THE REVERSIBILITY OF TYPE 2 DIABETES

Sarah Steven

Submitted for Doctor of Philosophy

Institute of Cellular Medicine

February 2016

Abstract

The incidence of both obesity and type 2 diabetes continues to rise, creating a major worldwide public health challenge. Understanding the mechanisms determining the normalisation of blood glucose levels and the limitations to complete reversal of diabetes by bariatric surgery or a very low calorie diet (VLCD) has important implications for the treatment of type 2 diabetes, but also for improving our understanding of the pathophysiology. A unifying hypothesis to explain the major pathophysiological changes in type 2 diabetes, hepatic insulin resistance and pancreatic beta cell insufficiency, involves excess triglyceride accumulation in liver and pancreas. This is supported by *in vitro* demonstration of impaired insulin signalling (and thereby insulin resistance) and defective insulin secretion, induced by the toxic metabolites of fat. Triglyceride content in liver and pancreas can now be measured non-invasively and precisely using the three point Dixon magnetic resonance technique. This thesis presents data on a direct comparison of bariatric surgery and VLCD, suggesting that the mechanisms involved in the reversibility of type 2 diabetes using these two interventions are similar. Pancreatic triglyceride content appears to decrease with substantial weight loss in obese individuals with type 2 diabetes but not those with normal glucose tolerance. The clinical characteristics which limit reversal of diabetes are investigated, particularly the effect of longer diabetes duration. Both a retrospective study of bariatric surgery and a prospective study using VLCD suggest that long duration diabetes is reversible, however, normal blood glucose levels are less likely to be achieved than in short duration disease. Diabetes duration may be a surrogate marker for beta cell reserve. Finally, the longer term durability of the beneficial effects of an 8 week very low calorie diet is demonstrated. Over a subsequent 6 month weight maintenance period the decrease in hepatic and pancreatic triglyceride content, and the improvements in hepatic insulin sensitivity and first phase insulin secretion are maintained. The latter appears to be a key mechanism for determining a glucose response to VLCD.

Dedication

Most importantly, I would like to thank my supervisor Professor Roy Taylor for his guidance and support throughout this project. His eternal positivity and unwavering enthusiasm have been invaluable to me.

I would also like to thank Dr Kieren Hollingsworth for his time and for sharing his insights and expertise with me over the last 5 years.

I would like to express my sincere gratitude to all of the participants for their remarkable stamina and dedication to the research.

I would also like to thank all of the research radiographers that I have had the pleasure of working with throughout these studies. Their skill, dedication and patience have been integral to the success of the work.

Finally, I wish to acknowledge the support and patience of my friends and family, especially Harry Shaw.

Acknowledgements

own work. The research contained within this thesis has not been previously submitted elsewhere for the degree of Doctor of Philosophy.

Table of Contents

List of original publications

1) *Reversal of type 2 diabetes after bariatric surgery is determined by the degree of achieved weight loss in both short- and long-duration diabetes.*
   Steven S, Carey PE, Small PK, Taylor R. Diabetic Medicine 2015; 32 (1): 47-53.

2) *Restoring normoglycaemia by use of a very low calorie diet in long- and short-duration Type 2 diabetes.*
   Steven S, Taylor R. Diabetic Medicine 2015; 32 (9): 1149-1155.

3) *Weight loss decreases excess pancreatic triacylglycerol specifically in type 2 diabetes.*
   Steven S, Hollingsworth KG, Small PK, Woodcock SA, Pucci A, Aribisala B, Al-Mrabeh A, Daly AK, Batterham RL, Taylor R. Diabetes Care 2015; Published online before print December 1, doi: 10.2337/dc15-0750.

4) *Durable Reversal of Type 2 Diabetes using a Very Low Calorie Diet.*
   Steven S, Hollingsworth KG, Al-Mrabeh A, Avery L, Aribisala BS, Caslake M, Taylor R. Diabetes Care 2015; accepted.

5) *Increased GLP-1 at 7 days following RYGB does not translate into improved insulin secretion rates or glucose control compared to 7 days of VLCD*
   Steven S, Hollingsworth KG, Small PK, Woodcock SA, Pucci A, Aribasala B,Al-Mrabeh A, Batterham RL, Taylor R. Diabetic Medicine; submitted.

List of published abstracts

1) *Reversal of type 2 diabetes following bariatric surgery is not limited by duration of diabetes.*
   Steven S, Coates JA, Ogilvie P, Carey PE, Small PK and Taylor R. Diabetic Medicine 2012; 29: (Supplement 1), 15 – abstract from oral presentation at Diabetes UK Annual Professional Conference March 2012, Glasgow.

2) *Reversal of type 2 diabetes following gastric bypass surgery is determined by the degree of achieved weight loss and is not limited by disease duration.*
   Steven S, Coates JA, Ogilvie P, Carey PE, Small PK, Taylor R. Diabetologia 2012; 55: (Supplement 1), S10 - abstract from oral presentation at 48th Annual Meeting of EASD October 2012, Berlin.

3) *Glucose lowering and anti-hypertensive effects of a very low calorie diet: comparison of response in short and long duration type 2 diabetes.*
   Steven S, Taylor R. Diabetic Medicine 2014; 31: (Supplement 1), 2 - abstract from oral presentation at Diabetes UK Annual Professional Conference March 2014, Liverpool.

4) *Direct comparison of the early change in fasting glucose levels after Roux-en-Y gastric bypass surgery or very low calorie diet.*
   Steven S, Small PK, Woodcock S, Hollingsworth KG, Taylor R. Diabetic Medicine 2014; 31: (Supplement 1), 41 - abstract from poster presentation at Diabetes UK Annual Professional Conference March 2014, Liverpool. Nominated for Eli Lilly Clinical Science Poster Award.

5) *The Glucose Lowering Effects of a Very Low Calorie Diet Are Heterogeneous in Long-Duration Compared to Short-Duration Type 2 Diabetes.*

Steven S, Hollingsworth KG, Caslake MJ, Taylor R. Diabetes 2014; 63: (Supplement 1), A79 - abstract from oral presentation at American Diabetes Association 74th Scientific Session June 2014, San Francisco.

6) *The glycaemic and anti-hypertensive effects of a very low calorie diet are maintained over 6 months in both short- and long- duration type 2 diabetes.*
Steven S, Taylor R. Diabetic Medicine 2015; 24: (Supplement 1), 74 – abstract from poster presentation at Diabetes UK Annual Professional Conference March 2015, London.

7) *Maintenance of Blood Glucose Control and Stable Weight over 6 Months after a Very Low Calorie Diet in Short- and Long-Duration Type 2 Diabetes.*
Steven S, Hollingsworth KG, Al-Mrabeh A, Caslake MJ, Taylor R. Diabetes 2015; 64: (Supplement 1), A555 - abstract from poster presentation at American Diabetes Association 75th Scientific Session June 2015, Boston.

List of tables

List of figures

Abbreviations

ALT	alanine transaminase
APE	atom percent excess
ATP	adenosine triphosphate
AUC	area under the curve
BMI	body mass index
BSA	body surface area
CT	computed tomography
CV	coefficient of variation
DPP-4	dipeptidyl peptidase-4 inhibitor
ffm	fat free mass
FPG	fasting plasma glucose
GC-MS	gas chromatography-mass spectrometry
GGT	gamma-glutamyl transferase
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide-1
HGP	hepatic glucose production
ISR	insulin secretion rate
Kcal	kilocalories
LADA	latent autoimmune diabetes in adults
MET	metabolic equivalent of task
MF	metformin

MODY	maturity onset diabetes of the young
MR	magnetic resonance
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NAFLD	non-alcoholic fatty liver disease
NEFA	non-esterified fatty acids
NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
PET	positron emission tomography
PNPLA3	patatin-like phospholipase 3
PPAR-γ	peroxisome proliferator-activated receptor gamma
ROI	region of interest
RYGB	Roux-en-Y gastric bypass surgery
SAT	subcutaneous adipose tissue
SD	standard deviation
SEM	standard error of the mean
SU	sulphonylurea
TZD	thiazolidinedione
VAT	visceral adipose tissue
VLCD	very low calorie diet
VLDL	very low density lipoprotein
WHO	World Health Organisation

Chapter 1. Introduction

1.1 Global importance of type 2 diabetes and obesity

The increasing prevalence of type 2 diabetes is precipitating a public health crisis in the UK and worldwide. The disease causes considerable personal morbidity but also causes a significant societal burden, with just over 8% of the annual NHS budget being spent on the treatment of type 2 diabetes and its complications (NICE, 2012). In 2012 in England, 7.4% of people aged over 16 years had diabetes, 90% of which is type 2 diabetes, and the burden of unrecognised disease is also thought to be high (PHE, 2014). In the last 2 decades type 2 diabetes has also been recognised as a disease of childhood and adolescence; this has important implications for future health service requirements (Haines *et al.*, 2007). The increasing prevalence seems to be due to the increasing rates of obesity. In England in 2013, 26.0% of men and 23.8% of women were regarded as obese (defined as a body mass index (BMI) of more than 30kg/m^2) (HSCIC, 2015). The last 60 years in the history of man has been unique in terms of being the only period in human history where food availability for the majority has been surplus to requirements. This positive calorie balance has not been matched by increased energy expenditure, in fact technologies have inadvertently decreased physical activity levels. This has resulted in a shift in the BMI curve of the population to the right over time (Figure 1-1).
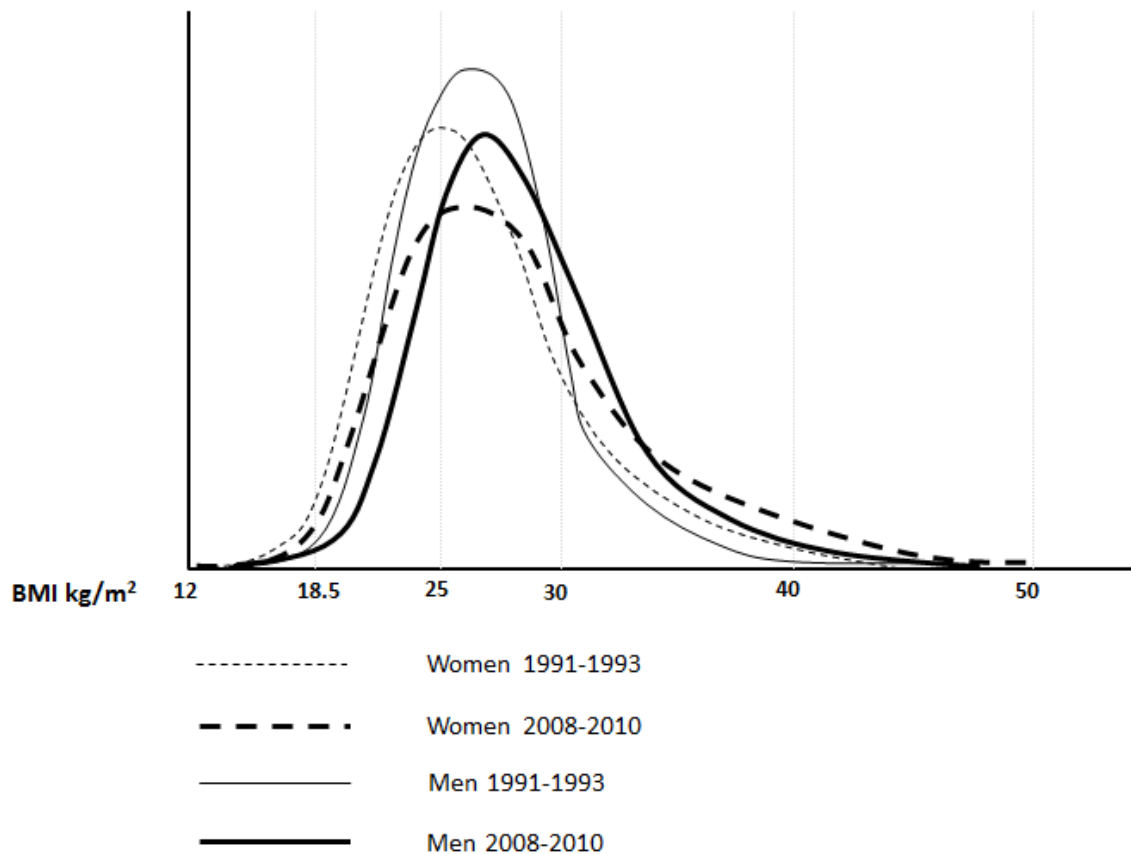
Figure 1-1 Change in the adult BMI distribution for England 1991-1993 and 2008-2010. Adapted from Public Health England Health Survey Data.

Obesity is a major predictor of risk of type 2 diabetes: compared to those of healthy weight, there is a seven times greater risk of diabetes in obese individuals and a threefold increased risk in those who are overweight (Abdullah *et al.*, 2010). Despite this, type 2 diabetes also exists in those with a BMI apparently indicating healthy weight. This may in part result from the fact that BMI is an epidemiologically defined measurement of obesity, which is a relatively insensitive marker in metabolic terms, particularly in some racial groups. Also, it does not reflect body composition, which is an important determinant of the metabolic sequelae of obesity, particularly fat mass and distribution. It is probable that on an individual level, susceptibility factors determine the development of the metabolic consequences of obesity; only about 50% of people with a BMI of more than

$40kg/m^2$ will develop diabetes (Steven and Taylor, 2012). It has been hypothesised that each individual has a personal fat threshold, which if exceeded, makes the development of type 2 diabetes probable (Taylor and Holman, 2015).

The natural history of type 2 diabetes is characterised by a progressive deterioration in blood glucose control despite an initial response to oral therapy; the failure rate of maintaining an HbA1c target of < 7% (53 mmol/mol) is 5-10% per year (UKPDS, 1995a; UKPDS, 1995b; UKPDS, 1998). There is also progressive weight gain, which may be partly due to the natural history of the disease, but is also compounded by medications, particularly sulphonylureas, thiazolidinediones and insulin (UKPDS, 1998; Kahn *et al.*, 2006). There is now a plethora of drug therapies available to treat type 2 diabetes, targeting different identified pathophysiological defects in the condition (Nathan *et al.*, 2009). However, all have only modest effects on glucose levels, and it is this parameter (specifically HbA1c) which has a very clear association with both the microvascular and macrovascular complications of type 2 diabetes (Stratton *et al.*, 2000). In the UK, bariatric surgery used to be considered a treatment option for people with type 2 diabetes and a BMI of more than $35kg/m^2$, but the most recent guidelines include individuals with a BMI of $30-34.9kg/m^2$ and recent onset diabetes (NICE, 2014). However, financial and resource limitations mean that this treatment can only be applied to a very small percentage of the eligible population. The importance of weight management in the treatment of type 2 diabetes is now recognised and despite the scarcity of anti-obesity medications there are now multiple glucose lowering medications which can aid weight loss, including glucagon-like peptide-1 (GLP-1) receptor agonists, and sodium glucose co-transporter 2 (SGLT2) inhibitors (Wilding, 2014).

1.2 Pathophysiology of type 2 diabetes

Type 2 diabetes is characterised by two pathophysiologic defects: insulin resistance and reduced insulin secretory capacity (Ferrannini *et al.*, 2011). Insulin resistance is a concept describing the phenomenon whereby normal concentrations of insulin produce less than the normal biological response

(Kahn, 1978). Insulin resistance usually precedes a diagnosis of type 2 diabetes by many years, and although insulin resistance is the first detectable defect in type 2 diabetes (Petersen *et al.*, 2012), this defect is insufficient on its own to cause blood glucose levels to rise (Taylor, 2012). It is the decline in insulin secretion that determines the onset of hyperglycaemia (Ferrannini *et al.*, 2004; Festa *et al.*, 2006). The ability to maintain normal glucose tolerance in these pre-diabetic states is determined by the ability of pancreatic beta cells to acutely increase insulin secretion in response to an increase in plasma glucose levels. Although fasting glucose levels have been shown to steadily rise for many years prior to a diagnosis of type 2 diabetes, there is a rapid rise in glucose levels relating to deteriorating beta cell function just prior to diagnosis (Sattar *et al.*, 2007; Tabák *et al.*, 2009).

1.2.1 Fasting and postprandial metabolism in health

Plasma glucose concentration is determined by the rate of release of glucose into the bloodstream and the rate of uptake of glucose by cells, both of which are under the control of insulin. There are 3 main target cells for insulin action: hepatocytes, skeletal myocytes and adipocytes. The major effect of insulin in the fasting state is to regulate first hepatic glycogenolysis and then hepatic gluconeogenesis in order to maintain a stable glucose level, thereby determining fasting blood glucose levels. In the fasting state, when insulin levels are low, there is lipolysis of triglycerides within adipose tissue releasing non-esterified fatty acids (NEFA). These NEFA are the primary source of energy through beta-oxidation in mitochondria, the tricarboxylic acid cycle and the respiratory chain.

In the fed state, metabolism switches to use glucose as the primary fuel source. The absorption of glucose and other substrates from the intestine exceeds the body's energy requirements. The maintenance of glucose homeostasis depends on storage of glucose as glycogen in the muscles and the liver, suppression of hepatic glucose production and an increase in glucose oxidation; all of which are facilitated by the actions of insulin. In

health, hepatic glucose production is suppressed within 30 minutes of eating a meal (Singhal *et al.*, 2002). Under normal physiologic conditions, insulin concentrations tightly control the balance between postprandial fatty acid storage as triglycerides and their release into plasma during the fasting state (Cusi, 2010). Adipose tissue is extremely sensitive to insulin induced suppression of lipolysis, inhibiting lipolysis at concentrations much lower than those required to suppress hepatic glucose production or stimulate glucose uptake in skeletal muscle. Thus, after eating plasma NEFA levels fall.

Glucose released as a monosaccharide in the intestine enters hepatocytes from portal blood through glucose transporter 2 (GLUT2). Within hepatocytes, glucose is directed towards glycolysis and adenosine triphosphate (ATP) production and to replenish glycogen stores. When glycogen stores are full, glucose is diverted towards lipid synthesis where it serves as a carbon donor for *de novo* lipogenesis. Fatty acids are esterified as triglyceride associated with apolipoprotein B100 and exported as very low density lipoproteins (VLDL). When glucose is metabolised to produce ATP (through the Krebs cycle) within mitochondria, citrate is formed. Within the cytoplasm this inhibits carnitine palmitoyltransferase the transporter of long chain fatty acyl coenzyme As into the mitochondria (Cusi, 2010). Malonyl CoA therefore acts as the metabolic switch for insulin sensitivity between the fasted and fed states.

1.2.2 Insulin resistance

Insulin resistance is defined as the inability of insulin to produce its usual biological actions at any given concentration. Resistance of target organs to the effects of insulin explains the metabolic abnormalities seen in type 2 diabetes. Hepatic insulin resistance results in decreased hepatic glycogen storage in the postprandial state and increased hepatic glucose production in both the fasting and the fed state. Skeletal muscle insulin resistance in the postprandial state results in less glucose being stored as glycogen. Magnetic resonance spectroscopy studies have shown fasting muscle glycogen levels to be almost 20% lower in those with type 2 diabetes

compared to age and BMI matched non-diabetic controls (Carey *et al.*, 2003), and that after day-long eating there is no change in muscle glycogen levels in those with diabetes compared to a 17% increase in non-diabetic controls (Macauley *et al.*, 2015c). Resistance of adipocytes to insulin results in impaired suppression of lipolysis and plasma NEFA levels are typically elevated. The cellular mechanisms of insulin resistance are complex. In the liver, the mechanism of insulin resistance appears to involve increased hepatic diacylglycerol levels, leading to protein kinase–Cε activation and subsequent inhibition of insulin receptor tyrosine kinase activity (Perry *et al.*, 2014) (Figure 1-2).
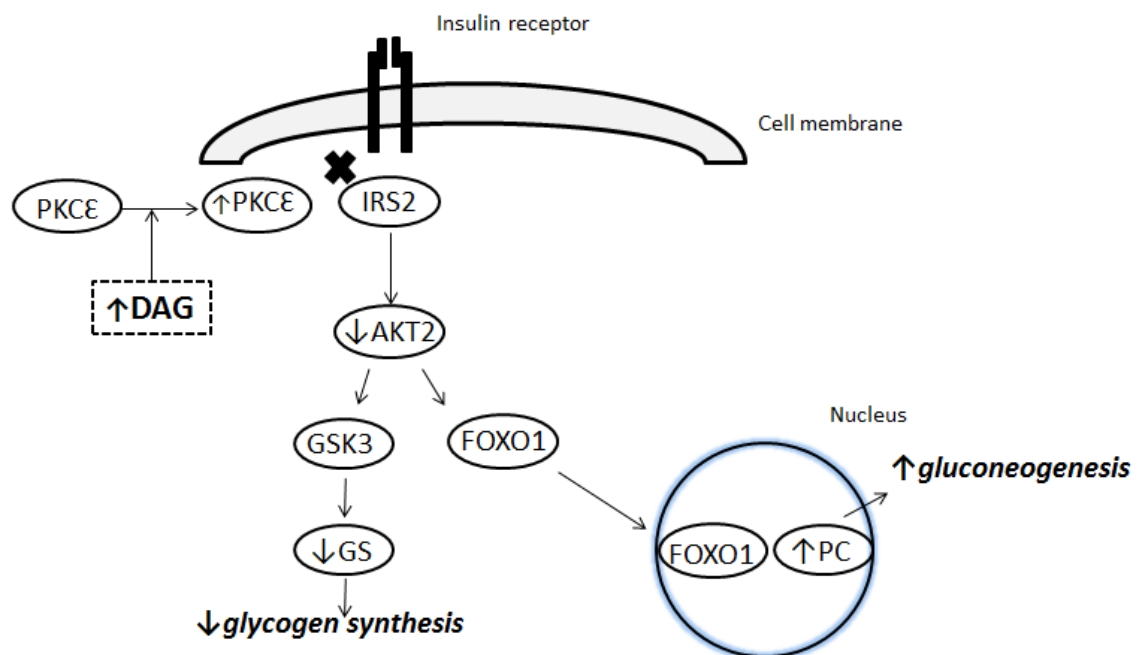
Figure 1-2 Excess diacylglycerol interferes with the insulin signalling pathway and results in insulin resistance, which in the liver results in decreased glycogen synthesis and increased gluconeogenesis. PKCε=protein kinase Cε, DAG=diacylglycerol, IRS2=insulin receptor substrate 2, GSK3=glycogen synthase kinase-3, GS=glycogen synthase, FOXO1=Forkhead box protein O1, PC=pyruvate carboxylase. Adapted from (Perry *et al.*, 2014)

1.2.3 Insulin secretion

Insulin is synthesised as pre proinsulin and processed to proinsulin. Proinsulin is then converted to insulin and C-peptide and stored in secretory granules awaiting release on demand. Insulin synthesis is regulated at both the transcriptional and translational level. *In vivo* approximately 50% of

daily insulin production is released in a constant, pulsatile manner to achieve control of basal metabolism, with the remainder being released to control postprandial metabolism. The release of insulin from pancreatic beta cells is coupled to ATP production due to increased substrate supply, most notably glucose but also amino acids and fatty acids. Glucose is transported into beta cells via GLUT2 receptors. It is then phosphorylated to glucose-6-phosphate with subsequent glycolysis and oxidation to produce ATP. ATP stimulates the closure of ATP-dependent cell membrane potassium channels, which depolarises the cell membrane and results in an influx of calcium ions. In response to this rise in intracellular calcium levels, the insulin containing secretory granules fuse with the cell membrane releasing insulin and C-peptide in equimolar amounts. The early response to a stimulus lasts about 10 minutes and is defined as the first phase insulin response when the stimulus is intravenous glucose. It is this early insulin response which suppresses endogenous glucose production at the start of a meal (Luzi and DeFronzo, 1989). Defective first phase insulin secretion is a characteristic feature of type 2 diabetes (Ferner *et al.*, 1986) and impairments in first phase insulin secretion may serve as a marker of risk for type 2 diabetes (Pimenta *et al.*, 1995). A study in a population of individuals with impaired glucose tolerance found that the absence of the peak of first phase insulin secretion was the best predictor of incident type 2 diabetes (Nijpels *et al.*, 2007). In contrast, the insulin response in established type 2 diabetes to the non-glucose secretagogue arginine is only mildly reduced (Anello *et al.*, 2005). Using fasting plasma glucose and insulin data, beta cell function has been shown to be reduced by about 50% at the time of diagnosis of type 2 diabetes and it is widely believed that there is an inexorable decline in insulin secretion thereafter (UKPDS, 1995a). However, a clinical scenario suggests that beta cells can recover and in fact can then have the capacity to hyperfunction. That is women who have had type 2 diabetes reversed by gastric bypass surgery, then subsequently maintain normal glucose tolerance during pregnancy, in the face of markedly increased insulin resistance (Steven *et al.*, 2011).

Fatty acids are required for normal beta cell function; mice with deletion of the fatty acid receptor GPR40 have 50% decreased insulin secretion (Alquier *et al.*, 2009). Acute exposure to fatty acids stimulates insulin secretion, however, it is well recognised that prolonged exposure *in vitro* increases basal insulin release but decreases glucose stimulated insulin secretion (Lee *et al.*, 1994). This seems to relate to endoplasmic reticulum stress, mitochondrial dysfunction and initiation of apoptosis (Morgan, 2009). Apoptosis is likely to be mediated by chronic exposure to the metabolites of fatty acids, such as diacylglycerols and ceramides (Shimabukuro *et al.*, 1998b). Not all fatty acids are equal in their chronic action to inhibit glucose mediated insulin secretion. Saturated fatty acids are particularly potent, whereas monounsaturated fatty acids have actually been shown to have some protective effects upon beta cells (Diakogiannaki *et al.*, 2007).

Chronic exposure to glucose and fatty acids contributes to beta cell failure, and this is referred to as glucotoxicity and lipotoxicity respectively. Lipotoxicity appears to be dependent on hyperglycaemia and elevated levels of glucose and fatty acids have a synergistic effect on impairing beta cell function. The term glucolipotoxicity is therefore often used. One hypothesis is that in the presence of physiological glucose concentrations, excessive fatty acids are readily disposed of through mitochondrial β-oxidation. However, when both glucose and fatty acids are elevated, there is accumulation of metabolites derived from fatty acid esterification which impair beta cell function (Robertson *et al.*, 2004). With regards to the mechanisms of pancreatic beta cell glucotoxicity specifically, the increased flux of glucose places a burden on mitochondrial oxidation, leading to the production of reactive oxygen species (Bensellam *et al.*, 2012). Oxidative stress can suppress insulin gene expression and can activate mitochondrial inner membrane protein uncoupling protein 2 (UCP2) leading to decreased ATP production (Fu *et al.*, 2013). The high demand for insulin secretion as a result of chronic hyperglycaemia places stress on the endoplasmic reticulum of the beta cells. Endoplasmic reticulum stress interferes with the insulin secretory pathway and insulin biosynthesis, mainly through excessive stimulation of the unfolded protein response (UPR) pathway.

Defects in beta cell mass as well as beta cell function contribute to the pathophysiology of type 2 diabetes (Marchetti *et al.*, 2004). The interplay between these factors is evident when it is recognised that many patients undergoing pancreatic surgery can maintain normal glycaemic control after resection of large volumes of pancreatic tissue. At 1 year following hemipancreatectomy only 25% have abnormal glucose tolerance (Kendall *et al.*, 1990). Studies of cadaveric pancreata have shown that beta cell mass is decreased by about 50% in individuals with type 2 diabetes (Butler *et al.*, 2003). It appears that the beta cell mass declines progressively over the years in type 2 diabetes (Rahier *et al.*, 2008) and that this decline is more pronounced in obese individuals (Hanley *et al.*, 2010). As beta cells turnover continuously during adult life, alteration of the balance of apoptosis and generation of new cells could restore beta cell function (Menge *et al.*, 2008). This understanding of beta cell dynamics may explain the increase in beta cell mass seen in adult life in the context of obesity and also during the insulin resistance associated with pregnancy (Butler *et al.*, 2010). Even more complex is the phenomenon of beta cell dedifferentiation whereby beta cells convert to non-beta pancreatic endocrine cell types in response to chronic pathophysiologic stress (Weir and Bonner-Weir, 2004; White *et al.*, 2013). Recent rodent work has suggested that beta cell dedifferentiation and reprogramming might explain the association of beta cell dysfunction and increased alpha cell function (and therefore hyperglucagonaemia) in early type 2 diabetes (Talchai *et al.*, 2012). Reprogramming of beta cells to alpha cells has been seen in human *ex-vivo* pancreata of individuals with recently diagnosed type 2 diabetes (White *et al.*, 2013). Understanding these mechanisms gives hope that targeting the inhibition of beta cell apoptosis and dedifferentiation, and the promotion of beta cell neogenesis and redifferentiation may offer new treatments for type 2 diabetes in the future.

1.2.4 Dyslipidaemia in type 2 diabetes

Long chain fatty acids become esterified with glycerol to form triglycerides, the major storage form of excess energy (Frayn, 2003). NEFA are the

transport form of lipid energy from adipose storage sites to sites of utilisation and oxidation. NEFA levels turnover very rapidly and reflect the rate of release from adipose tissues, which in turn determines the rate of NEFA utilisation by other tissues. Plasma NEFA levels are increased in obesity and elevated NEFA levels in experimental studies using lipid emulsions and a heparin infusion cause acute insulin resistance and activation of proinflammatory pathways (Boden, 2011). However, the direct association between elevated NEFA concentrations and insulin resistance *in vivo* has been questioned (Karpe *et al.*, 2011). NEFA are presented to cells either bound to albumin, or liberated from triglyceride in the plasma by lipoprotein lipase and carried as lipoproteins. Insulin resistance results in increased lipoprotein lipase activity in adipose tissue, thus increasing NEFA release. Unlike the tight regulation of glucose metabolism, the release of fatty acids into plasma is not regulated by substrate requirement, but rather by the rate of lipolysis of triglyceride stored in adipose tissue. Plasma NEFA are the main substrate for hepatic triglyceride production in the form of VLDL and NEFA turnover is a determinant of VLDL-triglyceride secretion and plasma triglyceride concentrations (Figure 1-3).
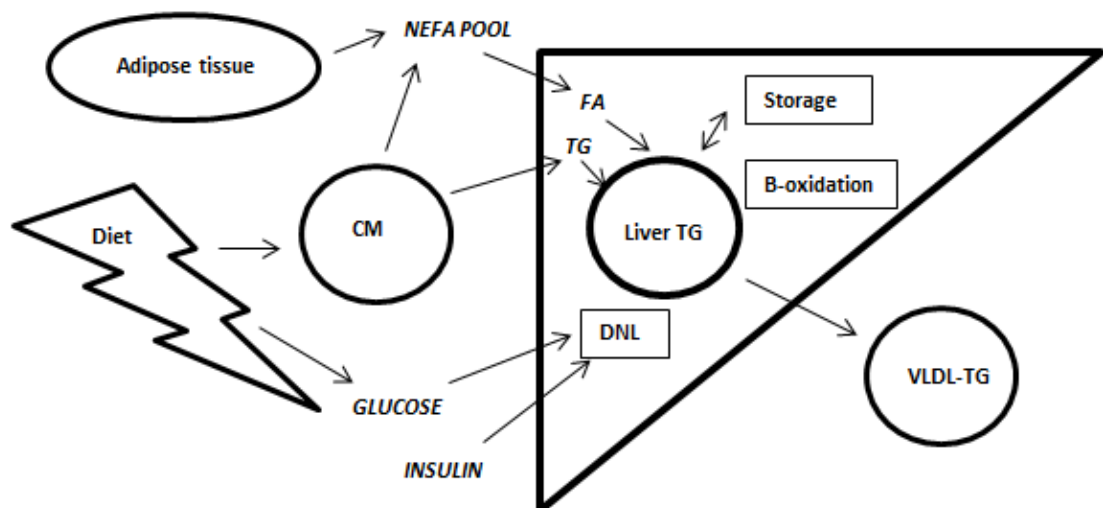
Figure 1-3 Sources of fatty acids for liver and VLDL-triglyceride. CM=chylomicron, DNL=de novo lipogenesis, TG=triglyceride, FA=fatty acids, NEFA=non-esterified fatty acids. Adapted from (Adiels *et al.*, 2008)

Plasma VLDL-triglyceride level is determined by the balance of the hepatic secretion of VLDL-triglyceride and its catabolism in peripheral tissues. A marked reduction in VLDL-triglyceride export from the liver would limit the delivery of fatty acids to peripheral tissues, but would be expected to result in accumulation of triglycerides in hepatocytes and the development of fatty liver. VLDL-triglyceride is synthesized and secreted by hepatocytes after a series of complex intracellular events involving the synthesis of apolipoprotein B100 (apoB100) and lipid and their assembly into lipoprotein particles. VLDL-triglyceride indicates the rate of lipid production, whereas VLDL-apoB100 indicates $VLDL_1$ particle production. Triglyceride is the main lipid component of VLDL and VLDL-triglyceride can either be secreted from the cell as $VLDL_2$ or can be lapidated to $VLDL_1$, this latter process requiring the further addition of triglycerides. $VLDL_1$ levels determine plasma triglyceride levels whereas $VLDL_2$ levels determine LDL cholesterol levels. VLDL-triglyceride is hydrolysed by lipoprotein lipase at the luminal surface of capillaries in peripheral tissues. In healthy individuals, the majority of VLDL exported by the liver is in the form of small triglyceride poor $VLDL_2$, whereas in conditions such as type 2 diabetes where there are large amounts of NEFA and triglyceride, the formation of large triglyceride rich $VLDL_1$ is increased (Verges, 2010). Although each VLDL particle has one apoB particle it is known that there can be dissociation of VLDL apoB and triglyceride secretion rates resulting in VLDL particles with variable triglyceride to apoB ratios. Weight loss has been associated with decreased VLDL-triglyceride levels; following gastric bypass surgery (Klein *et al.*, 2006) and following 10% loss in body weight by dietary restriction in women with abdominal obesity (Mittendorfer *et al.*, 2003). This is thought to relate to a decrease in the contribution of non-systemic fatty acids to VLDL-triglyceride production, from the lipolysis of intrahepatic and visceral fat. The dyslipidaemia of type 2 diabetes is therefore characterised by high

serum triglycerides, low HDL cholesterol concentrations and the appearance of small dense low density lipoproteins (Adiels *et al.*, 2008).

1.3 Intra-organ fat accumulation

1.3.1 Hepatic steatosis

Fatty liver is physiological in migrating birds as the energy stores are required to overcome prolonged fasting (Capeau, 2008). However, in humans excess liver fat is pathological and represents the presence of triglyceride as lipid droplets in the cytoplasm of hepatocytes. A population study using localised proton magnetic resonance spectroscopy showed that a hepatic triglyceride content of greater than 5.56% corresponds to the 95th percentile in "normal" subjects (Szczepaniak *et al.*, 2005) and above this level is considered to be non-alcoholic fatty liver disease (NAFLD) in the absence of alternative causative factors. NAFLD is now the most common cause of chronic liver disease in the Western world (Bedogni *et al.*, 2005), and can progress to non-alcoholic steatohepatitis (NASH) and cirrhosis. Hepatic steatosis results from an imbalance between triglyceride synthesis and degradation. The primary source of fatty acids (accounting for ~60%) is from lipolysis of adipose tissue, which is unsuppressed in insulin resistant states (Cusi, 2010). *De novo* lipogenesis is also increased in conditions of hyperinsulinaemia and hyperglycaemia (Adiels *et al.*, 2008). More than 90% of obese patients with type 2 diabetes have NAFLD (Tolman *et al.*, 2007). The extent of portal hyperinsulinaemia determines how much fatty acid is produced from glucose in the liver, hence why despite hyperglycaemia, individuals with type 1 diabetes do not develop excess liver fat (Perseghin *et al.*, 2005). Approximately 15% of the fatty acid supply comes from lipolysis of dietary chylomicrons (Capeau, 2008). These fatty acids can either be stored in the liver as triglyceride, undergo ß-oxidation in mitochondria, or can be exported into the circulation in the form of VLDL-triglyceride (Figure 1-3). In insulin resistant states, triglyceride is preferentially directed towards storage or export because the transport of fatty acid into mitochondria for oxidation is inhibited by malonyl Co A produced during lipogenesis. Therefore, positive energy balance in the context of portal

hyperinsulinaemia in individuals predisposed to insulin resistance results in the accumulation of liver fat (Figure 1-4).
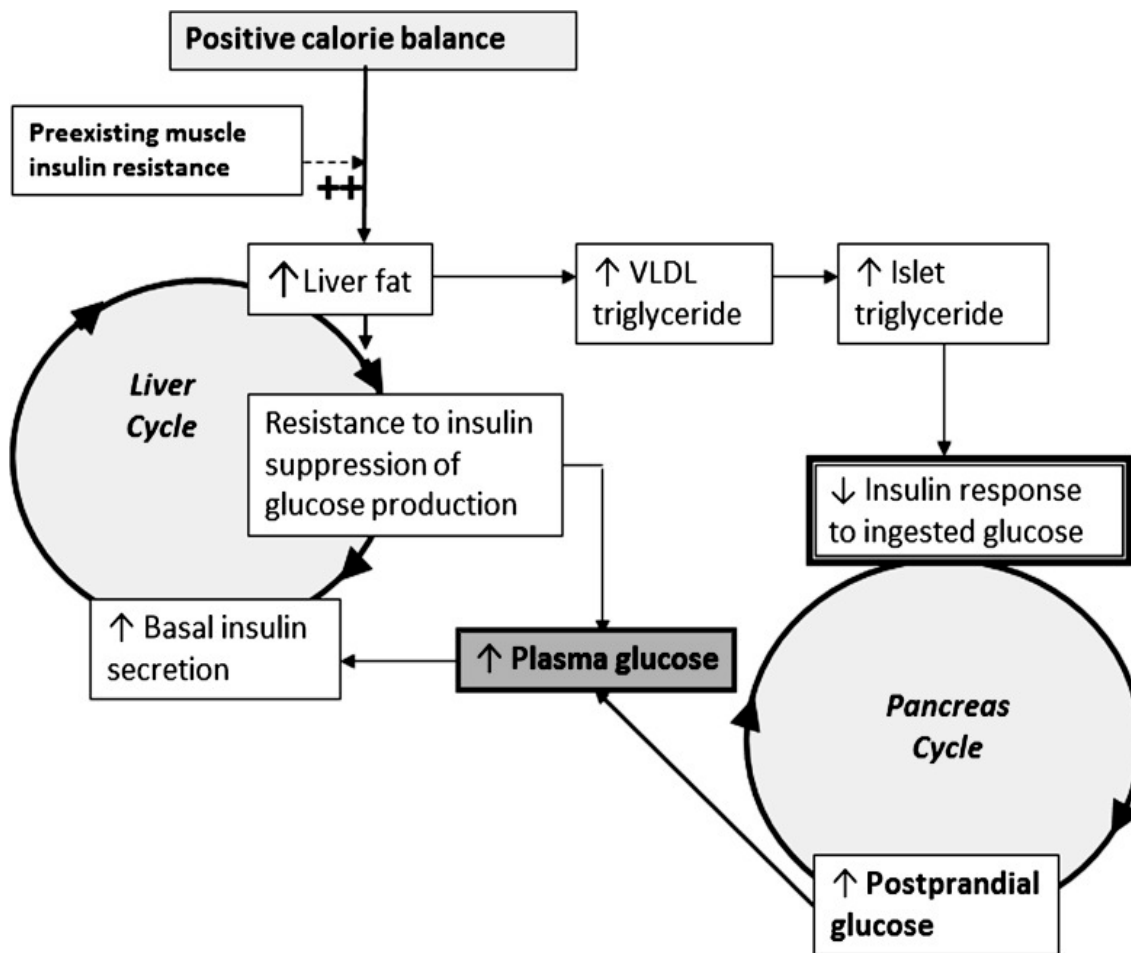
Figure 1-4 The twin cycle hypothesis on the pathogenesis of type 2 diabetes. Reproduced with permission (Taylor, 2013).

Hepatic steatosis correlates with the failure of insulin to suppress hepatic glucose production (Seppala-Lindroos *et al.*, 2002), which is the major determinant of fasting blood glucose levels. The presence of hepatic steatosis can actually predict the development of type 2 diabetes, even when adjustments are made for BMI. An ultrasound study of liver fat levels in Japanese males showed that a steady increase in liver fat precedes a diagnosis of type 2 diabetes with an age adjusted hazard ratio of 4.8 (Shibata *et al.*, 2007). The West of Scotland Coronary Prevention Study demonstrated an increase in the liver enzyme alanine transaminase (ALT) in the 18 months prior to a diagnosis of type 2 diabetes, indicating the

burden of excess fat on hepatocytes (Sattar *et al.*, 2007). There is a bi-directional relationship between excess calorie intake and liver fat accumulation. Overfeeding obese individuals by 1000kcal/day for 3 weeks results in 2% weight gain and 27% increase in hepatic fat content (Sevastianova *et al.*, 2012). During calorie restriction it appears that intra-hepatic fat stores are mobilised rapidly, and before visceral or subcutaneous fat stores are affected. Within 7 days of a very low calorie diet in individuals with type 2 diabetes, hepatic triglyceride content decreased by 30% and simultaneously the suppression of hepatic glucose production by insulin infusion improved from 43% at baseline to 74% (Lim *et al.*, 2011). As well as caloric restriction (Petersen *et al.*, 2005), increased physical activity (Cassidy *et al.*, 2016) and PPAR-γ agonists (Ravikumar *et al.*, 2008) can reduce liver fat content in individuals with type 2 diabetes.

Although the relationship between hepatic steatosis, metabolic syndrome and type 2 diabetes is strong, there is considerable interindividual variation. Data arising from genome-wide association studies identified that a single nucleotide polymorphism in the the adiponutrin (PNPLA3) gene rs738409G was associated with NAFLD and plasma levels of liver enzymes. The G allele in the PNPLA3 gene results in a missense mutation I148M that renders the protein incapable of triglyceride hydrolysis (He *et al.*, 2010). This gene mutation has been postulated as an explanation for the dissociation of liver fat and insulin sensitivity in some individuals (Romeo *et al.*, 2008; Speliotes *et al.*, 2010). The minor allele frequency of the 148M allele is approximately 0.26 in Caucasian populations (Petit *et al.*, 2010). Despite increased liver fat content in individuals with the mutation, there was no relationship between liver fat content and visceral adiposity, BMI or insulin resistance (Kantartzis *et al.*, 2009). It is hypothesised that sequestration of diacylglycerols and other fat metabolites as inert triglyceride may prevent inhibitory effects on insulin sensitivity in the liver in individuals with this mutation.

1.3.2 Pancreatic steatosis

The relevance of excess pancreatic lipid accumulation in the pathogenesis of type 2 diabetes in humans is controversial, but a role for excess pancreatic fat in causing the islet cell dysfunction has been postulated (Lee *et al.*, 1994; Taylor, 2008) (Figure 1-4). Human autopsy studies have reported a relationship between pancreatic fat content and BMI but not diabetes status (Clark *et al.*, 1988; Saisho *et al.*, 2007) . However, these post-mortem studies are limited by the scarcity of pancreatic biopsies, and the fact that pancreatic tissue undergoes autolysis by lipases with some fat being removed during histological processing. Imaging modalities used to measure pancreas fat content *in vivo* also have limitations; magnetic resonance spectroscopy studies are imprecise due to movement of the organ during the respiratory cycle and hence inadvertent inclusion of surrounding visceral fat in measurements. Finally, determining whether the fat is present within beta cells, acinar cells or intra-pancreatic adipocytes is a challenge. Current imaging techniques are unable to differentiate beta cells, which constitute only 1-2% of pancreatic mass, from non-insulin secreting parenchymal cells. Measurement of whole organ pancreas fat content represents parenchymal intracellular fat accumulation, including the accumulation of fat in islet and acinar cells.

Despite these caveats, Magnetic Resonance Spectroscopy (MRS) studies in obese rodents have shown an increase in pancreatic triglyceride accumulation prior to the onset of type 2 diabetes (Lee *et al.*, 2009). Imaging studies in humans have suggested increased pancreatic fat content in those with type 2 diabetes compared to normal glucose tolerance (Tushuizen *et al.*, 2007; Lim *et al.*, 2011). Studies looking at the potential relationship between pancreatic fat levels and insulin secretion have yielded conflicting results; a negative association between pancreatic fat and insulin secretion in some (Tushuizen *et al.*, 2007; Heni *et al.*, 2010; Szczepaniak *et al.*, 2012) and only a relationship with BMI not beta cell function in others (Saisho *et al.*, 2007; van der Zijl *et al.*, 2011) and another with age alone (Begovatz *et al.*, 2015). However, interpretation of results and comparisons between studies have been complicated by the fact that heterogeneous populations have been

studied, with inclusion of some individuals with impaired glucose tolerance and impaired fasting glycaemia meaning that some individuals will actually have increased insulin secretion compared to those with normal glucose tolerance. These studies have also used a variety of imaging modalities (^{1}H-MRS, MRI and computed tomography (CT)). In one study the poor coefficient of variation (14%) for pancreas fat is likely to relate to the use of MRS and inclusion of visceral fat due to respiratory motion during the long image acquisition time, resulting in insufficient specificity to detect differences between subject groups (van der Zijl *et al.*, 2011).

In vitro studies have established that beta cells avidly import fatty acids through CD36 transporters (Noushmehr *et al.*, 2005; Lalloyer *et al.*, 2006) and that these can be stored as intra-cellular triglyceride (Diakogiannaki *et al.*, 2007). Sustained exposure to elevated NEFA (a mixture of palmitate and oleate representative of the *in vivo* plasma composition of NEFA) concentrations has been demonstrated to result in increased triglyceride content in human islets and decreased glucose stimulated insulin secretion (Dubois *et al.*, 2004). Mice given a high fat diet rapidly accumulate adipocytes between pancreatic exocrine cells and close to islet cells (Pinnick *et al.*, 2008). In human post-mortem tissue, the degree of adipocyte infiltration in the pancreas is correlated with triglyceride content, confirming that there is storage of triglyceride in adipocytes within the pancreas (Pinnick *et al.*, 2008). During lipolysis of adipocyte triglyceride, fatty acids released into the extracellular space would be likely to surround the islets at high concentrations which may influence insulin secretion. In addition, there may be paracrine effects of adipocyte products, such as adipocytokines and leptin, which may influence islet cell function.

1.3.3 Use of the 3 point Dixon method to measure intra-organ fat
Principles of Magnetic Resonance (MR) imaging

Within a strong magnetic field, atomic nuclei align themselves and produce a measurable magnetic moment or spin. Also, spinning nuclei precess at a characteristic frequency that is proportional to the strength of the external

field. Because of the abundance of hydrogen nuclei compared to other nuclei in most tissues, the proton signal from hydrogen is used in clinical magnetic resonance scanning. Applying radiofrequency energy alters the fraction of protons aligned with the magnetic field. When this radiofrequency is removed, the protons release the energy they absorbed. This energy is released in the form of an oscillating magnetic field detected as an electric current in the receiver coil. The density of protons as well as the rate at which magnetisation returns to baseline can be measured by the radiofrequency signal emitted by the protons as they relax.

The advantage of using magnetic resonance imaging over CT or positron emission tomography (PET) for clinical studies include that it is non-invasive and there is no exposure to ionising radiation. This allows multiple scans to be performed in the same individual over time. The disadvantages include the fact that MR scanning is contraindicated in individuals with certain implantable metals. Also, as most scanners are enclosed claustrophobia can limit tolerability, particularly in obese individuals. In this latter group other technical challenges arise, such as increased noise resulting in reduced signal to noise ratio, longer scan times due to the larger cross-sectional area and this scan time can be detrimental in terms of image quality due to movement artefact, particularly in relation to respiratory motion (Uppot *et al.*, 2007).

The 3 point Dixon method

The power of magnetic resonance is based on small differences in the microenvironment of different tissues, which allow different molecules with the same nucleus to be differentiated. The resonant frequency of a nucleus is modified by the surrounding electrons, called chemical shielding. The fact that lipid protons and water protons have different resonant frequencies (water slightly faster than fat) is termed chemical shift. The chemical shift difference between fat and water is about 3.4ppm: at 3 Tesla this translates into a frequency difference of 435Hz. When a radiofrequency pulse is applied, the fat and water signals are in-phase (additive signals) but then they move out-of-phase (reduced signal as the fat signal subtracts from the

water signal) after 1.15ms (=1/(2x435Hz)). Using a subtraction technique fat and water only images can be produced from two images with in and out of phase echo times on a pixel wise basis (Dixon, 1984). The 3 point Dixon technique refers to the correction applied using a third image to correct magnet inhomogeneity, that is the fact that a magnetic field is never perfectly uniform (Glover and Schneider, 1991). This is because within the body, tissues are affected by neighbouring structures of dissimilar magnetic susceptibility such as the boundaries between soft tissue and air pockets and bone, for example. For measurement of organs which move during the respiratory cycle such as the pancreas, the 3 point Dixon method has advantages over magnetic resonance spectroscopy. Because of the fast acquisition time, images can be acquired during long breath holds, to try to prevent any inclusion of surrounding visceral fat in calculations. Also, all of the data processing can occur after image acquisition. Therefore quantitation of whole pancreas fat content can be performed by manually drawing regions of interest in an anatomically defined area within the scan slice, rather than analysis of pre-selected volumes in space such as in MR spectroscopy (Figure 1-5). The three point Dixon technique has been shown to have an inter-scan repeatability co-efficient of 0.5% for the liver and 0.9% for the pancreas (Lim *et al.*, 2011).
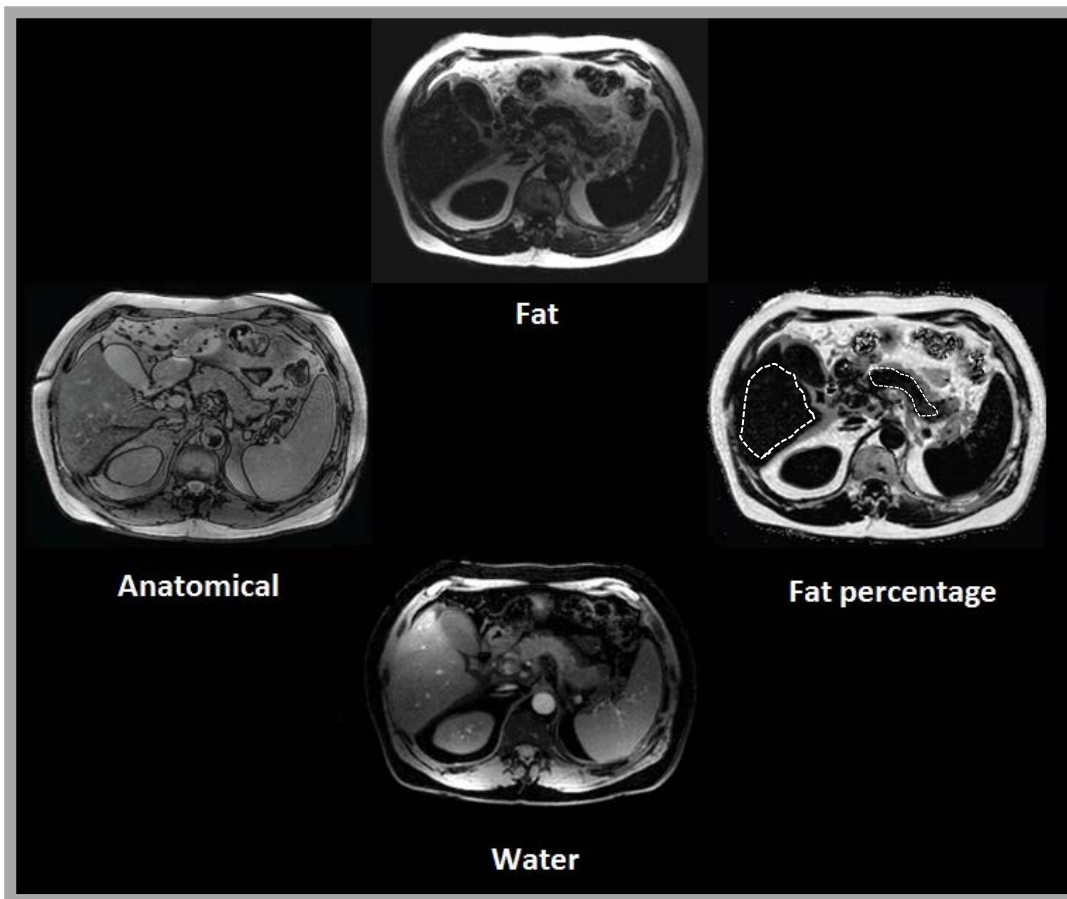
Figure 1-5 Three point Dixon MR method, showing the anatomical image and separated fat and water Dixon images, then the derived fat percentage image on which regions of interest (shown in dotted white) are drawn to measure hepatic and pancreatic triglyceride content.

