DEFINING THE LONG TERM PATHOPHYSIOLOGICAL DETERMINANTS OF TYPE 2 DIABETES

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Abstract

The prevalence of type 2 diabetes has increased significantly worldwide in recent decades along with growing rates of obesity. This presents a huge challenge to the health care systems all over the globe as well as ill health in millions of individuals.

Defining the long-term pathophysiological determinants of type 2 diabetes and the mechanisms underlying the normalisation of blood glucose levels have great implications for the effective management of early type 2 diabetes. The Twin Cycle hypothesis which explains the major pathophysiological changes in type 2 diabetes has been further validated in this work.

This thesis presents metabolic and clinical data from Tyneside arm of Diabetes Remission Clinical Trial (DiRECT), unravelling the mechanisms behind the remission of type 2 diabetes in people with a known duration of the disease of less than 6 years. The main culprits for non-reversal of type 2 diabetes were the lack of substantial weight loss and longer diabetes duration. Over 24 months of successful weight maintenance period the sustained decrease in VLDL-TG production by liver was followed by a reduction in liver and pancreatic fat content, a rise in first phase insulin secretion, and a near normalisation of maximum insulin secretory capacity. These factors have permitted to keep the normal blood glucose control in participants who did not have major weight regain. A group of non-diabetic individuals were studies specifically to allow comparison with the Tyneside DiRECT cohort after weight loss to evaluate just how far the underlying pathophysiological processes returned towards normal.

Weight loss induced decrease in liver enzymes levels reflected the reduction in liver fat content. The changes were analysed in detail. In routine clinical practice sequential monitoring of liver enzymes following weight loss can provide a simple assessment of normalisation of liver fat.

In conclusion, a low-calorie diet intervention induced a durable remission in early type 2 for up to 24 months for most people who achieved substantial weight loss. Overall, the metabolic findings of this thesis provide clinically important insights into the pathophysiologic basis of remission of type 2 diabetes.

Dedication

I would like to thank my educational supervisor Professor Roy Taylor for being a source of inspiration and for providing his invaluable support during this work.

I would also like to thank Dr Kieren Hollingsworth, Dr Ahmad Al-Mrabeh, and Ms Alison Barnes for sharing their expertise with me during my research work.

I would like to express my sincere gratitude to all Tyneside participants for their dedication to this research.

I would like to thank all research nurses at Newcastle Magnetic Resonance Centre and especially Ms Helen Pilkington. Their skills and patience have been crucial to the success of the metabolic research studies.

I would also like to thank all the research radiographers that I have had the pleasure of working with during this study.

I would like to thank my father Valeri Prakapovich who passed away in 2020 and to my mother Galina Prakapovich for their encouragement and moral support during this project.

Finally, I would like to express my thanks to Anatoli Zhyzhneuski for his support and especially to my daughters Elizabeth and Maria Zhyzhneuskaya for being the source of fun and inspiration for me during my research work.

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Acknowledgements

I conducted all the studies presented in this thesis at the Newcastle Magnetic Resonance Centre from 2015-2018. The design and organisation of DiRECT metabolic studies were performed by my predecessor in the job Dr Carl Peters. My contribution to the design of the metabolic studies was the advertising, screening, and recruitment of 25 non-diabetic comparators at Tyneside. Carl Peters had started performing the metabolic studies on Tyneside subgroup participants from the middle of 2014 till September 2015 when I have joined the project as Clinical Research Associate and took over the metabolic studies. The participants' care during metabolic studies, and the majority of Tyneside participants data analysis were undertaken by me with the following assistance. All magnetic resonance imaging studies were performed by senior research radiographers: Louise Ward, Dorothy Brown, and Timothy Hodgson. Three-point Dixon MR analyses for liver content were performed by me and Carl Peters on MatLab and ImageJ software and overseen by Dr Kieren Hollingsworth. The pancreatic fat MR analyses were performed by a single investigator Dr Ahmed Al-Mrabeh who was blinded to subjects' scans during the study. He had also subsequently analysed subcutaneous and visceral adipose content. The research nurses from the Clinical Ageing Research Unit (CARU) at Newcastle Magnetic Resonance Centre provided nursing assistance during the clinical studies with the key nurse to this study being Helen Pilkington. Me and Carl Peters had performed the Insulin secretion and VLDL secretion metabolic tests at baseline and during 2 years of follow up on each of the Tyneside Intervention and Control participants. Me and Carl had also performed the centrifugation and the chylomicron separation at the lab followed by Dr Ahmad Al-Mrabeh work who performed the ultracentrifugation and quantification of VLDL1-TG production rates. The palmitic acid quantification and analysis was performed by Dr Shaden Melhem from Newcastle University. Dr Benjamin Aribisala who is based at Lagos State University in Nigeria, performed the mathematical modelling analyses of C-peptide data for insulin secretion rates. Two research Psychologists Dr Lucia Rehackova and Angela Rodrigues performed the psychological evaluation of the subgroup of DiRECT study participants. Research dietitian Ms Alison Barnes had provided the teaching of Cambridge weight Plus low-calorie diet intervention to Primary Care practice nurses and played an important role in

follow up of the Tyneside subjects in the community. Ms Louise Burnip and Ms Alison Burnett (Department of Clinical Biochemistry, Newcastle upon Tyne Hospital NHS Foundation Trust) performed c-peptide and insulin levels quantification.

The main laboratory tests for the whole DiRECT study from both Scotland and Tyneside were carried centrally at biochemistry laboratory in Glasgow with the exception for the biochemical blood tests for Tyneside non-diabetes comparators done locally at the Royal Victoria Hospital biochemistry laboratory, Newcastle upon Tyne. The statistical analyses for the total DiRECT cohort main primary and secondary clinical outcomes were done at the Glasgow Centre for Biostatistics. The statistical analyses of Tyneside metabolic studies were done by me with exception of the statistical analyses of the VLDL-TG production rates and pancreatic fat performed by Dr Ahmed Al-Mrabeh.

The composition of this thesis is my own work. The research work in this thesis has not been previously submitted elsewhere for the degree of Doctor of Philosophy.

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Chapter 1. Introduction

1.1. Natural history of type 2 diabetes

Type 2 diabetes is widely regarded as a complex metabolic disease and characterised by an “ominous octet” (decreased insulin secretion, decreased incretin effect, increased lipolysis, increased glucose reabsorption and uptake, increased hepatic glucose production, increased glucagon secretion, and neurotransmitter dysfunction) with 2 main pathophysiological defects being insulin resistance in muscle and liver and progressive beta cell failure (Defronzo, 2009).

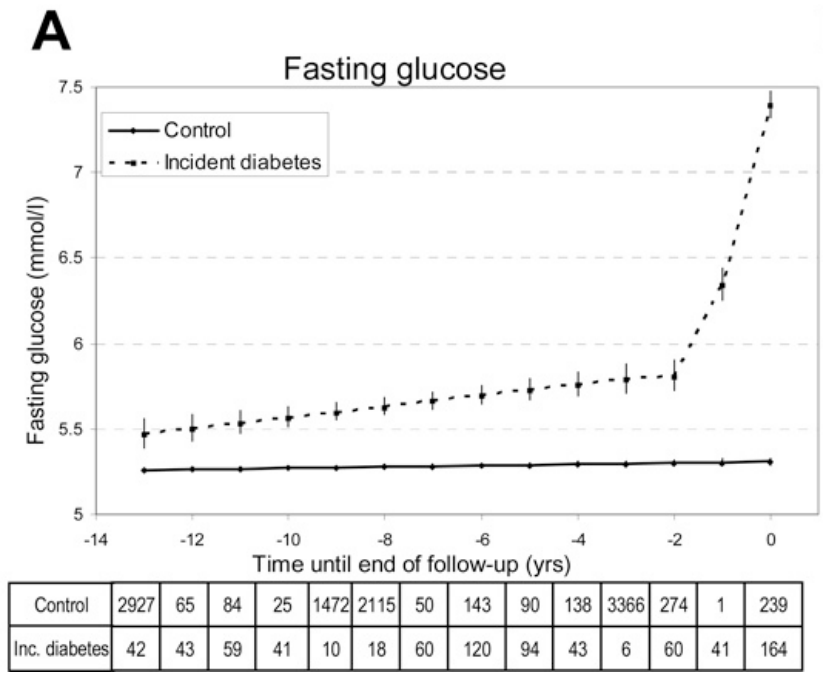
Failure of approximately 50 percent of the beta cells occurs before the development of overt type 2 diabetes (Holman, 1998). However, the first detectable abnormality relating to the development of type 2 diabetes is an insulin resistance (Defronzo, 2009).

Genetic predisposition plays a part in determining individual whole body insulin resistance (Groop and Lyssenko, 2008, Ahlqvist et al., 2011). Genome-wide association scans and candidate gene approaches have identified over 70 genes so far that have been linked with type 2 diabetes and more than 100 genes linked with obesity (Buniello et al., 2019, Sun et al., 2014, Ke et al., 2021). The majority of type 2 diabetes genes have been associated with impairment of beta cell function although some of these genes have been related to insulin resistance independent of obesity (Scherag et al., 2010). It is estimated that the genes identified already can predict only 15 % of type 2 diabetes and 5 % of obesity and environmental factors clearly play a major role (Bogardus, 2009). Insulin resistance in liver manifests by an overproduction of glucose during the basal state in spite of fasting hyperinsulinemia (DeFronzo et al., 1989). There is also impaired suppression of hepatic glucose production (HGP) by insulin following a meal (Groop et al., 1989, Ferrannini et al., 1988). Insulin resistance in the muscle is reflected in impaired glucose uptake of glucose after consuming a meal with a carbohydrate, resulting in postprandial hyperglycaemia (Ferrannini et al., 1988, Groop et al., 1989, DeFronzo et al., 1985, Pendergrass et al., 2007).

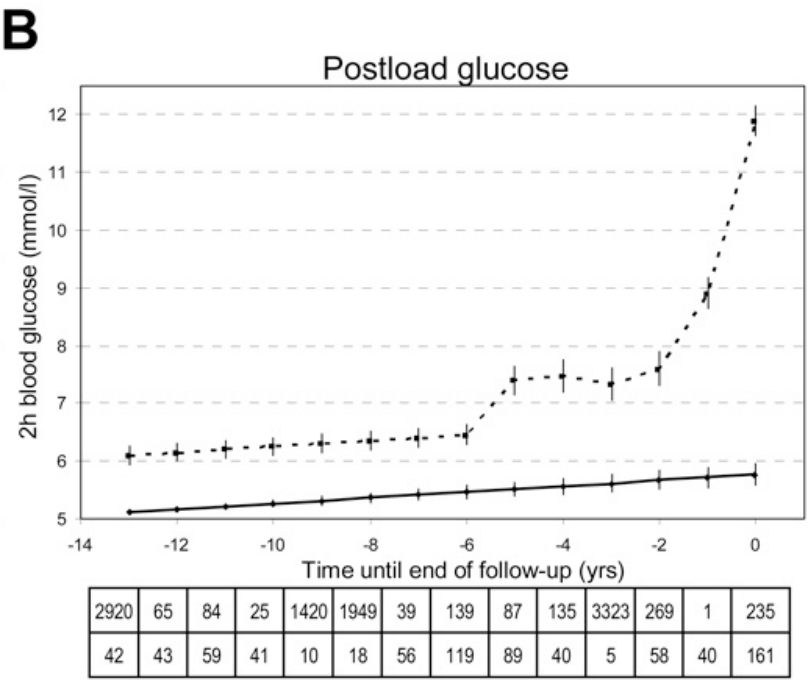
The epidemics of obesity and sedentary lifestyle are closely related to the current growth in the type 2 diabetic population (James, 2008). Both of them are well

recognised insulin-resistant states (DeFronzo et al., 1978). Glucose tolerance remains normal while beta cells are still able to augment insulin secretion to offset the insulin resistance (Schernthaner et al., 2010, Guardado-Mendoza et al., 2009, DeFronzo, 1988). As time goes on the beta cells begin to fail and postprandial glucose levels rise at the beginning followed by fasting plasma glucose rise and the development of overt type 2 diabetes as a consequence of these events (Guardado-Mendoza et al., 2009, Schernthaner et al., 2010, DeFronzo, 1988).

The Whitehall II study described the 13-year trajectories of fasting and postload blood glucose, insulin sensitivity and insulin secretion until type 2 diabetes diagnosis in a large, middle-aged, metabolically healthy population at baseline. Among subjects who developed type 2 diabetes, the levels of fasting and postload glucose and insulin secretion were higher and insulin sensitivity was lower than those among the controls 13 years before the diagnosis (Figure 1.1.). In the incident diabetes cases, linear increases in fasting and postload glucose were followed by a fast elevation in the last 6 to 3 years prior to a diagnosis of type 2 diabetes. For HOMA insulin sensitivity, there was a steeper decrease during the last 5 years prior to the diagnosis and HOMA beta cell function showed an increase between years 4 and 3 prior to the diagnosis and then a decrease until the diagnosis (Figure 1.2.)(Tabak et al., 2009).



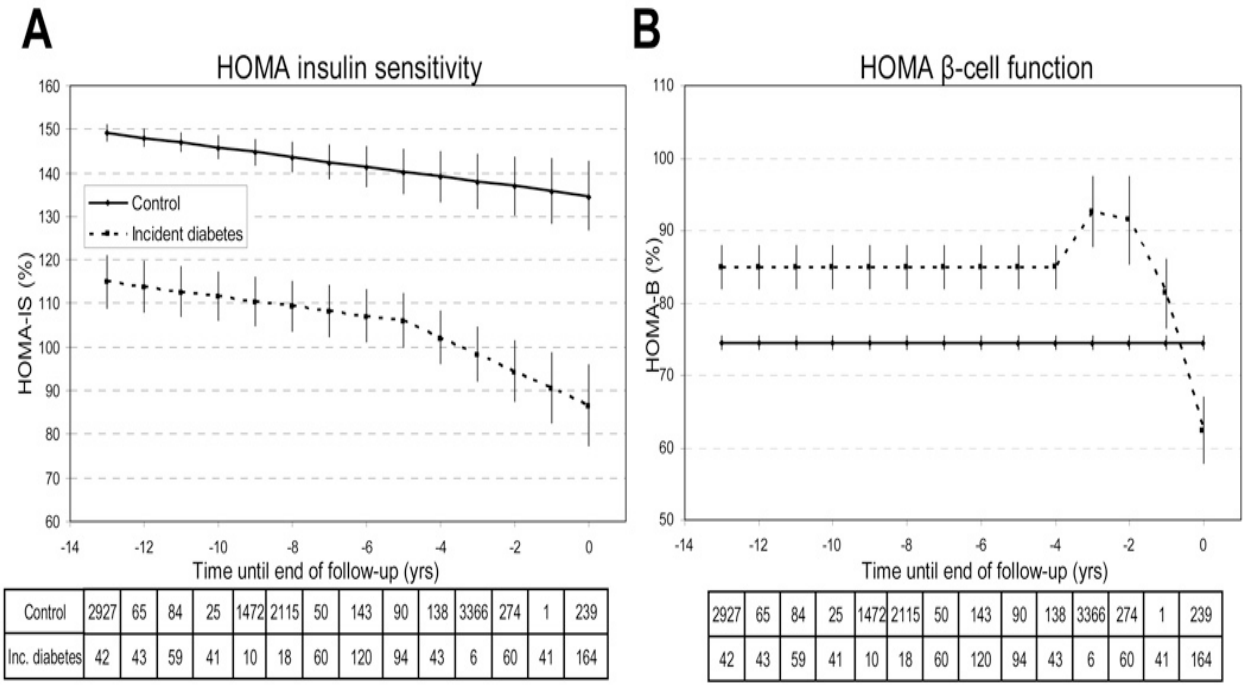
Control	2927	65	84	25	1472	2115	50	143	90	138	3366	274	1	239
Inc. diabetes	42	43	59	41	10	18	60	120	94	43	6	60	41	164



2920	65	84	25	1420	1949	39	139	87	135	3323	269	1	235
42	43	59	41	10	18	56	119	89	40	5	58	40	161

Figure 1.1

Fasting and 2-hour postload glucose trajectories (panels A and B) before the diagnosis of type 2 diabetes mellitus or the end of follow-up in 505 incident diabetes cases compared to 6033 non-diabetic controls (reproduced from Tabak et al 2009).



Control	2927	65	84	25	1472	2115	50	143	90	138	3366	274	1	239
Inc. diabetes	42	43	59	41	10	18	60	120	94	43	6	60	41	164

Figure 1.2

Homeostasis model assessment insulin sensitivity (HOMA2-%S) and HOMA beta cell function (HOMA2-%B) trajectories (panels A and B) before the diagnosis of type 2 diabetes mellitus or the end of follow-up in 505 incident diabetes cases compared to 6033 non-diabetic controls.

Multilevel longitudinal modeling using either linear growth model for non-diabetic and non-piecewise or piecewise approach including linear or quadratic terms for time for incident diabetic subjects with HOMA2-%S (A) and HOMA2-%B (B) as outcomes. Adjusted for age, sex, ethnicity and study phase. Estimated for a hypothetical population of 72 % male, 91 % Caucasian aged 63 years at time 0. Error bars show 95% confidence intervals for the fixed effects (reproduced from Tabak et al 2009).

The United Kingdom Prospective Diabetes Study (UKPDS) in 1995 demonstrated an impairment of insulin secretion and a reduction of beta cell by 50 percent at the time of diagnosis of overt type 2 diabetes. It was also shown that this progression of beta cell failure is not able to be modified by any of the current available blood glucose lowering treatment (Holman, 2006).

The Belfast Diet Study started in 1972 and aimed to study the effects of intensive dietary management of newly diagnosed diabetes, has shown the small but progressive rise in fasting plasma glucose during the first 10 years after the diagnosis while being on diet treatment alone associated with a progressive fall in beta cell function (Levy et al., 1998).

The beta cell function data were modelled from fasting plasma glucose and insulin observations, and the model tends to reflect insulin resistance in addition (Figure 1.3.).

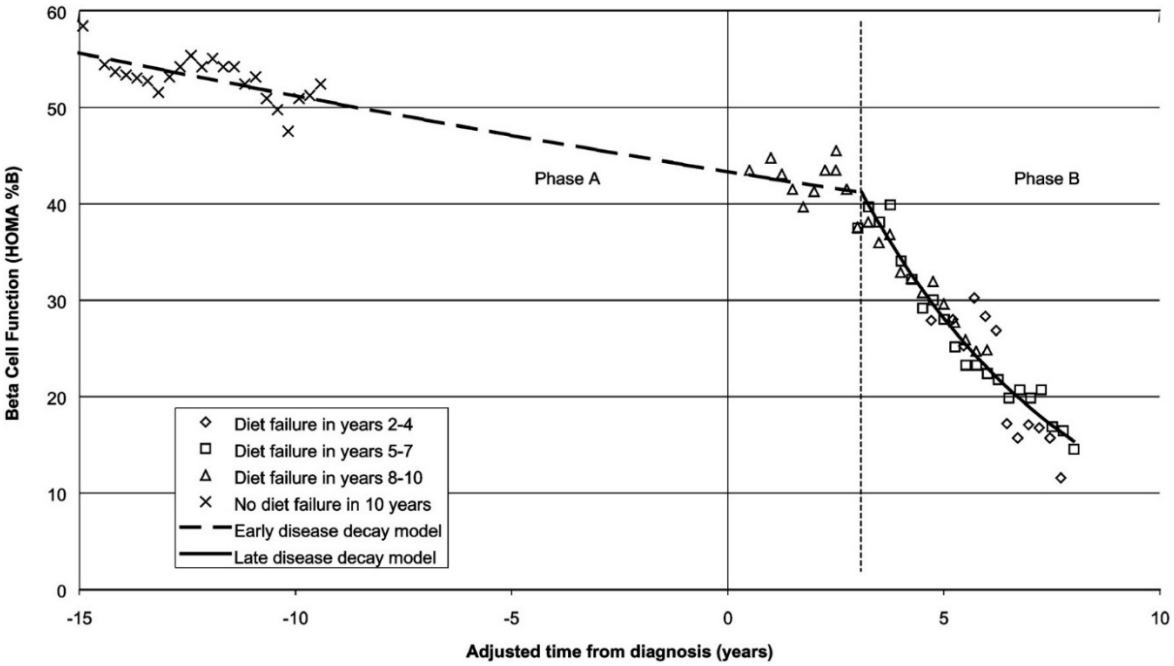


Figure 1.3 Final spline function model fitted to Belfast Diet Study results, showing two phases of beta cell function decay (reproduced from Levy et al 1998).

The autopsy studies of patients with type 2 diabetes have reported that the defective insulin secretion could be partially due to the reduced beta cell mass (Butler et al., 2003c).The progressive reduction in beta cells in type 2 diabetes has been suggested to be caused by beta cell apoptosis (Butler et al., 2003c).

The earliest defect of insulin secretion in type 2 diabetes is a loss of first phase insulin secretion in response to intravenous glucose (Vaag, 1999). A reduction in second phase insulin secretion in hyperglycaemic clamp studies is also present early in the natural history of type 2 diabetes (van Haeften et al., 1989).

1.2 Beta cell dysfunction

The United Kingdom Prospective Diabetes Study (UKPDS) famously demonstrated an impairment of insulin secretion and a reduction of beta cell function as assessed by HOMA by 50 percent at the time of diagnosis of overt type 2 diabetes. It also showed that this progression of beta cell failure was not able to be modified by any of the current available blood glucose lowering treatment (Holman, 2006). As discussed above, the Belfast Diet Study to evaluate the effects of intensive dietary management of newly diagnosed diabetes also showed the steady, progressive rise in fasting plasma glucose associated with a progressive fall in beta cell function during the first 10 years after the diagnosis during diet treatment alone (Levy et al., 1998). For both studies, beta cell function data were modelled from fasting plasma glucose and insulin observations, permitting an overall estimate in the fasting state.

The earliest defect of insulin secretion in type 2 diabetes is a loss of first phase insulin secretion in response to intravenous glucose (Vaag, 1999). It had been accepted for many years that this was irreversible and could not be restored to a useful degree by any pharmacological treatment. However, by 2008 clear evidence of the reversibility of hepatic insulin resistance had emerged and together with other observations led to the Twin Cycle Hypothesis being postulated (Taylor, 2008). This hypothesis was tested in a Counterpoint study which showed in 2011 that not only hepatic insulin resistance but

also beta cell dysfunction could be reversed in the first few years of diagnosis of type 2 diabetes if the fat-induced stress on the beta cells was removed (Lim et al., 2011b).

Historically, several different hypotheses have been proposed to attempt to explain the development of beta cell dysfunction in type 2 diabetes. Firstly, beta cell exhaustion due to the increased secretory demand was thought to arise from an insulin resistance (DeFronzo et al., 1992). Long continued hyperinsulinaemia due to obesity related insulin resistance is entirely compatible with long term normal glucose tolerance (Kahn, 2001). Also, the longitudinal data from the Pima Indians points out that beta cell function is enhanced in apparently healthy subjects as insulin resistance progresses (Weyer et al., 1999).

