METABOLIC EFFECTS OF DIPEPTYL PEPTIDASE 4 INHIBITOR IN TYPE 2 DIABETES

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ABSTRACT

Insulin resistance and abnormal insulin secretion are central to type 2 diabetes. The relationships have been established between lipid accumulation in the liver and muscle and insulin resistance, and that of lipid accumulation in the pancreas and β cell dysfunction. Therefore it is vital to assess the effect of therapies on intra-organ lipid concentration.

DPP-4 inhibitor vildagliptin is a new therapeutic agent in type 2 diabetes. Its known primary mechanism of action is to delay GLP-1 and GIP degradation, and enhance their endogenous concentration. GLP-1 and GIP increase the sensitivity of the α- and β-cells to glucose concentration. Although DPP-4 inhibitors have been found to decrease postprandial triglyceride levels and lipolysis, the effect on intra-organ lipid concentration is unknown.

Glycogen storage in the liver and muscle in type 2 diabetes is subnormal, nonetheless, the extent of glycogen storage abnormality after a whole day of normal eating has not been previously examined.

Although abnormal β cell function has been recognised as a feature of type 2 diabetes, the whole pancreas has not been extensively studied previously.

To test the hypothesis that DPP-4 inhibitors modulate intra-organ lipid accumulation, 44 well-controlled type 2 diabetes subjects treated only with metformin were randomised in a double-blinded manner to receive either vildagliptin or placebo for 6 months. In order to assess the extent of change, in intra-organ lipid content, and abnormal glycogen storage, and to provide comparative data for pancreas morphology, 14 normal glucose tolerant subjects matched for weight, BMI, sex and age were studied.

A clinically significant decrease in liver triglyceride content after 6 months treatment with vildagliptin was observed. Additionally, the studies demonstrate that the pancreas volume in type 2 diabetes was smaller compared to match controls, and that muscle did not contribute to glycogen storage during normal daily eating in type 2 diabetes.

DECLARATION

I carried out the following tasks:

- Recruitment and care of subjects, organisation and daily administration of the study.

- Combined metabolic tests including hyperinsulinaemic euglycaemic clamp, 6' 6' - dideuterated glucose infusion, and indirect calorimetry.

- Anthropometric measurements and body composition.

- Analysis of intramyocellular lipid (IMCL), hepatic lipid and pancreas volume MR data.

- Data management and analysis.

Dr Ahmad Al-Mrabeh quantified plasma 6' 6' - dideuterated glucose with gas chromatography mass spectrometry.

All metabolite and hormone assays were done in the Diabetes Research Laboratory, except insulin, triglyceride and free fatty acid done by the Royal Victoria Infirmary Biochemistry Laboratories and glucagon by King's College Hospital Clinical Biochemistry department.

MR Physics team developed the MR methods. Dr Pete Thelwall provided general expertise, Dr Kieren Hollingsworth developed the Dixon and Balanced Turbo Field Echo methods. Dr Fiona Smith analysed ^{13}C magnetic resonance spectroscopy and ^{1}H magnetic resonance spectroscopy data and Miss Katie Percival analysed the pancreas triglyceride data. The research contained within this thesis has not previously been submitted as part of any other thesis.

ACKNOWLEDGEMENTS

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LIST OF ORIGINAL PUBLICATIONS

Macauley M, Smith FE, Thelwall PE, Hollingsworth KG, Taylor R. Diurnal variation in skeletal muscle and liver glycogen in humans with normal health and type 2 diabetes. Clinical Science 2015 May 1; 128(10):707-13. doi: 10.1042/CS20140681

Macauley M, Hollingsworth KG, Smith FE, Thelwall PE, Al-Mrabeh A, Schweizer A, Foley JE, Taylor R. Effect of vildagliptin on hepatic steatosis. Journal of Clinical Endocrinology and Metabolism 2015 April; 100(4): 1578-1585. doi: 10.1210/jc2014-3794

Macauley M, Percival K, Thelwall PE, Hollingsworth KG, Taylor R. Altered volume, morphology and composition of the pancreas in type 2 diabetes. PLoS One 2015 May; 10(5): e0126825. doi: 10.1371/journal.pone.0126

LIST OF PUBLISHED ABSTRACTS

Marked changes in size and shape of the pancreas in type 2 diabetes. Macauley M, et al. Diabetes 2014; 63: Supplement 1 A534-A560; doi:10.2337/db14-2093-2206 (Abstract from poster presentation showcased in the Guided Audio Poster Tour at ADA 74thScientific Sessions San Francisco 2014)

Failure of skeletal muscle to contribute to diurnal glucose homeostasis in type 2 diabetes. Macauley M, et al. Diabetologia 2014; 57: Issue 1 supplement (Abstract from poster presentation in EASD Annual Meeting Austria 2014)

Precise non-invasive quantification of pancreas volume and relationship with insulin secretion in type 2 diabetes. Macauley M, et al. Diabetic Medicine 2014;31: Supplement s1 (Abstract from poster presentation at Diabetes UK Professional Conference, Liverpool 2014)

Relationship between fat accumulation in liver and pancreas in type 2 diabetes. K Percival, Macauley M, Steven S, Hollingsworth K, Taylor R. Diabetic Medicine 2014;31:Supplement s1 (Abstract from poster presentation at Diabetes UK Professional Conference, Liverpool 2014)

Diurnal variation of skeletal muscle and liver glycogen concentration in well controlled type 2 Diabetes Macauley M, et al. Diabetic Medicine 2012; 29:Supplement s1 (Abstract from poster presentation at Diabetes UK Professional Conference, Glasgow 2012)

Diurnal variation of skeletal muscle and liver glycogen concentration in well controlled type 2 Diabetes Macauley M, et al. Diabetes 2012; 61:Supplement 1 A443-A522; doi:

10.2337/db12-1721-2041 (Abstract from poster presentation at ADA 72thScientific Sessions San Francisco 2014

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CHAPTER 1 INTRODUCTION

1.1 Natural history of type 2 diabetes

Although diabetes mellitus was first recognised as a disease more than 3500 years ago, understanding its pathology and development in managing the disease have been revolutionised only in the last century. The great Greek physician Aretaeus was amongst the first to document polyuria as a symptom of diabetes mellitus (30 - 90 CE). The term diabetes is translated from the Greek for a siphon–rapid passage of fluid out and in; mellitus refers to the sweet taste of urine. Much later (980 - 1037) the Persian physician Aricenna gave a more detailed description of symptoms such as sweet taste in a diabetic urine, abnormal appetite and loss of sexual function. Although Von Mering and Minkowski first described the role of the pancreas in normal glucose metabolism in 1889 (Von Mering, 1890), Banting and Best were the first to purify insulin for clinical use. The latter was an important milestone in developing the treatment for diabetes.

It was initially believed that only one form of the disease existed and this was due to insulin deficiency. However, the distinction between insulin dependent (type 1) and non-insulin dependent diabetes (type 2) was first recognised as far back as 400 - 500 CE by Indian physicians Sushrata and Charaka. The distinction between both types was demonstrated and documented by Harold Percival Himsworth in 1936 using oral glucose tolerance tests with or without injected insulin (Himsworth, 1936). The development of radioimmunoassay to measure endogenous insulin concentration by Yalow and Berson (Yalow and Berson, 1960) allowed characterisation of hyperinsulinaemia, a marker of insulin resistance in non-insulin dependent (type 2) diabetes. The United Kingdom Prospective Diabetes Study (UKPDS) gave further insight into the pathophysiology of type 2 diabetes. Firstly, it was recognised that blood glucose increased with the duration of type 2 diabetes in the tight glycaemic control group; and secondly, fasting hyperinsulinaemia was a distinctive feature in the type 2 diabetes (UKPDS, 1999). This allowed the description of insulin resistance as the earliest detectable abnormality, and insulin secretory defect as a later abnormality in type 2 diabetes (Taylor, 2013).

1.2 New insights in the aetiopathophysiology of type 2 diabetes

Insulin resistance and subnormal glucose stimulated insulin secretion underpin the aetiopathophysiology of type 2 diabetes. Previously, it was thought that these two processes were inevitably progressive; and multiple oral agents and insulin therapy would be required to maintain normal glucose control as type 2 diabetes progressed. Recent work contributing to better understanding of the disease, has led to the development of interventions which can cause disease remission or in some cases retard progression (Pories *et al.*, 1992; Dixon *et al.*, 2008; Lim *et al.*, 2011a). Therapeutic interventions such as bariatric surgery and very low calorie diet have proved efficient in disease remission; while incretin based therapies with GLP-1 agonists have been shown to improve glycaemic control without weight gain.

It is now known that insulin resistance is present well before the diagnosis of type 2 diabetes is confirmed (Ferrannini *et al.*, 2004), but plasma glucose rises considerably only when pancreatic β cells fail to secrete insulin in response to nutrient (Cali *et al.*, 2009). This suggests that the onset of insulin resistance and β cell dysfunction differ and the two concepts should be considered separately. Although muscle insulin resistance is commonly present in type 2 diabetes, it does not fully explain the entire pathophysiological process of type 2 diabetes. If muscle insulin resistance is solely accountable, it would be expected that all individuals with muscle insulin resistance would develop type 2 diabetes, however this is not the case (Taylor, 2012). Additionally, early normalization of plasma glucose achieved by improving hepatic insulin sensitivity and not muscle insulin sensitivity (Lim *et al.*, 2011a) suggested that liver insulin sensitivity plays a very important role in the development of type 2 diabetes, irrespective of the presence of muscle insulin resistance.

Over time, β cell mass decreases and about 50% is present at the time of diagnosis (Butler *et al.*, 2003). However, there is growing evidence to suggest a common aetiology to both β cell dysfunction and insulin resistance in liver and muscle. Abnormal lipid accumulation in the pancreas, liver and muscle has been implicated in β cell dysfunction and insulin resistance respectively. Although individual genetic predisposition has to be present for the development of type 2 diabetes, chronic calorie surfeit is crucial to lipid accumulation in all three organs.

1.2.1 Insulin resistance in muscle

Muscle insulin resistance has been recognised to be active at the early stages of metabolic syndrome and onset of type 2 diabetes (Petersen *et al.*, 2007). After a meal, insulin-stimulated glucose uptake by the muscle is impaired and glycogen storage is negligible (Carey *et al.*, 2003). In order to prevent postprandial hyperglycaemia resulting from muscle insulin resistance, there is an initial physiological response to increase basal insulin secretion. If excess calorie intake continues, basal hyperinsulinaemia is exacerbated and normal or near-normal plasma glucose concentration is initially maintained. Nevertheless, chronic basal hyperinsulinaemia causes persistently high insulin concentration in the portal circulation, which enhances *de novo* hepatic lipogenesis and predisposes to increased liver fat synthesis and storage.

The inability of insulin resistant individuals to store glucose in the muscle has been reported to be associated with defects in insulin signalling, glucose transport receptors, glucose phosphorylation, and a reduction in glucose oxidation and glycogen synthesis (Bajaj and Defronzo, 2003; Bouzakri *et al.*, 2005; Karlsson and Zierath, 2007). Even though a mitochondrial defect was thought to account for muscle insulin resistance in type 2 diabetes (Petersen *et al.*, 2004), present evidence suggests that primary mitochondrial function is not defective in muscle insulin resistance (Schrauwen-Hinderling *et al.*, 2007). However it is suggested that secondary mitochondrial defect from hyperglycaemia and muscle lipid accumulation may potentially play a role (Lim *et al.*, 2011b; Lim *et al.*, 2011c).

1.2.2 Insulin resistance in liver

There is growing evidence to suggest that liver fat accumulation is an important contributing factor to the aetiology and pathogenesis in type 2 diabetes. Not only has the incidence of type 2 diabetes been shown to be higher in individuals with fatty liver (Shibata *et al.*, 2007), but also the association between hepatic insulin resistance and lipid accumulation has been amply demonstrated (Seppala-Lindroos *et al.*, 2002; Petersen *et al.*, 2007; Ravikumar *et al.*, 2008; Lim *et al.*, 2011a). In type 2 diabetes, fatty liver has been found to be associated with the failure to suppress hepatic glucose

production, an indicator of hepatic insulin resistance (Petersen *et al.*, 2007; Ravikumar *et al.*, 2008).

The supply of fatty acid to the hepatocyte can be from two main sources: extracellularly, the uptake of NEFA and LDL; and intracellularly, *de novo* lipogenesis and the breakdown from intracellular triacylglycerol and diacylglycerol stores. The fate of intracellular fatty acid is crucial to intra-hepatic triglyceride accumulation and needs to be considered. Intra-hepatic lipids can either be safely stored as triacylglycerol, metabolised in the mitochondria for energy, or exported as VLDL-triacylglycerol to other organs. Mitochondrial oxidation of fatty acid is reduced in type 2 diabetes. In well controlled type 2 diabetes subjects (HbA$_{1c}$ 7.1±1.0%), a 25% reduction in hepatic mitochondrial oxidation of fatty acid has been reported when compared to age, sex and BMI matched controls (Szendroedi *et al.*, 2009). Considering that the study group were well controlled type 2 diabetes subjects, it is likely that the defect in mitochondrial lipid oxidation is present before the diagnosis of type 2 diabetes. As mitochondrial fatty acid oxidation is suppressed, intracellular concentration of diacylglycerol and fatty acids rises (Kumashiro *et al.*, 2011). The accumulation of diacylglycerol activates protein kinase C (PKCε), which in turn prevents the intra-hepatic transport protein insulin receptor substrate 1 (IRS1) from binding to the transmembrane insulin receptor (Samuel *et al.*, 2010). Additionally, excess fatty acids in the hepatocyte promote the synthesis of ceramide, which is thought to play a role in the deactivation of Akt, thus affecting the insulin-signalling pathway (Kumashiro *et al.*, 2011) Figure 1.1.

In a small proportion of individuals with non-alcoholic fatty liver disease (NAFLD) who were found to have a G allele mutation of the PNPLA3 or rs738409 gene, insulin sensitivity was found to be normal (Speliotes *et al.*, 2010). PNPLA3 is a patatin-like phospholipase or adiponutrin gene expressed in adipose tissue, and functions as a lipase for hydrolysis of triacylglycerol. Expression of the mutant gene prevents the breakdown of triacylglycerol and allows safe storage within the hepatocyte. As such the accumulation of diacylglycerol and fatty acid is prevented and insulin signalling is preserved.

The accumulation of lipids in the liver causes metabolic strain on the hepatocytes, which results in a rise in alanine transaminase (ALT) (Sevastianova *et al.*, 2012). ALT, an enzyme present in high concentration within hepatocytes is released into plasma after stress or injury to the hepatocyte. Therefore it can be considered as a marker of hepatocyte health.

As such, a rise in ALT can be considered as a clinical surrogate marker for fatty liver and an indicator for insulin resistance in the liver in most individuals without the G allele of PNPLA3 (Tushuizen *et al.*, 2006).
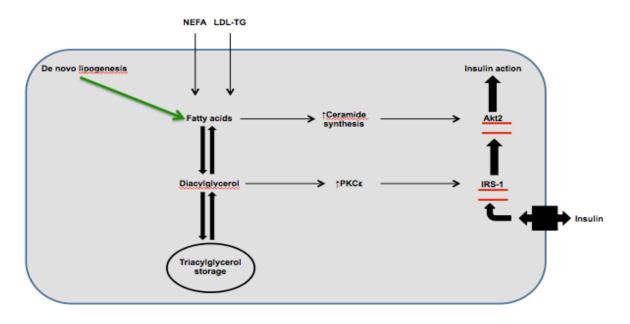
Figure 1.1 Intrahepatic activity of fatty acid

1.2.3 Pancreas dysfunction in type 2 diabetes

The β cells of the pancreas have a supple but precise characteristic that enables normal blood glucose concentrations to be maintained. They are nutrient sensing cells, which respond to the presence of nutrient by insulin biosynthesis and secretion. Adenosine triphosphate (ATP) produced from glucose metabolism is required for exocytosis of insulin. However intra-pancreatic fatty acid is likely to impair ATP production by inhibiting pyruvate dehydrogenase (Zhou *et al.*, 1996), and as such glucose stimulated insulin secretion is impaired. During chronic excess calorie surfeit, lipids are predominantly exported to the pancreas from the liver as VLDL-TG. The uptake of fatty acid by the β cells is mediated through an integral CD36 membrane protein (Noushmehr *et al.*, 2005).

When β cells are cultured *in vitro* in very low concentration of fatty acid (0.33mmol// palmitate) for 48 hours, they avidly take up fatty acid and decrease glucose stimulated

insulin secretion (Lalloyer *et al.*, 2006). It is now known that lipid accumulates in the β cells of the pancreas (Pinnick *et al.*, 2008). Very little is known about the effect of pancreatic lipid accumulation on α cells. However, in type 2 diabetes the inverse relationship between insulin and glucagon secretion is lost and glucagon concentration is inappropriately high. This suggests that abnormal α cell function is likely to be secondary to β cell dysfunction which is associated with pancreatic lipid accumulation. Rodent studies have provided robust evidence for the differentiation of the β cells to glucagon-secreting 'α - like' cells in response to hyperglycaemia, providing a unifying explanation for the complementary twin hormone abnormalities seen in type 2 diabetes (Talchai *et al.*, 2012; Brereton *et al.*, 2014).

The association between pancreatic lipid accumulation and β cells dysfunction has been recognised for some time. When *in vitro* β cells are incubated in 2mmol/l fatty acid for 48 hours, intra-pancreatic fatty acid content rise by 65% (Lupi *et al.*, 2002b). Additionally, *in vivo* studies demonstrated that the pancreatic triglyceride content in type 2 diabetes subjects was associated with a blunted acute phase β cells response to nutrient (Tushuizen *et al.*, 2007; Lim *et al.*, 2011a). In the latter study, the first phase insulin response gradually normalised over 8 weeks of 600kcal diet as pancreas triglyceride reduced to levels comparable with matched controls.

Additionally, the accumulation of fatty acid in the pancreas inhibits β cells proliferation and promotes apoptosis. The inhibition of β cells proliferation is mediated through the stimulation of p16 and p18 cell cycle inhibitors (Pascoe *et al.*, 2012). Cadaveric examination of the pancreas revealed a 50% reduction in β cells; the mechanism for β cell loss was thought to be due to an increased rate in apoptosis (Butler *et al.*, 2003). The association between lipid accumulation in the β cells and increased rate of apoptosis has been previously described (Shimabukuro *et al.*, 1998a).

1.3 Incretin hormone biology

1.3.1 Background to incretin hormones

As early as 1906, hormones produced by the intestine were thought to have an effect on insulin secretion. McIntyre *et al* were able to prove that oral glucose induced a larger insulin secretory response when compared with a similar intravenous glucose concentration (McIntyre *et al.*, 1964). This led to a new concept called the incretin effect, which is defined as the difference in β cell insulin secretory response to equal moles of glucose administered either orally or intravenously (Rehfeld, 2011). The incretin effect has been reported to account for 50 - 70% of the total insulin secreted after a meal (Baggio and Drucker, 2007).

1.3.2 Secretion and metabolism of incretin hormones

The two main hormones contributing to the incretin effect are Glucagon-Like Peptide 1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP). Incretin hormones are released autonomously from the entero-endocrine cells of gastrointestinal tract in response to nutrient absorption. Plasma concentration start to rise within 10 - 15 minutes of oral glucose ingestion, reaching a peak at 2 hours, and slowly decline thereafter. They enhance meal stimulated insulin secretion, through a neuro-endocrine pathway (Ahren and Holst, 2001; Drucker and Nauck, 2006).

Dipeptidyl peptidase-4 (DPP-4) enzyme inactivates and degrades incretin hormones by splitting the di-peptide bond from the N terminus (Deacon *et al.*, 1995). The enzyme is soluble in plasma and widely distributed in various tissues. As a result of rapid local and peripheral inactivation, only 10 - 15 % of intact GLP-1 reaches the pancreas (Deacon *et al.*, 1995) Figure 1.2. This suggests that part of their effect may be mediated through the local afferent intestine neurons or through the portal vein. Sympathetic nervous system activation by DDP-4 inhibition has been shown to be associated with postprandial lipid mobilization and oxidation (Boschmann *et al.*, 2009). The inhibition of DDP-4 increases endogenous GLP-1 and GIP concentration and thus potentiates the physiological effect of intact incretin hormones on insulin secretion, resulting in an improvement in glycaemic control. Consequently, DDP-4 inhibitors are now established as a treatment option in type 2 diabetes.
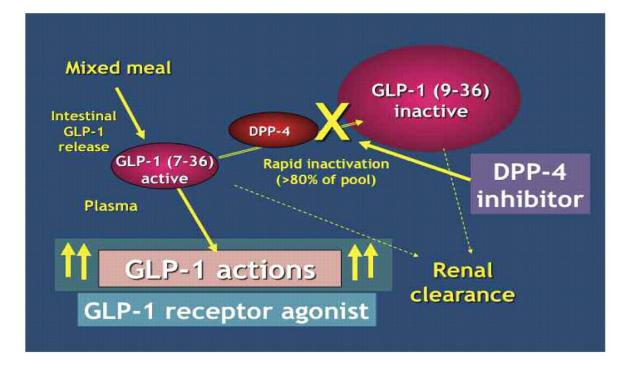
Figure 1.2 Secretion and metabolism of GLP-1 (Deacon *et al.*, 1995)

1.3.3 Glucagon-Like Peptide 1 (GLP-1)

GLP-1 is a pro-glucagon derived peptide (PGDP) derived from the main precursor pro-glucagon (Baggio and Drucker, 2007), which is a product of the glucagon gene translation (Carson *et al.*, 1983). The glucagon gene is expressed on α cell of the pancreas and the neuro-endocrine L cells of the distal intestine (Mojsov *et al.*, 1986).

GLP-1 is secreted from the L cells of the distal intestine in response to nutrient absorption (Eissele *et al.*, 1992). The most important nutrients stimulating GLP-1 release are glucose and triacylglycerol (TAG) (Orskov *et al.*, 1991). Neurotransmitters and peptide such as acetylcholine and gastrin-releasing peptide may contribute to the pulsatile GLP-1 secretion (Herrmann-Rinke *et al.*, 1995; Balks *et al.*, 1997; Rocca and Brubaker, 1999). The rapid rise in GLP-1 immediately after a meal may suggest that the secretion of the incretin hormones is partially mediated by neurotransmitters and neuro-peptides (Ahren and Holst, 2001; Nauck *et al.*, 2011). The proximal intestine may also contribute to GLP-1 release through the neuro-endocrine pathway. Intact GLP-1 diffuses across the basal membrane and lamina propia. On entering the capillaries of the intestine, GLP-1 is rapidly degraded by DPP-4 enzyme, which lines the endothelial cells (Staehr *et al.*, 2001).

1.3.4 L cells

The L cells are predominantly found in the distal colon (Eissele *et al.*, 1992), but they have also been located in the lamina propia of the villi lining the small intestines. They have G-protein coupled receptors, which are specific for different substrates (Brubaker, 2010). The following receptors of the L cells have been identified and described:

- Sweet taste and G-protein (gustducin) receptor: specific for glucose (Jang *et al.*, 2007).

- NEFA or G protein receptor (GPR) 119: specific for long chain monosaturated NEFA (Lauffer *et al.*, 2009).

- GPR40 receptor: specific for long chain saturated fatty acids (Hirasawa *et al.*, 2005; Zhao *et al.*, 2008).

- GPR120 receptor: specific for long chain polyunsaturated fatty acids (Hirasawa *et al.*, 2005; Edfalk *et al.*, 2008).

- GPR131 or TGR 5-GPR131 receptor: specific for bile acids (Evans *et al.*, 2009).

The first four receptors are likely to be directly involved in the stimulation of incretin secretion.

1.3.5 Glucose-Dependent Insulinotropic Polypeptide (GIP)

GIP is a 42 amino-acid peptide (Holst and Orskov, 2004), derived from the glucagon-secretin group of peptide (Takeda *et al.*, 1987). It is predominantly secreted from the K-cells in the duodenum and jejunum (Mortensen *et al.*, 2000; Baggio and Drucker, 2007). The most important stimulant of GIP secretion is dietary lipid, but carbohydrates also contribute significantly (Baggio and Drucker, 2007). A 10 - 20 fold increase in GIP secretion in response to a meal has been documented (Holst and Orskov, 2004). The half-life of GIP in normal subjects is about 7 minutes but it is reduced to 5 minutes in patients with type 2 diabetes (Deacon *et al.*, 2000).

1.3.6 Incretin hormone receptors

Incretin hormones exert their effect by interacting with receptors which are widely distributed in various organs. The GLP-1 receptor can be found on α, β, and δ cells of the pancreas, the central and peripheral nervous system, the lung, the heart, and the gastrointestinal tract (Drucker and Nauck, 2006; Baggio and Drucker, 2007). Although the role of GLP-1 receptors in adipose tissue has been poorly documented, it has recently been suggested that GLP-1 receptor is over- expression in obesity (Vendrell *et al.*, 2011).

The GIP receptor is a transmembrane G-protein predominantly found on β cells of the pancreas, stomach, small intestines, pituitary and adrenal gland, vascular endothelium, bone, central nervous system and adipocytes (Usdin *et al.*, 1993; Yip *et*

al., 1998; Basu *et al.*, 2003; Drucker and Nauck, 2006). After ingestion of a meal, release of incretin hormones occurs and these are transported in plasma to the pancreas. They interact with the receptors on the β cells and stimulate cyclic adenosine monophosphate (cAMP). This allows calcium to flow into the cell, thereby increasing the intracellular calcium concentration, which inhibits ATP-sensitive potassium channels. Intracellular calcium enhances the release of preformed insulin granules by exocytosis (Holst and Gromada, 2004).

1.3.7 Physiological Effects of incretin hormone

The physiological effects of GLP-1 and GIP can be classified as pancreatic and extra-pancreatic.

Pancreatic effects

GLP-1 stimulates glucose-dependent insulin secretion. It may enhance β cell action, by inducing the insulin gene and consequently increasing insulin synthesis. Additionally, it may inhibit β cell apoptosis (Farilla *et al.*, 2003). Throughout life, β cell mass is maintained by equal rates of regenerating new cells and apoptosis. Therefore an imbalance of this relationship with increased apoptosis will lead to lower the β cell numbers. As such, prospective therapeutic options with incretin therapy aimed at reducing β cell apoptosis have been considered.