1.4 Reversal of type 2 diabetes following bariatric surgery

The first recognition that type 2 diabetes is a reversible condition came from observations from bariatric surgery; the Greenville gastric bypass could correct glucose metabolism in those with type 2 diabetes (Pories *et al.*, 1992). Initially it was assumed that the mechanism was through achieving significant weight loss, however, two observations suggest that this is not the case. Firstly, there is disparity between the resolution rates of obesity and diabetes following surgery; the metabolic benefits occur despite many patients remaining obese post-operatively. Secondly, the timeframe of reversal of diabetes differs from that of weight loss. In a group of superobese women undergoing biliopancreatic diversion, it was found that within one week of surgery and prior to any significant weight loss, there was

normalisation of blood glucose levels (Guidone *et al.*, 2006). This observation, that glycaemic control occurs prior to any significant weight loss associated improvement in insulin sensitivity, prompted further investigation into the pathophysiological mechanisms behind the reversal of type 2 diabetes after bariatric surgery. The direct association between weight loss and improved insulin sensitivity is clear, but the mechanism of the early improvement in glucose metabolism requires explanation.

The effect of the surgery on foregut anatomy and nutrient absorption is an important consideration (Figure 1-6): in laparoscopic Roux-en-Y gastric bypass surgery (RYGB) the majority of the stomach is stapled off to leave a 30-50ml gastric pouch. A Roux limb of jejunum measuring 50-70cm is then anastomosed to the gastric pouch, with the biliopancreatic limb (100-150cm) being re-attached by jejuno-jejunostomy. This results in more rapid delivery of nutrients directly to the jejunum following surgery, as demonstrated by studies of paracetamol (Falkén *et al.*, 2011) and xylose absorption (Salehi *et al.*, 2011).
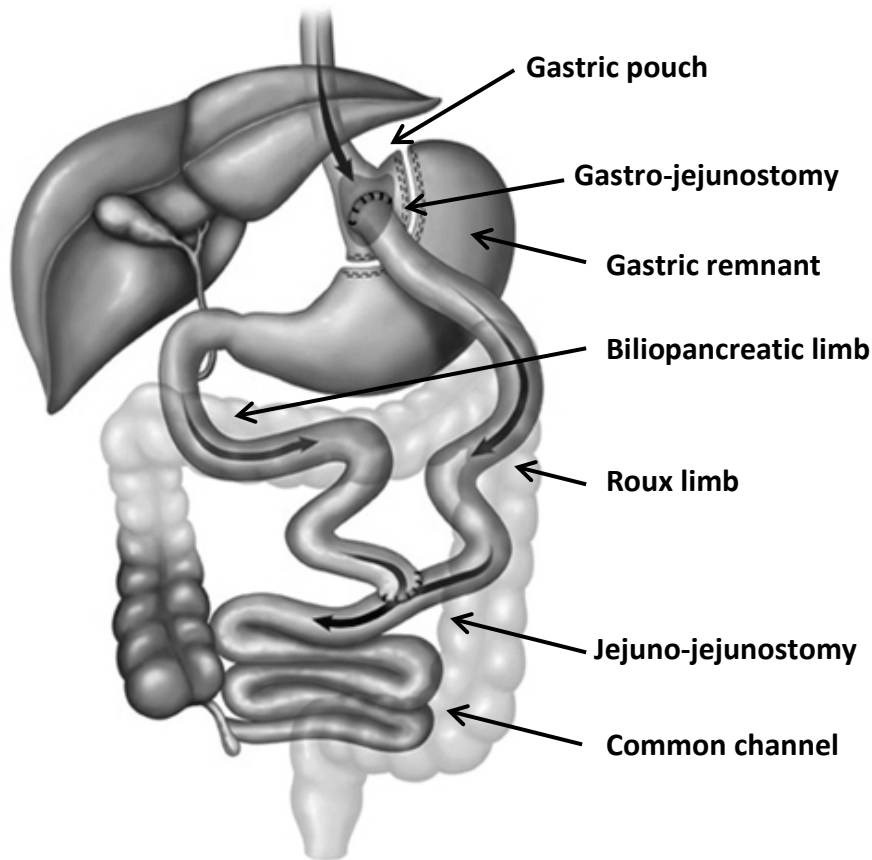
Figure 1-6 Representation of the altered foregut anatomy in a Roux-en-Y gastric bypass.

The incretin effect is the increased stimulation of insulin secretion elicited by oral compared to intravenous administration of glucose under similar plasma glucose levels. This effect is known to be diminished in type 2 diabetes (Vilsbøll *et al.*, 2001) and secretion of these incretin hormones, particularly glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) is markedly altered following manipulation of the gut during gastric bypass surgery (Dirksen *et al.*, 2012). The incretin hormones have been postulated as key mediators of the beneficial effects of gastric bypass surgery on glucose metabolism (Holst and Gromada, 2004). GLP-1 exerts glucoregulatory actions by enhancing insulin secretion in response to oral glucose stimulation, delaying gastric emptying and inhibiting glucagon secretion.

However, various observations suggest that alterations in secretion of these secondary glucoregulatory hormones are insufficient to explain the marked changes in glucose metabolism seen after surgery. The increase in postprandial GLP-1 secretion which occurs without change in fasting GLP-1 secretion cannot explain the marked changes in fasting physiology (Yousseif *et al.*, 2014). In clinical diabetology, GLP-1 receptor agonists can achieve supraphysiological circulating levels of hormone with only a modest effect on glycaemic control (DeFronzo *et al.*, 2005). Finally, studies using Exendin 9-39 to specifically antagonise the GLP-1 receptor, have demonstrated a blunted maximal insulin secretion rate in response to a meal. However it is important to note that this did not negate the beneficial effect of gastric bypass surgery on the insulin secretion profile (Salehi *et al.*, 2011; Jimenez *et al.*, 2013).

The major change after bariatric surgery is obligate caloric restriction. The effect of different surgical procedures on fasting glucose levels appears to correlate with the degree of caloric restriction imposed. A systematic review suggested resolution rates of type 2 diabetes of 95.1% for biliopancreatic diversion, 80.3% for gastric bypass, 79.7% for gastroplasty and 56.7% for laparoscopic adjustable gastric band (Buchwald *et al.*, 2004). Numerous studies have tried to directly compare the effects on metabolism of RYGB and dietary caloric restriction with the majority demonstrating no difference (Jackness *et al.*, 2013; Lingvay *et al.*, 2013; Lips *et al.*, 2014). Other studies have been inconclusive due to differences between groups in achieved weight loss (Isbell *et al.*, 2010), calorie intake (Laferrere *et al.*, 2008) or glucose status (Hofso *et al.*, 2011). Some of these studies have used a non-representative stimulus for investigating insulin secretion, typically an oral glucose tolerance test, which does not reflect the semi-solid high protein diet recommended to individuals after bariatric surgery.

Despite the lack of full understanding regarding the mechanisms determining reversal of type 2 diabetes following bariatric surgery, the superiority over intensive medical therapy in achieving glycaemic control in type 2 diabetes is clear. In a randomised clinical trial the primary outcome

of achieving HbA1c <6% (42 mmol/mol) at 12 months post-operatively was achieved in 12% of the medical group and 42% of the gastric bypass group (Schauer *et al.*, 2012). In the UK gastric bypass surgery is currently the most commonly performed procedure (NBSR, 2014). Given the fact that the population fulfilling eligibility criteria for bariatric surgery in the UK far exceeds capacity from a resource and financial point of view, establishing the individuals most likely to benefit from surgery and thereby understanding the limitations to complete reversal of type 2 diabetes will be crucial.

1.5 Reversal of type 2 diabetes using calorie restriction

The first insight into the importance of calorie restriction in the early metabolic changes following gastric bypass surgery was actually made in one of the original series on the Greenville gastric bypass. An individual with type 2 diabetes underwent a 'sham' procedure: the procedure had to be abandoned due to excess stomach contents at the time of surgery (Pories *et al.*, 1995). This individual followed the same post-operative diet as those who had undergone successful gastric bypass, and experienced a similar rapid reduction in plasma glucose levels as post-surgery individuals. In fact, the effect of famine on resolution of glycosuria has been described as far back as the 19th century (Bouchardat, 1875). Using a 40 day period of 330kcal/day dieting in obese individuals with type 2 diabetes, Henry *et al.* demonstrated that although weight reduced gradually over the study period, the majority (87%) of the reduction in mean fasting plasma glucose levels occurred during the first 10 days of dieting (Henry *et al.*, 1985). The rapid improvement in glucose levels during a VLCD prior to significant weight loss is a consistent finding (Anderson *et al.*, 2003). A study using a 1200kcal diet found that after an average of 7 weeks of dieting and 8kg weight loss, fasting plasma glucose dropped significantly, whole body insulin sensitivity increased approximately 2 fold and there was a marked increase in the suppression of hepatic glucose production associated with an 81% reduction in hepatic triglyceride content (Petersen *et al.*, 2005). Using a VLCD alongside gold standard tests of beta cell function, a more recent study gave

further insight into the mechanisms determining reversal of type 2 diabetes. After 7 days of VLCD in individuals with short duration type 2 diabetes, fasting plasma glucose levels had normalised, hepatic insulin sensitivity had normalised and hepatic triglyceride content decreased by 30% (Lim *et al.*, 2011). More gradually, and by the end of the 8 week VLCD first phase insulin secretion had increased to become indistinguishable from that of age and weight matched glucose tolerant controls. Alongside this, pancreatic triglyceride levels had decreased from 8.0±1.6% to 6.2±1.1%, with weight-matched glucose tolerant control levels being 6.0±1.3%. The normalisation of first phase insulin secretion was novel and has been influential in terms of understanding the pathophysiology of type 2 diabetes but also in guiding clinical management. Following publication of that study there was intense interest from people with diabetes and health care practitioners (Steven *et al.*, 2013). Previous studies of VLCD have suggested that weight recidivism is the norm once calorie restriction is lifted (Paisey *et al.*, 2002). Important clinical questions were therefore raised, questioning the durability of the reversal of the pathophysiological defects after return to normal eating after the VLCD, and the applicability of the findings to the wider type 2 diabetes population.

1.6 Limitations to reversal of type 2 diabetes

The extent of the improvement in glucose control following bariatric surgery in individuals with type 2 diabetes is thought to be influenced by the degree of achieved weight loss, subsequent weight regain, duration of diabetes, pre-surgery anti-diabetic therapy requirements, and the choice of bariatric procedure (Dixon *et al.*, 2011). In addition, poorer pre-operative diabetes control, indicated by an elevated HbA1c (Schauer, 2003) or the need for insulin therapy (Arterburn *et al.*, 2013), is associated with lower post-operative diabetes remission rates. It has been suggested that having had type 2 diabetes for more than 10 years is associated with less weight loss after surgery, less improvement in diabetes control and a lower likelihood of diabetes resolution (Renard, 2009). A prospective study on reversibility of type 2 diabetes following RYGB showed remission of diabetes, defined as

achieving an HbA1c <6.5% (48 mmol/mol) off anti-diabetic medication, was seen in 71% if diabetes duration was less than 8 years but only 44% when diabetes duration was more than 8 years (Hall *et al.*, 2010). Another group investigated obese subjects with and without type 2 diabetes before and then at 45 days and 1 year following RYGB (Nannipieri *et al.*, 2011). It was found that insulin sensitivity improved in proportion with post-operative decrease in BMI whereas beta cell glucose sensitivity improved early then levelled off with plasma glucose levels. In that study beta cell sensitivity to glucose was the only predictor of remission of diabetes. This information concurs with the fact that in individuals who do not achieve diabetes reversal following surgery, glucose levels gradually improve over time in parallel with weight loss (Kashyap *et al.*, 2011). One hypothesis is that the probability of diabetes reversal following bariatric surgery depends on the potential for recovery of beta cell function. In contrast, the improvement in glucose control post-operatively is determined by the increase in insulin sensitivity and therefore the degree of weight loss achieved.

The influence of diabetes duration on the effect of calorie restriction on glycaemic control has also been investigated using a minimum of 4 weeks of 500kcal/day dieting in individuals with type 2 diabetes of recent onset or longer duration (Nagulesparan *et al.*, 1981). Initial mean fasting plasma glucose was 14.4mmol/l if type 2 diabetes was of recent onset (≤2 years) and 14.8mmol/l if of long duration (≥6 years). After the diet period this decreased to 6.6mmol/l and 9.7mmol/l respectively. However, there was considerable heterogeneity in the long duration group; particularly with regards BMI, which ranged from 25.4–56.9kg/m² with 50% of individuals having a BMI of 25-30kg/m². Whether diabetes duration is of importance *per se* or whether it is a surrogate marker for beta cell function is unclear. Two small studies have looked at insulin secretion alone in long-standing type 2 diabetes, one using C-peptide response to glucagon (Zangeneh *et al.*, 2006) and the other C-peptide measurements during oral glucose tolerance testing following calorie restriction (Jain *et al.*, 2008). Both concluded that although a decline in beta cell function over time is characteristic, it is not inevitable and there was much inter-individual variation.

1.7 Aims of the studies

The aims of this thesis are to understand the mechanisms determining the improvement in glycaemic control in type 2 diabetes and the limitations to complete reversal, through studies using the interventions of gastric bypass surgery and very low calorie diet. A specific aim is to understand the pathophysiologic processes in the liver and pancreas and the potential connection with excess hepatic and pancreatic triglyceride accumulation as measured using the non-invasive 3 point Dixon MR technique. The study described in Chapter 3 looks at diabetes duration as a potential limiting factor in the reversal of type 2 diabetes following bariatric surgery and the possible interaction between achieved weight loss and diabetes duration. The literature previously discussed challenges the widely held belief that type 2 diabetes is an inevitably progressive disease, and laboratory data on beta cell replication, neogenesis and redifferentiation gives theoretical reasons to believe that reversal of diabetes could be possible at any stage of the disease. I postulated that achieving significant weight loss and relieving the burden of excess fat on the liver and pancreas might be more important than duration of diabetes in terms of achieving remission of diabetes. The second study, described in Chapters 4 and 5, relates to the mechanisms determining reversal of type 2 diabetes following gastric bypass surgery. In Chapter 4, comparison of the effects of bariatric surgery on hepatic and pancreatic triglyceride content and metabolic function in individuals with type 2 diabetes and those with normal glucose tolerance aims to investigate disease specific effects as opposed to consequences of significant weight loss alone. In chapter 5 the role of calorie restriction in the early improvement in glucose metabolism following gastric bypass surgery is investigated by direct comparison of metabolic effects at day 7 after RYGB or VLCD. The third study, described in Chapters 6 and 7 was designed to investigate the limits of reversibility of type 2 diabetes using an 8 week VLCD and the durability of the effect of the VLCD on glucose metabolism after return to normal eating. In Chapter 6, the pathophysiological differences between short and long duration diabetes and the reversibility of each using an 8 week VLCD are described. Chapter 7 describes the pathophysiological

mechanisms which determine diabetes reversal and what characterises those who achieve normalisation of glucose levels following a VLCD compared to those who do not. Finally, the durability of the beneficial effects of a VLCD after a 6 month period of weight maintenance is described.

Chapter 2. Methods

2.1 Research participants

2.1.1 Recruitment

Participants with an established diagnosis of type 2 diabetes were recruited. Detailed history taking was used to clinically exclude individuals with alternate diagnoses to type 2 diabetes, such as slow onset type 1 diabetes or maturity onset diabetes of the young (MODY). Diabetes duration was verified from general practitioner records. Individuals were excluded if they had a contraindication to MR scanning, untreated thyroid disease, evidence of renal dysfunction (defined as serum creatinine >150μmol/l), if they consumed more than 3 units of alcohol per day for women or more than 4 units per day for men, or if they were taking steroids or atypical anti-psychotic medication. Participants were recruited from advertisement displayed in local hospitals, university, council buildings and retinopathy screening clinics. Those who responded to the advert were sent a full participant information sheet detailing the purpose, nature and potential risks of the study. The potential participant was then invited for a screening visit at the Magnetic Resonance Centre.

Participants with no known personal history or family history (in a first degree relative) of type 2 diabetes and with no contra-indication to the study were also recruited following confirmation of normal glucose tolerance using a standard oral glucose tolerance test.

For all clinical studies, ethical permission was obtained from the Newcastle and North Tyneside Local Research Ethics Committee. All studies were explained in detail, through written and verbal explanation, to participants who expressed an interest in taking part and all queries were answered. Written informed consent was obtained from all participants recruited in the studies. After completion of the studies, all participants received feedback on personal and overall results.

2.1.2 Anthropometric measurements

Body weight was measured to the nearest 0.1kg (wearing indoor clothing only after removal of footwear) using a calibrated upright pedestal digital

scale (Seca Ltd., Birmingham, UK). Height was measured to the nearest 0.5cm using a stadiometer (Seca Ltd., Birmingham, UK). Body mass index was determined from weight and height measurements [BMI = weight (kg)/ height^2 (m)].

Waist and hip circumferences were measured to allow calculation of waist:hip ratio. A standard non-distensible tape measure was used for all measurements, which were taken with the participants standing and in a relaxed posture. The waist was taken as the half way point between the anterior superior iliac spine and the lower edge of the rib-cage. Hip measurements were taken from the level of the participant's greater trochanter.

2.1.3 Body composition
Body composition was determined after an overnight fast by measuring whole body impedence using a Bodystat®1500 (Bodystat Ltd, Isle of Man, UK). Bioelectrical impedence analysis is an indirect measurement of total body water volume made by measuring the body resistance to a sensationless excitatory current of 500μ root mean square at 50kHz. This is calculated from the equation: total body water = specific resistivity of the body fluid (ohm-cm) x (height^2)/measured impedence (ohm). Four electrodes are attached: two placed on the participant's right hand and two on the right foot. This information, along with gender, age, activity level, weight and height, are used to calculate body composition.

2.1.4 Energy balance
Indirect calorimetry

Whole body oxygen uptake (VO_2) and carbon dioxide production (VCO_2) were measured using an open circuit calorimeter (Quark RMR; COSMED, Rome, Italy) and a canopy hood. The canopy dilution technique is used to extract expired gases. A clear plastic canopy is placed over the participant's head and air is drawn through the canopy at a variable but continuously monitored flow rate. Carbon dioxide is measured by a non-dispersive

infrared sensor and oxygen by a paramagnetic sensor. The precision of the Quark monitor used in this study has previously shown to have a repeatability coefficient of variation of 1.2% on an ethanol burning test (Blond *et al.*, 2011).

Prior to each study and after a 15 minute warm-up period, the gas analysers were calibrated using a certified calibration gas (O_2 16% and CO_2 5%) to ensure precise measurement. Calibration of the digital turbine flowmeter was performed using a 3 L calibration syringe. Software uses the Weir equation to assess energy expenditure at rest (Weir, 1949).

Following an overnight fast and avoidance of physical activity, caffeine, nicotine and any other stimulants, participants were asked to lie supine on a bed and relax (but not sleep) without speaking for 20 minutes. The purpose of the test was fully explained to maximise cooperation. The first 5 minutes were considered the acclimatisation period and excluded from analysis.

Measurements of physical activity

Physical activity levels were measured objectively using a validated multi-sensor array (SenseWear Pro$_3$; BodyMedia Inc., Pittsburgh, USA). This 3-axis accelerometer incorporates a heat flux sensor, galvanic skin response sensor, skin temperature sensor, and a near-body ambient temperature sensor. Participants were asked to wear this for 5 days, including at least one weekend day, removing the armband only for bathing. Participants were asked not to change their daily routine while wearing the armband and received no feedback from the monitor during use. Data were analysed using the InnerView® Professional software which contains activity detection and lifestyle algorithms to apply appropriate formula to estimate energy expenditure from the sensor data as well as gender, age, weight, height, smoking status and hand dominance. Parameters reported include total energy expenditure, active energy expenditure, resting energy expenditure, metabolic equivalent of task (MET), total number of steps, sedentary time and physical activity duration (in METs): light (1.5-3.0);

moderate (3.0-5.9); vigorous (6.0-9.0); and very vigorous (>9.0). The Sensewear armband has been validated for the calculation of energy expenditure and quantification of metabolic physical activity on free-living individuals (Fruin and Rankin, 2004) and results show reasonable concordance with measurement of energy expenditure by doubly labelled water (St-Onge *et al.*, 2007).

Subjective reports of physical activity levels were determined using the International Physical Activity Questionnaire (IPAQ) (*International Physical Activity Questionnaire*; Hagströmer *et al.*, 2006). This questionnaire asks participants to recall physical activity levels over the preceding 7 days encompassing the following domains: job-related physical activity, transportation, housework and recreation/leisure time. Participants were instructed on how to complete the different sections of the questionnaire and were allowed as much time as required to complete it. Comparison with accelerometry data has found the long form of the IPAQ to have acceptable validity when assessing levels and patterns of physical activity in healthy adults (Hagströmer *et al.*, 2006) and reasonable consistency across different populations (Craig *et al.*, 2003).

Calorie intake

Self-report food diaries were collected; participants were asked to record all the food and beverages consumed during 3 consecutive days. This method has the advantage over 24hr recall food questionnaires of not relying on memory, however, the limitations of food diaries include uncertainty of portion size, completeness of diary and whether the 3 day period is representative of habitual diet. This method was selected as a compromise between precision, researcher burden (time for coding/analysis of diaries) and participant compliance. An in-house custom built database using the McCance and Widdowson food composition tables was used to analyse data (Dr E Foster) (FSA, 2002).

2.2 Magnetic resonance imaging

Magnetic resonance data were acquired using a 3 Tesla Philips Achieva scanner (Philips, Best, The Netherlands) with a 6 channel cardiac array (Philips) used for imaging by preference, or four large surface coils (large and medium, Philips) if required due to body habitus.

2.2.1 Three point Dixon magnetic resonance imaging

Three gradient echo scans were acquired with adjacent out-of-phase, in-phase and out-of-phase echoes. For human studies, the fat-water frequency difference at 3 Tesla is 435 Hz, and echo times of 3.45 ms, 4.60 ms and 5.75 ms were used to acquire adjacent out-of-phase, in-phase and out-of-phase images respectively. A repetition time (TR) of 50ms was used with a flip angle of 5 degrees for the liver, and 9 degrees for the pancreas. A breath hold time of less than 17 seconds was used to acquire 6 slices to cover the liver with slice thickness 10mm and of the pancreas with slice thickness of 5mm. Participant cooperation was maximised by careful explanation from research radiographers. The real and imaginary data (not magnitude corrected data) were then uploaded to a custom MATLAB (Mathworks, Cambridge, UK) script, following an algorithm to produce separate fat and water images (Glover and Schneider, 1991). The fat content of the image was then expressed as a percentage of the original signal, composed of both the water and fat signals (Figure 2-1). The fat percentage in the visible MR signal is referred to as the "fat content". Region-of-interest (ROI) definition was performed using the polygon ROI tool in ImageJ 1.43 software which is freely available from NIH (Abramoff *et al.*, 2004).
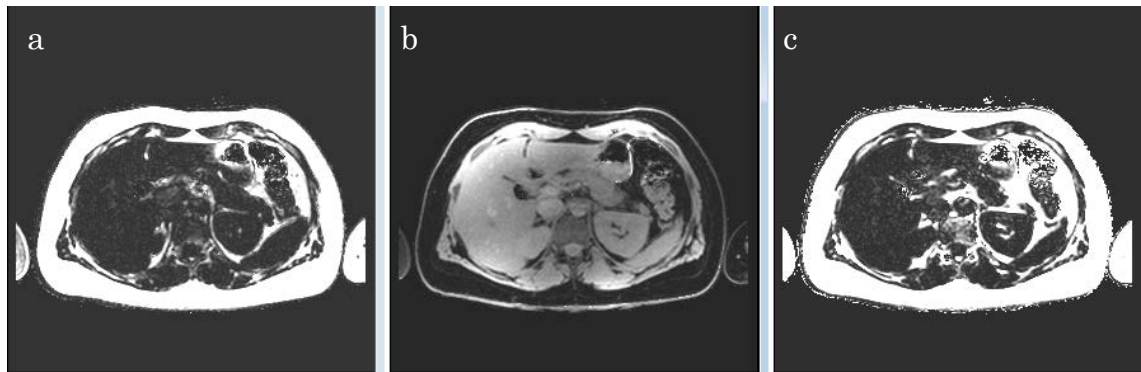
Figure 2-1 3 point Dixon technique to measure liver and pancreas fat content: (a) fat image, (b) water image, and (c) fat percentage image.

2.2.2 Hepatic and pancreatic triglyceride

The anatomical positions of the liver and pancreas were first ascertained by axial sections using a single shot balanced turbo-field echo (BTFE) sequence (TR = 2.8 ms, TE = 1.4 ms, number of averages = 1, flip angle = 40°, matrix of 172 x 192, median field of view (liver) = 420 mm; range 400-450 mm, median field of view (pancreas) = 400 mm; range 380-450 mm to suit participant size with 70% phase field of view, turbo factor 101, 18 slices, slice thickness 5mm and scan duration 6 s. A parallel acceleration factor of two was used in the phase direction. After completion of the automated reconstruction of the three-point Dixon images, the images were then independently analysed. The signal intensity in the images was calculated with investigator-defined regions of interest (ROI) using ImageJ (Abramoff *et al.*, 2004). A total of five ROIs for the liver and two ROIs for the pancreas (on consecutive slices) were acquired and averaged. These ROIs were chosen to reflect as closely as possible the same anatomical area across each consecutive scan for each individual. ROI placement in the liver was carefully performed to avoid contamination from blood vessels, gallbladder, falciform ligament and visceral fat. A smaller number of ROIs were used in pancreas analysis given the smaller volume of this organ and its lobulated structure, and care was taken to place ROI in pancreatic parenchyma rather than encroaching adipose tissue. The analysis was performed by a single trained investigator (myself). Pancreas image analysis was performed blind to both participant identity and visit number. Validation of the three point

Dixon technique for measuring liver and pancreas fat content by this method has been established previously and the inter-scan Bland-Altman repeatability coefficients were 0.5% for the liver and 0.9% for the pancreas (Lim *et al.*, 2011). The Bland-Altman repeatability for intra-observer pancreatic triglyceride analysis in these studies is shown in

Figure **2-2**. To assess the reproducibility of this process, 20% of the pancreas scans were independently analysed by another trained investigator (Dr K Percival). The Bland-Altman repeatability for inter-observer pancreatic triglyceride analysis is shown in Figure 2-3.
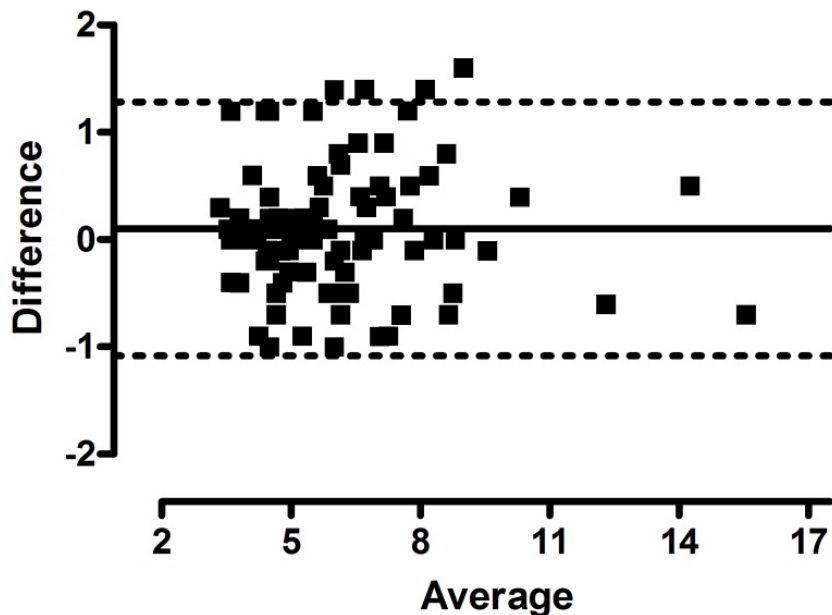
Figure 2-2 Bland-Altman plot for intra-observer difference in pancreatic triglyceride analysis. Bias is shown as solid line and 95% confidence intervals as dotted lines.
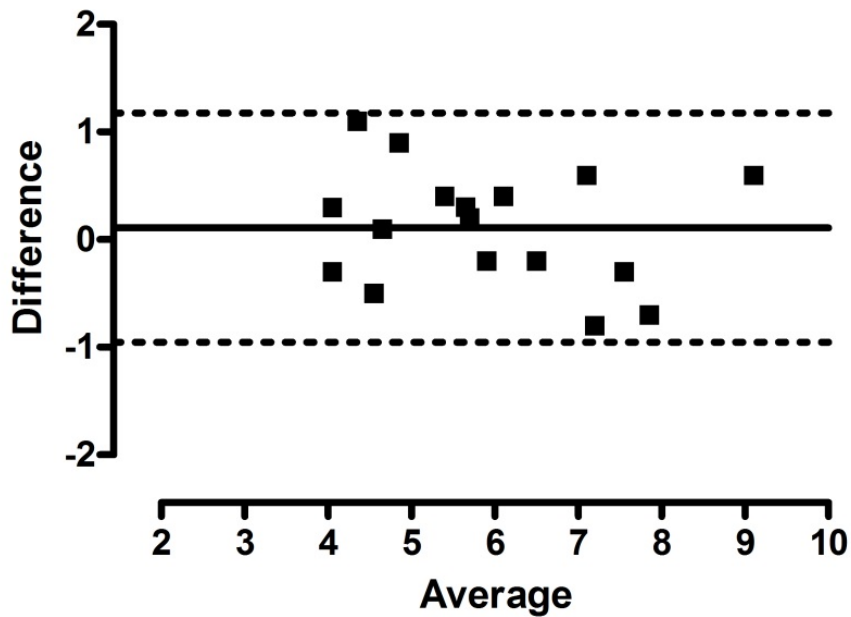
Figure 2-3 Bland-Altman plot for inter-observer difference in pancreatic triglyceride analysis. Bias is shown as solid line and 95% confidence intervals as dotted lines.

2.2.3 Subcutaneous and visceral adipose tissue areas

Subcutaneous and visceral fat measurements were made by acquiring images at the L2/L3 level using a three point Dixon sequence (TR=50 ms, TE=3.45/4.60/5.75 ms, number of averages = 1, flip angle = 30° matrix of 160 x 109, median field of view = 460 mm; range 400-520 mm. Single-slice visceral adipose tissue (VAT) areas at 5–10 cm above L4/L5 (approximately L2/L3) have been shown in overweight individuals to have a higher correlation with VAT volume than does the VAT area at the traditional L4/L5 location (Abate *et al.*, 1997; Shen *et al.*, 2004). Also, for participant comfort this level is less likely to require repositioning within the scanner following the liver/pancreas scans than the L4/L5 level to achieve adequate signal. The slice was acquired during a breath hold of 17s and had a slice thickness of 10mm. Fat and water were separated, and using ImageJ (Abramoff *et al.*, 2004), binary gating was applied to produce a map of structures containing more than 50% fat (

Figure **2-4**). A watershed algorithm was applied to divide the image into distinct areas allowing easy identification of subcutaneous and visceral compartments. The subcutaneous adipose tissue (SAT) compartment was

segmented and measured, and this area was subtracted from the total adipose tissue area to give the visceral adipose tissue area. However, in cases where the field of view was insufficient to include the entire SAT area, a T1 image at the L2/L3 level was used to measure total fat area. A region of interest was then manually drawn to measure intra-abdominal fat volume and the difference between the total fat area and the intra-abdominal fat area was taken to represent SAT area. VAT area in these individuals was measured using the three-point Dixon method and watershed algorithm as described above.
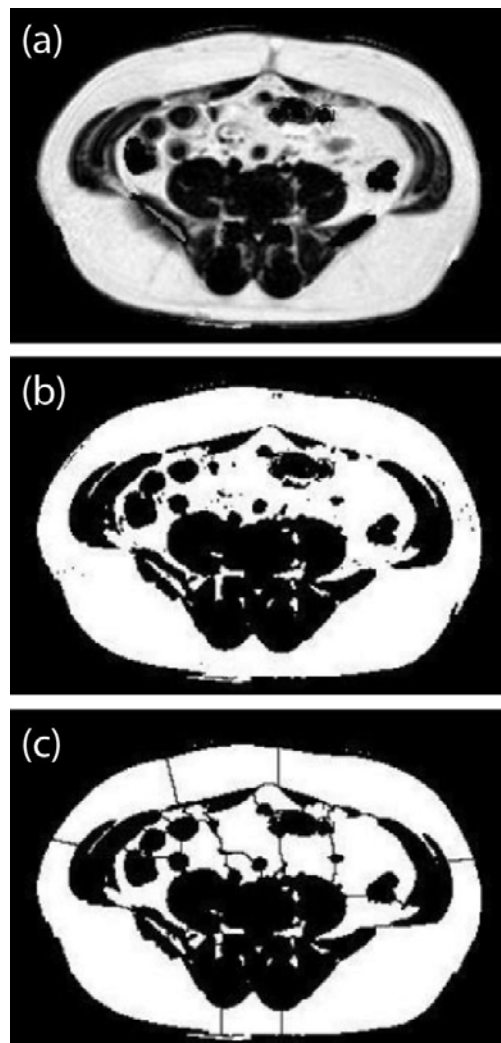
Figure 2-4 Quantifying subcutaneous and visceral fat: a) Dixon image at L2/L3 level, b) binary threshold applied to show structures containing >50% fat and total fat area in image measured, c) watershed algorithm applied to allow selection and measurement of subcutaneous fat area. Visceral fat area is then calculated from total fat area – subcutaneous fat area.

2.3 Metabolic studies

All metabolic studies were performed after an overnight fast. Metformin and sulphonylureas were discontinued for 72 hours before the study and DPP-4 inhibitors for 2 weeks. No rapid-acting insulin was administered within 12 hours of studies and long-acting insulin was withheld for 36 hours. Participants were asked to avoid unusual physical activity, alcohol or nicotine for 24 hours prior to the studies.

2.3.1 Blood sampling

Venous cannulation for arterialised blood sampling was performed by the insertion of an 18-gauge cannula (BD Venflon IV catheter, Oxford, UK) into a distal vein of the participants' dominant arm. The venous blood was 'arterialised' by placing the hand between microwaveable heat packs which were regularly reheated to ensure continuous vasodilatation. The intravenous line was flushed with 0.9% sodium chloride after taking each sample to keep the line patent. Dilution of samples was avoided by discarding the initial dead space line volume of blood withdrawn, and by using a fresh syringe for each sample. A second cannula was inserted into a large antecubital vein in the contralateral arm for administration of intravenous glucose, insulin or Intralipid. This line was flushed with 0.9% sodium chloride. Whole blood samples were centrifuged at 3000rpm at 4°C for 10 minutes (Harrier 18/80, MSE, London, UK), with the supernatant separated and then frozen at -40°C at the Magnetic Resonance Centre before being transported on ice for assaying.

2.3.2 Oral glucose tolerance test

After an overnight fast, blood glucose levels were measured. A standard 75g glucose load (Lucozade Original; GlaxoSmithKline, Coleford, UK) was consumed within 5 minutes and blood glucose levels were then measured 2 hours later with the participant relaxing in between with no exercise or nicotine. Glucose tolerance was then classified according to the 2006 WHO/IDF diagnostic criteria (WHO/IDF, 2006).

2.3.3 Hepatic glucose production

Hepatic glucose production was assessed by a primed continuous infusion of [6-6-^{2}H]-glucose (dideuterated glucose) over 270 min with priming dose adjusted for ambient fasting plasma glucose level (Hother-Nielsen and Beck-Nielsen, 1990; Ravikumar *et al.*, 2008). The priming dose is used to instantaneously label the whole glucose pool to the steady state level that would eventually be reached with a constant infusion alone. Achievement of steady state is crucial because at this point, with constant tracer level, the tracer disappearance rate (Rd) must equal the tracer infusion rate (Ra).

6'6'-dideuterated glucose powder (Cambridge Isotope Laboratories; Andover, MA) was made up into 10% strength solutions in sterile water using sterile technique. The priming dose (10% strength) was given intravenously and calculated to the priming dose for a fasting plasma glucose level of 5mmol/l being 300mg therefore:

$$\text{Bolus dose (10\% strength) in (ml)} = \frac{\text{fasting plasma glucose (mmol/l)} \times 300\text{mg}}{5}$$

After the participant had rested for 30 min the bolus injection was administered. Immediately thereafter a continuous infusion of 2% strength (20mg/ml) 6'6'-dideuterated glucose at a rate of 0.04 mg/kg/min was commenced to achieve equilibrium of 6'6'-dideuterated glucose.

In order to prevent any fall in atom percent excess (APE) of 6'6'-dideuterated glucose during the insulin-glucose clamp, the 6'6'-dideuterated glucose infusate was continued for the duration of the clamp. In addition, the variable 10% glucose infusion used to maintain isoglycaemia during the clamp was enriched with 2% 6'6'-dideuterated glucose (Figure 2-5).
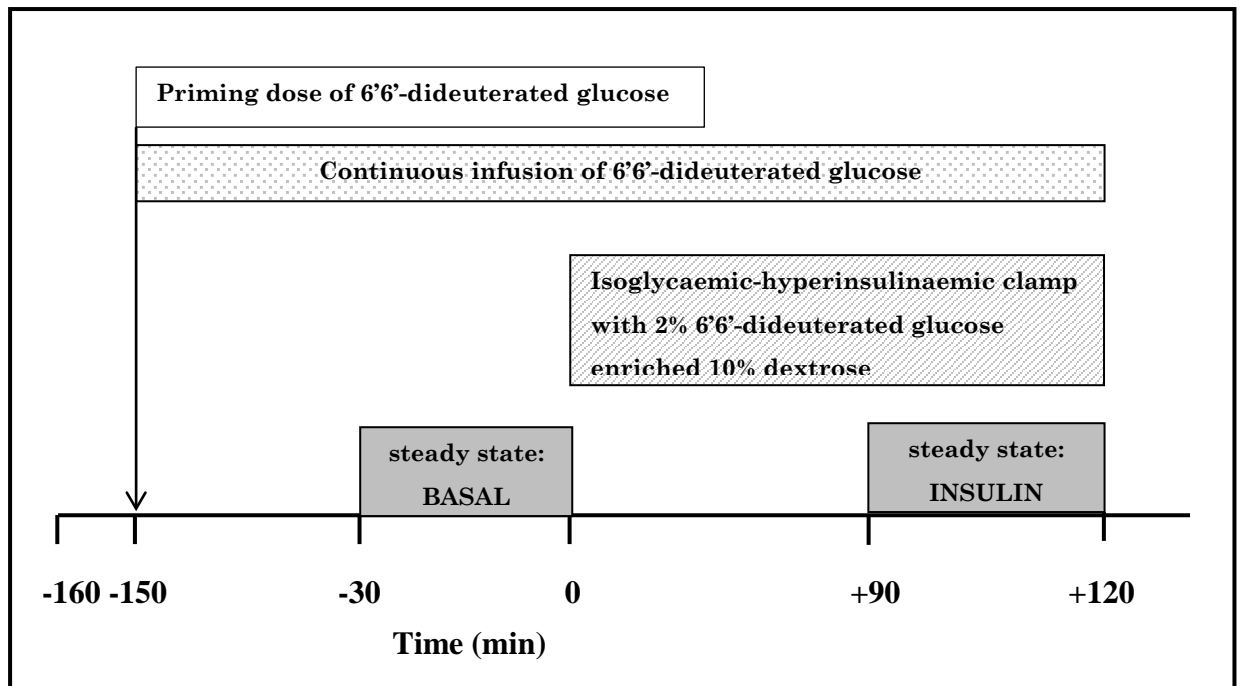
Figure 2-5 Schematic of hyperinsulinaemic isoglycaemic clamp protocol showing steady state basal period and steady state hyperinsulinaemic period.

Blood samples were taken every 5 min for determining plasma glucose concentration and frequently for determining plasma enrichment of 6'6'-dideuterated glucose, insulin and C-peptide concentration according to a schedule (Table 2-1). 1ml samples of 2% strength 6'6'-dideuterated glucose infusate (labelled NINF) and 2% 6'6'-dideuterated enriched 10% glucose (labelled GINF) were taken at each study for analysis.

Time (min)	Plasma glucose	Plasma [6-6-^{2}H]-glucose APE	Insulin	C-peptide
-160	X	X	X	X
-150	X	X		
-90	X	X		
-30	X	X		
-20	X	X		
-10	X	X		
0	X	X	X	X
5	X			
10	X			
15	X			
20	X			
25	X			
30	X	X		
35	X			
40	X			
45	X			
50	X			
55	X			
60	X	X	X	
65	X			
70	X			
75	X			
80	X			
85	X			
90	X	X	X	
95	X			
100	X	X		
105	X		X	
110	X	X		
115	X			
120	X	X	X	X

Table 2-1 Blood sampling schedule for hyperinsulinaemic isoglycaemic clamp

Gas chromatography-mass spectrometry analysis

Frozen samples were allowed to thaw and then underwent a vortex mix (Dr A Al-Mrabeh). Approximately 30μl plasma was transferred to an Eppendorf tube before 200μl acetonitrile:ethanol (2:1) (TraceSELECT Fluka Analytical Sigma-Aldrich, St. Louis, MO, USA) was added. Following a vortex mix, the sample was spun in a micro-centrifuge at 13000 rpm for 5 min. 200μl was

then transferred to a 5ml universal glass vial and evaporated to dryness at 90°C for 45 min under a gentle stream of dry air in a fume hood. 60μl pyridine anhydrous 99.8% (Sigma Aldrich, St. Louis, MO, USA) and 20μl acetic anhydride (Sigma-Aldrich, St. Louis, MO, USA) were added to dry the residue. The vial was capped, gently mixed and heated in an incubator at 90°C for 30 min. The samples were evaporated to dryness for 5 min at 90°C under a gentle stream of dry air. The pentaacetate derivative of glucose was then dissolved in 75μl acetonitrile and then a 1 in 4 dilution was prepared with acetonitrile. The samples were then analysed using gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS analysis was carried out on a Thermo 'Voyager' single quadruple mass spectrometer interfaced to a Thermo 'Trace' 2000 version 1.3 GC, with automated injection via a Thermo 'AS2000' autosampler v1.3 (Thermo Scientific, Waltham, MA, USA). Samples for glucose analysis were analysed under Electron Ionisation – Single Ion Monitoring (EI-SIM) conditions with separation of the pentaacetate derivatives carried out on a Zebron 30m x 0.25mm x 0.25μm ZB-5 ms capillary column (Phenomenex, Torrance, CA, USA). Injections of 3μl were made in the split mode with a 30:1 split and the injection port was maintained at a constant 220°C. The carrier gas was Helium at a constant flow of 1 ml/min. GC conditions were: 200°C ramping to 240°C and 290°C to a maximum of 350°C with a terminal hold of 5 min; MS Trace MS Plus 1.0 SPI for Xcaliber version 1.3 (Thermo Finnigan): the mass detected were 200 and 202 (6,6-dideuterated glucose). The scanning time was 2-8 min with a dwell time of 0.75 and 450 multiplier voltage. The CV for the precision of plasma $^2H_2$ APE measurements was 3.3%. The APE of the $^2H_2$ infusate was measured similarly and the CV was 0.3%. All samples for each study were analysed together along with the corresponding NINF and GINF samples and a 6'6'-dideuterated glucose standard was derivatised and analysed in addition.

Calculations

Hepatic insulin sensitivity can be assessed by the suppression of hepatic glucose production by insulin infusion. That is comparing basal hepatic

glucose production (under fasting steady state conditions of physiological plasma insulin) and clamp hepatic glucose production (under steady state supraphysiological insulin concentrations). Under non-steady state conditions created by a glucose-insulin clamp hepatic glucose production is estimated indirectly using a single compartment model (Steele *et al.*, 1956) (Figure 2-6).
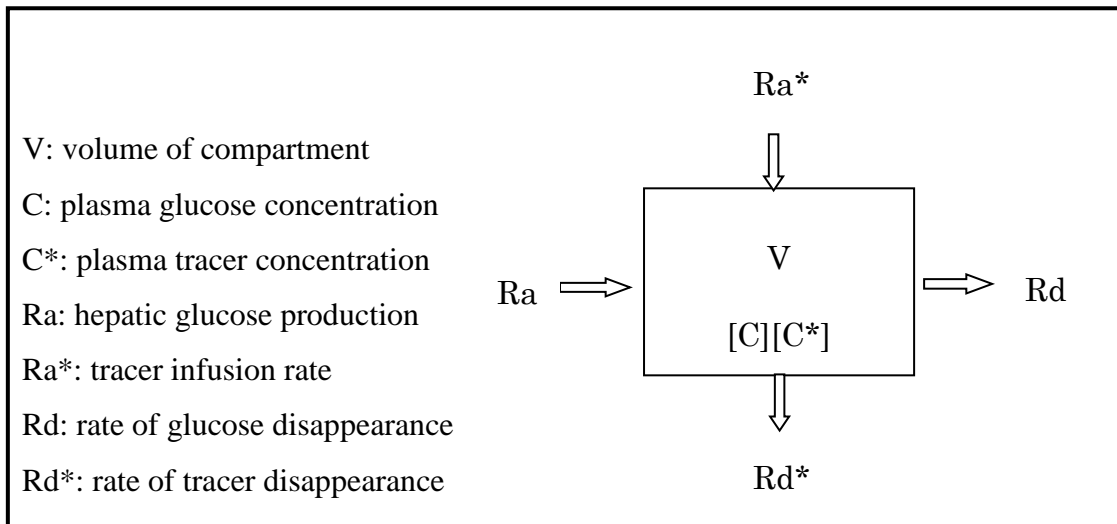
Figure 2-6 Single compartment model for estimating hepatic glucose production

This assumes that during steady-state glucose infusion, the only source of endogenous glucose production is the liver, and thus that HGP represents the difference between the rate of glucose appearance in the circulation (Ra) and its disappearance (Rd).

Basal hepatic glucose production was calculated during the last 30 min of the 150 min basal period using the following equation (Maggs *et al.*, 1998):

$$\text{Basal HGP} = \frac{(\text{basal [dideuterated glucose]GIR}}{\text{BSA}} \times \frac{(\text{enrichment}^{\text{inf}} - 1)}{\text{enrichment}^{\text{plasma}}}$$

Basal HGP= Basal hepatic glucose production (mg.m^{-2}.min^{-1})

GIR = basal dideuterated glucose infusion rate (mg/min)

BSA = body surface area (m^{2})

44

Enrichment = the fraction of isotope of glucose to naturally occurring glucose (%)

Enrichment ^{inf} = APE of glucose in infusate (%)

Enrichment ^{plasma} = APE of plasma glucose during steady state basal conditions

During the isoglycaemic clamp, clamped hepatic glucose production was calculated during the last 30 min of the clamp using the following equation:

$$\text{Clamp HGP} = \text{GIR}^{mean} \left(\frac{\frac{\text{mg}}{\text{m}^2}}{\text{min}} \right) \times \frac{(\text{enrichment}^{inf} - 1)}{\text{enrichment}^{plasma}}$$

Clamp HGP= Clamp hepatic glucose production (mg.m^{-2}.min^{-1})

GIR^{mean} = mean glucose infusion rate over the last 30 min of the clamp

Enrichment ^{inf} = APE of glucose in infusate (%)

Enrichment ^{plasma} = APE of plasma glucose during steady state conditions of the clamp (%)

Hepatic insulin sensitivity has also been calculated as the hepatic insulin resistance index; the product of the basal rate of hepatic glucose production and the fasting insulin concentration (Gastaldelli *et al.*, 2007). This measurement is based on the principles that fasting insulin concentration is a strong inhibitory stimulus for hepatic glucose production and that the insulin infusion rate used in the hyperinsulinaemic clamp suppresses hepatic glucose production by 90-95% in the majority of subjects. Use of this index of hepatic insulin resistance has been validated against the euglycaemic insulin clamp (Groop *et al.*, 1989).

2.3.4 Peripheral insulin sensitivity

An isoglycaemic hyperinsulinaemic clamp was used to quantitate peripheral insulin sensitivity. Isoglycaemic hyperinsulinaemia was induced using the insulin-glucose clamp technique (DeFronzo *et al.*, 1979). Isoglycaemia was selected over euglycaemia to avoid the alteration in insulin secretion and

hepatic glucose production that would occur over successive studies due to the change in the basal condition of each participant and hence fasting glucose levels. Human insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) was administered as a primed continuous infusion (40mU/m²/min) for 120 minutes. Insulin infusions were prepared by drawing up the required number of units of Actrapid insulin in 50ml of 0.9% sodium chloride. Fasting isoglycaemia was maintained using a 10% dextrose infusion 2% enriched with 6'6'-dideuterated glucose to maintain steady the plasma atoms percent excess of isotopic enrichment according to the 'hot GINF' protocol (Finegood *et al.*, 1987).

A primed constant insulin infusion was calculated according to body surface area at 40mU/m²/min to achieve plasma concentrations of ~400pmol/l. Body surface area (m²) was calculated according to the Dubois formula: 0.007284 x (height in cm)$^{0.725}$ x (weight in kg)$^{0.425}$ (Dubois and Dubois, 1916).

The units of insulin required in 50ml 0.9% sodium chloride for a dose of 40mU/m²/min was determined as follows (Brehm and Roden, 2007):

$$\text{Insulin (units)} = \frac{0.04\,(U) \times BSA\,(m^2) \times 60\,\text{min} \times 50\text{ml (syringe volume)}}{\frac{15ml}{hr}(constant\ infusion\ rate)}$$

The insulin infusion was primed for 7 min as follows: 0-4 min 60ml/hr (4x constant infusion rate); 4-7 min 30ml/hr (2x constant infusion rate) then for the rest of the 120 min of the clamp ran at 15ml/hr. Samples for serum insulin and C-peptide concentrations were taken according to the schedule (Table 2-1) to ensure that steady state hyperinsulinaemia was achieved during the final 30 minutes of the clamp and to ensure that endogenous insulin secretion was inhibited. Preventing endogenous insulin secretion abolishes the usual physiological gradient in insulin concentration between the portal and peripheral venous plasma.

The mean CV of plasma glucose level and glucose infusion rate in the last 30 min of the 159 clamp studies was 3.2% and 6.5% respectively.

Calculation of whole body glucose metabolism

Whole body glucose metabolism (M) was determined from the glucose infusion rate (GIR) in the last 30 min of the clamp corrected for glucose space (SC) and urinary loss (UC) (DeFronzo *et al.*, 1979; Rizza *et al.*, 1981). Where blood glucose levels were greater than 10 mmol/l at the commencement of insulin infusion, urinary glucose was determined. Participants were asked to void at the beginning and end of the clamp period. The entire urine volume from this 120 min clamp period was collected and the urinary glucose mass determined. Whole body glucose metabolism is corrected for fat free mass (M_{ffm}) to indicate insulin action on glucose uptake in each unit mass of metabolically active tissue. This helps to account for gender related differences in fat mass and is more reliable than correcting for body weight only in obese individuals. Glucose metabolic clearance rates (ml/kg_{ffm}/min) were calculated by dividing whole-body insulin sensitivity (in mg/kg_{ffm}/min) by steady state plasma glucose (Ferrannini and Mari, 1998) to correct for the difference in glucose levels for isoglycaemia between study timepoints. Muscle insulin sensitivity was calculated as the sum of M value and basal hepatic glucose production minus the urinary glucose loss (Rothman *et al.*, 1992; Petersen *et al.*, 2005).