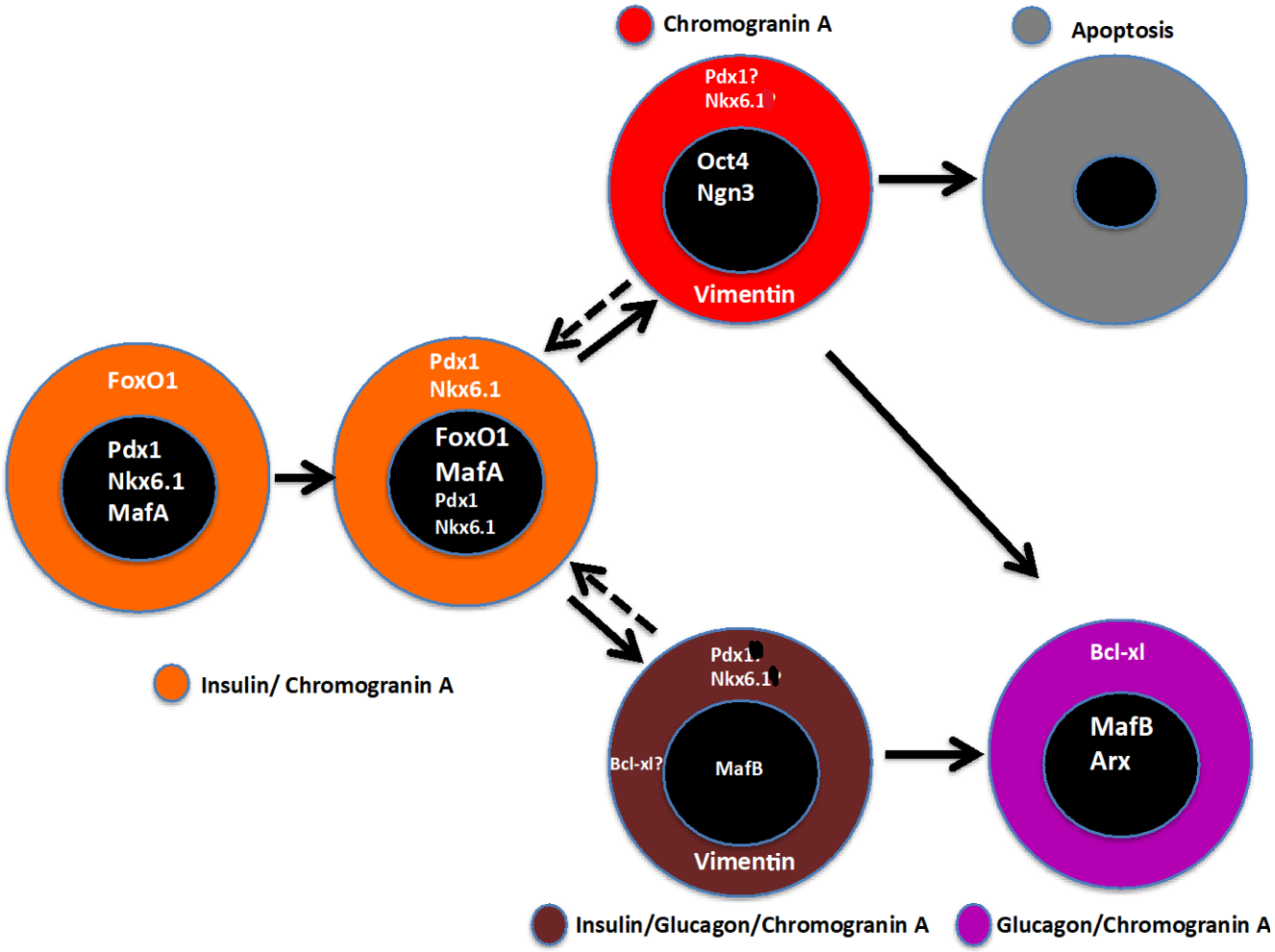
Secondly, desensitization of the beta-cell due to the elevation of the glucose levels or "glucose toxicity" had also been proposed (Yki-Jarvinen, 1992, Robertson et al., 1994). UKPDS data suggest that in the early stages of type 2 diabetes glucose is unlikely to be a critical factor determining the beta cell dysfunction progression based on observation that the disease progressed or worsened despite the "normalization" of glucose levels and continuation of the therapy (UKPDS, 1998, UKPDS, 1999).

Thirdly, the deposition of amyloid (Kahn et al., 1999, Opie, 1901) and apoptosis as a result of the deranged metabolic state (Efanova et al., 1998, Shimabukuro et al., 1998) have been implicated in a reduction of beta cell mass. The deposition of amyloid in the islets has been reported in a high proportion with type 2 diabetes (Westermark and Wilander, 1978, Rocken et al., 1992, Johnson et al., 1989). The islet amyloid polypeptide (IAPP) is a protein component of fibrils that are forming the amyloid deposits (Westermark et al., 1987, Cooper et al., 1987). IAPP is getting produced in the islet cells and released together with insulin (Kahn et al., 1990). When islet amyloid increases in the monkey, the glucose tolerance gets worse (Howard, 1986). The reduction of the beta cell mass seems to be associated with significant islet amyloidosis (Howard and Van Bueren, 1986, de Koning et al., 1993). Amyloid fibrils have been shown to be cytotoxic to the beta cells in vitro resulting in death by apoptosis (Lorenzo et al., 1994, Janson et al., 1999). Wang et al have studied islet amyloidosis by computerized fluorescent microscopy in transgenic mice bearing the amyloidogenic human IAPP gene and

developing typical islet amyloid (Wang et al., 2001). However, type 2 diabetes occurs without accumulation of amyloid, and it appears unlikely to be the cause of decreased beta cell function in type 2 diabetes.

Fourthly, endoplasmic reticulum (ER) stress response, an adaptive mechanism used to align ER functional capacity and demand, occurs in obesity and type 2 diabetes and in the beta cell prolonged ER stress has been suggested to impair the synthesis of insulin (Cnop et al., 2012). Data obtained in animal models of diabetes have suggested that the changes in lipid metabolism could contribute to the development of beta cell dysfunction (Unger, 1995). Also, a high energy diet, associated with a high fat intake, may contribute to the decrease in beta cell function (Tsunehara et al., 1990). These observations were nicely brought together by the studies of Anne Clark and others. When fatty acid concentrations are elevated *in vitro*, lipid synthesis and storage within the beta cell is favoured and chronic exposure of the beta cell to fatty acid excess directly impairs glucose-stimulated insulin secretion (Elks, 1993a, Lalloyer et al., 2006, Zhou and Grill, 1994). Long term exposure to increased levels of fatty acids *in vitro* directly results in beta cell stress and dysfunction (Pinnick et al., 2010). Exposure of the INS1 beta cell line to oleic acid brings about storage in intracytoplasmic vacuoles, whereas the saturated fatty acid palmitate induces expansion of the endoplasmic reticulum producing dramatic 'splits' or widening in the endoplasmic reticulum (Pinnick et al., 2010). This is associated with markers of endoplasmic reticulum stress, typically increased in human beta cells from individuals with type 2 diabetes (Laybutt et al., 2007, Marchetti et al., 2007). The exposure to a more physiological mixture of saturated and unsaturated fatty acids decreases insulin secretion, and removal of fatty acid from the medium allows return of insulin secretion over 24 hours (Pinnick et al., 2010). This observation lays the basis for understanding the *in vivo* reversal of type 2 diabetes and restoration of the non-diabetic first phase insulin response (Lim et al., 2011b, Steven et al., 2016b). Human islets cells are known to take up fatty acids avidly, and incubation in 0.33 mmol/l palmitate brings about both a large increase in islet triglyceride content and major impairment of function (Lalloyer et al., 2006). Once hyperglycaemia occurs, the additional stress of elevated glucose is likely to compound the metabolic insult (Poitout et al., 2010).

Autopsy studies of patients with type 2 diabetes have reported reduced beta cell mass, and this has long been accepted as the reason for decreased insulin secretory function (Butler et al., 2003c, Saito et al., 1979, Kloppel et al., 1985, Westermark and Wilander, 1978). However, in such histological studies, the apparent progressive reduction in beta cells in type 2 diabetes was judged by decreased insulin immunostaining. Such assessments were based on insulin secretory function rather than definitively identified beta cells. Very recently, the loss of beta cell function in type 2 diabetes was shown to be explained by beta cell dedifferentiation rather than beta cell death (Brereton et al., 2014, Spijker et al., 2015, Talchai et al., 2012, Wang et al., 2014, White et al., 2013). Chronic positive energy balance may result in reduced expression of beta cell transcription factors such as Pdx1, Nkx6.1 and MafA (Brereton et al., 2014, Spijker et al., 2015, Talchai et al., 2012). This leads to the loss of end-differentiated genes including insulin and induction of dismissed genes like lactate dehydrogenase and hexokinase (Weir et al., 2013b). It was proposed that enhanced FoxO1 nuclear translocation able to maintain the activation of some of beta cell transcription factors like MafA which preserves glucose oxidation and suppresses fatty acid oxidation resulting in limitation of mitochondrial stress (Accili et al., 2016). FoxO1 can initiate a compensatory response that leads to preservation of beta cell function under metabolic stress (Figure 1.4).



Healthy	**Compensation**	**Dedifferentiation/**	**Apoptosis/complete**
	Reversible	**Plasticity**	**alpha-cell reprogramming**
		Potentially reversible	Non-reversible
Non-diabetic	**Pre-diabetes**	**Early type 2 diabetes**	**Late type 2 diabetes**

Figure 1.4

Schematic representation of possible stages of beta cell fate changes associated with type 2 diabetes progression (reproduced from White et al.(White et al., 2016a)).

Lineage tracing studies in mice with beta cell specific deletion of FoxO1 exposed to metabolic stressors including ageing and multiple pregnancies demonstrated that loss of beta cell mass was not due to death but rather due to dedifferentiation (Talchai et al., 2012). Loss of insulin staining was encountered together with induction of genes not normally expressed in adult beta cells including mesenchymal marker vimentin and pancreatic progenitor marker neurogenin-3.
A further change in the stressed, dedifferentiated beta cells is highly relevant metabolically. Following loss of beta cell specific transcription factors as a result of chronic hyperglycaemia, glucagon production by beta cells becomes switched on (Marroqui et al., 2015, Brereton et al., 2014). Following reversal of type 2 diabetes *in vivo*, a fall to normal of fasting plasma glucagon levels occurs at the same time as return of normal beta cell function (Steven et al., 2015).

Genetic predisposition plays a part in determining individual susceptibility to type 2 diabetes (Groop and Lyssenko, 2008, Ahlqvist et al., 2011). It is likely that genetic factors underlie the susceptibility of the beta cell to fat-related metabolic stress. Genome-wide association scans and candidate gene approaches have identified more than 70 genes so far that have been linked with type 2 diabetes (Sun et al., 2014). The majority of type 2 diabetes genes have been associated with an impairment of beta cell function. The overall importance of the environmental factors in bringing type 2 diabetes is underscored by the estimation that the genes identified already can predict only 15 % of type 2 diabetes cases (Bogardus, 2009).

1.3 Insulin resistance

The definition of insulin resistance is an inability of insulin to increase glucose uptake and utilization comparable to the action of insulin in the normal population.

In type 2 diabetes the liver and the muscle are severely resistant to insulin action (Guardado-Mendoza et al., 2009). After an overnight fast in type 2 diabetes the liver produces glucose at a rate of approximately 2.5 mg/kg/min in comparison to 2

mg/kg/min in a normal subject (DeFronzo et al., 1989, Schernthaner et al., 2010). This adds up around 25-30 g of glucose to the circulation every night and is responsible for the increased fasting plasma glucose concentration (DeFronzo et al., 2013). The overproduction of the glucose by liver occurs in spite of two to threefold increase in fasting insulin levels which indicates severe hepatic insulin resistance (DeFronzo et al., 2013).

The euglycaemic insulin clamp with limb catheterisation studies have shown that lean and obese type 2 diabetes subjects have severe insulin resistance in skeletal muscles (Groop et al., 1989, Coletta et al., 2009, DeFronzo et al., 1985, Schernthaner et al., 2010, Pendergrass et al., 2007)

Multiple defects in insulin action has been documented including impaired glucose transport and phosphorylation (Groop et al., 1989), reduced glycogen synthesis (Shulman et al., 1985), and decreased oxygen oxidation (Groop et al., 1989).

At high physiological insulin concentration the skeletal muscle may account for approximately 85 to 90 percent of total body disposal of the glucose (DeFronzo et al., 1985). Yki-Jarvinen with colleagues estimated that the 70 percent of the whole body glucose uptake goes to muscles (Yki-Jarvinen et al., 1987). Under this conditions the liver and the gut (DeFronzo et al., 1981), and adipose tissue (Marin et al., 1987) account only for a very small proportion of glucose uptake. However, in obese subjects the adipose tissue will account for a higher proportion, and the early studies demonstrating the extent of skeletal muscle contribution to disposal of intravenous glucose were conducted on non-obese subjects.

Study of The Pima Indian population in Arizona, who have the highest reported prevalence and incidence of type 2 diabetes in the world, has revealed much about basic pathophysiology of type 2 diabetes (Bogardus et al., 1991).

Insulin resistance was found to be the primary abnormality that predisposes Pima Indians to develop type 2 diabetes at an increased rate and it leads to the impaired glucose tolerance followed by pancreatic failure with marked fasting hyperglycemia (Bogardus et al., 1991). Pima Indians were found to have lower insulin sensitivity and

higher plasma insulin levels (both fasting and in response to intravenous glucose) versus Caucasians (Bogardus et al., 1984).

1.3.1 Substrate level effect on measured insulin sensitivity

Unger has described the deleterious effect of fat accumulation on glucose metabolism as “lipotoxicity” (Unger, 2003). Experiments on elevation of non-esterified fatty acids (NEFA) to the levels in type 2 diabetes resulted in severe muscle and liver insulin resistance (Kashyap et al., 2004, Richardson et al., 2005, Dresner et al., 1999), inhibition of insulin secretion (Kashyap et al., 2003) and reproducing the core defects of type 2 diabetes (DeFronzo, 2010). It was demonstrated by the magnetic resonance spectroscopic studies that the organ specific insulin resistance was closely associated with intramyocellular and intrahepatic fat accumulation (Mayerson et al., 2002, Belfort et al., 2006, Miyazaki et al., 2002, Bajaj et al., 2003). Toxic intracellular metabolites of triacylglycerol and non-esterified fatty acids (NEFA) metabolism (fatty acyl CoA, diacylglycerol, ceramides) impairing insular signaling and multiple intracellular steps of glucose metabolism and causing severe insulin resistance (Kashyap et al., 2004, Belfort et al., 2005, Griffin et al., 1999).

Inside the hepatocyte (Figure 1.5), fatty acids could appear as a result of de novo lipogenesis, uptake of non-esterified fatty acids and low density lipoproteins, or lipolysis of intracellular triacylglycerol (Taylor, 2013). The lower ability to oxidize fat within the hepatocyte might be one of the factors for the accumulation of the liver fat (Belfort et al., 2006). Diacylglycerol excess has a deleterious effect on the activating of protein kinase C epsilon type which inhibits the signaling pathway from the insulin receptor to insulin receptor substrate 1 (IRS-1) (Samuel et al., 2010) which is the first post receptor step in intracellular insulin action. When there is a chronic excess of energy intake with food, a raised level of diacylglycerol inside the cell prevents the normal action of insulin, and therefore the production of glucose by the liver gets out of control (Taylor, 2013). The excess of fatty acids stimulates the ceramide synthesis by esterification with sphingosine

and the ceramides in turn cause the sequestration of Akt2 and activation of gluconeogenic enzymes (Taylor, 2013).

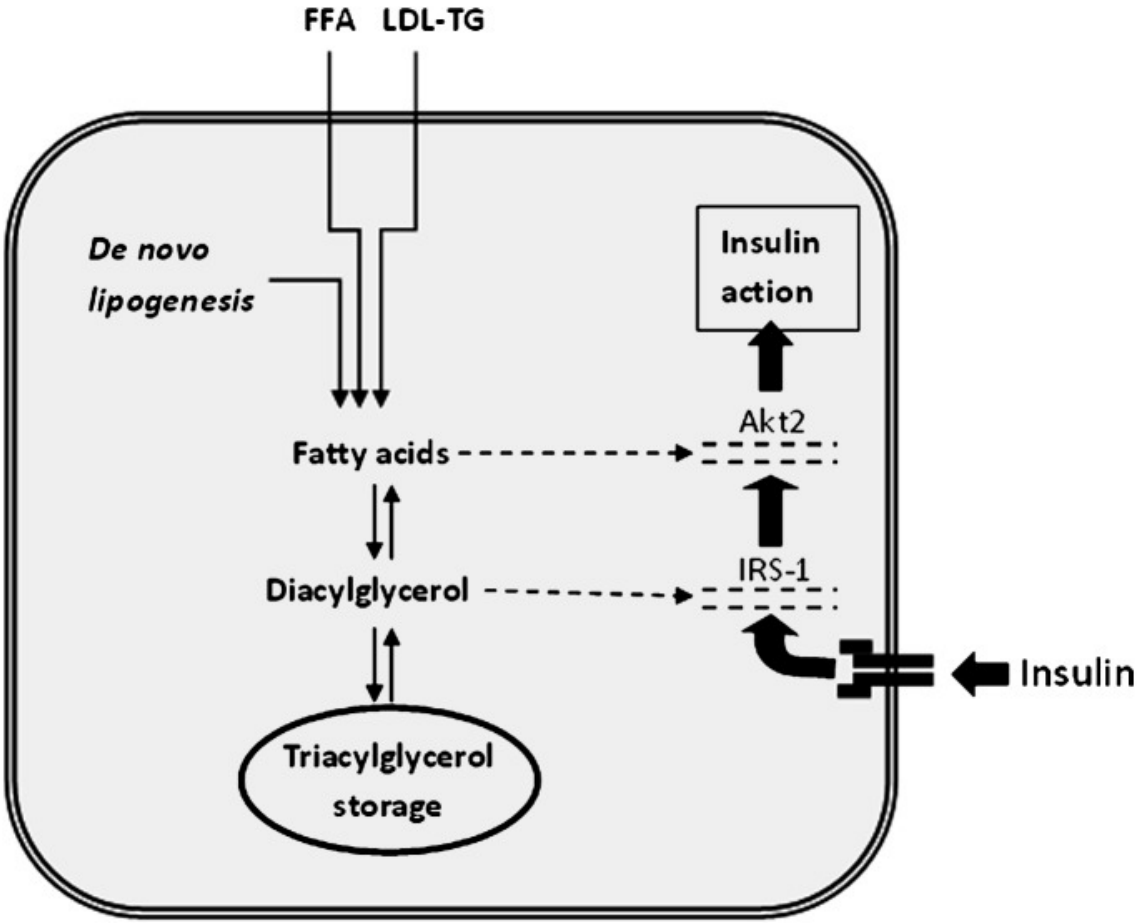


Figure 1.5

Mechanism of interaction between excess amounts of fatty acids, diacylglycerol, and ceramide and insulin action within the hepatocyte (reproduced from Taylor, R. 2013).

1.2.1 Molecular mechanisms of insulin action

Insulin is a hormone secreted by the pancreatic beta cells in response to increased circulating levels of the glucose and amino acids after a meal. In order to cause a biological effect, insulin must first to bind to specific cell surface receptors (Taniguchi et al., 2006, DeFronzo, 2010). This is followed by the activation of the 'second messengers' which initiates a phosphorylation-dephosphorylation cascade that stimulates glucose transport via GLUT-4, glucose phosphorylation via hexokinase II, glycogen synthase , and both phosphofructokinase and pyruvate dehydrogenase that regulates glycolysis and glucose oxidation (White et al., 1988). IRS-2 phosphorylation mediates the Insulin action in liver (Figure 1.6)(DeFronzo, 2010).

In muscle, insulin binds to its receptor (Taniguchi et al., 2006, White et al., 1988) which leads to tyrosine phosphorylation of IRS-1 mediating the effect of insulin on glucose metabolism. IRS-1 activates PI-3 kinase (Sun et al., 1992), which catalyses 3' phosphorylation of PI, PI-4 phosphate and PI-4,5 diphosphate, and augments glucose transport and glycogen synthase (Ruderman et al., 1990, Brady et al., 1997, Dent et al., 1990).

The molecular causes of insulin resistance are the impaired insulin signaling through the phosphoinositol-3 kinase pathway with intact signaling through the mitogen-activated protein kinase pathway (DeFronzo, 2010).

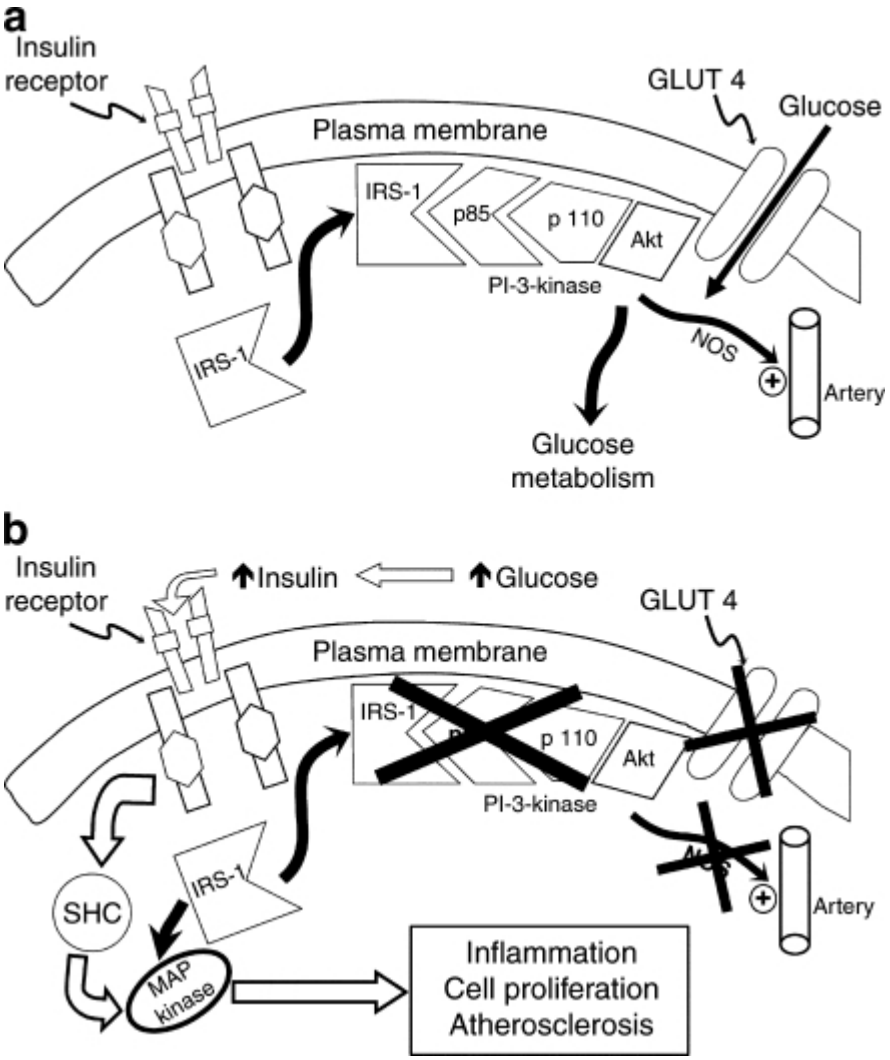


Figure 1.6

A Insulin signal transduction system in individuals with normal glucose tolerance (see text for a detailed discussion). NOS, nitric oxide synthase. **B** In type 2 diabetes participants insulin signaling is impaired at the level of IRS-1 leading to decreased glucose transport/phosphorylation/metabolism and impaired nitric oxide synthase activation/endothelial function. At the same time, insulin signaling through the MAP kinase pathway is normally sensitive to insulin. The compensatory hyperinsulinaemia (due to insulin resistance in the IRS-1/PI-3 kinase pathway) results in excessive stimulation of this pathway, which is involved in inflammation, cell proliferation and atherogenesis (see text for a detailed discussion). SHC, Src homology collagen. (Reproduced from DeFronzo 2010).