GLP-1 inhibits glucagon release from the α cells in a glucose-dependent manner as with the β cells (Brubaker, 2010), but it does not affect the counter regulatory effects in hypoglycaemia. GIP enhances glucagon secretion (Baggio and Drucker, 2007; Van Gaal *et al.*, 2008).

Extra-pancreatic effect

GLP-1 has a modest effect on inhibiting gastric emptying, which may contribute to the suppression of appetite (Brubaker, 2010). The inhibition of gastric emptying was seen most clearly during pharmacological elevation of GLP-1 concentration with exenetide therapy. Additionally, it inhibits gastrointestinal fat absorption; stimulates the central nervous system to mediate insulin release and may potentiate post-meal satiety (Baggio and Drucker, 2007). GLP-1 may offer some benefits in the immediate post angioplasty state by improving ventricular systolic function, global and regional wall motion, respectively (Nikolaidis *et al.*, 2004). GIP inhibits gastric acid secretion (Baggio and Drucker, 2007), and also increases fat deposition (Baggio and Drucker, 2007). These hormones have been claimed to have an immune-modulatory and neuro-protective effect. (Ma *et al.*, 2009; Lee *et al.*, 2010).

1.3.8 Incretin effect in insulin resistance and type 2 diabetes

Although the physiological basis for a defect in the incretin effect is elusive, there is sufficient evidence to suggest that the incretin effect is impaired in insulin resistant state and type 2 diabetes. GLP-1 secretion has been found to be reduced in type 2 diabetes after a mixed meal (Toft-Nielsen *et al.*, 2001; Vilsboll *et al.*, 2001) and attenuated in obese subjects after a carbohydrate meal (Ranganathan and Davidson, 1996). Additionally, an accelerated deactivation of incretin hormones in type 2 diabetes has been reported (Kjems *et al.*, 2003). It was previously thought that the defect in incretin system was a feature of pre-diabetic state, rather than a consequence of diabetes. However, the demonstration of a normal incretin effect in first degree relatives of type 2 diabetes patients, when compared to normal controls, may suggest that the defect is a sequel of type 2 diabetes (Nauck *et al.*, 2004).

DPP-4 inhibitors have been shown to decrease fasting lipolysis (Azuma *et al.*, 2008) and postprandial triglyceride (Matikainen *et al.*, 2006), and increase glucose utilization (Ahren *et al.*, 2005) in individuals in type 2 diabetes. However, the underlying mechanism for those changes have not been defined.

1.4 Treatment of type 2 diabetes

Previous treatment of type 2 diabetes was glucose-centric; and therapeutic interventions were mainly focused on reducing fasting blood glucose and HbA$_{1c}$. This was usually achieved with early initiation of insulin therapy at the expense of weight gain. Recent insights into the pathophysiology of type 2 diabetes have revealed that intra-organ lipid accumulation is central to type 2 diabetes, and that the condition can be reversed by decreasing intra-organ lipid accumulation. This heralds a new approach to the management of type 2 diabetes and it is relevant to discuss how present treatment options influence intra-organ lipid accumulation and overall glycaemic control.

1.4.1 Lifestyle modification

Lifestyle modification with weight loss and exercise are fundamental first line therapeutic strategies in the management of impaired glucose tolerance and type 2 diabetes (Knowler *et al.*, 2002). Very often, the clinical relevance of their metabolic benefits on glycaemic control is overlooked or unsustainable by patients in clinical practice. Weight loss can be efficiently achieved by low calorie diet and exercise plays a role in weight maintenance. In some health-motivated subjects, type 2 diabetes can be reversed with weight loss and weight gain can be prevented.

In overweight type 2 diabetes subjects moderate weight loss can improve insulin sensitivity and glycaemic control (Bogardus *et al.*, 1985). Weight loss reduces intra-hepatic lipid content and this is associated with an improvement in hepatic insulin sensitivity (Dube *et al.*, 2011; Lim *et al.*, 2011a). In the latter study, a 30% reduction in liver triglyceride content within the 7 days was associated with normalization of fasting blood glucose; and the reduction in pancreatic fat over 8 weeks was associated with normalization of β cell function. Exercise improves peripheral insulin sensitivity but does not affect hepatic insulin sensitivity (Dube *et al.*, 2011). It is also known that dietary habits are an important risk factor for developing type 2 diabetes (McNaughton *et al.*, 2008). Although the most appropriate dietary composition for maintaining normal glycaemic control remains unclear, there is growing evidence to suggest that a high monounsaturated fatty acid (MUFA) diet may improve insulin sensitivity and β cell function (Lopez *et al.*, 2008).

1.4.2 Biguanide

Metformin is the only biguanide presently used in clinical practice and it is considered as the first drug of choice for the treatment of type 2 diabetes (Inzucchi *et al.*, 2012). Although it has been in clinical use for more than five decades, the exact mechanism of action of metformin remains elusive, and several mechanisms of action have been proposed. It was previously thought that the mechanism of action was mediated through activation of adenosine monophosphate-activated kinase (AMPK) (Foretz *et al.*, 2010), which phosphorylates the CREB binding protein. Phosphorylated CREB binding protein causes the dissociation of the CREB-CBP-TORC2 complex required for the gene expression of gluconeogenic enzymes. Recent work suggests that metformin inhibits the glucagon signalling pathway in the liver mediated by adenylate cyclase; and this results in a reduction in hepatic glucose production (Miller *et al.*, 2013) Figure 1.3.

However, both mechanisms failed to explain a change in hepatic adenine nucleotide levels (Hawley *et al.*, 2002). More recent evidence suggests that metformin reduces hepatic glucose production by inhibiting mitochondrial glycerophosphate dehydrogenase; which regulates both cytosolic and mitochondrial redox states (Madiraju *et al.*, 2014). Metformin decreases non-esterified fatty acid (NEFA) and cholesterol synthesis via AMPK mediated inhibitory effect on the rate limiting steps of the lipid and cholesterol synthetic pathways. Additionally, metformin was found to reduce the risk of macrovascular complications in patients with type 2 diabetes (UKPDS, 1998).

In skeletal muscle, metformin increases glucose uptake by 23% during a hyperinsulinaemic euglycaemic clamp, via the non-oxidative glucose metabolic pathway and GLUT4 translocation (Johnson *et al.*, 1993). An extended release formulation limits some of the gastrointestinal side effects commonly seen with the standard metformin preparation. Liver triglyceride content does not change with metformin therapy (Tiikkainen *et al.*, 2004), and there is no information on the effect on pancreas triglyceride content.

As type 2 diabetes progresses metformin mono-therapy combined with lifestyle modification fails to achieve optimal glycaemic control in about 40 - 50% of patients after 2 years (Brown *et al.*, 2010). Treatment failure rises to 70% after 3 years (Turner *et al.*, 1999; Cook *et al.*, 2007); and 90% after 9 years (Turner *et al.*, 1999). It is often

considered clinically necessary to combine metformin with other oral or injectable anti-diabetic medications, tailored to the individual need of the patient.
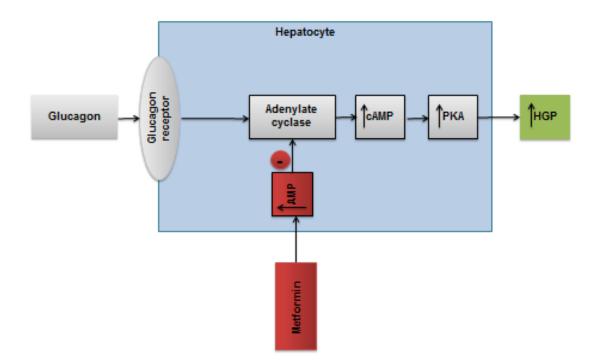
Figure 1.3 AMPK pathway in hepatocyte (Miller et al., 2013)

1.4.3 Sulfonylurea (SU)

SU is usually added if lifestyle modification and metformin fail to maintain adequate glycaemic control. Sulfonylurea promotes insulin secretion by interacting with specific sites on the SU receptor on the β cells of the pancreas. This causes the KATP channels to close, resulting in the accumulation of intracellular potassium concentration and consequently depolarisation of the β cell membrane. Membrane depolarisation causes an influx of calcium into the cells, which in turn enhances the exocytosis of insulin from the vesicle. There is no evidence to suggest that SU stimulates proinsulin biosynthesis. Unlike DPP-4 inhibitors, insulin secretion with SU is not glucose dependent and the risk of hypoglycaemia is greater. This is a particular problem if food intake is irregular, as may be the case for older people. SU have no effect on intra-organ lipid content. Side effects include hypoglycaemia and weight gain. The latter may possibly be as a result of the orexogenic effect of high insulin levels; therefore weight typically rises. It

has been shown that SU does not lower liver lipid content in type 2 diabetes (Phielix *et al.*, 2013). Common side effects associated with SU are hypoglycaemia and weight gain.

SU increases endogenous insulin production irrespective of glucose concentration; as such the liver is exposed to high concentration of insulin compared to the peripheral metabolic organs. Additionally there is an increase flux of lipids to the liver in type 2 diabetes. In the presence of hyperinsulinaemia and increase lipid flux, it is likely that *de novo* lipogenesis may increase modestly with SUs. This will happen only as long as the β cells are able to produce such elevated levels of insulin. In contrast with exogenous therapeutic insulin administration, there is no portal - peripheral gradient of insulin concentration and hence this is relatively lower in the hepatic circulation compared to that in the periphery. Incretin based therapies decrease lipid flux to the liver and reduce hyperinsulinaemia as a result of increased sensitivity to the β cells. These fundamental differences in mechanism of action may account for the differences in liver triglyceride with SU compared to therapeutic insulin and incretin based therapies.

1.4.4 Thiazolinedione (TZD)

TZD is an agonist for the nuclear receptor, peroxisome proliferate activator receptor ɣ (PPAR ɣ). Activated PPAR ɣ increases the transcription of specific genes which promote insulin sensitivity. PPAR ɣ are expressed abundantly in the adipocytes but they are sparsely located in skeletal muscle (Vidal-Puig *et al.*, 1997). Even though the distribution of PPAR ɣ receptors has led to some debate about the site of action of TZD, there is enough evidence to suggest that in type 2 diabetes the predominant site of action is the liver. Ravikumar *et al.*, demonstrated that a 50% reduction in hepatic fat with pioglitazone was associated with a 26% suppression of fasting hepatic glucose production and a two-fold reduction of postprandial hepatic glucose production (Ravikumar *et al.*, 2008). In addition, pioglitazone decreases glucagon and NEFA concentration in type 2 diabetes (Ravikumar *et al.*, 2008) (Basu *et al.*, 2008). Pioglitazone reduces intramyocellular lipid (IMCL) by 34% in subjects with impaired glucose tolerance, associated with a two-fold increase in peripheral insulin sensitivity during a glucose tolerance test (Rasouli *et al.*, 2005). In type 2 diabetes, a 55%

reduction in IMCL has been reported (Ravikumar *et al.*, 2008). The significant decrease in visceral fat to subcutaneous fat ratio, coupled with weight gain, suggests that TZD predominantly improves insulin sensitivity by mobilising and redistributing visceral and intra-hepatic fat to the subcutaneous tissues. The common side effects of TZD include water retention, oedema and weight gain.

1.4.5   Sodium glucose co-transporter 2 (SGLT2) inhibitor

SGLT2 inhibitor is a new class of oral hypoglycaemic agent. The mechanism of action is independent of insulin secretion and insulin resistance. SGLT2 inhibitor is a potent competitive inhibitors of the SGLT2 receptors located in the proximal tubules of the kidney, therefore preventing glucose reabsorption (Han *et al.*, 2008; Grempler *et al.*, 2012), as such renal glucose excretion is increased and plasma glucose falls. It can either be used as mono-therapy or combined with other anti-diabetes medications. Combined therapies with SGLT2 inhibitor and metformin have a low risk of hypoglycaemia, improve HbA_{1c} by 0.7%, and achieve weight loss of 2.5kg (Haring *et al.*, 2014). The weight loss achieved with SGLT2 therapy (Zhang *et al.*, 2010), either alone or as part of a combined therapy, may have an indirect effect in reducing hepatic steatosis by increasing calorie loss through the kidneys (Tahara *et al.*, 2013). The common side effects are urinary tract and genital infections, hypoglycaemia, and dehydration.

1.4.6   Bariatric surgery

Bariatric surgery is now recognised as a therapeutic option in obese type 2 diabetes to bring about durable weight loss, reduction in intra-organ lipid content and decrease in blood glucose concentration for the long term. It is a safe surgical procedure performed to reduce the size of the stomach and oral calorie intake with the aim of achieving weight loss in obese patients with or without diabetes. Remission of type 2 diabetes, or a significant improvement in glycaemic control, was observed in a majority of obese type 2 diabetes patients after bariatric surgery (Buchwald *et al.*, 2009). Marked weight loss after bariatric surgery in obese type 2 diabetes subjects has been

found to be associated with resolution of hepatic steatosis in more than 80% of cases; and this was associated with an improvement in other metabolic parameters (Clark *et al.*, 2005; Furuya *et al.*, 2007). The benefits of bariatric surgery are maintained long after surgery as weight gain is inhibited by calorie restriction. Weight loss of about 25% of body weight has been found to be associated with significant reduction in cardiovascular risks 12 months after surgery (Schauer *et al.*, 2012). Additionally, up to 42% of patients were free from type 2 diabetes at 1 year (Schauer *et al.*, 2012) and 38% of patients at 3 years (Schauer *et al.*, 2014). It was previously considered that the improvement in metabolic indices observed with bariatric surgery might be mediated through the incretin hormones (Laferrere *et al.*, 2007). On the contrary, there is growing evidence to suggest that the improvement in glycaemic control post bariatric surgery is mediated through a sudden reduction in calorie intake resulting from the procedure (Gumbs *et al.*, 2005; Lingvay *et al.*, 2013). Common side effects after bariatric surgery are dumping syndrome, hypoglycaemia, and vitamin and mineral deficiencies.

1.4.7 GLP-1 agonist

GLP-1 agonist is a synthetic analogue of the exendin-4 hormone, which is resistant to DPP-4 degradation. Exendin-4 hormone was originally isolated from the salivary gland of the gila monster, Heloderma suspectum. GLP-1 agonist enhances glucose-stimulated insulin secretion (Fehse *et al.*, 2005), suppresses glucagon secretion (Kolterman *et al.*, 2003), decreases gastric emptying and postprandial hyperglycaemia (Zhao *et al.*, 2008) and potentiates weight loss in obese subjects with or without type 2 diabetes (Brons *et al.*, 2009; Vilsboll *et al.*, 2012). In addition, GLP-1 agonist may have some benefits on cardiovascular risk factors.

GLP-1 agonist has been found to have a clear effect on reducing hepatic lipid content. In subjects with type 2 diabetes, 24 weeks of GLP-1 agonist treatment reduced intra hepatic lipid content by 42% (Cuthbertson *et al.*, 2012). In a case report, a 66% reduction in hepatic lipid content was observed in a poorly controlled type 2 diabetes patient after 44 weeks of treatment with GLP-1 agonist (Tushuizen *et al.*, 2006).

GLP-1 agonist is administered by subcutaneous injection and its low risk of hypoglycaemia presents a favourable choice as either monotherapy or in combination with either metformin, SU, TZD or with insulin. Exenetide and pioglitazone have been

found to have a greater effect in the reduction of hepatic triglyceride content compared to pioglitazone only treatment (61% vs. 41%) (Sathyanarayana *et al.*, 2011). It has been suggested that GLP-1 agonist promotes tissue lipolysis and this may possibly account for the reduction in liver triglyceride accumulation (Vendrell *et al.*, 2011). The most common side effects associated with GLP-1 agonist are nausea and vomiting. Commencing GLP-1 agonist at a lower dose reduces the potential risk of side effects.

1.4.8 DPP-4 inhibitor

DPP-4 inhibitor enhances insulin secretion from the β cells only in a glucose-dependent manner such that they do not enhance insulin secretion when blood glucose is low. In addition, it has a modest ability to improve the ability of α cells to sense hypoglycaemia and respond appropriately with an increased secretion of glucagon (Ahren *et al.*, 2009). These unique characteristics account for the low risk of hypoglycaemia, which would otherwise drive defensive eating as a compensatory mechanism. This may be the major reason why weight gain is not associated with the DPP-4 inhibitors. They can be regarded as weight neutral drugs (Ahren *et al.*, 2010). There is sufficient evidence to suggest that maximum clinical benefits are attained when a DPP-4 inhibitor is combined with metformin. Weight gain is negligible and glycaemic control is comparable to that of other agents (SU, TZD) when used singly or in combination with metformin (Ahren *et al.*, 2004; Bosi *et al.*, 2007). The effect of DDP-4 inhibitor on human liver, muscle and pancreas triglyceride accumulation has not been previously studied.

Vildagliptin is the DPP-4 inhibitor examined in this thesis. It is administered orally and was approved for clinical use in 2007 (Nauck *et al.*, 2011). It exerts similar pharmacokinetics in normal subjects and in patients with type 2 diabetes (Scheen, 2010). Vildagliptin is a nitrile-based compound which forms a covalent bond with the catalytic site of the DPP-4 enzyme. This process involves two steps: the formation of a reversible enzyme-inhibitor complex followed by slow dissociation of the covalent bond (Nauck *et al.*, 2011). The slow dissociation of the vildagliptin-DPP-4 bond explains the increased potential of this drug to augment the amounts of intact endogenous GLP-1 and GIP concentration (Ahren *et al.*, 2011). GLP-1 and GIP increase the sensitivity of the α- and β-cells to glucose and this is accepted as its major mechanism of action (Ahren *et al.*, 2011; JE, 2014). Vildagliptin is known to reduce or delay postprandial lipid absorption (Matikainen *et al.*, 2006) as well as reduce postprandial triglyceride concentration and lipolysis (Matikainen *et al.*, 2006; Derosa *et al.*, 2014).

1.5 Magnetic Resonance Imaging

Nuclear magnetic resonance (NMR) is a physical phenomenon that arises from the interactions between an atom's nucleus and a magnetic field. This phenomenon is the fundamental principle of magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), which allows the detection and identification of some nuclei/metabolites. As tissues differ in composition and physical properties, NMR can be used to differentiate them and also provide a non-invasive, non-ionising method of obtaining images and making *in vivo* measurements of metabolites in humans. These unique properties make it well suited to the present purpose of performing repeated measurements of liver and pancreas fat content, liver and skeletal muscle glycogen concentration, and pancreas volume.

1.5.1 BASIC PRINCIPLE OF NMR

An atom consists of negatively charged electrons revolving around a nucleus that is made up of neutrons (neutrally charged) and protons (positively charged). Some nuclei possess a magnetic property called 'spin', which results in an interaction between the nucleus and a scanner's magnetic field. Nuclei with spin will resonate (absorb) energy, allowing detection by the scanner at a frequency determined by the strength of the scanner's magnetic field, the type of nucleus and the chemical identity of the molecule containing the nucleus. NMR allows us to identify and quantify metabolites in a tissue from their different resonant frequencies.

When a patient is positioned in a scanner, the nuclei of metabolites interact with the scanner's magnetic field by aligning its field to that of the scanner. Applying a radio-frequency (RF) pulse specific for a particular nucleus through a transmitter coil excites the nucleus. This will consequently produce a signal, which can be detected by the receiver coil. The NMR signal detected by the scanner is called Free Induction Decay (FID); it represents the oscillating signals from the nuclei in the sample. Fourier transformation of the FID produces a spectrum, which is a plot of signal intensity against frequency. The spectrum shows signals from the nuclei with different resonant frequencies as individual peaks, thus separating signals from different molecules in a tissue Figure 1.4 (Butler, 2002).
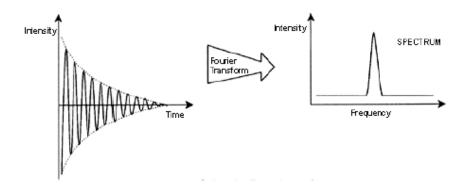
Figure 1.4 Signal emitted by an excited nucleus displayed in free induction decay (FID) format and transformed to intensity-frequency domain by Fourier transformation (Butler, 2002)

The area under the peak can be used to calculate the concentrations of the metabolite of interest. The concentration of the metabolite can be determined by either comparing it with that from a standard solution (phantom), with known concentration of the metabolite of interest, or an intrinsic compound, with known concentration, e.g. water peak of skeletal muscle.

1.5.2 FUNDAMENTAL PROPERTIES OF NMR SIGNAL

Chemical shift, spin-spin coupling and relaxation are NMR properties determined by the nuclei, metabolite and tissue that influence the detected NMR signal.

1.5.2.1 Chemical shift

Chemical shift is a characteristic that allows us to distinguish between different molecules / chemicals that contain the same nuclei. The resonant frequency of a nucleus is determined by the chemical composition of its molecules as well as the magnetic field of the scanner, as the molecule alters the magnetic field at the nucleus. To illustrate this concept, ^{1}H NMR spectrum of acetic acid (CH_3COOH) can be considered. The protons from the methyl ($-CH_3$) moiety resonate at a higher frequency than the proton of the carboxyl ($-COOH$) moiety, because the moieties experience a different chemical environment Figure 1.5. The signal intensity is also dependent on

the number of nuclei contributing to that signal (three protons from the -CH3 moiety and one from the -COOH moiety). Therefore, the intensity from the -CH3 moiety is three times greater than that from the -COOH moiety. Additionally, the electrons continuously revolving around the nucleus provide a shielding effect to the nuclei. The shielding effect can be described as the magnetic field effect of the electrons surrounding the carbon nucleus, hence, the magnetic effect of the carboxyl group on its carbon nucleus will exert a greater shielding effect, contributing to the higher resonance frequency of the -COOH moiety when compared with the signal frequency of the -CH3 moiety, which has less shielding effect. The relative displacement of the spectral peaks is measured in parts per million (ppm).
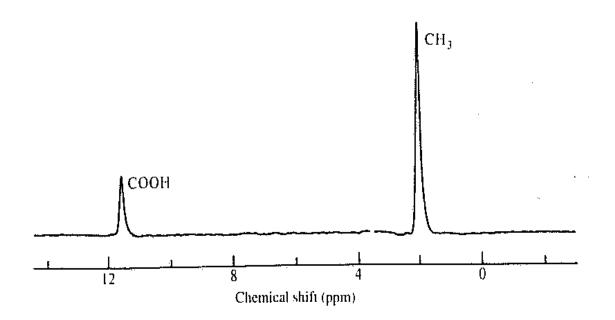
Figure 1.5 ^{1}H NMR spectrum of acetic acid (CH_3COOH) showing relative areas of the signal with a ratio of 3:1 (Gadian, 1985)

1.5.3 Chemical shift

Chemical shift can be used to differentiate between triglyceride within the myocytes i.e. intramyocellular lipids (IMCL) and that out of the myocytes i.e. extramyocelluar lipids (EMCL). The alignment of muscle fibres will influence the magnetic field around the IMCL and consequently produce a different lipid proton resonant frequency from

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that of EMCL. This has enabled investigation of the effect of IMCL on insulin sensitivity in skeletal muscle.

1.5.3.1 Spin decoupling

When a RF pulse excites ^{13}C nuclei in carbon compounds, the signal produced contains multiple peaks for a carbon nucleus that is covalently bonded to one or more hydrogens. This phenomenon, referred to as spin-spin coupling, results from the interaction between the magnetic fields of each proton, their neighbouring ^{13}C atoms and the scanner. The complex multi-peak spectrum can be simplified by applying a ^{1}H RF pulse during ^{13}C signal acquisition. This process is referred to as spin decoupling. Spin decoupling is used in ^{13}C spectroscopy to simplify the spectra and aid identification and quantification of carbon-based metabolites such as glycogen.

1.5.3.2 Relaxation

When protons are excited by a radio-frequency pulse (RF), they absorb energy and resonate, generating the NMR signal. The protons relax back to equilibrium by two processes: T1 and T2 relaxation. T2 relaxation represents the de-phasing of spins after their phase coherence; and T1 represents the return of the nuclei to their original realignment / energy levels after excitation by the RF pulse.

1.5.4 The effect of noise on signals

During acquisition of NMR images, the receiver coil detects signal from the sample and radiofrequency noise. In humans, normal physiological activities of charged ions such as nerve conduction or cell function creates a current. The current generates an alternating magnetic field which produces a noise signal that can also be detected by the receiver coil. Noise produced by these electrical activities described is displayed together with the signal from the nuclei of the desired molecule, hence the images and measurements of the metabolites of clinical interest are obscured. Increasing the

magnetic field strength can increase the magnitude of the metabolite signal relative to the noise, and scan protocols used for the study are optimised to ensure sufficient data quality can be obtained.

1.5.5 Surface coils

Surface coils are sensors used to detect signals from nuclei in a defined volume of tissue such as the liver, pancreas and skeletal muscle. They are held in place directly over the anatomical area and are required to be perpendicular to the scanner's magnetic field for signals to be detected. Surface coils are used to study organs close to the skin because the intensity of received MR signals decreases as the distance from the coil increases. In the studies required in this thesis, surface coils were used to measure liver and skeletal muscle glycogen concentration.

1.5.6 Magnetic Resonance Spectroscopy

In metabolic studies, MRS is a safe, non-invasive alternative to organ biopsy to measure glycogen and lipid concentration in the liver and skeletal muscle. This property enables the assessment of the effect of a new DPP-4 inhibitor on dynamic glycogen storage in skeletal muscle and liver, and measure any change in lipid concentration in skeletal muscle.

1.5.6.1 ^{1}H nucleus and MRS

^{1}H spectroscopy can be used to identify and measure the concentration of lipid in the different cellular components (intramyocellular and extramyocellular) of skeletal muscle (Schick et al., 1993). ^{1}H has a natural abundance of 99.98% and is the most sensitive nucleus for MRI compared with that of ^{13}C and ^{31}P. ^{1}H signal from fat and water can be adequately obtained and quantified in man, given that the human body contains fat and water, both of which are proton rich. ^{1}H spectroscopy is well suited to

measure and quantify the fat content of muscle in type 2 diabetes and also to evaluate the effect newer therapies such as DPP4-inhibitors may have on these fat stores.