2.3.5 Measuring insulin secretion
Stepped insulin secretion test with arginine

In order to characterise first phase insulin response and maximal insulin secretory capacity a modified protocol was used (Toschi *et al.*, 2002). Two consecutive 30 min square wave steps of hyperglycaemia (at 2.8mmol then 5.6mmol above fasting glucose) were achieved by a priming 20% dextrose bolus followed by a variable 20% dextrose infusion (Figure 2-7). Finally, following the second step of hyperglycaemia, the response to a bolus of 5g arginine (Amargine L-Arginine Hydrochloride 50% w/v 5g in 10ml; Martindale Pharmaceuticals, Essex, UK) was characterised.
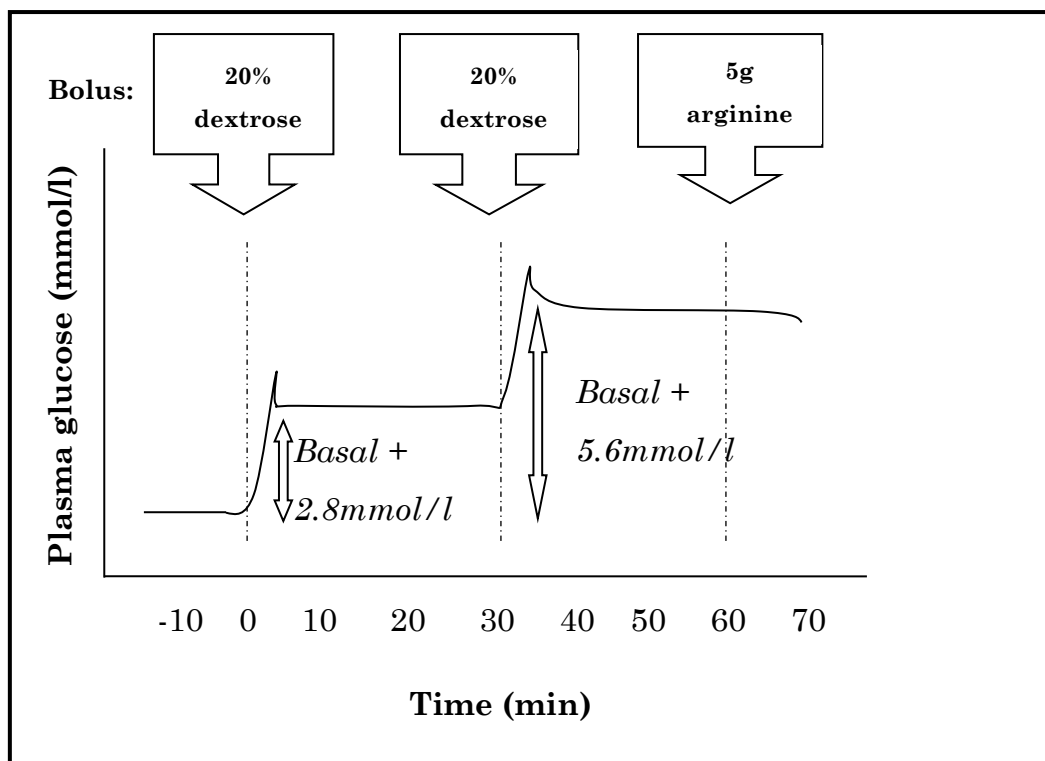
Figure 2-7 Schematic of protocol for stepped insulin secretion test with arginine

The priming doses of 20% dextrose for each step were calculated by multiplying the desired increments in plasma glucose concentration by the body glucose space (150ml per kg body weight). The 20% dextrose boluses were administered within 60 seconds. Glucose levels were then maintained at the desired plateau using a variable 20% dextrose infusion according to the glucose clamp technique (DeFronzo *et al.*, 1979). Blood samples for determination of plasma glucose, insulin and C-peptide concentrations were obtained every 2 min for 6 min then every 5 min from 10 min until the end of each step. After the arginine bolus blood samples were taken at 2, 4 and 6 min and the test ended after the 10 min blood sample was taken.

Calculation of insulin secretion rates

C-peptide concentrations represent insulin secretion more precisely than insulin concentrations; C-peptide is excreted in equimolar amounts, it is not subject to significant first pass hepatic extraction, and has relatively constant kinetics. Using C-peptide concentrations and a population model of

C-peptide kinetics (Van Cauter *et al.*, 1992) a mathematical modelling technique called deconvolution is implemented to calculate insulin secretion rates. Each participants' gender, age, diabetes status, height, weight, body surface area and BMI was entered along with C-peptide concentrations into ISEC computer program (Hovorka *et al.*, 1996) which implements a regularisation method of deconvolution (Twomey, 1965) giving an output of insulin secretion rate in nmol/min/m^2 of body surface area.

2.3.6 Incretin meal test

Each test was performed with the participant semi-reclined at a 45° angle in bed to avoid positional change resulting in variable gastric emptying between studies. After baseline blood samples at -10 and 0 min (for glucose, insulin, C-peptide, glucagon, total glucagon-like peptide-1 [GLP-1] and total glucose-dependent insulinotropic polypeptide [GIP]) participants were asked to consume the test meal within 3 minutes. A semisolid test meal was designed in accordance to the expected volume and consistency of diet consumed one week following gastric bypass surgery (10g Mornflake Instant Porridge Oats, 64g whole milk and 6g acacia honey: containing 100 kcal; 57% carbohydrate; 28% fat and 13% protein). Samples were taken every 10 min for the first 30 min, then every 30 min until the end of the 2 hr study. Samples for incretin hormones were taken into chilled tubes containing 0.25ml aprotinin/EDTA mix (Aprotinin 10,000 KIU/ml Nordic Pharma Ltd.; Reading, UK). At all times during processing samples were kept and transported on ice. The tubes were immediately centrifuged at 4°C (3000 rpm for 10 min) and the plasma separated into aliquots and frozen at -40°C until analysis. Positive incremental area under the curve was calculated for glucose, insulin, GLP-1 and GIP (Wolever *et al.*, 1991).

2.3.7 PNPLA3 genotyping

To look for the nonsynonymous variant I148M (rs738409 C/G, chr22:42656060-42656060) located in human patatin-like phospholipase domain containing 3 gene (PNPLA3, also known as adiponutrin) 10ml of

whole blood was collected at the final visit for each participant. The sample was collected in EDTA and after thorough mixing was then stored at -40°C. Genotyping was performed (Dr Yang-Lin Liu) blind to clinical parameters using TaqMan SNP Genotyping Analysis (Applied Biosystems, USA) on Applied Biosystems StepOne™ Real-Time PCR Systems. Each study was executed using the instructions provided and quality controls were included in every 48 well plate (duplication of negative controls, wild-type controls, mutant controls, and heterozygous controls).

2.3.8 Very low density lipoprotein-triglyceride production rates

$VLDL_1$-triglyceride production rates were measured using a validated technique which exploits the fact that VLDL accumulates during intravenous infusion of a chylomicron-like triglyceride infusion (Intralipid) due to competition for lipolysis by lipoprotein lipase (Bjorkegren et al., 1996; Al-Shayji et al., 2007). Following an overnight fast, two 18G intravenous cannulae were inserted: one for blood sampling and one for administration of Intralipid (purified soybean oil emulsion; Fresenius Kabi Ltd, Runcorn, UK). Two baseline blood samples were taken 10 min apart. For each sample, 9ml of whole blood was collected into EDTA tubes and kept at ambient temperature. A bolus of 20% Intralipid (0.1g/kg body mass) was injected within 60 seconds and was immediately followed by a continuous infusion of 10% Intralipid at 0.1g/kg/hr. At 5 min, 15 min, 30 min, 45 min, 60 min and 75 min further samples were collected and the infusion was stopped at 75 min. Samples were stored at ambient room temperature and were analysed within 72 hours of collection. Firstly, the plasma was separated from the cell pellet by centrifugation at 3000 rpm (Mrs J Cooney). Chylomicrons were separated from plasma by a short ultracentrifugation at density 1.006g/ml. The same method was used for the separation of Intralipid. Then a further ultracentrifugation step was used to separate $VLDL_1$ and $VLDL_2$. The $VLDL_1$ was then characterised using centrifugal analysis for full compositional analysis. Triglyceride and apoB concentrations were then measured in these fractions.

VLDL$_1$-triglyceride production rates (PR) were calculated from the gradient of the linear increase in their concentrations over time as follows:

$$\text{VLDL1TG PR (mg/hr)} = \frac{\text{Gradient (mg/dl)} \times \text{ plasma volume (dl)} \times 60}{\text{Time (min)}}$$

$$\text{VLDL1TG PR (mg/kg/day)} = \frac{\text{VLDL1TG production rate (mg/hr)} \times 24}{\text{Body weight (kg)}}$$

Where plasma volume (dl) = 4% of body mass

2.3.9 Metabolic and hormone assays

Blood samples for glucose measurement were taken into fluoride oxalate preservative. Whole blood and plasma glucose were measured by the glucose oxidase method (Yellow Springs glucose analyzer YSI 2300 STAT Plus; Yellow Springs Inc., Yellow Springs, OH: CV for measurement of glucose= 2%). Serum insulin was measured using ELISA kits (DAKO; Ely, Cambridge, UK: inter-assay CV [insulin range 36.9 - 47.3 pmol/l]: 6.2%). Serum C-peptide was initially measured using ELISA kits (DAKO; Ely, Cambridge, UK: CV for measurement [C-peptide range 1.20-2.00 nmol/l]: 7.1%) then by ELISA kits (Mercodia; Uppsala, Sweden: inter-assay CV for measurement [C-peptide range 1.16 – 1.40 nmol/l]: 4.4%). Mercodia results were adjusted to be in-line with DAKO results based on an in-house method evaluation of the correlation between the 2 assays: R^2 = 0.9466 therefore C-peptide (Dako) = C-peptide (Mercodia) + 0.0345)/0.7824. Plasma NEFA concentration was measured using a FLUOstar Omega microplate reader (BMG labtech; Ortenberg, Germany) by a commercially available enzymatic calorimetric kit (NEFA HR Reagent 1 and 2; Alpha laboratories, Eastleigh, Hampshire, UK: CV using 1mmol standard = 6.7%). Plasma glucagon concentration was measured by radioimmunoassay (Millipore Corporation; Billerica, MA, USA). The intra-assay CV and inter-assay CV at a mean

concentration of 111pg/ml were 2.9% and 7.1% respectively. ß-Hydroxybutyrate levels were measured using an Optium Exceed ketone meter (Abbott Diabetes Care; Oxfordshire, UK). HbA1c, liver function tests, γ glutamyl-transferase, thyroid function, full blood count and lipid profile were measured at a Clinical Pathology Accredited laboratory (Newcastle upon Tyne Hospitals NHS Foundation Trust, Department of Clinical Biochemistry). Incretin assays were batch assayed at the end of the study to reduce inter-assay variability (Dr A Pucci). All samples were run in duplicate and all of one participant's samples were run on the same plate. Human Total GLP-1 (7-36, 9-36) was measured using ELISA kits (Alpco Diagnostics; Salem, NH, USA) with intra-assay variation 4.1% and inter-assay variation 5.0%. Human Total GIP was measured using ELISA kits (Merck Millipore; Watford, UK). Intra-assay and inter-assay variability were 3.0% and 3.5% respectively.

Chapter 3. Effect of Diabetes Duration & Achieved Weight Loss on Reversal of Type 2 Diabetes Following Bariatric Surgery

3.1 Introduction

Duration of disease is considered to be an important clinical parameter which influences treatment selection and glycaemic outcomes in type 2 diabetes. The published data suggest that 2-3 years of type 2 diabetes does not usually result in irreversible beta cell damage, but it is widely believed that long duration disease results in an inevitable and irreversible decline in insulin secretion with the inevitable requirement for insulin therapy after an average of 10 years (UKPDS, 1998). Treatment guidelines for type 2 diabetes are based on this seemingly inevitable deterioration in glucose control, with algorithms of sequential addition of oral agents or non-insulin injectable therapies prior to commencement of insulin therapy (NICE, 2015). Diabetes duration has also been shown to be an important factor in determining remission of type 2 diabetes following bariatric surgical procedures (Schauer, 2003; Renard, 2009). This is acknowledged in the most recent UK guideline for obesity management which recommends expedited assessment for bariatric surgery for individuals with a BMI>35 kg/m^2 who have recent onset type 2 diabetes, considered to be within 10 years of diagnosis (NICE, 2014). A prospective study found that remission rates following gastric bypass surgery were 71% if diabetes duration was 8 years or less and 44% if diabetes duration was greater than 8 years (Hall *et al.*, 2010). However, a prediction model found that low pre-operative HbA1c and no requirement for insulin therapy were the pre-operative variables with the highest independent predictive value for resolution of type 2 diabetes after gastric bypass surgery (Hayes *et al.*, 2011). Other factors such as the extent of weight loss, weight regain and pre-surgery anti-diabetic therapy requirements have been postulated as predictors of outcomes. A randomised prospective study comparing remission of type 2 diabetes following gastric banding or intensive medical treatment found that remission was associated with the degree of weight loss and therefore was achieved more effectively in the surgery group (Dixon *et al.*, 2008). Another study of individuals undergoing gastric bypass surgery with less severe obesity showed a much higher remission rate at 88% in a group with relatively longstanding

diabetes, mean duration 12.5±7.4 years (Cohen *et al.*, 2012) suggesting that diabetes duration *per se* may not limit the reversibility of type 2 diabetes.

It is recognised that hyperglycaemia in type 2 diabetes is determined by impaired insulin secretion (Tabák *et al.*, 2009) on a background of longstanding insulin resistance (Martin *et al.*, 1992). Alongside the impairment in acute insulin secretion, characteristically a loss of first phase insulin response, there is also a reduction in beta cell mass in type 2 diabetes. In obese individuals with type 2 diabetes, relative beta cell volume is decreased by approximately 60% compared to obese people without diabetes (Butler *et al.*, 2003). This loss of beta cell mass seems to increase as duration of diabetes increases (Rahier *et al.*, 2008). The mechanism is likely to be due to apoptosis of beta cells induced by prolonged exposure to the toxic metabolites of fatty acids such as ceramides (Shimabukuro *et al.*, 1998a; Shimabukuro *et al.*, 1998b). *In vitro* work suggests that the functional impairment in insulin secretion can be reversed (Boucher *et al.*, 2004), and beta cell replication and neogenesis continue into adult life, suggesting the potential for recovery of both beta cell function and mass (Butler *et al.*, 2003; Butler *et al.*, 2010). The relative contribution of beta cell dysfunction and true decrease in beta cell mass is not clear. Recent rodent work has suggested that beta cell dedifferentiation and reprogramming might explain the association of beta cell dysfunction and increased alpha cell function in early type 2 diabetes (Talchai *et al.*, 2012). In human beta cells obtained from individuals with type 2 diabetes, the presence of mesenchymal cells and alpha cell phenotypic markers support this theory (White *et al.*, 2013).

Recently it has been demonstrated that reversal of type 2 diabetes can be achieved by acute calorie restriction alone through the use of a very low calorie diet (Lim *et al.*, 2011). In this study, recovery of first phase insulin response commenced after 7 days of a very low calorie diet and continued to improve, becoming indistinguishable from obese non-diabetic control subjects at 8 weeks. The participants all had a diabetes duration of less than 4 years. A differential effect of acute calorie restriction (minimum of 4 weeks

of 500kcal/day) on glycaemic control between individuals with short (≤2 years) and long duration (≥6 years) diabetes has been demonstrated, with a much more modest effect on fasting glucose in the long duration group (Nagulesparan *et al.*, 1981). However, this was a very heterogenous cohort, particularly regarding BMI which ranged from 25.4 to 56.9 kg/m². Whether diabetes duration is of importance *per se* or whether it is a surrogate marker for beta cell function is unclear.

Both duration of type 2 diabetes and extent of achieved weight loss are thus likely to influence the chance of diabetes reversal following bariatric surgery. However, the interaction between these two factors requires definition. The hypothesis that the degree of weight loss achieved after surgery is more important than disease duration in determining reversal of diabetes was tested in a heterogenous cohort representative of the bariatric surgery population in the UK.

3.2 Study design

3.2.1 Participants

148 individuals undergoing any bariatric surgical procedure in a specialist bariatric centre in North East England between 2009 and 2011 were identified through consecutive referrals to the diabetes team. All participants were seen by a member of the diabetes team, with clinical verification of the diagnosis of type 2 diabetes. Inclusion criteria were pre-operative HbA1c of ≥6.1% (43 mmol/mol) and a further HbA1c value measured 3 or more months after surgery.

3.2.2 Experimental protocol

This study was a retrospective data collection. Follow-up information on post-operative HbA1c was requested from the participants' primary care doctor. Post-operative HbA1c and weight data were taken at the furthest available time point from surgery. Post-operative HbA1c values are all from within the first 2.5 years following surgery, median 11 months (3-29). HbA1c is known to remain stable over this period (Mingrone *et al.*, 2012; Schauer *et al.*, 2012). Diabetes duration was pre-defined as the time

between diagnosis of type 2 diabetes and the pre-operative assessment. Reversal of diabetes was defined stringently as achieving a post-operative HbA1c <6.1% (43 mmol/mol) (Buse *et al.*, 2009). Diabetes duration was grouped as follows: short: <4 years; medium: 4-8 years; long: >8 years.

3.2.3 Statistical analysis

Parametric data are presented as mean ± SD or SEM and non-parametric as median and range apart from

Figure **3-2** where median and interquartile range is displayed. Statistical analyses were performed using Minitab 16 Statistical Software (Minitab Inc.;State College, PA: www.minitab.com). Parametric data were analysed using Student's t-test and non-parametric data were analysed using Mann Whitney U, Spearman rank correlation coefficient or Kruskal-Wallis.

3.3 Results

3.3.1 Clinical characteristics

Complete data on diabetes duration, achieved HbA1c and achieved post-operative weight loss were available for 89 eligible individuals. There was no statistically significant difference in baseline age, weight, BMI, or diabetes duration in those excluded from the analysis (n=59). The cohort consisted of 26 individuals with short, 36 with medium and 27 with long duration type 2 diabetes (Table 3-1). Pre-operative anti-diabetic treatments were: metformin: 75; sulphonylurea: 32; GLP-1 agonist: 19; thiazolidinedione: 18; insulin: 15; diet control only: 5; DPP-4 inhibitor: 4. The commonest surgical procedure was Roux-en-Y gastric bypass: n=57. Other procedures were laparoscopic adjustable gastric band (LAGB): n=16; sleeve gastrectomy (SG): n=8; and gastric balloon: n=8.

	Short duration diabetes (<4 yr)	Medium duration diabetes (4-8 yr)	Long duration diabetes (>8 yr)	Overall
n	26	36	27	89
M:F	6:20	13:23	9:18	28:61
Age (yr)	45.5 (32.0-62.0)	46.0 (18.0-64.0)	55.5 (37.0-74.0) *	49.0 (18.0-74.0)
Weight (kg)	136.3 ± 27.6	136.0 ± 27.4	133.0 ± 21.6	135.2 ± 25.9
Median BMI (kg/m²)	47.0 (39.3-66.8)	45.9 (37.7-67.1)	46.4 (37.2-75.6)	46.4 (37.2-75.6)
Median diabetes duration	2yr (1mo–3yr)	5yr (4yr–8yr)	12yr (9yr–31yr) *	5yr (1mo–31yr)
Median HbA1c (%)	7.5 (6.3-9.8)	7.4 (6.1-12.0)	7.8 (6.2-14.0)	7.5 (6.1-14.0)
(mmol/mol)	58 (45-84)	57 (43-108)	62 (44-130)	58 (43-130)
Diabetes treatment: n (%)				
Diet control:	4 (15.4)	1 (2.8)	0 (0.0)	5 (5.6)
OHA only:	22 (84.6)	31 (86.1)	16 (59.3)	69 (77.5)
Insulin:	0 (0.0)	4 (11.1)	11 (40.7)	15 (16.9)
Primary surgery: n (%)				
RYGB:	18 (69.2)	21 (58.3)	18 (66.7)	57 (64.0)
LAGB:	4 (15.4)	8 (22.2)	4 (14.8)	16 (18.0)
SG:	3 (11.5)	4 (11.1)	1 (3.7)	8 (9.0)
Balloon:	1 (3.8)	3 (8.3)	4 (14.8)	8 (9.0)

Table 3-1 Pre-operative participant characteristics. Results are displayed as mean ± SD or median and (range). * = $p<0.05$ comparing short and long duration diabetes.

3.3.2 Effect of degree of weight loss on achieved blood glucose control

Overall 48% (43/89) of this cohort achieved a post-operative HbA1c of <6.1% (43 mmol/mol). The median post-operative weight loss was 21.8kg (-4.2 - 64.7) and the mean percentage weight loss was 17.6 ± 1.1%. For the group achieving >25kg weight loss, there was a 61% (20/33) diabetes reversal rate compared to 41% (23/56) in the group achieving <25kg weight loss. The relationship between weight loss (%) and achieved HbA1c (Figure 3-1) showed a clear effect of greater weight loss bringing about lower HbA1c (R_s =-0.53; p<0.0001). In the group achieving weight loss of >25kg there was a modest difference between the short and long duration groups in achieved HbA1c (5.5±0.2 vs. 6.7±0.5 %; p=0.096) (37 vs. 50 mmol/mol) (

Figure **3-2**, panel e) compared to those who achieved <25kg weight loss (6.1±0.3 vs. 7.5±0.4 %; p=0.004) (43 vs. 58 mmol/mol) (

Figure **3-2**, panel f). In those who achieved HbA1c <6.1% (43 mmol/mol), post-operative weight loss was 29.4±2.4 kg (21.8±1.4 %) vs. 18.6±2.1 kg (13.6±1.5 %) in those who remained in the diabetic range (p=0.001).
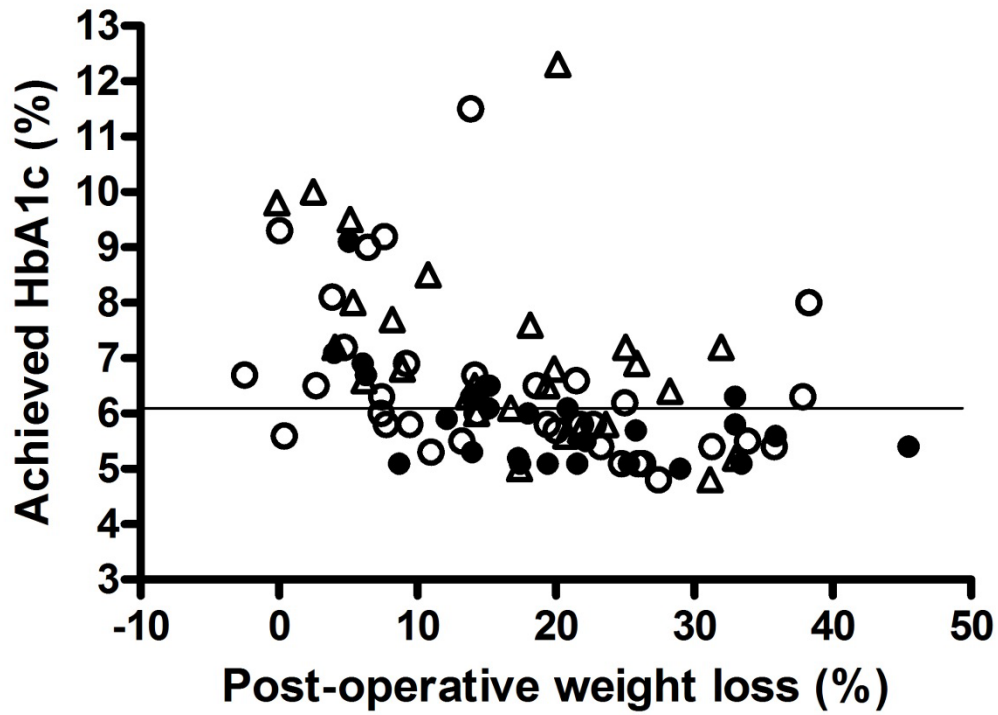
Figure 3-1 The relationship between achieved post-operative weight loss (%) and achieved HbA1c (%) in short (closed circles), medium (open circles) and long (triangles) duration type 2 diabetes. The solid line represents an HbA1c of 6.1 % (43 mmol/mol) and the cut-off for defining diabetes reversal.
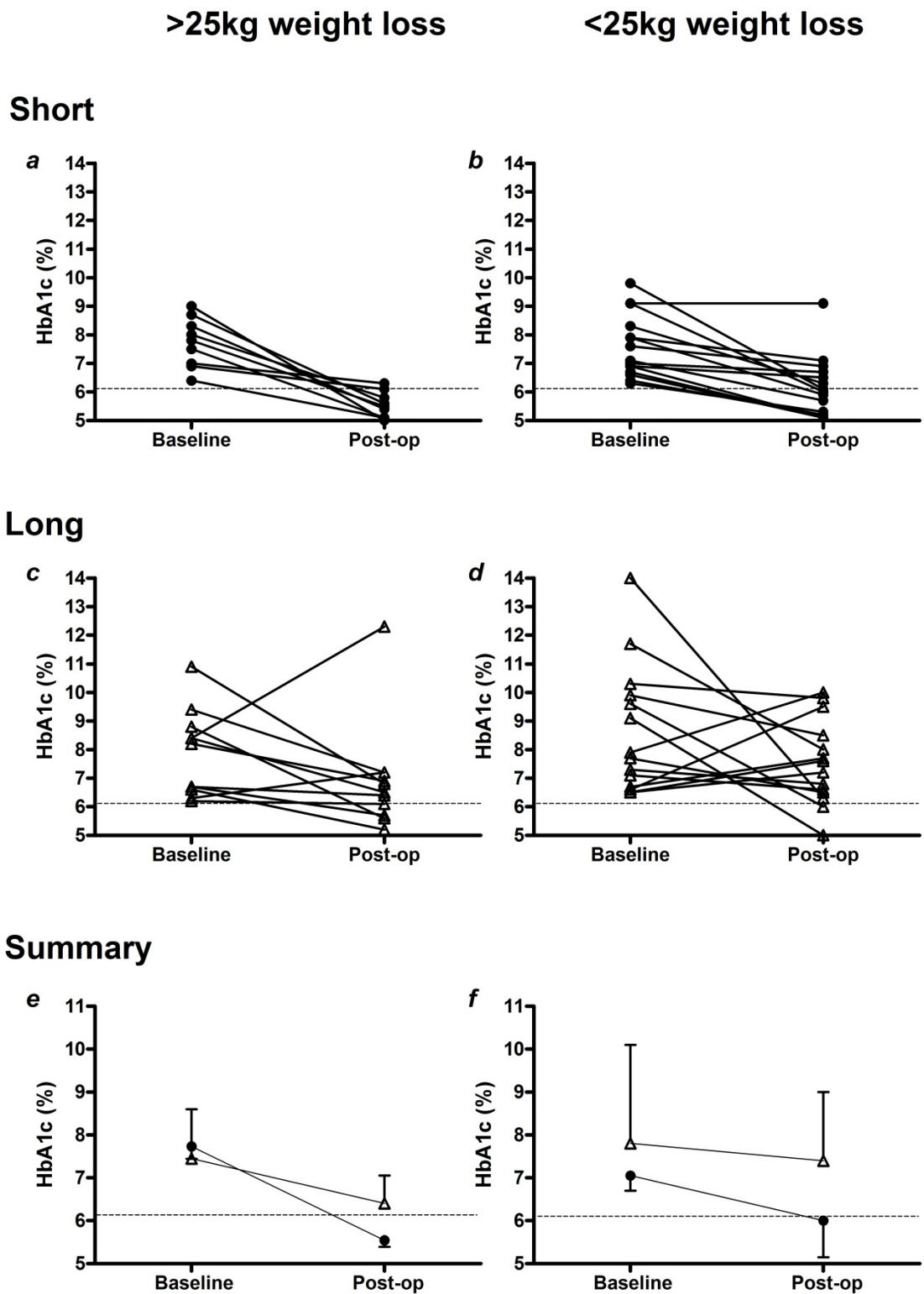
Figure 3-2 Change in HbA1c in each individual according to diabetes duration and achievement of more/less than 25 kg weight loss following bariatric surgery (panels *a-d*). Median and IQR HbA1c for long duration diabetes (triangles) and short duration diabetes (circles) (panels *e-f*). (n=11 for >25 kg short, n=13 for >25 kg long, n=17 for <25 kg short, n=14 for <25 kg long).

The characteristics of individuals who achieved >25kg weight loss in relation to post-operative HbA1c of less than or greater than 6.1% (43 mmol/mol) are shown in Table 3-2. There were no significant differences between the two groups at baseline.

	Achieved HbA1c <6.1% (43 mmol/mol)	Achieved HbA1c ≥6.1% (43 mmol/mol)
n	20	13
M:F	10:10	5:8
Age (yr)	49.0 (18.0-74.0)	47.0 (38.0-63.0)
Weight (kg)	145.9 ± 22.0	143.2 ± 22.2
Median BMI (kg/m²)	49.5 (39.7-67.1)	50.7 (40.1-75.6)
Median diabetes duration	5.5 yr (6 mo – 15 yr)	10.0 yr (2 yr – 19 yr) *
Median pre-operative HbA1c		
(%)	7.4 (6.2-9.3)	7.3 (6.2-10.9)
(mmol/mol)	57 (44-78)	56 (44-96)
Median achieved HbA1c		
(%)	5.4 (4.8-5.8)	6.5 (6.1-12.3) *
(mmol/mol)	36 (29-40)	48 (43-111)
Mean achieved weight loss		
(kg)	41.5 ± 12.2	37.2 ± 10.0
(%)	28.3 ± 6.7	26.3 ± 7.1
Diabetes treatment [n (%)]		
Diet controlled:	2 (10.0)	0 (0.0)
Non-insulin agents only:	16 (80.0)	8 (61.5)
Insulin:	2 (10.0)	5 (38.5)
Diabetes medications [n (%)]		
0	2 (10.0)	0 (0.0)
1	7 (35.0)	3 (23.1)
2	9 (45.0)	7 (53.8)
3	2 (10.0)	3 (23.1)
Primary surgery [n (%)]		
RYGB:	16 (80.0)	10 (76.9)
LAGB:	1 (5.0)	1 (7.7)
SG:	2(10.0)	2 (15.4)
Balloon:	1 (5.0)	0 (0.0)

Table 3-2 Characteristics of individuals who achieved >25kg weight loss according to achieved post-operative HbA1c. RYGB = Roux-en-Y gastric bypass surgery; LAGB = laparoscopic adjustable gastric band; SG = sleeve gastrectomy. Results are shown as mean ± SD or median (range). *=p<0.05 for between group difference.

3.3.3 Effect of duration of diabetes on achieved blood glucose control

HbA1c <6.1% (43mmol/mol) was achieved in 62% (16/26), 56% (20/36) and 26% (7/27) of the short, medium and long duration groups respectively. The diabetes durations in individuals who achieved a post-operative HbA1c <6.1% (43mmol/mol) overlapped with those who did not but was shorter [median 4 years (range: 1 month – 18 years) vs. median 6½ years (range: 9 months – 31 years); p=0.010]. The degree of achieved weight loss appeared to be more important in those with long duration compared to short duration diabetes (Figure 3-2, panels e and f). There was a notable lack of achievement of normoglycaemia in those individuals who had long duration diabetes and also lost less than 25kg (Figure 3-2, panel f). The relative effects of duration of type 2 diabetes and degree of achieved weight loss are shown in Figure 3-3. Despite increasing degrees of weight loss there is a plateau of diabetes reversal above about 20% post-operative weight loss.
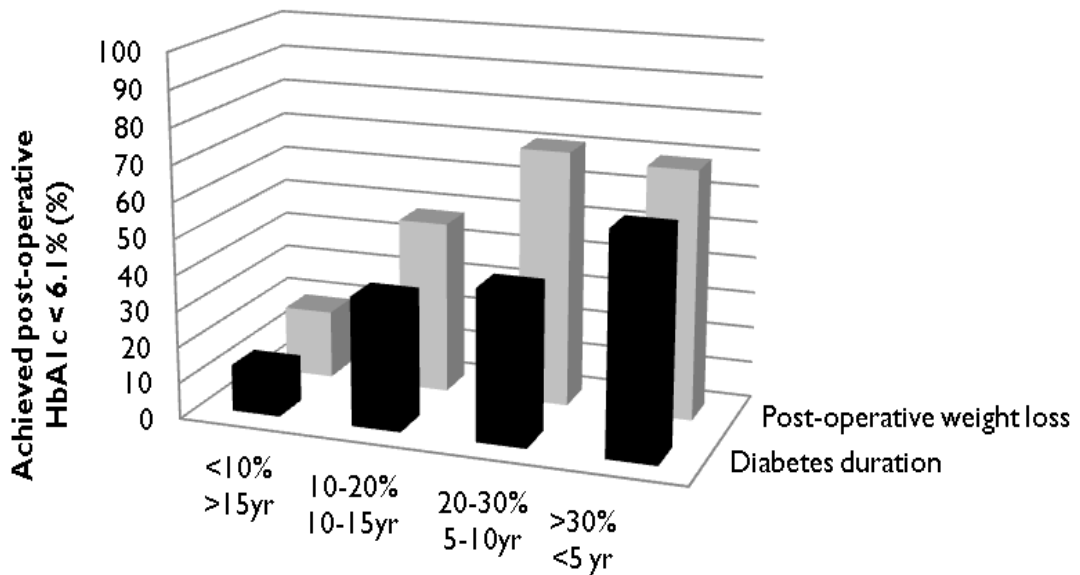
Figure 3-3 Reversal of diabetes according to duration of diabetes and degree of achieved post-operative weight loss following any bariatric procedure.

3.3.4 Predictors of achieved blood glucose control

Using regression analysis, it was found that maximum percentage achieved weight loss (ß=0.013, *p*<0.0001) and baseline HbA1c (ß=0.093, *p*=0.014) were significant predictors of achieved HbA1c. Diabetes duration (ß=0.028, *p*=0.118), age (ß=0.015, *p*=0.770) and initial BMI (ß=0.014, *p*=0.496) were not significant predictors.

3.4 Discussion

The study demonstrates that degree of achieved weight loss is the major determinant of reversal of diabetes and that in long duration diabetes, greater weight loss is required to achieve reversal. In this cohort, the longest duration of diabetes in which a post-operative HbA1c of <6.1% (43 mmol/mol) was achieved was 18 years. This individual was treated with insulin therapy pre-operatively with an HbA1c of 9.1% (76 mmol/mol) and achieved a post-operative HbA1c of 5% (31 mmol/mol) off insulin therapy after losing 21.4kg (17.4% of body weight) following RYGB. The results of the study challenge the widely held belief that as duration of type 2 diabetes increases, there is a steady, inexorable and irreversible decline in beta cell function. The data provide further evidence that it is the removal of fat that allows return of normal metabolic function.

A prospective study on reversibility of type 2 diabetes reported that remission of type 2 diabetes was seen in 68% following RYGB with a trend towards reduced remission rates as diabetes duration increased (Hall *et al.*, 2010). The higher remission rate in this study compared to the present study is likely to relate to two main differences: only participants undergoing RYGB were included and the definition of remission was achieving an HbA1c <6.5% (48 mmol/mol). More recently, diabetes remission was investigated in a group of 66 individuals with type 2 diabetes undergoing RYGB with a relatively low BMI at 30-35 kg/m² (Cohen *et al.*, 2012). In this study 88% of individuals achieved a post-operative HbA1c of <6.5% (48 mmol/mol) off anti-diabetic therapies, with the mean diabetes duration in those who achieved diabetes remission being 8.0±2.5 years. A recent retrospective study devised a scoring system to aid patient selection

for bariatric surgery incorporating pre-operative age, BMI, C-peptide and diabetes duration (Lee *et al.*, 2015). Similar to our study they concluded that weight loss has the dominant effect on type 2 diabetes remission.

The mechanisms determining the reversal of type 2 diabetes following bariatric surgery are becoming clear (Knop and Taylor, 2013). Calorie restriction alone can reverse type 2 diabetes (Lim *et al.*, 2011), and the acute negative calorie balance must be the primary driver for the early rapid improvement in glucose levels (Taylor, 2008). Studies directly comparing the change in glycaemic control following RYGB compared to calorie restriction have demonstrated no additive effect of surgery over calorie restriction suggesting that alterations in incretin hormones (the foregut or hindgut hypotheses) are not a primary mechanism (Isbell *et al.*, 2010; Lingvay *et al.*, 2013). In the first seven days of hypocaloric intake liver triglyceride content falls and hepatic insulin sensitivity increases (Guidone *et al.*, 2006). Although an improvement in peripheral insulin sensitivity may be expected with sustained weight loss, the majority of individuals remain clinically obese following surgery and the published data suggest that changes in beta cell function underlie the reversal of type 2 diabetes. The present study involves use of different surgical procedures. However, when the RYGB group are looked at separately (short duration: n=18; medium: n=22; long: n=17) the correlation between achieved HbA1c and percentage weight loss remains (Spearman rank -0.324; *p*=0.014) and mean post-operative weight loss was 31.1±2.7 kg in those who achieved a post-operative HbA1c of <6.1% vs. 23.6±2.3 kg in those who remained in the diabetic range. In this group, the mean duration of diabetes was 3.7±0.4 yr in those who achieved a post-operative HbA1c <6.1% (43 mmol/mol). A recent paper found no significant difference between RYGB and sleeve gastrectomy in terms of improvement in HbA1c (Schauer *et al.*, 2012). Overall, there is no convincing evidence for a specific effect of bypassing the duodenum separate from induction of negative calorie balance.

The recovery in beta cell function is likely to relate to removal of the burden of the toxic metabolites of saturated fatty acids with or without decrease in

beta cell apoptosis (Taylor, 2013). The demonstration that new beta cells can be developed from adult exocrine pancreatic ducts (Butler *et al.*, 2003) and the possibility that beta cell dedifferentiation is reversible (White *et al.*, 2013) suggests that recovery of insulin secretion in long duration diabetes is at least possible. Clinical observations suggest that beta cell function can recover and even hyperfunction; for example women who have had type 2 diabetes reversed by RYGB who do not develop gestational diabetes in subsequent pregnancies despite the increased insulin resistance associated with pregnancy (Steven *et al.*, 2011). Two studies have looked at insulin secretion in long-standing type 2 diabetes, using C-peptide response to glucagon (Zangeneh *et al.*, 2006) or fasting C-peptide measurements (Jain *et al.*, 2008). Both concluded that although a decline in beta cell function over time is characteristic, this is not inevitable and is highly variable between individuals. In our cohort, individuals who did not achieve a post-operative HbA1c of <6.1 % (43 mmol/mol) despite weight loss of more than 25 kg had a longer diabetes duration and were more likely to be on insulin or GLP-1 agonist therapies than those who did achieve a post-operative HbA1c of <6.1% (43 mmol/mol).

In the current study, there was a notable lack of normoglycaemia in individuals with long duration diabetes achieving <25 kg weight loss. Individuals with long duration disease are likely to have gained more weight since the time of diagnosis, partly due to the natural history of the disease but also compounded by the use of medications such as sulphonylureas and insulin (UKPDS, 1998). It is hypothesised that these individuals with long duration diabetes need to lose more weight than those with short duration disease in order to reach a weight at which beta cell function can return with normalisation of blood glucose levels. The negative correlation between duration of type 2 diabetes and beta cell function has previously been reported to be more apparent in obese individuals than in lean individuals (Funakoshi *et al.*, 2008; Saisho *et al.*, 2012).

Of note, one individual in the current study with diabetes duration of 9 years (

Figure **3-2**, panel c) demonstrated a marked deterioration in diabetes control post-operatively despite achieving 26.8kg (20.2%) weight loss 9 months after RYGB. Pre-operative HbA1c was 8.4% (68 mmol/mol) on metformin, gliclazide and pioglitazone therapies, and then 9 months post-operatively this was 12.3% (111 mmol/mol) on metformin and gliclazide. No specific cause for this paradoxical deterioration in control could be identified.

Limitations of the current study must be considered. The date of onset of diabetes is notoriously inaccurate, with the potential for a long period of unrecognised hyperglycaemia preceding a diagnosis. This would have the effect of underestimating diabetes duration, which if anything would increase the reversal rates in the long duration group. As fasting glucose levels were not available in this study the ADA Consensus criteria for defining diabetes remission (Buse *et al.*, 2009) were not used, although our criterion for defining diabetes reversal was consistent and stringently applied. In this study, as all individuals were reviewed as in-patients by the diabetes team, the risk of inclusion of individuals with alternate diagnoses including slow-onset type 1 diabetes and maturity onset diabetes of the young (MODY) has been minimised. Inclusion of such individuals would have the effect of reducing remission rates in general. As the type of bariatric procedure performed is decided by the surgeon, a possible confounder is that higher risk patients (such as those with poor diabetes control) were selected to have lower risk but less "effective" procedures. This could result in the association between lower achieved weight loss and less improvement in glucose control. However, the association between achieved weight loss and achieved HbA1c seen in the whole group was also evident when the RYGB cohort was examined separately.

This study demonstrates that degree of achieved weight loss is the major determinant of reversal of diabetes. Reversal to normoglycaemia can be achieved in long duration type 2 diabetes following bariatric surgery, but greater weight loss is required to achieve reversal compared to short

duration disease. A minority of individuals appear not to achieve diabetes reversal despite achieving significant weight loss even with short duration diabetes. The pathophysiologic limitations to complete reversal of type 2 diabetes require investigation.

Chapter 4. Comparison of the Effects of Bariatric Surgery on Glucose Metabolism in Type 2 Diabetes & Normal Glucose Tolerance

4.1 Introduction

Bariatric surgery is currently the most effective treatment for reversal of type 2 diabetes; a randomised trial of intensive medical therapy alone versus medical therapy plus bariatric surgery demonstrated an HbA1c <6% after 12 months in 12% of those medically treated compared to 42% of those having gastric bypass surgery (Schauer *et al.*, 2012). We do not fully understand the pathophysiologic mechanisms which define diabetes reversal, and this is crucial to understanding the aetiology of the disease and overcoming the limits to full reversibility. Major changes in incretin hormones occur after Roux-en-Y gastric bypass surgery (RYGB) which could potentially contribute to the observed increase in meal related insulin secretion (Guidone *et al.*, 2006). In type 2 diabetes, there is a diminished glucagon-like peptide-1 (GLP-1) response to food ingestion (Vilsbøll *et al.*, 2001), and some believe that RYGB exerts a specific effect in type 2 diabetes which differs from that in subjects with normal glucose tolerance. Some studies have supported this concept (Jorgensen *et al.*, 2013; Bojsen-Møller *et al.*, 2014; Manning *et al.*, 2015) although similar restoration of normoglycaemia has been observed after calorie restriction alone (Isbell *et al.*, 2010; Lingvay *et al.*, 2013). Previously, an 8 week very low calorie diet (VLCD) was used to test the hypothesis that acute negative calorie balance alone reverses type 2 diabetes by normalising both beta cell function and insulin sensitivity. In that study, over the first week of calorie restriction, hepatic insulin sensitivity normalised and hepatic triglyceride content decreased by 30% (Lim *et al.*, 2011). After 8 weeks of calorie restriction, first phase insulin secretion and pancreatic triglyceride content had normalised. These observations have confirmed some aspects of the twin cycle hypothesis of the aetiology of type 2 diabetes (Taylor, 2013).

The importance of hepatic triglyceride in the pathogenesis of type 2 diabetes is clear (Perry *et al.*, 2014). In contrast, it remains uncertain whether the change in pancreatic triglyceride seen during a VLCD is specific to diabetes itself, reflecting a disease related abnormality or whether it simply reflects decrease in whole body fat content that would occur during substantial

weight loss irrespective of the metabolic state. Human studies have yielded conflicting results; a negative association between pancreatic fat and insulin secretion in some (Tushuizen *et al.*, 2007; Heni *et al.*, 2010; Szczepaniak *et al.*, 2012); only a relationship with BMI not beta cell function in others (Saisho *et al.*, 2007; van der Zijl *et al.*, 2011); and another with age alone (Begovatz *et al.*, 2015). However, these studies have used a variety of imaging modalities, some of which are likely to have included visceral fat in the measurement of pancreatic fat. Also the use of heterogeneous populations in terms of glucose tolerance, including some subjects with impaired glucose tolerance and impaired fasting glycaemia who are likely to have increased insulin secretion, makes interpretation of the correlation with beta cell function challenging.

Investigation of intracellular pancreatic fat content from autopsy specimens has been limited by potential post-mortem pancreatic lipase activity. However, mechanistically, the importance of fat to beta cells is clear: *in vitro* studies have demonstrated that acute exposure of beta cells to fatty acids acts as a stimulus for insulin secretion, however, chronic exposure results in decreased glucose stimulated insulin secretion (Lee *et al.*, 1994). This seems to relate to endoplasmic reticulum stress, mitochondrial dysfunction and initiation of apoptosis (Morgan, 2009). Inhibition by excess intracellular fatty acids or their metabolites is a potential mechanism (Lee *et al.*, 1994; Lalloyer *et al.*, 2006; Diakogiannaki *et al.*, 2007). Currently available imaging techniques are unable to specifically measure triglyceride within beta cells, and it is likely that most pancreatic fat measured is present within adipocytes in exocrine tissue or in adipose tissue in the interlobular space. However, in animal models, islet fat content correlates with whole organ fat content (Lee *et al.*, 2009). Although the triglyceride measured is actually an inert storage form of fat, it may serve as a biomarker for the toxic metabolites of fat that are in the close environment of the beta cell.

Comparison of changes in pancreatic triglyceride in type 2 diabetes and normal glucose tolerance during acute weight loss could define those changes specific to the recovery of insulin secretory capacity. Achieving

equivalent dietary weight loss in normal glucose tolerant individuals who do not have the motivation of potentially reversing their diabetes to normal would be challenging. Bariatric surgery, specifically RYGB for obesity produces reliable weight loss and permits detailed comparison of the pathophysiologic changes in these two groups. The aim of this study was to compare groups of individuals with type 2 diabetes and normal glucose tolerance before and 8 weeks after RYGB; specifically the changes in pancreas triglyceride content, incretin-independent intravenous glucose mediated insulin secretion, and physiologic incretin secretion. Changes in hepatic triglyceride content and hepatic insulin sensitivity were also assessed.

4.2 Study Design

4.2.1 Participants

Individuals listed for laparoscopic RYGB were identified from two regional bariatric surgery centres. Individuals with type 2 diabetes (n=18) were recruited with diabetes duration <15 yr, aged 25-65 yr, BMI up to 45 kg/m^2 (due to scanner constraints), and no significant renal or hepatic dysfunction (creatinine <150 μmol/l; ALT <2.5-fold above upper limit of normal). Exclusion criteria were: contraindication to magnetic resonance scanning; alcohol consumption >14 units/wk; previous bowel surgery; or treatment with steroids. At the time of study enrolment, 4 participants were taking metformin only, 6 participants were treated with metformin and a sulphonylurea, 2 participants were on metformin and insulin, 1 participant was taking metformin, a sulphonylurea and a GLP-1 agonist, 1 metformin, sulphonylurea and a DPP-4 inhibitor, 1 metformin and GLP-1 agonist, 1 metformin, sulphonylurea and a thiazolidinedione, 1 metformin and a thiazolidinedione, 1 metformin, GLP-1 agonist and insulin. Participants were asked to discontinue DPP-4 and thiazolidinediones at least 1 month prior to starting the study and GLP-1 agonists 2 weeks prior. Metformin and sulphonylureas were stopped 72 hours prior to studies. No insulin was given within 24 hours of the first study. Statin therapy was continued throughout the study. The type 2 diabetes group (n=18) consisted of 11

females and 7 males, aged 49.1±7.0 years with diabetes duration 6.9±1.0 years. A group with normal glucose tolerance and no family history of type 2 diabetes in a first degree relative (n=9) was also studied, and were similar in age and weight. Glucose tolerance was confirmed by a standard 75g oral glucose tolerance test (section 2.3.2). This group consisted of 7 females and 2 males, aged 46.3±2.1 years. The groups were recruited by invite letter from the surgical team and participants understood that they were free to withdraw from the study at any point. The study protocol was approved by the Newcastle upon Tyne 1 Research Ethics Committee. All participants provided written informed consent. One individual in the normal glucose tolerance group did not undergo surgery following baseline studies due to the diagnosis of an unrelated medical problem.

4.2.2 Experimental protocol

All participants were studied just before surgery and at 8 weeks post-operatively. Pre-operatively all participants were on a liver reduction diet, as advised by their surgeon, based on low dietary fat and carbohydrate intake, with an approximate overall energy intake of 1200 kcal/day for 7-10 days. At both time points, fasting anthropometry and assessments of first phase insulin secretion, pancreatic and hepatic triglyceride content, insulin sensitivity and incretin response to a semi-solid meal test were made. All metabolic studies were performed after an overnight fast. Individuals were asked to avoid intensive physical activity or unusual alcohol or caffeine intake in the 48 hours prior to each study and to avoid nicotine intake on the day of studies. Studies took place over one full day (assessments of insulin sensitivity and insulin secretion) and one half day (assessment of body composition and meal test). On the morning of the first day, a cannula was inserted into an antecubital vein for infusions and a second cannula inserted into the contralateral wrist for blood sampling. A 4.5 hr isoglycaemic hyperinsulinaemic clamp study was performed with stable isotope dilution technique to assess basal hepatic glucose production and hepatic and peripheral insulin sensitivity (sections 2.3.3 and 2.3.4). Following the clamp study, participants rested for a minimum of 60 min (or

until steady state fasting glucose levels were achieved) before undergoing the stepped insulin secretion test with arginine to assess first phase insulin response and maximal insulin secretory capacity (section 2.3.5). The following morning, participants underwent measurement of body composition using electrical bioimpedence (Bodystat®1500; Bodystat Ltd., Isle of Man, UK) and then had hepatic and pancreatic triglyceride content (section 2.2.2) and visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) areas (section 2.2.3) measured using the three point Dixon magnetic resonance method on a 3 Tesla Achieva scanner (Philips; Best, The Netherlands). Waist and hip circumferences were measured using a standard non-distensible tape measure, taken with the participants standing and in a relaxed posture by one observer (SS). Following this the 2 hour semi-solid meal test was performed with the participant semi-reclined at a 45° angle in bed to avoid positional change affecting gastric emptying (section 2.3.6). Baseline blood samples were taken at -10 and 0 min (glucose, insulin, C-peptide, glucagon, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)). Participants were then asked to consume a semi-solid meal within 3 minutes (10g Mornflake Instant Porridge Oats, 64g whole milk and 6g acacia honey: 100 kcal; 57% carbohydrate; 28% fat; 13% protein), designed in accordance with the expected volume and consistency of diet consumed in the initial post-RYGB phase. Whole blood samples were collected for PNPLA3 genotyping (section 2.3.7).

4.2.3 Surgery

RYGB was performed laparoscopically in all patients. A gastric pouch (30-50ml) was fashioned on the lesser curve of stomach using an ETS 45 mm linear stapler firing blue (6R45B) cartridges (Ethicon Endosurgery; Cincinnati, OH). A biliopancreatic limb of 50-70cm from the duodenojejunal flexure was anastomosed to the gastric pouch. An alimentary limb of 100-150cm was then measured and a side to side antimesenteric jejuno-jejunostomy carried out. The roux construction was completed by dividing the omega loop close to the gastrojejunostomy. At the time of operation, 2

participants with type 2 diabetes underwent sleeve gastrectomy instead of RYGB due to the presence of significant intra-abdominal adhesions. The data from these 2 participants have been excluded from the incretin analyses.

4.2.4 Statistical analysis

Data are presented as mean ± SEM for parametric and median (range) for non-parametric data. Data was tested for normality using Kolmogorov Smirnov test. Insulin secretion rates are given as median with 25th and 75th percentile. Comparisons between the type 2 diabetes group and the normal glucose tolerance group were made using Student's unpaired t-test or Mann Whitney U test. Within group differences between baseline and week 8 were determined using Student's paired t-test or Wilcoxon Rank. Correlations were examined using Pearson or Spearman rank test. Minitab 16 statistical program was used (Minitab Inc.; State College, PA: www.minitab.com). Statistical significance was accepted at $p<0.05$.

4.3 Results

4.3.1 Weight loss and body composition

Pre-operative weight did not differ between the type 2 diabetes and normal glucose tolerance groups (121.1±3.0 vs. 114.5±5.0 kg; p=0.244). There was no difference in VAT or SAT areas between the two groups at baseline (Table 4-1). The VAT:SAT ratio at baseline was higher in the diabetes group but this did not reach statistical significance (p=0.084). At 8 weeks post-operatively weight loss was similar in the two groups (13.6±0.7 and 12.8±0.8 % respectively; p=0.286) as was change in total body fat content (Table 4-1). There was a significant decrease in SAT and VAT areas in both groups after surgery. The VAT:SAT ratio did not change in either group after surgery. The waist:hip ratio was lower in individuals with normal glucose tolerance at baseline and after surgery.

	T2DM Baseline	T2DM After surgery	p	NGT Baseline	NGT After Surgery	p
Weight (kg)	121.1±3.0	104.5±2.7	<0.001	114.5±5.0	99.7±4.6	<0.001
BMI (kg/m²)	42.7±0.7	36.9±0.7	<0.001	41.3±1.0	36.4±0.8	<0.001
Fat mass (kg)	56.6±2.4	43.0±2.4	<0.001	56.7±3.3	45.4±2.3	<0.001
Waist:Hip ratio	0.97±0.02	0.94±0.02	0.006	0.90±0.03 *	0.87±0.03 #	0.066
VAT area (cm²)	300.4±17.5	241.3±11.0	<0.001	244.5±28.4	187.9±28.3	0.010
SAT area (cm²)	453.8±28.9	393.2±26.8	<0.001	496.4±16.0	409.7±26.0	0.016
VAT:SAT ratio	0.74±0.09	0.70±0.09	0.213	0.50±0.07	0.47±0.07	0.159
Plasma glucose (mmol/l)	9.4±0.8	6.4±0.4	<0.001	5.2±0.2 *	4.9±0.1 #	0.089
Serum insulin (mU/l)	15.3 (4.3-61.2)	11.3 (2.9-27.0)	<0.001	11.0±1.6	6.7±0.7 #	0.008
ALT (U/l)	37.7±4.1	25.7±2.4	0.009	24.3±2.8 *	22.1±3.7	0.542
GGT (U/l)	33 (13-148)	15 (7-69)	0.002	23 (8-39)	11 (5-122)	0.234
NEFA (mmol/l)	0.85±0.08	0.77±0.05	0.207	0.72±0.09	0.80±0.07	0.263
Glucagon (ng/l)	74.6±9.8	58.0±8.3	0.001	49.4±5.0	48.1±5.0	0.865
Triglycerides (mmol/l)	1.5 (0.6-3.7)	1.1 (0.5-2.2)	0.011	1.2±0.2	1.1±0.2	0.156

Table 4-1 Anthropometric and fasting metabolic data before and at 8 weeks post-operatively in the type 2 diabetes (T2DM) and normal glucose tolerance (NGT) groups. * indicates a statistically significant difference between the 2 groups at baseline. # indicates a statistically significant difference between the 2 groups at week 8.