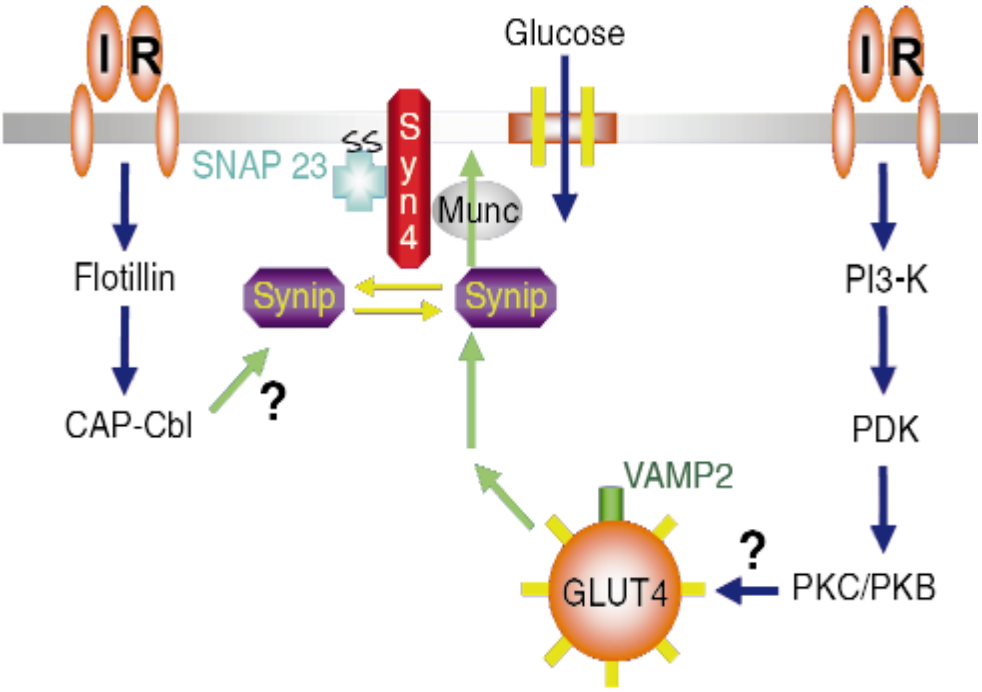


Figure 1.7

Schematic model indicating the presence of two potential insulin receptor–dependent signal transduction pathways. In this model, insulin stimulation results in the activation of a PI 3-kinase–dependent pathway that is necessary but not sufficient to induce GLUT4 translocation. In parallel, the insulin receptor activates an additional pathway leading to Cbl tyrosine phosphorylation through its interaction with the CAP protein, Syn4, syntaxin 4; PI3-K, PI 3-kinase (reproduced from Pessin et al.)(Pessin and Saltiel, 2000).

1.3.3 Muscle insulin resistance

Because muscle is the early pre-diabetic site of insulin resistance (Ferrannini et al., 1999, Cline et al., 1999, Kahn, 1994) and is widely regarded as accounting for the largest proportion of insulin stimulated glucose uptake, the muscle-specific insulin receptor knockout mice (MIRKO) were created with almost complete ablation of insulin receptor expression in all skeletal muscles (Bruning et al., 1998). It was a surprise that the MIRKO mice were able to maintain normal blood glucose levels up to at least 20 months of age and plasma insulin concentration was also normal with no increased incidence of type 2 diabetes (Bruning et al., 1998). In response to insulin the glucose uptake into muscles was severely decreased but it was normal in response to exercise (Wojtaszewski et al., 1999). However, the insulin stimulated glucose transport in adipose tissue was increased by approximately 3-fold in MIRKO mice and as a result of the substrate shift the lipid phenotype of the metabolic syndrome was developed (Kahn, 2003). In man, Savage et al. identified a PPP1R3A FS variant, which encodes a truncated protein that is mistargeted within the cell and decreases muscle glycogen synthesis activity. It increases phosphorylase activity resulting in the decrease of muscle glycogen content in human, but without hyperglycaemia (Savage et al., 2008). This mutation is present in approximately 1 in 70 UK Caucasians which increases the potential relevance of that finding (Savage et al., 2008). Like the MIRKO mouse studies, this indicates that lack of insulin responsiveness of muscle does not necessarily cause type 2 diabetes.

The study of Petersen et al. proved the hypothesis that insulin resistance in skeletal muscle promotes the atherogenic dyslipidemia by converting the energy from the carbohydrate into the hepatic de novo lipogenesis and increase production of very low density lipoproteins (Petersen et al., 2007). The data of this study had also demonstrated that skeletal muscle insulin resistance develops before the hepatic insulin resistance and that the triglyceride synthesis by the liver is increased after the high-carbohydrate meal in insulin resistant subjects may predispose them to the nonalcoholic fatty liver disease (NAFLD). Approximately 30 % of meal carbohydrate is taken up my muscle and stored as glycogen in the first 5 hours after eating, and that such storage is

negligible in insulin resistant subjects (Carey et al., 2003, Taylor et al., 1993). As *de novo* lipogenesis is the only other pathway for glucose storage if conversion to glycogen is relatively blocked, therefore it can be seen that insulin resistance in muscle predisposes to build up of liver fat.

Intramyocellular fat content can be raised without affecting insulin sensitivity. The example of this would be the fact that in the trained athletes the high insulin sensitivity is associated with the raised intramuscular triacylglycerol (Taylor, 2013).

Many individuals with no diabetes but a degree of muscle insulin resistance similar to the ones with type 2 diabetes maintain normal blood glucose levels (Taylor, 2012). Though the defect in mitochondrial function is associate with insulin resistance in skeletal muscle (Petersen et al., 2004) this does not seem to be relative in the etiology of type 2 diabetes (Taylor, 2013). The question, whether insulin resistance causes mitochondrial dysfunction or the other way round, has been debated. The former seems to be more likely on the current evidence basis (Taylor, 2012).

Exercise may reduce the insulin resistance and improve mitochondrial dysfunction (Ritov et al., 2010). On the other hand established mitochondrial dysfunction does not always produce insulin resistance in the humans or animal models (Zechner et al., 2010, Maassen et al., 2004).

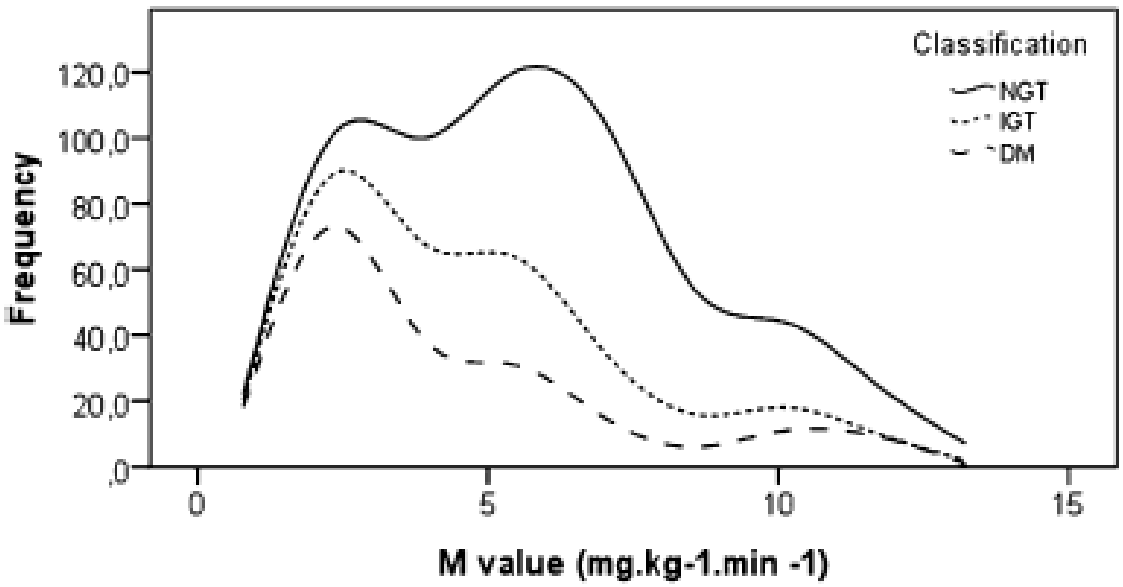


Figure 1.8

Distribution curves of insulin sensitivity as measured by the euglycemic-hyperinsulinemic clamp showing that people with type 2 diabetes sit within the range of the nondiabetic distribution, but toward the lower range. Identification of factors underlying muscle insulin resistance itself can be investigated by comparing groups drawn from the extremes of the total population distribution. Such factors may not be clearly discernible when type 2 diabetic individuals are compared with normoglycemic control subjects matched for weight and physical activity. The data are from previously published population studies of normal glucose tolerance (n = 256), impaired glucose tolerance (n = 119), and type 2 diabetes (n = 194) (Groop et al., 1996, Tripathy et al., 2004).

1.3.4 Liver insulin resistance

Moderate calorie restriction achieving weight reduction by approximately 8 kg is accompanied by reversal of hepatic steatosis and hepatic insulin resistance in type 2 diabetes. In turn, these changes lead to a normalization of basal rates of hepatic glucose production and improvement in fasting plasma glucose (Petersen et al., 2005, Ravikumar et al., 2008b).

By carefully separating the contribution of muscle and liver to insulin resistance it has been shown that the early improvement in fasting plasma glucose control is only associated with improvement in hepatic insulin sensitivity (Petersen et al., 2005, Lim et al., 2011c).

The close relationship between the liver fat, insulin resistance, and raised liver enzymes is routinely observed in type 2 diabetes (Nobili et al., 2006). People with type 2 diabetes have high normal or raised levels of plasma ALT (Taylor, 2013). The West of Scotland Coronary Prevention Study revealed that plasma triacylglycerol and ALT were moderately increased 2 years prior to the diagnosis of type 2 diabetes and also the steady rise of this liver enzyme towards the time of the diagnosis (Sattar et al., 2007). The overfeeding with sucrose for 3 weeks showed the increase of liver fat content by 30 percent as well as 30 percent rise in ALT but liver fat and serum ALT were going back to normal during the subsequent low calorie diet (Sevastianova et al., 2012). It was found that obese or non-obese subjects with raised plasma insulin levels have significantly increased *de novo* lipogenesis in the liver (Petersen et al., 2012, Rabol et al., 2011, Schwarz et al., 2003). The beginning of insulin therapy in type 2 diabetes reduces the portal insulin delivery by suppression of insulin secretion and as a result reducing the liver fat (Juurinen et al., 2007). The low calorie diet (Nobili et al., 2007), physical exercise (Perseghin et al., 2007), and thiazolidinedione therapy (Ravikumar et al., 2008b, Belfort et al., 2006) reduce the liver fat content by decreasing insulin secretion.

The insulin-resistant related excess in free fatty acids is directly toxic to hepatocytes. Commonly believed mechanisms include cell membrane disruption, toxin formation, mitochondrial dysfunction, and activation and inhibition of key steps in the regulation of metabolism (Neuschwander-Tetri and Caldwell, 2003). There are some other plausible explanations for the elevated liver transaminases in insulin-resistant conditions namely oxidant stress from reactive lipid peroxidation, peroxisomal beta-oxidation, and recruited inflammatory cells. In non-alcoholic steatohepatitis (NASH) patients there was an increased frequency of specific TNF-alpha-promoter polymorphism found suggesting a possible genetic link or predisposition to fatty liver found in insulin-resistant states (Grove et al., 1997).

GGT (gamma-glutamyl transpeptidase) is a nonspecific marker that is known to be elevated in subjects with type 2 diabetes. GGT has positive associations with alcohol intake, cigarette smoking, coronary heart disease, BMI, systolic blood pressure, serum triglyceride, heart rate, uric acid, haematocrit and an inverse association with the level of physical activity in epidemiological studies (Wannamethee et al., 1995).

In a prospective cohort study of 7,458 nondiabetic men aged 40–59 years conducted for 12 years mean serum GGT at the start was significantly higher in the 194 men who developed type 2 diabetes than in the rest of the cohort who did not develop diabetes (20.9 vs. 15.3 units/l, $p < 0.0001$) and that association was independent of serum glucose and BMI (Perry et al., 1998).

Ohlson et al. found elevated ALT to be a risk factor for developing type 2 diabetes, independent of obesity, body fat distribution, plasma glucose, lipid, AST, bilirubin concentrations, and family history in Swedish men with no prior history of diabetes (Ohlson et al., 1988). Salmela studied the prevalence of abnormal LFTs and their relationship to clinical findings in 175 unselected diabetic outpatients in Finland (Salmela et al., 1984). In this study 57 % (100 subjects) had at least one abnormal LFT and 27 % (48 subjects) had at least two abnormal tests. The type 2 diabetic patients more frequently had elevated ALT (22.0 vs. 5.3 %) and GGT (23.7 vs. 10. %) levels than those with type 1 diabetes. Elevated ALT and GGT were associated with elevated BMI and poor

glycaemic control in this study. Raised ALT was also associated with onset of diabetes within last 4 years, mature onset (35-51 years), and the use of diet and sulfonylurea.

The Counterpoint study employed a very low calorie diet in people within four years of type 2 diabetes diagnosis and demonstrated the rapid reduction of liver fat, by 30 % within the first 7 days of the study after eight weeks (Lim et al., 2011a).

1.4 Newer insights into the nature of type 2 diabetes

Several observational studies have suggested that the weight loss achieved post bariatric surgery could cause a significant benefit for the treatment of type 2 diabetes (Dixon et al., 2008). Originally, Pories and Albrecht suggested that the speed of the correction of the blood glucose within few days post bariatric surgery could be resulting from the exclusion of the food from the intestinal transit which is altering the incretin signals to the pancreatic beta cells (Pories and Albrecht, 2001). A randomized controlled trial by Dixon et al. showed that the weight loss secondary to the adjustable gastric banding resulted in the remission of type 2 diabetes in the majority of the recently diagnosed obese patients (Dixon et al., 2008) . A very important discovery of study done by Dixon et al is that the major driver of the improvement of glycaemic control is a degree of the weight loss (Dixon et al., 2008). It was also found that to produce the remission of type 2 diabetes a body weight loss of more than 10 percent was required (O'Brien et al., 2006).

Guidone et al studied 10 people with history of obesity and type 2 diabetes post biliopancreatic diversion (BPD) and observed a very early normalization of fasting blood glucose after surgery (Guidone et al., 2006). They suggested that BPD operation may affect the entero-insular axis by diverting the food away from the proximal gastrointestinal tract and therefore delivering the incompletely digested nutrients to the ileum which in turn enhanced the secretion of the GLP-1 in the transposed ileum. They postulated that the exclusion of the duodenum and jejunum might be responsible for the down-regulation of GIP and other gut hormones involved in the insulin sensitivity regulation (Guidone et al., 2006).

Lim et al tested the hypothesis (Figure 1.9) that insulin resistance and beta cell failure can be reversed by the dietary energy restrictions (Lim et al., 2011a). As a part of that study 11 people with type 2 diabetes have been put on a very low-calorie diet of 600 kcal a day for 8 weeks. Just after one week it was discovered that fasting plasma glucose have normalized. This study has revealed that by week 8 hepatic triacylglycerol decreased from 12.8±2.4 % to 2.9±0.2 %, pancreatic triacylglycerol fell from 8±1.6 % to 6.2±1.1 % and the first-phase insulin response increased and approached the control values (Lim et al., 2011a).

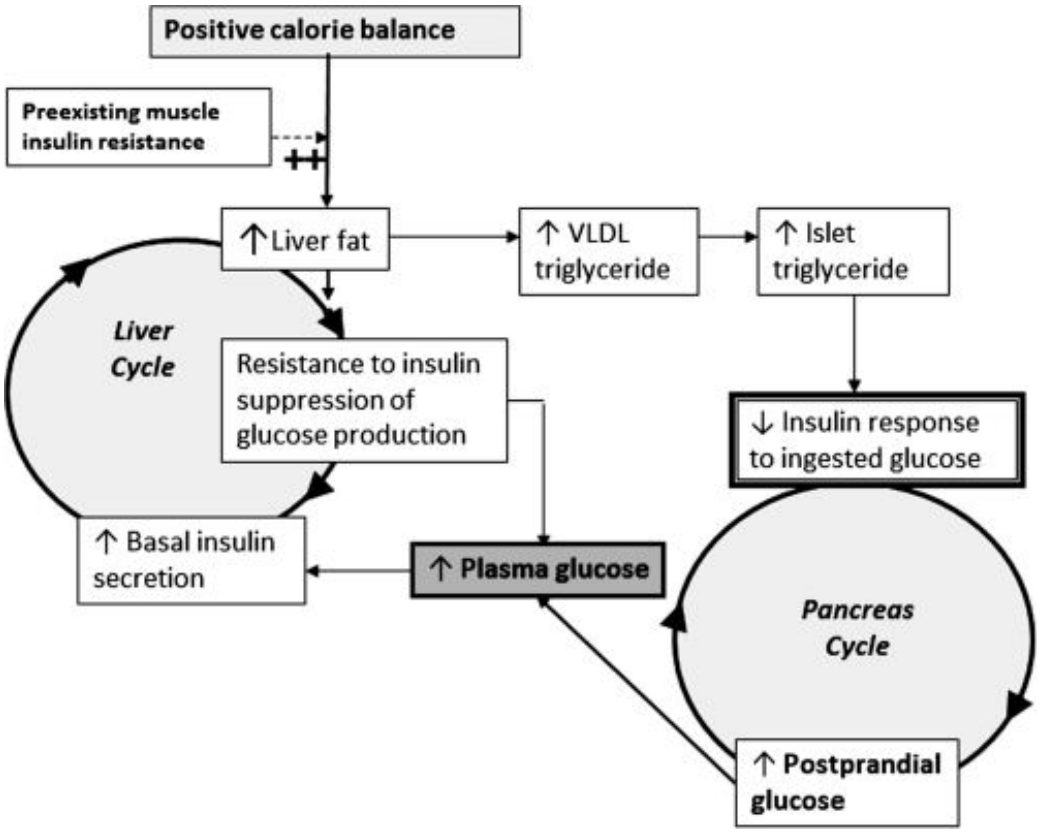


Figure 1.9

The Twin Cycle Hypothesis of etiology of type 2 diabetes (reproduced from Roy Taylor).

1.5 Application of the Magnetic Resonance to metabolic research

Magnetic Resonance occurs when the nuclei of some atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. There are 2 techniques based on this phenomenon: magnetic resonance imaging and magnetic resonance spectroscopy. The first method is used for revealing the anatomical structures while the second offers the biochemical information about the living tissues.

Several atomic nuclei have magnetic properties. In the magnetic field the nuclei of these atoms move into alignment, but after the application of the radio signal of a certain frequency the energy absorbed by the nucleus causes it to get out of the alignment. After the termination of the radio signal the nuclei go back into their alignment and release the energy in the form of radio waves which could be detected and subsequently analyzed. The nuclear magnetic resonance is a condition that the excitation field must be at the same frequency at which the sample oscillates. There is a decay of the signal due to the energy being lost from the excited nuclei which is called relaxation. Different materials have different surroundings hence different relaxation. There are 2 relaxation rates with different mechanisms of energy loss: T1 and T2. T1 is an energy loss to the molecular net and T2 is representing the energy exchange between the neighboring spins.

1.5.1 The advantages and disadvantages of MR methods

There are numerous advantages of the MR methods in relation to the clinical practice and the metabolic research. They are attractive for studies of human physiology and pathophysiology *in vivo*. These methods are non-invasive and have almost completely replaced the biopsy use in the metabolic research. MR techniques are non-radioactive and therefore could be safely applied to human subjects including children and pregnant

ladies. MR studies could be repeated as many times as needed which is allowing for a longitudinal monitoring during the metabolic studies. Organs such as liver, skeletal muscle, pancreas, adipose tissue, and brain are suitable for either MR spectroscopy or MR imaging or both. Natural abundance studies are possible by using the stable isotopes.

However, there are number of disadvantages of these MR methods worth mentioning. First, there are some practical difficulties like the availability and access to these scans. The cost associated with using the MR imaging in the research purposes is also quite substantial which is usually beyond the means of a single project. Subjects with metal implants must be excluded for obvious safety reasons. Claustrophobia is another important issue. It can prevent a subject from getting into the scanner as well as the large body habitus. The intrinsic insensitivity is the greatest disadvantage of NMR spectroscopy and imaging compared with other modalities therefore the substances present in micromolar concentrations cannot be detected directly in tissues. It should be mentioned that exogenous contrast agents can be detected indirectly at much lower concentrations through their effects on the relaxation of the water signal. Movement of the subject during the acquisition can lead to smearing effects in phase direction of images and spectral line distortion leading to an inaccurate quantification. Also signal to noise ratio must be optimal to be able to record enough signal for a reliable calculation. The real danger associated with MR is the potential for ferromagnetic objects that are not held in place to be attracted to the magnet therefore these things if accidentally brought into the MR scanner room are becoming a flying object with potential life-threatening consequences. The good thing is that there is a lot of surgical and monitoring equipment available now which is non-ferromagnetic.