The coefficient of variation for measuring IMCL with ^{1}H spectroscopy in obese and non-obese subjects is 7.9% and 11.8%, respectively (Schick *et al.*, 1993). ^{1}H spectroscopy provides a feasible and accurate methodology for quantifying IMCL when compared to other conventional methods such as electron microscopic morphometry and biochemical analysis from muscle biopsy (Schick et al., 1993).

1.5.6.2 ^{13}C MR spectroscopy

^{13}C spectroscopy can be used to measure glycogen concentration in liver and skeletal muscle. ^{13}C has a natural abundance of 1.1% and is less sensitive than ^{1}H nucleus in MRS. As the MR signal from glycogen is small, the measurement of liver glycogen is extremely difficult in obese subjects with significant subcutaneous fat, as this increases the separation between RF coil and the liver. Measuring glycogen concentration with ^{13}C MR spectroscopy methodology has been validated against direct biochemical assay of muscle biopsy, and a very strong correlation between the two methodologies has been demonstrated (r = 0.95; p< 0.0001) (Taylor *et al.*, 1992). Additionally, ^{13}C MR spectroscopy was shown to be more precise than biochemical assays of needle biopsy with a coefficient of variation of 4.3 ± 2.1% compared with 9.3 ± 5.9% with the biopsy (Taylor et al., 1992).

1.5.7   Image-based measurements of tissue fat content: Three-point Dixon MRI

Based on the chemical shift differences between fat and water protons, MRI can be used to quantify intra-organ fat content. The Dixon method exploited these concepts and employed a two-echo approach (in-phase and out-of phase) known as two-point Dixon technique to acquire separate fat and water images (Dixon, 1984). Over the years, this method has been extended to a three-echo approach to acquire fat and water images corrected for homogeneity of the magnetic field, B_0 and poor shimming (Glover and Schneider, 1991). Dixon MRI of the liver requires a breath hold during acquisition of images, thus preventing contamination of the liver and pancreas MR

signal by a signal from surrounding structures. The Dixon method allows separation of fat and water signals to accurately quantify the fat portion in organs. It provides sufficient spatial resolution and anatomical detail from which the volume of organs or intra-organ fat can be estimated. Figure 1.6 shows images obtained by the three-point Dixon method with water percentage and fat percentage images of both the liver and pancreas, respectively. It has been demonstrated that there is a strong correlation and agreement between the Dixon method and MRS methodology in quantifying liver fat (r = 0.95; p <0.0001) and pancreas fat (r = 0.51; p < 0.02) (Hu et al., 2010). It was suggested that the lower correlation co-efficient observed with pancreas fat measurement was due to technical difficulty and poor operator expertise in identifying the region of interest in the pancreas with the Dixon method while movements with respiration contributed modestly (Hu et al., 2010). To circumvent this problem, operators require to be fully trained in drawing regions of interest (ROIs) in the Dixon images and need to have background knowledge in human anatomy. Also, the MRS methodology does not offer the luxury of identifying the structure from which the spectra was obtained, making it impossible to identify any contamination from neighbouring structures. Respiratory movements are very likely to allow visceral fat to enter the ROI identified for signal collection from the pancreas. There was no statistically significant bias between the two methods in measuring liver fat. The bias between Dixon and MRS methodology in measuring liver fat calculated with a Bland-Altman plot, was reported as 0.38% (95% confidence interval (-3.5% to 4.3%, p = 0.4) (Hu *et al.*, 2010). The reproducibility of liver and pancreas fat measurement with the Dixon technique is higher than that with the MRS methodology, thus making it suitable for our study requiring repeated measurements. Tushuizen *et al.*, reported a Bland-Altman repeatability coefficient of variation for liver and pancreas fat analysis with MRS as 4.7% and 15.2%, respectively (Tushuizen *et al.*, 2007). Additionally, in another study, Lim *et a*l., also reported that the repeatability coefficient of variation for liver and pancreas fat measurements with the Dixon technique was 0.5% and 0.9%, respectively (Lim *et al.*, 2011).
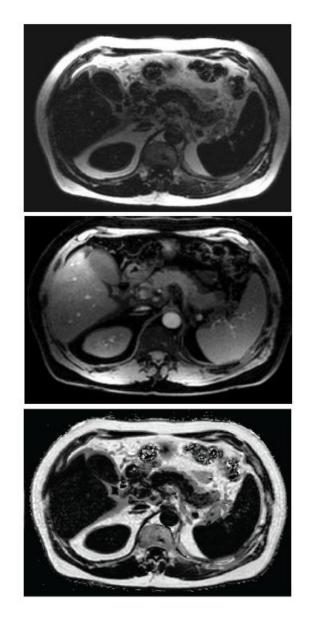
Figure 1.6 Demonstrates three point Dixon images of the liver and pancreas with fat (top), water (middle) and percentage of fat in MR signal (bottom) of the liver and pancreas

1.5.9 Balanced Steady State Free Precession (BTFE) Images

As the measurement of pancreas volume with NMR has not been previously documented, new techniques for measuring pancreas volume with Balanced Steady State Free Precession (BTFE) structural MRI scans were devised. Application of magnetic resonance technology to the pancreas is in its infancy. However, it holds great promise as a tool to investigate both changes in pancreas volume in response to therapy and long term changes during the natural history of type 2 diabetes.

Although type 2 diabetes does not develop without dysfunction of β cells, the pancreas is the least studied organ in this condition.

1.6   Aim of the study

The aim of this work is to elucidate the overall physiological effects of the DPP-4 inhibitor, vildagliptin in view of suggested effects on insulin sensitivity and lipid metabolism.

Specifically, effects upon insulin sensitivity of the liver and skeletal muscle were to be evaluated using the euglycaemic hyperinsulinaemic clamp and isotopically labelled glucose infusion. Both oxidation and non-oxidative metabolism must be examined to allow understanding of the mechanism of action, as any change in insulin sensitivity of a tissue may be caused by change in intracellular fatty acid availability. The triglyceride content of liver and skeletal muscle was to be examined using magnetic resonance methodology.

The question of whether DPP-4 inhibition could affect progression of the disease towards the need for insulin therapy has been raised. The possible effect of DPP-4 inhibition on pancreas fat content was to be defined. To achieve this aim, a group of 44 patients with type 2 diabetes was studied over a 6-month period. Successful recruitment and retention of volunteers was central to the success of the whole project.

CHAPTER 2 METHODS

2.1 Research subjects

2.1.1 Subject recruitment

A double blinded, controlled, randomised trial was designed in order to evaluate possible additional mechanism of a DPP-4 inhibitor (vildagliptin). As vildagliptin takes about 3 - 4 months to exert maximum clinical benefits, the type 2 diabetes subjects were studied for 6 months to ensure that assessments were made after optimal benefits of vildagliptin were achieved Figure 2.1. A control group of glucose tolerant subjects was also studied on one occasion to define the extent of change toward normal observed during vildagliptin therapy in the type 2 diabetes group. Normal glucose tolerance was demonstrated in all control subjects by normal response to 75g oral glucose tolerance test (mean fasting plasma glucose 5.3±0.1mmol/l; 2h 5.5±0.4mmol/l).

Inclusion criteria for the type 2 diabetes group were: $HbA_{1c} \leq 7.6\%$ (60mmol/mol), a BMI of 22 - 38kg/m^2, age 18 - 70 years and at least a 6 month history of type 2 diabetes. Eligible subjects must have been on a stable metformin dose of at least 1000mg 4 weeks prior to the screening visit and have been taking metformin for at least 3 months. Exclusion criteria were: previous insulin therapy and chronic oral or parenteral steroid therapy; significant ischaemic heart disease in the previous 6 months; kidney dysfunction indicated by creatinine levels ≥132μmol/l for males and ≥123μmol/l for females; liver impairment indicated by alanine aminotransferase (ALT) >90U/L and or aspartate aminotransferase (AST) >82U/L, total bilirubin >42μmol/l and or conjugated bilirubin >7μmol/l; fasting triglyceride >5.62mmol/l; ventricular arrhythmias; second and third degree AV block or prolonged QTc. It is worth noting that a cut off HbA_{1c} >7.6% (60mmol/mol) was chosen to avoid any big change in plasma glucose concentration. Subjects were recruited by newspaper adverts in Tyne and Wear and Durham. In addition, searches from General Practice (GP) and primary care research network databases in the same regions were carried out to identify potential volunteers.

For the normal control group, glucose tolerant subjects were recruited to match the weight, BMI sex and age of subjects in the diabetic group. These volunteers were similarly recruited by newspaper adverts and only had baseline MR studies.
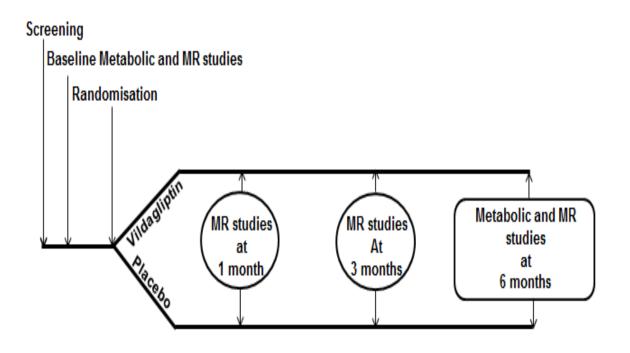
Figure 2.1 General overview of the study design for type 2 diabetes subjects

2.1.2 Informed consent procedures

Subjects wishing to participate in the study expressed their interest via telephone or by returning reply slips. Patient information sheets containing full description of the study were mailed out. They were then offered an informal visit to the Newcastle Magnetic Resonance Centre (NMRC) to discuss the study and matters arising from the information sheet. If they agreed to participate, subjects were invited for a screening visit, during which further explanation was given. Informed, written, witnessed consent was obtained. Prior to obtaining informed consent, it was explained that withdrawal from the study at any time was permitted and signing of the consent form did not oblige binding them to participate. Out of 65 volunteers screened, 44 met the inclusion criteria and were enrolled in the study. The most common reason for exclusion was $HbA_{1c} \leq$ 7.6% (60mmol/mol). The study protocol was reviewed and approved by the Newcastle and North Tyneside 2 Research Ethics Committee.

2.2 Statistical methods

Statistical analysis was performed with Graphpad Prism 6.0d software. Data are expressed as mean±SEM. Students two-tailed paired t-test or the Mann-Whitney test, and one-way ANOVA were used to compare groups as appropriate. Pearson correlation was used to assess relationship between variables. Statistical significance was defined as $p<0.05$.

Sample size calculation was based on detecting a difference in the mean change from baseline to 6 months in whole body insulin sensitivity (Rd) during the hyperinsulinaemic euglycaemic clamp between the metformin/vildagliptin treated and metformin/placebo treated group. 30% (1.65mg/kg/min) increase in Rd in the metformin/vildagliptin treated was considered clinically significant. Standard deviation was assumed as 1.55kg/mg/min (Bogardus *et al.*, 1984), 2 sided significant level of 5% and a desired power of 90% was considered appropriate. A sample size of 40 patients was allocated to either metformin/vildagliptin treated and metformin/placebo treated group in a 1:1 ratio. Additionally 4 patients were recruited to allow for a 10% drop out rate without any significant effect on the power of the study. Power calculation were performed using the nQuery Advisor® version 6.01 software. Based from observation from a previous study (Azuma *et al.*, 2008), it was felt that 6 months of treatment was appropriate to

examine the effect of vildagliptin on whole body insulin sensitivity and also investigate chronic changes in triglyceride and glycogen storage in the liver and muscle.

2.3   Anthropometric measurements and body composition

2.3.1   Body Mass Index

Subjects were weighed using an accurate weighing scale (SECA SCALES, Germany) wearing indoor clothing only. Prior to weighing, it was ensured that the scale was horizontal by the use of an integral spirit level and was not disrupted during measurements. Height was measured with a stadiometer (SECA SCALES, Germany) and BMI was calculated as weight (kg) divided by the square of height (m^2).

2.3.2   Waist-hip ratio

All measurements were made with a non-distensible tape measure, with subjects in a relaxed standing position. The waist was assumed to be the mid-point between the lower edge of the ribcage superiorly and the anterior superior iliac spine inferiorly. Hip measurements were taken at the level of the greater trochanter. The waist and hip measurements were expressed as a ratio.

2.3.3   Bio-electrical impedance

Bioelectrical impedance was used to determine percentage body fat with a Bodystat 1500 machine. This required input of the height, weight and activity level of the volunteer. This technique depends on measuring the resistance to a very small current applied through the electrodes. The resistance of the tissues is proportional to the water content as the electrical current is conducted through the electrolyte solution. Fat does not contribute to this, therefore the impedance of water and fat is differentiated and can be measured. The repeatability of the Bodystat 1500 machine was assessed for a single volunteer on 10 different occasions. The intra-subject coefficient of variation (CV) was calculated by dividing the standard deviation of these results by the mean, then multiplying by 100 to express as a percentage. The CV was found to be 6.0%.

2.4 Metabolic studies

2.4.1 Cannulation and blood sampling

An 18 gauge cannula was inserted into a vein in the subject's hand or wrist, and flushed with 5mls of 0.9% normal saline to maintain patency of the cannula. This hand was placed in an in-house built hand-warming device, heated to 55°C to achieve arterialisation of venous blood. Discarding the initial 2mls of blood withdrawn and using a new syringe to obtain blood samples, the dilution of samples with saline flushes was avoided. The samples were centrifuged and separated; plasma was stored immediately at -40°C at the NMRC and later transferred to the Diabetes Research Laboratory in ice for assaying of plasma insulin, glucagon, NEFA, triglyceride, atom per cent excess (APE) of 6' 6' - dideuterated glucose. A second 18 gauge cannula was inserted in a large ante-cubital vein in the contra-lateral arm to administer infusions (6' 6' - dideuterated glucose, insulin and 10% Dextrose as appropriate). Care was taken to minimise any discomfort to the subject during cannulation.

2.4.2 Combined metabolic investigations

Subjects were advised to abstain from vigorous exercise and smoking for 3 days prior to metabolic test days. On a metabolic test day combined assessments for hepatic glucose production (HGP), glucose and lipid oxidation were made at specific time points. Hepatic insulin sensitivity and whole-body insulin sensitivity (Rd) were calculated from measuring plasma tracer concentration and glucose infusion rate, respectively, as described Figure 2.2. A bolus of 10% 6' 6' - dideuterated glucose was given as the priming dose at time -180 minutes. The 2% 6' 6' - dideuterated glucose infusion was commenced immediately and continued to the end of the insulin infusion period (time 180 minutes). During the last 30 minutes of both the basal and insulin infusion period, it was assumed that a steady-state milieu is achieved, thereby allowing assessments to be made. Samples for basal HGP, plasma glucose, insulin and glucagon analysis were obtained over the last 30 minutes of the basal period (from -30 minutes to 0 minutes) and the insulin infusion period (time 150 minutes to 180 minutes). Similarly, indirect calorimetry for measuring glucose and lipid oxidation was done during the last 20 minutes of the basal period (from time -20 minutes to 0 minute)

and insulin infusion period (from time 160 minutes to 180 minutes). Insulin infusion was commenced at time 0 minute and continued for 180 minutes.

Hepatic insulin sensitivity was calculated as the difference between HGP measured during the basal and insulin infusion periods, expressed as a percentage. The glucose infusion rates recorded during the last 30 minutes of the insulin infusion period was used to calculate Rd.
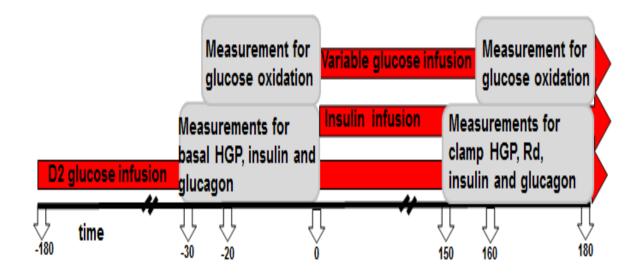
Figure 2.2 Metabolic test day demonstrating the time points for measuring HGP (basal and clamp), glucose oxidation and peripheral insulin sensitivity (Rd)

2.5 Hepatic glucose production

2.5.1 Clinical procedure

The quantification of hepatic glucose production in the basal and hyperinsulinaemic periods serves as a useful marker for assessing hepatic insulin sensitivity. The preferred method of assessing hepatic glucose production with tracers is now established. 6' 6' - dideuterated glucose was infused from 2.5 hours before samples were taken for analysis in the basal state, thus allowing proper equilibrium with the subject's plasma. During steady-state conditions the rates of glucose utilization and production are equal and consequently plasma glucose concentration remains stable. Endogenous glucose concentration was calculated by subtracting the calculated exogenous component and 6' 6' - dideuterated glucose concentration from the measured total glucose concentration. During the hyperinsulinaemic phase 6' 6' - dideuterated glucose was infused as in the basal state. In the clamp phase unlabelled glucose is added to the plasma pool by variable 10% Dextrose infusion (known) and by HGP (unknown), hence HGP can be calculated.

Under sterile conditions, 6' 6' - dideuterated glucose was made up to 10% strength solution. In order to achieve early steady-state isotope enrichment in plasma, a priming dose of 10% strength 6' 6' - dideuterated glucose adjusted for fasting blood glucose was given to the subjects at the start of the basal period (Hother-Nielsen and Beck-Nielsen, 1990; Ravikumar *et al.*, 2008). The priming dose was calculated as fasting plasma glucose multiplied by 0.6. The rest of the 10% strength 6' 6' - dideuterated glucose solution was made up to 2% strength. After infusing the bolus, 2% strength 6' 6' - dideuterated glucose was infused at 0.04mg/kg/min until the end of the insulin clamp period to achieve equilibrium of plasma atom per cent excess (APE) of 6' 6' - dideuterated glucose. A 1ml sample of the 2% strength 6' 6' - dideuterated glucose labelled 'NINF' was taken for analysis for tracer enrichment (enrichment$_{inf}$). The 10% glucose used to maintain euglycaemia during the clamp was enriched with 2% 6' 6' - dideuterated glucose to prevent loss of plasma atom per cent excess (APE) of 6' 6' - dideuterated glucose. A 1ml of 10% glucose enriched with 2% 6' 6' - dideuterated glucose labelled 'GINF' was taken for analysis.

2.5.2 Gas chromatography mass spectrometry analysis

Frozen plasma samples for 6' 6' - dideuterated glucose were allowed to thaw for 30 minutes and then vortex mixed to ensure homogeneity of the sample. 200µl of acetonitrile:ethanol (2:1) was added to 30µl of thawed plasma sample. This solution was vortex mixed again, then spun in a micro-centrifuge at 13000rpm for 5 minutes at room temperature, to facilitate complete precipitation of plasma proteins and separation of the supernatant. 200µl of supernatant was pipetted into a 5ml glass vial, which was then transferred into a thermoblock sited in a fume hood. The thermoblock was set at 90ºC and gentle steam of dry air was made to pass through the thermoblock from the top of the 5ml glass tube for 45 minutes to ensure complete dryness of the 5ml glass vial. The 5ml glass vial was then removed from the thermoblock, but held in the fume hood. 60µl of 99.8% anhydrous Pyridine (SIGMA, St Louis USA) and 20µl 98% anhydrous Acetic Anlydride (SIGMA, St Louis USA) was added to the 5ml glass vial in succession. The 5ml glass vial was capped tightly, agitated and transferred to an incubator set at 90ºC to dry for 30 minutes until a pale yellow hue in the vial was noted. The 5ml glass vial was re-transferred to the fume hood, de-capped and put in the thermoblock to dry for 5 minutes. The 5ml glass vial was removed from the fume hood and allowed to cool at room temperature for 5 minutes. 75µl of Acetoniitrile (SIGMA, ST Louis USA) was added to the 5ml glass vial and gently agitated. 20µl of solution was transferred from the 5ml glass vial to a mass spectroscopy vial (CHROMACOL 03-FIV, UK) containing 80µl of Acetonitrile (SIGMA, ST Louis USA) to allow 1:5 dilution. The 1:5 dilution was essential to prevent overloading of the column in the mass spectrometer.

 A Thermo 'Voyager' single quadruple mass spectrometer, attached to a Thermo 'Trace' gas chromatography with automated injection Thermo 'AS2000' auto sampler (Thermo Scientific – Waltham MA, USA) was used for gas chromatography mass spectrometry analysis. The Xcaliber version 1.3 (Thermo Finnigan 1998-2001) software was used to make automated settings.

The auto sampler was set as follows: volume of sample injected 3µl, injection delay 0.2 seconds, injection speed 50µl/sec. The auto sampler was set to complete a series of 2 pre-injection and 5 post injection washes with 5µl of Ethyl Acetone (BDH Chemicals Ltd, Poole UK) each time. The split mode was set at 30:1 and the injection port was maintained at 220ºC.

The gas chromatography was set as follows: gradient length 12.5 minutes, preparation run time 10 minutes; equilibration time 30 seconds; temperature range of 200ºC - 350ºC. Two ramps were set to run at 5ºC/min and 30ºC/min, respectively. Helium was used as a carrier gas and the flow rate was set at 1ml/min.

The mass spectrometer was set in electron ionisation mode with the following settings: source temperature 200ºC, interface temperature 250ºC, multiplier voltage 450 volts, scan time 0.05 seconds, peak format centroid. The full scan method was used to detect all ions. The Qual Browser software was used to filter, identify and record ion mass at 200 (normal glucose) and 202 (6' 6' - dideuterated glucose) APE values, respectively.

2.5.3 Calculation of hepatic glucose production (HGP)

HGP was calculated by using the model described by Steele *et al* (Steele *et al.*, 1965). The only tissues capable of de-phosphorylating glucose-6-phosphate and releasing glucose are the liver and the kidney. The kidney contributes very little to gluconeogenesis; and in practice, the liver accounts for about all endogenous glucose production (Ekberg *et al.*, 1999). Basal HGP or glucose appearance (Ra) was assessed during the last 30 minutes of the 180 minutes basal period and calculated as follows (Inzucchi *et al.*, 1998).

Basal HPG = {[TIR $\times$ enrichment$_{tracer}$ (NINF)] $\div$ tracer enrichment in plasma} – TIR

Where TIR is the tracer infusion rate, enrichment$_{tracer}$ (NINF) is the APE of 2% strength 6' 6' - dideuterated glucose infusion. Clamp HGP was assessed during the last 30 minutes of the 180 minutes hyperinsulinaemic euglycaemic clamp and calculated as follows (Inzucchi *et al.*, 1998).

Clamp HGP = GIF$_{mean}$ $\times$ [(enrichment$_{inf}$ $\div$ enrichment$_{plasma}$)] – 1

Where GIF$_{mean}$ is the mean glucose infusion rate during the last 30 minutes of the hyperinsulinaemic euglycaemic clamp, enrichment$_{inf}$ is the APE of 2% strength 6' 6' - dideuterated glucose in the 10% Dextrose, and enrichment$_{plasma}$ is the APE of plasma 6' 6' - dideuterated glucose in the last 30 minutes of the clamp when steady state conditions are achieved.

2.6 Measurement of whole-body insulin sensitivity

2.6.1 Euglycaemic hyperinsulinaemic clamp

The euglycaemic hyperinsulinaemic clamp was employed to measure overall insulin sensitivity (Rd). After an overnight fast, the subjects were transported to the NMRC by taxi. Cannulation was normally completed by 0845h. Insulin (Actrapid: Novo Nordisk, Bagsvaerd, Denmark) dose was calculated according to body surface area (BSA) and infused at 40mU/m^2/min.

BSA was calculated with the Du Bois formula (Dubois and Dubois, 1989) as:

BSA (m^2) = 0.20247 × Height 0.725 (m) × Weight 0.425 (kg)

Then, the insulin dose (ID) calculated as:

$$ID = \frac{BSA\ (m2) \times 0.04\ (U) \times 60\ (min) \times 50ml\ (syringe\ volume)}{15\ ml/h (constant\ infusion\ rate)}$$

The measured dose of insulin was transferred to a labelled syringe containing 50ml of 0.9% normal saline. Insulin was infused in two stages: priming and continuous infusion. The priming stage was further divided into two phases, A and B. In the priming part 'A' phase, the calculated insulin dose rate was multiplied by 4 to determine the priming dose 'A'. Insulin was infused at this rate for the first 4 minutes. In the priming part 'B' phase the calculated insulin dose rate was multiplied by 2, to determine the priming dose 'B'. Insulin was infused at this rate from 4 to 7 minutes. In the continuous stage from 7 to 180 minutes, insulin was infused at the calculated dose.

Plasma glucose concentration was allowed to reach euglycaemia defined as 5.5mM by 150 minutes and maintained at this level until completion of the clamp at 180 minutes (DeFronzo *et al.*, 1979). Euglycaemia was maintained by varying the rate of infusion of 10% Dextrose enriched with 2% 6' 6' - dideuterated glucose. To achieve this, blood samples were taken at 5 minute intervals and glucose concentration was measured with Yellow Springs glucose analyser (YSI, Ohio, USA). The mean coefficient of variation of plasma glucose concentration and glucose infusion in the last 30 minutes of the clamp were 3.3% and 10.0%, respectively.

2.6.2 Calculation of whole body insulin sensitivity (Rd)

Whole body insulin sensitivity (Rd) was determined during the last 30 minutes of the euglycaemic hyperinsulinaemic clamp (DeFronzo *et al.*, 1979). During the insulin infusion period, the rate of infusion of 10% Dextrose enriched with 2% 6' 6' - dideuterated glucose was adjusted as required to maintain euglycaemia of 5.5mmol/. Rd was calculated from the mean of glucose infusion rate during this period and expressed in (mg/kg/min) (Rizza *et al.*, 1981).