4.3.2 Plasma glucose, insulin, glucagon and metabolites

Fasting plasma glucose decreased from 9.4±0.8 to 6.4±0.4 mmol/l in the diabetes group ($p<0.001$) and from 5.2±0.2 mmol/l to 4.9±0.1 mmol/l in the normal glucose tolerance group ($p=0.196$). HbA1c decreased from 7.6±0.4 % (59±4 mmol/mol) at baseline to 6.2±0.2 % (45±2 mmol/mol) at week 8 in those with diabetes ($p<0.001$) compared to 5.4±0.1 % (35±1 mmol/mol) to 5.2±0.1 % (34±1 mmol/mol) in those without ($p=0.010$). Fasting insulin levels fell in both groups (T2DM: 15.3 (4.3-61.2) to 11.3 (2.9-27.0) mU/l ($p<0.001$); NGT: 10.7±1.4 to 6.7±0.7 mU/l ($p=0.008$). There were significant decreases in fasting plasma glucagon, triglyceride, ALT and GGT in the diabetes group following surgery but not the normal glucose tolerance group (Table 4-1). There was no significant change in fasting NEFA concentration after surgery in either group.

4.3.3 Pancreatic triglyceride content

Pancreatic triglyceride content was higher at baseline in those with type 2 diabetes compared to normal glucose tolerance (6.6±0.5 vs. 5.1±0.2 %; $p=0.009$). After surgery, pancreatic triglyceride content had decreased in the diabetes group to levels similar to the normal glucose tolerance group (6.6±0.5 to 5.4±0.4 %; $p=0.007$). Pancreatic triglyceride content did not change after surgery in those without diabetes (5.1±0.2 to 5.5±0.4 %; $p=0.437$) (Figure 4-1) despite a comparable decrease in whole body fat mass (Table 4-1).
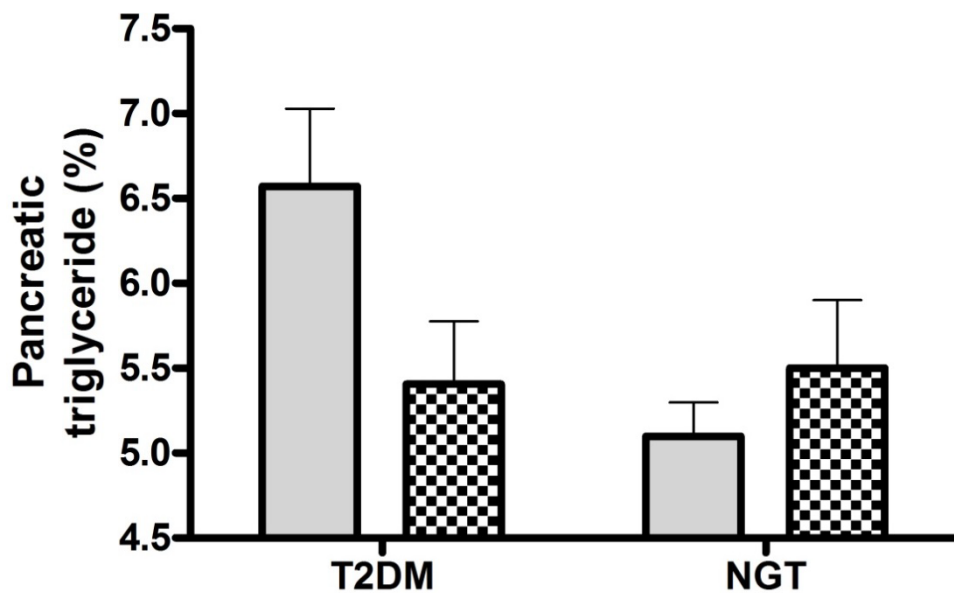
Figure 4-1 Change in pancreatic triglyceride content (shown as mean and SEM) in the diabetes (T2DM) and normal glucose tolerance (NGT) groups at baseline (grey) and then at 8 post-operative weeks (chequered).

4.3.4 Change in insulin secretion

The planned stepped increases in plasma glucose during the insulin secretion test were achieved (Figure 4-2A). At baseline, the first phase insulin response (baseline to 6 min insulin secretion rate) in the diabetes group was severely impaired compared to those with normal glucose tolerance (0.08 (-0.01-0.10) vs. 0.24 (0.13-0.46) nmol min^{-1} m^{-2}; $p=0.011$; Figure 4-2B). There was marked restoration of the first phase insulin response in those with diabetes after surgery: increasing to 0.22 (0.07-0.30) nmol min^{-1} m^{-2}; $p=0.005$; Figure 4-2C). There was no change in first phase insulin response in those with normal glucose tolerance: 0.24 (0.13-0.46) at baseline and 0.23 (0.19-0.37) nmol min^{-1} m^{-2} after surgery ($p=0.464$; Figure 4-2B&C). The first phase insulin response was not significantly different in the diabetes group compared to the normal glucose tolerance group at week 8 ($p=0.453$). Arginine induced maximal insulin secretory capacity (baseline to peak insulin secretion rate) did not change after surgery in the diabetes group (0.68 (0.61-0.84) at baseline to 0.64 (0.48-1.11) nmol min^{-1} m^{-2} after surgery; $p=0.663$) or in the normal glucose tolerance group (0.96 (0.81-1.16) and 0.70 (0.63-1.25) nmol min^{-1} m^{-2} respectively; $p=0.834$).
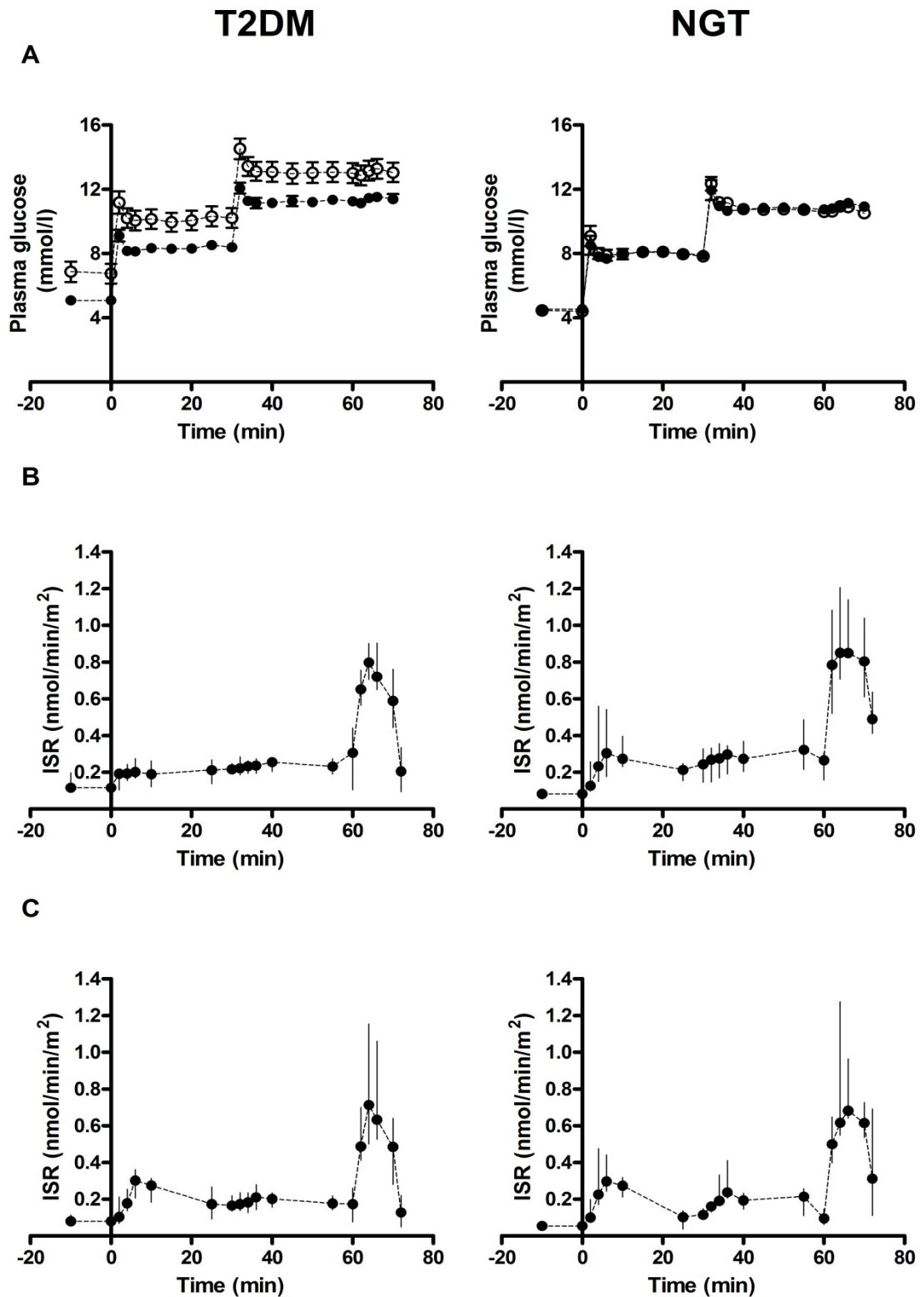
Figure 4-2 Stepped insulin secretion test with arginine, showing (A) the induced change in plasma glucose at baseline (open circles) and week 8 (closed circles), (B) baseline insulin secretion rates (median and 25th-75th percentile), and (C) 8 week post-operative insulin secretion rates (median and 25th-75th percentile) for the diabetes group (T2DM) and the normal glucose tolerance group (NGT).

4.3.5 Hepatic triglyceride content and hepatic enzymes

At baseline, hepatic triglyceride content was over 2-fold higher in those with diabetes compared to the normal glucose tolerance group (9.3±1.5 vs. 4.2±1.4 %; p=0.022) (Figure 4-3A). After surgery, hepatic triglyceride content decreased to a greater extent in those with diabetes (9.3±1.5 to 5.2±0.8 %; p=0.018) compared to those with normal glucose tolerance (4.2±1.4 to 2.3±0.6 %; p=0.059). These changes were reflected in the fall in serum ALT and GGT following surgery in the diabetes group only (Table 4-1). There was marked variability in baseline hepatic triglyceride content in both groups, but particularly those with diabetes (Figure 4-3B & C). There was no correlation between baseline hepatic triglyceride and pancreatic triglyceride contents in those with diabetes (Pearson=-0.096; p=0.714) or those with normal glucose tolerance (Pearson=-0.320; p=0.484). Likewise there was no correlation between the change in hepatic and pancreatic triglyceride contents in those with diabetes (Pearson=-0.224; p=0.387) or without (Pearson=-0.504; p=0.249).
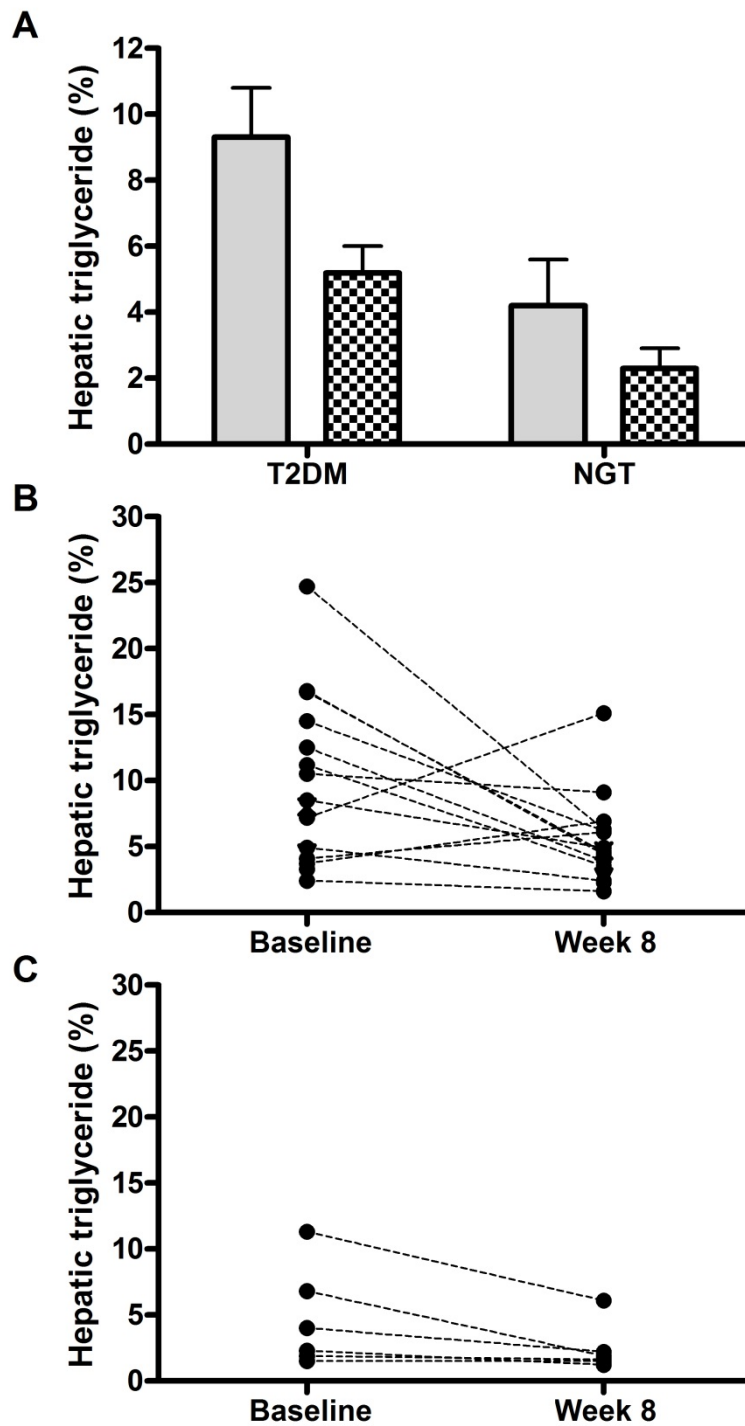
Figure 4-3 Effect of weight loss on change in hepatic triglyceride content in the diabetes (T2DM) and normal glucose tolerance (NGT) groups at baseline (grey) and at 8 post-operative weeks (chequered) (A). The change in individual hepatic triglyceride content between baseline and 8 post-operative weeks in participants with type 2 diabetes (B) and normal glucose tolerance (C).

4.3.6 Hepatic insulin sensitivity

Basal hepatic glucose production in the diabetes group decreased after surgery (3.60 ± 0.24 to 2.69 ± 0.12 mg/kg$_{ffm}$/min; $p<0.001$). There was no significant change in those with normal glucose tolerance (2.60 ± 0.08 to 2.51 ± 0.20 mg/kg$_{ffm}$/min; $p=0.555$). Hepatic insulin sensitivity improved in both groups but the change was only significant in those with diabetes: hepatic IR index 2.76 ± 0.41 to 1.33 ± 0.23 mmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} ($p=0.002$); normal glucose tolerance group: 1.18 ± 0.19 to 0.70 ± 0.07 mmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} ($p=0.062$). The insulin induced suppression of hepatic glucose production was greater in the type 2 diabetes group at 8 post-operative weeks: 67 ± 4 to 85 ± 3 %; $p<0.001$, with no change in the normal glucose tolerance group: 84 ± 4 to 77 ± 8 %; $p=0.339$.

4.3.7 Peripheral tissue insulin sensitivity

Insulin stimulated glucose disposal decreased in the type 2 diabetes group after surgery (3.23 ± 0.34 to 2.51 ± 0.32 mg/kg$_{ffm}$/min; $p=0.020$) and was unchanged in those with normal glucose tolerance (3.47 ± 0.50 to 3.58 ± 0.52 mg/kg$_{ffm}$/min; $p=0.784$). Insulin stimulated glucose metabolic clearance rates (to correct for the difference between clamp glucose levels between studies) did not change in either group: 2.46 (0.86-8.80) to 2.69 (0.45-10.07) ml/kg$_{ffm}$/min in the diabetes group ($p=0.223$) and 4.51 ± 0.63 to 4.79 ± 0.70 ml/kg$_{ffm}$/min in those with normal glucose tolerance ($p=0.572$). Both before surgery ($p=0.033$) and after surgery ($p=0.024$) peripheral insulin sensitivity was significantly lower in the diabetes group.

4.3.8 Change in meal tolerance test

The rise in plasma glucose over the first 20 min of the meal test was greater in both the diabetes and normal glucose tolerance groups following surgery (0.6 ± 0.1 at baseline to 1.8 ± 0.1 mmol/l post-operatively; $p<0.001$ and 0.5 ± 0.1 to 1.7 ± 0.2 mmol/l; $p=0.004$, respectively) (

Figure 4-4A). The positive incremental area under the curve for glucose increased after surgery only in the diabetes group (Table 4-2). 2 hour post-meal glucose was lower following surgery in both groups: 9.4 ± 0.8 to 6.4 ± 0.3

mmol/l in the diabetes group ($p<0.001$) and 5.5±0.2 to 5.0±0.0mmol/l in the normal glucose tolerance group ($p=0.022$).

The incremental rise in plasma insulin over the first 20 minutes increased in both groups after surgery, with a higher and earlier peak plasma insulin being achieved. In the diabetes group the peak insulin at baseline was 35.2±4.9 mU/l achieved at 60 (10-120) min compared to 47.7±5.8 mU/l at 20 (10-30) min post-operatively; $p=0.010$. In those with normal glucose tolerance the peak insulin at baseline was 37.0±5.0 mU/l achieved at 60 (30-120) min compared to 58.1±10.0 mU/l at 20 (20-30) min 8 weeks post-operatively; $p=0.032$ (

Figure **4-4**B). The positive area under the curve for insulin increased in both groups after surgery but this was only significant in the diabetes group (Table 4-2).

Peak GLP-1 levels during the meal test increased from 5.0±0.3 at baseline to 12.7±1.3 pmol/l after surgery in the type 2 diabetes group ($p<0.001$) and from 5.1±0.6 to 12.9±1.2 pmol/l in the normal glucose tolerance group respectively ($p=0.001$) (

Figure **4-4**C). There was a five-fold increase in positive incremental area under the curve (iAUC) of GLP-1 following surgery in those with diabetes and four-fold increase in those with normal glucose tolerance (Table 4-2). Peak GIP levels increased from 197.6±18.1 to 246.2±24.4 pg/ml in the type 2 diabetes group (p=0.051) but did not change in those with normal glucose tolerance: 206.7±28.6 to 231.2±32.6 pg/ml ($p=0.482$). There was an earlier rise in GIP in both groups post-operatively (

Figure **4-4**D). There was no significant change in positive iAUC of GIP following surgery in either group (Table 4-2).
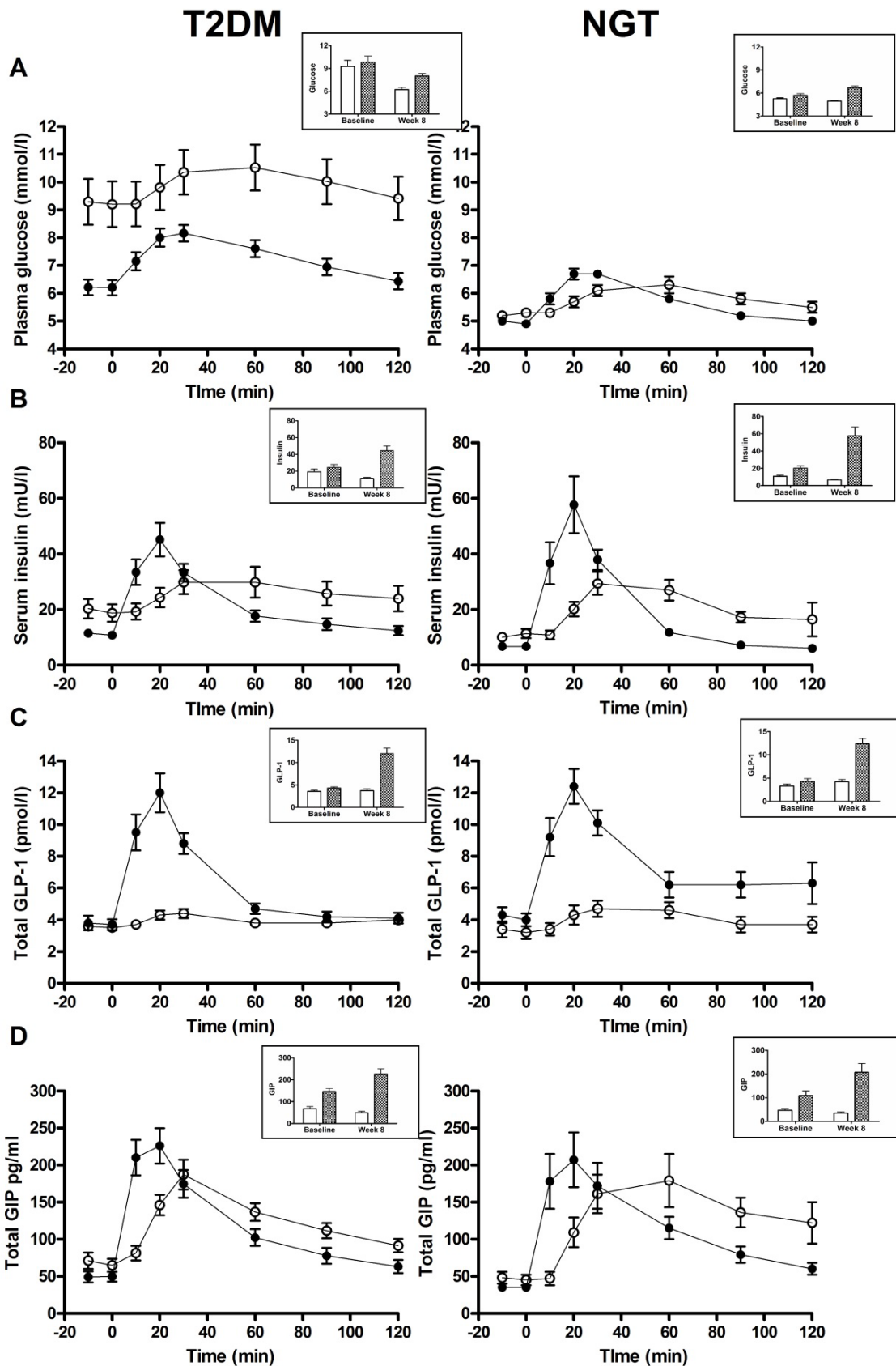
Figure 4-4 Glucose (A), insulin (B), total GLP-1 (C) and total GIP (D) levels (mean ± SEM) during the 2 hour meal test in the diabetes (T2DM) and normal glucose tolerance (NGT) groups at baseline (open circles) and week 8 (closed circles). Inset graph displays fasting (open bars) and 20 min (hatched bars) levels at baseline compared to week 8.

	Type 2 Diabetes			Normal Glucose Tolerance		
	Baseline	**Week 8**	***p***	**Baseline**	**Week 8**	***p***
Glucose (mmol.l^{-1}.min)	96.2±12.3	132.8±12.2	0.002	72.7±12.7	95.6±9.3	0.167
Insulin (mU.l^{-1}.min)	667.1 (22.5-3705.1)	1310.2 (500.9-2806.0)	0.005	1183.8±103.7	1578.1±172.9	0.082
GLP-1 (pmol.l^{-1}.min)	62.6±12.7	324.1±43.4	<0.001	101.6±28.1	409.1±64.2	<0.001
GIP (pg.ml^{-1}.min)	7136.5±749.2	8444.1±1042.5	0.265	10417.2±1544.1	10489.2±1656.3	0.972

Table 4-2 Positive incremental area under the curve during the semi-solid meal test at baseline then at 8 post-operative weeks in individuals with type 2 diabetes and normal glucose tolerance.

4.3.9 PNPLA3

In the whole group (n=26), the rs738409 C to G adiponutrin/PNPLA3
genotype (coding for I148M) was found in 9 individuals: 8 were heterozygous
for the SNP: CG (148I/M) and 1 homozygous: GG (148M/M), 6 of whom had
type 2 diabetes and 3 of whom were glucose tolerant (

Figure 4-5). In the diabetes group, mean baseline hepatic triglyceride
content was 8.7±1.9 % in CC wild type compared to 10.6±2.3 % (p=0.590) in
those a mutation (CG/GG). At 8 weeks post-operatively this was 3.9±0.5 and
8.2±1.9 % respectively (p=0.006). The GG homozygous individual with type 2
diabetes had a hepatic triglyceride content of 4.1% at baseline and 6.1%
post-operatively.
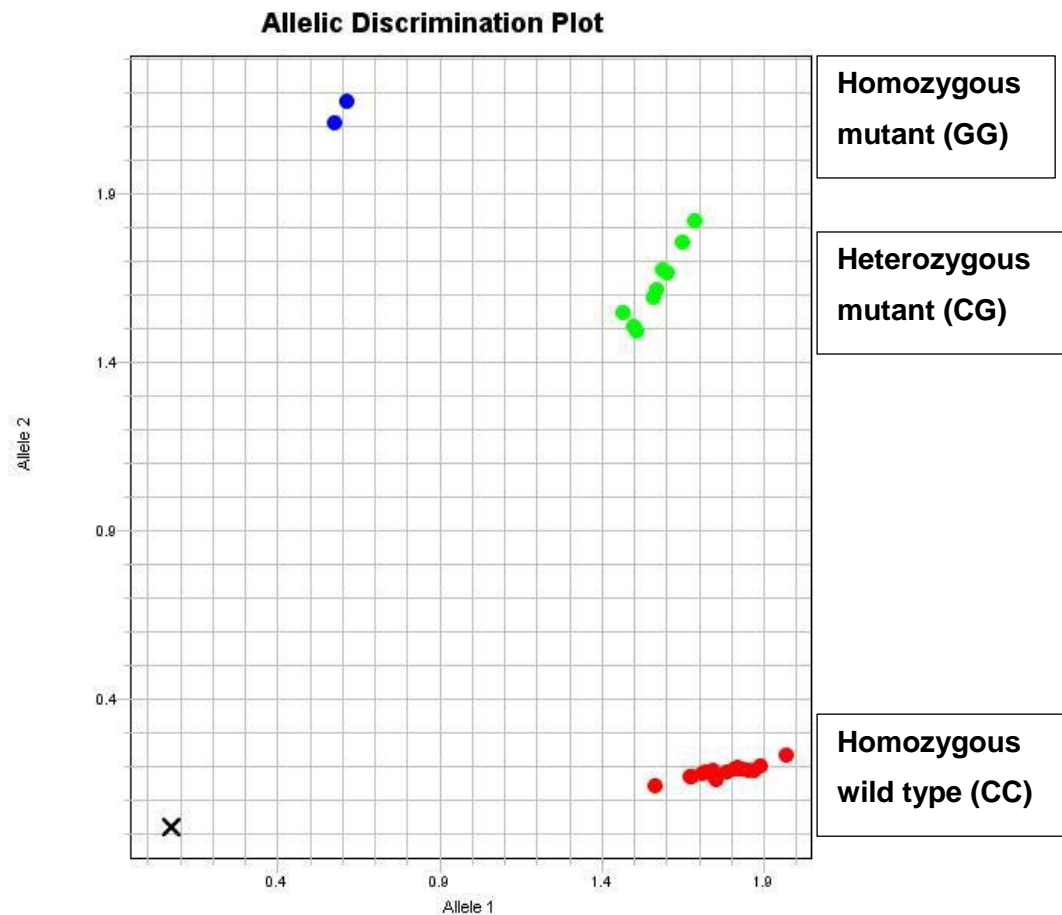
Figure 4-5 Allelic discrimination plot from Taqman using RT-PCR.
Controls were blank (cross), one homozygous mutant and one heterozygous
mutant.

4.4 Discussion

Despite similar achieved weight loss following bariatric surgery in groups of well-matched individuals with type 2 diabetes or normal glucose tolerance, pancreatic triglyceride content decreased uniquely in those with diabetes. This was associated with normalisation of first phase insulin secretion in the diabetes group. There was no change in pancreatic triglyceride in the normal glucose tolerance group despite a 5 unit decrease in BMI. Hepatic insulin sensitivity both fasting and during insulin stimulation normalised in those with diabetes in step with a greater decrease in liver triglyceride compared to those with normal glucose tolerance. The meal-related rise in glucose and insulin was faster in both groups after surgery and there was a similarly enhanced GLP-1 response.

Type 2 diabetes develops as a consequence of positive calorie balance over many years and ectopic fat storage appears to be central to the disease process (Taylor, 2013). The importance of pancreatic triglyceride in the pathogenesis of the disease was initially demonstrated by a study in obese rodents (Lee *et al.*, 2009). In humans significantly greater pancreatic triglyceride content in individuals with type 2 diabetes compared to glucose tolerant individuals has been demonstrated (Tushuizen *et al.*, 2007). Histological studies show no adipose tissue expansion within the limiting membrane of the pancreas (Saisho *et al.*, 2008) and exposure to even modest concentrations of fatty acids causes marked triglyceride accumulation in human islets *in vitro* (Lalloyer *et al.*, 2006). The change in pancreas fat content demonstrated in type 2 diabetes is small in absolute terms (1.2% in 56ml pancreas volume, or less than 700mg) and consistent with change in intracellular triglyceride content. The difficulty in demonstrating these modest differences in cross sectional studies has been identified and ethnic differences in pancreatic triglyceride content may exist (van der Zijl *et al.*, 2011; Szczepaniak *et al.*, 2012). Some of the variability in results may come from the variety of methods used to quantify pancreatic triglyceride content. Using the 3 point Dixon method to measure pancreatic triglyceride should overcome some of the disadvantages of using MR spectroscopy by using the image post-acquisition to guide definition of the region of interest. However

the technique must be carefully applied to avoid serious errors such as deriving negative values for percentage triglyceride (Begovatz *et al.*, 2015; Hollingsworth *et al.*, 2015).

In this study we have demonstrated that the increased pancreatic triglyceride is specifically decreased in type 2 diabetes after bariatric surgery whereas no change in pancreatic triglyceride occurred despite equivalent weight loss in those with normal glucose tolerance. The relative changes shown in Figure 4-1 are striking. After surgery there was a 15% decrease in pancreatic triglyceride content such that the 8 week post-operative levels in the diabetes group were the same as those with normal glucose tolerance, demonstrating that the increase in the fat content of the pancreas is specific to the condition rather than being a reflection of obesity *per se*. These data are in keeping with a previous study which demonstrated reduction in pancreatic fat content, as measured by magnetic resonance spectroscopy at 6 months after bariatric surgery and also dissociation of the reduction in hepatic and pancreatic triglyceride contents with weight loss (Gaborit *et al.*, 2015). This post-surgery decrease in pancreatic triglyceride content occurred at the same time as the recovery in first phase insulin secretion, similar to that previously observed following an 8 week very low calorie diet (Lim *et al.*, 2011). It is likely that in type 2 diabetes local lipolysis of pancreatic triglyceride brings about interstitial and intracellular concentrations of fatty acids sufficient to inhibit beta cell function. Recently it has been shown that fatty acid receptors are expressed in mouse and human pancreatic beta cells and when knocked out allow recovery of insulin secretion (Tang *et al.*, 2015).

Fasting plasma glucose concentration is determined by the rate of hepatic glucose production (Ravikumar *et al.*, 2008) which in turn is controlled by insulin (Prager *et al.*, 1986). Hepatic insulin sensitivity is known to be impaired by increased hepatic triglyceride content (Seppala-Lindroos *et al.*, 2002; Gastaldelli *et al.*, 2007). Short term carbohydrate overfeeding can induce hepatic triglyceride accumulation (Sevastianova *et al.*, 2012) and furthermore, weight loss with consequent reduction in liver fat by dietary

means or medication is associated with improvements in insulin sensitivity and fasting plasma glucose levels (Petersen *et al.*, 2005; Ravikumar *et al.*, 2008). The present study demonstrates a greater reduction in hepatic triglyceride content post-surgery in individuals with type 2 diabetes compared to normal glucose tolerance. Prior to surgery, hepatic triglyceride in those with diabetes was higher and hepatic insulin sensitivity lower compared with the glucose tolerant, and the post-surgery decrease in hepatic triglyceride content from 9.3 to 5.2% was associated with normalisation of hepatic insulin sensitivity. The toxic burden of fat within the hepatocytes is represented by the elevated hepatic enzymes (ALT and GGT) in the diabetes group and these normalise alongside the reduction in hepatic triglyceride content following surgery. In participants with normal glucose tolerance, hepatic glucose production was normal and the observed decrease in hepatic triglyceride from 4.2 to 2.3% may be assumed to be below the threshold for inhibition of insulin sensitivity by fat. A recent study reported similar reductions in endogenous glucose production in diabetes and glucose tolerant groups after bariatric surgery, but the latter group was heavier and also had higher hepatic triglyceride pre-operatively (Immonen *et al.*, 2014).

The range of baseline hepatic triglyceride in both groups is likely to reflect individual differences in susceptibility to the adverse metabolic effects of hepatic fat. Data from the UKPDS on individuals with normal BMI supports the concept of a variable personal fat threshold of susceptibility (Taylor and Holman, 2015). As one specific and known underlying factor, it appeared in the current study that the PNPLA3 polymorphism blunted the weight-loss associated decrease in hepatic triglyceride content. The G allele is thought to code for a lipase that is ineffective at hydrolysing triglyceride. In individuals with the mutation, it may be that sequestration of triglyceride in the liver despite weight loss might actually prevent the deleterious effects of metabolites of triglyceride such as diacylglycerol on the insulin signalling pathway.

The markedly increased nutrient-stimulated secretion of GLP-1 after RYGB is well recognised, and the present observations confirm this. However, data are lacking to indicate that this is the major mechanism underlying the improved blood glucose control. In the present study we observe normalisation of the first phase insulin response to a controlled intravenous infusion of glucose, demonstrating that the improvement was independent of acute incretin stimulation. This is consistent with a recent study in individuals with type 2 diabetes at 3 months after RYGB which observed a marked (2.6-fold) increase of insulin secretion in response to intravenous glucose as reflected by disposition index (Bojsen-Møller *et al.*, 2014). One of the major changes after RYGB is that the usual function of the stomach to retain and process food is lost and nutrients pass rapidly into the mid-jejunum. The dramatic extent of this change is demonstrated by studies of acetaminophen absorption (Falkén *et al.*, 2011) and more rapid and complete absorption of simple sugars after RYGB has also been demonstrated (Salehi *et al.*, 2011). A recent study compared beta cell function following comparable weight loss achieved by VLCD or RYGB. Acute insulin secretion increased similarly in both groups despite a marked increase in GLP-1 in the RYGB group only (Jackness *et al.*, 2013). A specific GLP-1 receptor antagonist, Exendin 9-39, has been shown not to affect the change in post RYGB insulin secretion profile (Salehi *et al.*, 2011; Jimenez *et al.*, 2013). Most studies examining the incretin effect of RYGB have used an oral glucose challenge (Guidone *et al.*, 2006; Bojsen-Møller *et al.*, 2014) or a liquid mixed meal (Isbell *et al.*, 2010) although an oral glucose load passes immediately into the small intestine via the gastroenterostomy, producing both rapid absorption and a non-physiological incretin stimulus (Salehi *et al.*, 2011). The present study used a semi-solid meal to minimise the rapid entry of nutrients into the jejunum, but even so a more rapid rise in glucose levels was observed after surgery in both those with and without type 2 diabetes. The GIP peak after the test meal was earlier and greater in both groups post-RYGB. Overall, it appears that acute post-meal enhanced incretin secretion does not explain the improved beta cell function in type 2 diabetes following bariatric surgery. A possible longer term impact of the

augmented nutrient stimulated GLP-1 response upon beta cell function requires specific study.

The limitations of the study must be discussed. Use of gold standard, validated methods for measuring hepatic triglyceride, pancreatic triglyceride and detailed insulin secretion studies have allowed detailed comparisons of the early changes in glucose metabolism after surgery in response to experimental conditions designed to reflect usual dietary intake. The group sizes were sufficient to achieve clear statistical significance, and although smaller numbers of normal glucose tolerant individuals were studied the range of responses within this group was small. Although the diabetes group were unselected in terms of diabetes duration and treatments, this is representative of the truly heterogeneous population undergoing bariatric surgery. Although there may have been heterogeneity between individuals in the exact nature of the liver reduction diet and how stringently this was followed, a significant variation between those with type 2 diabetes or normal glucose tolerance would not be expected. We measured total rather than active GLP-1, although the responses of active and total GLP-1 are tightly correlated (Heijboer *et al.*, 2011).

In summary, this study has demonstrated that individuals with severe obesity and type 2 diabetes exhibit an attenuated first phase insulin response and increased pancreatic triglyceride compared to BMI-matched glucose tolerant individuals. 8 weeks after bariatric surgery both first phase insulin response and pancreatic triglyceride normalise uniquely in the diabetes group. GLP-1 response after a semi-solid meal improved equally in both groups. These observations support the concept of pancreatic triglyceride and the resulting metabolites being central to the aetiology of type 2 diabetes. Understanding that type 2 diabetes is a disease of fat accumulation above a personal threshold lays the foundation for more appropriate clinical management.

Chapter 5. Comparison of the Early Metabolic Changes Following RYGB vs. VLCD in Individuals with Type 2 Diabetes

5.1 Introduction

It has been recognised since the 1950s that the metabolic derangements in type 2 diabetes can be ameliorated by bariatric surgery (Friedman *et al.*, 1955; Buchwald *et al.*, 2004). The defects in beta cell function and insulin sensitivity are reversed after biliopancreatic diversion (Camastra *et al.*, 2007). The variable remission rates following different surgical procedures was initially thought to relate to the relative degrees of restriction to calorie intake and nutrient malabsorption induced by the procedure, and therefore ultimately determined by the degree of weight loss achieved (Buchwald *et al.*, 2004). However, two observations suggest that this is not the case. Firstly, there is disparity between the resolution rates of obesity and type 2 diabetes following surgery. Secondly, the timeframe of reversal of diabetes differs from that of weight loss. In a group of superobese women undergoing biliopancreatic diversion, it was found that the major improvement in metabolism occurred within one week of surgery (Guidone *et al.*, 2006). The observation that glycaemic control occurs prior to any significant weight loss prompted further hypotheses regarding the pathophysiological mechanisms behind the reversal of type 2 diabetes by bariatric surgery. The major changes in incretin hormone secretion which occur after Roux-en-Y gastric bypass surgery (RYGB) became widely believed to result in the observed increase in meal related insulin secretion which thereby normalises glucose levels (Laferrere *et al.*, 2008; Knop, 2009). The hindgut hypothesis postulated that rapid exposure of the distal small intestine to nutrients enhances the secretion of incretin hormones such as glucagon-like peptide-1 (GLP-1). GLP-1 is released from L-cells in the ileum when stimulated by glucose, lipids or protein and levels have been shown to increase substantially after RYGB (Roux *et al.*, 2006; Korner *et al.*, 2007; Laferrere *et al.*, 2007). The foregut hypothesis postulated that duodenal exclusion of nutrients suppresses secretion of anti-incretin hormones. Studies on the effect of gastric bypass on glucose-dependent insulinotropic polypeptide (GIP), secreted by K-cells in the duodenum, have shown inconsistent results (Rubino *et al.*, 2004; Laferrere *et al.*, 2008). Several observations suggest that changes in incretin hormone secretion are insufficient to adequately

explain the dramatic change in glucose metabolism which occurs early after gastric bypass surgery. Firstly, the lack of change in fasting GLP-1 secretion after surgery cannot explain the marked improvements in fasting glucose metabolism (Falkén *et al.*, 2011). Secondly, the supraphysiological levels of GLP-1 achieved in clinical practice using GLP-1 receptor agonists result in only a modest effect on glucose levels (DeFronzo *et al.*, 2005). Thirdly, although studies have shown that the use of GLP-1 receptor antagonists is associated with a blunting of insulin secretion after bariatric surgery, the antagonists do not negate the beneficial effect of surgery on the insulin secretion profile or glucose tolerance (Salehi *et al.*, 2011; Jiménez *et al.*, 2014).

An alternative hypothesis to explain the profound change in metabolism following bariatric surgery, is that it is primarily a result of obligate calorie restriction enforced by the surgery (Knop and Taylor, 2013). This would be consistent with the original observations on the Greenville bypass (Pories *et al.*, 1995). One individual, in whom the procedure was abandoned due to a full stomach at the time of surgery, followed the same post-operative dietary restrictions as those who had undergone the procedure and achieved the same dramatic improvement in blood glucose levels. It has now been demonstrated that 7 days of calorie restriction alone, using a very low calorie diet (VLCD), can normalise fasting glucose levels in individuals with short duration type 2 diabetes (Lim *et al.*, 2011). Alongside the normalisation of glucose levels, in the first 7 days of calorie restriction, there was a marked reduction in hepatic triglyceride content and a normalisation of hepatic insulin sensitivity. Over the 8 week VLCD there was restoration of first phase insulin secretion alongside a reduction in pancreatic triglyceride content. Several studies have attempted to directly compare the early metabolic effects of acute caloric restriction and bariatric surgery (Isbell *et al.*, 2010; Jackness *et al.*, 2013; Lingvay *et al.*, 2013; Lips *et al.*, 2014); however the use of indirect measurements of insulin secretion and insulin sensitivity, use of tests involving non-physiological incretin stimulus, and heterogenous populations in terms of beta cell function has meant that no consensus has been reached.

This study aimed to directly compare the immediate metabolic responses to very low calorie diet and gastric bypass surgery in individuals with type 2 diabetes. Specifically, it aimed to directly compare the impact of GLP-1 secretion following a semi-solid meal 7 days after bariatric surgery with that after 7 days of a VLCD. Hepatic insulin sensitivity, hepatic triglyceride content, insulin secretion (both first phase and in response to a mixed meal) and pancreatic triglyceride content were also quantified.

5.2 Study design

5.2.1 Participants

Individuals with type 2 diabetes listed for laparoscopic RYGB were identified from two regional bariatric surgery centres. Inclusion criteria were: diabetes duration <15 yr; aged 25-65 yr; BMI up to 45 kg/m²; and listed for laparoscopic gastric bypass surgery. Exclusion criteria were: contraindication to MR scanning; alcohol consumption >14 units per week; previous bowel surgery; steroid treatment, or significant renal or hepatic dysfunction (creatinine <150 μmol/l; ALT <2.5-fold above the upper limit of normal). The study protocol was approved by the Newcastle upon Tyne 1 Research Ethics Committee. The groups were recruited by invitation letter from the surgical team and participants understood that they were free to withdraw from the study at any point. All participants provided written informed consent. Participants were asked to discontinue DPP-4 and thiazolidinediones at least one month prior to starting the study and GLP-1 agonists at least 2 weeks prior. Metformin and sulphonylureas were stopped 72 hours prior to studies. No insulin was given within 24 hours of the first study. Participants were asked to avoid intensive physical activity and excess alcohol or caffeine intake in the 48 hours prior to each study. Participants were also asked to avoid nicotine on study days. All metabolic studies were performed after an overnight fast. The baseline participant characteristics and anti-diabetic medications at recruitment are shown in Table 5-1.

.

	Surgery (n=9)	*VLCD* (n=9)	*p value*
Gender	3M:6F	4M:5F	
Age (yr)	45.9±2.5	52.3±1.6	0.046
Weight (kg)	120.8±5.0	121.2±3.7	0.946
BMI (kg/m²)	43.0±1.1	42.3±0.9	0.604
HbA1c (mmol/mol)	53 (38-92)	58 (40-103)	0.427
Diabetes duration (yr)	7.1±1.4	6.7±1.4	0.828
Diabetes medications:			
MF only	2	2	
MF + SU	4	2	
MF + Insulin	1	1	
MF + GLP-1	1	0	
MF + TZD	1	0	
MF + SU + GLP-1	0	1	
MF + SU + TZD	0	1	
MF + SU + DPP-4	0	1	
MF + Insulin + GLP-1	0	1	

Table 5-1 Baseline characteristics of the surgery and VLCD groups

5.2.2 Experimental protocol

At recruitment, participants were randomised using online software (www.sealedenvelope.com) into two groups: Surgery or VLCD. Participants randomised to VLCD were seen prior to the first study with spouse as appropriate to discuss the dietary requirements in detail. All participants were asked to follow their surgical pre-operative liver reduction diet plans up to the first study point, based on a low fat and low carbohydrate diet of approximately 1200 kcal per day, thus all were in modest negative calorie balance at the time of the first study. Both groups had assessments at baseline and then after 7 days of intervention; Surgery group (studied before and then 7 days after surgery) or VLCD group (studied before and after a 7 day VLCD, just prior to surgery). The post-operative diet was as advised by

the surgical team: liquids on day 1, then a semi-solid diet for the rest of the first post-operative week. At each study time point, a semi-solid meal test was used to assess metabolic and incretin responses. Hepatic and peripheral insulin sensitivity were also quantified using an isoglycaemic hyperinsulinaemic clamp with tracer studies, first phase and maximal insulin secretory capacity were measured using a stepped insulin secretion test, and pancreatic and hepatic triglyceride content were quantified by magnetic resonance imaging. Studies took place over 1 full day (assessment of insulin sensitivity and insulin secretion) and one half day (assessment of body composition and meal test). On the morning of the first day, after an overnight fast, a cannula was inserted into an antecubital vein for infusions and a second cannula was inserted into the contralateral wrist vein for blood sampling. An isoglycaemic clamp study was performed (sections 2.3.3 & 2.3.4); isoglycaemia was chosen over euglycaemia due to the anticipated change in fasting glucose levels over the two study time points, and as it was intended to study individuals in their current metabolic state. Following the clamp study, participants rested for at least 60 minutes, or until stable fasting glycaemia had resumed. The stepped insulin secretion test with arginine was then performed (section 2.3.5). On day 2, after an overnight fast, participants underwent assessment of body composition using electrical bioimpedence (section 2.1.3), had an MR scan using the 3 point Dixon technique (section 2.2.2) and then had a cannulae inserted into an antecubital fossa for blood sampling during the incretin meal test (section 2.3.6).

5.2.3 Surgery

RYGB was performed laparoscopically in all patients. A gastric pouch (30-50 ml) was fashioned on the lesser curve of stomach using an ETS 45 mm linear stapler firing blue (6R45B) cartridges (Ethicon Endosurgery; Cincinnati, OH). A biliopancreatic limb of 50-70 cm from the duodenojejunal flexure was anastomosed to the gastric pouch. An alimentary limb of 100-150 cm was then measured and a side to side antimesentericjejuno-jejunostomy carried out. The roux construction was completed by dividing

the omega loop close to the gastrojejunostomy. At the time of operation, 2 patients with type 2 diabetes underwent sleeve gastrectomy instead of RYGB due to the presence of significant intra-abdominal adhesions. These 2 patients have been excluded from the incretin analyses (Yousseif *et al.*, 2014). 3 day food diary analyses calculated that calorie intake in the first post-operative week was 449±53 kcal/day.

5.2.4 Very low calorie diet

Following baseline assessments the VLCD group commenced a prescribed 7 day VLCD using a liquid meal replacement product (Optifast; Nestle Nutrition, Croydon, UK) providing 624 kcal/day (2·6 MJ/day); 43% carbohydrate, 34% protein and 19.5% fat plus the recommended daily intake of vitamins, minerals and trace elements. In addition, participants were encouraged to take up to 240g of non-starchy vegetables to add fibre and variation with the intention of aiding compliance with the diet. A list of appropriate vegetables and recipe ideas was provided. Participants were also encouraged to drink at least 2L of calorie free beverages per day and to continue usual levels of physical activity. Support was provided to all participants by means of daily contact via e-mail/telephone call or text message.

5.2.5 Statistical analysis

Data are presented as mean ± SEM for parametric and median (range) for non-parametric data. Insulin secretion rates are given as median with 25th and 75th percentile. Statistical analysis used Student's t-test, paired t-test, Mann-Whitney U test, Wilcoxon Rank and Spearman Rank correlations as appropriate using Minitab 16 statistical program (Minitab Inc.; State College, PA: www.minitab.com).

5.3 Results

5.3.1 Weight loss, body composition and fat distribution

Weight loss was greater 7 days following surgery compared to after 7 days of VLCD (6.2±0.7 kg (5.1±0.5 %) vs. 4.3±0.5 kg (3.5±0.4 %); *p*=0.030). The

components of weight loss were notably different in the surgery vs. VLCD groups (

Table **5-2**), with the additional weight loss in the surgery group consisting of lean mass (-2.5±0.3 vs. -1.3±0.6 kg; p=0.108). Decrease in fat mass was almost identical after surgery vs. VLCD (3.0±0.3 vs. 3.0±0.7 kg). Waist and hip circumference decreased significantly in both groups. There was no significant change in either subcutaneous or visceral adipose tissue areas at 7 days in either group.

	Before VLCD	*7 days after VLCD*	*p*	*Before Surgery*	*7 days after Surgery*	*p*
Weight (kg)	121.4±3.7	117.1±3.6	<0.001	120.8±5.0	114.6±4.7	<0.001
BMI (kg/m²)	42.3±0.9	40.9±0.9	<0.001	43.0±1.1	40.9±1.2	<0.001
Fat mass (kg)	55.7±4.0	52.8±4.2	0.002	57.5±3.3	54.5±3.3	<0.001
Lean mass (kg)	65.6±3.7	64.3±3.7	0.075	62.1±4.6	59.6±5.1	<0.001
Body water (L)	49.4±2.4	47.8±2.1	0.012	46.7±3.3	44.3±3.1	<0.001
Waist circ (cm)	128.6±3.1	125.8±3.0	0.010	127.5±3.1	124.1±2.8	<0.001
Hip circ (cm)	132.0±2.1	129.9±2.2	0.021	131.9±3.5	129.4±3.6	<0.001
Waist:Hip ratio	0.97±0.02	0.97±0.02	0.612	0.97±0.04	0.97±0.04	0.287
VAT area (cm²)	313.2±24.1	303.5±19.2	0.371	283.0±30.0	272.6±21.9	0.437
SAT area (cm²)	403.8±26.5	410.9±30.6	0.587	510.2±48.0	496.5±48.8	0.361

Table 5-2 Anthropometric data before and then after 7 days of VLCD or 7 days after Surgery. There were no significant differences in any parameters between the groups either at baseline or at day 7. Values are shown as mean ± SEM. VAT = visceral adipose tissue and SAT = subcutaneous adipose tissue.

5.3.2 Plasma glucose, insulin, glucagon and metabolites

Fasting plasma glucose levels fell modestly and similarly in both groups (1.2±0.6 mmol/l following VLCD and 0.9±0.5 mmol/l following Surgery; p=0.739). The decrease in 2 hour glucose during the meal test was only significant in the Surgery group (Table 5-3). Fasting and 2 hour insulin levels were higher at baseline in the VLCD group, but appeared to decrease in both groups after intervention although this was not statistically significant. Fasting ß-hydroxybutyrate levels increased almost 3-fold in both groups at day 7. There was no significant change in fasting NEFA or glucagon in either group. Serum ALT appeared to increase in both groups after 7 days of intervention, however, GGT decreased only following the VLCD. The change in serum triglycerides was divergent and this difference was significant: after surgery (0.3 (-0.3-0.8) mmol/l and after VLCD (-0.1 (-2.4-0.2) mmol/l); p=0.039.