1.5.2 NMR spectroscopy

The nuclei in the different chemical compounds will resonate at different frequencies and this is called a chemical shift. The multiple signals are being generated in different chemical groups after excitation. Fourier transform is used to decode the frequencies of the signal and forms the basis of NMR spectroscopy. The Fourier transform breaks up a

function of time (a signal) into the frequencies that make it up. This term covers the frequency domain representation and the mathematical operation that associates the frequency domain representation to a function of time (Figure 1.10).

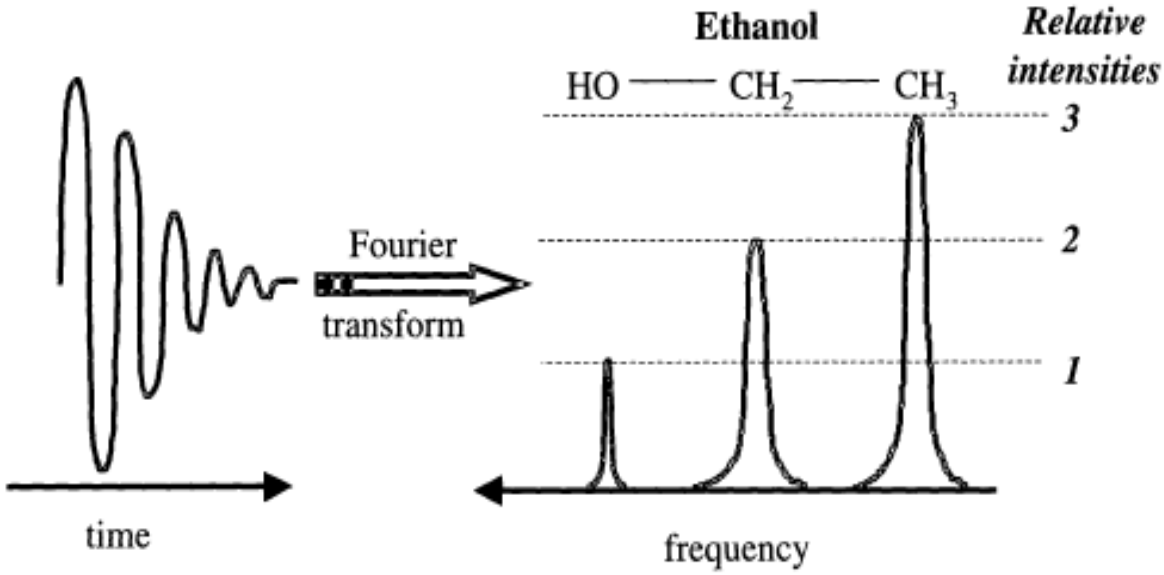


Figure 1.10

Fourier transform example. The hydrogen NMR spectrum of ethanol contains 3 frequencies corresponding to the 3 hydrogen groups (reproduced from Chatham et al) (Chatham and Blackband, 2001).

MR spectroscopy makes possible to perform a continuous monitoring of tissue concentrations of metabolites and metabolic fluxes. Various NMR-sensitive nuclei could be used in vivo. From the perspective of metabolic research, the most important of them are H ^{1}, ^{13} C, ^{31}P. ^{1}31P, ^{1} H, and ^{13}C-NMR spectroscopy are mainly used to investigate cellular bioenergetics and metabolism.

^{1}H is most sensitive of the resonant nuclei with natural abundance of almost 100 %. Proton MRS and MRI are the commonly used methods in metabolic research and clinical investigations. It is widely used for the assessment of the liver lipids that are being estimated in the percentage by ^{1}H NMR spectroscopy. *In vivo* ^{1}H MR spectroscopy measurement of intramyocellular triglycerides is comparable in accuracy to biochemical analysis from the muscle biopsy and electron microscopic morphometry (Schick et al., 1993). ^{1}H MR spectroscopy is currently the gold standard measurement of the liver fat. The separate signals from the water and the lipid can be detected easily. The signals from the lipids could be transformed into percent lipid relative to water.

^{31}P nuclei demonstrate a high signal to noise ratio due to the 99 % natural abundance of the ^{31}P isotope. ^{31}P MRS can detect metabolites of the phosphorus including ATP, phosphocreatine (PCr), phosphocholine, glycerophosphocholine, glucose-6-phosphate (G6P) and some others (Laufs et al., 2014). The rates of ATP synthesis were assessed by using saturation transfer ^{31}P MR spectroscopy and found to be reduced in muscles of the older people and in the offspring of type 2 diabetes patients (Petersen et al., 2004, Petersen et al., 2003). ^{31}P MRS has been used to investigate the mechanism behind the defect in glycogen synthesis in the muscles of people with type 2 diabetes (Rothman et al., 1992). That study has found reduced G6P concentration concentrations in in these subjects which lead to the conclusion of the defect in glucose transport and phosphorylation in type 2 diabetes.

Stable ^{13}C isotope is detectable by MR but naturally found only in 1.1 % of all carbon isotopes therefore the signal to noise ratio is much lower in comparison to ^{1}H and ^{31}P MRS. ^{13}C MR spectroscopy has a wide chemical shift range and primarily used for the detection of glycogen. The measurement of the muscle glycogen by ^{31}P MRS has been found to have a strong correlation with the muscle biopsy and direct biochemical assay for glycogen concentration (Taylor et al., 1992).

1.5.3 Three-point Dixon MR imaging

Three-point Dixon MRI is an effective means of separating the signals from the hydrogen nuclei in fat and in water. Dixon has published in 1984 the method where the fat and water were separated by 2 separate image acquisitions with modified spin echo pulse sequence so called two-point technique (Dixon, 1984). Fat quantification gets achieved by the summation and subtraction of the 2 sets of images (water-only and fat-only). The three-point Dixon technique with the third data acquisition has been developed in order to correct the phase error which is a result of the magnetic field inhomogeneity (Glover and Schneider, 1991). The method uses three measurements with phase shifts of 0, π and − π between the fat and water resonances. The additional information provided by the third measurement is used to calculate an image of the field inhomogeneity in addition to true water and fat images. The signal-to-noise ratio (SNR) in the decomposed images is equivalent to that of a 2.7 NEX acquisition (instead of 3 NEX), yielding an SNR imaging efficiency of 95 %. In addition, the B_0 image which is provided may have diagnostic value in its own right (Glover and Schneider, 1991).

Three-point Dixon MR imaging method has been found to have a high correlation with spectroscopy in the comprehensive study of the liver fat (Yokoo et al., 2009) but that strong correlation between the methods was not found to be the case in the imaging of the pancreas (Hu et al., 2010). This is most likely to reflect the main weakness of MR spectroscopy which relies upon setting in advance the volume from which signal is acquired. During the 15-20 minutes of the acquisition, respiratory movements allow visceral fat to move in and out of the volume of interest in an uncontrolled fashion. The main advantages of the three-point Dixon technique are the ability to be performed within the breath-hold and not requiring the respiratory gating. While the image being processed, it is allowing to avoid the non-tissue structures and blood vessels.

1.6 Relationship between type 2 diabetes and obesity

Type 2 diabetes is often regarded as a disease related to obesity. However, the majority (72 %) of people with BMI over 40 kg/m^2 have no diabetes (Gregg et al., 2007). Conversely, half of all newly diagnosed people with type 2 diabetes are not obese (Logue et al., 2013). The relationship between body weight and type 2 diabetes is nicely illustrated by data from the Nurses' Health Study. This showed that there is a four-fold increase in type 2 diabetes mellitus prevalence for women of BMI 23-25 compared with those of BMI less than 22 kg/m^2 (Hu et al., 2001). The implications of this striking observation have not been widely appreciated. As would be expected, the study also confirmed the exponential relationship between type 2 diabetes and rising BMI such that the most obese category had a 37-fold increased prevalence. It is clear, that obesity *per se* is permissive but not sufficient to cause type 2 diabetes.

The distribution of BMI for the 5102 people with newly diagnosed type 2 diabetes recruited into the United Kingdom Prospective Diabetes Study (UKPDS) is shown in Figure 1.11. (1991).

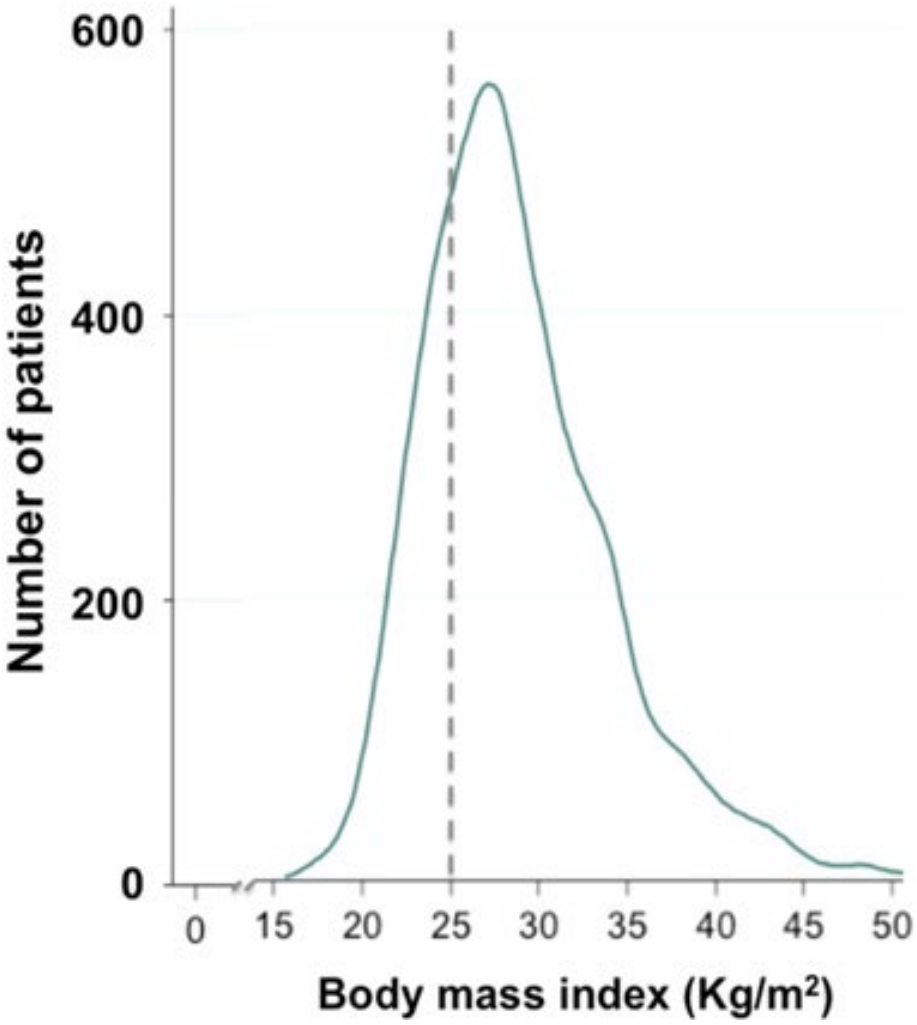


Figure 1.11

Population BMI distribution frequency plot for the entire 1977–1991 UKPDS cohort with newly diagnosed type 2 diabetes (reproduced from Taylor R. et al 2015).

The distribution is unimodal with a slight skew to the right. It demonstrates that only a minority of the newly diagnosed individuals with type 2 diabetes have a BMI greater than 35 kg/m^2 and 36 % of the subjects had a BMI less than 25 kg/m^2. This distribution is right-shifted from that during the time of recruitment for UKPDS (between 1977 and 1991) when 64 % of the adult UK population had a BMI less than 25 kg/m^2 (Rosenbaum et al., 1985).

From today's perspective, it is remarkable that so many people with newly diagnosed type 2 diabetes had normal BMIs those days. Indeed, careful reviews in that era concluded that obesity did not have a major influence on type 2 diabetes (Jarrett et al., 1979, Taylor, 1989, Leslie and Pyke, 1985). Given that the risk of type 2 diabetes rises steeply at higher BMI and that higher body mass indexes are now more prevalent, it is not surprising that the association between obesity and type 2 diabetes mellitus is much more evident today.

These observations have been explained by the Personal Fat Threshold (PFT) hypothesis which focusses upon the individual rather than the population mean (Taylor and Holman, 2015a). This is explained diagrammatically in Figure 1.12.

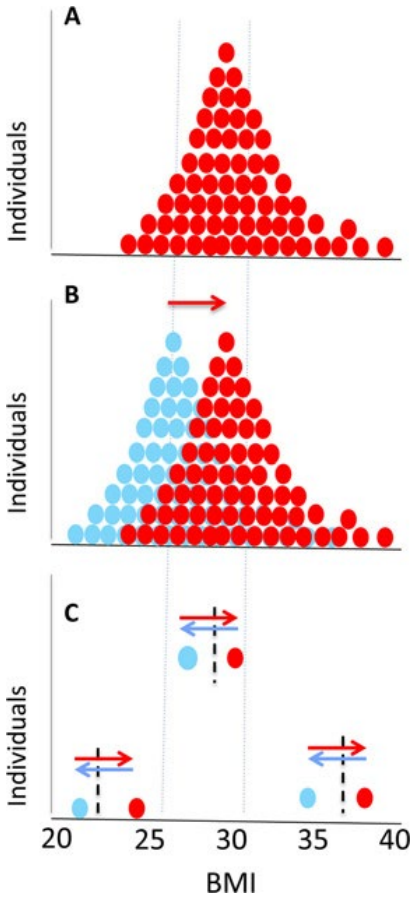


Figure 1.12 The personal fat threshold versus population metric: (A) Representative frequency distribution of BMI for a group of individuals with T2DM. (B) Frequency distribution of BMIs in blue for the individuals depicted in (A) before they gained weight. The red frequency distribution, when diabetes had developed, is right shifted (red arrow) and usually interpreted as indicating a higher prevalence of obesity. (C)Three illustrative individuals from (B) are shown demonstrating their relative positions within the population BMI distribution. One is obese, one overweight and one normal weight. Weight loss of 15 kg in each case resulted in return to normal glucose tolerance although their classification by the population measure of BMI did not change. It is hypothesized that each individual has a PFT (dotted line) above which excess fat is stored within the liver and the pancreas. This individual susceptibility has no relationship to BMI despite the higher probability of diabetes being precipitated in the obese range. For each individual, moving to the right of their PFT triggers T2DM (red arrows) and moving to the left of the line restores normal glucose tolerance (blue arrows). (Reproduced from Taylor and Holman et al. 2011) (Lim et al., 2011a).

When individuals exceed their personal fat threshold, they become likely to develop type 2 diabetes. It is clear, that the hypothesized PFT is independent of BMI. Most of obese individuals are well equipped to store large quantities of fat in a metabolically safe fashion in subcutaneous adipose tissue. But some apparently slim individuals have a low capacity in this depot, and ectopic fat builds up at low BMI's. As an extreme example, in generalised lipodystrophy and effective absence of subcutaneous fat, gross fatty liver disease occurs, and diabetes is common (Reitman et al., 2000). Depending upon the genetically determined susceptibility of the beta cells to exhibit endoplasmic reticulum stress and consequent beta cell de-differentiation, diabetes may or may not occur (Lee et al., 1994b, Talchai et al., 2012, White et al., 2016b). Prolonged Intralipid infusion in people predisposed to develop type 2 diabetes is known to severely impair beta cell function (Storgaard et al., 2003). If a person with recent onset type 2 diabetes loses the excess weight, going down below his or her PFT, that makes likely a return to normal glucose control and reversal of type 2 diabetes (Lim et al., 2011b, Taylor and Holman, 2015a, Steven et al., 2016b). Moderate calorie restriction achieving weight reduction by approximately 8 kg is accompanied by reversal of hepatic steatosis and hepatic insulin resistance leading to a normalization of basal rates of hepatic glucose production and improvement in fasting plasma glucose (Petersen et al., 2005, Ravikumar et al., 2008a). The Counterpoint study employed a very low calorie diet in recently diagnosed people with type 2 diabetes and demonstrated reduction of liver fat by 30 % within the 7 days and normalisation of fasting plasma glucose (Lim et al., 2011b). Continuation of the weight loss brings about decrease in pancreatic fat and return of glucose stimulated insulin secretion in type 2 diabetes (Lim et al., 2011b, Taylor and Holman, 2015a, Steven et al., 2016b). Importantly, the Counterpoint study demonstrated that weight loss of around 15 kg was required to achieve remission of type 2 diabetes and also that this could readily be achieved using a low-calorie liquid diet.

Appreciation of the individual susceptibility to type 2 diabetes appears to be determined both by the relative inability to store fat safely in subcutaneous tissues at any given BMI and by the relative susceptibility of beta cells to de-differentiate in the presence of metabolic insult. This allows understanding of the phenomenon that type 2 diabetes can

occur at any BMI reflecting a degree of weight gain excessive for the individual. It also allows understanding that weight loss resulting in a BMI above 30 kg/m^2 can achieve metabolic normality.

1.7 Management of body weight in type 2 diabetes

1.7.1 Goals

The twin goals of management of body weight in type 2 diabetes are achievement of weight loss and, very importantly, long term avoidance of weight regain. Wing and Hill proposed criteria for successful weight loss and maintenance: loss at least 10 % of the body weight and weight stability for at least one year (Wing and Hill, 2001). Six key strategies were proposed based on the data from the National Weight Control Registry: high level of physical activity, low energy and low fat diet, eating breakfast, self-monitoring weight on the regular basis, keeping the consistent eating pattern, and catching "slips" before they turn into lager weight regain (Wing and Phelan, 2005). However, application of cross-sectional data introduces confounders and care is required in interpreting such data. Different strategies are required to achieve weight loss, as well as to achieve long term weight stability.

How much weight loss is sufficient to lose type 2 diabetes and put it into the long-term remission? Conventional guidelines suggest aiming for 5 % weight loss as a general help with management of hyperglycaemia. In the Counterpoint study where participants have lost on average 15 kg of weight during 8 weeks of very low calorie diet (800 kcal) and all 11 people reversed type 2 diabetes (Lim et al., 2011a). It must be noted that only people with duration of type 2 diabetes of less than 4 years were studied. Reversal of diabetes was not observed with weight loss of less than 8 kg.

1.7.2 Reported dietary weight loss interventions in people with type 2 diabetes

Dietary management for the people with type 2 diabetes has been evolving over many decades. It differs from primary prevention advice to be applied to populations at risk in that there is a potent motivator for people who have been diagnosed – to escape from diabetes entirely, and avoid the risk of blindness, amputation, and premature death.

In UKPDS the response to the diet was reported in the 3,044 newly diagnosed who had fasting plasma glucose of 12.1±3.7 mmol/l and weight of 130±26 % ideal body weight (1990). Initial body weight did not determine the glycaemic response to weight loss, as could be predicted from the Personal Fat Threshold hypothesis (Taylor and Holman, 2015a). In this study 16 % of the group reached a normal fasting plasma glucose less than 6 mmol/l after 3 months (1990).

The Belfast Diet Study showed that treatment with diet alone for the first 10 years after the diagnosis of type 2 diabetes is associated with progressive rise in fasting plasma glucose but this study concentrated on composition of food rather than quantity (Levy et al., 1998).

The longest randomised controlled study to date of an intensive lifestyle intervention for weight management is Look AHEAD (Action for Health in Diabetes Study). This study showed that over 8 years overweight or obese people with type 2 diabetes lost 4.7 % of initial body weight in the intensive lifestyle intervention group (versus 2.1 % in usual care group) (LookAhead, 2014). 26.9 % of the intervention group lost > 10 % of initial body weight by the end of a trial. The degree of weight loss in predicting remission was notable, with those achieving weight loss of > 6.5 % having a remission rate of 16.4 % at one year. However, these results appeared less impressive than may have been desired in view of the intensive and expensive nature of the intervention. There was an emphasis on exercise in Look Ahead, and this may have been counterproductive.

Very low-calorie diets rapidly improve plasma glucose control. The old extremely low energy diets (330 kcal/day) brought about weight loss of 10.5±0.4 kg with improvement in fasting plasma glucose (Henry et al., 1985). Using a modern ~700 kcal/day liquid formula diet, superior average weight loss (15.2kg) has been reported, with complete normalisation of plasma glucose within 7 days (Lim et al., 2011a). Although it has been assumed that rapid weight loss is always followed by weight regain, this concept developed in the absence of appropriate continuing support programmes. Ongoing weight stability following rapid weight loss and a careful step-wise reintroduction of normal foodstuffs has been shown to be achievable (Steven et al., 2016b).

1.7.3 Approaches to avoid long term weight regain

The principal dietary interventions for long term use which are supported by evidence will be considered: low-fat diet, restricted carbohydrate diet, Mediterranean diet, and intermittent energy restriction.

A low-fat diet (< 30 % total energy from fat) has long been widely advised. The idea became popularised by an epidemiological association between different countries of high fat intake with cardiovascular death (Keys, 1953). Such associations from cross-sectional studies have repeatedly been shown not represent cause and effect (Feinman et al., 2015), but the belief in a low fat diet for health is very widespread and reflected in current guidelines for type 2 diabetes. A head-to-head comparison of low fat diet with an energy restricted diet showed no significant difference in weight loss (Jeffery et al., 1995), whereas combination of the low-fat plus low energy diet did show a significant difference versus low fat diet alone (Schlundt et al., 1993, Pascale et al., 1995).

Low carbohydrate diets continue to arouse strong feelings, possibly as a backlash against more extreme carbohydrate avoidance diets (Feinman et al., 2015, Spiro and Stanner, 2016). A restricted carbohydrate diet brings about an increase in the proportion of calories from fat, conflicting with long-held beliefs about the risks of higher fat diets. However, the practical outcome has been shown to be beneficial for both weight

management and improvement in cardiovascular risk factors (Bazzano et al., 2014). The macronutrient composition of diet, for equivalent weight loss, does not affect liver fat content nor any other aspect of fat distribution (de Souza et al., 2012). These points have been incorporated into evidence-based nutrition guidelines (Dyson et al., 2011).

The Mediterranean diet consistently has been reported to be advantageous in terms of weight control and cardiovascular health (Estruch et al., 2016, Garcia-Fernandez et al., 2014, Martinez-Gonzalez and Martin-Calvo, 2016), with a decreased diabetes incidence independent of weight (Salas-Salvado et al., 2011). A combination of Mediterranean with carbohydrate restriction may be beneficial (Esposito et al., 2014).

Time-limited approaches to eating (such as alternate day or intermittent fasting) appear to be very suitable for some individuals as an alternative to daily calorie restriction. This is as effective as calorie restriction for weight loss and maintenance for up to 12 months (Davis et al., 2016). The proportion of people losing more than 5 % in weight has been reported to be higher with intermittent energy reduction (60-65 %) compared to daily energy restriction (37 %) (Harvie et al., 2013). For ongoing avoidance of weight regain, one day of energy restriction per week was found to be successful. Using the 5:2 approach in people with type 2 diabetes achieves comparable reductions in weight and HbA1c to calorie restriction with no adverse effects on exercise levels or appetite (Carter et al., 2016, Harvie and Howell, 2016). Longer term weight maintenance outcomes are currently lacking.