2.6.3 Rd/Insulin

During a hyperinsulinaemic euglycaemic clamp, insulin primarily drives glucose disposal to muscle. Therefore the rate of insulin clearance will influence the rate of glucose disposal to muscle. The Rd/Insulin eliminates the inter-personal variation of insulin clearance in a subject. Rd/insulin (ml/kg/min/pM) was calculated as Rd divided by steady state plasma insulin concentration. Steady state plasma insulin was calculated as the mean plasma insulin concentration obtained at 150, 165 and 180 minutes. In practical terms it should be considered that the relationship between Rd and steady state plasma insulin concentration is not linear, but sigmoidal.

2.6.4 Homeostatic model assessment (HOMA)

The homeostatic model assessment of insulin resistance (HOMA-IR) and β cell function (HOMA-β) are simple tools used to quantify insulin resistance and β cell function in the basal fasting state. It has been demonstrated that insulin resistance quantified by (HOMA-IR) in type 2 diabetes and normal glucose tolerant subjects correlated with that estimated by hyperinsulinaemic euglycaemic clamp (r=0.88; p=0.0001); likewise β cell function (HOMA-β) correlated with hyperinsulinaemic hyperglycaemic clamp (r=0.61; p<0.01) (Matthews *et al.*, 1985). HOMA-IR and HOMA-β are calculated as follows:

$$\text{HOMA-IR (µU/mol/L}^3) = \frac{\text{Fasting plasma insulin x Fasting plasma glucose}}{22.5}$$

$$\text{HOMA-}\beta \text{ (\%)} = \frac{\text{Fasting plasma insulin} \times 20}{\text{Fasting plasma glucose -3.5}}$$

It would have been impractical to perform monthly hyperinsulinaemic euglycaemic clamps. Consequently, these parameters were used to describe month by month change, thereby allowing comparison of any rate of change of insulin resistance and sensitivity between groups.

2.7 Substrate metabolism with indirect calorimetry

Indirect calorimetry allows measurement of glucose and lipid oxidation by quantitation of O_2 uptake and CO_2 production. Subjects were made comfortable in the supine position. A clear Perspex canopy hood was placed over the head of the subjects. They were asked to breathe normally and to avoid talking and sleeping. A pump extracted expired air and VO_2 and VCO_2 were analysed by metabolic cart sensors at 5 second intervals. VCO_2 is measured by an infrared digital sensor, with a measuring range of 0 - 10% and an accuracy of 0.02% (Blond *et al.*, 2011). Oxygen was measured with a paramagnetic sensor with a measuring range of 0 - 30% and with similar accuracy to CO_2 (Blond *et al.*, 2011). The flow rate was adjusted to maintain the Fractional inspired carbon dioxide ($FiCO_2$) of 0.8% - 1.0%, when measurements of VO_2 and VCO_2 are optimal. Measurements were made over 20 minutes during the last 30 minutes of both the basal period (-30 to 0 minute) and the insulin infusion period (150 to 180 minutes). Prior to commencing the measurements of glucose and lipid oxidation, the metabolic monitor was calibrated using a standard gas to ensure consistency of the flow meter and gas analysers.

2.7.1 Measuring substrate metabolism with the Quark RMR

The Deltratac ‖TM metabolic monitor which had previously been regarded as the gold standard for indirect calorimetry, was no longer serviced by the manufacturers. Malfunction of the Deltratac ‖TM metabolic monitor after the first 6 months of metabolic studies caused replacement by the Quark RMR (COSMED, Italy). Prior studies by Blond *et* al had demonstrated that the Quark RMR has good accuracy and consistency when compared with the Deltratac ‖TM Figure 2.3 (Blond *et al.*, 2011). The correlation has been shown to be good for VCO_2 (R2=0.87), VO_2 (R2=0.90), and resting metabolic rate (RMR) (R^2 = 0.90). The correlation and Bland-Altman plot for RMR is shown in Figure 2.3.
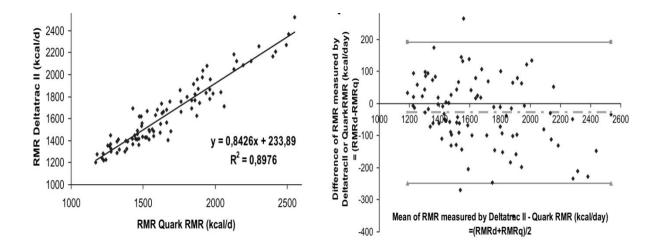
Figure 2.3 Correlation and Bland-Altman representation of plot RMR (Blond *et al.*, 2011)

2.7.2 Calculation of substrate oxidation

Even though the indirect calorimetry technique was primarily developed to measure metabolic rate, it was demonstrated some two centuries ago that measurements of oxygen consumption, carbon dioxide and nitrogen production can also be used to calculate glucose (G) and lipid (L) oxidation rates, respectively (Frayn 1983).

$$G = 4.55\ VCO_2 - 3.21\ VO_2 - 2.87\ N$$

$$L = 1.67\ (VO_2 - VCO_2) - 1.92\ N$$

Data obtained in the first 5 minutes of the measured period was excluded and only data obtained during steady state was included. Steady state is defined as less than a 10% CV in for VO_2 and VCO_2 (Compher *et al.*, 2006). $FiCO_2$ less than 0.8% or greater than 1.0% were excluded to ensure that only optimal measurements of VO_2 and VCO_2 were included in the final analysis. The calculated mean values for VO_2 and VCO_2 were used to estimate glucose and lipid oxidation rate. The CV for VO_2, VCO_2 and RQ were 5.4%, 5.5% and 2.4%, respectively. Previously validated assumptions on protein oxidation were used to calculate energy expenditure and carbohydrate and lipid oxidation rates (Wolfe, 1992).

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2.8 Metabolite and hormone analysis

Plasma glucose concentration was measured with a Yellow Springs glucose analyser (YSI, Ohio, USA) by the glucose oxidase method. The CV of the YSI for measuring glucose concentration in a sample taken during the steady state period of the hyperinsulinaemic euglycaemic clamp was found to be 1.0%.

HbA_{1c} was measured by high performance liquid chromatography (Bio-rad): the standard error of the mean (SEM) for HbA_{1c} of 5.5%:2.2 and 9.9%:1.6, respectively.

Plasma insulin concentration was measured with Dako Insulin enzyme linked immunoabsorbent assay (DAKO; Denmark) using a spectrophotometric analyser. For this method plasma insulin sample is added to a monoclonal anti-insulin antibody coated micro-plate well and incubated with anti-insulin conjugate at 20°C - 30°C for 60 minutes to form an antibody-insulin-conjugate complex. The micro-plate well undergoes three automated washes to remove unbound anti-insulin conjugate. 3, 3', 5, 5' - tetramethylbenzidine (TMB) is then added to the antibody-insulin-conjugate complex and incubated at 20°C - 30°C for 10 minutes. Sulphuric acid is then added to stop the reaction between the antibody-insulin-conjugate complex and TMB. The change in colour observed after adding sulphuric acid is proportional to the plasma insulin concentration and is measured by a spectrophotometer (CV for three mean concentrations are as follows: [15.1mU/L:5.6%; 42.1mU/L:6.2%; 153mU/L:9.1%]).

Plasma glucagon concentration was measured with Millipore Glucagon Radioimmunoassay (RIA) kit (Millipore Corporation; Massachusetts SUA), and this assay is specific for quantifying pancreatic glucagon. For this method, the kit employs labelled tracer antigen (I125 - labelled Glucagon) and Glucagon antiserum to determine the glucagon concentration in plasma (CV for three mean concentrations are as follows: [82.7.1pg/ml:9.4%; 111.1mU/L:2.9%; 542.6mU/L:3.6%]).

Plasma NEFA concentration was measured with enzymatic colorimetric method assay using the Wako NEFA-HR (2) reagent (Wako Chemical, Neuss Germany) and an automated analyser. For this method, plasma NEFA underwent three enzymatic reactions, ultimately resulting in a blue purple pigment, the intensity of which is proportional to the plasma NEFA concentration, and this is measured by photometric means to derive the concentration of NEFA (CV for measurement [range 0.01 - 4.0mmol/l: ≤1.5%.]).

Plasma triglyceride concentration was measured with the Triglyceride GPO-PAP spectrophotometric assay (Roche Diagnostics, Germany), using Roche/Hitachi MODULARa analyser. For this method triglyceride is hydrolysed by a combination of microbial lipases to produce glycerol. Glycerol is phosphorylated and oxidized to produce a red dyestuff called chromophore. The plasma triglyceride concentration is proportional to the colour intensity of chromophore which is measured by photometric means (CV at three mean concentration variety is as follows: [0.81mmol/l:3.7%; 1.34mmol/l:3.7%; 2.11mmol/l:1.4%]).

HDL cholesterol concentration was measured by Roche WAKO Direct Homogenous assay (SD for measurement [range 0.74-1.76mmol/l:2.7%]). LDL, VLDL and non-HDL cholesterol concentrations were calculated from the total and HDL cholesterol measurements.

2.9 Magnetic resonance methods

2.9.1 Magnetic resonance spectroscopy (MRS) for measuring glycogen concentration

A magnetic safety check was carried out on every study participant on each scan day, immediately prior to the MR scan. All magnetic resonance data were acquired using a Philips 3 Tesla Achieva scanner (Philips, Best, The Netherlands). Muscle glycogen concentration was obtained using a 6cm diameter ^{13}C surface coil with an integral ^{1}H decoupling surface coil (PulseTeq, Worton under Edge, UK). Liver glycogen was measured with an in-house built 12cm diameter ^{13}C/^{1}H surface coil.

2.9.1.1 Skeletal muscle glycogen measurements

The subjects were comfortably positioned in the supine position inside the scanner with the widest part of the gastrocnemius (calf) muscles lying directly over the RF coil. For each study, the coil was held in position against the calf with fabric straps to prevent movement. Scout images were obtained at the start of each study to ensure identical coil position over the gastrocnemius muscle and also to measure the distance between the surface of the coil and the muscle. This distance varied with the amount of subcutaneous fat and was used in the calculation of tissue glycogen concentration. The amplitude of the glycogen signal measured was determined by tissue glycogen concentration and the distance between the coil and muscle.

^{13}C RF pulse power was calibrated to a nominal value of 80° by observing the power dependent variation in signal from a fiducial marker located in the coil housing, containing a sample exhibiting ^{13}C signal with short T_1 (213mM [2-13C]-acetone and 25mM GdCl$_3$ in water). In order to ensure that the magnetic field within the scanner was uniform over the active volume of the ^{13}C coil, automated shimming was carried out by the scanner. The ^{13}C spectra were acquired over 15 minutes using a non-localised ^{1}H decoupled ^{13}C pulse acquires sequence (TR 120ms, spectral width 8 kHz, 7000 averages, WALTZ decoupling). ^{1}H decoupling was applied for 60% of the ^{13}C signal acquisition, to allow a relatively fast TR of 120ms to be used within scanner power Specific Absorption Rate (SAR) limitations.

2.9.1.2 Liver glycogen measurements

The subjects were comfortably positioned in the supine position with the ^{13}C liver RF coil centrally placed over the right lobe of the liver. While in the scanner, scout images were used to confirm correct coil positioning of the liver and measure the distance between the coil and liver surfaces. The calibration of pulse power, automated shimming and manual coil tuning was carried out. Spectra showing glycogen [1- ^{13}C] resonance were acquired over 15 minutes using a non-localised ^{1}H decoupled ^{13}C pulse acquired sequence (TR 300ms, spectral width 8kHz, 2504 averages, WALTZ decoupling, nominal ^{13}C tip angle of 80°).

2.9.1.3 Calibration and analysis of spectra

^{13}C spectra were calibrated by comparing *in vivo* [1- ^{13}C] glycogen signal amplitude with that obtained from a standard glycogen solution containing 100mM oyster glycogen, 70mM potassium chloride and 0.05% sodium azide (Sigma Aldrich Ltd, Gillingham, UK). The same coil, tip angles and pulse sequences used to obtain signals from in vivo [1- ^{13}C] glycogen were used to obtain signals from the standard glycogen solution (Sigma Aldrich Ltd, Gillingham, UK).

Spectra were analysed with Java Based Magnetic Resonance User Interface (jMRUI) version 3.0 and the AMARES algorithm (Naressi *et al.*, 2001) Figure 2.4. Glycogen concentration was determined from the magnitude of the natural abundance ^{13}C signal from the C1-glycogen carbon at a frequency of 100.3ppm. The signal from the C1 carbon of glycogen is well separated from signals from other carbon containing molecules. For each subject the separation between RF coil and muscle / liver tissue was measured from ^{1}H images, and ^{13}C coil loading assessed from ^{13}C flip angle calibration data.
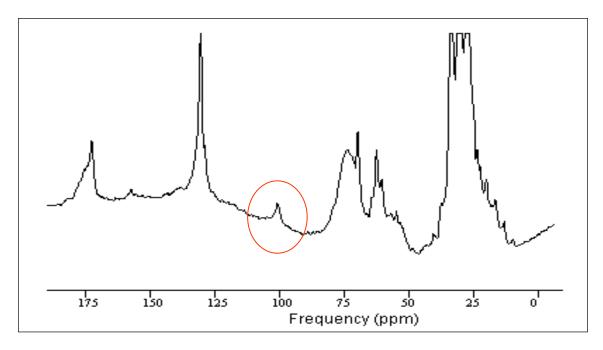
Figure 2.4 A typical ^{13}C spectrum acquired from the calf of a volunteer

2.9.2 Measurement of liver and pancreas fat with Three-point Dixon MRI

A six-channel cardiac coil (Philips Healthcare, Best, the Netherlands) was used to measure liver and pancreas triglyceride content. Data were acquired using a three-point Dixon method (Glover and Schneider, 1991) with three gradient-echo scans acquired with adjacent out-of-phase and in-phase echos during a 17 second breath hold (repetition time (TR)/echo time /averages/flip angle = 50ms/3.45, 4.60, 5.75ms/1/5°. A matrix size of 160×109 and field view of 400 - 480mm were deemed suitable for volunteer size. This size of matrix enables acquisition of six slices within a 17 second breath hold, to cover the liver and pancreas with slice thickness of 10mm and 5mm, respectively. The fat and water contributions of the MRI signal were separated using an in-house programme written in MATLAB, with the triglyceride content in the images expressed as a percentage of the total signal from fat and water in each pixel. Five liver and two pancreas regions of interest were defined on the fat images and their averages were used to estimate intra-organ fat percentage. Using the polygon region of interest (ROI) tool in the imaging software Image-J (Abramoff *et al.*, 2004) the ROIs were carefully drawn to prevent contamination of data from blood vessels, gall bladder or any peripheral tissue so that liver fat data solely represented intrahepatic fat. For intra-pancreatic fat analysis, identical ROIs were drawn on both fat and water images as demonstrated in Figure 2.5. Again, care was taken to avoid contamination from surrounding tissue. The measurements were averaged to calculate the triglyceride content in liver and pancreas. The inter-scan Bland–Altman repeatability coefficient have previously been reported as 0.5% (Lim *et al.*, 2011a).
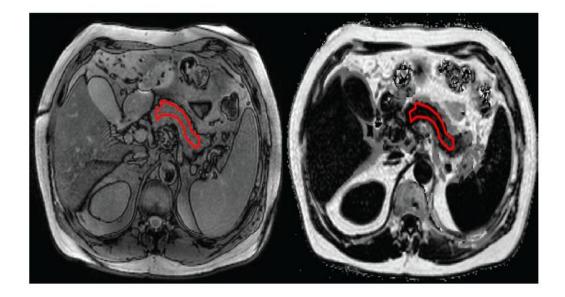
Figure 2.5 Manually drawn pancreas ROI shown on a BTFE structural scan (left) and fat percentage in the MR signal (right) image. The ROI was drawn to avoid contamination from surrounding structures

2.9.3 Measurement of pancreas volume with Balanced Turbo Field Echo (BTFE) images

A new technique to measure pancreas volume with (BTFE) structural scans was developed. BTFE images contain both T_1 and T_2 contrast, which distinguishes high signal intensity from vessels and visceral fat with lower intensity signals from the pancreas. It can be used to clearly delineate the boundaries of the pancreas from adjacent surrounding tissue including the splenic vein, the superior mesenteric vessels, the inferior vena cava, the duodenum, and the surrounding visceral fat. Twelve axial sections of 5mm thickness were imaged during an eight second breath hold (repetition time/echo time/flip angle = 3.1ms/1.6ms/40°, field of view 400 - 480x300mm according to patient size, zero-filled to give resolution 1.39mm x 1.40mm, turbo factor 95, parallel imaging factor 2, bandwidth 1156Hz per pixel). MRIcro software was used to convert the images to a format that can be read by Image-J. The polygon ROI tool in the freely-available imaging software Image-J (Schneider *et al.*, 2012) was used to draw regions of interest around the circumference of the pancreatic tissue in each slice as demonstrated in Figure 2.6. The sum of the surface areas of interest in all the regions of interest was multiplied by the slice thickness of 5mm to obtain pancreas volume in mm^3. This was then converted to cm^3 by dividing by 1000. The intra-observer bias (calculated using measurements by a trained researcher) to be $0.8cm^3$ with a 95% limit of agreement of $9.2cm^3$ ($p>0.05$) and inter-observer bias was $8cm^3$ with a 95% limit of agreement of $13.1cm^3$ ($p>0.05$). Bland-Altman plots for 20 randomly selected scans for intra-observer and 53 randomly selected scans for inter-observer analysis demonstrated reliability of volume measurement Figure 2.7
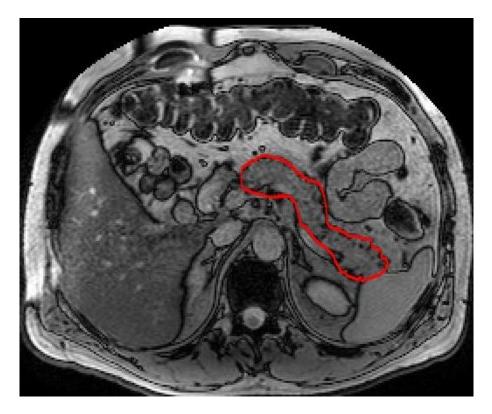
Figure 2.6 Demonstrates a BTFE image of the pancreas with a manually drawn ROI around the circumference of the pancreas
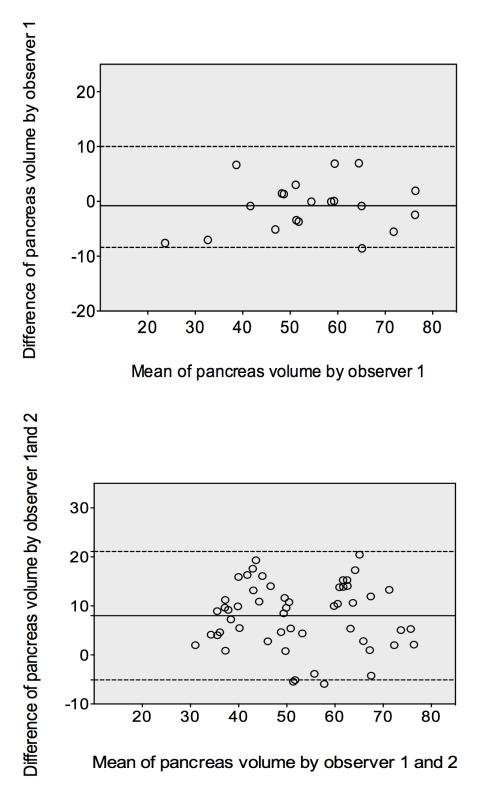
Figure 2.7 Bland-Altman plot of intra-observer difference (top) and inter-observer difference (bottom)

2.9.4 Measurement of skeletal muscle fat with ^{1}H MR spectroscopy

A 12cm ^{1}H transmitter/receiver coil was used to obtain ^{1}H spectra to measure intramyocellular lipid content in muscle Figure 2.8. The PRESS (Point Resolved Spectroscopy) (Bottomley, 1987) sequence was used to acquire ^{1}H spectra from the gastrocnemius muscle with a 2 × 2 × 2cm voxel, using an echo time of 25ms, spectral resolution of [1]Hz and repetition time of 5000ms with 32 acquisitions. Spectra were analysed with jMRUI version 3.0 using the least square fitting AMARES algorithm (Bottomley, 1987; Naressi *et al.*, 2001). The inter-observer bias was 0.09mmol/l with a 95% limit of agreement of 0.8mmol/l (p>0.05).
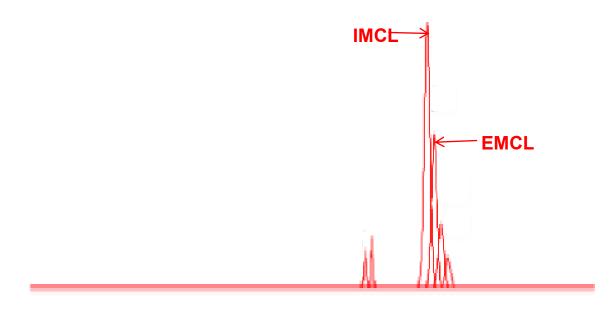
Figure 2.8 Spectral model fitted to a leg muscle ^{1}H spectrum showing calculated IMCL and EMCL peaks

CHAPTER 3 Evaluating metabolic effects of DDP-4 inhibition in type 2 diabetes

3.1 Introduction

Following identification of the therapeutic effect of GLP-1, the DPP-4 inhibitors were developed specifically to delay its rapid degradation in plasma and hence enhance the incretin effect in type 2 diabetes (Ahren *et al.*, 2005; Mari *et al.*, 2005; Ahren *et al.*, 2009). Vildagliptin achieves prolonged and almost complete DPP-4 inhibition resulting in the extension of meal-induced increases in GLP-1 and GIP over 24 hours. GLP-1 and GIP increase the sensitivity of the α- and β-cells to glucose, and this is accepted as its major mechanism of action (Ahren *et al.*, 2011; JE, 2014). As a result of an increase in sensitivity of α cells to glucose, inappropriate glucagon secretion is suppressed, and consequently, hepatic gluconeogenesis would be expected to fall (Ahren *et al.*, 2010). However, vildagliptin brings about changes that would not be predicted from its actions in the pancreas.

Vildagliptin has been shown to increase glucose utilization as assessed during a two-step hyperinsulinaemic euglycaemic clamp at the high insulin dose (80mU), and this might accompany or underlie an improvement in whole body insulin sensitivity (Ahren *et al.*, 2005; Mari *et al.*, 2005; Utzschneider *et al.*, 2009). If peripheral insulin sensitivity is increased by vildagliptin, the lower rates of lipolysis in adipose tissue would decrease the rate of delivery of non-esterified fatty acids to the liver and muscle, with potential decrease in hepatic and muscle triglyceride storage, and consequently improve insulin sensitivity in both organs.

It is known that the increase in fatty acid concentration is associated with a reduction in overall glucose utilisation in healthy young glucose tolerant subjects (Thiebaud *et al.*, 1982). Additionally lipid accumulation in the liver reduces hepatic insulin sensitivity; and in the muscle, it reduces peripheral glucose utilisation, respectively. Pioglitazone has been found to reduce fasting and postprandial hepatic glucose production, a marker of hepatic insulin sensitivity (Ravikumar *et al.*, 2008). Hepatic insulin sensitivity normalised within 7 days of calorie restriction, while peripheral insulin sensitivity improved by 36% at 8 weeks (Lim *et al.*, 2011a). The latter suggests that normalization of hepatic and peripheral insulin sensitivity may occur at a different time course of a therapeutic intervention and this warrants independent assessment. In both these studies, the change in hepatic insulin sensitivity was found to be associated with a reduction in liver triglyceride content.

The effect of vildagliptin on hepatic and peripheral insulin sensitivity has not been examined previously. Therefore a randomized, placebo-controlled study was designed to examine the possible effects of vildagliptin on these parameters as well as liver triglyceride content. In order to minimize any indirect metabolic effects, due to a large change in ambient plasma glucose levels, people with type 2 diabetes well controlled on metformin alone were studied.

3.2   Study design

3.2.1   Subjects

44 subjects with well-controlled type 2 diabetes (HbA$_{1c}$ ≤ 7.6% or 60mmol/mol) were recruited by newspaper advertisement. All were taking a stable dose of metformin and no other oral hypoglycaemic agent or insulin. Inclusion criteria included age 18 - 70 years; HbA$_{1c}$ ≤ 7.6% or 60mmol/mol; and BMI of 22 - 38kg/m^2. Exclusion criteria were previous insulin and chronic oral or parenteral steroid therapy; significant ischaemic heart disease and cardiac arrhythmia, renal and liver impairment; any acute infection which may affect blood glucose control. The main reason for excluding 10 volunteers was HbA$_{1c}$ > 7.6% or 60mmol/mol. The characteristics of the study group is summarised in Table 3.1, and mean duration of type 2 diabetes was 5.9±0.8years. Age and weight were significantly different in the vildagliptin treated and placebo treated group by chance. Recruiting via the primary care research network identified 54 volunteers.