	Before VLCD	7 days after VLCD	p	Before Surgery	7 days after Surgery	p
Fasting glucose (mmol/l)	10.5±1.4	9.4±1.2	0.078	8.0±0.7	7.1±0.6	0.105
2hr glucose (mmol/l)	10.4±1.4	9.5±1.0	0.175	8.4±0.7	7.3±0.6	0.040
Fasting insulin (mU/l)	18.5 (13.5-44.6)	14.8 (10.6-38.8)	0.813	13.5 (4.3-61.2) *	9.8 (5.2-23.2) #	0.076
2hr insulin (mU/l)	22.3 (18.3-78.9)	19.5 (10.1-76.2)	0.363	14.4 (5.2-70.5) *	11.0 (5.6-28.3)	0.124
Fasting β-hydroxybutyrate (mmol/l)	0.22±0.07	0.63±0.17	0.005	0.29±0.06	0.86±0.18	0.019
Fasting NEFA (mmol/l)	0.85 ± 0.13	0.91 ± 0.08	0.402	0.85 ± 0.11	0.96 ± 0.08	0.087
Fasting glucagon (ng/l)	63.9 (27.3-117.4)	51.0 (37.7-129.3)	0.636	61.3 (33.3-203.8)	58.5 (40.3-124.7)	0.124
Fasting triglycerides (mmol/l)	1.7 (0.6-3.7)	1.3 (0.8-2.4)	0.193	1.3 (1.0-2.0)	1.8 (0.9-2.0)	0.124
Fasting ALT (U/l)	34.7 ± 6.4	39.9 ± 7.2	0.020	40.8 ± 5.2	61.4 ± 9.9	0.108
Fasting GGT (U/l)	33 (20-113)	27 (15-81)	0.014	39 (13-148)	40 (29-241)	0.155

Table 5-3 Metabolic response to 7 days of either VLCD or Surgery. Values are mean ± SEM or median (range). * p<0.05 for baseline difference between groups, # p<0.05 for day 7 difference between groups.

5.3.3 Incretin meal test

Following surgery, the gastroenterostomy caused a significantly greater early rise in post-meal plasma glucose (Figure 5-1A & Figure 5-2A). This was associated with a greater early rise in insulin (4.5±2.2 to 22.5±6.9 mU/l; p=0.063) which was unchanged after VLCD (5.7±2.3 to 3.8±2.3 mU/l; p=0.493). The early rise in insulin correlated with the rise in plasma glucose (Spearman rank=0.867; p=0.002). The resulting greater early insulin secretion did not fully compensate as shown by the overall glucose AUC, and indeed there was a modest increase in glucose AUC in the surgery group (Figure 5-1B). There was no significant change in insulin AUC in either group. If GLP-1 was driving the insulin response, then it would be expected to be directly proportionate to the change in insulin/glucose ratio. No such relationship was observed (Spearman rank: Surgery: =-0.214; p=0.610; VLCD: -0.190; p=0.651; Whole group: 0.365; p=0.165). The considerable extent and duration of the postprandial rise in GLP-1 can be appreciated from the plasma hormone profile (Figure 5-2C). The GIP response to the meal test appeared to increase at 7 days after Surgery but the change in AUC was not significant. The AUC for GIP was not altered by VLCD however, there did seem to be a temporal shift in the GIP profile (Figure 5-2D).
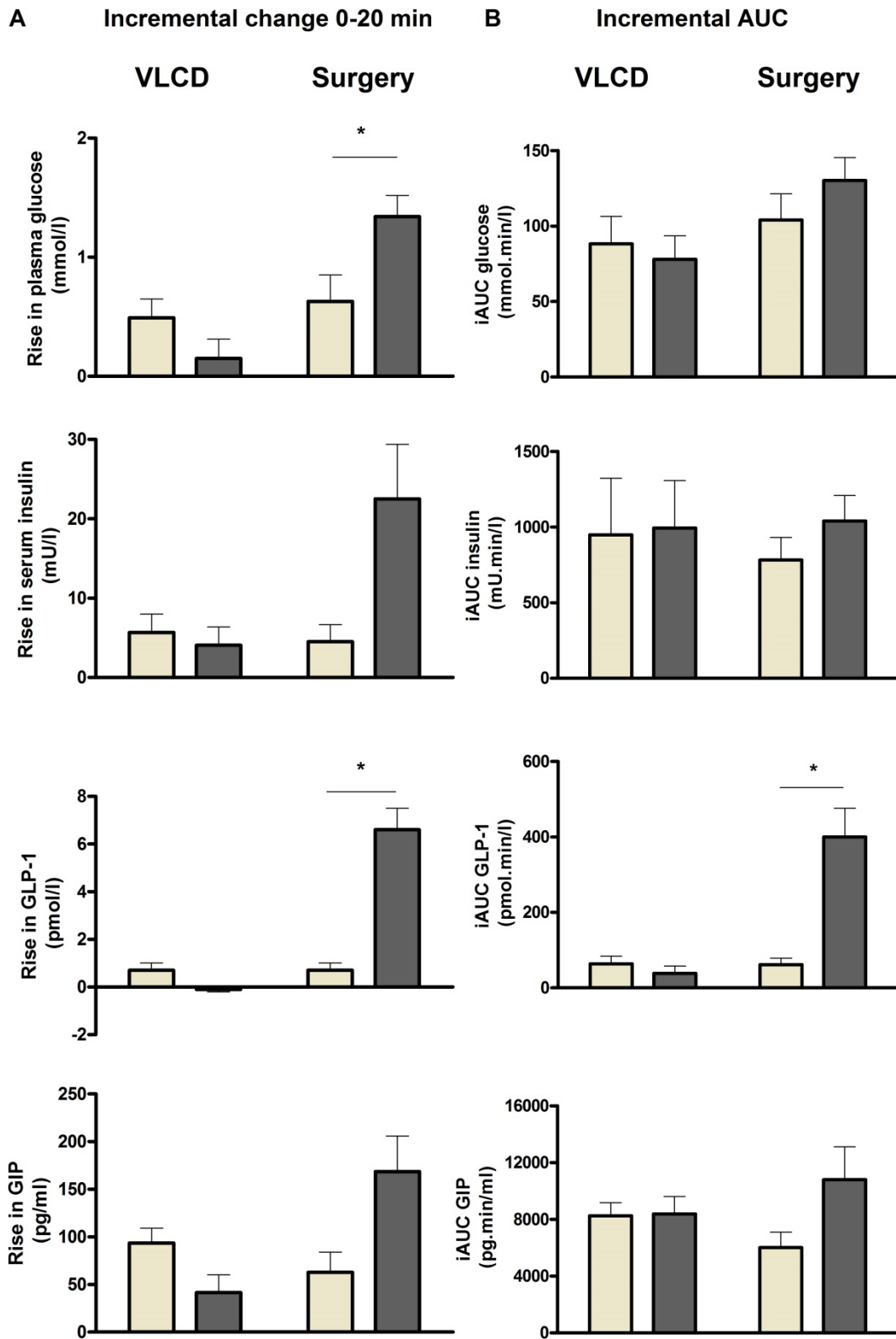
Figure 5-1 Incremental change in glucose, insulin, total GLP-1 and total GIP from fasting to 20 minutes (A) and change in positive incremental area under the curve (B) during the semi-solid meal test before (pale) and 7 days after (dark) intervention (VLCD or Surgery). Data are shown as mean ± SEM. *=p<0.05 for baseline to day 7 difference.
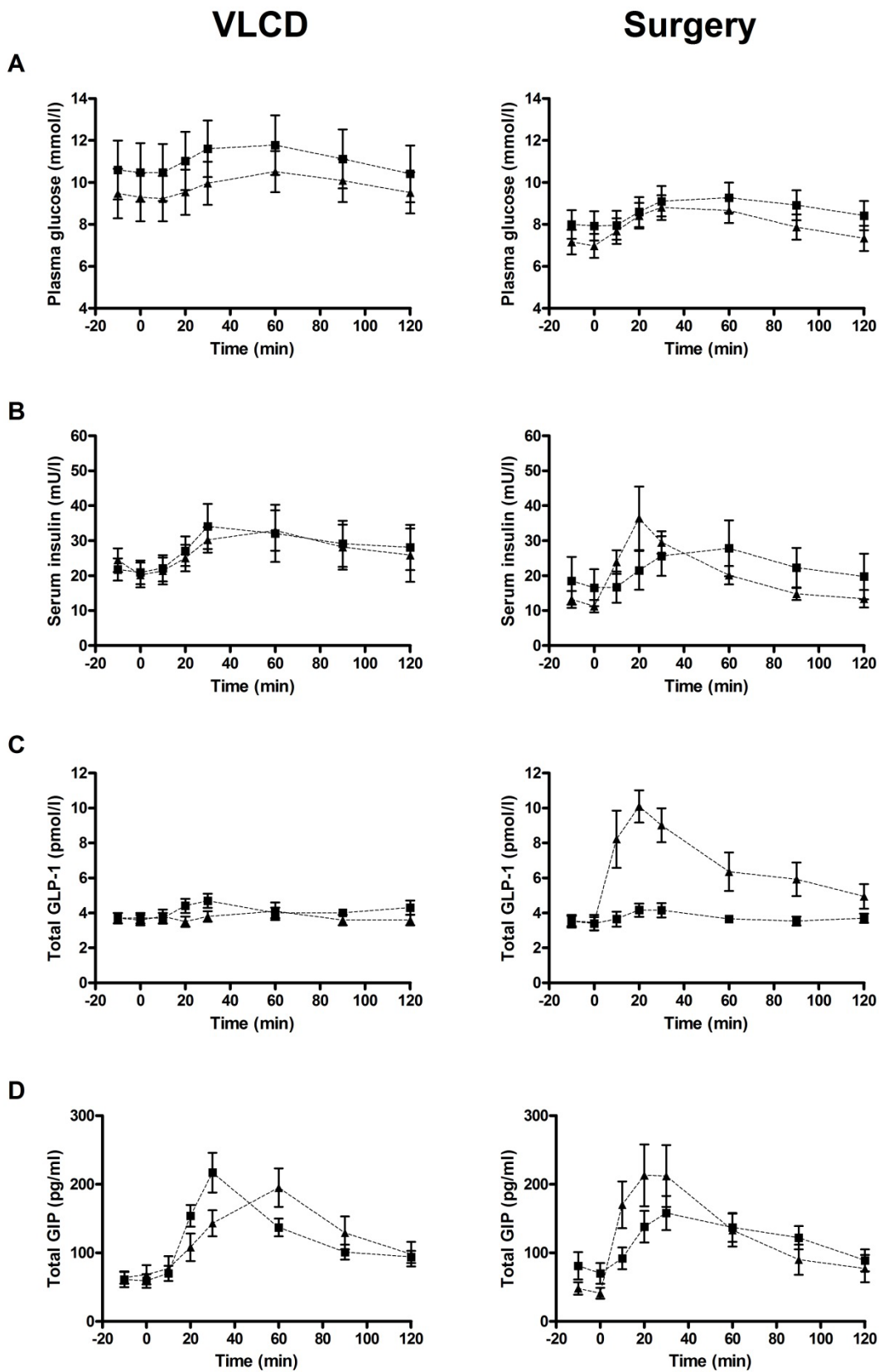
Figure 5-2 Glucose (A), insulin (B), total GLP-1 (C) and total GIP (D) levels (mean ± SEM) during the 2 hour meal test in the VLCD and Surgery groups at baseline (squares) and day 7 (triangles).

5.3.4 Insulin response to intravenous stimulus

First phase insulin secretion (baseline to 6 min increment in insulin secretion rate in response to an intravenous glucose challenge) was unchanged at day 7 compared to baseline in both groups: 0.08 (0.07-0.10) to 0.06 (0.00-0.13) nmol min^{-1} m^{-2} in the Surgery group (p=0.722) and 0.04 (-0.03-0.10) to 0.10 (0.08-0.14) nmol min^{-1} m^{-2} in the VLCD group (p=0.155). There was no change in maximal insulin secretory capacity in response to an arginine bolus (baseline to peak insulin secretion rate) in either the Surgery group (0.80 (0.63-0.84) to 0.67 (0.54-1.46) nmol min^{-1} m^{-2}; p=0.906) or the VLCD group (0.67 (0.53-0.70) to 0.54 (0.43-0.72) nmol min^{-1} m^{-2}; p=0.477).

5.3.5 Hepatic insulin sensitivity

Concordant with the modest change in fasting plasma glucose levels, basal hepatic glucose production did not change significantly in either group: 3.48±0.32 to 3.43±0.63 mg/kg$_{ffm}$/min in the VLCD group (p=0.906) compared to 3.71±0.36 to 3.16±0.33 mg/kg$_{ffm}$/min in the Surgery group (p=0.083). Hepatic insulin sensitivity did not change significantly in either the VLCD or Surgery group although there was a trend toward improvement; measured either as hepatic IR index: 2.38 (1.07-4.62) to 1.97 (0.60-6.98) mmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} ($p$=0.427) and 2.15 (0.61-8.02) to 1.25 (0.20-3.14) mmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} ($p$=0.077) respectively, or percentage suppression of hepatic glucose production induced by insulin: 61.7±5.5 to 73.0±5.7 % (p=0.173) and 73.2±4.6 to 78.9±7.2 % (p=0.448) respectively.

5.3.6 Peripheral tissue insulin sensitivity

There was no significant change in peripheral insulin sensitivity expressed as glucose disposal rates during either 7 day intervention. Insulin-stimulated glucose disposal was 3.48±0.54 then 3.21±0.83 mg/kg$_{ffm}$/min (p=0.594) in the VLCD group and 2.97±0.43 then 2.37±0.37 mg/kg$_{ffm}$/min (p=0.186) in the Surgery group. Glucose metabolic clearance rate (MCR) was calculated to correct for the difference in fasting plasma glucose between the 2 study time points. There was no significant change in glucose MCR after

either intervention; VLCD: 2.21 (0.91-8.80) to 1.83 (1.10-13.79) ml/kg$_{ffm}$/min (p=0.791) and Surgery: 2.55 (0.86-5.15) to 2.40 (0.59-3.94) ml/kg$_{ffm}$/min (p=0.724).

5.3.7 Intra-organ triglyceride content

Hepatic triglyceride content decreased in both groups; VLCD by 18.6±4.0 % and Surgery by 29.8±3.7 % (p=0.058) (VLCD: 11.8±2.2 to 9.8±1.9 %; p=0.003 and Surgery: 6.5±1.6 to 4.5±1.0 %; p=0.029). However, there was a strikingly different relationship to weight loss within each group. After VLCD, the fall in hepatic triglyceride was directly related to the extent of weight loss (Pearson r=0.798; p=0.010), whereas after surgery the greatest reduction in hepatic triglyceride occurred in participants with modest weight loss (Pearson r=-0.833; p=0.010) (Figure 5-3). This effect was not explained by PNPLA3 genotype: the rs738409 C to G adiponutrin/PNPLA3 genotype (coding for I148M) was found in 4 individuals in the VLCD group (one homozygous and 3 heterozygous; change in hepatic triglyceride 4.1 to 3.2% and 13.9±1.8 to 12.2±1.7% respectively) and 1 individual in the Surgery group (heterozygous; change in hepatic triglyceride 7.2 to 4.8%). There was no correlation between the reduction in hepatic triglyceride content and the reduction in lean mass in the Surgery group (Spearman rank=-0.094; p=0.840). There was no correlation between the change in hepatic triglyceride content and change in either serum triglycerides or serum ALT in either group. There was no change in pancreatic triglyceride content after 7 days of either VLCD or Surgery (6.4±0.6 to 6.5±0.7 %; p=0.781 and 6.7±0.7 to 6.7±0.6 %; p=0.885 respectively).
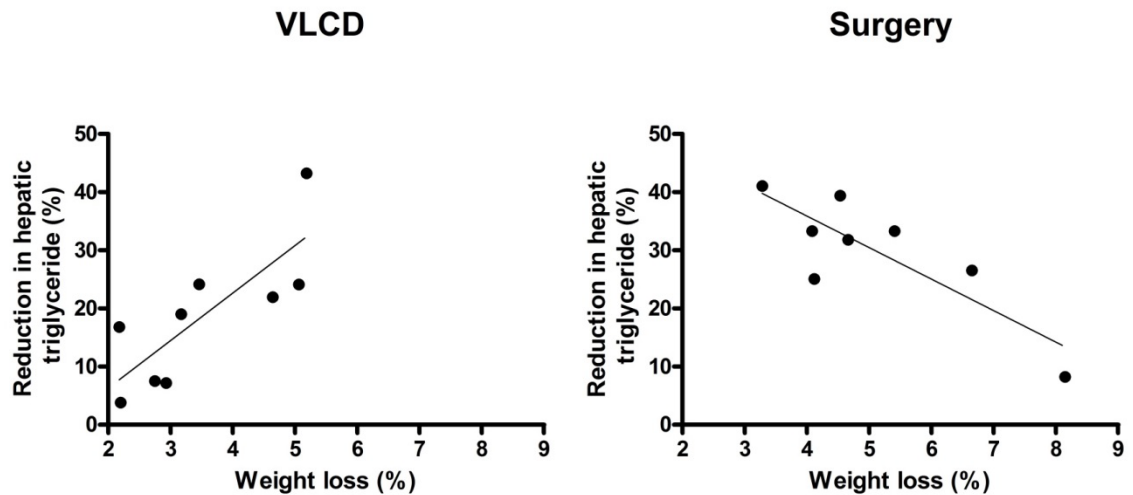
Figure 5-3 Relationship between achieved weight loss and reduction in hepatic triglyceride content after 7 days of VLCD or 7 days after Surgery.

5.4 Discussion

This study demonstrates that despite greater weight loss at 7 days after surgery, the improvement in fasting plasma glucose was modest and not significantly greater than 7 days after VLCD. After surgery, a greater early rise in plasma glucose after the test meal was associated with a greater 0-20 minute rise in both plasma insulin and GLP-1. The latter was 7 fold greater in the surgery group compared to the VLCD group and plasma levels remained higher throughout the test meal period. Despite this, the incremental AUCs for insulin and glucose were not significantly different between the VLCD and Surgery groups. There was no association between the extent of GLP-1 rise and insulin secretion independent of the glucose stimulation and the marked increase in GLP-1 did not confer a clear benefit on post-meal glucose levels or insulin secretion. There was no change in non-incretin dependent beta cell function as assessed by the stepped intravenous insulin secretion test in either Surgery or VLCD groups. Differences were observed between the 7 day response to RYGB or VLCD in components of body weight change and also in the relationship between extent of weight loss and change in hepatic triglyceride content.

The increased meal stimulated GLP-1 noted only in the surgery group did not seem to be accompanied by any additional benefits over the diet group. This is not in keeping with the widespread acceptance that GLP-1 has a determinant role in improving postprandial insulin secretion after RYGB (Holst, 2011; Naslund and Hellstrom, 2013; Vella, 2013; Goldfine and Patti, 2014). It may be that the changes in GLP-1 secretion are secondary to the rapid rerouting of nutrients through the gut. Studies have demonstrated that d-xylose (Salehi *et al.*, 2011) and acetaminophen (Falkén *et al.*, 2011) are absorbed more rapidly and reach higher peak levels following gastric bypass surgery. The alterations in insulin and incretin hormone secretion may be secondary phenomena related to change in post-operative glucose profile. The greatest metabolic change seen after bariatric surgery is decreased fasting glucose, which would not be expected to be affected by postprandial secretion of GLP-1. A previous comparison of a matched group of obese individuals with type 2 diabetes before and one month after RYGB and a matched group undergoing 10kg dietary weight loss demonstrated that stimulated GLP-1 and GIP levels increased after surgery but not after diet induced weight loss (Laferrere *et al.*, 2008). There was a similar reduction in fasting glucose, insulin, C-peptide and HOMA-IR in both groups. The authors concluded that the incretin effect explained the improvement of glucose control after RYGB, however the data show that the peak plasma glucose was higher after surgery and the calculated incretin effect did not differ after surgery or diet.

In contrast to the lack of definitive data on the role of GLP-1 in the post RYGB improvement in glycaemic control, several studies have demonstrated the greater improvement in fasting plasma glucose by calorie restriction alone (Isbell *et al.*, 2010; Lingvay *et al.*, 2013). The earlier effects of RYGB on glucose metabolism, that is within 4 days of surgery, have been compared to calorie restriction using replication of the post-RYGB diet in a group which included some individuals with type 2 diabetes (Isbell *et al.*, 2010). The increase in meal stimulated GLP-1 after surgery was not accompanied by any additional benefits over the diet group. Another study compared the effects of VLCD and surgery in individuals with type 2

diabetes due to have RYGB in a paired design using individuals as their own controls (Lingvay *et al.*, 2013). After 10 days of VLCD there was a significant improvement in fasting glucose, peak glucose and glucose AUC during a mixed meal challenge test but not in the early post RYGB phase despite a greater GLP-1 response after surgery. These studies, together with the present data, indicate that the major mechanism underlying the change in glucose control in the early post-operative period is severely restricted oral calorie intake. This does not preclude a role for the ongoing cumulative effects of markedly increased postprandial GLP-1 in the more extended post-operative period, which could include augmentation of beta cell mass and/or function. This requires further investigation.

This study has demonstrated some differences in the metabolic state at 7 days after RYGB compared to VLCD. There were similar differences in the decrease in fat mass, but surgery appeared to induce a larger decrease in lean mass, which may represent a catabolic response to the surgery. The differing relationship between fall in hepatic triglyceride and achieved weight loss following VLCD and bariatric surgery has not, to my knowledge, been demonstrated previously. At 7 days after surgery, participants with the greatest weight loss had the smallest reduction in hepatic triglyceride content. Exacerbation of fatty liver disease following jejuno-ileal bypass has been reported, ranging from transient worsening of inflammation and fibrosis to steatosis and rarely to fulminant hepatic failure (Peters *et al.*, 1975) and it is possible that the underlying mechanism for this could disturb the relationship. One hypothesis would be that this is due to massive fatty acid mobilisation from visceral stores, reaching the liver through the portal vein, which would also fit with the elevated NEFA and triglycerides resulting in a metabolic insult to hepatocytes and an elevated ALT. Another possibility is that the GLP-1 peak after every meal might produce a cumulative effect on liver fat content. A decrease in liver fat has been seen with therapeutic use of both GLP-1 agonists (Cuthbertson *et al.*, 2012) and DPP-4 inhibitors (Macauley *et al.*, 2015a). In view of this possible relationship, the extent of post-meal GLP-1 elevation and fall in liver fat in the surgery group was examined. The strong correlation (Spearman rank

0.590; p=0.016) between peak GLP-1 and fall in hepatic triglyceride content is suggestive of a causal relationship and this requires further examination.

Ectopic fat storage is central to the pathophysiology of type 2 diabetes (Taylor, 2013). The importance of pancreatic triglyceride in the pathogenesis of diabetes was suggested by a study in obese rodents showing an increase in triglyceride accumulation in the pancreas prior to the onset of type 2 diabetes (Lee *et al.*, 2009). In humans significantly greater pancreatic fat content in individuals with type 2 diabetes compared to those with normal glucose tolerance has been demonstrated (Tushuizen *et al.*, 2007). However, the data on the relationship between pancreatic triglyceride content and beta cell function in humans is discordant (Tushuizen *et al.*, 2007; Heni *et al.*, 2010; van der Zijl *et al.*, 2011). Pancreatic triglyceride content has been shown to correlate with hepatic triglyceride content and hepatic insulin resistance (Hannukainen *et al.*, 2011). A previous study demonstrated a significant improvement in first phase insulin secretion after 8 weeks of VLCD alongside a reduction in pancreatic triglyceride content, with no meaningful change after 7 days (Lim *et al.*, 2011). The present data confirm this for VLCD and show that the same is true following bariatric surgery. Similarly, a decrease in pancreatic triglyceride content and improvement in first phase insulin secretion has been demonstrated at 8 weeks after bariatric surgery (data in chapter 4). It may be that although hepatic triglyceride stores are utilised early during calorie restriction, pancreatic triglyceride stores may decrease more gradually.

In this study, change in beta cell function does not explain the early improvement in glucose control seen after bariatric surgery. In contrast, both hepatic triglyceride content and hepatic insulin sensitivity improve rapidly during calorie restriction. Fasting plasma glucose concentration is determined by the rate of hepatic glucose production (Ravikumar *et al.*, 2008) and elevated liver fat concentration is associated with decreased insulin sensitivity to suppression of hepatic glucose production (Seppala-Lindroos *et al.*, 2002; Gastaldelli *et al.*, 2007). Short term carbohydrate overfeeding can induce liver fat accumulation (Sevastianova *et al.*, 2012)

and furthermore, weight loss with consequent reduction in liver fat by dietary means (Petersen *et al.*, 2005) or by Pioglitazone (Ravikumar *et al.*, 2008) is associated with improvements in insulin sensitivity and fasting plasma glucose levels. The design of this study, with pre-operative calorie restriction due to surgical requirements, minimised change in hepatic insulin resistance, but it is known that liver fat content continues to fall beyond the first week of calorie restriction (Lim *et al.*, 2011). In the latter study, all eleven participants achieved a fasting glucose level of <7mmol/l after 7 days of VLCD. The lower rates of achieving a normal fasting glucose level seen in the current study (33% (3/9) of the VLCD and 44% (4/9) of the Surgery group) is likely to relate to the difference in the populations studied. The cohort reported by Lim *et al.* had less severe obesity (mean BMI 33.6 vs. 42.7 kg/m^2), a shorter diabetes duration (all < 4yr vs. median 6.25 yr), and required less anti-diabetic treatment at baseline (Metformin and Sulphonylurea only vs. insulin and triple oral therapy).

The limitations of the study must be discussed. There was baseline heterogeneity between the VLCD and Surgery groups; both groups were in negative calorie balance prior to their surgery but the degree of compliance with the pre-operative diet advised by the surgical teams was not able to be quantified and may have been highly variable. The baseline heterogeneity does not preclude comparison of the changes in the first 7 days of either intervention. The fact that body water content decreased similarly in the 2 groups after 7 days suggests both groups were in a similar phase of calorie restriction, with glycogen stores being utilised first (Henry and Gumbiner, 1991). The lack of change in peripheral insulin sensitivity in the surgery group and the similar changes between the surgery and VLCD groups makes it very unlikely that there has been any significant blunting of the effect of surgery by a temporary post-operative insulin resistance. The potential effect of the different macronutrient content of the dietary intake of the two groups during the 7 day interventions must be considered. This could be a potential explanation for the greater loss of lean mass in the surgery group. However, over this short period of time, any effect is likely to be minimal when compared to that of the major reduction in overall energy

intake, which was similar with the meal replacement product and the high protein semi-solid post-operative diet advised by the surgical dieticians. In this study, the unselected population in terms of diabetes duration and treatments is representative of the truly heterogeneous population undergoing bariatric surgery. The small numbers in each group were necessary given the practical time constraints of this study and the complex physiological studies undertaken. The potential carry-over effect of anti-diabetic medications is a possibility; however, this would be the same in both the Surgery and VLCD group and is unlikely to have significantly influenced the baseline results. The study design differs from the majority of studies after RYGB in that an oral glucose challenge was not used and this must be borne in mind when comparing with results from earlier studies. However, the use of a semi-solid test meal allows assessment of post-meal physiology following RYGB in response to experimental conditions designed to reflect usual dietary intake.

This study suggests that the more rapid entry of food into the small intestine causes both a more rapid rise in plasma glucose and a more rapid rise in insulin. No relationship of this with the large postprandial rise in plasma GLP-1 could be detected. There were similar reductions in glucose levels and hepatic triglyceride content achieved by 7 days of VLCD and at 7 days after RYGB. No change was observed in either beta cell function or pancreatic triglyceride content at 7 days after either intervention.

Chapter 6. Pathophysiology & Reversibility of Long Duration Type 2 Diabetes Compared to Short Duration Disease

6.1 Introduction

The landmark UK Prospective Diabetes Study demonstrated that glucose control in type 2 diabetes steadily worsened towards an inevitable requirement for insulin treatment despite best possible therapy (UKPDS, 1995a). Since this study the progressive and irreversible nature of type 2 diabetes has been widely accepted. Moreover, the approach to management has developed around this concept with guidelines laying out the sequential addition of therapies prior to initiation of insulin therapy (NICE, 2015). It has been recognised for over 3 decades that normalisation of glucose control can occur following bariatric surgery. More recently it has been demonstrated that a very low calorie diet can also reverse the pathophysiological defects of type 2 diabetes and normalise glucose control (Lim *et al.*, 2011). This latter study selected individuals with type 2 diabetes of duration less than four years. Studies investigating the factors associated with diabetes remission following bariatric surgery have identified duration of diabetes of more than 10 years as a potential limiting factor (Renard, 2009; Hall *et al.*, 2010). In line with this, the most recent NICE guidance on bariatric surgery has recommended expedited assessment for surgery in individuals with a BMI in excess of $35kg/m^2$ or who have recent onset type 2 diabetes, which was considered to be within 10 years from diagnosis (NICE, 2014). In chapter 3, a retrospective data collection showed that reversal to normoglycaemia can be achieved in long duration type 2 diabetes following bariatric surgery, but that greater weight loss is required to achieve reversal compared to short duration disease.

There is compelling evidence that of the multiple pathophysiological defects seen in type 2 diabetes, it is beta cell dysfunction that determines the onset of hyperglycaemia and whether or not blood glucose control can be achieved (Ferrannini *et al.*, 2004). Studies investigating C-peptide response to glucagon (Zangeneh *et al.*, 2006) or during oral glucose tolerance testing following calorie restriction (Jain *et al.*, 2008) in long duration type 2 diabetes concluded that although a decline in beta cell function over time is characteristic, it is not inevitable and there was much variation between

individuals. A recent study on outcomes of gastric bypass surgery at 2 years in individuals with short duration (<5 yr) and long duration (>10 yr) diabetes demonstrated that the better glycaemic outcomes in the former group were related to greater improvement in beta cell function post-operatively (Khanna *et al.*, 2015).

In vitro work suggests a key role for fat in causing beta cell dysfunction in type 2 diabetes, in particular, the metabolites of triglyceride such as ceramides and diacylglycerol. Lipid-induced reversible inhibition of glucose sensitive insulin secretion has been demonstrated (Lee *et al.*, 1994; Cnop, 2008). Furthermore, it appears likely that the toxic effects of lipid metabolites on beta cells could explain both the onset and reversibility of beta cell dysfunction (Kashyap *et al.*, 2003; Morgan *et al.*, 2008; Taylor, 2013). These same inhibitory molecules can induce beta cell apoptosis (Shimabukuro *et al.*, 1998a). As well as beta cell dysfunction, a time-dependent reduction of functional beta cell mass could limit the capacity to return to normal glucose control in longer duration type 2 diabetes (Rahier *et al.*, 2008). Recent work demonstrating ongoing beta cell replication and neogenesis in adult life gives theoretical hope of diabetes reversal at any stage of the disease. Removal of the toxic milieu caused by ectopic lipid deposition could allow existing beta cells to recover function and then with downregulation of apoptotic pathways there is potential for beta cell mass expansion and redifferentiation (White *et al.*, 2013).

The pathways by which fat metabolites can impair insulin sensitivity in liver and muscle have now been defined. Activation of protein kinase C epsilon type inhibits the signalling pathway from the insulin receptor to insulin receptor substrate 1 (Samuel *et al.*, 2010). There is a clear relationship between liver fat and hepatic insulin sensitivity, and weight loss achieved either by diet (Petersen *et al.*, 2005) or bariatric surgery (Immonen *et al.*, 2014) reduces liver fat and improves hepatic insulin sensitivity, the latter being the major determinant of fasting glucose levels.

There is now accumulating evidence that pancreatic fat content could be a biomarker for beta cell dysfunction. Pancreatic fat levels rise prior to the

development of type 2 diabetes in rodents (Lee *et al.*, 1994). In humans supranormal pancreas triglyceride content has been associated with beta cell dysfunction (Tushuizen *et al.*, 2007). More recently normalisation of first phase insulin secretion has been seen alongside a reduction in pancreatic triglyceride content induced by weight loss (Lim *et al.*, 2011) Identification of the location of triglyceride within the pancreas has been hampered by rapid post-mortem autolysis, but study of pancreata retrieved but not used for pancreas transplantation has provided clear information (Pinnick *et al.*, 2010). Intracellular fat droplets are widely distributed within the exocrine pancreatic cells, in addition to widely scattered isolated adipocytes. Recently it has been shown that fatty acid receptors are expressed in human pancreatic beta cells (Tang *et al.*, 2015) and *in vitro* exposure of human islet cells to fatty acids causes marked triglyceride accumulation (Lalloyer *et al.*, 2006). It is hypothesised that the export of excess fat from the liver in the form of VLDL-triglyceride may be one of the mechanisms by which excess fat accumulates in and around beta cells causing dysfunction (Taylor, 2013). It is likely that local lipolysis could result in interstitial and intracellular concentrations of fatty acids sufficient to inhibit beta cell function.

The aims of this study were to establish the pathophysiological differences between long and short duration type 2 diabetes, and to establish how effectively a VLCD could improve glucose control in long duration compared to short duration type 2 diabetes. This has not previously been investigated and the results could have important implications for the management of type 2 diabetes in everyday clinical practice.

6.2 Study design

6.2.1 Participants
Individuals with type 2 diabetes were identified in response to local advertisement; 15 with short duration disease (defined as <4yr duration) and 15 with long duration disease (defined as >8yr duration) were recruited. Inclusion criteria were age 25-80 years; type 2 diabetes treated by diet, metformin, sulphonylurea, DPP-4 inhibitors and/or insulin; and BMI 27-45

kg/m². Exclusion criteria were loss of more than 5kg body weight in the preceding 6 months, treatment with thiazolidinediones, GLP-1 agonists, steroids or atypical anti-psychotic medications, untreated thyroid disease, renal dysfunction (serum creatinine >150 μmol/l) or alcohol consumption >3 units per day for women and >4 units per day for men. The history of the diagnosis of diabetes was taken carefully to clinically exclude alternate diagnoses than type 2 diabetes. Date of diagnosis of type 2 diabetes was confirmed from primary care medical records. The study protocol was approved by Newcastle and North Tyneside 2 Ethics Committee and all participants gave informed written consent. Participants were asked to remain on their usual dose of lipid lowering treatment throughout the study. Anti-hypertensive medications were decreased as necessary throughout the study. Participants were asked to discontinue all anti-diabetic therapy prior to the baseline study: metformin and sulphonylureas for 72 hours; long acting insulin for >36 hours and short acting insulin for >12 hours. Dietary adherence was assessed using blood ketones. As in a previous study, participants were excluded if they were unable to achieve weight loss targets of 3·8% body weight at week one of the VLCD and 9·3% at week four (Lim *et al.*, 2011). Only one participant in the long duration group did not meet the weight loss target and left the study after week one. Hence the final numbers were 15 in the short duration group and 14 in the long duration group. The baseline characteristics for the participants who completed the VLCD are shown in Table 6-1.

.

	All (n=29)	*Short duration (n=15)*	*Long duration (n=14)*	*p value*
Gender	15M:14F	7M:8F	8M:6F	
Age (yr)	56.7±1.8	52·1±2·6	61·6±2·0	0·007
Weight (kg)	98.0±2.6	99·0±3·7	96·9±3·8	0·683
BMI (kg/m²)	34.2±0.7	34·1±0.8	34.3±1.2	0·862
Fat mass (kg)	39.1±1.9	38.8±2.3	39.4±3.3	0.881
Body water (L)	43.8±1.5	44.4±2.2	43.2±2.1	0.705
Lean mass (kg)	58.9±2.3	60.2±3.2	57.5±3.3	0.549
Diabetes duration (yr)	7.3±1.1	2·3±0·3	12·7±1·2	<0·001
Fasting plasma glucose (mmol/l)	11.4±0.6	9·6±0·7	13·4±0·8	0·001
HbA1c (mmol/mol) (%)	62.5±2.7 (7.9±0.3)	55±2 (7.2±0.2)	70±4 (8.6±0.4)	0·004
Diabetes treatment (n):				
Diet	7	4	3	
MF	21	11	10	
SU	11	3	8	
Insulin	3	0	3	
Anti-hypertensives (n)	17	6	11	
Statins (n)	19	9	10	

Table 6-1 Baseline participant characteristics and anthropometrics for the short and long duration groups. Data are mean ± SEM.

6.2.2 Experimental protocol

Participants were asked to continue their habitual pattern of eating until the start of the study. They were seen prior to the first study with family/friends as appropriate to discuss the very low calorie diet requirements in detail. They were asked to avoid intensive physical activity and excess alcohol or caffeine intake in the 48 hours prior to each study and to avoid nicotine on study days. Assessments of body composition, beta cell function, hepatic and peripheral insulin sensitivity, hepatic and pancreatic triglyceride content, and hepatic $VLDL_1$-triglyceride production were carried out at baseline. Assessments took place over 1 half day then 1 full day. All studies were performed after a 12 hour overnight fast. On the morning of the half day, anthropometric and body composition measurements were made using electrical bioimpedence (section 2.1.3) and hepatic and pancreatic triglyceride measured using the 3 point Dixon MR method (section 2.2.2). Resting metabolism was assessed using indirect calorimetry (section 2.1.4). A cannula was then inserted into an antecubital fossa for infusion of Intralipid to measure $VLDL_1$-triglyceride production rate, and a second cannula was inserted in the contralateral arm for blood sampling (section 2.3.8). On the morning of the full day, a cannula was inserted into an antecubital fossa for infusions and a second cannula inserted into the contralateral wrist for arterialised blood sampling. Assessments of hepatic and peripheral insulin sensitivity were made using an hyperinsulinaemic isoglycaemic clamp study (sections 2.3.3 & 2.3.4). Following the clamp, participants rested for a minimum of 60 min and until stable fasting glucose levels were re-established. Insulin secretion was then assessed using a stepwise insulin secretion test with arginine (section 2.3.5).

6.2.3 Very low calorie diet

Following completion of baseline assessments, all participants commenced an 8 week very low calorie diet. This was provided using a meal replacement liquid formula diet (43% carbohydrate, 34% protein and 19.5% fat; vitamins, minerals and trace elements; 2·6 MJ/day [624 kcal/day]); Optifast; Nestlé Nutrition, Croydon, UK). In addition, participants were asked to consume

up to 240g of non-starchy vegetables per day, such that the total energy intake per day was 624-700 kcal. Participants were provided with detailed information on vegetable and recipe ideas. They were also encouraged to drink at least two litres of calorie-free beverages per day and to maintain their habitual levels of physical activity. In order to maximise adherence to the protocol, one-to-one support was provided weekly by telephone, e-mail, text or face-to-face contact. Additional face-to-face contact was provided if requested, but this was rare and similar between the groups. Participants were seen at week 1, week 4 and then at the end of the 8 week diet for fasting blood tests, anthropometry and blood pressure measurement.

Statistics

Statistical analyses were performed using Minitab 16 Statistical Software (Minitab Inc.; State College, PA: www.minitab.com). Data are presented as mean ± standard error of the mean, or median (range) for non-parametric data. Insulin secretion rates and hepatic insulin resistance indices are presented as median with 25th and 75th percentile. Statistical comparisons between the short and long duration groups were performed using the two-tailed Student's *t*-test or Mann Whitney U test, while within-group differences before and after the VLCD were determined using a two-tailed Student's paired *t*-test or Wilcoxon rank test if non-parametric. Correlations were examined using the Spearman rank test. Statistical significance was accepted at *p*<0·05.

6.3 Results

6.3.1 Pathophysiological differences between long and short duration type 2 diabetes

Fasting metabolism

Individuals with long duration type 2 diabetes had poorer baseline glycaemic control represented by a higher fasting plasma glucose and HbA1c (Table 6-1). There was a wide and overlapping range of baseline C-peptide levels but the long duration group had significantly lower fasting insulin and C-peptide levels along with elevated plasma NEFA levels,

suggestive of a relatively insulin deficient state (Table 6-2). There were no differences in resting fasting metabolism between the short and long duration groups; resting energy expenditure: 2008.1±96.8 vs. 1958.4±80.3 kcal/day; $p=0.696$ respectively and respiratory quotient: 0.78±0.01 vs. 0.79±0.01; $p=0.694$.

	Short duration	Long duration	p value
Insulin (mU/l)	17.4 (3.9-48.9)	7.0 (4.1-31.9)	0.006
C-peptide (nmol/l)	1.1 (0.4-2.8)	0.6 (0.4-1.2)	0.006
β-hydroxybutyrate (mmol/l)	0.1 (0.1-0.3)	0.2 (0.1-1.1)	0.371
NEFA (mmol/l)	0.55±0.04	0.69±0.05	0.044
Serum triglycerides (mmol/l)	1.5 (0.7-8.1)	1.3 (0.5-3.3)	0.348
Total cholesterol (mmol/l)	4.6±0.2	4.8±0.3	0.589
Non-HDL cholesterol (mmol/l)	3.5±0.2	3.4±0.3	0.925
HDL cholesterol (mmol/l)	1.1±0.1	1.4±0.1	0.042
ALT (U/l)	27 (11-118)	23 (12-151)	0.472
GGT (U/l)	31 (11-185)	31 (11-62)	0.407

Table 6-2 Fasting metabolites in short and long duration diabetes. Values are mean ± SEM or median (range).

Liver

Hepatic triglyceride content was 11.8±2.2 % in those with short duration diabetes and 8.2±1.4 % in those with long duration diabetes ($p=0.182$). There was no correlation between diabetes duration and hepatic triglyceride content (Spearman rank -0.281; $p=0.139$). Hepatic triglyceride content and fasting insulin levels were strongly positively correlated (Spearman rank=0.636; $p<0.001$). Hepatic insulin sensitivity was lower at baseline in those with short duration compared to long duration diabetes: hepatic IR index: 2063 (1202-2389) vs. 1219 (888-1409) μmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} respectively; $p=0.034$. There was no difference in hepatic VLDL$_1$-triglyceride

production rate between the groups (136.5±19.3 vs. 145.4±14.0 mg/kg/day; p=0.719).

Pancreas

There was no difference between first phase insulin response (baseline to 6 min increment in insulin secretion rate) between short and long duration diabetes (0.02 (0.00-0.18) and 0.01 (0.00-0.03) nmol min^{-1} m^{-2}; p=0.230). The difference in maximal insulin secretory capacity (baseline to peak increment in insulin secretion rate) following arginine bolus was not significant (0.41 (0.20-0.67) and 0.23 (0.18-0.33) nmol min^{-1} m^{-2}; p=0.102). Pancreatic triglyceride content was greater in those with long duration compared to short duration disease: 5.7% (3.8-15.9) vs. 4.7% (3.3-8.0); p=0.043).

6.3.2 Effect of a VLCD in individuals with short vs. long duration type 2 diabetes

Weight loss

At baseline the groups were well matched for weight and BMI (Table 6-1). Weight loss during the first week for the short duration vs. long duration group was 4·0±0·2 % vs. 3·7±0·2 % (p=0.384) and at week four was 9·0±0·5 % vs. 8·4±0·4 % (p=0.355). The mean weight loss over the eight week diet period was very similar in the two groups at 14·8±0·8 % and 14·4±0·7 % respectively (p=0·662). Absolute weight loss and changes in waist and hip circumference are shown in

Table 6-3. Body mass index decreased from 34·1±0·8 to 29·1±0·9 kg/m^2 in the short duration group (p<0·001) and from 34·3±1·2 to 29·4±1·1 kg/m^2 in the long duration group (p=0·001). Overall, the conditions were established to allow direct comparison of the glucose response to weight loss in short and long duration type 2 diabetes.

		Baseline	*Week 1*	*Week 4*	*Week 8*
Weight (kg)	**Short**	99·0±3·7	95·1±3·6	90·2±3·6	84·5±3·5
	Long	96·9±3·8	93·2±3·6	88·6±3·4	83·0±3·2
Waist circ. (cm)	**Short**	110·0±2·3	107·6±2·2	102·9±2·4	98·0±2·5
	Long	113·8±3·0	112·3±2·9	107·0±2·9	101·4±2·9
Hip Circ. (cm)	**Short**	115·7±2·4	114·3±2·5	112·2±2·5	107·9±2·4
	Long	117·3±3·0	115·8±3·1	113·1±3·2	109·9±3·1
Waist:Hip Ratio	**Short**	0·95±0·02	0·94±0·02	0·93±0·02	0·91±0·02
	Long	0·97±0·02	0·97±0·02	0·95±0·02	0·92±0·02

Table 6-3 Change in anthropometry over the 8 week VLCD in both short and long duration type 2 diabetes.

Glucose control

Over the eight week VLCD fasting plasma glucose decreased from 9·6±0·7 to 5·8±0·2 mmol/l in the short duration group ($p<0·001$) and 13·4±0·8 to 8·4±1·1 mmol/l in the long duration group ($p<0·001$). The glucose response to acute calorie restriction was strikingly heterogeneous in long duration diabetes (Figure 6-1B), with some responding within one week just as in the short duration group (Figure 6-1A) and some not at all. In 3/14 (21%) of the long duration group, a slow, steady return to non-diabetic fasting plasma glucose levels occurred and this was not observed in any of the short duration group. This is reflected in the difference in the fall in fasting glucose levels between week one and week eight of the VLCD between the groups; this being significantly greater in the long duration group compared to the short duration group: 2·6±0·6 vs. 1·1±0·3 mmol/l ($p=0·039$).
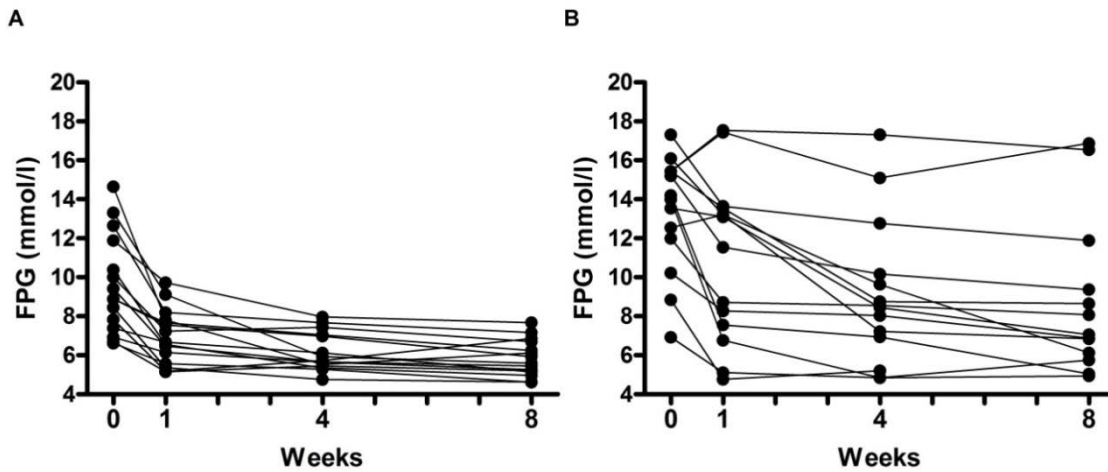
Figure 6-1 Change in fasting plasma glucose levels over the eight week VLCD for each individual with short duration type 2 diabetes (A) and long duration type 2 diabetes (B).

At week 8, 87% of the short duration group and 50% of the long duration group achieved a fasting plasma glucose level of <7 mmol/l. The baseline differences in those with long duration diabetes between responders and non-responders are shown in

Table **6-4**. The non-responders had higher baseline fasting plasma glucose levels and lower maximal insulin secretory capacity.

	Long duration Responders (n=7)	Long duration Non-responders (n=7)	p value
HbA1c (mmol/mol)	67.3 ± 6.8	73.3 ± 4.7	0.483
Baseline FPG (mmol/l)	11.8 ± 1.2	14.9 ± 0.6	0.048
ALT (U/l)	26.0 (12.0-151.0)	22.0 (19.0-61.0)	0.277
Triglycerides (mmol/l)	1.3 ± 0.1	1.7 ± 0.3	0.263
Insulin (mU/l)	12.0 ± 3.5	7.1 ± 1.2	0.212
C-peptide (nmol/l)	0.77 ± 0.08	0.67 ± 0.10	0.426
B-hydroxybutyrate (mmol/l)	0.2 (0.1-0.2)	0.2 (0.1-1.1)	0.482
NEFA (mmol/l)	0.61 ± 0.07	0.77 ± 0.06	0.113
Hepatic triglyceride (%)	9.6 ± 2.0	6.8 ± 1.9	0.338
Pancreatic triglyceride (%)	7.0 ± 1.5	6.0 ± 0.6	0.534
Hepatic IR index ($mmol.min^{-1}.kg_{ffm}^{-1}.pmol.l^{-1}$)	1.45 ± 0.26	1.01 ± 0.15	0.160
Basal HGP ($mg/kg_{ffm}/min$)	3.77 ± 1.75	3.77 ± 0.41	0.994
Hepatic VLDL-TG production rate (mg/kg/day)	147.8 ± 22.9	143.4 ± 18.7	0.882
First phase insulin response ($nmol\ min^{-1}\ m^{-2}$)	0.037 ± 0.026	0.005 ± 0.003	0.234
Maximal insulin secretory capacity ($nmol\ min^{-1}\ m^{-2}$)	0.352 ± 0.055	0.169 ± 0.042	0.022

Table 6-4 Baseline differences in the long duration group between those achieving a fasting plasma glucose <7 mmol/l following the VLCD (responders) and those achieving a fasting plasma glucose >7 mmol/l (non-responders).

Diabetes duration correlated with fasting plasma glucose at week 8 (Spearman rank 0·501; p=0·006;

Figure **6-2**). If diabetes duration was <4 yr, mean achieved fasting plasma glucose level at week eight of the VLCD was 5·8±0·2 mmol/l; for diabetes

duration 8-12 yr this was 6·2±0·7 mmol/l; and for diabetes duration ≥12 yr this was 10·6±1·7 mmol/l.
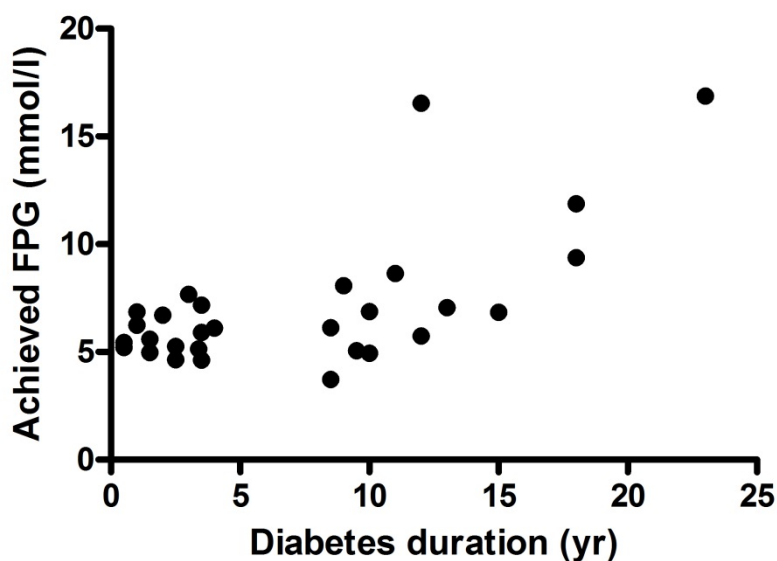
Figure 6-2 Achieved fasting plasma glucose levels at the end of the 8 week VLCD according to type 2 diabetes duration.

HbA1c decreased from 55±2 mmol/mol (7.2±0.2 %) to 44±2 mmol/mol (6.1±0.2 %) in the short duration group ($p<0·001$) and from 70±4 mmol/mol (8.6±0.4 %) to 64±6 mmol/mol (8.0±0.5 %) in the long duration group ($p=0·276$). Although the HbA1c levels would not completely reflect change over the relatively short period of the study, non-diabetic levels (<43 mmol/mol) were achieved at week eight in 6/15 (40%) of the short duration group and 2/14 (14%) of the long duration group. Fasting beta-hydroxybutyrate levels increased from 0.1 (0.1-0.3) to 0.8 (0.2-4.6) mmol/l; $p=0.001$ in those with short duration disease and from 0.2 (0.1-1.1) to 1.0 (0.2-4.6) mmol/l; $p=0.001$ in those with long duration disease. There was no difference between the fasting beta-hydroxybutyrate levels in the groups at week 8 ($p=0.300$).

Basal energy expenditure decreased in both groups after the VLCD (Short: 2008.1±96.8 to 1708.2±93.2 kcal/day; $p<0.001$ and Long: 1958.4±80.3 to 1587.5±39.6 kcal/day; $p<0.001$). There was no change in respiratory quotient in either group (Short: 0.78±0.01 to 0.77±0.01; $p=0.795$ and Long: 0.79±0.01 to 0.78±0.02; $p=0.393$).

Blood pressure control

Blood pressure improved markedly and similarly in both groups (short vs. long duration): systolic: 144±5 to 125±5 mmHg (p=0·003) vs. 160±7 to 133±6 mmHg (p<0·001); and diastolic: 91±2 to 82±3 mmHg (p=0·007) vs. 90±2 to 80±3 mmHg (p=0·003). This improvement was sufficient to allow antihypertensive dose reduction in 1/6 (17%) and 7/11 (64%) of the short duration and long duration groups respectively who were on anti-hypertensives at baseline.