Omission of breakfast runs counter to beliefs about this meal, although the latter mainly derived from cross-sectional studies, often with a potential commercial bias (Brown et al., 2013). Clearly the approach of not eating before noon suits some people and not others. Prospective study suggests a major energy advantage of this pattern of eating with no disbenefit in terms of eating later in the day (Clayton et al., 2016, Kealey, 2016).

Over recent years guidelines have moved away from enforcing any particular macronutrient composition to acknowledging that there is no ‘one best diet’ for every individual with diabetes (Dyson et al., 2011). In practice, long term energy intake can be minimised by using an approach suited to the individual. Taken together, these studies illustrate important points. Clear separation of a limited duration weight loss phase

followed by a weight maintenance phase of both calorie limitation and increased physical activity may be a more successful approach (Steven et al., 2016b). Confirmation of this in a large population is currently being sought (Leslie et al., 2016). The nature of support and advice about eating during long term weight maintenance clearly deserves close study.

1.7.4 Exercise

Relatively low levels of physical activity contribute to the positive energy balance over many years as obesity develops, and obesity itself completes the vicious circle by decreasing the ability to undertake physical activity. The increase in inactivity rates among the population is a growing problem especially in the economically rich countries.

Physical inactivity is associated with increased insulin resistance (Sigal et al., 2004). The evidence from the Finnish Diabetes Prevention Study demonstrates that weight loss and maintenance of this through the diet and physical activity reduces the incidence of type 2 diabetes by more than a half (Tuomilehto et al., 2001). Lifestyle interventions combining limitation of quantity of food with increased daily physical activity are the mainstay of programmes to manage body weight (Sigal et al., 2004). Men with low physical activity levels and type 2 diabetes have much higher mortality rates compared with their fitter counterparts (Wei et al., 2000).

The energy expenditure achieved by the amount of exercise feasible for overweight, older people is modest and easily cancelled out by a snack. To maximise weight loss, the initial approach must recognise the dangers of compensatory eating brought about by any sudden increase in exercise (Finlayson et al., 2009, Hopkins et al., 2014, King et al., 2012). This increase in energy intake, partly conscious and partly sub-conscious is counterproductive and underlies the common observation that exercise in overweight

people does not result in weight loss. The impact of compensatory overeating varies between individuals (Hopkins et al., 2014) but can be entirely avoided. Studies focussed on decreased energy intake with no additional exercise achieve ~15 % weight loss in 8 weeks. In contrast, the intensive exercise advised in Look AHEAD, only achieved a maximum weight loss of 8.5 % despite dietary input (LookAhead, 2014). This matter must be seen as distinct from the extremely important role of increased physical activity in achieving long term weight control (Wing and Phelan, 2005).

A sustained increase in physical activity is without doubt vital for the long term avoidance of weight regain, and is the single most solid outcome of research across the weight maintenance field (Pronk and Wing, 1994, Kayman et al., 1990). A combination of diet and exercise achieves better weight loss compared with diet alone after 20 weeks of treatment (8.3 kg vs 5.6 kg respectively) and in one year (7.9 vs 3.8 kg)(Wing, 1989). It is possible that the effect of increasing daily physical activity on food limitation is greater in men (Wood et al., 1991).

1.7.5 Bariatric surgery

For individuals who are not able to achieve weight loss by overall restriction of energy intake, bariatric surgery is an effective option. The overall effects of surgery – including involuntary restriction of food intake, rapid weight loss, post-prandial hypoglycaemia, risk of surgical complications - must be discussed with the individuals and their partners. Randomised studies comparing outcomes are not informative, as individuals most suited to surgery are not the same people as those most suited to an effective dietary approach. The multicentre Swedish Obese Subjects Study (SOS) is important as an observational non-randomised study comparing different types of bariatric surgeries with medical weight loss treatment (Sjostrom et al., 2004). The remission was 3 times greater and the risk of type 2 diabetes development was more than 3 times lower for the bariatric surgery group at 10 years of follow up (Sjostrom et al., 2004).

Bariatric surgery is very successful in achieving sustained major weight loss (Buchwald et al., 2009, Dixon et al., 2008). Indeed, it is the only successful weight loss intervention which can be done by doctors to patients irrespective of the degree of motivation to lose weight. The nature of the operation is important only in the degree of energy restriction enforced, as illustrated by the lesser effect of gastric banding or the equivalent effects of gastric sleeve surgery compared with Roux-en-Y gastric bypass (Dixon et al., 2008, Schauer et al., 2012). Any procedure which results in rapid food entry into the ileum will bring about a greatly increased GLP-1 response after, and many studies have drawn attention to the association with metabolic changes (Jorgensen et al., 2012, Laferrere et al., 2007, Guidone et al., 2006). However, such studies do not indicate any causal relationship and matched feeding studies demonstrate very precisely that identical metabolic changes in type 2 diabetes are achieved by pair feeding studies (Lingvay et al., 2013). Detailed examination of the metabolic changes demonstrated no detectable effect of the GLP-1 spike itself (Steven et al., 2016c, Isbell et al., 2010b, Jimenez et al., 2013). These observations solely concern the early metabolic response to bariatric surgery and other potential effects of the enhanced postprandial GLP-1 response, such as on appetite in the long term, remain to be definitively established.

Other effects of bariatric surgery include changes in bile acid handling and in the gut microbiota, and the consequences of these await precise evaluation. The re-routing of nutrients after bariatric surgery may affect enterohepatic recirculation of bile acids with potential effects upon glucose metabolism (Pournaras et al., 2012). Obesity alters the gut microbiota, and conversely distinct changes occur after successful treatment by bariatric surgery (Palleja et al., 2016, Zhang et al., 2009). Other hypotheses concerning the metabolic effects of bariatric surgery have been postulated, based largely upon rodent studies. The foregut hypothesis that exclusion of nutrients from the proximal small bowel is unlikely to be relevant to humans, given the striking similarity between the metabolic effects of sleeve gastrectomy and Roux-en-Y gastric bypass for any given degree of weight loss (Schauer et al., 2014). The hindgut hypotheses of incretin production effect is effectively ruled out by the observations on paired feeding studies and other direct human observations (Isbell et al., 2010a, Lingvay et al., 2013, Steven et al., 2015).

Bariatric surgery is associated with certain mortality (Omalu et al., 2007). However, weight loss achieved by bariatric surgery decreases mortality in type 2 diabetes by up to 92 % (Adams et al., 2007). On the basis of all the evidence to date, The International Diabetes Organisation have issued a treatment algorithm for bariatric surgery for type 2 diabetes, supporting use in people with BMI 35-39.9 kg/m^2 when hyperglycaemia is inadequately controlled by lifestyle and optimal medical therapy and in those with BMI ≥ 40 kg/m^2 (Rubino et al., 2016). The assessment for a bariatric surgery should be also thought in patients with BMI of 30 to 34.9 with recent onset type 2 diabetes receiving a Tier 3 service as per NICE CG189 guidance (Stegenga et al., 2014). Nonetheless, bariatric surgery should be considered in patients who fail to achieve their aims with lifestyle behavioural intervention.

Chapter 2. Methods

2.1 Introduction

The detailed metabolic research was performed on a subgroup of DiRECT (Diabetes in Remission Clinical Trial) who could access the Newcastle Magnetic Resonance Centre. The DiRECT study used a cluster-randomised controlled design, with GP practice being the unit of randomisation. Recruitment of the Tyneside practices was conducted centrally and independently of the local research team, by the Robertson Centre for Biostatistics, University of Glasgow. Practices were allocated using a minimisation method to maintain the required balance across intervention groups within each study region (Scotland or England) and practice list size (>5700 or≤5700). Ethical approval was obtained from the West of Scotland Research Ethics Committee (reference number: 13/WS/0314).

2.2 Organisation

2.2.1 Overall organisation of DiRECT (Diabetes Remission Clinical Trial)

Recruitment of general practices was undertaken in multiple Health Board areas in Scotland and in the Newcastle upon Tyne NHS Foundation Trust area in the Northeast of England. Practices from across Scotland and Tyneside were invited to participate by the Primary Care Research Network (PCRN). Practices expressing interest in participation were contacted by research team and recruited to the study.

It was planned to recruit 280 participants (140 per arm of the study) from Tyneside and Scotland general practices. As the study proved to be extremely popular, 297

participants were recruited in total. Potential participants were identified by a computerised search of GP records, undertaken by staff from Primary Care Research Network. Individuals who responded to the invitation as interested in participating were contacted by a member of the research team and invited to attend for a screening appointment. At this initial appointment, the study was fully explained and discussed with prospective participants. Informed consent was obtained from all participants, for 2-years on the trial protocol and for indefinite long-term data collection from their medical records and through national records linkages.

2.2.2 Tyneside organisation of DiRECT

The original aim was to recruit 68 Intervention and 20 Control participants from Tyneside. 15 General practices were randomised to be whether on Intervention (8 practices) or on Control side (7 practices). The Tyneside recruitment of subjects with type 2 diabetes was done by letters from GP to their patients meeting the study criteria.

Inclusion criteria:

- men and women aged 20 – 65 years old
- known duration of type 2 diabetes mellitus 0–6 years (diagnosis based on 2 recorded diagnostic-level tests, HbA1c and/or blood glucose)
- HbA1c ≥ 48 mmol/mol at the last routine clinical check within last 12 months if on diet alone
- HbA1c ≥ 43 mmol/mol if on treatment with oral hypoglycaemic agents
- Body Mass Index > 27 kg/m^2 and < 45 kg/m^2
- all ethnicities

Exclusion criteria:

- current insulin use
- recent routine HbA1c ≥ 108 mmol/mol
- Weight loss of >5 kg within the last 6 months
- recent eGFR < 30 mls/min/1.73 m^2
- history of substance abuse
- known oncology diagnosis
- myocardial infarction within previous 6 months
- severe heart failure defined as equivalent to the New York Heart Association grade 3 (NYHA)
- learning difficulties
- current treatment with anti-obesity drugs
- eating disorder or purging
- being pregnant or considering pregnancy
- patients who have required previous hospitalisation for depression or having been prescribed with antipsychotic treatment
- current participation in another clinical research trial
- contraindications for MR scanning

Practices randomised to control (7 practices) continued to deliver usual type 2 diabetes and obesity management as per current clinical guidelines. Detailed baseline clinical and medication information was recorded for each of the participants on eCRF (electronic Central Research Facility) website. Participants recruited in these practices were followed for 2 years and seen on 3 occasions by the trained GP practice nurses. The outcome data collected by GP nurses included weight change and capillary blood glucose measurements.

Practices randomised to intervention (8 practices) continued with their usual guideline-based care and delivered a Total Diet Replacement (TDR) with Counterweight Plus diet provided by Cambridge Weight Plan Ltd. TDR phase

followed by Food Reintroduction (FR) phase and a structured support programme for long term weight loss maintenance. The intervention was delivered to each participant individually, at their general practice by a practice nurse and sometimes by a research dietitian to cover for the absence of a practice nurse. Participants attended for 35 appointments over the 2-year intervention period.

2.2.3 Non-diabetic comparator group

In order to be able to fully interpret the novel metabolic parameters, a non-diabetic comparator group was recruited at Tyneside. This group was intended to match the intervention group post TDR and weight loss for BMI, age, and gender. Participants from non-diabetic comparator group were not to have a personal history of type 2 diabetes or a history of type 2 diabetes in the first-degree relatives. Those individuals also were not to have contraindications to the study or MR scanning. Non-diabetic comparator participants were recruited through the advertisement in the local newspapers. 25 individuals were recruited in total. Following that they attended for an initial screening visit to Magnetic Resonance centre. The screening visit included the explanation of the study to the participant, taking the medical and family history, and obtaining the informed consent. Then participants had a standard oral glucose tolerance test to confirm their normal glucose tolerance.

2.3 Intervention

2.3.1 Total Dietary Replacement phase

A commercial micronutrient-replete 825–853 kcal/d liquid formula diet (soups and shakes) were provided by Cambridge Weight Plan to replace usual foods, with ample

fluids (2.25 L a day), for up to 12-20 weeks. Oral hypoglycaemic agents (OHA), antihypertensive and diuretic drugs were withdrawn on commencement of TDR, and reintroduced (as per study protocols) if type 2 diabetes or hypertension returns. Aspirin was continued if prescribed because of a previous myocardial infarction (prior to the previous 6 months) but discontinued if prescribed solely because of type 2 diabetes. Beta-blockers prescribed for the management of angina were continued. A soluble fibre supplement (Fybogel 2 × 3.5 g/day) was prescribed to reduce constipation. Participants were returning for review one week after commencement on the TDR and at 2 weekly intervals thereafter until the commencement of the FR stage. To allow some flexibility for patients whose commitments, or life events, prevent achievement of 15 kg at 12 weeks, or if individuals wished to achieve more weight loss, the TDR phase was continued for up to 20 weeks. If BMI was falling below 23 kg/m^2 during the TDR phase, participants were moved forward to the food reintroduction and weight loss maintenance phases.

2.3.2 Food reintroduction phase

The food reintroduction (FR) phase included a stepped transition to a food diet based on the "Eatwell" guidelines [Food Standards Agency. The eat-well plate. 2010. http://food.gov.uk/healthiereating/eatwellplate/] while reducing TDR. To allow flexibility for participants with different level of confidence, the FR phase was varied between protocol-defined limits of 2–8 weeks before switching to full food-based weight loss maintenance. Participants were monitoring weight on a weekly basis and comparing this with caloric intake and activity levels. Participants were returning for review at 2 weekly intervals throughout the FR phase.

A stepped transition to food-based Weight Maintenance, replacing low energy liquid diet with meals which contain 30 % of energy from fat. During this phase there was a possibility that some further modest weight loss to occur.

Week 12: step down on to 400 kcal/d low energy liquid diet plus 1 low-fat meal a day (360-400 kcal) and 2 servings of fruit, 200 mls skimmed milk and free vegetables. Total intake was 1000 kcal/day.

Week 14: step down to 200 kcal/d low energy liquid diet plus 2 low-fat meals a day (720-800 kcal) plus 2 servings of fruit, 200 mls skimmed milk and free vegetables. Total intake was 1200 kcal/day.

Week 16: 3 low-fat meals per day (1080-1200 kcal) plus 2 servings of fruit, 200mls skimmed milk and free vegetables. Total intake was 1400kcal/day.

2.3.3 Weight loss maintenance phase

Subjects were advised by dietitians and practice nurses to follow a food-based diet and were provided with an individually tailored energy prescription, to support weight stabilisation and prevent weight regain. The option of using one sachet of formula diet per day for the duration of weight loss maintenance was also available to participants. Participants were reviewed at the monthly intervals. All participants allocated to the intervention were provided with printed support materials describing the management plan and support for each phase of the intervention. Physically capable participants were advised to increase daily physical activity.

2.3.4 Relapse management for weight regain or re-emergence of diabetes

If weight regain occurred, or if diabetes was found to have returned (HbA1c $\geq$ 48 mmol/mol) at any time during the 18-month weight loss maintenance stage, 'rescue plans' to reverse weight gain was offered.

1) Weight re-gain of > 2 kg: the use of TDR was offered to replace one or two main-meal per day for 4 weeks, offer Orlistat 120 mg three times a day, with each meal.

2) Weight gain of > 4 kg, or to < 15 kg below starting weight or if diabetes recurred: 4 weeks TDR were offered with fortnightly practice nurse/dietitian review and then a 2–4 weeks FR (as described above). Individualised dietary advice, based on the Eatwell guidelines, and physical activity targets were reinforced for weight loss maintenance phase. Orlistat treatment was offered for the remainder of the weight loss maintenance period, with repeat advice to restrict dietary fat. Relapse management included an exploration of the reasons for weight regain, and anticipatory support to prevent recurrence.

2.4 Baseline Measurements

Height was measured to the nearest 1 mm, with the Frankfort plane horizontal, using a portable stadiometer (Chasmors Ltd, London) during the first appointment with GP practice nurse. Prior to the appointment for metabolic studies at the Magnetic Resonance Centre in the morning, the body weight was measured in the fasting sate to the nearest 100 g without shoes in light clothing using an approved calibrated upright pedestal digital scale (Seca Ltd., Birmingham, UK). At the GP practices waist circumference was measured halfway between the point of the lowest rib and the iliac crest and hip circumference was measured at the maximum circumference around the buttocks. Those measurements were done by a standard non-distensible measuring tape.

Blood pressure was measured by automatic blood pressure monitor Dinamap V100 (GE Medical systems information technologies Inc, Wisconsin, USA) with the patient seated, at rest, with legs uncrossed for at least 5 min just prior to the start of metabolic tests.

2.5 Accelerometry

Physical activity was assessed in all participants at baseline, 12-, and 24-month follow-up, using GENEActiv accelerometers, a fully waterproof wrist-worn triaxial, raw data accelerometer, for activity and sleep tracking in free living studies (http://www.geneactiv.org/actigraphy/geneactiv-original). The device was worn by participants for 9 days at each data collection time point.

Participants were instructed to wear a tri-axial, wrist-worn, raw accelerometer (GENEActiv, Activinsights Ltd, United Kingdom) continuously (24 hours) on their non-dominant wrist. Data was recorded at a sampling frequency of 50Hz under free-living conditions at baseline, 1 and 2 years. The accelerometers were pre-programmed to start recording immediately after and instructions were provided on wearing the device.

Accelerometer data was processed in R (www.cran.r-project.org) using the Raw Accelerometer Data Analysis software package GGIR (https://cran.r-project.org/web/packages/GGIR)(van Hees et al., 2013).

All analysis was performed with the operator blinded to treatment allocation. Valid data (≥ 16 hours/day) extracted between the first and last midnight for a maximum of 9 days were retained for analysis. If included in the analysis period, the first and last hours of data were excluded as they were expected to be influenced by the monitor distribution and collection procedure. Monitor non-wear time was detected and replaced by mean acceleration calculated on the other days of the measurement (van Hees et al., 2014).

The average metric Euclidean norm (magnitude) of the 3 raw signals minus 1 g (Euclidian Norm Minus One) per 5 second interval was used to quantify acceleration due to movement and expressed in milligravity (m*g*) units (van Hees et al., 2013).

Calculated Euclidian Norm Minus One was used to derive time spent in active and inactive mode per day.

Time spent being inactive (minutes) was time per day with Euclidian Norm Minus One < 50 m*g*. Time spent in low levels of physical activity (minutes), representing a mixed zone between inactive and active behaviour (for instance, sitting while moving arms, or slow walking while keeping hands still) was time per day with Euclidian Norm Minus One ≥ 50 m*g* and < 100 m*g*. The time spent in moderate-to-vigorous physical activity (MVPA, minutes) was defined as ≥ 100 m*g* based on walking at 4 km/h being classified as moderate physical activity (Ainsworth et al., 2011) and was equivalent to an acceleration of 100 m*g* in a laboratory-based study on 30 adults (Hildebrand et al., 2014). To qualify as moderate-to-vigorous activity, ≥80% of the activity needed to be ≥ 100 m*g* for at least 10 minutes.

The mean Euclidian Norm Minus One for the least active 5 hour period of the day (L5, m*g*) and the most active 5 hour period (M5, m*g*) along with the difference between the two (ΔM5-L5, m*g*) were calculated (Innerd et al., 2015, Anderson et al., 2014).

2.6 Measurement of the Liver and Pancreas fat

Participants attended the Magnetic Resonance Centre in Newcastle upon Tyne in the morning fasting from 10 pm previous day. To avoid any metabolic stress, transport to the MR centre by taxi was offered. Only still water was allowed prior to the test.

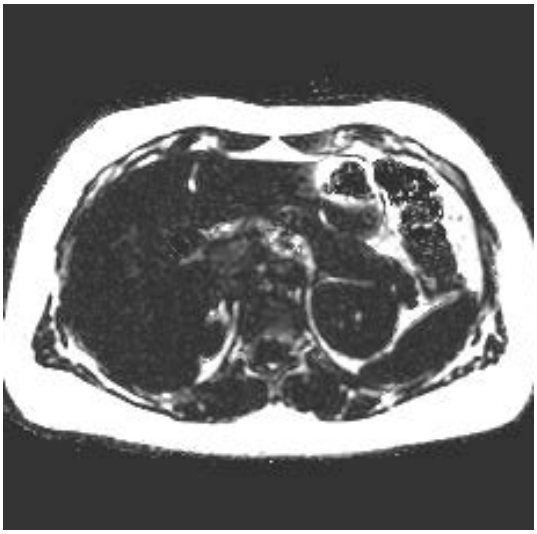
Participants were explained by a radiographer all safety procedures for MR scanning and filled in the MR questionnaire.

Magnetic resonance images were collected by means of using of 3 Tesla Phillips Intera Achieva Scanner (Phillips, best, The Netherlands). A standard 6 channel cardiac coil (Phillips) was used for taking images in most cases. In the circumstances of the large body habitus the so-called Flexi Coil (Picture 2.1.) or four large surface coils (Phillips) were used instead.

Figure 2.1 Standard Cardiac and Flexi Coils (Phillips).

Liver and pancreas fat data were gathered by using the Newcastle modification of the 3-point Dixon MR scanning method (Lim et al., 2011a). 3 gradient echo scans were taken with adjacent out-of-phase, in-phase, and out-of-phase echoes. 3 Tesla fat-water difference for human studies is 435 Hz. Echo times of 3.45 ms for out-of-phase, 4.60 ms for in-phase and 5.75ms for out-of-phase were used. 6 slices were acquired to cover liver with thickness of 10 mm and pancreas with thickness 5 mm. To get these images the breath hold time of less than 17 seconds was applied.

The MATLAB software (Mathworks, Cambridge, UK) was used in the measurement of the liver and pancreas fat concentration. The MR data were uploaded to a custom MATLAB script. The special algorithm was used to produce separate fat and water images (Schneider and Glover, 1991). The fat content was expressed as a percentage of the original signal collected from both water and fat signals. Therefore, the fat content is the fat percentage in the visible MR signal. The Image J 1.43 software was used afterward to define the Region-of-Interest (ROI) by utilising the Image J polygon tool (Schneider et al., 2012).



