
Thr : 2.19 / 2
Ala : 3.36 / 3
Arg : X
Tyr : 2.07 / 2
Cys : X
Val : 1.07 / 1
Met : 0.98 / 1
Phe : 2.08 / 2
Leu : 2.27 / 2
Lys : 5.10 / 5
Pro : X

**COMMENTS** : Storage at -20°C
Solubility tested: 0.4 mg/ml in distilled water.
Trp destroyed during hydrolysis done for AA analysis.
Vial sealed under argon.

Date : 23/11/2011

[Signature]
Production

[Signature]
QAO

---

PolyPeptide Laboratories France - SAS au capital de 5,000,000 Euros - 414 477 569 RCS Strasbourg - NAF 7292Z - VAE. TV 88 344 287 995
Siège social : 7 av de Boulogne - 67100 Strasbourg - France - Tél. : +33 (0)3 88 78 69 79 - Fax : +33 (0)3 88 78 56 66 - E-mail : spsi@polypeptide.fr
www.polypeptide.com
Appendix C

Additional details on gene expression qPCR techniques

Methods for gene expression of lipid enzymes LPL, ATGL and HSL

Total RNA was extracted from adipose tissue using RNeasy Lipid Tissue Mini Kit (Qiagen Ltd, UK) following the manufacturer’s instructions. cDNA was generated using SuperScript VILO cDNA Synthesis Kit (Invitrogen, UK). Quantitative PCR was performed using a Bio-Rad CFXConnect Real-time PCR system (Bio-Rad Laboratories Ltd., UK). The PCR reactions were performed in triplicate for each sample, each containing predesigned TaqMan Gene Expression Assays (optimized mixture of primers and probes) and TaqMan Gene Expression Master Mix, with the manufacturer’s instructions on the thermal cycling conditions (Applied Biosystems, UK).

Negative controls omitting reverse transcriptase were included. These assays were:
Hs00386101_m1 (pnpla2 encoding ATGL),
Hs00193510_m1 (lipe encoding HSL),
Hs00173425_m1 (lpl encoding LPL).
Relative gene expression was quantified using ΔΔCq method normalized for the transcript level of a housekeeping gene β-actin (Hs99999903_m1).

qPCR thermal cycling conditions used SAT gene expression of lipid enzymes (Applied Biosystems, UK):

- Hold 50°C, 2 min
- Hold 95 °C, 10
- 95 °C, 15 sec
- 60 °C for 1min
- Go to step 3 and repeat 39 cycles.

qPCR thermal cycling protocol used SAT gene expression of adipokines (BioRAd)

- 95°C for 3 minutes
- 95°C for 30 seconds
- 58°C for 30 seconds, temperature differed for different gene primer pairs.
- 72°C for 30 seconds
- The specificity of amplification reactions was confirmed by melt curve analysis.
Primer sets used for SAT gene expression of adipokines (TNF-α, MCP-1, osteopontin and adiponectin)

Primer sets used with their respective annealing temperatures were:

**TNF-alpha:**
Forward: 5′CTCTTCTGCTGCTGCACTTTG3′
Reverse: 5′ATGGGCTACAGGCTTGTCACTC3′
Annealing temperature: 62°C

**Osteopontin:**
Forward: 5′TTGCAGCCTTCTCAGCCAA3′
Reverse: 5′GGAGGCAAAGCAATCCTG3′
Annealing temperature: 65°C

**MCP-1:**
Forward: 5′TCAGCCAGATGCAATCAATGCC3′
Reverse: 5′GGTGGTCCATGGAATCCTGA3′
Annealing temperature: 62.5°C

**Adiponectin:**
Forward: 5′GACCAGGAAACCACGACTCA3′
Reverse: 5′CCTTAGGACCAATAAGACCTGGA3′
Annealing temperature: 58°C
Appendix D

Visual analogue scale (VAS)

PARTICIPANT: DATE:
TEST DAY: Visit 1/ visit 3 Time

INSTRUCTIONS FOR PARTICIPANTS: Please read each question and then put a mark through the line that best represents how you are feeling in relation to that sensation at this moment.

EXAMPLE:
How TIRED do you feel at this moment?
Not at all tired ___________________________ Extremely tired

PLEASE ANSWER THE FOLLOWING QUESTIONS:

How HUNGRY do you feel at this moment?
Not at all hungry ___________________________ Extremely hungry

How STRONG is your desire to eat at this moment?
Not at all strong ___________________________ Very strong
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Publications from research

Copies of the two original articles published from this research (indicated below) are attached to the hardbound copy of this thesis.

  


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For distinction in scholarship in the American Journal of Physiology - Endocrinology and Metabolism for the article

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