Serum lipid profile

Total cholesterol improved from 4·6±0·2 to 3·6±0·2 mmol/l (p=0·004) and 4·8±0·3 to 3·7±0·3 mmol/l (p<0·001) in the short and long duration groups respectively. The reduction was due to a decrease in non-HDL cholesterol from 3·5±0·2 to 2·5±0·2 mmol/l (p=0·004) and from 3·4±0·3 to 2·4±0·3 mmol/l (p<0·001) in the short and long duration groups respectively. There was no change in HDL cholesterol in either group (1·1±0·1 to 1·1±0·1 mmol/l; p=0·322 and 1·4±0·1 to 1·3±0·1 mmol/l; p=0·187). Triglycerides improved from 1.5 (0.7-8.1) to 0.9 (0.6-1.9) mmol/l (p=0·002) and 1·3 (0.5-3.3) to 1·0 (0.6-2.1) mmol/l (p=0·005). There was no correlation between decrease in fasting triglycerides and glucose response in either the short duration (Pearson 0·371; p=0·174) or long duration groups (Pearson 0·077; p=0·793).

6.4 Discussion

Long duration type 2 diabetes is characterised by higher fasting glucose levels and pancreatic triglyceride content, and lower fasting insulin and C-peptide levels, hepatic triglyceride content and hepatic insulin resistance compared to short duration type 2 diabetes. The glucose response to a VLCD correlated with diabetes duration, and at week 8, 50% of people with long duration and 87% with short duration type 2 diabetes returned to non-diabetic fasting glucose levels despite withdrawal of all anti-diabetic therapies. The glucose response to acute calorie restriction in long duration diabetes was heterogeneous in contrast to the uniformly early fall in plasma glucose levels seen in those with short duration diabetes. Irrespective of

diabetes duration, clinically important improvements in blood pressure and lipid profile occurred. In those with long duration type 2 diabetes, lack of glucose response was characterised by higher baseline fasting glucose levels and decreased maximal insulin secretory capacity compared to those who responded.

The observation of steady loss of beta cell function in the UK Prospective Diabetes Study was made during the usual natural history of type 2 diabetes, that is during progressive weight gain (UKPDS, 1995a). Reports of normalisation of blood glucose control after bariatric surgery raised the possibility that negative calorie balance could restore normal physiology, however this was widely assumed to be surgery-specific and related to change in gut hormone profiles (Guidone *et al.*, 2006; Laferrere *et al.*, 2007; Bradley *et al.*, 2012). Direct comparison of gastric bypass with negative calorie balance alone has suggested that the latter is primarily responsible for the improvement in glucose handling (Jackness *et al.*, 2013; Lingvay *et al.*, 2013). The Counterpoint study was designed to test the hypothesis that negative calorie balance would decrease fat levels in liver and pancreas and that this would be associated with normalisation of liver insulin sensitivity and beta cell insulin secretion (Lim *et al.*, 2011). A return to normal of both parameters was observed in step with a fall in intra-organ fat levels, with complete normalisation of liver within seven days and return of beta cell function gradually over eight weeks. However, that study had been designed to examine the pathophysiological mechanisms underlying reversibility of type 2 diabetes, and to ensure a homogenous study group all were selected to have diabetes duration of less than four years. The clinical question of whether the observations may have more widespread applicability was raised immediately on publication (Yki-Järvinen, 2011). The present therapeutic study was therefore carried out to determine how people with longer term type 2 diabetes would respond to a very low calorie diet.

In keeping with the findings from the Counterpoint Study, the current study demonstrated a rapid fall in fasting plasma glucose in all participants with short duration type 2 diabetes. This is in contrast with the variable response

in those with long duration diabetes, ranging from rapid normalisation of fasting glucose levels to no glucose response to acute calorie restriction. Four individuals with long duration diabetes showed a distinct, intermediate response of a slow steady decrease in glucose levels over the 8 week VLCD which ultimately entered non-diabetic levels. The demonstration of potential for diabetes reversal using a VLCD in approximately half of those with longstanding disease is in keeping with observations following bariatric surgery. A study of 110 individuals with type 2 diabetes undergoing Roux-en-Y gastric bypass found a remission rate of 44% for those with diabetes duration of more than eight years (Hall *et al.*, 2010).

Beta cell dysfunction and relative insulin deficiency (demonstrated by lower fasting insulin and higher NEFA levels and reduced maximal insulin secretory capacity) seem to be the key features defining both longer duration type 2 diabetes and individuals less likely to achieve non-diabetic fasting glucose levels after VLCD. In non-diabetic humans senescence has been associated with a progressive decline in basal insulin release (Iozzo *et al.*, 1999). However, small studies of stimulated C-peptide response in diabetes of longer duration have suggested significant inter-individual variation (Zangeneh *et al.*, 2006; Jain *et al.*, 2008). It may be that diabetes duration is simply a surrogate and insensitive marker for insulin secretory capacity, which is the major determinant of the potential therapeutic utility of a VLCD from a glucose perspective.

A previous post-mortem study on human pancreata demonstrated that adipose tissue content was positively correlated with age (Schmitz-Moormann *et al.*, 1981). Another study using multidetector-row CT scanning with measurement of Hounsfield units demonstrated that as duration of type 2 diabetes increases, pancreatic volume decreases and pancreatic fat content increases, with mean age being the same in all groups at 53 years (Lim *et al.*, 2014). In the current study, the long duration group had higher pancreatic triglyceride content and lower hepatic triglyceride content when compared with the short duration group. This is in keeping with a previous

study using the same 3 point Dixon method, which showed that hepatic and pancreatic triglyceride content were positively correlated in those with normal glucose tolerance but that this association was lost in type 2 diabetes (Macauley *et al.*, 2015b). As diabetes duration increases and insulin secretory capacity declines, lower portal insulin levels are likely to result in diminution of hepatic *de novo* lipogenesis. In this situation, ongoing export of $VLDL_1$-triglyceride from the liver could result in this divergence of hepatic and pancreatic triglyceride levels. Higher pancreatic triglyceride content coupled with decreased beta cell mass in individuals with long duration diabetes may be an explanation for variable gradual glucose response to the VLCD in some individuals in the long duration group. It could be hypothesised that it would take longer to mobilise the increased fat content in the pancreata of those with long duration disease. If this were to relieve the toxic burden on the beta cells, and if there was sufficient remaining beta cell mass, a critical level of insulin secretion necessary to regain normal fasting glucose levels could be recovered.

Although a VLCD strategy has been incorporated into NICE guidelines on obesity, this has not yet been widely taken up despite demonstration of efficacy and durability when applied in routine primary care (Ross *et al.*, 2008; NICE, 2014). This apprehension in using a VLCD may relate to perceived low adherence, concerns about sustainability of effects and theoretical concerns about detrimental effects on lipid profile (RCGP, 2002). The use of nutritionally complete diet products has been extensively reviewed and are considered safe (Mustajoki and Pekkarinen, 2001). The present study provides clear information on acceptability and metabolic state. Compliance with the VLCD is high, at least in motivated individuals, as judged by the hard end-point of substantial weight loss, with only one of 30 participants unable to complete the study. Follow-up of the Counterpoint study at three months after completing the VLCD showed that 7 of the 11 participants were not diabetic on oral glucose tolerance testing despite weight gain of 4kg (Lim *et al.*, 2011). That study had not been designed as a therapeutic study and no follow up support had been provided. Following publication of the Counterpoint study the extent of the enthusiasm of some

people with type 2 diabetes to take major steps to escape from diabetes became clear in the very large scale email feedback (Steven *et al.*, 2013). Amongst the email cohort of those who lost weight and returned to normal glucose tolerance, duration of normal glucose control in some now approaches three years (Peters *et al.*, 2015). Provided that weight loss is maintained, there is hope that diabetes will not return at least over several years and it is now important to establish the durability of the effect on glucose control.

The overall health benefits of the VLCD were both striking and appreciated by all participants. These included lower blood pressure, improvement in lipid profile, enhanced general wellbeing, and better mobility. The 19-27 mmHg improvement in systolic blood pressure and 9-10 mmHg improvement in diastolic blood pressure is comparable to addition of two antihypertensive agents at usual dose (Arauz-Pacheco *et al.*, 2002). The improvement in total cholesterol seen in this study is similar to that observed with full dose statin therapy (MRC/BHF, 2003). Together these improvements in blood pressure and lipids are likely to make a consequent major reduction in cardiovascular events in individuals with diabetes.

The limitations of the current study must be considered. Diabetes duration is an imprecise entity and some individuals may have a prolonged period of unrecognised hyperglycaemia preceding a diagnosis. The best information suggests that fasting glucose levels rise only gradually over many years preceding a diagnosis, but in the final 12-24 months increase rapidly (Sattar *et al.*, 2007; Tabák *et al.*, 2009). In the current study, the date of diagnosis reported by participants was verified from medical records and any subclinical period would be equally likely in each group and would if anything, underestimate duration. Secondly, the diagnosis of type 2 diabetes itself depends upon exclusion of other possible diagnoses. Participants were recruited if diabetes was detected through a routine test and excluded if diagnosis was precipitated by severe osmotic symptoms. Thorough history taking was used to exclude those with clinical features suggestive of latent autoimmune diabetes in adults (LADA) or maturity onset diabetes of the

young (MODY). Thirdly, the baseline levels of fasting plasma glucose were necessarily different in the groups defined by duration of type 2 diabetes. Additional matching for glycaemic control or treatment for diabetes would have resulted in an atypical group as defined by duration of diabetes. Finally, the proportion of these research participants successfully completing the VLCD cannot be extrapolated to the general population with the condition. Individuals in the study were highly motivated to succeed in losing weight and a selection bias will have operated. However, even if only a modest proportion of the affected population was able to follow this treatment, the impact upon health service budgets would be substantial.

In conclusion, in individuals with type 2 diabetes of duration greater than eight years, a therapeutic trial of very low calorie diet may be undertaken with a 50% chance of achieving non-diabetic fasting plasma glucose levels off all anti-diabetic therapies. For those who do not achieve non-diabetic plasma glucose levels, blood pressure, lipids and general wellbeing, will be considerably improved. These insights provide quantitative evidence for the potential for use of a VLCD in long duration type 2 diabetes and carry implications for everyday clinical practice. The critical factor which seems to differentiate long duration from short duration type 2 diabetes, and determine reversibility in the long duration group is baseline glucose control and insulin secretory capacity. Individuals with long duration diabetes have higher pancreatic triglyceride content compared to short duration disease and the potential relationship of this to beta cell function requires further investigation.

Chapter 7. Durability of the Pathophysiological Changes Associated with Reversal of Type 2 Diabetes Following a VLCD

7.1 Introduction

The benefits of significant weight loss achieved through an intensive lifestyle intervention on glycaemic control in type 2 diabetes are well recognised (Henry *et al.*, 1985; Pi-Sunyer *et al.*, 2007; Gregg *et al.*, 2012). However, the major challenge to most patients and clinicians is long-term maintenance of weight loss rather than achieving initial significant weight loss. It is thought that only 20% of individuals manage to lose 10% of body weight and maintain this for at least 1 year (Wing and Hill, 2001). One advantage of bariatric surgery over a very low calorie diet (VLCD) in the management of type 2 diabetes is the increased propensity for maintenance of long term weight reduction. There are now long term studies going out to 15 years demonstrating only slight weight gain and that although diabetes remission rates decrease over time, bariatric surgery has superior sustained benefit compared to conventional lifestyle and pharmacological treatment with an odds ratio of 6.3 (Sjostrom *et al.*, 2014). Previously there has been clear demonstration of recidivism of weight loss following VLCD in individuals with type 2 diabetes (Paisey *et al.*, 2002). However, more recent reports suggesting the potential widespread applicability (Steven *et al.*, 2013) and durability of the benefit of a VLCD to at least 3 years (Peters *et al.*, 2015) have renewed interest in their potential role in the therapeutic armamentarium for the treatment of type 2 diabetes.

The rapid beneficial effect of a very low calorie diet on metabolism has been recognised for decades (Henry *et al.*, 1985), however, they have never been incorporated into routine diabetes clinical practice. Use of VLCD has recently become part of national guidance regarding obesity management in specific clinical scenarios, as part of a multidisciplinary weight management strategy (NICE, 2014). Initial safety concerns are no longer applicable with modern nutritionally complete VLCD products (Basciani *et al.*, 2015). There is now a clear understanding of the pathophysiological changes occurring during acute calorie restriction in individuals with type 2 diabetes (Lim *et al.*, 2011). Within 7 days, hepatic triglyceride content decreases markedly. Within the liver, the close relationship between triglyceride content and

hepatic glucose output, the primary determinant of fasting blood glucose, is well established (Petersen *et al.*, 2005; Ravikumar *et al.*, 2008) and within 7 days of VLCD hepatic insulin sensitivity improves to that of weight matched glucose tolerant controls. By the end of an 8 week VLCD recovery of first phase insulin secretion is seen alongside a reduction in pancreatic triglyceride content. However, these changes were demonstrated in the metabolic milieu of ongoing calorie restriction (Lim *et al.*, 2011). Also, this study involved 11 individuals with short duration, well controlled type 2 diabetes. Of great clinical relevance and import is the wider applicability of a VLCD as a treatment strategy in the wider, heterogeneous type 2 diabetes population. Also, whether the effect of the VLCD on metabolism is maintained after normal eating resumes, and crucially whether the benefit is maintained in the longer term with successful maintenance of weight loss.

The twin cycle hypothesis of type 2 diabetes links excess hepatic triglyceride and exposure of beta cells to excess fatty acid through the export of $VLDL_1$-triglyceride from liver to pancreas (Taylor, 2008). NEFA are major substrates for hepatic $VLDL_1$-triglyceride production and this may be a substrate driven process. Weight loss has been shown to reduce the secretion of $VLDL_1$-triglyceride from the liver (Klein *et al.*, 2006). However, this effect is variable suggesting that weight loss induced alterations in hepatic $VLDL_1$-triglyceride secretion are regulated by factors other than weight loss alone. There is now strong evidence for the detrimental effect of metabolites of triglyceride, namely diacylglycerol, on both insulin secretion and insulin action directly and via ceramide activation of novel and/or atypical protein kinase C's which affect insulin signalling and apoptosis (Samuel *et al.*, 2010; Stretton *et al.*, 2010).

Studies in bariatric surgery have identified clinical characteristics which are associated with reversal or persistence of diabetes, but understanding the pathophysiologic limitations to diabetes reversal is crucial in furthering our understanding of the pathophysiology of type 2 diabetes. Moreover, understanding the limitations to diabetes reversal will help to individualise

therapy in type 2 diabetes and target use of a VLCD and/or bariatric surgery towards those most likely to benefit.

The aim of this study was to test the hypothesis that individuals who achieve non-diabetic fasting blood glucose levels after a VLCD will remain normoglycaemic in the long term provided that weight regain is avoided. The physiological parameters defining reversibility of type 2 diabetes will be investigated along with the durability of these changes.

7.2 Study design

7.2.1 Participants

30 individuals with type 2 diabetes were identified in response to local advertisement (as per chapter 6). Exclusion criteria were loss of more than 5kg body weight in the preceding 6 months, treatment with thiazolidinediones, GLP-1 agonists, steroids or atypical anti-psychotic medications, untreated thyroid disease, renal dysfunction (serum creatinine >150 μmol/l), contraindication to MR scanning or alcohol consumption >3 units per day for women and >4 units per day for men. The history of the diagnosis of diabetes was taken carefully to clinically exclude alternate diagnoses than type 2 diabetes. Participants discontinued all anti-diabetic therapy prior to the baseline study (as described in chapter 6) but remained on their usual lipid lowering treatment. Anti-hypertensive medications were decreased as necessary throughout the study. As per a previous study, weight loss of >2.8% body weight at day 7 of the VLCD was considered necessary to continue in the study (Lim *et al.*, 2011). One participant did not meet this weight loss target and left the study after week one, hence the final participant number of 29: age 56.7±1.8 yr, weight 98.0±2.6 kg, BMI 34.2±0.7 kg/m², HbA1c 62.4±2.7 mmol/mol, diabetes duration 4 (0.5-23) yr. The study protocol was approved by Newcastle and North Tyneside 2 Ethics Committee and all participants gave informed written consent.

7.2.2 Experimental protocol

This prospective, longitudinal, single centre study comprised 3 phases: an 8 week weight loss phase using a VLCD; a stepped return to isocaloric intake

of normal food over 2 weeks; and a 6 month structured, individualised weight maintenance program. Assessments were carried out on 3 occasions: at baseline prior to the VLCD, at week 10 following a gradual return to a diet isocaloric with their achieved weight, and then at the end of the 6 month weight maintenance period. Studies were performed after an overnight fast and took place over 2 days. On the first half day, individuals underwent assessment of body composition with electrical bioimpedence using a Bodystat®1500 (Bodystat Ltd, Isle of Man, UK) (see section 2.1.3) and then measurement of pancreatic and hepatic triglyceride content and visceral and subcutaneous adipose tissue areas using the 3 point Dixon method on a 3 Tesla Achieva MRI scanner (Philips, Best, The Netherlands) (see section.2.2.2 & 2.2.3). Thereafter, cannulae were inserted into both antecubital veins for infusion and blood sampling respectively. Assessment of VLDL$_1$- triglyceride production was then performed using competitive blockade of lipoprotein lipase with an Intralipid infusion (see section 2.3.8). Baseline physical activity levels (MET-min/week) were measured using a standard questionnaire (*International Physical Activity Questionnaire*). On the second day, after an overnight fast, a cannula was inserted into an antecubital vein for infusions and a second cannula was inserted into the contralateral wrist vein for arterialised blood sampling. Assessment of hepatic glucose production and insulin sensitivity were carried out using an isoglycaemic hyperinsulinaemic clamp (see sections 2.3.3 & 2.3.4). Isoglycaemia rather than euglycaemia was selected to ensure that the true fasting condition of each participant could be observed at each time point given the anticipated change in fasting glucose between the first two studies. Following the clamp study participants rested until stable fasting glycaemia had been restored (and a minimum of 60 minutes) and then a stepped insulin secretion test was used to assess first phase insulin secretion and maximal insulin secretory capacity in response to arginine (see section 2.3.5). PNPLA3 genotyping was performed on DNA extracted from white blood cells. 10 ml of whole blood was collected in EDTA and after thorough mixing was then stored at -40°C. DNA was isolated and genotyping performed (blinded to the clinical parameters) using TaqMan

SNP Genotyping Analysis (Applied Biosystems, USA) as described previously (section 2.3.7) (Liu *et al.*, 2014).

7.2.3 Weight loss phase

The VLCD consisted of a liquid formula diet (43% carbohydrate, 34% protein and 19.5% fat; 2·6 MJ/day [624 kcal/day]; Optifast; Nestlé Nutrition, Croydon, UK) taken as 3 sachets per day. In addition, up to 240g of non-starchy vegetables were consumed, making total daily energy intake 624-700 kcal. Participants were given suitable vegetable recipes, were encouraged to drink at least two litres of calorie-free beverages per day and to maintain their habitual level of physical activity. To maximise adherence to the diet, one-to-one support was provided weekly and as required by telephone, e-mail, text message or face-to-face contact. Stepped reintroduction of food took place over one week with sachets gradually being replaced by solid foods. Isocaloric intake was determined from resting energy expenditure measured by indirect calorimetry using an open circuit calorimeter (Quark RMR; COSMED, Rome, Italy) and a canopy hood at week 8 (section 2.1.4). The post-VLCD studies were conducted a minimum of 6 days after full return to solid foods. At the week 10 studies, the standard threshold for remission of diabetes (fasting plasma glucose <7 mmol/l) was used to define the group of responders (Buse *et al.*, 2009).

7.2.4 Weight maintenance phase

The primary goal of this phase was to prevent weight regain through use of a structured individualised weight maintenance program. The isocaloric diet was individualised both in respect of personal food preferences and in relation to observed body weight. Dietary advice was guided by weight trajectory and participants were asked to weigh themselves weekly. The individualised program was delivered by two investigators (myself and a health psychologist (LA)), and was based on goal setting, action planning and barrier identification with reviews at monthly visits (Michie *et al.*, 2011). Participants were given verbal and written information including a daily calorie prescription, informed by indirect calorimetry. Individualised

daily meal plans, recipes and snack ideas were provided. During the 6 month weight maintenance phase, monthly assessments of weight, waist and hip circumference, blood pressure and fasting blood tests were made. In addition, all participants were encouraged to maintain telephone/email or text message contact between monthly visits with updates on progress or to discuss challenges. If fasting plasma glucose exceeded 10 mmol/l on 2 occasions, anti-diabetic agents were recommenced. Physical activity was encouraged but the primary focus was on weight maintenance. Physical activity levels were measured objectively using a validated multisensor array (SenseWear Pro$_3$; BodyMedia Inc., Pittsburgh, USA) worn for 5 days after the VLCD and then at the end of the weight maintenance period. Data were analysed using the InnerView® Professional software which contains activity detection and lifestyle algorithms based on gender, age, weight, height, smoking status and hand dominance.

7.2.5 Statistical analysis

Statistical analysis was performed using Minitab 16 statistical program (Minitab Inc.; State College, PA: www.minitab.com). Prior to any comparison, the data were tested for normality using the Kolmogorov Smirnov test. Data are presented as mean ± SEM for parametric and median (range) for non-parametric data. Insulin secretion rates and hepatic insulin resistance indices are expressed as median and 25th and 75th percentile. Statistical analysis used Student's paired and 2-sample t-test, Mann Whitney U, Wilcoxon Rank and Spearman Rank correlation as appropriate. Statistical significance was accepted at $p<0.05$.

7.3 Results

7.3.1 All participants: weight loss and glucose response

Weight fell from 98.0±2.6 kg at baseline to 83.8±2.4 kg following the VLCD ($p<0.001$) and remained stable for the subsequent 6 month period (84.7±2.5 kg). Fasting plasma glucose decreased from 11.9 (6.6-17.3) to 7.3 (5.4-22.3) mmol/l ($p<0.001$) then remained stable at 7.2 (4.7-15.9) mmol/l. HbA1c decreased from 62.5±2.7 to 54.2±3.7 mmol/mol ($p=0.020$) and then remained

stable at 53.7±2.8 mmol/mol. Resting energy expenditure decreased from 1983.3±61.9 at baseline to 1647.8±51.1 kcal/day at week 8 (end of the VLCD) (p<0.001) and remained stable over the weight maintenance period 1706.6±49.9 kcal/day. There was no change in physical activity levels during the weight maintenance period; measured as metabolic equivalents of task (METs): 1.1 (0.9-1.6) to 1.3 (0.9-1.7); p=0.355; daily step count: 8139±622 to 8450±617 steps; p=0.488; light activity time (1.5-3.0 METS) per day: 210±14 to 214±12 min; p=0.676; or as sedentary time per day: 19.5±0.4 to 19.1±0.3 hr; p=0.246.

After the VLCD, 41% of the group (12/29) achieved a fasting plasma glucose of <7.0 mmol/l and were defined as responders. At the end of the weight maintenance period, 45% of the group (13/29) had a fasting plasma glucose <7 mmol/l off all oral hypoglycaemic agents or insulin. In the responder group, 9 were from the short duration group and 3 from the long duration group.

7.3.2 Responders vs. non-responders: weight loss & glucose response

There was no significant difference in achieved weight loss after VLCD between the responders and non-responders (15.8±0.5 vs. 13.6±0.7 % respectively; p=0.057). Weight remained constant over 6 months in both responders (84.1±3.1 to 84.4±3.2 kg; p=0.826) and non-responders (83.6±3.5 to 84.8±3.7 kg; p=0.198) (Figure 7-1A). In the responders, fasting plasma glucose fell from 8.9±0.7 to 6.2±0.1 mmol/l (p=0.002) post-VLCD then remained constant at 6.2±0.3 mmol/l (Figure 7-1B). In the non-responders, fasting plasma glucose fell from 13.2±0.6 to 10.9±1.1 mmol/l (p=0.016) post-VLCD and remained constant at 9.4±0.7 mmol/l. In the non-responder group 6 individuals restarted medication during the 6 month weight maintenance period: metformin only (n=2), metformin and sulfonylurea (n=3) and insulin (n=1) but all required less treatment at the end of the study than at baseline. The increase in fasting plasma glucose during solid food reintroduction (between week 8 and week 10) was significantly greater in the non-responders compared to the responders (2.4±0.4 vs 1.0±0.3 mmol/l; p=0.019) (Figure 7-1B). This occurred despite no weight gain in the non-

responders and only minimal weight gain in the responders (-0.18±0.27 vs. 0.43±0.42 kg; p=0.208). HbA1c remained stable throughout the weight maintenance period in both groups (responders: 40±2 to 41±2 mmol/mol; p=0.543) and non-responders: 64±5 to 62±3 mmol/mol; p=0.505) (Figure 7-1C).
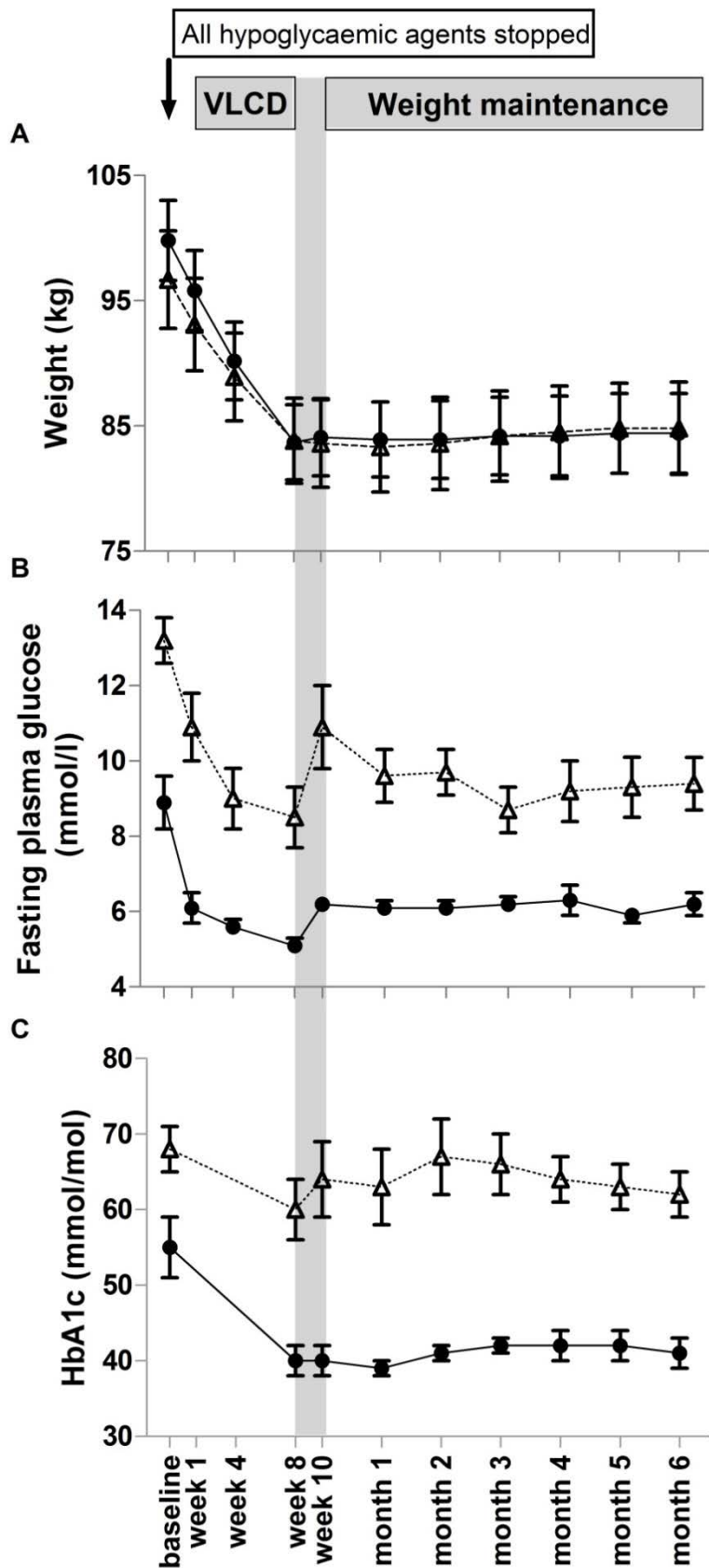
Figure 7-1 Change in weight (A), fasting plasma glucose (B) and HbA1c (C) over the study in responders (circles) and non-responders (triangles). The grey band represents the stepped transition from VLCD to isocaloric solid foods. Results are mean ± SEM.

7.3.3 Responders vs. non-responders: insulin secretion

Fasting insulin levels decreased in both groups following the VLCD and stayed stable during the weight maintenance period (Table 7-1). First phase insulin response improved after the VLCD in the responders: 0.11 (0.00-0.19) to 0.19 (0.15-0.23) nmol min^{-1} m^{-2} (p=0.025) and did not change over the weight maintenance period (0.21 (0.15-0.31) nmol min^{-1} m^{-2}) (Figure 7-2). There was only a marginal increase in first phase insulin secretion in the non-responder group (0.01 (0.00-0.02) to 0.03 (0.00-0.04) nmol min^{-1} m^{-2}; p=0.039, with no change during weight maintenance (0.02 (0.01-0.05) nmol min^{-1} m^{-2}). There was no significant change in maximal insulin secretory capacity (baseline to peak insulin secretion rate in response to arginine bolus) in the responders following the VLCD (0.57 (0.45-0.94) to 0.70 (0.46-0.92) nmol min^{-1} m^{-2}; p=0.969) or after the weight maintenance period (0.67 (0.57-1.09) nmol min^{-1} m^{-2}) (p=0.255 from baseline). There was no change in maximal insulin secretory capacity in non-responders either: 0.19 (0.15-0.30) to 0.18 (0.13-0.41) nmol min^{-1} m^{-2} following VLCD (p=0.538) and then 0.32 (0.10-0.37) nmol min^{-1} m^{-2} (p=0.242 from baseline).

	Responders			Non-Responders		
	Baseline	**Post VLCD**	**After 6 months**	**Baseline**	**Post VLCD**	**After 6 months**
BMI (kg/m²)	34.0±0.8	28.6±0.8 *	28.7±0.7 #	34.4±1.1	29.8±1.1 *	30.2±1.1 #
Waist:hip ratio	0.97±0.02	0.93±0.02 *	0.93±0.02 #	0.96±0.02	0.91±0.01 *	0.92±0.01 #
Fat mass (%)	36.2±1.9	30.1±2.0 *	31.5±1.9 #	42.6±2.2 ×	37.2±2.0 *	40.8±2.5
Plasma glucose (mmol/l)	8.9±0.7	6.2±0.1 *	6.2±0.3 #	13.2±0.6 ×	10.9±1.1 *	9.4±0.7 #
Serum insulin (mU/l)	20.4 (5.7-48.1)	7.9 (3.4-16.6) *	7.6 (3.1-31.6) #	9.3 (3.9-48.9)	5.5 (1.4-22.9) *	5.9 (1.2-14.9) #
Serum ALT (U/l)	43 (11-151)	26 (18-42) *	21 (7-27) #	22 (12-61) ×	19 (13-47)	18 (9-33) #
Serum GGT (U/l)	67±16	32±11 *	40±17 #	33±5 ×	19±2 *	27±5
Plasma NEFA (mmol/l)	0.51±0.05	0.46±0.05	0.46±0.04	0.69±0.04	0.53±0.04 *	0.53±0.03 #
β-hydroxybutyrate (mmol/l)	0.1 (0.1-0.3)	0.1 (0.0-0.9)	0.1 (0.0-0.2)	0.2 (0.1-1.1) ×	0.2 (0.1-1.5)	0.1 (0.0-0.3) #

Table 7-1 Fasting anthropometric and metabolic data in responders and non-responders at baseline, after VLCD, and then after a 6 month weight maintenance period (* = $p<0.05$ for baseline to post-VLCD difference; # = $p<0.05$ for baseline to month 6 difference, and × = $p<0.05$ for between group difference at baseline).
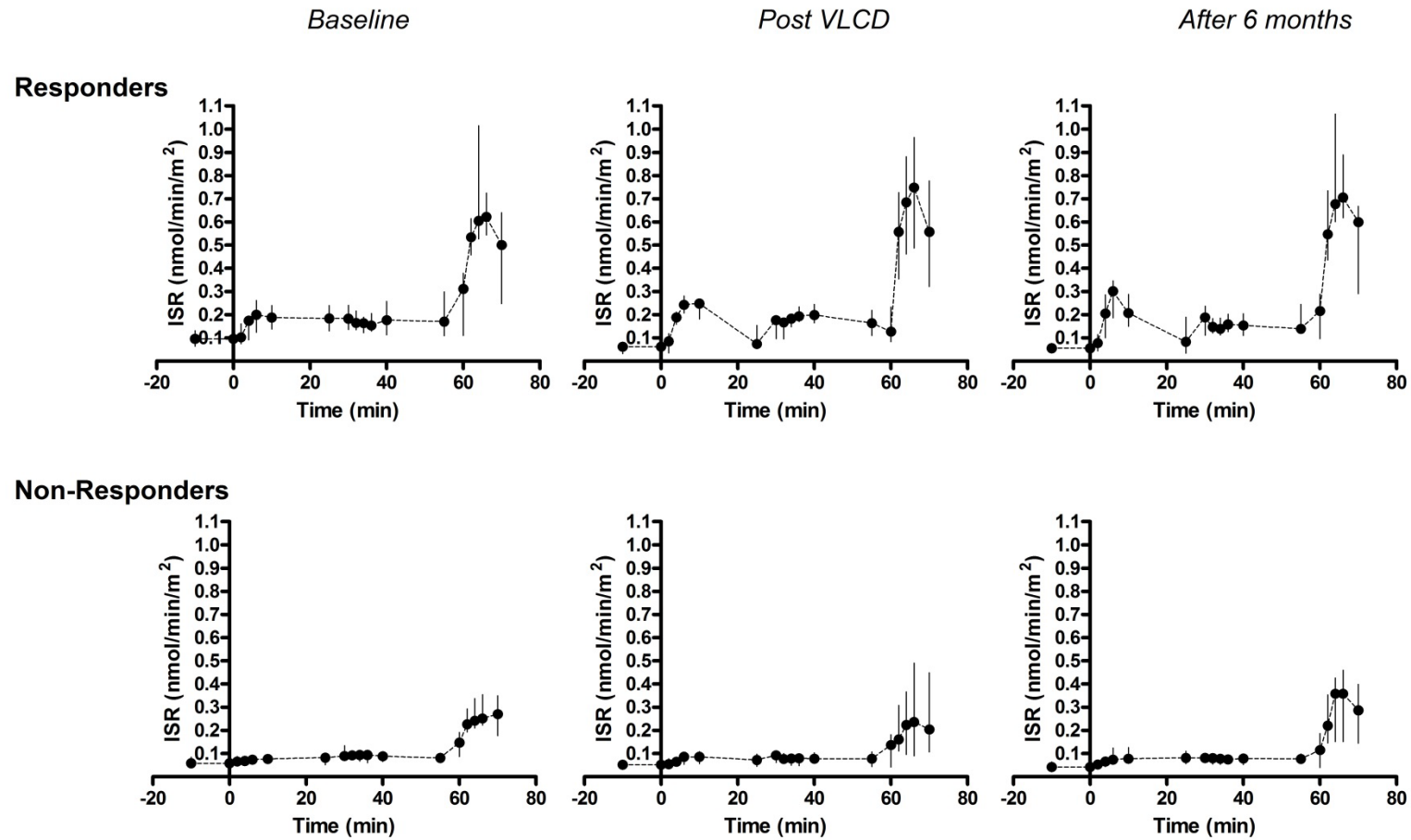
Figure 7-2 Insulin secretion rates (median and interquartile range) during the stepped insulin secretion test in responders and non-responders at baseline, after VLCD and after 6 months weight maintenance.

7.3.4 Responders vs. non-responders: liver metabolism

Serum ALT and GGT levels decreased in both groups and remained stable during weight maintenance (Table 7-1). Basal hepatic glucose production decreased in both groups after the VLCD (responders: 2.58 (2.23-4.01) to 2.38 (2.06-3.46) mg/kg$_{ffm}$/min; p=0.055 and non-responders: 3.33 (2.59-8.10) to 2.97 (2.41-4.46) mg/kg$_{ffm}$/min; p=0.020). Hepatic insulin resistance index also decreased in both groups after the VLCD and remained stable with weight maintenance (responders: 2153 (1199-2484) to 851 (641-1020); p=0.003 and then 750 (662-1010) μmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} and non-responders: 1237 (910-1475) to 773 (424-989); p=0.001 and then 755 (466-1209) μmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1} (

Figure 7-3). Hepatic VLDL$_1$-triglyceride production rate appeared to decrease in both groups following the VLCD and remained stable with maintenance of weight loss (responders: 125.3±22.9 to 102.7±19.6; p=0.148 then 102.0±18.9 mg/kg/day and non-responders: 150.0± 14.0 to 120.3±12.0; p=0.015 then 118.0±12.2 mg/kg/day (Figure 7-3).
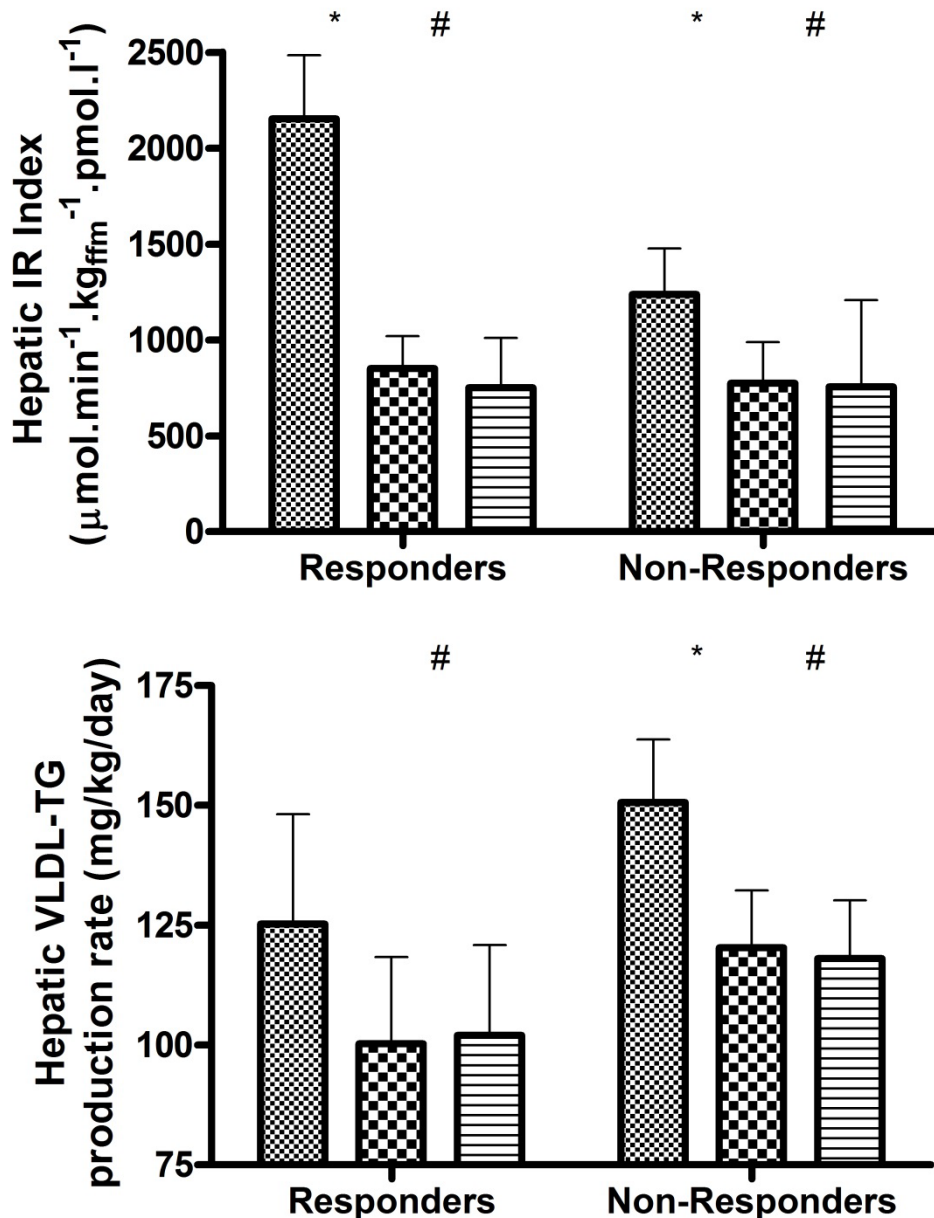
Figure 7-3 Hepatic insulin resistance index (upper panel) and hepatic VLDL₁-triglyceride production (lower panel) in responders and non-responders at baseline (hatched), after VLCD (chequered) and after 6 months weight maintenance (striped). * denotes $p<0.05$ for baseline to post-VLCD difference and # denotes $p<0.05$ for baseline to month 6 difference.

In the whole group (n=29), the rs738409 C to G adiponutrin/PNPLA3 genotype (coding for I148M) was found in 8 individuals: 6 were heterozygous for the SNP: CG (148I/M) and 2 homozygous: GG (148M/M). There was no difference in mean baseline hepatic triglyceride content in wild type compared to individuals with a mutation (9.9±1.2 vs. 10.6±3.9 % respectively; p=0.836). Similarly there was no difference in the achieved hepatic triglyceride content following the VLCD (2.3±0.1 and 2.0±0.1%

respectively; $p=0.189$). Baseline serum triglycerides appeared higher in wild type compared to those with a mutation but the difference was not significant (1.5 (0.7-8.1) vs. 1.2 (0.5-4.8) mmol/l; $p=0.242$). There was no difference in the serum triglycerides following the VLCD (1.2±0.1 in wild type and 1.2±0.2 mmol/l in those with a mutation; $p=0.694$).

7.3.5 Responders vs. non-responders: intra-organ triglyceride content and body composition

Marked normalisation in hepatic triglyceride content was seen in both the responders (12.8±2.7 to 2.2±0.2 %; $p=0.002$) and non-responders (8.2±1.1 to 2.2±0.1 %; $p<0.001$) following the VLCD (Figure 7-4A). There was no re-accumulation of hepatic triglyceride following the 6 month weight maintenance period in either responders or non-responders: 2.1±0.3 vs. 2.3±0.2 % respectively. Following the VLCD, there was a significant decrease in pancreatic triglyceride content in both groups (responders: 5.3±0.4 to 4.5±0.3 %; $p=0.039$ and non-responders: 5.9±0.7 to 5.3±0.5 %; $p=0.004$) (Figure 7-4B). Pancreatic triglyceride content remained stable during weight maintenance in responders and non-responders (4.4±0.3 % and 5.0±0.5 % respectively).

In responders, subcutaneous adipose tissue area decreased from 319.6±31.0 to 232.0±23.1 cm^2 ($p<0.001$) after VLCD then remained stable during weight maintenance at 238.6±20.3 cm^2. Visceral adipose tissue area decreased from 287.0±23.1 to 191.9±18.9 cm^2 ($p<0.001$) and then 179.5 ±22.3 cm^2. In non-responders, subcutaneous adipose tissue area decreased from 285.4±24.7 to 223.3±23.5 cm^2 ($p<0.001$) and remained stable at 219.3±22.8 cm^2 after 6 months and visceral adipose tissue area decreased from 289.6±23.7 to 209.5±22.1 cm^2 ($p<0.001$) and then 198.9±4.8 cm^2 after 6 months.

Hepatic VLDL$_1$-triglyceride production rate did not correlate with baseline hepatic triglyceride content (Pearson=0.143; $p=0.467$) even after exclusion of the main outlier with baseline hepatic triglyceride content of 36% (Pearson= -0.303; $p=0.124$). There was no correlation between hepatic VLDL$_1$-triglyceride production rate and pancreatic triglyceride content at baseline (Spearman = -0.049; $p=0.807$). There was no correlation between the change

in hepatic $VLDL_1$-triglyceride production rate and the change in hepatic triglyceride content or pancreatic triglyceride content over the VLCD (Whole group: Pearson=0.116; p=0.566 and Pearson=0.035; p=0.867 respectively; Responders only: Pearson=0.266; p=0.430 and Pearson=0.049; p=0.894). The absolute change in hepatic triglyceride and pancreatic triglyceride content over the VLCD was weakly and negatively correlated (Spearman rank=-0.384; p=0.044) (Figure 7-4C).

There was no significant correlation between the change in hepatic $VLDL_1$-triglyceride production rate and the change in first phase insulin response between baseline and following the VLCD (Spearman rank 0.316; p=0.109). There was no correlation between the change in hepatic $VLDL_1$-triglyceride production and the change in NEFA between baseline and following the VLCD (Pearson=0.069; p=0.731). Neither was there any correlation between the change in hepatic $VLDL_1$-triglyceride production rate and change in serum triglycerides after the VLCD (Spearman rank=0.269; p=0.175).

The correlation between hepatic $VLDL_1$-triglyceride production rate and fasting plasma glucose only became significant at month 6 (Spearman rank: baseline: 0.179; p=0.362; week 10: 0.322; p=0.095 and month 6: 0.463; p=0.011). There was no correlation between hepatic $VLDL_1$-triglyceride production rate and either fasting serum triglycerides or fasting insulin.
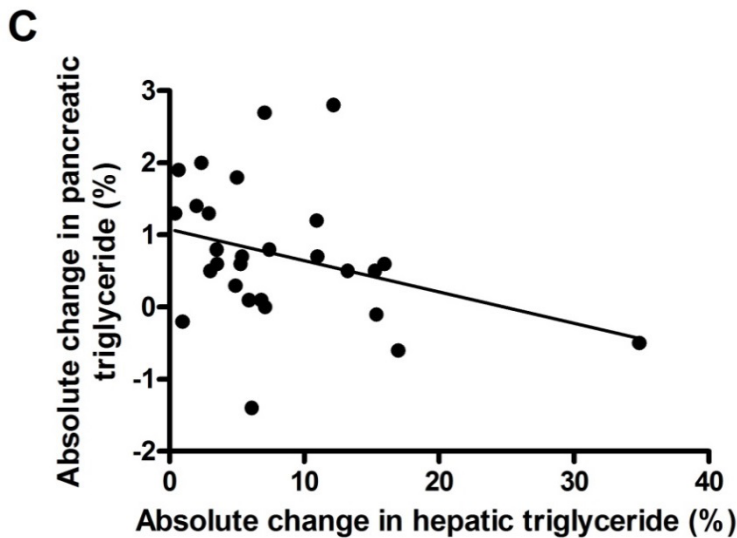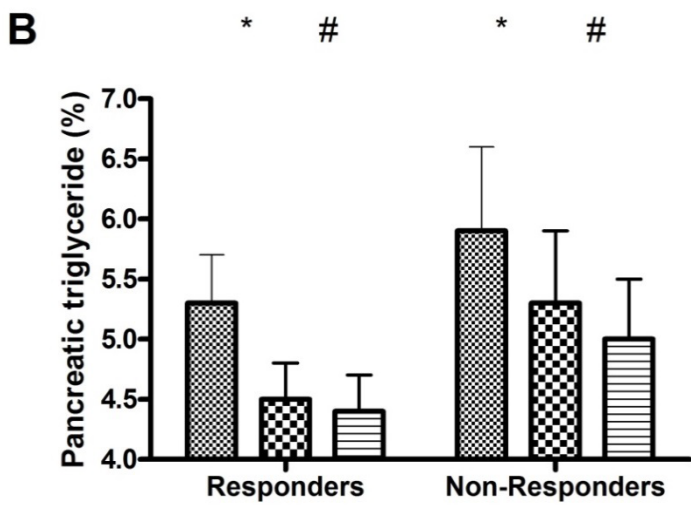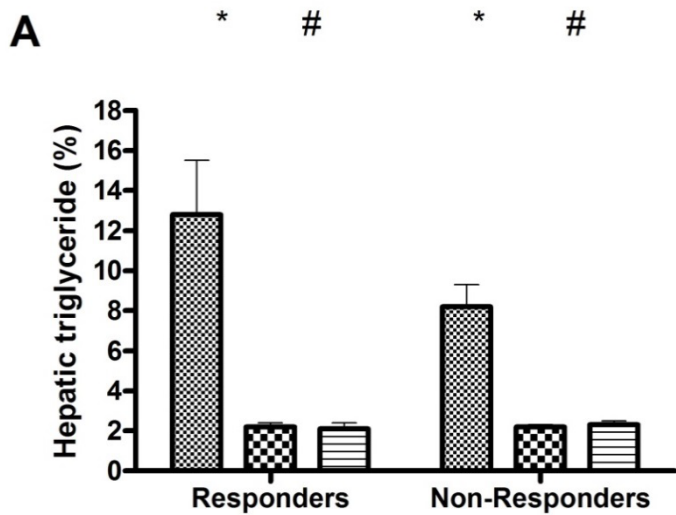
Figure 7-4 Change in hepatic (A) and pancreatic (B) triglyceride content in responders and non-responders at baseline (hatched), after VLCD (chequered) and after 6 months weight maintenance (striped). * denotes $p<0.05$ for baseline to post-VLCD difference and # denotes $p<0.05$ for baseline to month 6 difference. Correlation of absolute change in hepatic and pancreatic triglyceride during the VLCD (Spearman rank=-0.384; p=0.044) (C).

7.3.6 Responders vs. non-responders: peripheral insulin sensitivity

Peripheral insulin sensitivity increased in responders following the VLCD (M_{ffm}: 3.06 (1.55-4.55) to 3.93 (2.11-8.93) mg/kg$_{ffm}$/min; p=0.038) and was 3.29 (2.29-12.35) mg/kg$_{ffm}$/min after weight maintenance. In non-responders the increase following the VLCD was not significant (M_{ffm}: 3.97 (1.25-15.89) to 5.41 (2.12-14.85) mg/kg$_{ffm}$/min; p=0.421 and remained stable after 6 months weight maintenance 5.53 (2.43-22.99) mg/kg$_{ffm}$/min.

7.3.7 Responders vs. non-responders: lipids and blood pressure

Serum triglycerides decreased by 37% in the responders and 23% in the non-responders over the VLCD period (Table 7-2). The significant improvements in total cholesterol and non-HDL cholesterol were also maintained over 6 months. Although plasma NEFA appeared to decrease in both groups, the change was only significant in the non-responder group. HDL-cholesterol appeared to have increased in both groups by the end of the study. There was an approximately 40% reduction in VLDL$_1$ mass following the VLCD in both groups which was sustained with weight maintenance (Table 7-2). The decrease in VLDL$_1$ apoB in both groups was also sustained. The triglyceride to apoB ratio did not change significantly over the course of the study suggesting that the particle size stays the same. There was a clinically significant and sustained improvement in both systolic and diastolic blood pressure in both groups (Table 7-2).

	Responders			Non-Responders		
	Baseline	**Post VLCD**	**After 6 months**	**Baseline**	**Post VLCD**	**After 6 months**
$VLDL_1$-TG (mg/dl)	62.6±12.8	33.4±7.4 *	30.1±3.6 #	37.2 (4.4-431.8)	22.7 (5.0-77.6) *	21.3 (4.6-110.7) #
$VLDL_1$-apoB (mg/dl)	2.6±0.5	1.5±0.3 *	1.4±0.1 #	1.7 (0.3-15.7)	1.2 (0.3-3.2) *	1.0 (0.2-4.8) #
TG:apoB ratio	23.1±0.7	22.6±1.2	20.9±0.9	22.0±1.1	19.8±0.8	21.3±1.1
$VLDL_1$-TG production rate (mg/kg/day)	125.3±22.9	100.3±18.1	102.0±18.9 #	150.6±13.2	120.3±12.0 *	118.0±12.2 #
Triglycerides (mmol/l)	1.97±0.32	1.25±0.16 *	1.15±0.12 #	1.30 (0.50-8.10)	1.00 (0.60-2.00) *	1.20 (0.50-3.10) #
Total cholesterol (mmol/l)	4.7±0.3	3.9±0.2 *	4.2±0.3 #	4.7±0.3	4.0±0.3 *	4.2±0.2 #
Non-HDL cholesterol (mmol/l)	3.6±0.3	2.8±0.3 *	2.8±0.3 #	3.3±0.3	2.7±0.3 *	2.7±0.2 #
HDL cholesterol (mmol/l)	1.1±0.1	1.1±0.1	1.4±0.1 #	1.3±0.1 ˟	1.3±0.1	1.5±0.1
Systolic blood pressure (mmHg)	142±5	129±7 *	128±5 #	159±6	139±5 *	143±6 #
Diastolic blood pressure (mmHg)	91±2	84±4 *	82±2 #	90±2	84±2 *	85±2 #

Table 7-2 Changes in lipid profile and blood pressure in responders and non-responders. (* = $p<0.05$ for baseline to post-VLCD difference; # = $p<0.05$ for baseline to month 6 difference, and ˟ = $p<0.05$ for between group difference at baseline).