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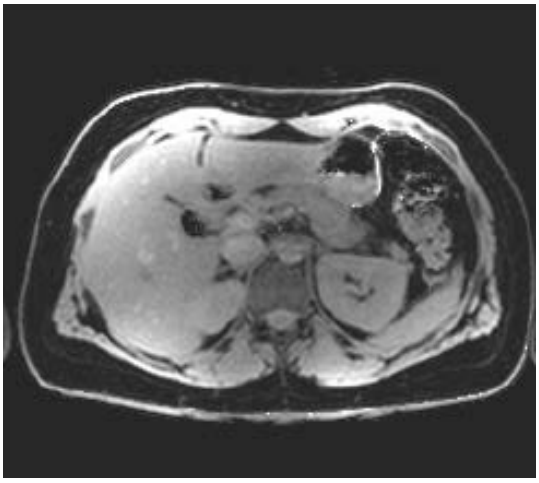

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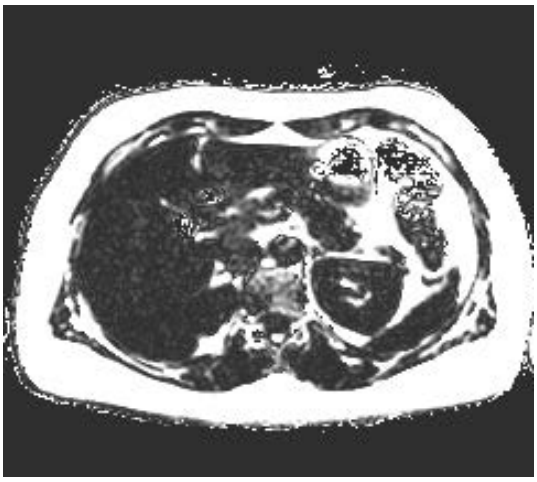

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Figure 2.2 3-point Dixon technique for measurement of liver and pancreas fat content (A. Fat image, B. Water image, C. Fat percentage image)**.**

The anatomical positions of the liver and pancreas were first established 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 .

The so-called SENSE factor or a parallel imaging 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 was calculated using ImageJ software. The investigator defined regions of interest (ROI) (Schneider et al., 2012). 5 ROIs for the liver and 2 ROIs for the pancreas were acquired and averaged. ROIs were defined to reflect as closely as possible the same anatomical area across each consecutive scan for every individual at different time point of the study. ROI in the liver was carefully selected to avoid contamination from blood vessels, gallbladder, falciform ligament and visceral fat. The liver fat analysis was performed by me and by my predecessor Dr Carl Peters. 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 for the pancreas fat was performed by a single trained investigator Dr Ahmad Al-Mrabeh. Pancreas image analysis was performed blind to both participant identity and visit number. Validation of the 3-point Dixon technique for measuring liver and pancreas fat content by this method has been verified previously. The inter-scan Bland-Altman repeatability coefficients were 0.5 % for the liver and 0.9 % for the pancreas (Lim et al., 2011a).

2.7 Very Low Density Lipoprotein - Triglyceride production rates

Very Low Density Lipoprotein- Triglyceride (VLDL1-TG) production rates were measured by plasma accumulation during inhibition of clearance which is a non-isotope competitive blocking method (Al-Shayji et al., 2007). An Intralipid (a chylomicron-like triglyceride) infusion was used to saturate lipoprotein lipase. This was allowing the accumulation of endogenous VLDL in plasma and enabling the calculation of production rate and plasma pool size of VLDL1 - triglycerides.

Subjects attended on a separate day from the MR studies after an overnight fast from 10 pm the day before the test. 2 cannulas (whether 18G or 20G depending on the subject's vein access) were inserted into the antecubital fossa veins of each arm and 3-way taps were attached. One cannula was used for blood drawing and the other one for the infusion of Intralipid (manufactured by Fresenius Kabi Ltd (Runcorn,UK)). Intralipid is a purified soybean oil emulsion which is a triglyceride rich substance.

Calculations:

Bolus Dose (0.1 g/kg of 20 % Intralipid)

0.1 g x body mass (kg) x 100 ml = Intralipid volume (ml)

~~Kg~~ 20 g

IV Infusion Rate (0.1 g/kg/hr of 10% Intralipid)

Dose (g/kg/hr) x body mass (kg) = Flow rate (ml/hr)

Concentration (g/ml)

➔ **0.1 (g/kg/hr) x body mass (kg) = Flow rate (ml/hr)**

0.1 (g/ml)

At 10 minutes prior to the start of Intralipid, one 9 ml EDTA sample was taken and stored at 4 degrees C. The calculated bolus of 20 % Intralipid (0.1 g/kg body mass) was injected through one cannula within 60 seconds. Immediately the continuous infusion of Intralipid was commenced by turning the stopcock to open the port of the infusion line and starting the infusion of 0.1 g/kg per hour of 10 % Intralipid.

The infusion was continued for 75 minutes. Further blood samples (collected in labelled 9ml EDTA vacutainers) were taken at intervals during the infusion (15, 30, 45, 60, 75 min). At the end of the test all study subjects were offered an appropriate snack/meal prior to leaving. In the first instance samples had undergone the plasma separation by centrifugation on Sanyo MSE Harrier 18/80 refrigerated centrifuge at 3000rpm, 4 degrees C, for 10 minutes to separate plasma from red cells.

Then 4 ml of plasma had been obtained at each time point of the study and gently covered (drop by drop) on the top with 4 ml Sodium Chloride solution with the density of 1.006 g/ml by using the Easy pump. That was followed by a further 30 minutes centrifugation at 10000 rpm at 15 degrees Celsius with the brake of 5 in the end to allow the separation of chylomicrons from plasma. 2ml of chylomicron-free plasma was obtained and frozen at minus 40 degrees Celsius.

The frozen samples were later thawed and ultracentrifugated on Beckman L7 Ultracentrifuge at 39000 rpm, 23C, for 82 minutes. Ultracentrifugation of the plasma samples allows quantification of VLDL1 - TG, and here is the accumulation of triglycerides can be seen after centrifugation starting form baseline and increasing

through the test as the milky colour ring on the top of the test tube which becomes more prominent towards the end of the test (see Picture 2.3).

Figure 2.3 VLDL1 - TG appears as a milky colour ring top layer on the top of the test tube.

Calculations

Production rates (mg/h) = slope (mmol/min) x 87.6 x plasma volume (dl) x 60

Production rates (mg/kg/day) = (production rates (mg/h) x24) / (weight (kg))

Production rates (mg/kg/day) = (slope (mmol/min) x 87.6 x plasma volume (dl)x60 x 24) / (weight (kg))

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

VLDL1 - TG (pools/day) was calculated from the gradient of the linear increase in their concentrations (mg/dl) over time (min) divided by fasting concentrations mg/dl) and then multiplied by 60 min and 24 h.

Pools/day = (slope (mg/dl)) / (fasting TG concentration (mg/dl)) x60x 24 or

Pools/day = (slope (mmol/min) x 87.6) / (fasting TG (mmol/l) x plasma vol (dl) x 87.6) x 60 x 24

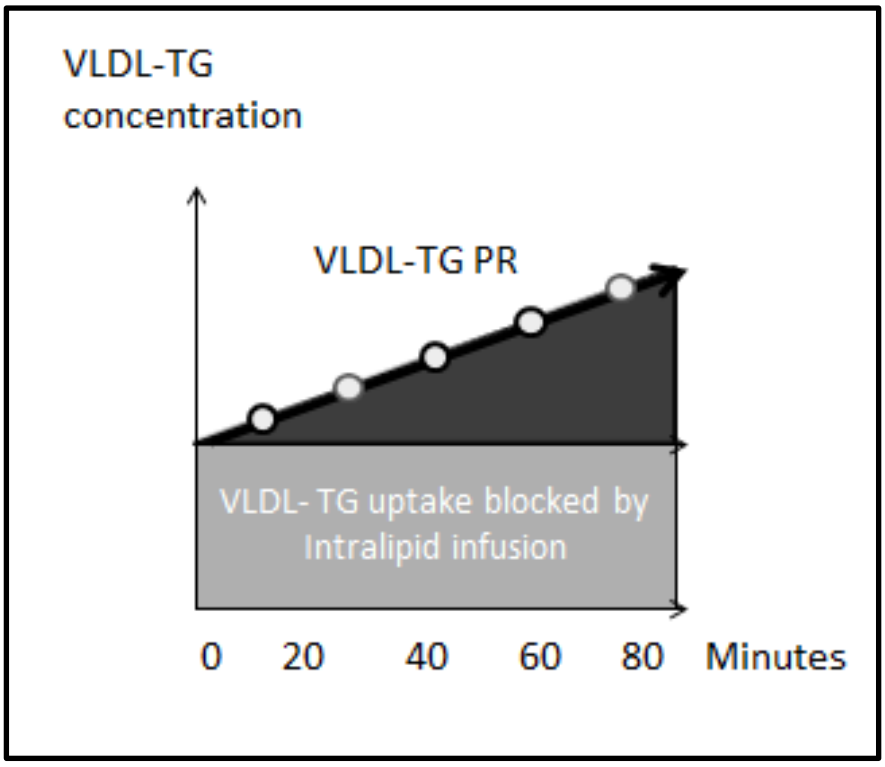

Figure 2.4 Schematic representation of VLDL - TG production.

2.8 Stepped Insulin Secretion Test with Arginine

2.8.1 Method description

Beta cell function was assessed using the stepped insulin secretion test with arginine (Lim et al., 2011a, Toschi et al., 2002).

The test was performed on participants in the fasting state immediately after the MR studies. Only still water was permitted to maintain hydration.

One large bore (18G) cannulas were inserted into each arm. To make venous blood richer in oxygen and closer to arterial the heat packs warmed up in the microwave were applied on each arm during the duration of the test.

20 % Glucose was infused to achieve two square wave step increase in plasma glucose level by 2.8 mmol/l during the first 30 min (first phase of insulin secretion) and 5.6 mmol/l above fasting blood glucose for the rest of the test.

10 minutes prior the start of the test the samples for fasting blood glucose, insulin and c-peptide were obtained. Immediately prior to the first bolus of 20 % dextrose another fasting blood sample was taken for blood glucose, insulin, and c-peptide. The first bolus was delivered intravenously at time 0 during approximately 20 seconds. During the rest of the test blood samples for insulin and c-peptide were obtained at 2-5 minutes intervals. At 30 minutes a second bolus of 20 % dextrose was given within 20 seconds to increase blood glucose by 5.6 mmol/l from fasting level and to induce the second phase of insulin response. That was followed by intravenous 5 g arginine stimulation at 60 minutes of the glucose infusion. Arginine was delivered over 30 seconds.

Bolus calculation

Desired glucose increment = 2.8 mmol/L

~= 2.8 x ~18 (mg/dL)

~= 2.8 x ~18 / 100 (mg/mL)

Volume to be incremented (ie. The glucose pool) is assumed to be 150 mL per kg

Volume to be incremented (mL) = 150 mL/kg x body weight (kg)

Glucose required (mg) = desired glucose increment (mg/mL) x volume to be incremented (mL)= 2.8 x ~18 / 100 mg/mL x 150mL/kg x body weight(kg)

20% dextrose strength = 200g/L of glucose = 200mg/mL

= 0.378 x bodyweight (kg)

Infusion rates:

20 % dextrose strength = 200 g/L of glucose = 200 mg/mL

Infusion rate (ml/min) = infusion rate (ml/hr) / 60 (min/hr)

Glucose infusion rate (mg/kg/min) = infusion strength (mg/mL) x infusion rate (ml/min) / body weight (kg)

Therefore, 1mg/kg/min infusion rate will be:

1mg/kg/min = 200 (mg/mL) x infusion rate (ml/min) / body weight (kg)

infusion rate (ml/min) = 1mg/kg/min x body weight (kg) / 200 (mg/mL)

= bodyweight / 200

infusion rate (ml/hr) = infusion rate (ml/min) x 60

= bodyweight x 60 / 200

= bodyweight x 0.3

2.8.2 Estimation of the insulin secretion rates by a deconvolution method

C-peptide is excreted in equimolar amounts, and it is not a subject to significant first pass hepatic extraction. C-peptide has also relatively constant kinetics.

Insulin secretion rates were estimated by a deconvolution from c-peptide concentration. This method was established by Van Cauter et al (Van Cauter et al., 1992). It is based on c-peptide kinetics and the mathematical modelling technique called deconvolution. It was implemented to calculate insulin secretion rates. This test allows to observe the rapid changes in insulin secretion namely in the first phase insulin response and total secretory capacity.

ISEC computer program (Hovorka et al., 1996) was used which applies a regularisation method of deconvolution described by Twomey. S. back in 1965 giving an output of insulin secretion rate in nmol/min/m_2 of body surface area. Participants' gender, age, diabetes status, height, weight, body surface area, BMI, and C-peptide concentrations into ISEC computer model.

The estimation of the insulin secretion rates was performed for us by Dr Benjamin Aribisala, Professor in Mathematics from Lagos State university, Nigeria.

2.9 Indirect calorimetry

2.9.1 Introduction

Indirect calorimetry was carried out to measure the rates of the whole-body lipid oxidation as part of the assessment of lipid dynamics. Whole body glucose oxidation was also measured (Frayn, 1983). The Quark indirect calorimeter exhibits excellent reproducibility (coefficient of variability 3.8 % from our study).

The whole body O2 uptake and the release of CO2 was measured by a Quark RMR indirect calorimeter (COSMED, Rome, Italy). Prior to each test the calibration was performed to assure that the system is acquiring the reliable measurements.

The 3-liter calibration syringe was used to preform flow/volume calibration. The syringe was connected to the flowmeter (a digital turbine). Flows and volumes are measured by the bidirectional digital turbine that offers very low resistance to flow. Air passing through the helical conveyors causes the spiral rotation of the turbine rotor. Quark RMR software was used to calibrate the flowmeter.

The gas analyser calibration was performed by Quark ERGO-RMR software which allows to automatically calibrate the zero, gain and delay of the gas sensors. Prior to calibration the warm-up period of 5 minutes was observed. The gas cylinder pressure was regulated to 5 bar as per the manual. The gas sampling line was disconnected from the reader at the back of the calorimeter and connected to the front panel of the Quark. During the calibration a graph with O2 and CO2 concentrations was displayed. The software gives the outcome display in the acceptable range. When both calibrations were satisfactory only than the indirect calorimetry test was attempted.

The bubble-hood canopy was connected to the wrinkled tube after inserting the disposable anti-bacterial filter. After entering the patient details (weight, height, age) into the Quark RMR software the bubble-hood with the canopy attached was put over the participant's head and shoulders to ensure that there are no leaks under the canopy from the outside. Patients were having the test while being fasted from 10 pm the night before. Measurements were conducted after 30 minutes of supine rest and the acquisition period will not commence until steady state has been achieved. Indirect calorimetry was measured for 20-minute time. The test for Resting Metabolic rate was performed. Participants were positioned on the bed, not conversating and listening to music. The initial 5-minute measurement was not counted and only last 15 minutes were analysed.

2.9.2 The principles of indirect calorimetry

Indirect calorimetry measures gas exchange (whole body O2 uptake and CO2 release).

The oxygen consumption (VO2 in L min^{-1}), carbon dioxide production (VCO2 in L min^{-1}), and respiratory quotient (RQ) are calculated by Quark RMR software every 10 seconds from these gas concentrations and constant air flow (Q). The flow rate (Q) is taken from the software. The expired CO2 (FeCO2) and the inspired CO2 (FiCO2) is used to calculate VCO2 (CO2 production):

$VCO2 = Q \times (FeCO2 - FiCO2)$ or simplified to

$VCO2 = Q \times (FeCO2)$

The respiratory quotient (RQ) can be calculated from the gas fractions alone. This takes into account the Haldane transformation (McLean, 1987) which states the relationship between the inspiratory and expiratory volume.

$$RQ = \frac{VCO2}{VO2} = \frac{1 - FiO2}{\frac{FiO2 - FiO2}{\frac{}{FeO2} - FiO2}}$$

When V02 and RQ were calculated, VO2 (the oxygen consumption) could be obtained from RQ.

$VO2 = VCO2 / RQ$

2.9.3 Substrate oxidation

The information about the type and rate of fuel oxidation can be obtained from the measurements of oxygen consumption (VO2) and carbon dioxide production (VCO2) (Frayn, 1983). The respiratory quotient (RQ) is 1.00 for glucose, 0.70 for lipids, and 0.80 for proteins.

When lipid is oxidised (typical fat palmitoyl – stearoyl – oleoyl - glycerol or PSOG) according to the equation:

PSOG ($C_{55}H_{104}O_6$) + 78 O_2 → 55 CO_2 + 52 H_2O

Glucose oxidation is given by the equation below where 6 mol of oxygen is consumed and 6 mol of carbon dioxide is produced for each mol of glucose.

Glucose ($C_6H_{12}O_6$) + 6 O_2 → 6 CO_2 + 6 H_2O

1 mol of gas occupies 22.4 litres therefore the above formulas can be converted into:

1 g of Glucose (G) + 0.746 L O_2 → 0.746 L CO_2 + 0.6 g H_2O

1 g of Lipid (L) + 2.029 L O_2 → 1.43 L CO_2 + 1.09 g H_2O

The protein oxidation varies within a narrow range according to the amino-acid composition of a given protein. The amount of protein being oxidised may be estimated from the urinary nitrogen excretion.

1 g protein (P) = 0.966 L O_2 → 0.782 L CO_2 + 0.45 g H_2O

Nitrogen is 16 % of the protein weight therefore 1g of urinary nitrogen is assumed to come from 6.25g of protein.

1 g Nitrogen (N) + 6.04 L O_2 → 4.89 L CO_2 + 2.81 g H_20

Therefore, for oxidation of 1 g of glucose and 1 g of Lipid and excreting N grams of urinary nitrogen per minute the total oxygen consumption and the total carbon dioxide production are:

$V02\ (L\ min^{-1}) = 0.746\ G + 2.03\ L + 6.04\ N$

$VCO2\ (L\ min^{-1}) = 0.746\ G = 1.43\ L + 4.89\ N$

The calculation of the substrate oxidation rates is based exclusively on the measurements of the gas exchange and urinary nitrogen excretion.

$G = 4.55\ VCO2 - 3.21\ VO2 - 2.87\ N$

$L = 1.67\ VO2 – 1.67\ VCO2 – 1.92\ N$

The rates of the glucose and lipid oxidation can also be calculated this way.

Quark RMR software gives the output of the calculated estimate of RQ, the Resting Metabolic Rate (RMR) in Kcal/day, and the average amount of fat and carbohydrates oxidised in Kcal/day.

Frayn postulates that oxidation of 1 g of fat liberates 39.4 KJ, oxidation of 1 g of glucose liberates 15.6 kJ, and 1 g of protein – 20.1 kJ. Keeping these figures in mind it is possible to do an indirect calculation of the substrate oxidation from an average number of kcal/day for each of them calculated by the Quark RMR software.

Fat oxidation rate

Average Fat (kcal/day) x 4.184 (conversion coefficient) = Average Fat (KJ/ day)

Fat (g/day) = Average fat (kJ/day) / 39.4

Fat oxidation rate (mg/kg/min) = Fat (g/day) / body weight/ 1440 x 1000

Glucose oxidation rate

Average Carbohydrate (CHO) (kcal/day) x 4.184 = Average CHO (KJ/day)

Carbohydrate (g/day) = Average CHO (KJ/day) / 15.6

CHO (mg/kg/min) = CHO (g/day) / body weight/ 1440 x 1000

Chapter 3. Clinical outcomes during and after weight loss in Tyneside subjects as a part of the DiRECT study

3.1 Introduction

Type 2 diabetes now affects 4.2 million people in UK and at least one in ten US adults (Whicher et al., 2020, Menke et al., 2015). It has long been considered as a steadily progressive, lifelong condition. However, the present work has demonstrated that nearly half of those with early (< 6 years) type 2 diabetes can be returned to long term non-diabetic glucose control using an effective method to achieve and maintain substantial weight loss (Lean et al., 2018). The initial period of weight loss was followed by weight maintenance and the data were reported at 5, 12, and 24 months. This population-based study built upon the results of earlier small studies which revealed the detailed physiological basis of the transition from type 2 diabetes to normal (Lim et al., 2011a, Petersen et al., 2005, Steven et al., 2016a, Steven et al., 2016d). However, whether these mechanisms operate in all individuals with type 2 diabetes and the critical factor(s) that determine the capacity to return to non-diabetic glucose metabolism remain uncertain. During a very low calorie diet in type 2 diabetes an initial study showed that liver fat content rapidly decreased, with normalisation of hepatic insulin sensitivity within 7 days (Lim et al., 2011a). Over an 8-week period, pancreas fat content decreased more slowly, as first phase insulin response gradually returned. A follow up study demonstrated that as duration of type 2 diabetes increased beyond 10 years, the possibility of restoring beta cell function decreased (Steven et al., 2016a). These observations led to a Twin Cycle hypothesis of the aetiology of type 2 diabetes (Taylor, 2013), consistent with earlier observations (Henry et al., 1986, Wing et al., 2016), in that linked but distinct mechanisms in liver and pancreas appeared to explain the condition. Recently, the molecular basis of the liver abnormalities in type 2 diabetes has been clarified (Perry et al., 2018). In addition, a major decline in beta cell function is necessary before type 2 diabetes develops. In the last few years, metabolic stress-induced beta cell de-differentiation and subsequent redifferentiation with significant weight loss have been demonstrated, potentially 4 explaining any return from type 2 diabetes to normal glucose tolerance (Cinti et al., 2016, Pinnick et al., 2010, Talchai et al., 2012, White et al., 2016a). A larger study was required to determine the extent to which this explains common type 2 diabetes. Detailed pathophysiologic studies were

carried out in a geographically pre-defined sub-group of DiRECT participants. These were designed to test the hypothesis that there would be differences between those who did or did not return to non-diabetic glucose control in some or all factors previously identified as underlying type 2 diabetes.

The irreversible nature of type 2 diabetes has been established in UK Prospective Diabetes Study which demonstrated that glucose control has progressively deteriorated to the point of a requirement for insulin treatment despite the best possible therapy (1995b).

Current guidelines for type 2 diabetes care concentrate on sequential introduction of antidiabetic medication followed by insulin to reduce blood glucose. Sufficient weight loss for remission, of over 10-15 kg, can be achieved in various ways, including bariatric surgery but also using a low-calorie formula for total diet replacement.

Some weight loss studies, that achieved at least 10 kg, demonstrated return to normal blood glucose control with short duration of type 2 diabetes but no previous dietary intervention trial managed to sustain type 2 diabetes in remission for at least one year (Henry et al., 1985, Sjostrom et al., 2014, Schauer et al., 2003).
The DiRECT study was set up to test the hypothesis that diabetes remission by weight loss would be achievable in routine clinical practice in the Primary Care setting. The further hypothesis to be tested was that effective weight management and avoidance of weight regain could produce sustained remission of type 2 diabetes. Confirmation of these hypotheses could have important implications for future management of type 2 diabetes in day-to-day clinical practice.

A low-calorie formula diet was used, followed by stepped food reintroduction and then long-term structured support for weight loss maintenance (Leslie et al., 2016). The primary outcomes were the achievement of ≥ 15kg weight loss and remission of type 2 diabetes at 12 months (Leslie et al., 2016).

A specific question was whether remissions of type 2 diabetes could be durable up to 24 months follow up. For this, the key issue was whether long term maintenance of weight

loss and remissions of diabetes could be achieved in Primary Care after rapid weight loss. In the past liquid formula diets were commonly regarded as only effective in the very short-term (Lean and Hankey, 2018).