	Baseline		
	Vildagliptin (n=22)	Placebo (n=22)	P value
Age (years)	65.1±0.7	58.7±1.5	0.0004
Weight (kg)	82.7±3.4	92.1±2.5	0.02
BMI (kg/m^2)	29.4±0.8	30.9±0.6	0.15
Body fat (%)	31.1±1.8	31.3±1.8	0.92
Waist hip ratio	0.95±0.02	0.96±0.01	0.68
FPG (mmol/l)	7.9±0.2	7.5±0.2	0.09
FPI (mU/L)	12.0±1.6	14.6±2.0	0.47
HOMA-IR (µU/mol/l^3	4.2±0.6	5.0±0.7	0.40
HbA$_{1c}$ (%)	6.6±0.1	6.5±0.1	0.21
Non-esterified fatty acid (mmol/l)	0.5±0.03	0.5±0.03	0.76

	6 months		
	Vildagliptin (n=22)	Placebo (n=22)	P value
Age (years)	-	-	
Weight (kg)	81.2±3.2	91.6±2.6	0.008
BMI (kg/m^2)	-	-	
Body fat (%)	28.7±1.9	30.0±2.1	0.64
Waist hip ratio	0.93±0.02	0.96±0.01	0.30
FPG (mmol/l)	7.0±0.3	7.7±0.4	0.07
FPI (mU/L)	11.8±1.4	13.2±1.8	0.25
HOMA-IR (µU/mol/l^3	3.7±0.5	4.4±0.6	0.16
HbA$_{1c}$ (%)	5.9±0.1	6.5±0.1	0.007
Non-esterified fatty acid (mmol/l)	0.5±0.04	0.5±0.03	0.76

Table 3.1 Characteristic of study subjects at baseline (top) and at 6 months (bottom)

Data are shown as mean ± SEM

3.2.2 Study protocol

All subjects were asked to avoid vigorous exercise and take no alcohol for 3 days prior to assessment. On the morning of study days, subjects were transported to the study centre by taxi, after a 10 hour overnight fast. Only water was allowed during the fasting period, and all subjects were advised to omit their morning dose of metformin at baseline, and both metformin and either vildagliptin or placebo on subsequent test days. Combined metabolic tests with hyperinsulinaemic euglycaemic clamp, 6' 6' - dideuterated glucose, and indirect calorimetry were used to measure overall insulin sensitivity, endogenous glucose production, and substrate (glucose and lipid) oxidation, respectively. The type 2 diabetes subjects were randomised (ratio 1:1) in a double-blinded fashion to receive either DPP-4inhibitor (vildagliptin 50mg bid) or placebo after completion of baseline studies. The three combined metabolic tests were repeated at 6 months. All subjects were asked to maintain their usual calorie intake over the 6-month period. Fasting plasma, glucose and insulin were measured monthly and Homeostatic Model Assessment of insulin resistance [HOMA-IR] calculated to describe month-by-month change in insulin resistance. Fasting plasma non-esterified fatty acid was measured at baseline, 1, 3, and 6 months, respectively. Weight was measured monthly and body composition was measured at baseline and 6 months Figure 3.1.

Compliance was assessed by pill count and handling of study medication was accurately recorded in an accountability ledger. Subjects were given 80 tablets of the study medication on each monthly visit and unused tablets were returned, counted and recorded on subsequent visits. Compliance was assessed by the difference between the number of tablets dispensed and returned.
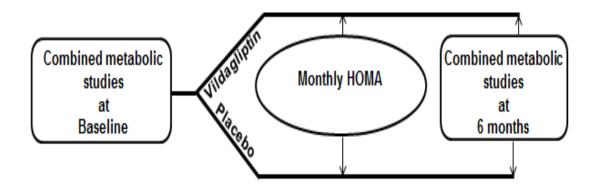
Figure 3.1 Test schedule of type 2 diabetes groups

3.3 Side effects of vildagliptin and placebo

Vildagliptin was well tolerated and there were no meaningful differences between the vildagliptin and placebo groups in the overall adverse events (AE) profile. AEs occurred in 15 patients (68.2%) in the vildagliptin group vs. 16 patients (72.7%) in the placebo group. Discontinuation due to AE happened in one vs. three patients, respectively. Serious adverse events (SAEs) (two vs. one patient respectively) were similar between the treatment groups with no deaths. Most frequent AEs were of minor infection (four vs. five patients) and musculoskeletal disorders (four patients in each group). Mild hypoglycaemia (<3.1mmol/l) was reported in one patient in the vildagliptin group and none was reported in the placebo group.

3.4 Results

3.4.1 HbA$_{1c}$ and plasma glucose

Mean HbA$_{1c}$ decreased by 0.5±0.1% (p<0.0001) from a baseline of 6.5±0.1% in the vildagliptin group, while a small numerical increase (0.2±0.1% from a baseline of 6.4±0.1%; p=0.14) was seen in the placebo group figure 3.2 (top). The between-group difference in change from baseline was -0.7±0.1% (p<0.001). In the vildagliptin group, HbA$_{1c}$ decreased at 3 months (6.1±0.1%; p<0.0001) and at 6 months (6.0±0.1%; p<0.0001); whereas in the placebo group there was little change over the entire study period (6.4±0.1; p=0.07 at 3 months and 6.5±0.2%; p=0.14 at 6 months, respectively). The time course of change is shown in Figure 3.4.

Mean fasting plasma glucose changed by -0.9±0.3mmol/l (p=0.001) from a baseline of 7.9±0.2mmol/l in the vildagliptin group and by +0.2±0.3mmol/l (p=0.24) from a baseline 7.5±0.2mmol/l in the placebo group over the study period figure 3.2 (middle). The between-group difference in change from baseline was 1.0±0.5mmol/l (p=0.018). In the vildagliptin group a sharp fall in fasting plasma glucose was observed at 1 month (7.4±0.3mmol/l; p=0.01) and at 2 months (6.9±0.2mmol/l; p<0.0001). Thereafter fasting plasma glucose did not change. In the placebo group, there was a small numerical increase in fasting plasma glucose at 3 months (7.6±0.2mmol/l; p=0.34) and at 4 months (7.7±0.3mmol/l; p=0.11). Thereafter fasting plasma glucose remained unchanged. The time course of change is shown in figure 3.3.

In the vildagliptin treated group, the decrease in fasting plasma glucose positively correlated with the decrease in fasting liver triglyceride content at 3 months (r=0.47; p=0.02) and 6 months (r=0.44; p=0.03).

65

3.4.2 Plasma insulin

Mean fasting plasma insulin decreased over 6 months by 0.2±0.2mU/L (p=0.22) in the vildagliptin group from a baseline 12.0±1.6mU/L, and by 1.4±0.2mU/L (p=0.21) in the placebo group from a baseline of 14.6±2.0mU/L. The between-group difference in change from baseline to 6 months was insignificant (p=0.95). Due to the loss of samples, subjects with missing data were excluded for further analysis. Complete data sets for 11 and 18 subjects in the vildagliptin and placebo treated groups respectively and 14 control subjects were analysed to compare the difference in fasting insulin concentration over 6 months. There was a significant difference in mean fasting insulin concentration at the $p < 0.05$ level for the vildagliptin, placebo and control groups [$F_{(2, 6)} = 56.8$, $p < 0.0001$]. Figure 3.2 shows fasting plasma insulin concentration in all (left) and complete (right) data sets in all groups, over 6 months.
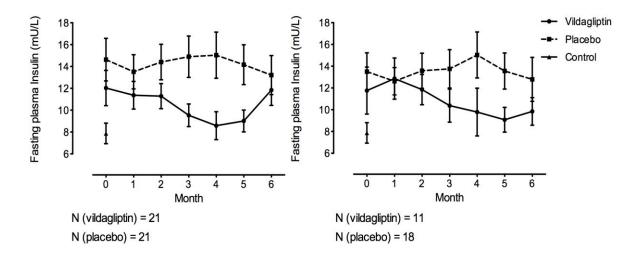
Figure 3.2 Fasting insulin concentration in the all (left) and complete (right) data sets in all groups

3.4.3 Plasma non-esterified fatty acid

Plasma NEFA remained unchanged over the study period in the vildagliptin (0.48±0.04 to 0.50±0.05mmol/l; p>0.05) and placebo (0.50±0.03 to 0.49±0.03mmol/l; p>0.05) groups.

3.4.4 Whole body insulin sensitivity assessed by euglycaemic (5.5mmol/l) hyperinsulinaemic (40mU insulin min-2min-1) clamp

Baseline mean glucose disposal rates were 3.24±0.30mg/kg/min in the vildagliptin group and 3.19±0.38mg/kg/min in the placebo group. Glucose disposal rates did not change meaningfully in either group from baseline to 6 months (3.24±0.30 to 3.50±0.31mg/kg/min and 3.19±0.38 to 3.52±0.40mg/kg/min, respectively) Figure 3.3 (bottom). There was no between-group difference in the change from baseline (-0.06mg/kg/min; p=0.86). When glucose disposal was expressed as a ratio to the clamp plasma insulin at steady state [M/I (ml/kg/min/pM)], insulin sensitivity did not change at 6 months in the vildagliptin (0.0065±0.0006 to 0.0071±0.0006ml/kg/min/pM) and placebo group (0.0074±0.0013 to 0.0078±0.0013ml/kg/min/pM). There was no between-group difference in the change from baseline (0.0001ml/kg/min/pM; p=0.89).

3.4.5 Hepatic glucose production (HGP)

Baseline fasting hepatic glucose production was the same in the vildagliptin and placebo group (1.95±0.10 vs. 1.95±0.07mg/kg/min). At 6 months, fasting hepatic glucose production was similar in both groups (1.97±0.1 vs. 2.01±0.1mg/kg/min; p=0.37). Baseline clamp hepatic glucose production was comparable between the vildagliptin and placebo groups (1.13±0.1 vs. 0.94±0.1mg/kg/min; p=0.16). At 6 months, clamp hepatic glucose production was also comparable between the two groups (1.16±0.1 vs. 1.03±0.1 mg/kg/min; p=0.09). Percentage suppression of HGP during the hyperinsulinaemic euglycaemic clamp at baseline and 6 months was similar in the vildagliptin (42.2±0.07 vs. 42.8±0.05%; p=0.89) and placebo group (54.7±0.06% vs. 48.4±0.07%; p=0.42).
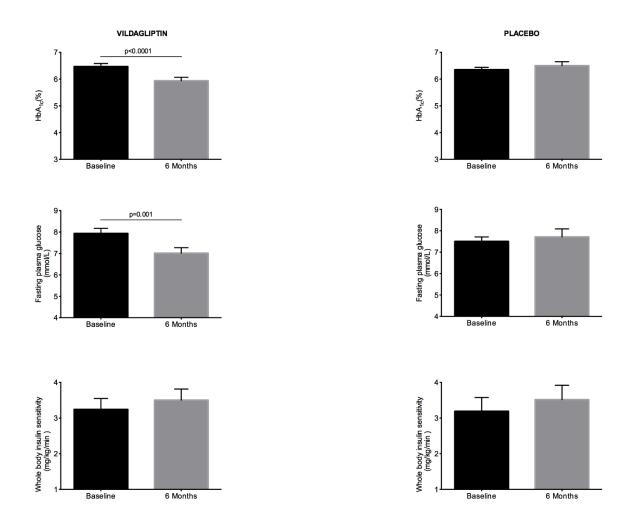
Figure 3.3 Change in HbA$_{1c}$ (top), fasting plasma glucose (middle) and whole body insulin sensitivity (bottom) at baseline and 6 months
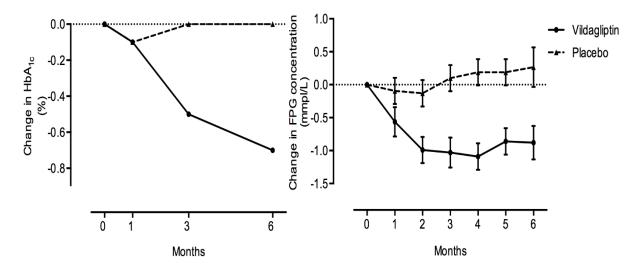
Figure 3.4 The effect of 6 months of vildagliptin on change in HbA$_{1c}$ (%) (left) and fasting plasma glucose (FPG) (mmol/l) (right) in the vildagliptin treated and placebo groups, respectively

3.4.6 Whole body glucose and lipid oxidation

Fasting whole body glucose oxidation rate did not differ between the vildagliptin and placebo groups either at baseline (0.99±0.16 vs. 0.87±0.10mg/kg/min) or at 6 months (0.77±0.19 vs. 0.95±0.14mg/kg/min), respectively. The between-group difference in change from baseline was insignificant; p=0.21. Similarly, insulin stimulated whole body glucose oxidation rate did not differ between the vildagliptin and placebo groups at baseline (1.52±0.21 vs. 1.50±0.08mg/kg/min) or at the end of the study (1.55±0.16 vs. 1.65±0.14mg/kg/min), respectively. The between-group change from baseline was insignificant; p=0.65.

Fasting whole body lipid oxidation rate did not differ between the vildagliptin and placebo groups either at baseline (1.08±0.07 vs. 1.13±0.05mg/kg/min) or at study endpoint (1.13±0.06 vs. 1.05±0.08mg/kg/min), respectively. There was no significant difference in clamp lipid oxidation between both groups at baseline (0.89±0.09 vs. 0.86±0.08mg/kg/min) and end of study (0.91±0.07 vs. 0.94±0.07mg/kg/min).

Fasting whole body energy expenditure did not change in the vildagliptin (1698±92.5 vs. 1673±89.6kcal/day; p=0.35) or placebo groups (1849±75.3 vs. 1729±94.9kcal/day; p=0.08) over 6 months. No change was observed in clamp whole body energy expenditure in both vildagliptin (1724±77.6 vs. 1676±104.8kcal/day; p=0.22) and placebo group (1877±96.9 vs. 1798±101kcal/day; p=0.23).

3.4.7 HOMA-IR

There was a numerical decrease in mean HOMA-IR over 6 months by $0.7 \pm 0.5 \mu U/mol/l^3$; p=0.22 in the vildagliptin group from a baseline of $4.2 \pm 0.6 \mu U/mol/l^3$, and by $0.3 \pm 0.5 \mu U/mol/l^3$; p=0.21 in the placebo group from a baseline of $5.0 \pm 0.7 \mu U/mol/l^3$. The between-group difference in change from baseline to endpoint was insignificant (p=0.54). Due to the loss of samples, subjects with missing data were excluded for further analysis. Complete data sets for 12 and 18 subjects in the vildagliptin and placebo treated groups respectively and 14 control subjects were analysed to compare the difference in HOMA-IR over 6 months. There was a significant difference in mean HOMA-IR at the p<0.05 level for the vildagliptin, placebo and control groups [F (2, 6) = 62.1, p=0.0001]. Figure 3.5 shows HOMA-IR in all (left) and complete (right) data sets in all groups, over 6 months.
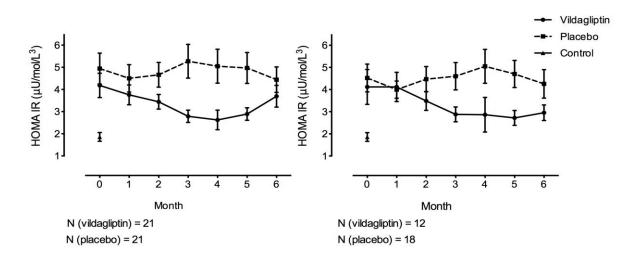
Figure 3.5 HOMA-IR in all (left) and complete (right) data sets in all groups

3.5 Discussion

The data demonstrate that DPP-4 inhibition can bring about clinically useful decreases in HbA_{1c}, fasting plasma glucose and insulin concentration in the patient group studied. Even though the patient group had well controlled type 2 diabetes and change in plasma glucose levels was expected to be modest, HbA_{1c} fell from an average of 6.5% to 6.0% in the group receiving vildagliptin, and 70% achieved a normal HbA_{1c} (<6.1% or 43mmol/mol). However, there was no change in whole body insulin sensitivity as assessed by the euglycaemic (5.5mmol/l) hyperinsulinaemic (40mU insulin $min^{-2}min^{-1}$). Additionally, there was no change in hepatic glucose production, which is a marker of hepatic insulin sensitivity.

Overall, blood glucose control improved on vildagliptin as reflected by mean HbA_{1c} falling into the non-diabetic range and mean fasting plasma glucose falling by 0.9mmol/l at 6 months. These results are particularly remarkable given the very low baseline HbA_{1c} of patients included in the study (mean 6.4%) and considering that oral hypoglycaemic agents in general have effects directly proportional to initial blood glucose levels. Mean HbA_{1c} was normalized following 6 months of treatment with vildagliptin 50mg bid (twice daily). Of note, the vildagliptin treatment effect on HbA_{1c} seen in this study is very robust when compared to previous data (Scherbaum *et al.*, 2008). In the present study, vildagliptin was added to stable metformin therapy and it is possible that this is relevant to the extent of normalization of blood glucose levels.

Although overall insulin sensitivity assessed by euglycaemic (5.5mmol/l) hyperinsulinaemic (40mU insulin $min^{-2}min^{-1}$) clamp did not change with 6 months of vildagliptin, insulin resistance assessed by HOMA-IR improved with vildagliptin treatment. A significant change in HOMA-IR was observed as early as the second month, and reached nadir at 4 months of vildagliptin treatment. In a recent, larger study, a significant reduction in HOMA-IR was observed at 9 months (Derosa *et al.*, 2013). In the later study, the baseline HbA_{1c} was higher when compared to this study group (8.1%); and this may account for the time difference in which significant reduction in HOMA-IR is achieved.

Basal hepatic glucose production did not change with vildagliptin treatment and this confirms previous observations with both vildagliptin and sitagliptin therapy (Azuma *et al.*, 2008; Solis-Herrera *et al.*, 2013). In contrast, in a single dose, post-meal and overnight study (Balas *et al.*, 2007), there was on average 0.3mg/kg/min less hepatic glucose production in the vildagliptin group relative to the placebo group from the

beginning of the dinner meal until the next morning. This contrasting result suggests that the effect of DPP-4 inhibition on the suppression of fasting hepatic glucose production differs from the suppression of postmeal hepatic glucose production. In the later study (Balas *et al.*, 2007), it is possible that maximal post meal concentration of GLP-1 and GIP preserved by vildagliptin enhanced the suppression of inappropriate glucagon secretion. In the fasting state, GLP-1 and GIP concentration is expected to be low and the incretin effect on glucagon suppression is negligible. Further work is required to evaluate the effect of vildagliptin on hepatic glucose production during low, physiological increase in insulin concentrations such as occurs after meals. The lack of improvement in fasting hepatic glucose production despite fall in liver fat (chapter 4) must be considered. This may relate to the very well controlled type 2 diabetes subjects under study, as hepatic glucose production in diabetes has been observed to decline to non-diabetic levels below a plasma glucose of approximately 8mmol/l (DeFronzo *et al.*, 1989). Also it must be noted that the clamp insulin levels were optimal to measure peripheral rather than hepatic insulin sensitivity. Although, ideally a two-step clamp would have been used with an initial low dose insulin concentration, this was not feasible in view of the duration of the studies and the number of test visits required of the subjects.

Treatment with vildagliptin added to metformin over 6 months resulted in a 1.6kg weight loss vs. baseline. While overall, treatment with DPP-4 inhibitors is weight neutral, it was previously reported that from a similarly low baseline level of glycaemia as in the present study (Scherbaum *et al.*, 2008), baseline mean HbA$_{1c}$ 6.6%) vildagliptin therapy reduced body weight by 1.1kg over a 2 year period (Bluher *et al.*, 2014). Furthermore, examination of weight as a function of fasting plasma glucose in a pooled analysis of more than 2000 patients demonstrated that negative calorie balance occurred in those with glucose levels below the renal threshold (Bluher *et al.*, 2014).

De novo lipogenesis was not assessed in this study, but overall a modest increase in this process is unlikely to account for the lower plasma glucose levels in the face of no decrease in hepatic glucose production. In this study, clamp lipid oxidation did not change with vildagliptin treatment. Hepatic export of lipid has to be considered. This is unlikely in view of the results on total hepatic lipid content presented in chapter 4 and the results from a previous study showed no effect of vildagliptin treatment on postprandial triglyceride levels secondary to reduced flux of lipoproteins from the liver

(Matikainen *et al.*, 2006). The fall in fasting plasma glucose is likely to reflect the prolonged effect of glucagon suppression during an overnight fast.

Possible limitations of the study must be considered. Examination of whole body insulin sensitivity by conducting euglycaemic hyperinsulinaemic clamp at a range of insulin concentrations would have been ideal. Similarly, detailed examination of hepatic insulin sensitivity within the physiological range of insulin concentrations would be of interest in relation to the substantial decrease in hepatic triglyceride. Data for whole body glucose and lipid oxidation rates measured by indirect calorimetry was collected for 11 and 8 subjects in the vildagliptin treated and placebo groups, respectively. Technical problems prevented more subjects being studied and therefore the small numbers in each group lacked the power required to detect any true difference.

In summary, the study has demonstrated that a clinically significant reduction in fasting glucose, insulin and HbA$_{1c}$ in well controlled type 2 diabetes is achieved on vildagliptin therapy. No effect of the drug on hepatic glucose production could be detected.

CHAPTER 4 Effect of vildagliptin on liver steatosis and muscle lipid content

4.1 Introduction

Lipid toxicity has been identified as a major contributing factor to insulin resistance (Lee *et al.*, 1994a; McGarry, 2002). Human studies have demonstrated the association between hepatic lipid accumulation and insulin resistance in subjects with metabolic syndrome (Kotronen *et al.*, 2008). This concept is supported by the observance of increased prevalence of type 2 diabetes in people with NAFLD (Shibata *et al.*, 2007). Hepatic lipid accumulation reduces insulin sensitivity, impairs glucose uptake, glycogen synthesis, and increases gluconeogenesis in the liver (Samuel *et al.*, 2004). The latter contributes to inadequate suppression of hepatic glucose suppression, which results in fasting hyperglycaemia.

In normal health, acute rises in plasma free fatty acid concentration have been shown to increase intramyocellular lipid content (IMCL) (Boden *et al.*, 2001), which is a marker for muscle insulin resistance. IMCL accumulation is now known to be associated with impaired skeletal muscle insulin sensitivity in normal glucose tolerance subjects (Krssak *et al.*, 1999), lean off-spring of type 2 diabetes (Jacob *et al.*, 1999) and also obese type 2 diabetes subjects (Ravikumar *et al.*, 2008). Additionally, hyperlipidaemia has been shown to reduce insulin stimulated glucose disposal in skeletal muscle during hyperinsulinaemic euglycaemic clamp (Johnson *et al.*, 1992; Kelley *et al.*, 1993). In a recent study, calorie restriction using a 600kcal diet has been shown to reduce liver triglyceride concentration by 30% in 7 days and normalise hepatic glucose production (Lim 2011).

As such, the effect of new therapeutic strategies on liver and muscle lipid content warrant further investigation. Although DDP-4 inhibitors were developed to delay the rapid degradation of GLP-1 and GIP, vildagliptin brings about changes that would not be predicted from its actions in the pancreas. It decreases postprandial triglyceride levels and decreases lipolysis as assessed *in vivo* by palmitate dilution more than can be accounted for by change in plasma insulin concentration (Matikainen *et al.*, 2006; Derosa *et al.*, 2014). This could result in a decrease in liver triglyceride concentration. Vildagliptin has also been shown to increase glucose utilization as assessed during a two-step hyperinsulinaemic euglycaemic clamp at the high insulin dose (80mU) and this might accompany or underlie a reduction in liver triglyceride concentration (Ahren *et al.*, 2005; Mari *et al.*, 2005; Utzschneider *et al.*, 2009). If peripheral insulin sensitivity is increased by vildagliptin, the lower rates of lipolysis in adipose tissue would decrease

the rate of delivery of non-esterified fatty acids to the liver with potential decrease in hepatic triglyceride storage.

Whether hepatic lipid metabolism is specifically affected has not previously been examined and there is no information on any modulation of liver triglyceride concentration. The randomized placebo-controlled study was designed to examine the possible effects of vildagliptin on liver and muscle triglyceride content in a group of individuals with type 2 diabetes subjects well controlled on metformin alone. In order to minimise any indirect effect due to a large change in ambient plasma glucose levels, well controlled type 2 diabetes subjects on metformin alone were studied.

4.2 Study design

4.2.1 Subjects

The 44 (28M; 16F) well controlled type 2 diabetes subjects were as previously described in CHAPTER 3. In order to assess the extent of any normalization of the parameters measured, a control group of subjects with normal glucose tolerance was recruited. The characteristics for the groups is summarised in Table 4.1. The study was approved by the Newcastle and North Tyneside 2 Research Ethics Committee.

	Vildagliptin (n=22)	Placebo (n=22)	Type 2 Diabetes group (n=44)	Controls (n=14)
Age (years)	65.2±0.7	58.9±1.6	61.8 ± 1.0	59.0 ± 2.2
Weight (kg)	83.0±3.2	91.8±2.4	87.7 ± 2.1	86.7 ± 3.7
BMI (kg/m^2)	29.4±0.8	31.1±0.6	30.0 ± 0.5	29.6 ± 1.0
Body fat (%)	31.5±1.6	30.7±1.8	31.6 ± 1.2	27.9 ± 2.6
WHR	0.95±0.01	0.95±0.01	0.95 ± 0.01	0.9 ± 0.02*
FPG (mmol/l)	7.7±0.2	7.8±0.3	7.7 ± 0.2	5.1± 0.1**
FPI (mUIL)	13.0±1.8	14.8±1.6	13.8 ± 1.2	7.9 ± 0.9**
HOMA-IR (μU/mol/l^3)	4.4±0.6	5.3±0.7	5.0 ± 0.4	1.9 ± 0.2**
HbA$_{1c}$ (%) (mmol/mol)	6.6±0.1	6.5±0.09	6.4 ± 0.07 (46.5 ± 0.8)	

Table 4.1 Characteristics of study groups

Data are shown as mean ± SEM. *P ≤ 0.05 and **P ≤ 0.01

4.2.2 Study protocol

The study protocol is described in CHAPTER 3. MR studies were carried out at baseline, and then at 1, 3, and 6 months of drug or placebo treatment.