7.3.8 Characteristics of responders vs. non-responders

The responders had a shorter diabetes duration, were younger, and had better glucose control at baseline (fasting plasma glucose and HbA1c) compared to the non-responders (Table 7-3). Achieved fasting glucose level after the VLCD was positively correlated with diabetes duration in the whole group (Spearman rank=0.59; p=0.001). Although there was no difference between non-responders and responders in terms of initial weight or BMI, the total fat mass appeared to be higher in non-responders at baseline (p=0.042). Prior to the study, the responders required less medication compared to non-responders (Table 7-3). There was no difference in baseline physical activity levels. Responders were characterised by higher baseline serum insulin levels compared to non-responders. This state of relative insulin deficiency in the non-responders is supported by higher baseline fasting ß-hydroxybutyrate and NEFA compared to the responders (Table 7-3).

First phase insulin response was diminished at baseline in non-responders compared to responders (0.01 (0.00-0.02) vs. 0.11 (0.00-0.19) nmol min^{-1} m^{-2}; p=0.060) (Figure 7-2). Baseline maximal insulin secretory capacity (baseline to peak ISR) was also markedly lower in the non-responders group (0.19 (0.15-0.30) vs. 0.57 (0.45-0.94) nmol min^{-1} m^{-2}; p<0.001). Pancreatic triglyceride content was similar in responders and non-responders: 5.3±0.4 vs. 5.9±0.7 %; p=0.494.

Hepatic triglyceride content appeared higher at baseline in the responders compared to the non-responders although this was not significant (12.8±2.7 vs. 8.2±1.1 %; p=0.092). Basal hepatic glucose production was higher in non-responders than responders at baseline (3.33 (2.59-8.10) vs 2.58 (2.23-4.01) mg/kg$_{ffm}$/min; p=0.010). At baseline, the responders tended to have greater hepatic insulin resistance (Hepatic IR index: 2153 (1199-2484) vs. 1237 (910-1475) μmol.min^{-1}.kg$_{ffm}$$^{-1}$.pmol.l^{-1}; p=0.060). Hepatic VLDL$_1$-triglyceride production rate was similar in the two groups at baseline (125.3±22.9 vs. 150.6±13.2 mg/kg/day; p=0.312). There was no difference at baseline between responders and non-responders in subcutaneous (319.6±31.0 vs.

285.4±24.7 cm^2; p=0.397) or visceral adipose tissue areas (287.0±23.1 vs. 289.6±5.7 cm^2; p=0.940). There was no significant difference at baseline between responders and non-responders in peripheral insulin sensitivity (M$_{ffm}$: 3.06 (1.55-4.55) vs. 3.97 (1.25-15.89) mg/kg$_{ffm}$/min; p=0.088).

	Responders	Non-Responders	p-value
Age (yr)	52.0±2.9	59.9±2.1	0.032
Gender	8M:4F	7M:10F	
Diabetes duration (yr)	3.8±1.0	9.8±1.6	0.007
Physical activity (MET-min/week)	4446 (509-24581)	5337 (378-29985)	0.900
Fasting plasma glucose (mmol/l)	8.9±0.7	13.2±0.6	<0.001
HbA1c (mmol/mol)	55±4	68±3	0.01
Fasting β-hydroxybutyrate (mmol/l)	0.10 (0.10-0.30)	0.20 (0.10-1.10)	0.02
Fasting insulin (mU/l)	20.4 (5.7-48.1)	9.3 (3.9-48.9)	0.005
Plasma NEFA (mmol/l)	0.51±0.05	0.69±0.04	0.011
Medications:			
diet control	5	2	
metformin only	6	4	
metformin and SU	1	8	
metformin, SU & insulin	0	2	
insulin only	0	1	

Table 7-3 Baseline clinical characteristics of responders (n=12) and non-responders (n=17).

7.4 Discussion

This study demonstrates that weight loss can be maintained for 6 months following a very low calorie diet using a structured individualised programme. Crucially, in those who respond to the VLCD and maintain

weight loss, normal blood glucose control is sustained at 6 months. Of the pathophysiological changes demonstrated following the VLCD, namely improved first phase insulin secretion, decreased pancreatic triglyceride content, decreased hepatic triglyceride content, improved hepatic insulin sensitivity and decreased hepatic $VLDL_1$-triglyceride production, restoration of first phase insulin secretion seems to be a prerequisite for normalisation of blood glucose control.

In this study, the low dropout rate during the VLCD (1/30) indicates that it is an acceptable therapeutic option, albeit in this self-selected and highly motivated population. Re-introduction of normal eating was done in a planned manner, with definitive prescription of food type and amount, with emphasis on continuing high vegetable intake as during the VLCD but adding in other foods up to the specified restricted daily calorie intake. The participants reported that the weight maintenance phase of the study was more challenging than the VLCD phase, but the structured, individualised weight maintenance program was successful in avoiding weight gain during the 6 month follow up period in the majority of individuals.

Clinical characteristics associated with a glucose response to the VLCD were younger age, shorter diabetes duration, better baseline glycaemic control and less requirement for diabetes therapies, however, the critical factor in determining outcome seems to be a degree of first phase insulin secretion at baseline and the recovery of this following VLCD. Moreover, the non-responders showed signs of insulin deficiency: lower fasting insulin levels and almost absent first phase insulin response, with higher fasting ketone and NEFA levels. The responders appeared to have higher hepatic triglyceride content, lower hepatic insulin sensitivity and lower hepatic $VLDL_1$-TG production, characteristics that would be consistent with an environment of hyperglycaemia and hyperinsulinaemia. It may be that once insulin secretion becomes significantly impaired, the insulinopaenic environment results in less *de novo* lipogenesis with lower hepatic triglyceride content and consequently improved hepatic insulin sensitivity. However, the improvements in these parameters do not seem to outweigh

the loss of insulin secretory capacity in terms of glucose levels. The presence of the PNPLA3 mutation is known to dissociate the relationship between hepatic triglyceride content and insulin resistance (Kantartzis *et al.*, 2009). In this study, the presence of the mutation did not affect either baseline hepatic triglyceride content or serum triglyceride levels, nor did it affect the capacity of the VLCD to reduce these parameters.

The metabolic change occurring during the VLCD that seems to define glucose response is an improvement in first phase insulin secretion. It has been hypothesised that the restoration of insulin secretion occurs due to resolution of the burden of toxic fatty acids on pancreatic islet cells allowing recovery of insulin secretion (Morgan *et al.*, 2008). However, it may be that there is a minimum functional beta cell mass required to allow regain of function. Theoretically, ongoing reduction in the exposure to toxic fat metabolites could suppress beta cell apoptosis and data demonstrating ongoing beta cell neogenesis and redifferentiation could mean that beta cell mass could recover over time, thereby improving insulin secretory capacity (Szabat *et al.*, 2012; White *et al.*, 2013). The importance of pancreatic triglyceride in the pathogenesis of type 2 diabetes was initially demonstrated in obese rodents (Lee *et al.*, 2009). Beta cells have been shown to take up fatty acids and store them as intracellular triglyceride (Pinnick *et al.*, 2010). In the human pancreas the magnetic resonance technique detects the total intra- and extra-cellular triglyceride in exocrine and endocrine cells of the pancreas (Pinnick *et al.*, 2008; Hollingsworth *et al.*, 2015). However, evidence is accumulating that whole organ pancreas fat may be a biomarker for the burden of toxic fat metabolites on beta cell function (Lim *et al.*, 2011; Szczepaniak *et al.*, 2012). Human islets *in vitro* accumulate triglyceride on exposure to low concentrations of fatty acids (Lalloyer *et al.*, 2006) and this will result in local lipolysis sufficient to bring about inhibition beta cell function (Lee *et al.*, 1994; Tushuizen *et al.*, 2007). Pancreatic triglyceride content is decreased with weight loss uniquely in type 2 diabetes compared to those with normal glucose tolerance (see data in chapter 4). The extent of decrease in pancreatic triglyceride content following the VLCD was similar in responders and non-responders, however

it appears that only in responders was the capacity for return of acute insulin secretory after release from fat-mediated inhibition by fat intact (Dubois *et al.*, 2004; Tang *et al.*, 2015).

The hypothesis that the decrease in pancreatic triglyceride is due to the decrease in delivery of hepatic $VLDL_1$-triglyceride from the liver to all extra-hepatic cells and tissues but specifically the pancreas is not supported by this study. However, a larger study of a responder group would be required to test the hypothesis definitively. There was a negative correlation between hepatic and pancreatic triglyceride content but no correlation between the change in hepatic triglyceride content and hepatic $VLDL_1$-triglyceride production nor between the change in pancreatic triglyceride content and hepatic $VLDL_1$-triglyceride production. In the study there was no correlation between hepatic $VLDL_1$-triglyceride production and fasting glucose levels until month 6. This is in contrast to a previous study which demonstrated, using multiple regression analyses, that in a group of 30 individuals (in which one third had type 2 diabetes) that plasma glucose was significantly associated with hepatic $VLDL_1$-triglyceride production (Adiels *et al.*, 2005). The reduction in hepatic $VLDL_1$-triglyceride production with weight loss seen in this study is in keeping with previous findings following weight loss achieved after gastric bypass surgery (Klein *et al.*, 2006). $VLDL_1$-triglyceride secretion is largely dependent on the availability of fatty acids which are derived from *de novo* lipogenesis (5-25%), lipolysis of triglyceride stores in adipose tissue (60-80%) or dietary fatty acids. A gradual increase in insulin levels can promote hepatic triglyceride production by upregulation of lipogenic enzymes which promotes *de novo* lipogenesis and results in an increased flux of fatty acids to the hepatic NEFA pool. This ultimately results in increased $VLDL_1$-triglyceride secretion. In contrast, acute increases in insulin secretion seem to inhibit hepatic $VLDL_1$-triglyceride production probably through suppression of plasma NEFA. The decreased fasting insulin levels seen following the VLCD in this study are likely to divert NEFA flux from lipogenesis to oxidation, thus decreasing hepatic triglyceride content. The potential relationship between hepatic $VLDL_1$-triglyceride production and intra-organ triglyceride

content is complex and likely to depend on NEFA concentration, degree and chronicity of hyperinsulinaemia, insulin sensitivity of lipolysis and current nutritional status.

The post-VLCD studies were performed at week 10 rather than at week 8 in order to report the change in metabolism during conditions of usual isocaloric diet as opposed to reflecting ongoing strict calorie restriction. There was a marked increase in glucose levels occurring between the end of the 8 week VLCD and the week 10 studies, most prominent in the non-responder group, despite negligible weight gain. This is likely to reflect a refeeding phenomenon; it has previously been demonstrated that after 10 days of an isocaloric diet following a very low calorie diet, basal hepatic glucose output increases and this correlates with fasting plasma glucose levels (Henry *et al.*, 1985).

Although both groups achieved significant weight loss during the VLCD phase and maintained this for 6 months, they remained similarly obese or overweight at the end of the study (BMI 28.7±0.7 in responders and 30.2±1.1 kg/m^2 in non-responders; p=0.308). It is notable that despite ongoing excess body fat there was no redistribution of fat to the liver from subcutaneous or other adipose tissue deposits over 6 months of weight stability. This supports the concept of a personal fat threshold rather than a genetically determined percentage of body fat being stored within the organs. Above a certain threshold, specific to each individual, excess triglyceride cannot be stored in adipose tissue and spills over into ectopic sites such as the liver (Taylor and Holman, 2015). It could be hypothesised that non-responders, despite achieving substantial weight loss during the VLCD, may not have crossed their personal fat threshold to regain normal metabolic function.

This study has demonstrated durability of the effect of a VLCD over a 6 month period. However, the permanency of the effect and potential impact on clinical outcomes of prolonged avoidance of weight recidivism remain to be demonstrated. Long-term follow-up data from the Swedish Obese Subjects Study demonstrated a diabetes remission rate of 72.3% in the bariatric surgery group at 2 years and only 30.4% at 15 years. Even in this

setting there was weight gain over the follow-up period, particularly in the gastric bypass group (Sjostrom *et al.*, 2014). We have previously reported one individual with type 2 diabetes who successfully lost 41kg and has maintained normal glucose levels and remission of painful neuropathy for over 3 years (Peters *et al.*, 2015). The current study did not aim to look at clinical outcomes from long term maintenance of weight loss and non-diabetic blood glucose levels. However, we know that risk and progression of long term complications from diabetes relates to ambient blood glucose levels, therefore durable reversal of diabetes would be expected to be associated with long term health (Stratton *et al.*, 2000). Furthermore, even if hyperglycaemia were to recur, the 'legacy effect' suggests that a period of normoglycaemia would confer substantial benefit in terms of risk reduction (Holman *et al.*, 2008). A previous study of 18 obese individuals with type 2 diabetes showed sustained improvement in cardiovascular risk profiles at 18 months following a 30 day VLCD (Jazet *et al.*, 2007). The clinical implication from information comparing responders and non-responders in this study is that a VLCD would be best attempted in individuals with preservation of some beta cell function and/or beta cell mass, as indicated by lower glucose levels and higher fasting insulin levels. However, there are still considerable benefits to be gained even in those individuals who do not have a glucose response to a VLCD. In the non-responder group the improvements in blood pressure and lipid profile are likely to confer long term benefit in terms of cardiovascular risk. In addition, the improvement in wellbeing and quality of life reported by participants should not be overlooked.

The limitations of the study must be considered. The use of gold standard imaging techniques and detailed metabolic tests necessitates the small numbers studied; however the group sizes have allowed significant differences to be detected. The heterogeneous population studied represents the spectrum of individuals with type 2 diabetes who may wish to undertake calorie restriction. Pancreatic triglyceride measurements reported are necessarily of both intra- and extra-cellular triglyceride as current methodology precludes measurement of potentially more relevant intra-islet

triglyceride content. To further investigate the refeeding phenomenon it would have been informative to have performed the detailed metabolic studies at week 8 in addition to week 10, however, the burden on participants was considered prohibitive. The inclusion of individuals with alternative diagnoses to type 2 diabetes is a possibility as we did not measure auto-antibodies as part of the screening process. However, detailed clinical history taking was performed to minimise this where possible.

This study has demonstrated durability of the effects of an 8 week VLCD for 6 months in individuals who maintain weight loss with 45% of participants achieving a fasting glucose <7 mmol/l at month 6. Restoration of first phase insulin secretion is the pathophysiological change which appears to define a glucose response to the VLCD. The study has indicated which individuals are more likely to achieve a glucose response: younger age, shorter diabetes duration, with higher fasting insulin and better glycaemic control on fewer diabetes medications. The decreases in hepatic and pancreatic triglyceride content are durable as are the improvements in cardiovascular risk factors, notably blood pressure and lipid profile.

Chapter 8. General Discussion

These studies confirm the previous observations that type 2 diabetes is a reversible syndrome and provide insight into how this knowledge may be applied in clinical practice. The insights could lead to a paradigm shift in the way that the condition is viewed. Rather than a chronic, progressive disease with a requirement for the sequential addition of therapies over time and the inevitable development of multi-system complications, type 2 diabetes could be tackled pro-actively with targeted strategies implemented at diagnosis.

Diabetes reversal using VLCD or bariatric surgery

Similar overall weight loss (on average 14% of initial body weight) was achieved over 8 weeks either by VLCD or bariatric surgery. Correspondingly, rates of reversal of diabetes (defined as achieving a fasting plasma glucose level of <7 mmol/l) were similar at week 8 following VLCD or bariatric surgery (69% vs. 67% respectively). The cohort studied was representative of the heterogeneous type 2 diabetes population. The mechanisms responsible for normalisation of glucose levels after either intervention appear similar: early change in fasting plasma glucose due to rapid change in hepatic insulin sensitivity, but primarily as a consequence of restoration of first phase insulin secretion. Work from this thesis supports the theory that negative calorie balance, particularly acute and significant calorie restriction, rather than weight loss *per se* has a profound role in regulating glucose metabolism. The bidirectional nature of this relationship is clear in the longer term study reported in Chapter 7. These showed significant improvement in fasting glucose levels after VLCD but an increase in fasting glucose levels following resumption of normal diet. These rapid changes go beyond that which can be explained by the modest change in body weight over these short time periods. The main advantage of bariatric surgery over VLCD is widely considered to be the higher success rates of achieving long term maintenance of weight loss (Sjostrom *et al.*, 2004) however, a study using the National Weight Control Registry showed comparable weight loss maintenance at 1 year following surgical and non-surgical interventions (Bond *et al.*, 2009). Data in this thesis demonstrate

that weight loss can be maintained for 6 months following a VLCD using a structured individualised programme based on goal setting, action planning and barrier identification.

Impact of decreased intra-organ fat on glucose metabolism

There are epidemiological, genetic and clinical data to support the importance of excess intra-organ fat as a causal risk factor for type 2 diabetes (Sattar and Gill, 2014). Data from this thesis are concordant with previous work showing higher intra-organ fat levels in individuals with type 2 diabetes compared to those with normal glucose tolerance, specifically triglyceride content in the liver (Kotronen *et al.*, 2008) and pancreas (Tushuizen *et al.*, 2007; Gaborit *et al.*, 2015). Use of the 3 point Dixon magnetic resonance technique rather than other methods such magnetic resonance spectroscopy or computed tomography, has allowed more precise determination of fat content particularly in the pancreas (Hu *et al.*, 2010). The latter is a small organ, which moves with respiration, therefore careful delineation of regions of interest after image acquisition is preferable to data obtained from pre-selected voxels which is likely to include surrounding visceral fat. An important observation has been made in these studies: hepatic and pancreatic triglyceride levels decrease with acute calorie restriction, achieved by bariatric surgery or VLCD, with no difference between those who achieved normalisation of fasting glucose levels and those who did not. The association between increased hepatic triglyceride content and decreased hepatic insulin sensitivity is clear (Nobili *et al.*, 2006; Gastaldelli *et al.*, 2007) and data from the present studies are in line with this; hepatic insulin resistance index and hepatic triglyceride content were positively correlated in all participants with diabetes (Spearman rank=0.386; *p*=0.008). However, in terms of achieving normalisation of glucose levels, a decrease in liver fat content with concurrent improvement in hepatic insulin sensitivity was insufficient by itself to ensure reversal of diabetes. Following 8 weeks of VLCD, individuals who did not achieve normal fasting glucose levels demonstrated a marked improvement in hepatic triglyceride content and hepatic insulin sensitivity,

similar to that seen in those who did achieve a glucose response. Also, in individuals with initially normal glucose tolerance, marked reduction in hepatic fat content after bariatric surgery was observed without a significant change in hepatic insulin sensitivity. In this latter group, it could be argued that the baseline levels of hepatic triglyceride could be below a threshold for inhibition of insulin action by the toxic metabolites of fat. However, in the former the lack of improvement in fasting glucose levels in some individuals despite the marked reduction in hepatic triglyceride content and hepatic insulin resistance index suggests that this mechanism is not of primary importance in the reversal of diabetes. Of great clinical importance for care of individuals with NAFLD and/or type 2 diabetes is the demonstration of longer term maintenance of low hepatic triglyceride levels. Weight loss achieved through lifestyle modification (Vilar-Gomez *et al.*, 2015) and bariatric surgery (Lassailly *et al.*, 2015) has been shown to result in improvement in the histological features of non-alcoholic steatohepatitis, a precursor to the development of cirrhosis.

The paradoxical relationship between the change in hepatic triglyceride content and achieved weight loss in the first week after bariatric surgery (chapter 5) has not been noted before to my knowledge. Individuals who achieved the greatest weight loss in the first 7 post-operative days had the smallest reduction in hepatic triglyceride content, but still achieved improved fasting glucose levels. This effect was not mediated by greater loss of lean mass compared to fat mass in these individuals, nor by the rs738409 C/G polymorphism of the PNPLA3 gene. The potential for bariatric surgery to cause hepatic steatosis is well recognised (Peters *et al.*, 1975; Pillai and Rinella, 2009), but it is also possible that the marked post-operative increase in GLP-1 could interrupt the usual relationship between weight loss and hepatic triglyceride content. Both GLP-1 agonist therapy and DPP-4 inhibition have been reported to bring about a greater fall in hepatic triglyceride than would be expected by the degree of weight change (Cuthbertson *et al.*, 2012; Macauley *et al.*, 2015a). This could also be a potential explanation for the differing effect of the rs738409 C/G polymorphism of PNPLA3 on weight loss induced reduction in hepatic

triglyceride content achieved by bariatric surgery compared to VLCD. Following bariatric surgery the presence of a mutation appeared to blunt the weight loss induced reduction in hepatic triglyceride content, but this effect was not seen with equivalent weight loss achieved through VLCD. A previous study also demonstrated effective decrease in liver fat content through weight loss regardless of PNPLA3 genotype (Sevastianova *et al.*, 2011). It compared the effect of a 6 day hypocaloric low-carbohydrate diet in individuals who were homozygous for either the wild type or the mutant rs738409 polymorphism. Given that the PNPLA3 mutation is thought to impair the hydrolysis of triglyceride without increasing hepatic insulin resistance, this suggests that the method by which calorie restriction is achieved may influence hepatic lipid metabolism to a degree.

Despite the wide scatter of absolute levels of pancreatic triglyceride content there were significantly higher levels in individuals with type 2 diabetes compared to weight matched individuals with normal glucose tolerance. This is in keeping with previous studies (Tushuizen *et al.*, 2007; Lim *et al.*, 2011; Macauley *et al.*, 2015b). Pancreatic triglyceride levels did not fall following bariatric surgery in individuals with normal glucose tolerance despite 15kg weight loss, suggesting that the change is disease specific rather than simply an effect of weight loss. In line with this specific link between pancreatic fat and diabetes, a recent study using magnetic resonance spectroscopy demonstrated higher pancreatic triglyceride content in individuals with type 2 diabetes, with no difference between lean and obese individuals with normal glucose tolerance (Gaborit *et al.*, 2015). Data in this thesis demonstrates reduction in pancreatic triglyceride content following VLCD in individuals with type 2 diabetes. In those with low fasting plasma insulin levels and an absent first phase insulin response, the decrease in pancreatic triglyceride content could not restore beta cell functionality. Other recent work following gastric bypass surgery has shown a relationship between decrease in pancreatic fat and improvement in beta cell function (Honka *et al.*, 2015). The cohort studied included obese non-diabetic subjects who would be expected to have minimal change in beta cell function or pancreatic fat levels (chapter 4) and the imaging modality used

was computed tomography. Overall, it appears that chronic exposure to high fat levels within the pancreatic islet may eventually cause irreversible loss of glucose mediated insulin secretion as long duration type 2 diabetes was associated with negligible first phase insulin secretion, and removal of fat did not change this. The rate at which irreversible changes occur is likely to differ between individuals, and this may account for apparent lack of reversibility in a small number of people with type 2 diabetes of duration less than 4 years.

Data from these studies indicate that the relationship between decreasing hepatic triglyceride content and return of hepatic insulin sensitivity, and decreasing pancreatic triglyceride and return of first phase insulin secretion are not linear and must be influenced by other factors. The absence of a technique to measure local concentrations of the metabolically inhibitory metabolites of fat, such as diacylglycerol, means that triglyceride content, as measured by MR, is being used as a biomarker: pancreatic triglyceride as a biomarker for the metabolic inhibition of insulin secretion by fat, and liver triglyceride as a biomarker of the metabolic inhibition of insulin action by fat. This indirect measure could explain some of the dissociation in the relationship between intra-organ fat content and metabolic function. Although removal of excess intra-organ fat is an essential step to move an individual below their personal fat threshold, the limiting factor appears to be the potential for restoration of insulin secretory capacity from beta cells, that is the ability to respond to a glucose stimulus when the ambient metabolic inhibition by lipid metabolites is lifted. Regardless of intra-organ fat levels, if that individual does not have sufficient beta cell capacity for appropriate insulin secretion, normalisation of glucose levels will not occur. This limitation to diabetes reversal seems likely to be determined by a combination of residual beta cell mass and the state of de- or re-differentiation of the beta cells (White *et al.*, 2013). Using a mouse model of insulin secretory deficiency, recovery of beta cell function through re-differentiation has been demonstrated by control of hyperglycaemia using insulin therapy (Wang *et al.*, 2014). A human study looking at the effect of early short-term insulin therapy or oral agents for initial rapid correction of

hyperglycaemia in individuals with newly diagnosed type 2 diabetes, demonstrated that glycaemic control (capillary blood glucose <6.1mmol/l) was achieved in 95.2% of the multiple dose insulin group in 5.8 days compared to 83.5% of the oral agent group in 9.3 days (Weng *et al.*, 2008). Remission rates at 1 year were significantly higher in the insulin group than the oral agent group. Also, the improvement in acute insulin response was sustained in the insulin group but significantly declined in the oral agent group. Further studies have found similar results and give support to the theory that early optimised glycaemic control with insulin may eliminate the deleterious effect of hyperglycaemia, and avoid irreversible loss of beta cell function and/or mass (Weng *et al.*, 2015). It is difficult to determine whether the effect of early insulin therapy is exclusively due to avoidance of hyperglycaemia or whether the optimised metabolic milieu, including the beneficial effect on fatty acids, might be mechanistically important. Also, a direct effect of insulin on beta cell mass or function cannot be excluded from these studies. However, it must be acknowledged that any intervention which improves glucose levels is likely to have a beneficial effect on beta cell function.

Weight loss results in decreased hepatic VLDL-triglyceride export

Hepatic VLDL export is the only known mechanism by which liver fat content can be reduced other than through increasing fat oxidation. Data in this thesis demonstrate a decrease in hepatic VLDL-triglyceride secretion with weight loss which was maintained with weight maintenance. Similar to a recent study looking at VLDL-triglyceride secretion after RYGB (Magkos *et al.*, 2010), there was no relationship between achieved weight loss and decrease in hepatic VLDL-triglyceride secretion seen following VLCD (Spearman rank=-0.048; *p*=0.812). Also, no relationship could be demonstrated between hepatic VLDL-triglyceride production rates and hepatic triglyceride content or hepatic insulin resistance index. This is in line with mouse models where inhibiting hepatic VLDL secretion increases hepatic triglyceride content but does not cause insulin resistance (Jacobs *et al.*, 2010; Niebergall *et al.*, 2011). One theory put forward to try to explain

this dissociation between liver fat and insulin resistance postulated that diacylglycerols sequestered in lipid droplets may not promote the PKC epsilon activation that has been shown to cause hepatic insulin resistance (Perry *et al.*, 2014). It was hypothesised that increased VLDL-triglyceride export might be the source of inhibitory fat metabolites reaching the pancreas; this would mechanistically link excess liver fat with excess pancreas fat in type 2 diabetes (Taylor, 2008). However, in these studies there was no association between the weight loss induced decrease in $VLDL_1$-triglyceride production and decrease in pancreatic triglyceride content or the improvement in insulin secretion rates in responders. It is important to acknowledge that the studies were not powered to test such a relationship and this needs to be investigated in a future study with a larger group size.

Liposusceptibility and the implications for diabetes reversal

The concept of liposusceptibility refers to an individual's threshold for tolerating the deleterious metabolic effects of excess fat. In these studies this is best illustrated by the participants undergoing bariatric surgery who have managed to maintain normal glucose tolerance despite class II or III obesity. The lack of change in hepatic insulin sensitivity in this group suggests that the degree of liver fat accumulation was well tolerated, and the concept of the personal fat threshold could explain the lack of response to calorie restriction in some individuals in the study (Taylor and Holman, 2015). This hypothesis suggests that individuals experience metabolically deleterious effects of intra-organ fat accumulation only when this exceeds a level of tolerability specific to an individual and unrelated to body weight or BMI. Despite achieving significant weight loss, this may have been insufficient for that individual to cross their personal fat threshold in order to regain normal metabolic function. Although baseline intra-organ fat levels were higher in those with diabetes compared to those with normal glucose tolerance, there was a wide range and overlap between the groups (hepatic triglyceride: 2.4-24.7 vs. 1.5-11.3 % and pancreatic triglyceride: 3.6-10.5 vs. 4.2-6.1 % respectively). One individual in the normal glucose

tolerance group who did not have a PNPLA3 mutation had a hepatic triglyceride content of 11.3%. However, differences in liposusceptibility are likely to be only one factor implicated in determining reversal of type 2 diabetes; the plateau in diabetes reversal rates following bariatric surgery, seen beyond 20% body weight loss (chapter 3), suggests that additional factors are important and specifically any irreversible loss of glucose sensitive insulin secretion.

Factors determining reversibility of type 2 diabetes

Adding to previous work showing reversibility of short duration type 2 diabetes, these studies have demonstrated that reversal of long duration type 2 diabetes is also possible, although reversal rates are less than in short duration disease. Following either VLCD or bariatric surgery, reversal rates at 8 weeks according to diabetes duration were remarkably similar: Short (<4yr) vs. Long duration (>8yr): VLCD 87 vs. 50 % and Surgery 100 vs. 40 %. These numbers are in keeping with previous findings on diabetes reversal rates after surgery where Hall *et al.* found a 75% reversal rate when diabetes duration was 0-4 yrs and a 44% reversal when diabetes duration was >8 yr (Hall *et al.*, 2010). The response to calorie restriction in long duration diabetes was heterogenous, with the longest diabetes duration to achieve normalisation of glucose control in these studies being 18 years (following 21.4kg weight loss after bariatric surgery). Long duration type 2 diabetes is characterised by higher fasting glucose levels and pancreatic triglyceride content, and lower fasting insulin, C-peptide, hepatic triglyceride content and hepatic insulin resistance compared to short duration type 2 diabetes. In individuals with longer duration disease, despite hyperglycaemia, the presence of diminishing portal insulin concentrations is likely to result in decreased hepatic *de novo* lipogenesis (Schwarz *et al.*, 2003). Lower hepatic triglyceride levels in long duration compared to short duration disease could therefore simply be a consequence of relative insulinopaenia. In these studies, fasting insulin levels and hepatic triglyceride content were highly correlated both before (Spearman

rank=0.599; $p<0.001$) and after weight loss (Spearman rank=0.553; $p<0.001$) in all participants (including those with normal glucose tolerance).

The metabolic characteristics of long duration type 2 diabetes were similar to those seen in individuals who did not achieve a glucose response to VLCD and are suggestive of relative insulin deficiency. However, the normalisation of glucose levels in a significant proportion of individuals with long duration disease through VLCD or bariatric surgery suggests that diabetes duration alone does not limit diabetes reversal. In fact diabetes duration may just represent a surrogate and insensitive marker for decreased insulin secretory capacity. Until specific interventions are developed specifically to augment beta cell mass/redifferentiation, it seems that bariatric surgery and VLCD would be best attempted earlier in the type 2 diabetes disease process given the higher diabetes reversal rates in short duration disease.

Implications for the whole population

One clinical implication of these studies is to direct public health strategies to focus on the prevention of weight gain in populations over time, recognising that, regardless of baseline weight, weight gain over many years in predisposed individuals is likely to result in metabolic disease. The remarkably low drop-out rate during VLCD in these studies demonstrates the viability of this intervention in general diabetes care. This is in accordance with a recent systematic review of VLCD in individuals with diabetes showing high tolerability and good safety outcomes (Sellahewa *et al.*, 2015). Although the individuals in these studies represent a selected and highly motivated population, there is evidence to suggest that a significant proportion of the diabetes population would be willing to attempt such dietary restriction (Steven *et al.*, 2013). A critical question for health care provision is whether truly long term reversal of diabetes can be achieved in Primary Care. To answer this question, a community based study (DiRECT: Diabetes REmission Clinical Trial) is now underway of individuals with type 2 diabetes randomised to VLCD with structured individualised weight maintenance or to best possible guideline-based care. Individuals can currently be counselled about the approximate chance of

achieving normal blood glucose levels with a VLCD. From data in Chapter 7 it can be simply calculated that there is an 80% chance of reversal of type 2 diabetes if duration is less than 10 years and fasting glucose is less than 9 mmol/l. Individuals can be reassured that even if normal glucose levels are not achieved with significant weight loss, they will reap benefits in terms of improved glucose control with decreased requirement for diabetes therapies, and improvement in cardiovascular risk factors such as blood pressure control and lipid profile. Finally, and of great importance, is the improvement in general well-being after significant weight loss which was universally reported by the participants studied.

Future work

Future work is required to establish whether modification of the VLCD could result in increased diabetes reversal rates. For example, could a more prolonged period of weight loss could result in a higher diabetes reversal rate? The mean BMI in individuals with diabetes in these studies at 8 weeks after surgery or VLCD was 36.9 ± 07 and 29.3 ± 0.7 kg/m^2 respectively. Based on the personal fat threshold theory, further weight loss in some of these individuals might result in normalisation of glucose levels. In a larger cohort, studying individuals who regain weight following a VLCD may help strengthen the association between intra-organ triglyceride content and metabolic dysfunction. A bidirectional relationship would be expected to be seen, with any re-accumulation of hepatic and pancreatic triglyceride being associated with elevation of fasting glucose levels and loss of first phase insulin secretion. Development of an imaging modality that is able to quantify functional islet cell mass would be of great benefit. Also, the effect of a more prolonged period of isocaloric weight maintenance will be important to define. It could be hypothesised that over time, in the context of avoidance of weight gain and the toxic effect of excess fat metabolites, re-differentiation of beta cells or neogenesis resulting in restoration of beta cell mass might restore insulin secretory capacity and result in higher diabetes reversal rates. Finally, to confirm the clinical relevance of these studies a long term prospective study evaluating rates of diabetes specific

complications and cardiovascular outcomes following a VLCD and weight maintenance programme is required.

Conclusion

The work presented in this thesis furthers understanding of both the mechanisms and the limits of reversibility of type 2 diabetes. Rather than diabetes duration itself being a limiting factor, it appears that the key factor determining reversibility is the potential for recovery of residual beta cell function. Substantial acute calorie restriction achieved by VLCD or bariatric surgery, with avoidance of weight regain, can restore normoglycaemia in a proportion of people with type 2 diabetes.

References

Abate, N., Garg, A., Coleman, R., Grundy, S.M. and Peshock, R.M. (1997) 'Prediction of total subcutaneous abdominal, intraperitoneal, and retroperitoneal adipose tissue masses in men by a single axial magnetic resonance imaging slice', *The American Journal of Clinical Nutrition*, 65(2), pp. 403-8.

Abdullah, A., Peeters, A., de Courten, M. and Stoelwinder, J. (2010) 'The magnitude of association between overweight and obesity and the risk of diabetes: a meta-analysis of prospective cohort studies', *Diabetes Res Clin Pract*, 89(3), pp. 309-19.

Abramoff, M.D., Magelhaes, P.J. and Ram, S.J. (2004) 'Image Processing with ImageJ', *Biophotonics International*, 11(7), pp. 36-42.

Adiels, M., Boren, J., Caslake, M.J., Stewart, P., Soro, A., Westerbacka, J., Wennberg, B., Olofsson, S.O., Packard, C. and Taskinen, M.R. (2005) 'Overproduction of VLDL1 driven by hyperglycemia is a dominant feature of diabetic dyslipidemia', *Arterioscler Thromb Vasc Biol*, 25(8), pp. 1697-703.

Adiels, M., Olofsson, S.-O., Taskinen, M.-R. and Boren, J. (2008) 'Overproduction of Very Low Density Lipoproteins Is the Hallmark of the Dyslipidaemia in the Metabolic Syndrome', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28, pp. 1225-1236.

Al-Shayji, I., Gill, J., Cooney, J., Siddiqui, S. and Caslake, M. (2007) 'Development of a novel method to determine very low density lipoprotein kinetics', *Journal of Lipid Research*, 48, pp. 2086-2095.

Alquier, T., Peyot, M.L., Latour, M.G., Kebede, M., Sorensen, C.M., Gesta, S., Ronald Kahn, C., Smith, R.D., Jetton, T.L., Metz, T.O., Prentki, M. and Poitout, V. (2009) 'Deletion of GPR40 impairs glucose-induced insulin secretion in vivo in mice without affecting intracellular fuel metabolism in islets', *Diabetes*, 58(11), pp. 2607-15.

Anderson, J., Kendall, C. and Jenkins, D. (2003) 'Importance of weight management in type 2 diabetes: review with meta-analysis of clinical studies', *Journal of the American College of Nutrition*, 22, pp. 331-339.

Anello, M., Lupi, R., Spampinato, D., Piro, S., Masini, M., Boggi, U., Del Prato, S., Rabuazzo, A.M., Purrello, F. and Marchetti, P. (2005) 'Functional and morphological alterations of mitochondria in pancreatic beta cells from type 2 diabetic patients', *Diabetologia*, 48(2), pp. 282-9.

Arauz-Pacheco, C., Parrott, M.A. and Raskin, P. (2002) 'The Treatment of Hypertension in Adult Patients With Diabetes', *Diabetes Care*, 25(1), pp. 134-147.

Arterburn, D.E., Bogart, A., Sherwood, N.E., Sidney, S., Coleman, K.J., Haneuse, S., O'Connor, P.J., Theis, M.K., Campos, G.M., McCulloch, D. and Selby, J. (2013) 'A multisite study of long-term remission and relapse of type 2 diabetes mellitus following gastric bypass', *Obes Surg*, 23(1), pp. 93-102.

Basciani, S., Costantini, D., Contini, S., Persichetti, A., Watanabe, M., Mariani, S., Lubrano, C., Spera, G., Lenzi, A. and Gnessi, L. (2015) 'Safety and efficacy of a multiphase dietetic protocol with meal replacements including a step with very low calorie diet', *Endocrine*, 48(3), pp. 863-70.

Bedogni, G., Miglioli, L., Masutti, F., Tiribelli, C., Marchesini, G. and Bellentani, S. (2005) 'Prevalence of and risk factors for nonalcoholic fatty liver disease: the Dionysos nutrition and liver study', *Hepatology*, 42(1), pp. 44-52.

Begovatz, P., Koliaki, C., Weber, K., Strassburger, K., Nowotny, B., Nowotny, P., Mussig, K., Bunke, J., Pacini, G., Szendrodi, J. and Roden, M. (2015) 'Pancreatic adipose tissue infiltration, parenchymal steatosis and beta cell function in humans', *Diabetologia*, 58(7), pp. 1646-55.

Bensellam, M., Laybutt, D.R. and Jonas, J.C. (2012) 'The molecular mechanisms of pancreatic beta-cell glucotoxicity: recent findings and future research directions', *Mol Cell Endocrinol*, 364(1-2), pp. 1-27.

Bjorkegren, J., Packard, C.J., Hamsten, A., Bedford, D., Caslake, M., Foster, L., Shepherd, J., Stewart, P. and Karpe, F. (1996) 'Accumulation of large very low density lipoprotein in plasma during intravenous infusion of a chylomicron-like triglyceride emulsion reflects competition for a common lipolytic pathway', *J Lipid Res*, 37(1), pp. 76-86.

Blond, E., Maitrepierre, C., Normand, S., Sothier, M., Roth, H., Goudable, J. and Laville, M. (2011) 'A new indirect calorimeter is accurate and reliable for measuring basal energy expenditure, thermic effect of food and substrate oxidation in obese and healthy subjects', *e-SPEN, the European e-Journal of Clinical Nutrition and Metabolism*, 6(1), pp. e7-e15.

Boden, G. (2011) 'Obesity, insulin resistance and free fatty acids', *Curr Opin Endocrinol Diabetes Obes*, 18(2), pp. 139-43.

Bojsen-Møller, K.N., Dirksen, C., Jørgensen, N.B., Jacobsen, S.H., Serup, A.K., Albers, P.H., Hansen, D.L., Worm, D., Naver, L., Kristiansen, V.B., Wojtaszewski, J.F.P., Kiens, B., Holst, J.J., Richter, E.A. and Madsbad, S. (2014) 'Early Enhancements of Hepatic and Later of Peripheral Insulin Sensitivity Combined With Increased Postprandial Insulin Secretion Contribute to Improved Glycemic Control After Roux-en-Y Gastric Bypass', *Diabetes*, 63(5), pp. 1725-1737.

Bond, D.S., Phelan, S., Leahey, T.M., Hill, J.O. and Wing, R.R. (2009) 'Weight-loss maintenance in successful weight losers: surgical vs non-surgical methods', *Int J Obes (Lond)*, 33(1), pp. 173-80.

Bouchardat, C. (1875) *De la Glycosurie ou Diabète Sucré* Paris: Baillière.

Boucher, A., Lu, D., Burgess, S.C., Telemaque-Potts, S., Jensen, M.V., Mulder, H., Wang, M.Y., Unger, R.H., Sherry, A.D. and Newgard, C.B. (2004) 'Biochemical mechanism of lipid-induced impairment of glucose-stimulated insulin secretion and reversal with a malate analogue', *J Biol Chem*, 279(26), pp. 27263-71.

Bradley, D., Conte, C., Mittendorfer, B., Eagon, J.C., Varela, J.E., Fabbrini, E., Gastaldelli, A., Chambers, K.T., Su, X., Okunade, A., Patterson, B.W.

and Klein, S. (2012) 'Gastric bypass and banding equally improve insulin sensitivity and ß cell function', *The Journal of Clinical Investigation*, 122(12), pp. 4667-4674.

Brehm, A. and Roden, M. (2007) 'Glucose Clamp Techniques', in Roden, M. (ed.) *Clinical Diabetes Research*. Chichester, England: John Wiley & Sons, p. pp. 60.

Buchwald, H., Avidor, Y., Braunwald, E., Jensen, M.D., Pories, W., Fahrbach, K. and Schoelles, K. (2004) 'Bariatric surgery: a systematic review and meta-analysis', *JAMA*, 292(14), pp. 1724-37.

Buse, J., Caprio, S., Cefalu, W., Ceriello, A., Del Prato, S., Inzucchi, S., McLaughlin, S., Phillips, G., Robertson, R., Rubino, F., Kahn, R. and Kirkman, M. (2009) 'How Do We Define Cure of Diabetes?', *Diabetes Care*, 32(11), pp. 2133-2135.

Butler, A., Cao-Minh, L., Galasso, R., Rizza, R., Corradin, A., Cobelli, C. and Butler, P. (2010) 'Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy', *Diabetologia*, 53, pp. 2167-2176.

Butler, A., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. and Butler, P. (2003) 'Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes', *Diabetes*, 52, pp. 102-110.

Camastra, S., Manco, M., Mari, A., Greco, A.V., Frascerra, S., Mingrone, G. and Ferrannini, E. (2007) 'Beta-cell function in severely obese type 2 diabetic patients: long-term effects of bariatric surgery', *Diabetes Care*, 30(4), pp. 1002-4.

Capeau, J. (2008) 'Insulin resistance and steatosis in humans', *Diabetes & Metabolism*, 34, pp. 649-657.

Carey, P., Halliday, J., Snaar, J., Morris, P. and Taylor, R. (2003) 'Direct assessment of muscle glycogen storage after mixed meals in normal and type 2 diabetic subjects', *American Journal of Physiology*, 284, pp. E286-294.

Cassidy, S., Thoma, C., Hallsworth, K., Parikh, J., Hollingsworth, K.G., Taylor, R., Jakovljevic, D.G. and Trenell, M.I. (2016) 'High intensity intermittent exercise improves cardiac structure and function and reduces liver fat in patients with type 2 diabetes: a randomised controlled trial', *Diabetologia*, 59(1 Epub ahead of print).

Clark, A., Wells, C.A., Buley, I.D., Cruickshank, J.K., Vanhegan, R.I., Matthews, D.R., Cooper, G.J., Holman, R.R. and Turner, R.C. (1988) 'Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes', *Diabetes Res*, 9(4), pp. 151-9.

Cnop, M. (2008) 'Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes', *Biochem Soc Trans*, 36(Pt 3), pp. 348-52.

Cohen, R.V., Pinheiro, J.C., Schiavon, C.A., Salles, J.E., Wajchenberg, B.L. and Cummings, D.E. (2012) 'Effects of Gastric Bypass Surgery in Patients With Type 2 Diabetes and Only Mild Obesity', *Diabetes Care*, 35(7), pp. 1420-1428.

Craig, C.L., Marshall, A.L., Sjostrom, M., Bauman, A.E., Booth, M.L., Ainsworth, B.E., Pratt, M., Ekelund, U., Yngve, A., Sallis, J.F. and Oja, P. (2003) 'International physical activity questionnaire: 12-country reliability and validity', *Med Sci Sports Exerc*, 35(8), pp. 1381-95.

Cusi, K. (2010) 'The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes', *Current Diabetes Reports*, 10, pp. 306-315.

Cuthbertson, D.J., Irwin, A., Gardner, C.J., Daousi, C., Purewal, T., Furlong, N., Goenka, N., Thomas, E.L., Adams, V.L., Pushpakom, S.P., Pirmohamed, M. and Kemp, G.J. (2012) 'Improved Glycaemia Correlates with Liver Fat Reduction in Obese, Type 2 Diabetes, Patients Given Glucagon-Like Peptide-1 (GLP-1) Receptor Agonists', *PLoS ONE*, 7(12), p. e50117.

DeFronzo, R.A., Ratner, R.E., Han, J., Kim, D.D., Fineman, M.S. and Baron, A.D. (2005) 'Effects of Exenatide (Exendin-4) on Glycemic Control and

Weight Over 30 Weeks in Metformin-Treated Patients With Type 2 Diabetes', *Diabetes Care*, 28(5), pp. 1092-1100.

DeFronzo, R.A., Tobin, J.D. and Anders, R. (1979) 'Glucose clamp technique: a method for quantifying insulin secretion and resistance', *American Journal of Physiology*, 237, pp. E214-E223.

Diakogiannaki, E., Dhayal, S., Childs, C., Calder, P., Welters, H. and Morgan, N. (2007) 'Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain acids in pancreatic beta-cells', *Journal of Endocrinology*, 194, p. 283.

Dirksen, C., Jorgensen, N.B., Bojsen-Moller, K.N., Jacobsen, S.H., Hansen, D.L., Worm, D., Holst, J.J. and Madsbad, S. (2012) 'Mechanisms of improved glycaemic control after Roux-en-Y gastric bypass', *Diabetologia*, 55(7), pp. 1890-901.

Dixon, J., Zimmet, P., Alberti, K. and Rubino, F. (2011) 'Bariatric surgery: an IDF statement for obese Type 2 diabetes', *Diabetic Medicine*, 28, pp. 628-642.

Dixon, J.B., O'Brien, P.E., Playfair, J., Chapman, L., Schachter, L.M., Skinner, S., Proietto, J., Bailey, M. and Anderson, M. (2008) 'Adjustable gastric banding and conventional therapy for type 2 diabetes: a randomized controlled trial', *Journal of the American Medical Association*, 299(3), pp. 316-23.

Dixon, W.T. (1984) 'Simple proton spectroscopic imaging', *Radiology*, 153(1), pp. 189-94.

Dubois, D. and Dubois, E. (1916) 'A formula to estimate the approximate surface area if height and weight be known', *Archives of Internal Medicine*, 17, pp. 863-871.

Dubois, M., Kerr-Conte, J., Gmyr, V., Bouckenooghe, T., Muharram, G., D'Herbomez, M., Martin-Ponthieu, A., Vantyghem, M.C., Vandewalle, B. and Pattou, F. (2004) 'Non-esterified fatty acids are deleterious for human

pancreatic islet function at physiological glucose concentration', *Diabetologia*, 47(3), pp. 463-9.

Falkén, Y., Hellström, P.M., Holst, J.J. and Näslund, E. (2011) 'Changes in Glucose Homeostasis after Roux-en-Y Gastric Bypass Surgery for Obesity at Day Three, Two Months, and One Year after Surgery: Role of Gut Peptides', *The Journal of Clinical Endocrinology & Metabolism*, 96(7), pp. 2227-2235.

Ferner, R.E., Ashworth, L., Tronier, B. and Alberti, K.G.M.M. (1986) 'Effects of short-term hyperglycaemia on insulin secretion in normal humans', *American Journal of Physiology*, 250, pp. E655-E661.

Ferrannini, E. and Mari, A. (1998) 'How to measure insulin sensitivity', *J Hypertens*, 16(7), pp. 895-906.

Ferrannini, E., Nannipieri, M., Williams, K., Gonzales, C., Haffner, S.M. and Stern, M.P. (2004) 'Mode of onset of type 2 diabetes from normal or impaired glucose tolerance', *Diabetes*, 53(1), pp. 160-5.

Ferrannini, E., Natali, A., Muscelli, E., Nilsson, P.M., Golay, A., Laakso, M., Beck-Nielsen, H. and Mari, A. (2011) 'Natural history and physiological determinants of changes in glucose tolerance in a non-diabetic population: the RISC Study', *Diabetologia*, 54(6), pp. 1507-16.

Festa, A., Williams, K., D'Agostino, R., Jr., Wagenknecht, L.E. and Haffner, S.M. (2006) 'The natural course of beta-cell function in nondiabetic and diabetic individuals: the Insulin Resistance Atherosclerosis Study', *Diabetes*, 55(4), pp. 1114-20.

Finegood, D.T., Bergman, R.N. and Vranic, M. (1987) 'Estimation of endogenous glucose production during hyperinsulinemic-euglycemic glucose clamps: Comparison of unlabelled and labelled exogenous glucose infusates', *Diabetes*, 36, pp. 914-924.

Frayn, K. (2003) 'Lipoprotein metabolism', in Frayn, K. (ed.) *Metabolic regulation. A human perspective.* 2nd edn. Oxford: Blackwell Science.

Friedman, M.N., Sancetta, A.J. and Magovern, G.J. (1955) 'The amelioration of diabetes mellitus following subtotal gastrectomy', *Surg Gynecol Obstet*, 100(2), pp. 201-4.

Fruin, M. and Rankin, J. (2004) 'Validity of a Multi-Sensor Armband in Estimating Rest and Exercise Energy Expenditure', *Medicine & Science in Sports & Exercise*, 36(6), pp. 1063-1069.

FSA (2002) *Food Standards Agency McCance and Widdowson's The Composition of Foods*. 6th summary edn. Cambridge: Royal Society of Chemistry.

Fu, Z., Gilbert, E.R. and Liu, D. (2013) 'Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes', *Curr Diabetes Rev*, 9(1), pp. 25-53.

Funakoshi, S., Fujimoto, S., Hamasaki, A., Fujiwara, H., Fujita, Y., Ikeda, K., Hamamoto, Y., Hosokawa, M., Seino, Y. and Inagaki, N. (2008) 'Analysis of factors influencing pancreatic beta-cell function in Japanese patients with type 2 diabetes: association with body mass index and duration of diabetic exposure', *Diabetes Res Clin Pract*, 82(3), pp. 353-8.

Gaborit, B., Abdesselam, I., Kober, F., Jacquier, A., Ronsin, O., Emungania, O., Lesavre, N., Alessi, M.C., Martin, J.C., Bernard, M. and Dutour, A. (2015) 'Ectopic fat storage in the pancreas using 1H-MRS: importance of diabetic status and modulation with bariatric surgery-induced weight loss', *Int J Obes*, 39(3), pp. 480-487.

Gastaldelli, A., Cusi, K., Pettiti, M., Hardies, J., Miyazaki, Y., Berria, R., Buzzigoli, E., Sironi, A.M., Cersosimo, E., Ferrannini, E. and DeFronzo, R.A. (2007) 'Relationship Between Hepatic/Visceral Fat and Hepatic Insulin Resistance in Nondiabetic and Type 2 Diabetic Subjects', *Gastroenterology*, 133(2), pp. 496-506.

Glover, G. and Schneider, E. (1991) 'Three-point Dixon technique for true water/fat decomposition with B0 inhomogeneity correction', *Magnetic Resonance in Medicine*, 18, pp. 371-383.

Goldfine, A.B. and Patti, M.E. (2014) 'Diabetes Improvement Following Roux-en-Y Gastric Bypass: Understanding Dynamic Changes in Insulin Secretion and Action', *Diabetes*, 63(5), pp. 1454-1456.

Gregg, E.W., Chen, H., Wagenknecht, L.E., Clark, J.M., Delahanty, L.M., Bantle, J., Pownall, H.J., Johnson, K.C., Safford, M.M., Kitabchi, A.E., Pi-Sunyer, F.X., Wing, R.R. and Bertoni, A.G. (2012) 'Association of an intensive lifestyle intervention with remission of type 2 diabetes', *JAMA*, 308(23), pp. 2489-96.

Groop, L.C., Bonadonna, R.C., DelPrato, S., Ratheiser, K., Zyck, K., Ferrannini, E. and DeFronzo, R.A. (1989) 'Glucose and free fatty acid metabolism in non-insulin dependent diabetes mellitus: evidence for multiple sites of insulin resistance', *Journal of Clinical Investigation*, 84, pp. 205-213.

Guidone, C., Manco, M., Valera-Mora, E., Iaconelli, A., Gniuli, D., Mari, A., Nanni, G., Castagneto, M., Calvani, M. and Mingrone, G. (2006) 'Mechanisms of recovery from type 2 diabetes after malabsorptive bariatric surgery', *Diabetes*, 55(7), pp. 2025-31.

Hagströmer, M., Oja, P. and Sjöström, M. (2006) 'The International Physical Activity Questionnaire (IPAQ): a study of concurrent and construct validity', *Public Health Nutrition*, 9(06), pp. 755-762.