3.2 Study design

3.2.1 Participants

General Practices in the Tyneside region of England were invited to participate by Northeast Commissioning Support (NECS) in Tyneside. Agreeable practices were randomised to Intervention or Control arms. Randomisation was conducted by the Robertson Centre for Biostatistics, University of Glasgow using a minimisation method to maintain balance for sex and practice list size (> 5700 or $\leq$ 5700) across intervention groups within each study region.

An invitation pack was sent by GP practice staff in Tyneside to eligible participants and they were asked to respond via post using a pre-paid envelope. Participants of the Control arm of the study were offered and incentive of a £50 Amazon voucher. At an initial appointment, the participants have signed the informed consent after detailed discussion.

Intervention participants were put on a Counterweight-Plus weight management program with a goal to reach and maintain ≥ 15 kg weight loss at 12 months which was a first co-primary DiRECT study outcome. Weight loss was achieved with a Total Diet Replacement phase using a low-calorie (825- 853 kcal/day) formula diet for 3 to 5 months depending on participant circumstances and individual response. This was followed by a food reintroduction during 4 to 6 weeks and an ongoing structured program with monthly visits for long term weight loss maintenance. All oral anti-diabetes and antihypertensive drugs were discontinued on Day 1 of the diet start.

Regular monitoring of blood glucose and blood pressure was performed at the practices and standard protocols for drug reintroduction under national clinical guidelines were followed if indicated. Participants were told to keep their usual physical activities during the diet. Intervention and Control participants were seen for data collection at baseline, 5 months (Tyneside subgroup only), 12 months, and 24 months. Second co-primary outcome was a remission of type 2 diabetes defined as HbA1c < 48 mmol/mol whilst being off all anti-diabetic medication for at least 2 months.

3.2.2 Statistics

The main outcomes of the DiRECT study from both Scotland and Tyneside centres study were analysed by the Glasgow Centre for Biostatistics given the formal randomised clinical trial and need for professional statistics advice. A separate post hoc analysis of the Tyneside subgroup parameters and metabolic results was carried out by me and Dr Ahmad Al-Mrabeh. Analyses were conducted on all subjects with paired data both before and after weight loss and weight maintenance phases. Data are presented as mean ± SEM for normally distributed data and median [IQR] for nonparametric data. Student paired or two-sample t test was used as appropriate for parametric data and Mann Whitney U test for nonparametric data.

For those participants who did not attend follow up study assessments, and data could not be obtained from General Practice records, the assumption was made that the primary outcomes were not met. No assumptions were made regarding missing data for the main analysis of secondary outcomes.

Sample size calculations indicated that recruitment of 280 participants from both Scotland and Tyneside sites would be required to achieve 80 % power. These calculations assumed: diabetes remission in 22 % of Intervention participants at one year compared with an estimated 5 % in the Control arm; 10 participants per practice (fixed); an intraclass correlation coefficient of 0.05 to account for cluster randomization; an estimated 25 % individual participant drop-out within 12 months.

3.3 Clinical outcomes during first 12 months of study

DiRECT study recruited 306 participants in Intervention and Control practices on both sites. Out of the total number 71 Intervention participants and 24 Control participants were recruited in the Tyneside area. One participant withdrew consent and permission to use his data. Subsequently 7 subjects were found to be ineligible after baseline HbA1C re-check (baseline HbA1c< 48mmol/mol without anti-diabetes medication). Results were presented for 298 participants, 149 Intervention and 149 Control (Figure 3.1). 64 Intervention and 24 Control participants in the Tyneside subgroup had at baseline no important differences in the distributions of data (Table 3.1).

128/149 (85.9 %) Intervention and 147/149 (98.7 %) were left at 12 months with mean loss to follow up of 23 (7.7 %). The data for the first primary outcome of weight loss $\geq$ 15 kg was available for 285 participants (137 Intervention, 148 Control), and for the second primary outcome (diabetes remission) for 290 participants (142 Intervention, 148 Control). For ITT analysis, the remaining participants with missing data were assumed to have not met each primary outcome (Figure 3.1).

Within the Intervention group, 6 participants (4.0 %) consented but thereafter never engaged with the intervention, and 26 (17.4 %) withdrew from treatment during the first 12 months (15 during the total diet replacement phase, 6 during the food reintroduction phase, 5 during the weight loss maintenance phase).

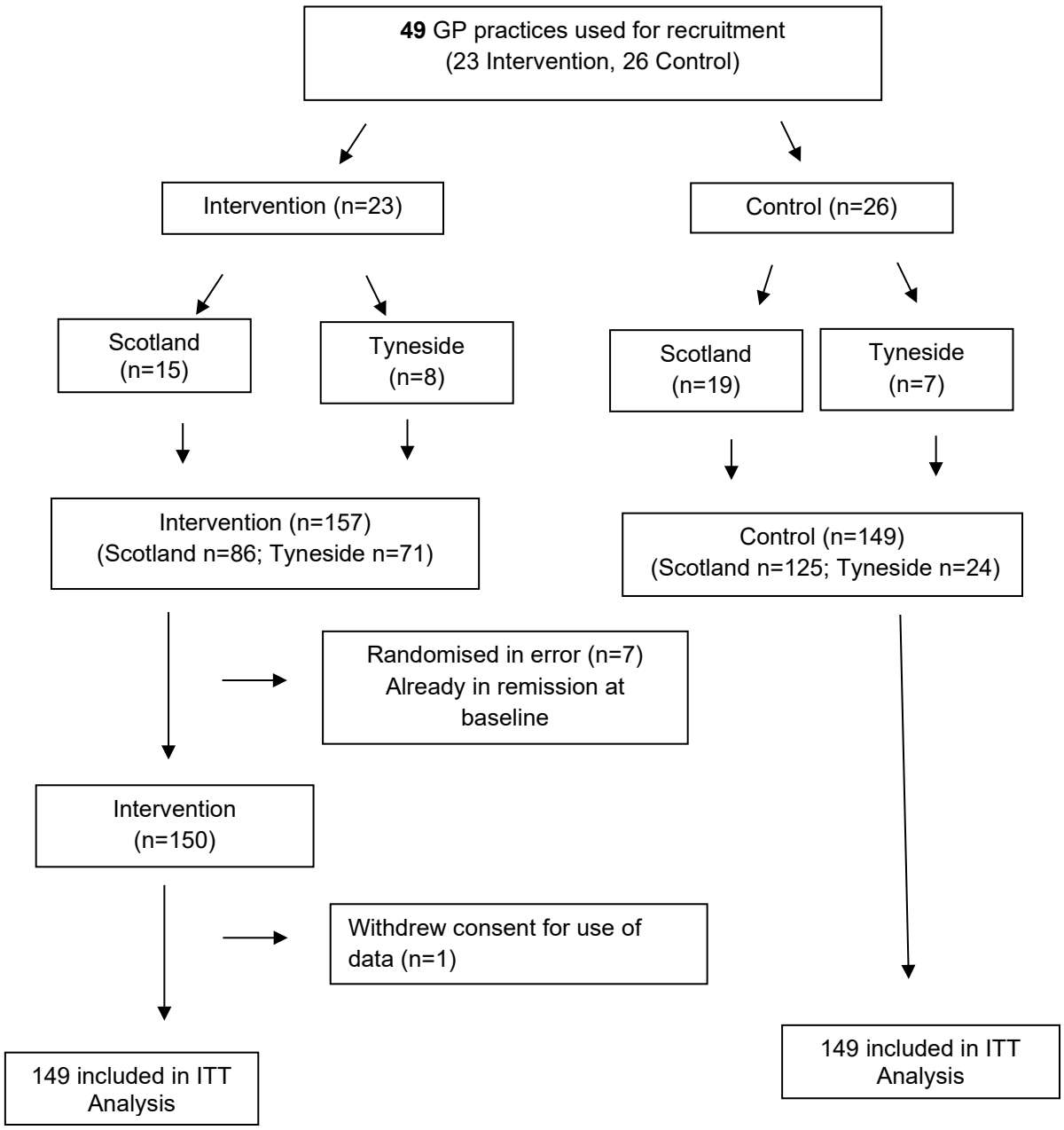

Figure 3.1 DiRECT Trial Profile.

	Control n=24	Intervention n=64
Sex (Male)	16 (66.7)	34 (53.1)
Age (years)	54.6 (7.7)	52.3 (8.0)
Weight (kg)	97.3 (11.8)	101.0 (17.8)
BMI (kg/m^2)	33.5 (3.5)	35.1 (4.5)
Systolic BP (mmHg)	137.2 (16.0)	132.7 (17.5)
Diastolic BP (mmHg)	85.5 (8.8)	84.6 (10.2)
Years since diabetes diagnosis	3.0 (1.9)	3.0 (1.7)
HbA1c (mmol/mol)	58 (11.9)	60 (11.0)
Fasting Glucose (mmol/l)	8.5 (2.2)	8.8 (2.8)
Number of oral anti-diabetic medications		
0	7 (29.2)	24 (37.5)
1	11 (45.8)	20 (31.25)
2+	6 (25.0)	20 (31.25)
Total Cholesterol (mmol/l)	3.80 (0.97)	4.25 (1.10)
HDL Cholesterol (mmol/l)	1.14 (0.39)	1.04 (0.25)
Triglycerides (mmol/l)	1.43 (0.86)	1.87 (0.83)

Table 3.1 Tyneside Baseline characteristics by randomised group. Data are mean (SD) or N (%).

3.3.1 Primary outcomes at 12 months

In the Tyneside subgroup of the study the weight loss ≥ 15 kg at 5 months was recorded for 29 out of 64 (45.3 %) subjects in the Intervention group, and by none in the Control group. Diabetes remission was achieved by 40 out of 64 (62.5 %) of Tyneside Intervention participants immediately after weight loss at 5 months and only by 1 out of 24 (4.2 %) of Controls.

In the Tyneside subgroup of the study the weight loss ≥ 15 kg at 12 months was recorded for 13 out of 64 (20.3 %) subjects in the Intervention group, and by none in the Control group. Type 2 diabetes remission was achieved by 29 out of 64 (45.3 %) of Tyneside Intervention participants and by 1 out of 24 (4.2 %) of Control participants at 12 months.

Mean HbA1c reduced by 9.6 mmol/mol in the whole DiRECT Intervention group while there was a rise of 1.4 mmol/mol in Control group with an adjusted difference of -9.3 (95% CI: -12.1, -6.5) mmol/mol ($p<0.0001$).

Remission of type 2 diabetes was achieved by none out of total DiRECT cohort who did not lose weight at 12 months. The type 2 diabetes was put into remission in 6/89 (7 %) of those who maintained 0-5 kg weight loss, 19/56 (34 %) with 5-10 kg loss, 16/28 (57 %) with 10-15 kg loss, and 31/36 (86 %) of those who lost $\geq$ 15 kg at 12 months (Figure 3.2.c). Mean weight decreased from 100.4 kg to 90.4 kg in the whole DiRECT Intervention group and from 98.7 kg to 97.7 kg in Controls with an adjusted difference between the groups at 12 months of -8.8 kg ((95% CI: -10.3, -7.3), $p<0.0001$). If total dietary replacement phase was completed, subjects showed more weight loss in comparison to those who did not manage to finish it. Similarly, if food reintroduction phase was completed by the participants, they showed less weight gain, compared with non-completers (Figure 3.3).

In the whole Intervention cohort of DiRECT the weight loss ≥ 15 kg at 12 months was achieved by 24.2 % of subjects, and by nobody in the Control group (Fisher's Exact test $p<0.0001$) (Figure 3.2.a). Diabetes remission was achieved by 45.6 % of Intervention participants and only by 6 out of 149 (4.0 %) of Control subjects (Figure 3.2.b).

In the Tyneside subgroup mean weight reduced from 101.1±17.8 kg at baseline (n=64) to 85.8±15.3 kg at 5 months (n=58) and to 88.7±16.5 kg at 12 months (n=48) ($p<0.0001$ for both). In Control Tyneside subgroup (n=24 at baseline) weight has not significantly changed (from 97.3±11.8 kg to 95.8±12.8 kg at 5 months (n=23) and 95.2±14.4 kg at 12 months (n=20).

Baseline clinical and metabolic features of the Tyneside subgroup (Responders with Hba1c<48 mmol/mol post intervention vs. Non-responders) are shown in the Table 3.2. Responders and Non-responders did not differ in age, gender, and weight (Table 3.2). Responders had a lower fasting plasma glucose than Non-responders (8.3±0.4 mmol/l vs. 9.3±0.7 mmol/l, $p=0.18$), and HbA1c was 7.4±0.2 % vs. 7.9±0.2 % respectively

(p=0.04). Baseline liver fat in the whole Intervention group was 16.0±1.3 % and was not different between Responders and Non-responders (16.7±1.5 vs. 14.5±2.6 % respectively; p=0.47). There was also no difference in VLDL1-TG production between Responders and Non-responders (560.7±30.9 mg/kg/day vs. 581.1±52.1 mg/kg/day, p=0.74) or total plasma triglyceride production (1.84±0.13 mmol/l vs. 1.91±0.25 mmol/l; p=0.76). Non-responders had longer duration of diabetes versus Responders (2.7±0.3 years vs. 3.8±0.4 years; p=0.02). They also had lower fasting plasma insulin (108.3±10.0 pmol/l vs. 77.2±8.5 pmol/l; p=0.02) and lower plasma ALT (34.1±2.8 pmol/l vs. 26.3±2.6 pmol/l; p<0.05).

Metabolic changes in Tyneside Responders and Non-responders summarised in the Table 3.3. During the weight loss phase, weight decreased in Responders (100.6±2.6 kg to 84.4±2.1 kg; p<0.0001, n=40) and Non-responders (102.1±4.4 kg to 88.7±4.4 kg; p<0.0001, n=18). The change during the weight loss phase did not differ significantly between those two groups (-16.2 ±1.2 kg vs. -13.4 ±1.4 kg; p=0.14). Weight in Responders increased by 3.3±0.8 kg to 86.2±3.0 kg (p<0.0001). In Non-responders increased by 4.9±0.8 kg to 92.5±4.6 kg but stayed lower at 12 months than baseline (p<0.0001). At 12 months the overall change was greater in the Responders (-14.1±1.5 kg vs. -9.4±1.3 kg; p=0.02).

Fasting plasma glucose went down in Responders from 8.3±0.4 mmol/l to 5.7±0.1 mmol/l (p<0.0001) with no significant change in Non-responders (9.3 ±0.7 mmol/l to 8.8 ±0.6 mmol/l; p=0.47). At 12 months there was no significant change in plasma glucose in both groups (Responders with 5.7±0.1 mmol/l and Non-responders with 8.4±0.4 mmol/l). HbA1c decreased in Responders to 41±1.1 mmol/mol (p<0.0001) but not in Non-responders (63.9±4.4 mmol/mol, p=0.67) post weight loss. At 12 months, HbA1c did not change further in either group so that differences were maintained (39.9±1.1 mmol/mol vs. 59.6±2.1 mmol/mol, p<0.0001).

	Responders			Non-responders		
	Baseline (n=40)	Post-weight loss (n=40)	12 months (n=29)	Baseline (n=18)	Post-weight loss (n=18)	12 months (n=16)
BMI (kg/m²)	34.9±0.7	29.4±0.6***	29.6±0.8***###	35.7±1.2	31.1±1.3***	32.4±1.4***###
Age (Year)	53.0±1.2	-	-	53.3±1.9	-	-
Sex (F/M)	17/23	-	-	9/9	-	-
Diabetes duration (years)	2.7±0.3	-	-	3.8±0.4†	-	-
VLDL1-TG pool(mg)	2445.9±267	1258.4±168***	1461.7±240**##	2775.4±505	1866.4±432*	2234.1±570
Fasting NEFA (mmol/l)	0.56±0.03	0.55±0.03	0.51±0.03	0.66±0.04	0.59±0.05	0.61±0.04
Alanine Aminotransferase (ALT) (U/l)	34.1±2.8	-	17.1±1.0***	26.3±2.6†	-	18.3±2.0**
Cholesterol (mmol/l)	4.3±0.2	-	4.3±0.2	4.1±0.3	-	4.0±0.2
HDL (mmol/l)	1.09±0.05	-	1.23±0.08**	0.99±0.05	-	1.11±0.06*
Ketone (mmol/l)	0.19±0.02	0.29±0.04**	0.26±0.03*	0.18±0.02	0.20±0.02	0.24±0.04
Lipid oxidation (mg/kg/min)	0.96±0.05	0.87±0.06	0.64±0.09***##	0.84±0.08	0.89±0.11	0.83±0.07
Glucose oxidation (mg/kg/min)	1.27±0.12	1.44±0.14	2.0±0.19***##	1.48±0.17	1.19±0.21	1.31±0.20†
Resting Energy Expenditure (kcal/day)	1996.5±57.0	1647.1±48.1***	1696.4±67.3***#	1981.7±108.0	1641.1±89.5**	1733.2±109.5**

*P<0.05 vs. baseline, **P<0.01 vs. baseline, ***P<0.0001 vs. baseline, # P<0.05 vs. 5 months, ## P<0.01 vs. 5 months, ### P<0.0001 vs. 5 months, † P<0.05 responders vs. non-responders, †† P<0.01 responders vs. non-responders. Paired data were presented (baseline to post-weight loss or baseline /post weight loss to 12 months).

Table 3.2

Clinical and metabolic features of Tyneside Responders and Non- responders at baseline and after intervention at 5 and 12 months.(Al-Mrabeh et al., 2019)

	Baseline to post-weight loss		Post weight loss to 12 months		Baseline to 12 months	
Δ change	Responders (n=40)	Non-responders (n=18)	Responders (n=29)	Non-responders (n=16)	Responders (n=29)	Non-responders (n=16)
Weight (kg)	-16.2±1.2	-13.4±1.4	3.3±0.8	4.9±0.8	-14.1±1.5	-9.4±1.3*
Fasting plasma glucose (mg/dl)	-46.6 ±7.3	-8.9±12.0**	1.6±2.6	-3.6±11.2	-47.9±8.7	-21.3±7.8*
HbA1c (%)	-1.5±0.2	0.2±0.4***	-0.1±0.05	-0.4±0.35	-1.6±0.2	-0.4±0.2***
Liver fat (%)	-13.4±1.4	-11.9±2.4	0.6±0.3	3.6±1.6	-13.5±1.9	-9.7±2.1
VLDL1-TG production (mg/kg/day)	-147.2 ±33.8	-59.2±52.7	43.1 ±26.7	155.8±50.8	-119.2 ±39.0	72.2±73.6*
Plasma VLDL1-TG (mmol/l)	-0.26±0.07	-0.19±0.12	0.10±0.05	0.16±0.10	-0.21±0.09	-0.04±0.08
VLDL1-TG pool (mg)	-1187.5 ± 245.9	-909.0 ± 385.4	391.5 ± 149.0	659.1 ± 364.7	-993.7 ± 303.8	-313.5 ± 301.1
Total plasma TG (mmol/l)	-0.54±0.12	-0.67±0.19	0.08±0.10	0.13±0.19	-0.58±0.18	-0.57±0.13
Fasting plasma insulin (pmol/l)	-69.7±9.3	-41.7±5.8*	7.2±3.9	10.7±5.2	-65.3±12.1	-32.7±5.7*
Pancreas fat (%)	-0.90±0.17	-0.78±0.23	-0.14±0.28	0.17±0.23	-1.31±0.28	-0.74±0.27

Table 3.3 Metabolic changes in Tyneside Responders and Non-responders during the first 12 months of follow up.(Al-Mrabeh et al., 2019)

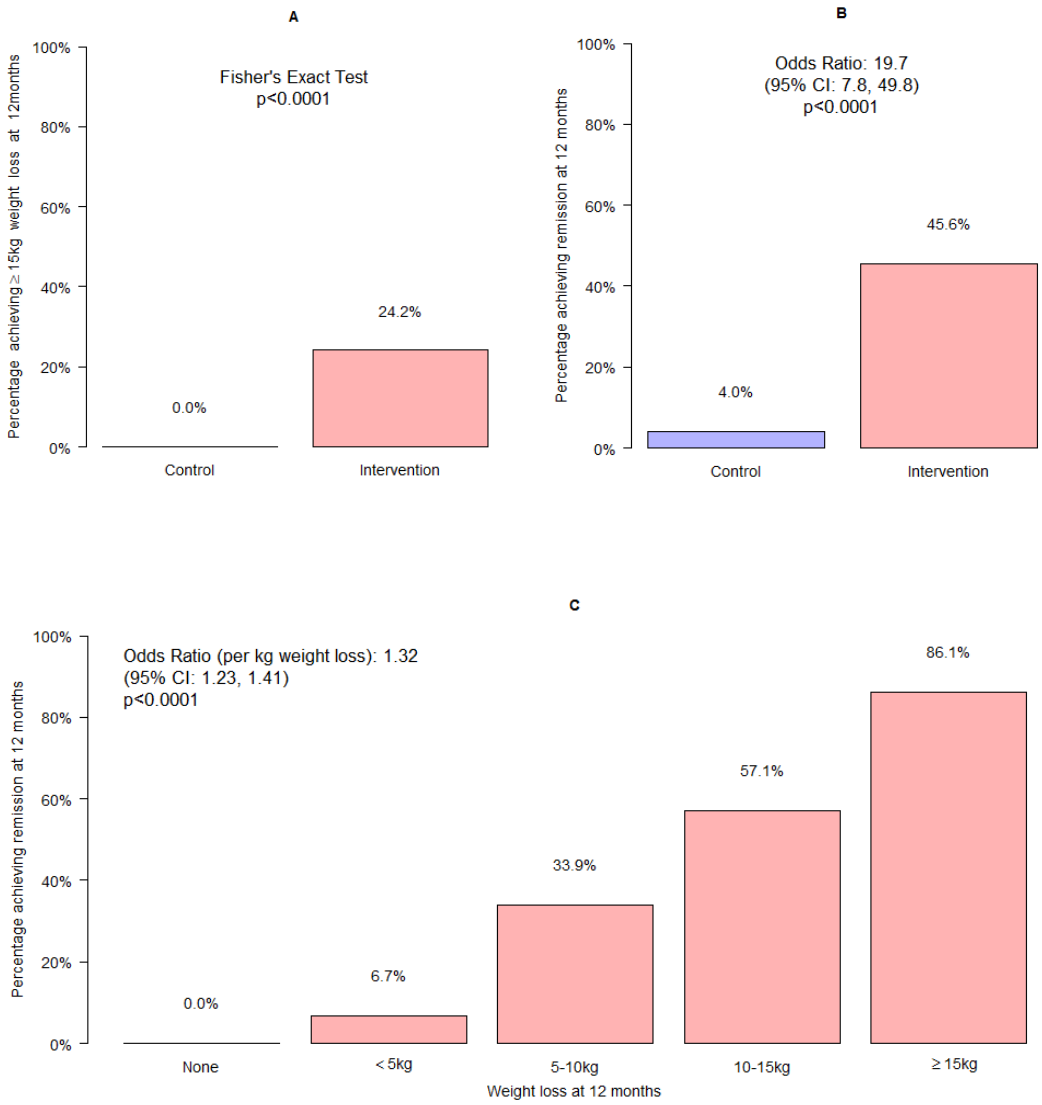

Figure 3.2 DiRECT study primary outcomes and remission of diabetes in relation to weight loss at 12 months.