4.3 Results

4.3.1 Liver triglyceride

At baseline, liver triglyceride was higher in the whole type 2 diabetes group compared with the control group (6.7±0.7 vs. 3.4±0.8%; p=0.0008). Mean fasting liver triglyceride decreased during vildagliptin treatment from 7.3±1.0% at baseline to 5.3±0.9% at 6 months (p=0.001). There was no change in the control group (5.4±0.7% to 5.4±1.0%; p=0.48). The between-group difference in change from baseline was significant (p=0.013), representing a clinically relevant improvement in liver triglyceride. The time course of change is shown in Figure 4.1. Within the vildagliptin treated group, liver triglyceride fell from baseline by 12% at 1 month (p=0.04), 29% at 3 months (p=0.001) and 27% at 6 months (p=0.0006). In the placebo group intra-hepatic triglyceride remained unchanged over 6 months (6.0±0.9 to 5.7±1.0%; p=0.96).
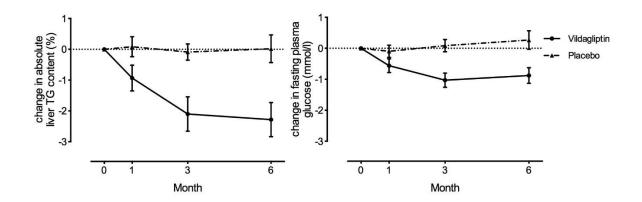
Figure 4.1 The effect of 6 months of vildagliptin on change in (left) liver triglyceride (TG) content (expressed as absolute change in the percent hepatic fat, not relative change and (right) fasting plasma glucose in the vildagliptin treated and placebo groups, respectively (*p<0.05; **p<0.005; ***p<0.0005)

4.3.2 Plasma alanine aminotransferase (ALT)

Mean plasma ALT fell from 27.2±2.8 to 20.3±1.4IU/l in the vildagliptin treated group (p=0.0007) and did not change in the placebo group (29.6±3.0 to 29.6±3.7IU/l; p=0.44). There was a positive correlation between the fall in ALT and the fall in liver triglyceride in the vildagliptin treated group (r=0.83; p<0.0001) Figure 4.2. The correlation between these remained significant even if the outlier was excluded (r=0.64; p=0.003).
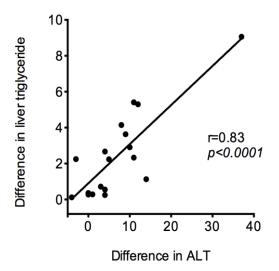
Figure 4.2 Relationship between change in liver triglyceride concentration and change in plasma ALT (r=0.83; p<0.0001). The correlation remained significant if the outlying point was excluded (r=0.64; p=0.003)

4.3.3 IMCL

At baseline IMCL was higher in the type 2 diabetes compared with the control group (22.3±2.0 vs. 16.2±1.1mmol/l; p=0.006). Due to the difficulty in analysing IMCL data, subjects with missing data were excluded for further analysis. Complete data sets for 15 and 10 subjects in the vildagliptin and placebo treated groups respectively and 10 subjects in the control group. There was a significant difference in the effect of vildagliptin on IMCL at the p<0.05 level for the vildagliptin, placebo and control groups [F (2, 3) = 18.5, p=0.02]. Figure 4.3 shows IMCL in all (left) and complete (right) data sets in all groups, over 6 months.
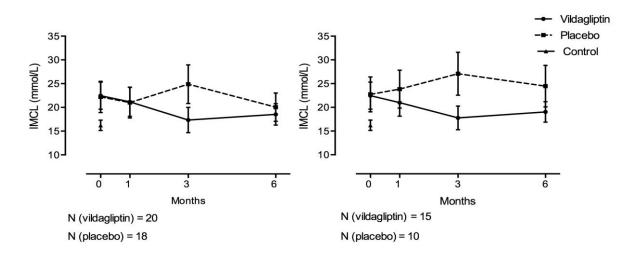
Figure 4.3 IMCL in all (left) and complete (right data sets in all groups

4.3.4 Fasting lipid parameters

Although there was a tendency for fasting triglyceride and total cholesterol to decrease with vildagliptin treatment in comparison with the placebo group, there was no between group difference in mean change. Fasting lipid parameters are summarized in Table 4.2 with p values for adjusted mean change.

	Vildagliptin		Placebo		p value
	Baseline	Endpoint	Baseline	Endpoint	
Triglyceride	1.5±0.1	1.3±0.1	1.4±0.1	1.4±0.1	0.17
Total cholesterol	4.2±0.2	3.9±0.2	4.2±0.2	4.2±0.2	0.57
LDL cholesterol	2.1±0.2	1.9±0.2	2.3±0.3	2.1±0.2	0.72
HDL cholesterol,	1.4±0.1	1.4±0.1	1.3±0.1	1.3±0.1	0.37
Non-HDL cholesterol	2.8±0.2	2.5±0.2	2.9±0.2	2.7±0.2	0.34
VLDL cholesterol	0.5±0.1	0.4±0.0	0.5±0.0	0.4±0.0	0.99
Non-esterified fatty acid	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.63

Table 4.2 Fasting lipid parameters with p values for adjusted mean change

Data are shown as mean ± SEM. *$P \leq 0.05$ and **$P \leq 0.01$

4.3.5 Weight

Mean body weight decreased by 1.6±0.5kg from a baseline of 82±3.4kg (p=0.002) in the vildagliptin group and by 0.4±0.5kg from a baseline of 92.1±2.5kg (p=0.41) in the placebo group over the study period. The between-group difference did not reach statistical significance (p=0.08). Modest weight loss in the placebo group was associated with no change in liver triglyceride content, and the time course of change in weight and change in liver triglyceride content were not congruent in either group, Figure 4.4.
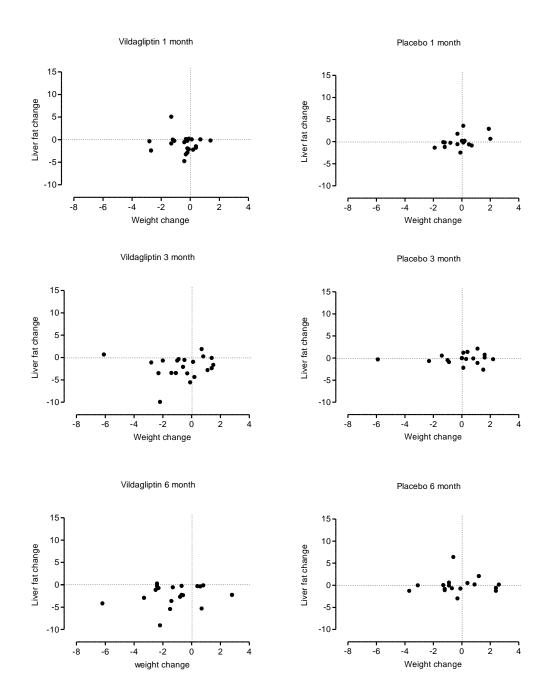
Figure 4.4 The relationship between change in liver triglyceride (fat%) and change in weight (kg) for each individual is shown for the vildagliptin (left hand column) and placebo (right hand column) at 1 months (top row), 3 months (middle row) and 6 months (bottom row)

4.4 Discussion

This study demonstrates for the first time that DPP-4 inhibition can bring about clinically useful decreases intra-hepatic triglyceride levels associated with a fall in plasma ALT and plasma glucose. The improvement in glucose control observed is unlikely to have had a direct effect upon liver triglyceride levels as shown by observation of no change in liver triglyceride levels during metformin or sulfonylurea therapy (Tiikkainen *et al.*, 2004; Phielix *et al.*, 2013).

The mechanism underlying both the discovery and development of DPP-4 inhibitors was the GLP-1 mediated enhancement of pancreatic islet function (Ahren *et al.*, 2005; Mari *et al.*, 2005; Ahren *et al.*, 2009). The GLP-1 mediated action brings about increased sensitivity of both α- and β-cells to glucose. However vildagliptin has been observed to cause changes, which suggest additional metabolic effects. These include decreases in rate of appearance of palmitate (i.e. lipolysis) and decreases in lipid oxidation rate during a euglycaemic clamp study (Azuma *et al.*, 2008). During fasting, almost 80% of fatty acid in the very low-density lipoprotein secreted from the liver derives from adipose tissue (Barrows and Parks, 2006). The rate of *de novo* lipogenesis rises sharply with increasing extent of liver triglyceride accumulation and accounts for over three-fold greater contribution to plasma triglyceride in subjects with non-alcoholic fatty liver disease (Lambert *et al.*, 2014).

It was therefore important to quantify the effect of DPP-4 inhibition upon ectopic triglyceride in liver, as this organ is central to lipoprotein homeostasis and whole body lipid metabolism.

The present study has demonstrated a substantial fall in fasting liver triglyceride (fat percentage) concentration during vildagliptin treatment, with levels becoming similar to that of the non-diabetic control group. The major influence on liver triglyceride is body weight; and weight and per cent body fat decreased to only a modest degree in the vildagliptin treated group. Figure 4.5 illustrates the lack of temporal relationship between change in body weight and change in liver triglyceride (fat percentage). Additionally, by applying the observation of Tiikkainen and colleagues that a 1kg loss of body weight at steady state is associated with a decrease in liver triglyceride (fat percentage) of approximately 0.58% (Tiikkainen *et al.*, 2003), the highly statistically significant fall in liver triglyceride on vildagliptin therapy remained (1 month $p<0.01$; 3 months $p<0.01$; 6 months $p<0.001$). The Tiikkainen correction was calculated as the product of change weight and correction factor (0.58), added to liver fat percentage (if

weight increased) or subtracted from liver fat percentage if weight decreased at that time point. The extent of the fall in liver triglyceride was clinically significant as reflected by the reduction in plasma ALT and also by the correlations between the decreases in liver triglyceride and those of both plasma ALT and fasting plasma glucose. Compared to the 50% reduction in liver triglyceride reported for thiazolidinedione therapy (Bajaj *et al.*, 2003; Ravikumar *et al.*, 2008), a 27% reduction was seen in the vildagliptin treated group and there was no change in the placebo treated group. However, when the analysis is restricted to those individuals who completed 6 months of vildagliptin therapy, intra-hepatic triglyceride fell by 42% (p=0.0003).

The mechanism underlying the fall in liver triglyceride on vildagliptin therapy must be considered. Either fat oxidation must have increased or the balance between net hepatic storage and export must have changed. The most likely explanation is a substantial decrease in storage due to the previously observed decrease in fasting lipolysis and fatty acid supply to the liver (Utzschneider *et al.*, 2009). *De novo* lipogenesis was not assessed in this study, but overall a modest increase in this process would account for the lower plasma glucose levels in the face of no decrease in hepatic glucose production and still be consistent with the observed change in hepatic triglyceride concentration. No change in whole body lipid oxidation was observed. Hepatic export of lipid is unlikely to have increased given the 13% fall in plasma triglyceride concentration and given the results from a previous study which showed no effect of vildagliptin treatment on postprandial triglyceride levels secondary to reduced flux of lipoproteins from the liver (Matikainen *et al.*, 2006).

It is known that the GLP-1 receptor is expressed on human hepatocytes (Svegliati-Baroni *et al.*, 2011) and a direct effect of increased GLP-1 levels is feasible. GLP-1 agonist therapy is known to decrease hepatic triglyceride levels in ob/ob mice (Gupta *et al.*, 2010) and DPP-4 inhibitors have been shown to reduce liver fat in obese mice models (Kern *et al.*, 2012). Furthermore, DPP-4 inhibition prevents fatty liver developing during overfeeding of both wild type and glucokinase deficient mice (Shirakawa *et al.*, 2011). In the latter study it was also demonstrated that fatty acid synthase expression was decreased. However, caution is required in drawing mechanistic information from studies on rodents which, unlike humans, are able to synthesize fatty acids from glucose in adipocytes (Shrago *et al.*, 1969).

Although there was a tendency for intramyocellular lipid to decrease with vildagliptin treatment, this was not statistically significant. Metformin and pioglitazone have been

shown to reduce intramyocellular lipid by 25% and up to 55%, respectively (Teranishi *et al.*, 2007; Ravikumar *et al.*, 2008). The exact mechanism through which intramyocellular lipid accumulation impairs skeletal muscle insulin sensitivity is not fully understood. Nonetheless, it has been suggested that the intermediaries of lipid oxidation such as DAG, ceramide and sphingosine (Samuel *et al.*, 2010; Dube *et al.*, 2011) may play a role in the impairment of GLUT 4 expression and insulin signalling (Greco *et al.*, 2002). Even though elevated intramyocellular lipid in endurance-trained athletes was found to be associated with an increase in muscle insulin sensitivity (Goodpaster *et al.*, 2001), intramyocellular lipid oxidative capacity is higher in endurance-trained athletes compared to lean sedentary control subjects (Goodpaster *et al.*, 2001; Perseghin *et al.*, 2002). This suggests that intramyocellular lipid oxidative capacity may also contribute significantly to normal muscle insulin sensitivity. Arguably it should be considered whether either reducing intramyocellular lipid accumulation or increasing oxidative reduction could increase muscle insulin sensitivity. Short-term low calorie diet has been shown to decrease IMCL by increasing its rate of oxidation. This led to improving skeletal muscle insulin sensitivity (Lara-Castro *et al.*, 2008).

The starting mean hepatic triglyceride level in the vildagliptin treated group was by random chance higher than that of the placebo group. It is therefore important to be sure that the observed change was not merely tendency to the mean. To exclude this possibility, when subjects were pair matched between the groups for baseline liver triglyceride level with values within 0.2%, 12 pairs were identified. In the vildagliptin treated subjects liver triglyceride levels fell over the 6-month period (6.3 ± 1.0 to $4.1\pm0.6\%$; $p<0.02$) whereas there was no change in the pair members on placebo (6.4 ± 1.0 to $6.6\pm1.6\%$; $p=0.73$).
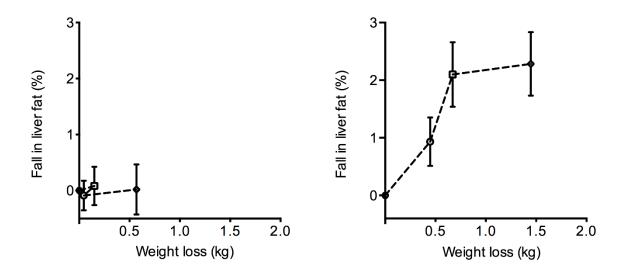
Figure 4.5 Lack of relationship between change in liver fat and change in weight for the placebo group (left) and the vildagliptin (right)

Weight loss of 0.5kg brought about no change in liver fat in the placebo group whereas the continuing steady weight loss in the vildagliptin group brought about no additional change in liver fat from 3 to 6 months.

CHAPTER 5 Morphology and composition of the pancreas

5.1 Introduction

Three basic aspects of the pancreas may be of relevance to the aetiopathogenesis of type 2 diabetes. Firstly, the overall size of the pancreas must be considered. By the time type 2 diabetes is diagnosed, the number of β cells would have decreased by 50% (Butler *et al.*, 2003; Rahier *et al.*, 2008). Studies using ultrasound or CT have suggested 7 - 22% decrease in pancreas volume (Alzaid *et al.*, 1993; Saisho *et al.*, 2007), but the groups studied have not been homogenous. Secondly, there has been no description of the overall appearance of the pancreas in type 2 diabetes. There is marked variation of morphology in the general population, with a more serrated boundary of the pancreas generally ascribed to ageing (Hahn, 1999). Thirdly, the pancreas triglyceride content has been reported to be related to β cell function (Tushuizen *et al.*, 2008; Szczepaniak *et al.*, 2012).

Short-term exposure of fatty acid to normal islet cell cultures has been found to increase glucose stimulated insulin secretion (Gravena *et al.*, 2002), and long-term exposure of fatty acid to normal islet cell cultures have been reported to have a contrasting effect (Dubois *et al.*, 2004). Both effects were thought to be independent of glucose concentration. However, long-term exposure of the pancreas to lipids facilitates intra-pancreatic lipid accumulation, and this may possibly be related to loss in first phase glucose stimulation insulin response (Lee *et al.*, 1994a; Lupi *et al.*, 2002a) and β cell apoptosis (Shimabukuro *et al.*, 1998b; Butler *et al.*, 2010). Measurement of pancreas fat content using magnetic resonance spectroscopy has been questioned (Saisho *et al.*, 2008). Spectroscopy requires pre-definition of the volume of tissue but the organ moves with respiration. To avoid possible inclusion of signal from surrounding adipose tissue, an imaging-based method of high precision (Lim *et al.*, 2011a) was developed. Using this method during rapid weight loss, the pancreas fat content was shown to decrease over the same time course of return of normal insulin secretory capacity (Lim *et al.*, 2011a). That longitudinal study was designed to evaluate the mechanisms underlying the return to normal glucose control during a very low calorie diet and was not large enough to quantitate precisely the difference in pancreas morphology between type 2 diabetes and age, weight and sex matched non-diabetic controls.

The present study was designed to define volume, morphology and triglyceride content of the pancreas. As DPP-4 inhibition may delay the decline in insulin secretion, and as

this may underlie atrophy of the pancreas, the possible effects of vildagliptin on pancreas volume in type 2 diabetes were determined during the course of 6 months of therapy.

5.2 Study design

5.2.1 Subjects

The clinical and metabolic descriptions of type 2 diabetes subjects are given in CHAPTER 3. Fourteen (8M: 6F) normal glucose tolerant subjects matched for age, weight and BMI, with no first - degree relative of diabetes were recruited as controls and studied only at baseline to provide comparative data. They were not on any drug therapy affecting glucose metabolism such as oral steroids and had no known first - degree relative with diabetes. The characteristics of both type 2 diabetes and control groups is summarised in Table 5.1.

	Type 2 diabetes (n=42)	Controls (n=14)	p value
Weight (kg)	87.2±2.0	86.7±3.7	0.81
BMI (kg/m^2)	30.3±0.5	29.6±1.0	0.67
Body fat (%)	31.6±1.2	27.9±2.6	0.11
Waist/Hip ratio	0.95±0.01	0.90±0.02	0.02
Age (years)	61.8±1.0	59.0±2.2	0.10
Fasting plasma glucose (mmol/l)	7.9±0.5	5.1±0.1	<0.0001
Fasting plasma insulin (mU/L)	13.3±1.3	7.9±0.9	0.002
HOMA-β (%)	72.0±7.1	97.3± 9.5	0.01
HOMA-IR (µU/mol/l^3)	4.7±0.4	1.9 ± 0.2	0.02
HbA$_{1c}$ (%)	6.4±0.1	-	
Duration of diabetes (years)	5.7±0.7	-	

Table 5.1 Anthropometry and metabolic characteristics of the type 2 diabetes and control groups

Data are shown as mean ± SEM

5.2.2 Study protocol

A non-invasive magnetic resonance method was developed to quantitate pancreas volume using Balanced Turbo Field Echo (BTFE) structural scans. BTFE images contain a mix of T_1 and T_2 contrast, which distinguishes high signal intensity from vessels and visceral fat with lower intensity signals from the pancreas. It can therefore be used to clearly delineate the boundaries of the pancreas from the adjacent structures, including the surrounding visceral fat, the splenic vein, the superior mesenteric vessels, the inferior vena cava and the duodenum. Twelve axial sections of 5mm thickness were imaged during an 8 second breath hold (repetition time/echo time/flip angle = 3.1ms/1.6ms/40°, field of view 400 - 480x300mm according to patient size, zero-filled to give resolution 1.39mm x 1.40mm, turbo factor 95, parallel imaging factor 2, bandwidth 1156Hz per pixel). The polygon ROI tool in the freely-available imaging software Image-J (Schneider *et al.*, 2012) was used to delineate the pancreatic tissue in each section. Integration over all the sections containing tissue allows the total pancreas volume in cm^3 to be obtained. A Bland-Altman analysis was carried out to define repeatability and reproducibility of the pancreas volume measurement, with independent inter-observer assessment of pancreas volume performed by two trained analysts (myself and another trained observer). A subset of 20 randomly selected measurements were analysed twice to define inter-observer statistics.

A pancreas volume index, defined as the pancreas volume in cm^3 divided by the body surface area calculated by the method of Dubois and Dubois (DuBOIS and DuBOIS, 1916) was used to examine possible effects of different anthropometry on observed measurements (Goda *et al.*, 2001). The ratio of the pancreas volume to the waist circumference (in cm^3/cm) was also calculated to evaluate the data corrected for potential excess visceral fat in type 2 diabetes subjects.

The anterior border of the pancreas was observed to be particularly variable in respect of degree of smoothness or irregularity. To quantify this, a five point semi-quantitative scale of irregularity (1 = most irregular, 5 = least irregular) using standardized outlines to define each grade was applied to score all pancreas scans. This was done blind to the type 2 diabetes and normal glucose tolerance status. Two observers agreed the score for each pancreas in consensus.

At baseline, 44 type 2 diabetes subjects and 14 normal glucose tolerant control subjects had pancreas volume and triglyceride content measured after a 10 hours overnight fast.

The type 2 diabetes subjects were randomised to receive either DPP-4 inhibitor or placebo in addition to metformin as described in CHAPTER 3. Measurements were also carried out at baseline, 1, 3 and 6 months of treatment, respectively Figure 4.1.

5.3 Results

5.3.1 Pancreas volume

Mean pancreas volume was smaller in the type 2 diabetes compared with the control group (55.5±2.8 vs. 82.6±4.8cm^3; p<0.0001) Figure 5.1. Hence the pancreas was one third smaller in the type 2 diabetes subjects. In the type 2 diabetes group, pancreas volume ranged from 20.8 to 99.7cm^3, and in the control group from 60.1 to 124.2cm^3. Expression of the data as pancreas volume index did not change the extent of difference between the groups (33%; 27.8±1.3 vs. 41.7±2.5cm^3/m^2; p<0.0001). When the data were expressed as ratio of pancreas volume to waist ratio, there was a 38% decrease in the type 2 diabetes group (0.53±0.03 vs. 0.85±0.06; p<0.0001).
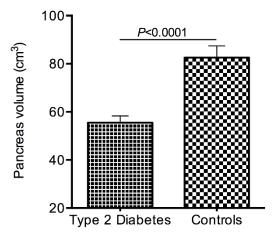
Figure 5.1 Mean pancreas volume in the type 2 diabetes and control groups

5.3.2 Relationship of pancreas volume to insulin secretion

There was a positive correlation between HOMA-B and pancreas volume in the type 2 diabetes (r=0.31; p=0.03), control (r=0.46; p=0.05), and in the whole population studied (r=0.37; p=0.003) Figure 5.2. Pancreas volume was inversely proportional to age in those less than 60 years in the type 2 diabetes (n=7; r=-0.56; p=0.02), but this could not be demonstrated in the control group, probably as a type 2 statistical error (n=6; r=-0.66; p=0.09). Pancreas volume did not correlate with the duration of type 2 diabetes (r=-0.15; p=0.19).
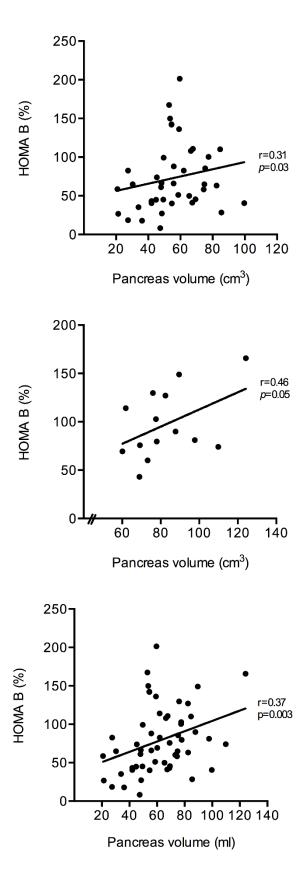
Figure 5.2 Relationship between HOMA-B (%) and pancreas volume (cm^3) in the type 2 diabetes group (top), control group (middle) and combined group (bottom).
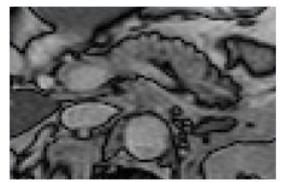
5.3.3 Effect of DDP-4 inhibition on pancreas volume

Pancreas volume remained constant over 6 months in both the placebo plus metformin treated (60.5 ± 2.0 to $60.6\pm4.5cm^3$) or the vildagliptin plus metformin treated type 2 diabetes groups (50.4 ± 4.1 to $51.8\pm4.2cm^3$; p=0.20).

5.3.3.1 Pancreas morphology

The pancreata of type 2 diabetes subjects were typically involuted, with a feathery, irregular border when compared with age, weight and sex matched glucose tolerant controls Figure 5.3. The semi-quantitative analysis of irregularity of the border of the pancreas is shown in Figure 5.4. Most type 2 diabetes subjects were deemed to have an irregular pancreatic outline (modal score 3). The distribution of scores was significantly different between type 2 diabetes and normal glucose tolerant subjects (p<0.002).
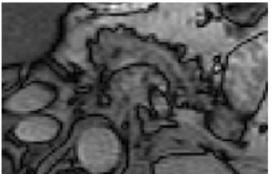
T2DM subjects	NGT subjects
58y female, 78.5kg	51y female, 78.0kg
64y male, 94.4kg	65y male, 93.0kg
64y male, 75.1kg	64y male, 74.7kg
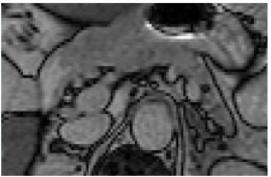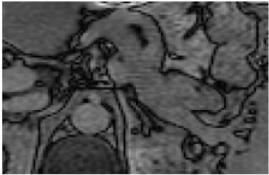
Figure 5.3 Comparison of the morphology of the pancreata of type 2 diabetes (left) and normal glucose tolerant subjects (right) demonstrating the more irregular anterior (uppermost) border of the pancreata. [TDM=type 2 diabetes subjects; NGT=normal glucose tolerant subjects]
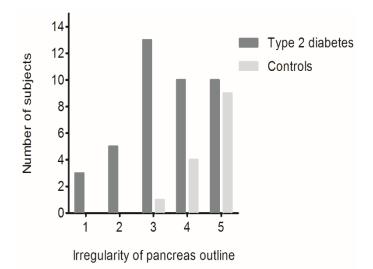
Figure 5.4 Semi-quantitative analysis of the irregularity of the pancreas outline. Most irregular = 1 and least irregular = 5. It can be seen that the most non-diabetic individuals have a smooth pancreas outline whereas many people with type 2 diabetes have an irregular outline. The distribution of scores is significantly different (p<0.002)

5.3.3.2 Intra-pancreatic triglyceride

Pancreas triglyceride content was 23% higher in type 2 diabetes compared with normal glucose tolerant (5.4±0.3 vs. 4.4±0.4%; p=0.02) Figure 5.5. There was a positive correlation between pancreas triglyceride and BMI in normal glucose tolerant (r=0.64; p=0.008) but this was not present in type 2 diabetes (r= -0.10; p=0.29). There was a positive correlation between pancreas and liver triglyceride in the normal glucose tolerant (r=0.63; p=0.01) but this was lost in type 2 diabetes (r=0.02; p=0.46), and in the whole group (r=0.18; p=0.10).