Haines, L., Wan, K.C., Lynn, R., Barrett, T.G. and Shield, J.P. (2007) 'Rising incidence of type 2 diabetes in children in the U.K', *Diabetes Care*, 30(5), pp. 1097-101.

Hall, T.C., Pellen, M.G.C., Sedman, P.C. and Jain, P.K. (2010) 'Preoperative Factors Predicting Remission of Type 2 Diabetes Mellitus After Roux-en-Y Gastric Bypass Surgery for Obesity', *Obesity Surgery*, 20(9), pp. 1245-1250.

Hanley, S., Austin, E., Assouline-Thomas, B., Kapeluto, J., Blaichman, J., Moosavi, M., Petropavlovskaia, M. and Rosenberg, L. (2010) '{beta}-Cell mass dynamics and islet cell plasticity in human type 2 diabetes', *Endocrinology*, 151(4), pp. 1462-72.

Hannukainen, J.C., Borra, R., Linderborg, K., Kallio, H., Kiss, J., Lepomäki, V., Kalliokoski, K.K., Kujala, U.M., Kaprio, J., Heinonen, O.J., Komu, M., Parkkola, R., Ahotupa, M., Lehtimäki, T., Huupponen, R., Iozzo, P. and Nuutila, P. (2011) 'Liver and pancreatic fat content and metabolism in healthy monozygotic twins with discordant physical activity', *Journal of Hepatology*, 54(3), pp. 545-552.

Hayes, M., Hunt, L., Foo, J., Tychinskaya, Y. and Stubbs, R. (2011) 'A Model for Predicting the Resolution of Type 2 Diabetes in Severely Obese Subjects Following Roux-en Y Gastric Bypass Surgery', *Obesity Surgery*, 21(7), pp. 910-916.

He, S., McPhaul, C., Li, J., Garuti, R., Kinch, L., Grishin, N., Cohen, J. and Hobbs, H. (2010) 'A Sequence Variation (I148M) in PNPLA3 Associated with Nonalcoholic Fatty Liver Disease Disrupts Triglyceride Hydrolysis ', *Journal of Biological Chemistry*, 285(9), pp. 6706-6715.

Heijboer, A.C., Frans, A., Lomecky, M. and Blankenstein, M.A. (2011) 'Analysis of glucagon-like peptide 1; what to measure?', *Clin Chim Acta*, 412(13-14), pp. 1191-4.

Heni, M., Machann, J., Staiger, H., Schwenzer, N., Peter, A., Schick, F., Claussen, C., Stefan, N., Haring, H.-U. and Fritshce, A. (2010) 'Pancreatic fat is negatively associated with insulin secretion in individuals with impaired fasting glucose and/or impaired glucose tolerance: a nuclear magnetic resonance study', *Diabetes/Metabolism Research and Reviews*, 26, pp. 200-205.

Henry, R.R. and Gumbiner, B. (1991) 'Benefits and limitations of very-low-calorie diet therapy in obese NIDDM', *Diabetes Care*, 14(9), pp. 802-23.

Henry, R.R., Schaeffer, L. and Olefsky, J.M. (1985) 'Glycaemic effects of intensive caloric restriction and isocaloric refeeding in non-insulin dependent diabetes mellitus', *Journal of Clinical Endocrinology and Metabolism*, 61, pp. 917-925.

Hofso, D., Jenssen, T., Bollerslev, J., Ueland, T., Godang, K., Stumvoll, M., Sandbu, R. and Roislien, J. (2011) 'Beta cell function after weight loss: a clinical trial comparing gastric bypass surgery and intensive lifestyle intervention', *European Journal of Endocrinology*, 164, pp. 231-238.

Hollingsworth, K.G., Al-Mrabeh, A., Steven, S. and Taylor, R. (2015) 'Pancreatic triacylglycerol distribution in type 2 diabetes', *Diabetologia*, 58(11), pp. 2676-2678.

Holman, R.R., Paul, S.K., Bethel, M.A., Matthews, D.R. and Neil, H.A. (2008) '10-year follow-up of intensive glucose control in type 2 diabetes', *N Engl J Med*, 359(15), pp. 1577-89.

Holst, J. and Gromada, J. (2004) 'Role of incretin hormones in the regulation of insulin secretion in diabetic and nondiabetic humans', *American Journal of Endocrinology and Metabolism*, 287, pp. E199-E206.

Holst, J.J. (2011) 'Postprandial Insulin Secretion After Gastric Bypass Surgery: The Role of Glucagon-Like Peptide 1', *Diabetes*, 60(9), pp. 2203-2205.

Honka, H., Koffert, J., Hannukainen, J.C., Tuulari, J.J., Karlsson, H.K., Immonen, H., Oikonen, V., Tolvanen, T., Soinio, M., Salminen, P., Kudomi, N., Mari, A., Iozzo, P. and Nuutila, P. (2015) 'The effects of bariatric surgery on pancreatic lipid metabolism and blood flow', *J Clin Endocrinol Metab*, 100(5), pp. 2015-23.

Hother-Nielsen, O. and Beck-Nielsen, H. (1990) 'On the determination of basal glucose production rate in patients with Type 2 (non-insulin dependent) diabetes mellitus using primed-continuous 3-3H-glucose infusion', *Diabetologia*, 33, pp. 603-610.

Hovorka, R., Soons, P.A. and Young, M.A. (1996) 'ISEC: a program to calculate insulin secretion', *Computer Methods and Programs in Biomedicine*, 50(3), pp. 253-264.

HSCIC (2015) *Statistics on Obesity, Physical Activity and Diet - England, 2015*. Available at: http://www.hscic.gov.uk/catalogue/PUB16988/obes-phys-acti-diet-eng-2015.pdf.

Hu, H.H., Kim, H.W., Nayak, K.S. and Goran, M.I. (2010) 'Comparison of fat-water MRI and single-voxel MRS in the assessment of hepatic and pancreatic fat fractions in humans', *Obesity (Silver Spring),* 18(4), pp. 841-7.

Immonen, H., Hannukainen, J.C., Iozzo, P., Soinio, M., Salminen, P., Saunavaara, V., Borra, R., Parkkola, R., Mari, A., Lehtimäki, T., Pham, T., Laine, J., Kärjä, V., Pihlajamäki, J., Nelimarkka, L. and Nuutila, P. (2014) 'Effect of bariatric surgery on liver glucose metabolism in morbidly obese diabetic and non-diabetic patients', *Journal of Hepatology*, 60(2), pp. 377-383.

International Physical Activity Questionnaire. Available at: http://www.ipaq.ki.se/index.htm.

Iozzo, P., Beck-Nielsen, H., Laakso, M., Smith, U., Yki-Jarvinen, H. and Ferrannini, E. (1999) 'Independent influence of age on basal insulin secretion in nondiabetic humans. European Group for the Study of Insulin Resistance', *J Clin Endocrinol Metab*, 84(3), pp. 863-8.

Isbell, J., Tamboli, R., Hansen, E., Saliba, J., Dunn, J., Phillips, S., Marks-Shulman, P. and Abumrad, N. (2010) 'The importance of caloric restriction in the early improvement in insulin sensitivity after Roux-en-Y gastric bypass surgery', *Diabetes Care*, 33, pp. 1438-1442.

Jackness, C., Karmally, W., Febres, G., Conwell, I.M., Ahmed, L., Bessler, M., McMahon, D.J. and Korner, J. (2013) 'Very low-calorie diet mimics the early beneficial effect of Roux-en-Y gastric bypass on insulin sensitivity and beta-cell Function in type 2 diabetic patients', *Diabetes*, 62(9), pp. 3027-32.

Jacobs, R.L., Zhao, Y., Koonen, D.P.Y., Sletten, T., Su, B., Lingrell, S., Cao, G., Peake, D.A., Kuo, M.-S., Proctor, S.D., Kennedy, B.P., Dyck, J.R.B. and Vance, D.E. (2010) 'Impaired de Novo Choline Synthesis Explains Why Phosphatidylethanolamine N-Methyltransferase-deficient Mice Are

Protected from Diet-induced Obesity', *Journal of Biological Chemistry*, 285(29), pp. 22403-22413.

Jain, R., Kabadi, U. and Kabadi, M. (2008) 'Is beta-cell failure in type 2 diabetes mellitus reversible?', *International Journal of Diabetes in Developing Countries*, 28(1), pp. 1-5.

Jazet, I.M., de Craen, A.J., van Schie, E.M. and Meinders, A.E. (2007) 'Sustained beneficial metabolic effects 18 months after a 30-day very low calorie diet in severely obese, insulin-treated patients with type 2 diabetes', *Diabetes Research and Clinical Practice*, 77(1), pp. 70-76.

Jimenez, A., Casamitjana, R., Viaplana-Masclans, J., Lacy, A. and Vidal, J. (2013) 'GLP-1 action and glucose tolerance in subjects with remission of type 2 diabetes after gastric bypass surgery', *Diabetes Care*, 36(7), pp. 2062-9.

Jiménez, A., Mari, A., Casamitjana, R., Lacy, A., Ferrannini, E. and Vidal, J. (2014) 'GLP-1 and Glucose Tolerance After Sleeve Gastrectomy in Morbidly Obese Subjects With Type 2 Diabetes', *Diabetes*, 63(10), pp. 3372-3377.

Jorgensen, N.B., Dirksen, C., Bojsen-Moller, K.N., Jacobsen, S.H., Worm, D., Hansen, D.L., Kristiansen, V.B., Naver, L., Madsbad, S. and Holst, J.J. (2013) 'Exaggerated glucagon-like peptide 1 response is important for improved beta-cell function and glucose tolerance after Roux-en-Y gastric bypass in patients with type 2 diabetes', *Diabetes*, 62(9), pp. 3044-52.

Kahn, C. (1978) 'Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction', *Metabolism*, 27(12), pp. 1893-1902.

Kahn, S.E.M.B.C., Haffner, S.M.M.D., Heise, M.A.P., Herman, W.H.M.D.M.P.H., Holman, R.R.F., Jones, N.P.M.A., Kravitz, B.G.M.S., Lachin, J.M.S., O'Neill, M.C.B., Zinman, B.M.D.F. and Viberti, G.M.D.F. (2006) 'Glycemic Durability of Rosiglitazone, Metformin, or Glyburide Monotherapy', *The New England Journal of Medicine*, 355(23), pp. 2427-43.

Kantartzis, K., Peter, A., Machicao, F., Machann, J., Wagner, S., Konigsrainer, I., Konigsrainer, A., Schick, F., Fritsche, A., Haring, H.U. and Stefan, N. (2009) 'Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene', *Diabetes*, 58(11), pp. 2616-23.

Karpe, F., Dickmann, J.R. and Frayn, K.N. (2011) 'Fatty acids, obesity, and insulin resistance: time for a reevaluation', *Diabetes*, 60(10), pp. 2441-9.

Kashyap, S., Belfort, R., Gastaldelli, A., Pratipanawatr, T., Berria, R., Pratipanawatr, W., Bajaj, M., Mandarino, L., DeFronzo, R. and Cusi, K. (2003) 'A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes', *Diabetes*, 52(10), pp. 2461-74.

Kashyap, S., Louis, E. and Kirwan, J. (2011) 'Weight loss as a cure for type 2 diabetes? Fact or fantasy', *Expert Reviews in Endocrinology & Metabolism*, 6(4), pp. 557-561.

Kendall, D., Sutherland, D., Najarian, J., Goetz, F. and Robertson, R. (1990) 'Effects of Hemipancreatectomy on Insulin Secretion and Glucose Tolerance in Healthy Humans', *New England Journal of Medicine*, 322, pp. 898-903.

Khanna, V., Malin, S.K., Bena, J., Abood, B., Pothier, C.E., Bhatt, D.L., Nissen, S., Watanabe, R., Brethauer, S.A., Schauer, P.R., Kirwan, J.P. and Kashyap, S.R. (2015) 'Adults with long-duration type 2 diabetes have blunted glycemic and beta-cell function improvements after bariatric surgery', *Obesity (Silver Spring)*, 23(3), pp. 523-6.

Klein, S., Mittendorfer, B., Eagon, C., Patterson, B., Grant, L., Feirt, N., Seki, E., Brenner, D., Korenblat, K. and McCrea, J. (2006) 'Gastric bypass surgery improves metabolic and hepatic abnormalities associated with nonalcoholic fatty liver disease', *Gastroenterology*, 130, pp. 1564-1572.

Knop, F. and Taylor, R. (2013) 'Mechanism of metabolic advantages after bariatric surgery - *it's all gastrointestinal factors vs. it's all food restriction*', *Diabetes Care*, 36(Suppl 2), pp. S287-S291.

Knop, F.K. (2009) 'Resolution of type 2 diabetes following gastric bypass surgery: involvement of gut-derived glucagon and glucagonotropic signalling?', *Diabetologia*, 52, pp. 2270-2276.

Korner, J., Bessler, M., Inabnet, W., Taveras, C. and Holst, J.J. (2007) 'Exaggerated glucagon-like peptide-1 and blunted glucose-dependent insulinotropic peptide secretion are associated with Roux-en-Y gastric bypass but not adjustable gastric banding', *Surg Obes Relat Dis*, 3(6), pp. 597-601.

Kotronen, A., Juurinen, L., Hakkarainen, A., Westerbacka, J., Cornér, A., Bergholm, R. and Yki-Järvinen, H. (2008) 'Liver Fat Is Increased in Type 2 Diabetic Patients and Underestimated by Serum Alanine Aminotransferase Compared With Equally Obese Nondiabetic Subjects', *Diabetes Care*, 31(1), pp. 165-169.

Laferrere, B., Heshka, S., Wang, K., Khan, Y., McGinty, J., Teixeira, J., Hart, A. and Olivan, B. (2007) 'Incretin levels and effect are markedly enhanced 1 month after Roux-en-Y gastric bypass surgery in obese patients with type 2 diabetes', *Diabetes Care*, 30(7), pp. 1709-16.

Laferrere, B., Teixeira, J., McGinty, J., Tran, H., Egger, J.R., Colarusso, A., Kovack, B., Bawa, B., Koshy, N., Lee, H., Yapp, K. and Olivan, B. (2008) 'Effect of weight loss by gastric bypass surgery versus hypocaloric diet on glucose and incretin levels in patients with type 2 diabetes', *Journal of Clinical Endocrinology and Metabolism*, 93(7), pp. 2479-85.

Lalloyer, F., Vandewalle, B., Percevault, F., Torpier, G., Kerr-Conte, J., Oosterveer, M., Paumelle, R., Fruchart, J.C., Kuipers, F., Pattou, F., Fievet, C. and Staels, B. (2006) 'Peroxisome proliferator-activated receptor alpha improves pancreatic adaptation to insulin resistance in obese mice and reduces lipotoxicity in human islets', *Diabetes*, 55(6), pp. 1605-13.

Lassailly, G., Caiazzo, R., Buob, D., Pigeyre, M., Verkindt, H., Labreuche, J., Raverdy, V., Leteurtre, E., Dharancy, S., Louvet, A., Romon, M., Duhamel, A., Pattou, F. and Mathurin, P. (2015) 'Bariatric Surgery Reduces Features

of Nonalcoholic Steatohepatitis in Morbidly Obese Patients', *Gastroenterology*, 149(2), pp. 379-88; quiz e15-6.

Lee, M.H., Lee, W.J., Chong, K., Chen, J.C., Ser, K.H., Lee, Y.C. and Chen, S.C. (2015) 'Predictors of long-term diabetes remission after metabolic surgery', *J Gastrointest Surg*, 19(6), pp. 1015-21.

Lee, Y., Hirose, H., Ohneda, M., Johnson, J.H., McGarry, J.D. and Unger, R.H. (1994) 'B-Cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-B-Cell relationships', *Proceedings of the National Academy of Science of U.S.A.*, 91, pp. 10878-10882.

Lee, Y., Lingvay, I., Szczepaniak, L.S., Ravazzola, M., Orci, L. and Unger, R.H. (2009) 'Pancreatic steatosis: harbinger of type 2 diabetes in obese rodents', *Int J Obes*, 34(2), pp. 396-400.

Lim, E.L., Hollingsworth, K.G., Aribisala, B.S., Chen, M.J., Mathers, J.C. and Taylor, R. (2011) 'Reversal of type 2 diabetes: normalisation of beta cell function in association with decreased pancreas and liver triacylglycerol', *Diabetologia*, 54(10), pp. 2506-14.

Lim, S., Bae, J.H., Chun, E.J., Kim, H., Kim, S.Y., Kim, K.M., Choi, S.H., Park, K.S., Florez, J.C. and Jang, H.C. (2014) 'Differences in pancreatic volume, fat content, and fat density measured by multidetector-row computed tomography according to the duration of diabetes', *Acta Diabetol*, 51(5), pp. 739-48.

Lingvay, I., Guth, E., Islam, A. and Livingston, E. (2013) 'Rapid Improvement in Diabetes After Gastric Bypass Surgery: Is it the diet or surgery?', *Diabetes Care*, 36(9), pp. 2741-2747.

Lips, M.A., de Groot, G.H., van Klinken, J.B., Aarts, E., Berends, F.J., Janssen, I.M., Van Ramshorst, B., Van Wagensveld, B.A., Swank, D.J., Van Dielen, F., Willems van Dijk, K. and Pijl, H. (2014) 'Calorie restriction is a major determinant of the short-term metabolic effects of gastric bypass

surgery in obese type 2 diabetic patients', *Clin Endocrinol (Oxf)*, 80(6), pp. 834-42.

Liu, Y.L., Patman, G.L., Leathart, J.B., Piguet, A.C., Burt, A.D., Dufour, J.F., Day, C.P., Daly, A.K., Reeves, H.L. and Anstee, Q.M. (2014) 'Carriage of the PNPLA3 rs738409 C >G polymorphism confers an increased risk of non-alcoholic fatty liver disease associated hepatocellular carcinoma', *J Hepatol*, 61(1), pp. 75-81.

Luzi, L. and DeFronzo, R.A. (1989) 'Effect of loss of first phase insulin secretion on hepatic glucose production and tissue glucose disposal in humans', *American Journal of Physiology*, 257, pp. E241-E246.

Macauley, M., Hollingsworth, K.G., Smith, F.E., Thelwall, P.E., Al-Mrabeh, A., Schweizer, A., Foley, J.E. and Taylor, R. (2015a) 'Effect of vildagliptin on hepatic steatosis', *J Clin Endocrinol Metab*, 100(4), pp. 1578-85.

Macauley, M., Percival, K., Thelwall, P.E., Hollingsworth, K.G. and Taylor, R. (2015b) 'Altered volume, morphology and composition of the pancreas in type 2 diabetes', *PLoS One*, 10(5), p. e0126825.

Macauley, M., Smith, F.E., Thelwall, P.E., Hollingsworth, K.G. and Taylor, R. (2015c) 'Diurnal variation in skeletal muscle and liver glycogen in humans with normal health and Type 2 diabetes', *Clin Sci (Lond)*, 128(10), pp. 707-13.

Maggs, D.G., Buchanan, T.A., Burant, C.F., Cline, G., Gumbiner, B., Hsueh, W.A., Inzucchi, S., Kelley, D., Nolan, J., Olefsky, J.M., Polonsky, K.S., Silver, D., Valiquett, T.R. and Shulman, G.I. (1998) 'Metabolic Effects of Troglitazone Monotherapy in Type 2 Diabetes MellitusA Randomized, Double-Blind, Placebo-Controlled Trial', *Annals of Internal Medicine*, 128(3), pp. 176-185.

Magkos, F., Fabbrini, E., McCrea, J., Patterson, B.W., Eagon, J.C. and Klein, S. (2010) 'Decrease in hepatic very-low-density lipoprotein–triglyceride secretion after weight loss is inversely associated with changes in circulating leptin', *Diabetes, obesity & metabolism*, 12(7), pp. 584-590.

Manning, S., Pucci, A. and Batterham, R.L. (2015) 'GLP-1: A Mediator of the Beneficial Metabolic Effects of Bariatric Surgery?', *Physiology (Bethesda)*, 30(1), pp. 50-62.

Marchetti, P., Del Guerra, S., Marselli, L., Lupi, R., Masini, M., Pollera, M., Bugliani, M., Boggi, U., Vistoli, F., Mosca, F. and Del Prato, S. (2004) 'Pancreatic islets from type 2 diabeteic patients have functional defects and increased apoptosis that are ameliorated by metformin', *The Journal of Clinical Endocrinology & Metabolism*, 89, pp. 5535-5541.

Martin, B.C., Warram, J.H., Krolewski, A.S., Soeldner, J.S., Kahn, C.R. and Bergman, R.N. (1992) 'Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study', *The Lancet*, 340(8825), pp. 925-929.

Menge, B., Tannapfel, A., Belyaev, O., Drescher, R., Müller, C., Uhl, W., Schmidt, W. and Meier, J. (2008) 'Partial Pancreatectomy in Adult Humans Does Not Provoke β-Cell Regeneration', *Diabetes*, 57, pp. 142-149.

Michie, S., Ashford, S., Sniehotta, F.F., Dombrowski, S.U., Bishop, A. and French, D.P. (2011) 'A refined taxonomy of behaviour change techniques to help people change their physical activity and healthy eating behaviours: the CALO-RE taxonomy', *Psychol Health*, 26(11), pp. 1479-98.

Mingrone, G., Panunzi, S., De Gaetano, A., Guidone, C., Iaconelli, A., Leccesi, L., Nanni, G., Pomp, A., Castagneto, M., Ghirlanda, G. and Rubino, F. (2012) 'Bariatric Surgery versus Conventional Medical Therapy for Type 2 Diabetes', *New England Journal of Medicine*, 366(17), pp. 1577-1585.

Mittendorfer, B., Patterson, B. and Klein, S. (2003) 'Effect of weight loss on VLDL-triglyceride and apoB-100 kinetics in women with abdominal obesity', *American Journal of Physiology Endocrinology and Metabolism*, 284, pp. E549-E556.

Morgan, N. (2009) 'Fatty acids and beta-cell toxicity', *Current Opinion in Clinical Nutrition and Metabolic Care*, 12, pp. 117-122.

Morgan, N.G., Dhayal, S., Diakogiannaki, E. and Welters, H.J. (2008) 'The cytoprotective actions of long-chain mono-unsaturated fatty acids in pancreatic beta-cells', *Biochem Soc Trans*, 36(Pt 5), pp. 905-8.

MRC/BHF (2003) 'MRC/BHF Heart Protection Study of cholesterol-lowering with simvastatin in 5963 people with diabetes: a randomised placebo-controlled trial', *The Lancet*, 361(9374), pp. 2005-2016.

Mustajoki, P. and Pekkarinen, T. (2001) 'Very low energy diets in the treatment of obesity', *Obes Rev*, 2(1), pp. 61-72.

Nagulesparan, M., Savage, P.J., Bennion, L.J., Unger, R.H. and Bennett, P.H. (1981) 'Diminshed effect of caloric restriction on control of hyperglycaemia with increasing known duration of type 2 diabetes mellitus', *Journal of Clinical Endocrinology and Metabolism*, 53, pp. 560-568.

Nannipieri, M., Mari, A., Anselmino, M., Baldi, S., Barsotti, E., Guarino, D., Camastra, S., Bellini, R., Berta, R. and Ferrannini, E. (2011) 'The role of beta-cell function and insulin sensitivity in the remission of type 2 diabetes after gastric bypass surgery', *Journal of Clinical Endocrinology and Metabolism*.

Naslund, E. and Hellstrom, P.M. (2013) 'Elucidating the mechanisms behind the restoration of euglycemia after gastric bypass surgery', *Diabetes*, 62(4), pp. 1012-3.

Nathan, D.M., Buse, J.B., Davidson, M.B., Ferrannini, E., Holman, R.R., Sherwin, R. and Zinman, B. (2009) 'Medical management of hyperglycemia in type 2 diabetes: a consensus algorithm for the initiation and adjustment of therapy: a consensus statement of the American Diabetes Association and the European Association for the Study of Diabetes', *Diabetes Care*, 32(1), pp. 193-203.

NBSR (2014) *The UK National Bariatric Surgery Registry: Second Registry Report 2014*. Available at: http://nbsr.co.uk/wp-content/uploads/2014/11/Extract_from_the_NBSR_2014_Report.pdf.

NICE (2012) *Type 2 diabetes: prevention in people at high risk. PH38.* Available at: https://www.nice.org.uk/guidance/ph38.

NICE (2014) *Obesity: identification, assessment and management of overweight and obesity in children, young people and adults.* [Online]. Available at: https://www.nice.org.uk/guidance/cg189.

NICE (2015) *Type 2 diabetes in adults: management [NG28].* Available at: https://www.nice.org.uk/guidance/ng28.

Niebergall, L.J., Jacobs, R.L., Chaba, T. and Vance, D.E. (2011) 'Phosphatidylcholine protects against steatosis in mice but not non-alcoholic steatohepatitis', *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1811(12), pp. 1177-1185.

Nijpels, G., Boorsma, W., Dekker, J., Hoeksema, F., Kostense, P., Bouter, L. and Heine, R. (2007) 'Absence of an Acute Insulin Response Predicts Onset of Type 2 Diabetes in a Caucasian Population with Impaired Glucose Tolerance', *The Journal of Clinical Endocrinology & Metabolism* 93, pp. 2633-2638.

Nobili, V., Marcellini, M., Devito, R., Ciampalini, P., Piemonte, F., Comparcola, D., Sartorelli, M.R. and Angulo, P. (2006) 'NAFLD in children: a prospective clinical-pathological study and effect of lifestyle advice', *Hepatology*, 44(2), pp. 458-65.

Noushmehr, H., D'Amico, E., Farilla, L., Hui, H., Wawrowsky, K.A., Mlynarski, W., Doria, A., Abumrad, N.A. and Perfetti, R. (2005) 'Fatty acid translocase (FAT/CD36) is localized on insulin-containing granules in human pancreatic beta-cells and mediates fatty acid effects on insulin secretion', *Diabetes*, 54(2), pp. 472-81.

Paisey, R.B., Frost, J., Harvey, P., Paisey, A., Bower, L., Paisey, R.M., Taylor, P. and Belka, I. (2002) 'Five year results of a prospective very low calorie diet or conventional weight loss programme in type 2 diabetes', *Journal of Human Nutrition and Dietetics*, 15(2), pp. 121-127.

Perry, R.J., Samuel, V.T., Petersen, K.F. and Shulman, G.I. (2014) 'The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes', *Nature*, 510(7503), pp. 84-91.

Perseghin, G., Lattuada, G., De Cobelli, F., Esposito, A., Costantino, F., Canu, T., Scifo, P., De Taddeo, F., Maffi, P., Secchi, A., Del Maschio, A. and Luzi, L. (2005) 'Reduced intrahepatic fat content is associated with increased whole-body lipid oxidation in patients with type 1 diabetes', *Diabetologia*, 48(12), pp. 2615-21.

Peters, C., Steven, S. and Taylor, R. (2015) 'Reversal of Type 2 Diabetes by Weight Loss Despite Presence of Macro- and Microvascular Complications', in Draznin, B., Wang, C.L. and Rubin, D. (eds.) *Diabetes Case Studies*. Alexandria, VA: American Diabetes Association, pp. 271-274.

Peters, R.L., Gay, T. and Reynolds, T.B. (1975) 'Post-jejunoileal-bypass hepatic disease. Its similarity to alcoholic hepatic disease', *Am J Clin Pathol*, 63(3), pp. 318-31.

Petersen, K., Dufour, S., Befroy, D., Lehrke, M., Hendler, R. and Shulman, G. (2005) 'Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes', *Diabetes*, 54(3), pp. 603-8.

Petersen, K.F., Dufour, S., Morino, K., Yoo, P.S., Cline, G.W. and Shulman, G.I. (2012) 'Reversal of muscle insulin resistance by weight reduction in young, lean, insulin-resistant offspring of parents with type 2 diabetes', *Proc Natl Acad Sci U S A*, 109(21), pp. 8236-40.

Petit, J.-M., Guiu, B., Masson, D., Duvillard, L., Jooste, V., Buffier, P., Terriat, B., Bouillet, B., Brindisi, M.-C., Loffroy, R., Robin, I., Hillon, P., Cercueil, J.-P. and Verges, B. (2010) 'Specifically PNPLA3-Mediated Accumulation of Liver Fat in Obese Patients with Type 2 Diabetes', *Journal of Clinical Endocrinology and Metabolism*, 95, pp. E430-E436.

PHE (2014) *Adult obesity and type 2 diabetes*.Public Health England.

Pi-Sunyer, X., Blackburn, G., Brancati, F.L., Bray, G.A., Bright, R., Clark, J.M., Curtis, J.M., Espeland, M.A., Foreyt, J.P., Graves, K., Haffner, S.M., Harrison, B., Hill, J.O., Horton, E.S., Jakicic, J., Jeffery, R.W., Johnson, K.C., Kahn, S., Kelley, D.E., Kitabchi, A.E., Knowler, W.C., Lewis, C.E., Maschak-Carey, B.J., Montgomery, B., Nathan, D.M., Patricio, J., Peters, A., Redmon, J.B., Reeves, R.S., Ryan, D.H., Safford, M., Van Dorsten, B., Wadden, T.A., Wagenknecht, L., Wesche-Thobaben, J., Wing, R.R. and Yanovski, S.Z. (2007) 'Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial', *Diabetes Care*, 30(6), pp. 1374-83.

Pillai, A.A. and Rinella, M.E. (2009) 'Non-Alcoholic Fatty Liver Disease: Is Bariatric Surgery the Answer?', *Clinics in Liver Disease*, 13(4), pp. 689-710.

Pimenta, W., Korytkowski, M., Mitrakou, A., Jenssen, T., Yki-Jarvinen, H., Evron, W., Dailey, G. and Gerich, J. (1995) 'Pancreatic beta-cell dysfunction as the primary genetic lesion in NIDDM. Evidence from studies in normal glucose-tolerant individuals with a first-degree NIDDM relative', *JAMA*, 273(23), pp. 1855-61.

Pinnick, K., Neville, M., Clark, A. and Fielding, B. (2010) 'Reversibility of metabolic and morphological changes associated with chronic exposure of pancreatic islet beta-cells to fatty acids', *J Cell Biochem*, 109(4), pp. 683-92.

Pinnick, K.E., Collins, S.C., Londos, C., Gauguier, D., Clark, A. and Fielding, B.A. (2008) 'Pancreatic ectopic fat is characterized by adipocyte infiltration and altered lipid composition', *Obesity (Silver Spring)*, 16(3), pp. 522-30.

Pories, W., Swanson, M., MacDonald, K., Long, S., Morris, P., Brown, B., Barakat, H., deRamon, R., Israel, G. and Dolezal, J. (1995) 'Who would have thought it? An operation proves to be the most effective therapy for adult-onset diabetes mellitus', *Annals of Surgery*, 222(3), pp. 339-352.

Pories, W.J., MacDonald, K.G., Jr., Morgan, E.J., Sinha, M.K., Dohm, G.L., Swanson, M.S., Barakat, H.A., Khazanie, P.G., Leggett-Frazier, N., Long,

S.D. and et al. (1992) 'Surgical treatment of obesity and its effect on diabetes: 10-y follow-up', *Am J Clin Nutr*, 55(2 Suppl), pp. 582S-585S.

Prager, R., Wallace, P. and Olefsky, J.M. (1986) 'In vivo kinetics of insulin action on peripheral glucose disposal and hepatic glucose output in normal and obese subjects', *J Clin Invest*, 78(2), pp. 472-81.

Rahier, J., Guiot, Y., Goebbels, R.M., Sempoux, C. and Henquin, J.C. (2008) 'Pancreatic β-cell mass in European subjects with type 2 diabetes', *Diabetes, Obesity and Metabolism*, 10, pp. 32-42.

Ravikumar, B., Gerrard, J., Dalla Man, C., Firbank, M.J., Lane, A., English, P.T., Cobelli, C. and Taylor, R. (2008) 'Pioglitazone decreases fasting and postprandial endogenous glucose production in proportion to decrease in hepatic triglyceride content', *Diabetes*, 57, pp. 2288-2295.

RCGP (2002) *Clinical guidelines for type 2 diabetes.* . University of Sheffield The Royal College of General Practitioners. Effective Clinical Practice Unit.

Renard, E. (2009) 'Bariatric surgery in patients with late-stage type 2 diabetes: expected beneficial effects on risk ratio and outcomes', *Diabetes & Metabolism*, 35, pp. 564-568.

Rizza, R.A., Mandarino, L.J. and Gerich, J.E. (1981) 'Dose response characteristics for effects of insulin on production and utilisation of glucose in man', *American Journal of Physiology*, 240, pp. E630-E639.

Robertson, R.P., Harmon, J., Tran, P.O.T. and Poitout, V. (2004) 'β-Cell Glucose Toxicity, Lipotoxicity, and Chronic Oxidative Stress in Type 2 Diabetes', *Diabetes*, 53(suppl 1), pp. S119-S124.

Romeo, S., Kozlitina, J., Xing, C., Pertsemlidis, A., Cox, D., Pennacchio, L., Boerwinkle, E., Cohen, J. and Hobbs, H. (2008) 'Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease.', *Nature Genetics*, 40(12), pp. 1461-1465.

Ross, H.M., Laws, R., Reckless, J., Lean, M. and Counterweight Project, T. (2008) 'Evaluation of the Counterweight Programme for obesity

management in primary care: a starting point for continuous improvement', *British Journal of General Practice*, 58(553), pp. 548-554.

Rothman, D.L., Shulman, R.G. and Shulman, G.I. (1992) 'P-31 Nuclear magnetic resonace measurements of muscle glucose-6-phosphate - Evidence for reduced insulin dependent muscle glucose transport or phsophorylation activity in NIDDM', *Journal of Clinical Investigation*, 89(4), pp. 1069-1075.

Roux, C.W.l., Aylwin, S.J.B., Batterham, R.L., Borg, C.M., Coyle, F., Prasad, V., Shurey, S., Ghatei, M.A., Patel, A.G. and Bloom, S.R. (2006) 'Gut Hormone Profiles Following Bariatric Surgery Favor an Anorectic State, Facilitate Weight Loss, and Improve Metabolic Parameters', *Annals of Surgery*, 243(1), pp. 108-114.

Rubino, F., Gagner, M., Gentileschi, P., Kini, S., Fukuyama, S., Feng, J. and Diamond, E. (2004) 'The early effect of the Roux-en-Y gastric bypass on hormones involved in body weight regulation and glucose metabolism', *Ann Surg*, 240(2), pp. 236-42.

Saisho, Y., Butler, A.E. and Butler, P.C. (2008) 'Pancreatic Fat Content and β-Cell Function in Men With and Without Type 2 Diabetes: Response to Tushuizen et al', *Diabetes Care*, 31(5), p. e38.

Saisho, Y., Butler, A.E., Meier, J.J., Monchamp, T., Allen-Auerbach, M., Rizza, R.A. and Butler, P.C. (2007) 'Pancreas volumes in humans from birth to age one hundred taking into account sex, obesity, and presence of type-2 diabetes', *Clin Anat*, 20(8), pp. 933-42.

Saisho, Y., Tanaka, K., Abe, T., Shimada, A., Kawai, T. and Itoh, H. (2012) 'Effect of obesity on declining beta-cell function after diagnosis in type 2 diabetes: a possible link suggested by cross-sectional analysis', *Endocrine Journal*, 59(3), pp. 187-195.

Salehi, M., Prigeon, R.L. and D'Alessio, D.A. (2011) 'Gastric bypass surgery enhances glucagon-like peptide 1-stimulated postprandial insulin secretion in humans', *Diabetes*, 60(9), pp. 2308-14.

Samuel, V., Petersen, K. and Shulman, G. (2010) 'Lipid-induced insulin resistance: unravelling the mechanism', *Lancet*, 375, pp. 2267-2277.

Sattar, N. and Gill, J. (2014) 'Type 2 diabetes as a disease of ectopic fat?', *BMC Medicine*, 12(1), p. 123.

Sattar, N., McConnachie, A., Ford, I., Gaw, A., Cleland, S.J., Forouhi, N.G., McFarlane, P., Shepherd, J., Cobbe, S. and Packard, C. (2007) 'Serial metabolic measurements and conversion to type 2 diabetes in the west of Scotland coronary prevention study: specific elevations in alanine aminotransferase and triglycerides suggest hepatic fat accumulation as a potential contributing factor', *Diabetes*, 56(4), pp. 984-91.

Schauer, P., Burguera, B, Ikramuddin, S, Cottam, D, Gourash, W, Hamad, G, Eid, GM, Mattar, S, Ramanathan, R, Barinas-Mitchel, E, Rao, RH, Kuller, L, Kelley, D (2003) 'Effect of laparoscopic Roux-en-Y gastric bypass on type 2 diabetes mellitus', *Annals of Surgery*, 238, pp. 467-484.

Schauer, P.R., Kashyap, S.R., Wolski, K., Brethauer, S.A., Kirwan, J.P., Pothier, C.E., Thomas, S., Abood, B., Nissen, S.E. and Bhatt, D.L. (2012) 'Bariatric Surgery versus Intensive Medical Therapy in Obese Patients with Diabetes', *New England Journal of Medicine*, 366(17), pp. 1567-1576.

Schmitz-Moormann, P., Pittner, P.M. and Heinze, W. (1981) 'Lipomatosis of the pancreas', *Pathology - Research and Practice*, 173(1), pp. 45-53.

Schwarz, J.M., Linfoot, P., Dare, D. and Aghajanian, K. (2003) 'Hepatic de novo lipogenesis in normoinsulinemic and hyperinsulinemic subjects consuming high-fat, low-carbohydrate and low-fat, high-carbohydrate isoenergetic diets', *Am J Clin Nutr*, 77(1), pp. 43-50.

Sellahewa, L., Khan, C., Lakkunarajah, S. and Idris, I. (2015) 'A systematic review of evidence on the use of very low calorie diets in people with diabetes', *Curr Diabetes Rev*, Epub ahead of print.

Seppala-Lindroos, A., Vehkavaara, S., Hakkinen, A., Goto, T., Westerbacka, J., Sovijarvi, A., Halavaara, J. and Yki-Jarvinen, H. (2002) 'Fat

accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men', *Journal of Clinical Endocrinology and Metabolism*, 87(7), pp. 3023-8.

Sevastianova, K., Kotronen, A., Gastaldelli, A., Perttilä, J., Hakkarainen, A., Lundbom, J., Suojanen, L., Orho-Melander, M., Lundbom, N., Ferrannini, E., Rissanen, A., Olkkonen, V.M. and Yki-Järvinen, H. (2011) 'Genetic variation in PNPLA3 (adiponutrin) confers sensitivity to weight loss–induced decrease in liver fat in humans', *The American Journal of Clinical Nutrition*, 94(1), pp. 104-111.

Sevastianova, K., Santos, A., Kotronen, A., Hakkarainen, A., Makkonen, J., Silander, K., Peltonen, M., Romeo, S., Lundbom, J., Lundbom, N., Olkkonen, V.M., Gylling, H., Fielding, B.A., Rissanen, A. and Yki-Järvinen, H. (2012) 'Effect of short-term carbohydrate overfeeding and long-term weight loss on liver fat in overweight humans', *The American Journal of Clinical Nutrition*, 96(4), pp. 727-734.

Shen, W., Punyanitya, M., Wang, Z., Gallagher, D., St-Onge, M.-P., Albu, J., Heymsfield, S.B. and Heshka, S. (2004) 'Visceral adipose tissue: relations between single-slice areas and total volume', *The American Journal of Clinical Nutrition*, 80(2), pp. 271-278.

Shibata, M., Kihara, Y., Taguchi, M., Tashiro, M. and Otsuki, M. (2007) 'Nonalcoholic fatty liver disease is a risk factor for type 2 diabetes in middle-aged Japanese men', *Diabetes Care*, 30(11), pp. 2940-4.

Shimabukuro, M., Higa, M., Zhou, Y.T., Wang, M.Y., Newgard, C.B. and Unger, R.H. (1998a) 'Lipoapoptosis in beta-cells of obese prediabetic fa/fa rats. Role of serine palmitoyltransferase overexpression', *J Biol Chem*, 273(49), pp. 32487-90.

Shimabukuro, M., Zhou, Y.T., Levi, M. and Unger, R.H. (1998b) 'Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes', *Proc Natl Acad Sci U S A*, 95(5), pp. 2498-502.

Singhal, P., Caumo, A., Carey, P., Cobelli, C. and Taylor, R. (2002) 'Regulation of endogenous glucose production after a mixed meal in type 2 diabetes', *Am. J. Physiol. Endocrinol. Metab.*, 283(2), pp. E275-E283.

Sjostrom, L., Lindroos, A.-K., Peltonen, M., Torgerson, J., Bouchard, C., Carlsson, B., Dahlgren, S., Larsson, B., Narbro, K., Sjostrom, C., Sullivan, M. and Wedel, H. (2004) 'Lifestyle, diabetes, and cardiovascular risk factors 10 years after bariatric surgery', *The New England Journal of Medicine*, 351, pp. 2683-2693.

Sjostrom, L., Peltonen, M., Jacobson, P., Ahlin, S., Andersson-Assarsson, J., Anveden, A., Bouchard, C., Carlsson, B., Karason, K., Lonroth, H., Naslund, I., Sjostrom, E., Taube, M., Wedel, H., Svensson, P.A., Sjoholm, K. and Carlsson, L.M. (2014) 'Association of bariatric surgery with long-term remission of type 2 diabetes and with microvascular and macrovascular complications', *JAMA*, 311(22), pp. 2297-304.

Speliotes, E., Butler, J., Palmer, C., Voight, B. and Hirschhorn, J. (2010) 'PNPLA3 Variants Specifically Confer Increased Risk for Histologic Nonalcoholic Fatty Liver Disease But Not Metabolic Disease', *Hepatology*, 52, pp. 904-912.

St-Onge, M., Mignault, D., Allison, D.B. and Rabasa-Lhoret, R. (2007) 'Evaluation of a portable device to measure daily energy expenditure in free-living adults', *The American Journal of Clinical Nutrition*, 85(3), pp. 742-749.

Steele, R., Wall, J., DeBodo, R. and Altszuler, N. (1956) 'Measurement size and turnover rate of body glucose pool by the isotope dilution method', *American Journal of Physiology*, 187, pp. 15-24.

Steven, S., Lim, E.L. and Taylor, R. (2013) 'Population response to information on reversibility of Type 2 diabetes', *Diabet Med*, 30(4), pp. e135-8.

Steven, S. and Taylor, R. (2012) 'Pathophysiology of type 2 diabetes', in Barnett, A. (ed.) *Type 2 Diabetes*. 2nd edn. Oxford: Oxford University Press.

Steven, S., Woodcock, S., Small, P. and Taylor, R. (2011) 'Type 2 diabetes, bariatric surgery and the risk of subsequent gestational diabetes', *Obstetric Medicine*, 4(4), pp. 171-173.

Stratton, I.M., Adler, A.I., Neil, H.A., Matthews, D.R., Manley, S.E., Cull, C.A., Hadden, D., Turner, R.C. and Holman, R.R. (2000) 'Association of glycaemia with macrovascular and microvascular complications of type 2 diabetes (UKPDS 35): prospective observational study', *BMJ*, 321(7258), pp. 405-12.

Stretton, C., Evans, A. and Hundal, H.S. (2010) 'Cellular depletion of atypical PKCλ is associated with enhanced insulin sensitivity and glucose uptake in L6 rat skeletal muscle cells', *American Journal of Physiology - Endocrinology And Metabolism*, 299(3), pp. E402-E412.

Szabat, M., Lynn, F.C., Hoffman, B.G., Kieffer, T.J., Allan, D.W. and Johnson, J.D. (2012) 'Maintenance of beta-cell maturity and plasticity in the adult pancreas: developmental biology concepts in adult physiology', *Diabetes*, 61(6), pp. 1365-71.

Szczepaniak, L.S., Nurenberg, P., Leonard, D., Browning, J.D., Reingold, J.S., Grundy, S., Hobbs, H.H. and Dobbins, R.L. (2005) 'Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population', *Am J Physiol Endocrinol Metab*, 288(2), pp. E462-8.

Szczepaniak, L.S., Victor, R.G., Mathur, R., Nelson, M.D., Szczepaniak, E.W., Tyer, N., Chen, I., Unger, R.H., Bergman, R.N. and Lingvay, I. (2012) 'Pancreatic steatosis and its relationship to beta-cell dysfunction in humans: racial and ethnic variations', *Diabetes Care*, 35(11), pp. 2377-83.

Tabák, A., Jokela, M., Akbaraly, T., Brunner, E., Kivimäki, M. and Witte, D. (2009) 'Trajectories of glycaemia, insulin sensitivity, and insulin secretion before diagnosis of type 2 diabetes: an analysis from the Whitehall II study', *Lancet*, 373, pp. 2215-2221.

Talchai, C., Xuan, S., Lin, Hua V., Sussel, L. and Accili, D. (2012) 'Pancreatic β Cell Dedifferentiation as a Mechanism of Diabetic β Cell Failure', *Cell*, 150(6), pp. 1223-1234.

Tang, C., Ahmed, K. and Gille, A. (2015) 'Loss of FFA2 and FFA3 increases insulin secretion and improves glucose tolerance in type 2 diabetes', *Nature Medicine*, 21(2), pp. 173-7.

Taylor, R. (2008) 'Pathogenesis of Type 2 diabetes: Tracing the reverse route from cure to cause', *Diabetologia*, 51, pp. 1781-1789.

Taylor, R. (2012) 'Insulin resistance and type 2 diabetes', *Diabetes*, 61(4), pp. 778-9.

Taylor, R. (2013) 'Type 2 diabetes: etiology and reversibility', *Diabetes Care*, 36(4), pp. 1047-55.

Taylor, R. and Holman, R.R. (2015) 'Normal weight individuals who develop type 2 diabetes: the personal fat threshold', *Clin Sci (Lond)*, 128(7), pp. 405-10.

Tolman, K.G., Fonseca, V., Dalpiaz, A. and Tan, M.H. (2007) 'Spectrum of liver disease in type 2 diabetes and management of patients with diabetes and liver disease', *Diabetes Care*, 30(3), pp. 734-43.

Toschi, E., Camastra, S., Sironi, A.M., Masoni, A., Gastaldelli, A., Mari, A., Ferrannini, E. and Natali, A. (2002) 'Effect of acute hyperglycemia on insulin secretion in humans', *Diabetes*, 51 Suppl 1, pp. S130-3.

Tushuizen, M.E., Bunck, M.C., Pouwels, P.J., Bontemps, S., van Waesberghe, J.H., Schindhelm, R.K., Mari, A., Heine, R.J. and Diamant, M. (2007) 'Pancreatic fat content and beta-cell function in men with and without type 2 diabetes', *Diabetes Care*, 30(11), pp. 2916-21.

Twomey, S. (1965) 'The application of numerical filtering to the solution of integral equations encountered in indirect sensing measurements', *Journal of the Franklin Institute*, 279(2), pp. 95-109.

UKPDS (1995a) 'U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. U.K. Prospective Diabetes Study Group', *Diabetes*, 44(11), pp. 1249-58.

UKPDS (1995b) 'United Kingdom Prospective Diabetes Study (UKPDS). 13: Relative efficacy of randomly allocated diet, sulphonylurea, insulin, or metformin in patients with newly diagnosed non-insulin dependent diabetes followed for three years', *BMJ*, 310(6972), pp. 83-8.

UKPDS (1998) 'Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)', *The Lancet*, 352(9131), pp. 837-853.

Uppot, R.N., Sahani, D.V., Hahn, P.F., Gervais, D. and Mueller, P.R. (2007) 'Impact of obesity on medical imaging and image-guided intervention', *AJR Am J Roentgenol*, 188(2), pp. 433-40.

Van Cauter, E., Mestrez, F., Sturis, J. and Polonsky, K.S. (1992) 'Estimation of insulin secretion rates from C-peptide levels. Comparison of individual and standard kinetic parameters for C-peptide clearance', *Diabetes*, 41(3), pp. 368-77.

van der Zijl, N., Goosens, G., Moors, C., van Raalte, D., Muskiet, M., Pouwels, P., Blaak, E. and Diamant, M. (2011) 'Ectopic fat storage in the pancreas, liver, and abdominal fat depots: impact on beta-cell function in individuals with impaired glucose metabolism', *Journal of Clinical Endocrinology and Metabolism*, 96(2), pp. 459-467.

Vella, A. (2013) 'Does caloric restriction alone explain the effects of Roux-en-Y gastric bypass on glucose metabolism? Not by a long limb', *Diabetes*, 62(9), pp. 3017-8.

Verges, B. (2010) 'Abnormal hepatic apolipoprotein B metabolism in type 2 diabetes', *Atherosclerosis*, 211, pp. 353-360.

Vilar-Gomez, E., Martinez-Perez, Y., Calzadilla-Bertot, L., Torres-Gonzalez, A., Gra-Oramas, B., Gonzalez-Fabian, L., Friedman, S.L., Diago, M. and Romero-Gomez, M. (2015) 'Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis', *Gastroenterology*, 149(2), pp. 367-78.e5; quiz e14-5.

Vilsbøll, T., Krarup, T., Deacon, C.F., Madsbad, S. and Holst, J.J. (2001) 'Reduced Postprandial Concentrations of Intact Biologically Active Glucagon-Like Peptide 1 in Type 2 Diabetic Patients', *Diabetes*, 50(3), pp. 609-613.

Wang, Z., York, N.W., Nichols, C.G. and Remedi, M.S. (2014) 'Pancreatic β-cell Dedifferentiation in Diabetes and Re-differentiation following Insulin Therapy', *Cell metabolism*, 19(5), pp. 872-882.

Weir, G.C. and Bonner-Weir, S. (2004) 'Five stages of evolving beta-cell dysfunction during progression to diabetes', *Diabetes*, 53 Suppl 3, pp. S16-21.

Weir, J.B. (1949) 'New methods for calculating metabolic rate with special reference to protein metabolism', *J Physiol*, 109(1-2), pp. 1-9.

Weng, J., Li, Y., Xu, W., Shi, L., Zhang, Q., Zhu, D., Hu, Y., Zhou, Z., Yan, X., Tian, H., Ran, X., Luo, Z., Xian, J., Yan, L., Li, F., Zeng, L., Chen, Y., Yang, L., Yan, S., Liu, J., Li, M., Fu, Z. and Cheng, H. (2008) 'Effect of intensive insulin therapy on beta-cell function and glycaemic control in patients with newly diagnosed type 2 diabetes: a multicentre randomised parallel-group trial', *Lancet*, 371(9626), pp. 1753-60.

Weng, J., Retnakaran, R., Ariachery, C.A., Ji, L., Meneghini, L., Yang, W. and Woo, J.T. (2015) 'Short-term intensive insulin therapy at diagnosis in type 2 diabetes: plan for filling the gaps', *Diabetes Metab Res Rev*, 31(6), pp. 537-44.

White, M.G., Marshall, H.L., Rigby, R., Huang, G.C., Amer, A., Booth, T., White, S. and Shaw, J.A.M. (2013) 'Expression of Mesenchymal and α-Cell

Phenotypic Markers in Islet ß-Cells in Recently Diagnosed Diabetes', *Diabetes Care*, 36(11), pp. 3818-3820.

WHO/IDF, C. (2006) *Definition and diagnosis of diabetes mellitus and intermediate hyperglycaemia.* [Online]. Available at: http://www.who.int/diabetes/publications/diagnosis_diabetes2006/en/.

Wilding, J.P.H. (2014) 'The importance of weight management in type 2 diabetes mellitus', *International Journal of Clinical Practice*, 68(6), pp. 682-691.

Wing, R.R. and Hill, J.O. (2001) 'Successful weight loss maintenance', *Annu Rev Nutr*, 21, pp. 323-41.

Wolever, T.M., Jenkins, D.J., Jenkins, A.L. and Josse, R.G. (1991) 'The glycemic index: methodology and clinical implications', *The American Journal of Clinical Nutrition*, 54(5), pp. 846-54.

Yki-Järvinen, H. (2011) 'Type 2 diabetes: remission in just a week', *Diabetologia*, 54(10), pp. 2477-2479.

Yousseif, A., Emmanuel, J., Karra, E., Millet, Q., Elkalaawy, M., Jenkinson, A.D., Hashemi, M., Adamo, M., Finer, N., Fiennes, A.G., Withers, D.J. and Batterham, R.L. (2014) 'Differential effects of laparoscopic sleeve gastrectomy and laparoscopic gastric bypass on appetite, circulating acyl-ghrelin, peptide YY3-36 and active GLP-1 levels in non-diabetic humans', *Obes Surg*, 24(2), pp. 241-52.

Zangeneh, F., Arora, P., Dyck, P., Bekris, L., Lernmark, A., Achenbach, S., Oberg, A. and Rizza, R. (2006) 'Effects of duration of type 2 diabetes on insulin secretion', *Endocrine Practice*, 12(4), pp. 388-393.