Pancreas triglyceride content remained unchanged at 6 months during placebo and metformin treatment of type 2 diabetes (5.6±0.4 vs. 5.5±0.5%; p=0.90), and there was also no difference between the vildagliptin and metformin treated group (5.1±0.4 to 5.0±0.2%; p=0.74).
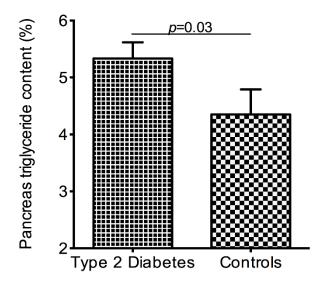
Figure 5.5 Pancreas triglyceride content in the type 2 diabetes and control group

5.4 Discussion

This study demonstrates that pancreas volume is 33% smaller in individuals with type 2 diabetes well controlled on metformin compared to normal glucose tolerant controls matched for age, weight and BMI. Insulin secretion assessed by HOMA-B was proportional to pancreas size in the type 2 diabetes, control and combined groups. A unique serrated boundary of the pancreas early in type 2 diabetes has been described for the first time. Not only is the pancreas small and involuted in appearance but also it has a 23% higher triglyceride content compared with that of well-matched glucose tolerant individuals.

Several previous studies have assessed the size of the pancreas in type 2 diabetes. A retrospective study of individuals who had undergone abdominal CT scan reported a small decrease in pancreas volume in type 2 diabetes (7%; p<0.05). However, the group was unusual in having a low BMI (25.4kg/m^2), and body weight was not matched between the type 2 diabetes and control groups (Saisho *et al.*, 2007). Two ultrasound studies have examined pancreas volume in type 2 diabetes. One observed an 11% decrease in pancreas volume compared to the controls (Alzaid *et al.*, 1993). However, the type 2 diabetes subjects studied had a longer duration of 9 years and fasting plasma glucose of 13mmol/l. The larger study of 84 type 2 diabetes and 80 controls subjects matched for age, sex and body mass observed a 22% decrease in area of the pancreas (Migdalis *et al.*, 1991). However, the abnormality has not been widely recognized. The present study used a new method of defined precision to demonstrate a 33% decrease in pancreas volume in people with type 2 diabetes who are well controlled on metformin.

Further analysis in the type 2 diabetes group demonstrated a significant difference in mean basal insulin concentration between those with pancreas volume <50cm^3 (n=16) compared to those with pancreas volume >50cm^3 (n=23); (7.9±2.2mU/L; p=0.0009), and this supports the association between pancreas volume and endocrine function. Although the endocrine pancreas consisting of the islet cells accounts for only 1% of total pancreas tissue, pancreas volume was found to be reduced by a third in this study. Therefore it is reasonable to speculate that the exocrine pancreatic tissue may also be reduced in type 2 diabetes. This is likely to be an effect of loss of the anabolic or trophic effects of insulin especially after each meal. Normally, plasma insulin concentrations rise markedly, and the rise in concentration of insulin within the interstitial tissue of the whole pancreas is likely to be much greater. The clear effect of loss of insulin secretory

capacity on the pancreas volume in type 1 diabetes supports this proposed mechanism (Foulis and Frier, 1984).

The question of cause and effect is immediately posed by the demonstration of a small, involuted pancreas early in the natural history of type 2 diabetes. Are people with a small pancreas predisposed to develop type 2 diabetes, or is the decrease in pancreas volume a consequence of the ongoing pathological processes? It has been postulated that cell mass may be subnormal in those predisposed to develop type 2 diabetes (Bagust and Beale, 2003). However, in the absence of the means of measuring this *in vivo*, it has not been possible to examine this possibility. It is known that low birth weight is a risk factor for type 2 diabetes (Barker *et al.*, 1993), and it may be considered that this could be associated with a smaller pancreas. If this is correct, it could be interpreted as indirectly supporting the possibility of low pancreas volume and hence low β cell mass as predisposing factors for type 2 diabetes.

It has been suggested that approximately 20 genes may be associated with predisposition to type 2 diabetes (Prokopenko *et al.*, 2008) and majority of these genes are thought to contribute to β cell dysfunction (Gloyn and McCarthy, 2008). Although the precise mechanism involved is unknown, TCF/L2 gene silencing has been strongly linked to an inhibitory effect on glucose-induced insulin secretion in type 2 diabetes (Grant *et al.*, 2006).

However, pancreas volume could secondarily be affected of the loss of normal post-meal rise in insulin concentration by a paracrine effect. After meals, the local intra-pancreatic rise in concentration must vastly exceed the 10 - 15 fold rise achieved in plasma concentration (Carey *et al.*, 2005). Therefore, pancreatic tissue is likely to be exposed to very high concentrations of insulin under normal circumstances. When there is no local insulin production as in type 1 diabetes, pancreas volume is known to be decreased by one third (Foulis and Frier, 1984) and this is evident within months of diagnosis (Williams *et al.*, 2012). As such it is reasonable to postulate that phasic secretion of insulin, with concentration at least 10 fold greater than required for metabolic effects (Poggi *et al.*, 1979) achieved with meals promotes growth locally by binding to IGF1 receptors. In type 2 diabetes basal insulin levels are raised but there is an absence of immediate and major insulin release after meals, which could explain a lack of growth promoting concentrations of the hormone. It is reasonable to postulate that normalisation of phasic insulin secretion in type 2 diabetes with calorie restriction may restore pancreas volume and outline. The recovery of β cell function after calorie

restriction is likely to depend upon release from the inhibitory effects of lipid metabolites. This relatively rapid process contrasts with longer time course likely to be required to change volume of the whole pancreas. Clinical studies will be required to examine the effect of calorie restriction on pancreas morphology. Studies in non-human primates have observed an approximate 10% decrease in pancreas volume 2 - 6 months after low dose streptozotocin, but at each time point only single animals were observed and statistical significance could not be demonstrated (Saisho *et al.*, 2011).

This work establishes a significant relationship between pancreas volume and HOMA-B in both type 2 diabetes and control groups, but the latter is an estimate of overall β cell insulin responsiveness and not a measure of glucose-mediated acute insulin secretion. Further work is required to define the precise relationship of first phase insulin response to pancreas volume.

The association of pancreas triglyceride with type 2 diabetes remains contentious. A cross-sectional study using ultrasound of 7,464 individuals showed higher pancreas triglyceride levels to be associated with both type 2 diabetes and pre-diabetes (Bi *et al.*, 2013). Magnetic resonance spectroscopy allows measurement of intra-organ triglyceride, although, this relies upon correction for respiratory movement in defining tissue which is definitely within the pancreas. Using this method, it has been shown that higher pancreas triglyceride levels are associated with raised basal insulin levels, and that this relationship is present in different ethnic groups (Szczepaniak *et al.*, 2012). The wide scatter of absolute levels of pancreas triglyceride with a tendency for higher levels in people with diabetes has been confirmed (Tushuizen *et al.*, 2007). The latter study showed that pancreas triglyceride correlated negatively with early glucose-stimulated insulin secretion.

Great care is required using the spectroscopic method as the fat signal could be contaminated by the stronger signal from surrounding visceral adipose tissue, and because of this an imaging-based method was developed (Lim *et al.*, 2011a). All analysis is conducted on an acquired image by this method and not on a pre-determined volume of the body, the contents of which may change with respiration or movement. This method permits higher precision of measurement (Lim *et al.*, 2011a). The present results apply the 3-point Dixon method of magnetic resonance imaging to well characterized individuals with type 2 diabetes subjects well controlled on metformin only and demonstrate that pancreas triglyceride content is 23% higher than

in age, weight and sex matched normal controls. Additionally, the data indicate that the normal positive correlation between pancreas and liver fat is lost in type 2 diabetes subjects.

The mechanism through which long-term lipid exposure to the pancreas impairs glucose stimulated insulin secretion has been examined. Although it has been suggested that the suppression of mitochondrial pyruvate cycling may play a role in the loss of glucose stimulated insulin secretion (Boucher *et al.*, 2004), a longitudinal study of aetiopathogenesis of type 2 diabetes using the new method has confirmed that weight-loss induced decrease in pancreatic triglyceride is temporally associated with restoration of normal glucose stimulated acute insulin secretion (Lim *et al.*, 2011a).

The limitations of the present observation must be considered. Firstly, although measurement of organ volume is routine in radiographic practice, it has not previously been applied to the study of the pancreas in diabetes. The accuracy and precision of the method applied to pancreas require to be examined. Accuracy of such methodology is rarely established, but one study has shown volumetry by magnetic resonance imaging to correspond almost exactly to water displacement for pancreata of mini-pigs (Szczepaniak *et al.*, 2013). Good intra- and inter-observer agreement as reflected in the Bland-Altman plots were demonstrated. Additionally, repeat measurements after 6 months of vildagliptin or placebo treatment were highly consistent. Thus the measurement of pancreas volume by magnetic resonance imaging with BTFE can be regarded as robust. Secondly, assessment of irregularity of shape of the pancreas can only be regarded, as semi-quantitative and further work is required to develop a mathematical method for measuring deviation from a smooth but variable curve. Thirdly, accuracy and precision of pancreas triglyceride must be considered. Data on accuracy of human pancreas triglyceride measurement is lacking, due to the rapid enzyme mediated autolysis of the pancreas post-mortem. However, the Newcastle MR group has previously reported an excellent inter-scan Bland-Altman coefficient (Lim *et al.*, 2011a), and the 3-point Dixon method used gives almost identical absolute measurements of intra-pancreatic triglyceride compared to that obtained by the centre with the largest experience of measurement using magnetic resonance spectroscopy (Szczepaniak *et al.*, 2013). Finally, the type 2 diabetes group was large for this type of detailed study. Although the group of subjects with proven normal glucose tolerance was smaller, the consistency of measured outcomes within

the relatively homogenous control group allowed confident identification of the major abnormalities associated with type 2 diabetes.

In summary, this study has demonstrated that in a group of type 2 diabetes individuals with good metabolic control on metformin the pancreas is 33% smaller, typically involuted and has raised triglyceride content compared to normal. These gross abnormalities could potentially yield prognostic information. Further work must elucidate their relationships with the pre-diabetes phase, their interaction with treatment modalities and changes during the natural history of type 2 diabetes.

CHAPTER 6 Direct assessment of diurnal glycogen storage

6.1   Introduction

In normal health, the control of postprandial plasma glucose depends upon timely storage of meal-derived glucose as glycogen in the liver and skeletal muscle. In young healthy humans, approximately 30% of carbohydrate absorbed is stored as skeletal muscle glycogen in the immediate postprandial period (Taylor *et al.*, 1993) and approximately 20% as liver glycogen (Taylor *et al.*, 1996). If no food is taken over the next 7 hours, glycogen concentration in both muscle and liver decline. Glucose carbon is glycolysed to lactate and exported to fuel hepatic gluconeogenesis in order to maintain glucose supply largely for brain and red blood cell oxidative needs. However, when meals are taken at intervals of 4 - 5 hours during the day, there is a sequential rise in muscle and liver glycogen concentration in healthy volunteers (Hwang *et al.*, 1995; Carey *et al.*, 2003). Tissue glycogen concentrations would be expected to be maximal after the evening meal prior to the overnight fall to fasting levels.

These data suggest that muscle and liver act to buffer the plasma changes in glucose concentration after meals by storing the osmotically active metabolite in an inert form, hence preventing large postprandial swings in plasma glucose concentration. In type 2 diabetes, inadequate glucose storage as glycogen has been previously reported, and the postprandial rise in plasma glucose concentration is excessive (Mitrakou *et al.*, 1990; Carey *et al.*, 2003; Krssak *et al.*, 2004). However, the extent of abnormality has not been defined for the buffering function of glycogen storage in muscle and liver during day-long eating in type 2 diabetes. Additionally, data on the effect of therapeutic DPP-4 inhibition on tissue glycogen storage in type 2 diabetes is lacking. The purpose of this study was to quantify the maximum daily flux of muscle and liver glycogen in people with well controlled type 2 diabetes compared to matched glucose tolerant controls, and to characterise the effect of vildagliptin on day-long eating.

6.1 Study design

6.1.1 Subjects

The clinical and metabolic description of 44 type 2 diabetes and 14 normal glucose tolerant subjects matched for age, weight and BMI subjects are given in CHAPTER 3. The characteristics for both groups is summarised in Table 5.1. The study was approved by the Newcastle and North Tyneside 2 Research Ethics Committee.

6.1.2 Study protocol

Fasting and post-evening meal muscle and liver glycogen concentrations were measured by ^{13}C MR spectroscopy as described in CHAPTER 2. All subjects were asked to avoid vigorous exercise and take no alcohol for 3 days prior to assessment. At baseline, all subjects attended for measurements of fasting and post-evening meal muscle and liver glycogen concentration, plasma glucose, insulin, glucagon and triglyceride concentration. The measurements were repeated at 6 months.

The type 2 diabetes subjects were randomised (ratio 1:1) in a double-blinded fashion to receive either vildagliptin 50mg bid or placebo. All committed to take metformin therapy at a stable dose. Further measurements of fasting muscle and liver glycogen concentration in the type 2 diabetes subjects (vildagliptin and placebo groups) were carried out at 1 and 3 months Figure 4.1.

On the morning of first day after a 10 hour overnight fast, subjects were transported to the study centre by taxi and measurements commenced at 0800h. Blood samples for plasma glucose, insulin, glucagon and triglyceride concentration were taken; then magnetic resonance (MR) measurements of fasting muscle and liver glycogen concentration were performed. Only water was allowed during the fasting period, and all subjects with type 2 diabetes were advised to omit their morning dose of metformin.

Postprandial measurements after a day of three standard meals were carried out at least 3 days after fasting assessments. All food for the study day had been previously provided and subjects were asked to consume all of the meals but nothing in addition to that provided. Meal composition is detailed in Appendix 2. Meals were eaten at 0800h, 1200h, at 1600h. The type 2 diabetes subjects took their usual doses of metformin tablets 5 minutes before meals. Blood samples were taken at 2000h, and

then MR measurements were performed immediately Figure 6.1. This ensured that muscle and liver post-meal glycogen concentrations were measured 4 hours after the third meal, when maximal net glycogen storage from the three meals would be expected.
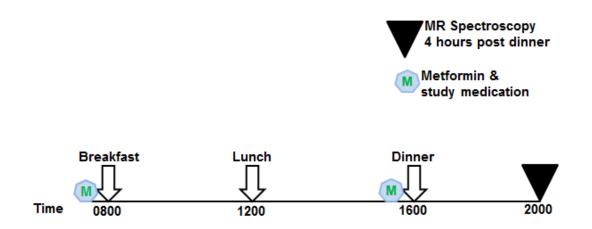
Figure 6.1 Post meal skeletal muscle and liver glycogen concentration measured by MR spectroscopy

6.2 Results

6.2.1 Muscle glycogen concentration

At baseline, mean fasting muscle glycogen concentration did not differ between the type 2 diabetes and normal glucose tolerant groups (68.3±2.6 vs. 68.1±4.8mmol/l; p=0.82). At 4 hours after the third meal, muscle glycogen concentration rose by 17.3% in the normal glucose tolerant group (68.1±4.8 to 79.7± 4.2mmol/l; p=0.0006) Figure 6.2. In the type 2 diabetes group, muscle glycogen concentration remained unchanged over the same time period (68.3 ± 2.6 to 67.1 ± 2.0mmol/l; p=0.62). The change in diurnal muscle glycogen concentration inversely correlated with HOMA-IR only in the normal glucose tolerant group (r = -0.56; p=0.02) Figure 6.3.
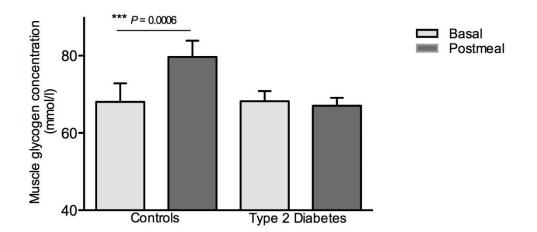
Figure 6.2 Diurnal change in muscle glycogen concentration. Fasting and post-three meal concentrations are shown for the normoglycaemic control and type 2 diabetes groups
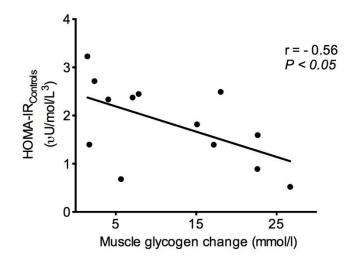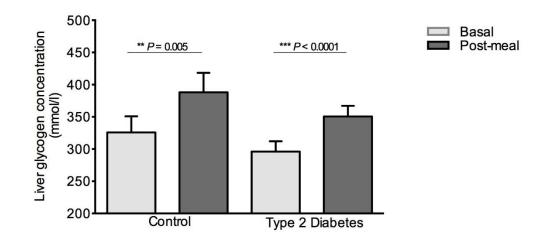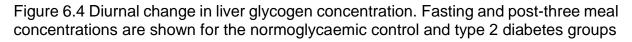
Figure 6.3 Correlation between HOMA-IR and change in muscle glycogen concentration in the normoglycaemic control subjects

Fasting muscle glycogen concentration was similar between the vildagliptin treated and placebo groups at baseline (70.5±4.3 vs. 65.9±23.0mmol/l; p=0.82). In the vildagliptin treated group, fasting muscle glycogen concentration remained unchanged at 1 month (68.6±3.6 vs. 64.8±3.3mmol/l p=0.43), at 3 months (66.4±2.9 vs. 66.0± vs. 3.2mmol/l), and at 6 months (67.5±2.4 vs. 64.4±3.0mmol/l; p=0.43). After 6 months of vildagliptin treatment, muscle glycogen concentration 4 hours after the third meal remain unchanged (69.0±2.5 to 66.0± 3.3mmol/l; p>0.05).

6.2.2 Liver glycogen concentration

At baseline, fasting liver glycogen concentration was similar in the type 2 diabetes and normal glucose tolerant groups (296.1±16.0 vs. 325.9±25.0mmol/l; p=0.24). At 4 hours after the third meal, liver glycogen concentration was higher in both groups (296.1±16.0 to 350.5±6.7mmol/l; p<0.0001 and 325.9±25.0 to 388.1±30.3mmol/l; p=0.005), respectively; and there was no significant difference between the groups (p=0.31). The per cent diurnal glycogen change was comparable between the type 2 diabetes and normal glucose tolerant groups (18 vs. 19%) Figure 6.4.

Figure 6.4 Diurnal change in liver glycogen concentration. Fasting and post-three meal concentrations are shown for the normoglycaemic control and type 2 diabetes groups

Fasting liver glycogen concentration was similar between the vildagliptin treated and placebo groups at baseline (303.3 ± 22.47 vs. 288 ± 23.4mmol/l). In the vildagliptin treated group, fasting liver glycogen concentration remained unchanged at 1 month (303.2 ± 21.1 vs. 299.9 ± 17.4mmol/l), at 3 months (296.9 ± 19.3 vs. $302.1\pm$ vs.21.9mmol/l), and at 6 months (308.5 ± 27.5 vs. 298.2 ± 19.8mmol/l). After 6 months of vildagliptin treatment, liver glycogen concentration 4 hours after the third meal was similar in both vildagliptin treated (313.7 ± 26.3 to 349.4 ± 25.9mmol/l) and placebo group (280.1 ± 18.2 to 354.0 ± 24.3mmol/l).

6.2.2.1 Plasma glucose, metabolites and hormone concentration

6.2.2.2 Plasma glucose concentration

Baseline plasma glucose concentration was higher in the type 2 diabetes group than the normal glucose tolerant group, at both fasting (7.7 ± 0.2 vs. 5.1 ± 0.1mmol/l; $p<0.0001$) and 4 hours after the third meal (6.5 ± 0.2 vs. 5.7 ± 0.2mmol/l; $p=0.03$). There was no difference in baseline fasting plasma glucose concentration between the vildagliptin treated and placebo groups (7.7 ± 0.2mmol/l vs.7.8 ± 0.3mmol/l).

At the 6 months time point, blood glucose concentration at 4 hours after the third meal was not significantly different between the vildagliptin treated and placebo group (6.3 ± 0.3 vs.6.8 ± 0.3mmol/l; $p=0.22$) Figure 6.5a. However, in the placebo group a

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significant rise had occurred (p<0.02), whereas in the vildagliptin group a significant fall (p=0.05) was observed.

6.2.2.3 Plasma insulin concentration

Baseline plasma insulin concentration was higher in the type 2 diabetes group than the normal glucose tolerant group at both fasting (13.8±1.2 vs. 7.9±0.9mU/L; p=0.01) and 4 hours after the third meal (31.9±2.9 vs. 18.8±3.3mU/L; p=0.03). Plasma insulin 4 hours after the third meal was higher than fasting in the normal glucose tolerant group (7.9±0.9 to 18.8±3.3mU/L; p=0.003). Similarly in the type 2 diabetes group, plasma insulin rose from 13.8±1.2 to 31.9±2.9mU/L (p<0.0001).

Plasma insulin concentration at baseline was comparable between the vildagliptin treated and placebo groups, both at fasting (13.0±1.8 vs. 14.8±1.6mU/L; p=0.33) and at 4 hours after the third meal (30.8±3.0 vs. 33.8±4.9mU/L; p=0.61).

At the 6 months time point, plasma insulin concentration after the third meal was significantly lower in the vildagliptin treated group (22.3±2.7 vs. 32.3±3.8mU/L; p=0.001) Figure 6.5b.

6.2.2.4 Plasma glucagon concentration

Baseline plasma glucagon concentration was higher in the type 2 diabetes group than the normal glucose tolerant group at both fasting (70.4±4.7 vs. 48.4±4.8ng/L; p=0.01) and 4 hours after the third meal (108.5±7.5 vs. 67.7±7.3ng/L; p<0.001).

Plasma glucagon concentration at baseline was not statistically different in the vildagliptin treated and placebo group at both fasting (64.1±5.8 vs. 73.7±7.1ng/L; p=0.31) and 4 hours post meal (107.8±10.2 vs. 110.2±12.0ng/L; p=0.88), respectively.

At the 6 months time point, plasma glucagon concentration was significantly lower in the vildagliptin treated group after the third meal (82.6±7.9 vs. 106.1±12.7ng/L; p=0.01) and also at fasting (47.7±6.2 vs. 60.1±5.8ng/L; p<0.0001) Figure 6.5c.

6.2.2.5 Plasma triglyceride concentration

At baseline, plasma fasting triglyceride concentration was comparable in the type 2 diabetes and normal glucose tolerance groups (1.4±0.1 vs. 1.5±0.3mmol/l; p=0.90). At 4 hours after the evening meal, triglyceride levels rose in both type 2 diabetes (1.4±0.1 to 2.2±0.1mmol/l; p<0.0001) and in the normal glucose tolerant group (1.5±0.3 to 2.3±0.3mmol/l; p=0.007), respectively.

Baseline plasma fasting triglyceride concentration was similar in the vildagliptin treated and placebo groups (1.5±0.1 vs. 1.5±0.1mmol/l). The change from fasting to post evening meal plasma triglyceride concentration was similar in both the vildagliptin and placebo groups at baseline (1.5±0.1 to 2.2±0.2mmol/l; p<0.001).

At the 6 months time point, plasma triglyceride concentration 4 hours after the third meal was significantly lower in the vildagliptin treated (2.2±0.2 vs. 1.9±0.1mmol/l; p=0.045) Figure 6.5d.
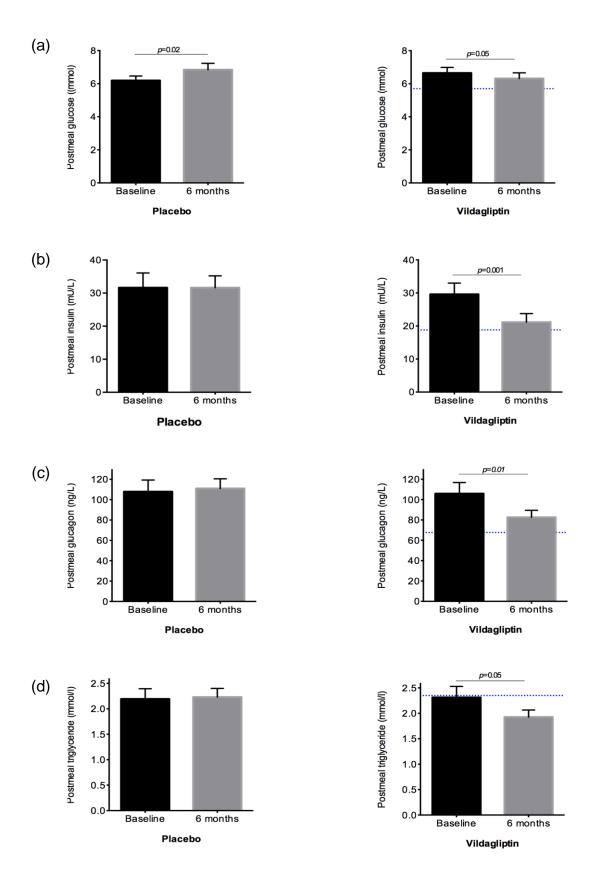
Figure 6.5 Postprandial changes in (a) glucose, (b) insulin, (c) glucagon and (d) triglyceride concentration for the control and vildagliptin treated groups at baseline and at 6 months. The dotted blue line indicates the postprandial concentration for the normal glucose tolerant subjects

6.3 Discussion

This study has defined the extent of daylong glycogen storage in muscle and liver during normal eating in both people with well controlled type 2 diabetes and with normal glucose tolerance. The data show that under normal everyday eating, skeletal muscle does not contribute to glycogen storage in people with early, well controlled type 2 diabetes. In contrast, daylong storage of glycogen in liver was similar between the type 2 diabetes and normal glucose tolerant groups. In normal glucose tolerant individuals muscle and liver were shown to act together as dynamic buffers, storing osmotically active glucose temporarily and allowing redistribution of carbon energy overnight.

Previous studies with either euglycaemic hyperinsulinaemic (Basu *et al.*, 2001) or oral glucose administration (Firth *et al.*, 1986; Kelley and Mandarino, 1990; Mitrakou *et al.*, 1990; Kelley and Simoneau, 1994) have shown that the rate of muscle glucose uptake is subnormal in subjects with type 2 diabetes, and this is reflected by higher blood glucose concentration in diabetic subjects. The present data demonstrate that under everyday conditions postprandial storage of glucose as glycogen in muscle is markedly impaired in type 2 diabetes. This sets into a physiological context the demonstration of direct observation of subnormal insulin stimulated rate of glycogen synthesis in type 2 diabetes subjects using labelled ^{13}C-glucose (Shulman *et al.*, 1990b).

Natural abundance ^{13}C MR spectroscopy has previously been applied to measure change in muscle and liver glycogen concentration in type 2 diabetes (Carey *et al.*, 2005). In a group of people with type 2 diabetes who had similar BMI but less tight blood glucose control than the present experimental group, muscle glycogen concentration did not rise significantly after two successive meals although there appeared to be a modest upward trend. The present data demonstrate that there is no net contribution of muscle glycogen to meal derived glucose storage during a full day of eating. The complete lack of increase in muscle glycogen following three meals is all the more striking when the ambient plasma insulin levels are considered. Both fasting and 4 hours after the third meal, plasma insulin levels were over 50% higher in type 2 diabetes, illustrating the practical everyday extent of muscle insulin resistance of type 2 diabetes.

The likely aetiology for the lack of glycogen storage in the skeletal muscle must be considered. The translocation of GLUT 4 membrane transporters has been shown to be impaired in individuals with insulin resistance (Garvey *et al.*, 1998). In skeletal muscle, glucose uptake via the GLUT 4 membrane transporters is the rate-limiting step

for glycogen storage (Yki-Jarvinen, 1990; Roden *et al.*, 1996; Cline *et al.*, 1999). Resistance to insulin stimulated glucose uptake by muscle has been shown to precede the onset of type 2 diabetes by decades (Lillioja *et al.*, 1988; Warram *et al.*, 1990). The defect specifically involves muscle glycogen synthesis as shown by clamp studies using 13-C labelled glucose infusion and MR spectroscopy (Shulman *et al.*, 1990a). As exercise training and weight loss both have the potential to ameliorate this defect, these interventions have been intensively studied. Although 6 weeks of intensive aerobic exercise increased insulin dependent muscle glucose uptake, this did not change relative to the non-diabetic control group (Perseghin *et al.*, 1996). Weight loss sufficient to restore durable normoglycaemia had no effect upon insulin sensitivity measured by insulin clamp (Lim *et al.*, 2011a). The defect in muscle glycogen synthesis appears to be a central aspect of the aetiopathogenesis of type 2 diabetes (Taylor, 2013).

The normality of the capacity of the liver to store glycogen in type 2 diabetes is striking in contrast to the absence of insulin stimulation of glycogen synthesis in muscle. This is likely to represent the very different glucose handling in this tissue compared with muscle. Unlike in skeletal muscle where glucose uptake via GLUT 4 glucose transporters is the rate-limiting step for glycogen storage, there is no insulin regulation of cellular glucose uptake by the hepatocyte. Liver glucose concentration inside the hepatocyte reflects that of the extracellular fluid, and hepatic glycogen synthesis is responsive to glucose concentration such that hyperglycaemia increases the rate of this process by mass action (Agius *et al.*, 1990). This will be reflected in the overall rate of storage in type 2 diabetes being expedited by the ambient hyperglycaemia following each meal, in addition to the effect brought about by insulin. Hence the normal diurnal increment in hepatic glycogen concentration cannot be interpreted as indicating normal insulin sensitivity of this metabolic pathway. When plasma glucose is clamped, hepatic glycogen synthesis rate has been shown to be subnormal (Krssak *et al.*, 2004). The same paper reported decreased rates of hepatic glycogen storage following a test meal in type 2 diabetes, and the reasons for the lack of accord with the present data must be considered. This is most likely to relate to the different groups studied. In the previously reported study (Krssak *et al.*, 2004), the type 2 diabetic subjects (n=7) were close to normal weight (BMI 26.9kg/m^2) with HbA$_{1c}$ of 7.1%; whereas in the present study the type 2 diabetic group (n=41) had a more typical mean BMI of 30kg/m^2 and were at an earlier stage of the condition with HbA$_{1c}$ of 6.4%. The latter point is important

to illustrate that type 2 diabetes develops whilst post-prandial hepatic glycogen metabolism is normal.

Knowledge of the physiological mechanisms involved in substrate storage and maintenance of glucose homeostasis is central to the appreciation of normal energy metabolism in humans (Mitrakou *et al.*, 1990). The present data quantify the ranges between which glycogen concentration in muscle and liver vary during diurnal substrate ingestion. The nocturnal usage of glycogen stores requires to be considered. In liver, the stored glucose will directly contribute to the ongoing hepatic glucose production overnight by glycogenolysis. In muscle, the carbon energy cannot be exported as glucose, due to the lack of glucose-6-phosphatase, and is instead exported as lactate or pyruvate. The process of peripheral export of these 3-carbon fragments and utilization for gluconeogenesis is described as Cori cycle activity (Dunn *et al.*, 1967). Although classically this is regarded as a futile cycle, moving glucose carbon to peripheral tissues and recycling this to liver, it can be seen that the temporal shift in storage and export allows a vital function to prevent postprandial hyperglycaemia to be satisfied. Hence, in normal health, glycogen stores in muscle and liver are built up during daytime eating and underpin the essential overnight maintenance of plasma glucose, to support brain glucose oxidation.

In the normal glucose tolerant control group there was a correlation between intramyocellular lipid and insulin sensitivity as quantified by HOMA-IR; higher intramyocellular lipid tended to be associated with lower postprandial increase in muscle glycogen as previously reported (Krssak *et al.*, 1999). In the type 2 diabetes subjects intramyocellular lipid was raised and there was no net increase in muscle glycogen. As a consequence there was no correlation between lipid stores and extent of diurnal glycogen cycling in muscle. In the liver, there was no relationship between lipid stores and extent of postprandial storage of glycogen in either group, most likely reflecting the lack of a lipotoxic effect upon control of cellular glucose uptake in the liver. This contrasts with the close relationship between intrahepatic lipid and insulin sensitivity of hepatic glucose production (Seppala-Lindroos *et al.*, 2002; Petersen *et al.*, 2005; Lim *et al.*, 2011a). Additionally, when the mass action effect of glucose on hepatic uptake is removed a significant inverse relationship can be demonstrated during hyperglycaemic hyperinsulinaemic clamp in type 2 diabetes and control subjects. The difference between this experimental fixed condition, designed to give

insight into mechanisms, and the everyday condition investigated in the present study is important to appreciate.

This study did not show any change in glucose storage in the muscle and liver with vildagliptin. It can be postulated that DPP-4 inhibition has no effect on the translocation of GLUT 4 transporters and this accounts for the lack of increase in muscle glycogen storage after 6 months treatment with vildagliptin. Although a small decrease in intramyocellular lipid was observed (22.5±2.9 to 18.5±2.3mmol/l) in the vildagliptin treated group, this was not statistically significant and did not influence muscle insulin sensitivity or glucose storage. The relatively small number of subjects analysed lacked sufficient power to allow definite conclusions to be made. Therefore a potential effect on muscle glycogen storage could not be seen. Intramyocellular lipid is technically difficult to measure in obese people and as such we were only able to obtain measurements for 14 vildagliptin treated and 12 placebo subjects.

The extent of diurnal liver glycogen storage was comparable between the type 2 diabetes and normal glucose tolerance group at baseline and at 6 months. Although vildagliptin has been shown to decrease hepatic triglyceride content, a marker of hepatic insulin sensitivity, hepatic glycogen storage is mostly dependent on extracellular glucose concentration (Agius *et al.*, 1990).

The study showed that vildagliptin lowers postprandial insulin, glucagon and TG concentrations in subjects with type 2 diabetes, and this is consistent with previous studies (Matikainen *et al.*, 2006; Vella *et al.*, 2007; Ahren *et al.*, 2010). Consequently insulin and glucagon are appropriately secreted to reflect normal physiology. The results suggest vildagliptin may restore normal postprandial insulin and glucagon profile in subjects with the type 2 diabetes. Such a profile would be characterised by earlier control of post - prandial glucose and lower the need for insulin in the late post - prandial phase. It has been suggested that the lower levels of insulin and glucagon are as a result of vildagliptin-mediated increase in α and β cell sensitivity to glucose concentration (Ahren *et al.*, 2010). Whereas metformin alone is thought to bring about a direct suppression on hepatic glucose production, DDP-4 inhibition has a clear effect through glucagon suppression (Solis-Herrera *et al.*, 2013). The combined effects of DDP-4 inhibitor and metformin may contribute to a clinical improvement in postprandial glucose concentration and overall glycaemic control.

The effect of vildagliptin on postprandial glucose excursion warrants careful consideration. If vildagliptin is taken immediately before an evening meal, postprandial

glucose excursion and inappropriate glucagon secretion are reduced, and these effects continue beyond the postprandial period (Balas *et al.*, 2007). As such postprandial hepatic glucose production is reduced and fasting plasma glucose falls.

This study has quantified the extent of day-long storage of food-derived glucose as muscle and liver glycogen in type 2 diabetic and matched normoglycaemic individuals. Muscle glycogen storage was completely inactive in type 2 diabetes whereas liver glycogen storage was normal. The data define the extent of post-prandial glucose storage as glycogen in health and identify an important contributor to post-prandial hyperglycaemia.

CHAPTER 7 General Discussion

7.1   Overview

The work reported in this thesis advances basic knowledge about type 2 diabetes and also demonstrates a previously unrecognised mechanism of action of a DPP-4 inhibitor drug. It was demonstrated that muscle glycogen storage was completely inactive in type 2 diabetes whereas liver glycogen storage was normal after a whole day of usual eating. The practical result of insulin resistance in muscle has not previously been made so evident and appeared to contribute significantly to the aetiopathophysiology of type 2 diabetes.

A central feature of type 2 diabetes concerns the abnormality of β cell function, although the whole pancreas has not been extensively studied previously. This work showed that the volume of the pancreas in type 2 diabetes was 33% smaller than that of age, weight and BMI matched glucose tolerant controls. Furthermore, the pancreas in type 2 diabetes subjects was found to be involuted with pancreatic triglyceride content 23% higher than that of matched controls.

It was demonstrated that vildagliptin (DPP-4 inhibition) therapy reduces hepatic triglyceride content by 27% in subjects with well controlled type 2 diabetes. The reduction in hepatic triglyceride remained significant even after applying correction of weight change, a major influence in hepatic triglyceride. The 27% fall in hepatic triglyceride was that observed when the data was analysed as described for a pharmaceutical trial; wherein a measured data at 3 months was carried forward if a subject had been in the study for at least 3 months but dropped out before 6 months. If non-completers were excluded, a 42% fall in hepatic triglyceride was observed; however this was not primary data. Postprandial plasma glucose, insulin, glucagon and triglyceride concentration also fell with 6 months of vildagliptin therapy.

7.2 Critique of methodology

The conclusions depend very much upon the robustness and appropriateness of the methodology used. Firstly, well-controlled type 2 diabetes subjects were studied (HbA$_{1c}$ ≤ 7.6% or 60mmol/mol) on metformin only, with a mean duration of 5.9±0.8 years and normal glucose tolerant subjects matched for age, weight and BMI. The subjects studied allowed vital conclusions to be drawn in early type 2 diabetes wherein lipotoxicity is a fundamental contributing factor to the aetiopathophysiology. MR techniques such as the three point Dixon (to measure liver and pancreas triglyceride concentration), ^{13}C spectroscopy (to measure muscle and liver glycogen concentration), ^{1}H spectroscopy (to measure IMCL concentration) are safe and have been shown to be robust. The techniques that have been described were used to measure intra-organ lipid concentration in the liver and muscle as a surrogate marker of insulin resistance. Diacylglyceride and ceramide have been directly implicated in abnormal insulin signalling, but it is presently impossible to measure their intra-organ concentration with MR techniques. It is hoped that further advancement in MR physics may permit direct in vivo measurement of metabolically active lipids. The measurement of IMCL concentration with ^{1}H spectroscopy poses practical difficulties in our type 2 diabetes group. Due to an increase in the surface to coil distance in our study population, it was difficult to obtain good quality spectra for analysis. As such, data was obtained in only 50% of the vildagliptin and placebo treated group, respectively, and therefore lacked sufficient power to detect any true difference. The euglycaemic hyperinsulinaemic clamp regarded as the gold standard method to assess insulin sensitivity was used to assess the effect of DPP-4 inhibitor in the defined study group.

7.3 Diurnal change in muscle and liver glycogen storage in type 2 diabetes

The process of storing meal-derived glucose as glycogen in the muscle and liver is vital to maintaining normal postprandial glucose concentration in healthy humans as such massive swings in plasma glucose concentration after meals are prevented. If tissue uptake did not occur, a meal containing 80g of carbohydrate can be calculated to bring about a rise in plasma glucose of 32mmol/l for a 70kg person. Previous studies have demonstrated that approximately 30% of carbohydrate absorbed is stored as skeletal muscle glycogen in the immediate postprandial period (Taylor *et al.*, 1993) and approximately 20% as liver glycogen (Taylor *et al.*, 1996) in young healthy humans. Therefore the muscle and liver can be considered to act together as dynamic buffers, storing osmotically active glucose temporarily and allowing redistribution of carbon energy overnight. Although liver glycogen storage is mainly dependent on plasma glucose concentration, saturation kinetics will only allow approximately 20% of meal carbohydrate to be stored as liver glycogen. Half of meal carbohydrate will be utilised to maintain normal cell function. The outstanding 30% of meal carbohydrate, which is not utilised in liver glycogen storage or energy production, contributes to postprandial hyperglycaemia. Consequently, basal insulin secretion increases and this in turn drives *de novo* hepatic lipogenesis and sets into motion the Twin Cycle Hypothesis in type 2 diabetes previously described Figure 7.1 (Taylor, 2013).
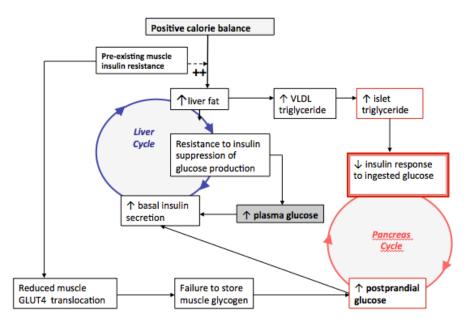
Figure 7.1 Adaptation of the twin cycle hypothesis of the aetiopathophysiology of type 2 diabetes (Taylor, 2013)

The lack of relationship between IMCL content and inability to store glycogen in the muscle in the type 2 diabetes group illustrates the role of muscle insulin resistance which precedes the onset of type 2 diabetes. Although muscle insulin resistance alone does not cause type 2 diabetes, it contributes to the pathogenesis of type 2 diabetes by causing a relative hyperinsulinaemic state which ultimately leads to hepatic triglyceride accumulation by stimulating hepatic *de novo* lipogenesis (Petersen *et al.*, 2007; Taylor, 2013).

7.4 Morphology and composition of the pancreas in type 2 diabetes

An important observation on pancreas morphology was made. The pancreas in type 2 diabetes subjects was observed to be small and involuted with a serrated boundary. It is remarkable that these combined abnormalities have not been previously detected and described.

Several important questions arise. Does having a small pancreas, potentially with proportionally decreased numbers of β cells, predispose to developing type 2 diabetes? Furthermore, could this be a consequence of inadequate intra-uterine development of the pancreas and hence explain the link with low birth weight (Barker *et al.*, 1993)? Alternatively, could it be that the pancreas involutes as post-prandial insulin spikes are diminished by a paracrine effect? Some animal studies have shown that the inhibition of insulin secretion by low dose streptozotocin resulted in a modest decrease in pancreas volume (Saisho *et al.*, 2011). After meals, the pancreas is exposed to insulin concentration far greater than the 10 - 15 fold increase observed in peripheral circulation (Carey *et al.*, 2005). Meal stimulated supra physiological insulin concentration may bring about a growth promoting effect of the pancreas. In type 1 diabetes, insulin secretion is negligible and pancreas volume is known to be decreased by one third (Foulis and Frier, 1984), and this change happens within months of diagnosis (Williams *et al.*, 2012). Although basal insulin concentration is raised in type 2 diabetes, there is complete lack of the acute first phase insulin secretory response after a meal. As such, peak intra-pancreatic supra physiological concentration of insulin required for a paracrine growth effect is not achieved.

The higher triglyceride content of the pancreas, compared with that of well-matched normal glucose tolerant individuals that was observed, is consistent with previous studies with either ultrasound (Bi *et al.*, 2013) or magnetic resonance spectroscopy (Szczepaniak *et al.*, 2012). As human islets cells dramatically increase triglyceride stores when incubation with excess fatty acids *in vitro* (Diakogiannaki *et al.*, 2007) and as islets cells exposed to excess fatty acids *in vitro* have been shown to decrease insulin secretion in response to an acute glucose stimulus (Lee *et al.*, 1994b; Tushuizen *et al.*, 2007), it is reasonable to postulate that the observed increased triglyceride content of the pancreas over time could be a direct cause of the loss of acute insulin secretory ability. The loss of acute insulin secretion due to intra-pancreatic lipid accumulation could account for a lack of a paracrine growth effect of the pancreas.

7.5   Effect of vildagliptin on the liver

Observation of improved post-prandial triglyceride handling during vildagliptin treatment suggested that other mechanisms other than an inhibitory effect on GLP-1 breakdown might be operating. As accumulation of hepatic lipid is such a characteristic feature of type 2 diabetes (Taylor, 2013), the question of whether vildagliptin affected this central aspect of triglyceride metabolism needed to be answered. The data revealed a clinically useful decrease in liver triglyceride concentration over 6 months of vildagliptin therapy. As the fall in liver triglyceride was associated with a fall in plasma ALT, a marker of metabolic stress in the hepatocytes, it appeared that hepatocyte function improved simultaneously. The correlation between improvement in liver triglyceride and in plasma glucose concentration suggests a direct mechanistic link. Fasting plasma glucose fell by 0.9mmol/l and HbA$_{1c}$ normalised to the non-diabetic range. Additionally, the falls in liver triglyceride content and fasting plasma glucose positively correlated at 3 months (r=0.47; p=0.02) and 6 months (r=0.44; p=0.03). This effect can be attributable to vildagliptin as the small effect of weight loss did not account for the change in liver triglyceride content. The fall in liver triglyceride content is highly unlikely to be secondary to improvement in glycaemic control, as it is not seen during therapy with other oral hypoglycaemic agents (Tiikkainen *et al.*, 2004; Phielix *et al.*, 2013).

Although previous work (Lim *et al.*, 2011a) demonstrated an association between a reduction in hepatic triglyceride content and normalization of fasting HPG, this study did not observe this association. The result of this work was consistent with previous observations during both vildagliptin and sitagliptin therapy (Azuma *et al.*, 2008; Solis-Herrera *et al.*, 2013). The lack of improvement in fasting HGP may relate to the very well controlled type 2 diabetes under study, as HGP in diabetes has been observed to decline to non-diabetic levels below a plasma glucose of approximately 8mmol/l (DeFronzo *et al.*, 1989). Secondly, it is reasonable to suggest that during fasting, GLP-1 and GIP concentrations are at a nadir and inhibition of DPP-4 is unlikely to enhance an incretin effect. Considering the known basic mechanism of DDP-4 inhibition, the extent of postprandial and fasting effects of vildagliptin on overall glycaemic control warrant consideration. Additionally, postprandial glucagon concentration fell by 22% (p=0.01) in the vildagliptin treated group, but remained unchanged in the placebo group. These observations may explain the difference in the effect of DPP-4 inhibition on fasting and postprandial HGP. Although postprandial insulin concentration reduced
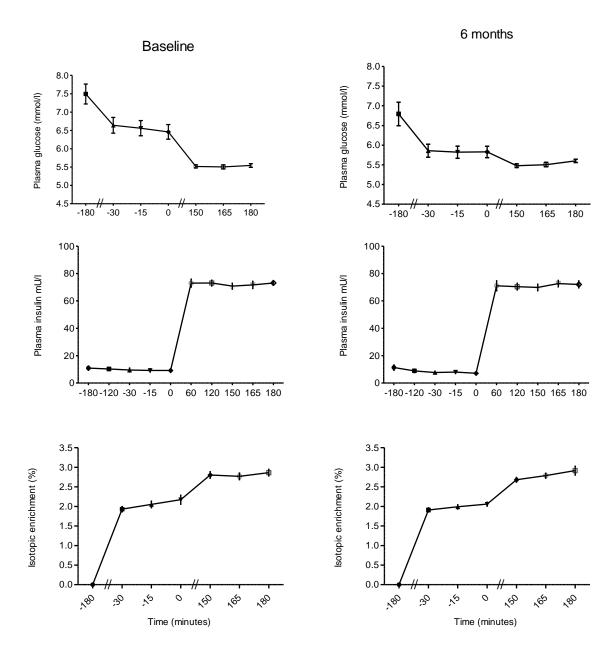
by 26% (p=0.001), there was no change in the fasting insulin concentration in the vildagliptin group.

7.6 Future research questions

The work in this thesis adds to the understanding of type 2 diabetes, and describes previously unknown mechanism of action of DPP-4 inhibitors. However, further questions arose from the conclusions drawn. The questions posed by observing smaller and involuted pancreas in type 2 diabetes are yet to be answered. The question of whether or not the smaller, involuted pancreas is secondary to the lack of post-prandial insulin excursion could be answered by examining the effect of reversal of type 2 diabetes on pancreas volume.
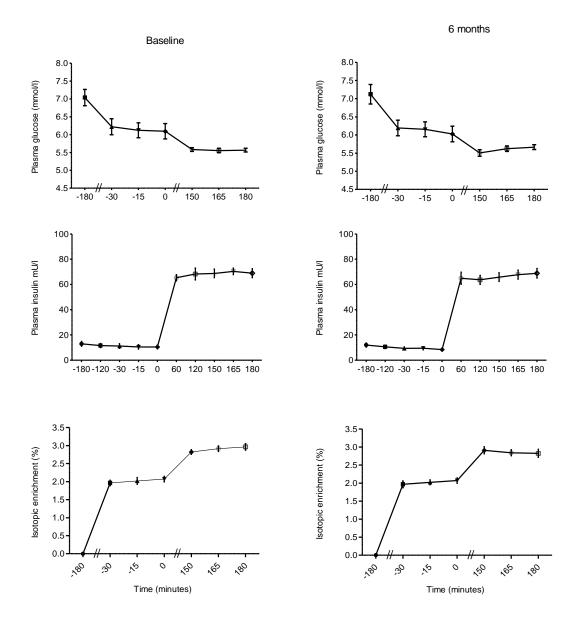
The failure of skeletal muscle to store food carbohydrate and contribute to maintaining postprandial glucose haemostasis has been identified as playing a role in the initiation of the twin cycle hypothesis of type 2 diabetes. Although vildagliptin did not show any improvement in muscle glycogen storage, it is important to investigate the effect of other therapeutic option such as high intensity training on muscle glycogen storage in type 2 diabetes. Further research questions arising from this work are:

- Can pancreas volume be restored if type 2 diabetes is reversed and normal insulin secretion is restored?

- Does a small pancreas in subjects with strong family history of type 2 diabetes and gestational diabetes act as a biomarker for future type 2 diabetes?

- Can a therapeutic intervention with high intensity training in type 2 diabetes improve muscle glycogen storage and postprandial hyperglycaemia?

- Does vildagliptin affect postprandial hepatic glucose production as a consequence of the decreased liver triglyceride in type 2 diabetes?

APPENDIX

APPENDIX 1:   CHANGE IN PLASMA GLUCOSE, INSULIN AND 6' 6' - DIDEUTERATED GLUCOSE ENRICHMENTS DURING THE CLAMPS

Changes in plasma glucose, insulin and 6' 6' - dideuterated glucose enrichments during clamps for vildagliptin treated group

Change in plasma glucose, insulin and 6' 6' - dideuterated glucose enrichments during clamps for control group

APPENDIX 2:   MEAL COMPOSITION FOR CHAPTER 6 - Direct assessment of diurnal glycogen storage

BREAKFAST

Male and female

	CHO(g)	FAT(g)	PRO(g)	ENERGY (kcal)
Porridge (40g)	33.9	4.8	8.9	365
banana (200g)	46	0.6	2.2	178
1 vanilla bean smoothie (250mls)	35.25	4.0	10	225
Tea / Coffee, semi-skimmed milk	8.0	3.0	7.0	7.4
Total	123.15	12.4	28.1	775.4

LUNCH

Male

	CHO(g)	FAT(g)	PRO(g)	ENERGY (kcal)
Chicken bacon sandwich	39.8	20.8	24.2	450
banana (100g)	23	0.3	1.1	89
2 vanilla bean smoothies (500mls)	70.5	8.0	20	450
Total	133.3	29.1	45.3	989

Female

	CHO(g)	FAT(g)	PRO(g)	ENERGY (kcal)
chicken stuffing sandwich	44	14	25	400
banana (100g)	23	0.3	1.1	89
2 tropical juices (500ml)	25.2	0.2	0.8	110
Total	92.2	14.5	26.9	599

DINNER

Male

	CHO(g)	FAT(g)	PRO(g)	ENERGY (kcal)
Beef lasagne	55.6	33.6	30.4	660
banana (100g)	23	0.3	1.1	89
1 banana smoothie (250mls)	41.5	0.3	6.5	200
Total	120.1	34.2	38.0	949

Female

	CHO(g)	FAT(g)	PRO(g)	ENERGY (kcal)
Spaghetti bolognaise	41.2	28.8	32	560
banana (100g)	23	0.3	1.1	89
1 vanilla bean smoothie (250mls)	35.25	4.0	10	225
Total	99.45	33.1	43.1	874

Carbohydrate (CHO), fat, protein (Pro); energy

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