A: First co-primary outcome, achievement of ≥ 15 kg weight loss at 12 months, by randomised group. **B:** Second co-primary outcome, remission of diabetes (HbA1c < 48 mmol/mol, off anti-diabetic medication for 2 months), by randomised group.
C: Remission of diabetes, in relation to weight loss achieved at 12 months (both randomised groups combined).(Lean et al., 2018)

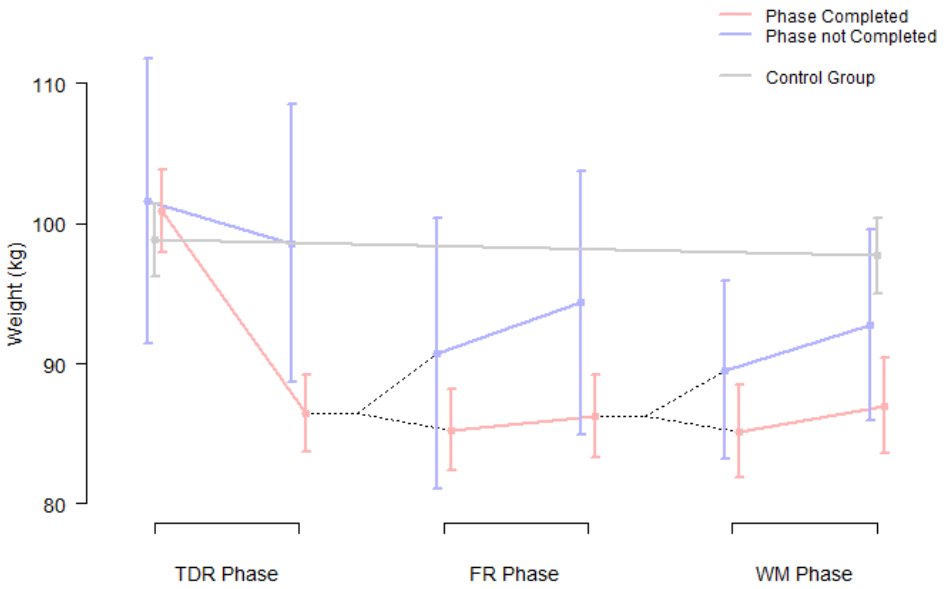

Figure 3.3 Changes in weight during each treatment phase over 12 months.

Data during TDR phase reported for all participants who started TDR; data during FR phase reported for all participants who successfully completed TDR; data during WM phase reported for all participants who successfully completed FR (plus one patient who progressed directly from TDR to WLM). Grey line shows weight changes over 12 months in the Control group, for reference.(Lean et al., 2018)

3.3.2 Secondary outcomes at 12 months

Medication: The proportions taking none, one, two or more anti-diabetes medications were similar in each group at baseline (Table 3.1). In the whole DiRECT Intervention group at 12 months, 109 participants (73.6 %) were taking no anti-diabetes medication with mean HbA1c at 46.8 mmol/mol (6.4 %), compared with 27 participants (18.2 %) in the Control group with mean HbA1c at 54.6 mmol/mol (7.2 %) (p=0.0032)(Lean et al., 2018).

Quality of life: The EQ-5D visual analogue scale improved by 7.2±21.3 points in the Intervention group and decreased by 2.9±15.5 points in the Control group while the adjusted difference between Interventions and Controls at 12 months was 6.4 points ((95 % CI: 2.5, 10.3), p=0.0012)(Lean et al., 2018).

Physical activity: There were no differences at 12 months observed between Intervention and Control groups in physical activity levels and sleep duration (Lean et al., 2018). Sleep time was slightly higher in Control group at baseline at 441.7 minutes/day versus 421.4 minutes/day.

Withdrawal: 21.48 % of Intervention participants withdrew prematurely from the intervention programme. The social reason was the main one for withdrawal in 30.8 % of those who commenced on dietary intervention (Lean et al., 2018).

Blood pressure at 12 months in the whole DiRECT study was similar in the Intervention and Control groups which is remarkable as antihypertensive medications were stopped in 48 % of those who were taking them at baseline in the Intervention group. Antihypertensives were continued in the Control group. Antihypertensive treatment was prescribed to 31.8 % (one agent in 29 subjects and two or more agents in 18 subjects) of the Intervention group versus 61.5 % (43 one agent, 48 two or more agents) of the Control group (p=0.0001)(Lean et al., 2018).

Serum triglyceride decreased by 0.31±1.33 mmol/l in the total DiRECT Intervention group and increased by 0.09±0.92 mmol/l in the Controls. Triglycerides were 20 % lower in the Intervention group at 12 months (95% CI: 11%, 28%); p<0.0001) (Lean et al., 2018).

Mean total serum triglyceride reduced similarly in Tyneside Responders and Non-responders post weight loss (1.84±0.13 mmol/l to 1.30 ±0.13 mmol/l, p<0.0001 and 1.91±0.25 mmol/l to 1.24 ±0.14 mmol/l respectively, p=0.002). This remained stable at 12 months (Responders: to 1.24±0.12 mmol/l, p=0.43; Non-responders: to 1.39±0.21 mmol/l, p=0.52).

Adverse events: Serious adverse events during the 12 months were reported in 8 participants in the total DiRECT Intervention group but only one biliary colic was considered potentially Intervention induced. Adverse events in DiRECT study are summarised in Table 3.4.
Commonly reported adverse events during dietary intervention phase were constipation (46.8 %), increased cold sensitivity (41.0 %), headache (38.1 %), and dizziness (35.3 %). Most adverse events were mild to moderate and resolved with time and only constipation required treatment. Few participants reported adverse events during the food reintroduction and weight maintenance phases. (Lean et al., 2018)

	TDR phase (12-20 weeks)				FR phase (4-6 weeks)				WLM phase (26-36 weeks)			
	Total (n=139)	Mild	Mod	Sev	Total (n=124)	Mild	Mod	Sev	Total (n=94)	Mild	Mod	Sev
Constipation	65 (46.8)	30 (21.6)	24 (17.3)	11 (7.9)	18 (14.5)	14 (11.3)	4 (3.2)	0 (0.0)	6 (6.4)	2 (2.1)	2 (2.1)	2 (2.1)
Sensitivity to cold	57 (41.0)	37 (26.6)	12 (8.6)	8 (5.8)	30 (24.2)	19 (15.3)	6 (4.8)	5 (4.0)	13 (13.8)	7 (7.4)	2 (2.1)	4 (4.3)
Headache	53 (38.1)	31 (22.3)	13 (9.4)	9 (6.5)	15 (12.1)	10 (8.1)	3 (2.4)	2 (1.6)	8 (8.5)	5 (5.3)	2 (2.1)	1 (1.1)
Dizziness	49 (35.3)	40 (28.8)	7 (5.0)	2 (1.4)	11 (8.9)	3 (2.4)	6 (4.8)	2 (1.6)	7 (7.4)	4 (4.3)	3 (3.2)	0 (0.0)
Fatigue	45 (32.4)	24 (17.3)	11 (7.9)	10 (7.2)	18 (14.5)	10 (8.1)	3 (2.4)	5 (4.0)	8 (8.5)	2 (2.1)	0 (0.0)	6 (6.4)
Mood change	35 (25.2)	16 (11.5)	12 (8.6)	7 (5.0)	10 (8.1)	4 (3.2)	4 (3.2)	2 (1.6)	4 (4.3)	1 (1.1)	2 (2.1)	1 (1.1)
Nausea	25 (18.0)	15 (10.8)	4 (2.9)	6 (4.3)	3 (2.4)	3 (2.4)	0 (0.0)	0 (0.0)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)
Diarrhoea	23 (16.5)	11 (7.9)	10 (7.2)	2 (1.4)	5 (4.0)	4 (3.2)	1 (0.8)	0 (0.0)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)
Indigestion	20 (14.4)	15 (10.8)	3 (2.2)	2 (1.4)	4 (3.2)	2 (1.6)	2 (1.6)	0 (0.0)	1 (1.1)	1 (1.1)	0 (0.0)	0 (0.0)
Hair Loss	19 (13.7)	10 (7.2)	7 (5.0)	2 (1.4)	13 (10.5)	3 (2.4)	6 (4.8)	4 (3.2)	8 (8.5)	4 (4.3)	3 (3.2)	1 (1.1)

Table 3.4 Adverse effects identified a priori as relevant to the intervention treatment, experienced by Intervention group participants during first year of DiRECT study visits in each phase of the weight management programme. The usual-care control group was seen only at baseline and 12 months (data reported as N (%))(Lean et al., 2018)

3.4 Clinical outcomes at 24 months

77.9 % of all Interventions and 94.0 % of all Controls attended the 24 months DiRECT study assessment. 14.1 % of randomised participants failed to attend the study at 24 months for various reasons. For some participants weight and HbA1c data were obtained from GP records where available. Therefore the 24 months for weight and HbA1c were available for 91.3 % DiRECT participants (Intervention n=129 and Control n=143). For ITT analysis, the remaining 26 participants with no data at 24 months, who did not attend at 12 or 24 months and GP records were not available for them, were assumed to have achieved primary outcomes.

In Tyneside subgroup of the study the weight loss ≥ 15 kg at 24 months was recorded for 8 out of 64 (12.5 %) subjects in the Intervention group, and by none in the Control group. Tyneside data were similar to the total DiRECT cohort data at 24 months where weight loss of 15 kg or more was recorded in 13.2 % of total Intervention group participants, and by only 2% in the Control group (p=0.0023; Figure 3.4.a). In the Intervention group 24.2 % maintained ≥ 10 kg weight loss at 24 months.

Diabetes remission as per ITT was achieved by 20 out of 64 (31.3 %) of Tyneside Intervention participants at 24 months and only by 1 out of 24 (4.2 %) of Control participants (p<0.0001) similar to the total DiRECT cohort outcomes where remission was seen in 35.6 % of participants in the Intervention and 3.4 % in the Control group (p<0.0001) (Figure 3.4.b)(Lean et al., 2019).

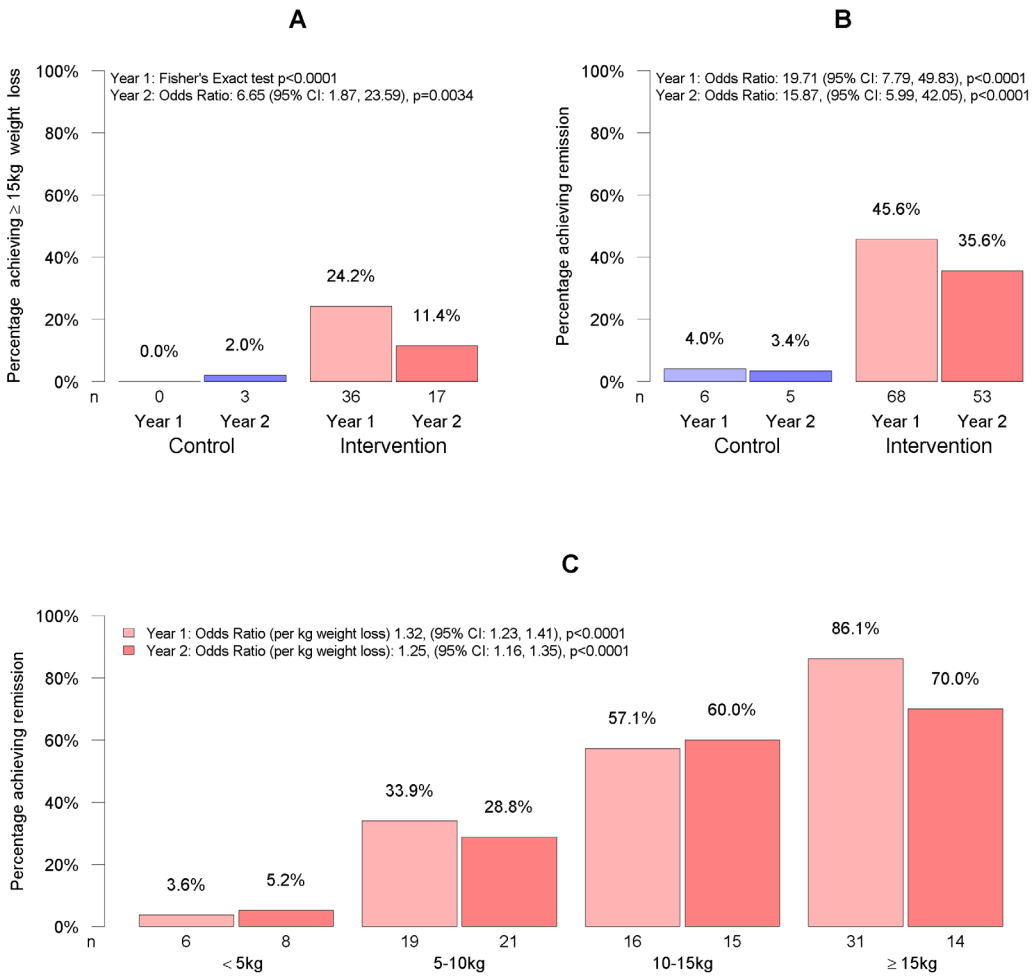

Figure 3.4 Primary outcomes and remission of diabetes in relation to weight loss at 1 and 2 years of DiRECT study. Regression models adjusting for practice list size, study centre and a random effect for practice.

A: First co-primary outcome, achievement of ≥15 kg weight loss, by randomised group.
B: Second co-primary outcome, remission of diabetes (HbA_{1c} < 48mmol/mol, off anti-diabetic medication for 2 months), by randomised group.
C: Remission of diabetes, in relation to weight loss achieved (both randomised groups combined).(Lean et al., 2019)

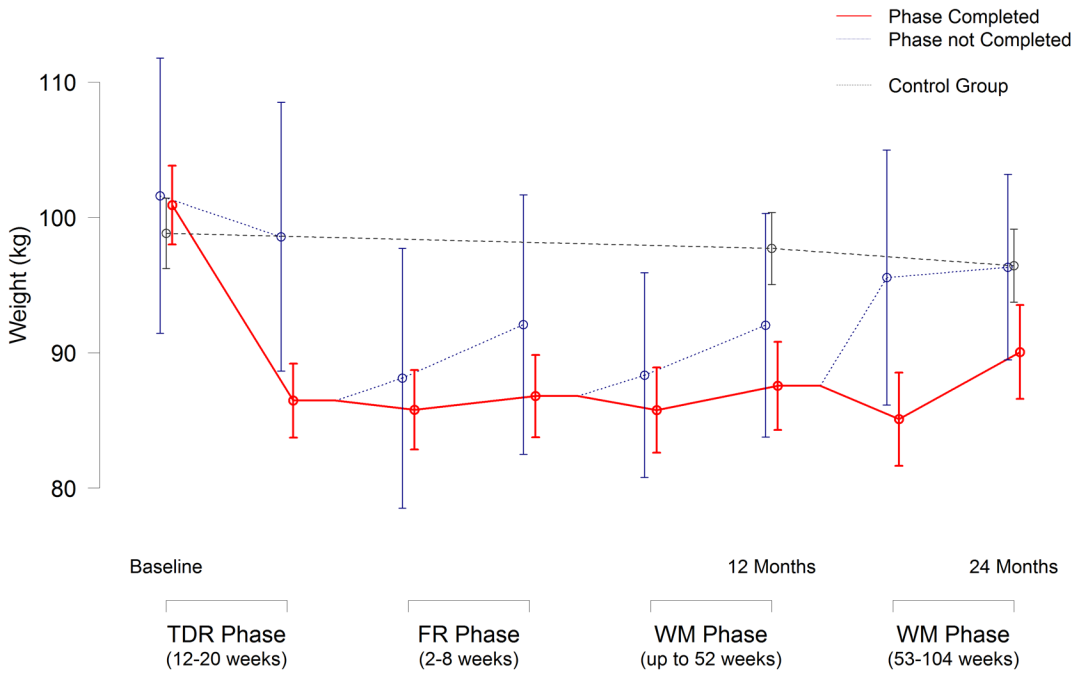

Figure 3.5 Changes in weight of participants who remained in the trial over 24 months follow up and those who dropped out during each phase of the intervention. Error bars represent 95% CI.(Lean et al., 2019)

3.4.1 Primary outcomes at 24 months

In Tyneside, the diabetes remission was achieved by 20 out of 64 (31.3 %) of Intervention participants and by 1 out of 24 (4.2 %) of Control participants at 24 months.

For the whole DiRECT study cohort, remission of type 2 diabetes at 24 months were achieved by 5.2 % participants who failed to achieve 5 kg weight loss, by 28.8 % of those who maintained 5–10 kg loss, by 60.0 % who maintained 10–15 kg loss, by 64.4 % who maintained ≥ 10 kg loss, and by 70.0 % of participants who lost 15 kg or more (Figure 3.4.c)(Lean et al., 2019). Four participants (out of 50 with weight gain (8.0 %)) were in remission at both 12 and 24 months despite small weight gains (0-2 kg) at 24 months. These individuals had baseline Hba1c between 47.5 mmol/mol and 49 mmol/mol.

Between baseline and 24 months, mean body weight fell by 7.6±6.5 kg in the whole DiRECT Intervention group and by 2.3±5.2 kg in the Control group ($p<0.0001$)(Lean et al., 2019).

Average body weight fell by 8.2±6.7 kg in the Tyneside Intervention group and by 1.6±5.8 kg in the Tyneside Control group between baseline and 24 months ($p<0.0001$).

Body weight increased by 2.6±5.0 kg in the total Intervention group and decreased by 1.3±4.2 kg in the Control group between 12 and 24 months, (adjusted difference in weight change between groups of 3.34 kg, 95% CI 2.18 to 4.50; $p<0.0001$). In the Intervention group, those maintaining remission between 12 and 24 months (n=48), after having lost on average 15.51±6.6 kg during first 12 months, regained 4.25±3.68 kg. In participants, who relapsed after 12 months (n=15), weight regain was greater (7.09±5.42 kg t-test $p=0.0732$), after having mean weight loss of 11.98±7.7 kg. Subjects who stayed in remission at 24 months lost an average of 10.4±6.8 kg from baseline. Those participants who were in remission at 12 months but relapsed at 24 months lost 3.7±5.9 kg and those who did not achieve remission at 12 or 24 months lost 3.2±5.2 kg. About half of 143 Intervention arm participants, who have data during treatment phases, required relapse management with brief total dietary replacement and the offer of orlistat during the 24 months follow up. Out of those 49.7 % had no rescue plan,

34.3 % had one rescue plan, 10.5 % had two and 5.6 % had three or more rescue plan phases. The total numbers of Intervention arm participants receiving orlistat at 12 and 24 months were 0 and 3 respectively (Lean et al., 2019).

Body weight increased by 3.9±3.8 kg ($p<0.0001$) in the Tyneside Intervention subgroup (n=46) and decreased by 1.2±3.5 kg in the Tyneside Control subgroup (n=19) between 12 and 24 months. 13 out of 40 (32.5 %) of original Tyneside Responders post immediate weight loss at 5 months on whom data is available, relapsed at 24 months of follow up. Those who relapsed gained on average 5.8±4.8 kg of weight between 12 and 24 months of follow up.

Average HbA1c reduced in the whole Intervention group between baseline (60.4± 13.7 mmol/mol) and 24 months (54.4±15.9 mmol/mol), adjusted mean difference -4.82, (-8.28, -1.36), p=0.0063). 74.5 % of Intervention participants were receiving anti-diabetes medications at baseline versus 39.5 % at 24 months. In the Control group, mean HbA1c remained similar between baseline (58.2±11.5 mmol/mol) and 24 months (58.6±14.4 mmol/mol). 77.2 % of Control subjects were receiving anti-diabetes medications at baseline and that figure increased to 83.9 % at 24 months (Lean et al., 2019).

In Tyneside subgroup Hba1c in Intervention arm reduced between baseline and 24 months by 5.6±16.4 mmol/mol ($p<0.0001$) and in Control group by 1.6±9.3 mmol/mol.

3.4.2 Secondary outcomes at 24 months

Systolic blood pressure at 24 months reduced by 4.3±18.7 mmHg in the total Intervention group and by 1.4±13.4 mmHg in the Control group (adjusted mean difference -3.43, (-6.70, -0.16), p=0.0397). 60.1 % in the Control group and only 47.3 % in the Intervention group were receiving antihypertensive medication at 24 months (adjusted odds ratio 0.31, (0.14, 0.71), p=0.0058)(Lean et al., 2019).

Serum triglycerides reduced from baseline by 0.3±0.8 mmol/l in the Tyneside Intervention subgroup and by 0.2±0.7 mmol/l in the Tyneside Control subgroup. That was a reduction on par with the whole DiRECT cohort (in Intervention group by 0.4±1.2 mmol/l and in Control group by 0.2±0.7 mmol/l (p=0.0055).

Serious adverse events reported for the first 24 months of DiRECT were 15 in the Intervention and 25 in the Control group. While there had been no significant difference at 12 months, in the second year of DiRECT, 6 participants in the Intervention group and 16 in the Control group suffered 9 and 22 serious adverse events respectively but those side effects did not lead to a withdrawal from the study. The serious adverse events included several vascular events in the Control group (two cerebral vascular accidents, one toe amputation, one aortic aneurysm rupture, and one sudden death), compared with one non-fatal MI in the Intervention group in a person who had not attended for review. Cholelithiasis, abdominal pain both in one participant during first year were potentially attributed to the intervention (Lean et al., 2019).
Notably, over 24 months of the study the newly diagnosed cancer was found in 5 (3%) of Control participants and not in anyone from the total DiRECT Intervention cohort. The cancers encountered were as follows: bladder cancer 1 (1%), colon cancer in 2 (1%), prostate cancer in 2 (1%), and renal cell carcinoma in 1 (1%) participant.

Quality of life has improved at 24 months more in the Intervention group in comparison to a Control group (change from baseline 10.0 (0.0, 20.0) than in controls 2.5 (-5.0, 9.0); p=0.03)).

3.4.3 Likelihood of remission

In the whole DiRECT study, likelihood of remission at 24 months (n=58/298, 19.5 %) was higher for male gender (adjusted odds ratio for female vs. male 0.44 (0.22, 0.88), p=0.0196) and increased with age (adjusted odds ratio 1.08 (1.03, 1.13) per year,

p=0.0020). The chances of remission were increased with weight loss from baseline (adjusted odds ratio 0.83 (0.77, 0.90) per kg, p<0.0001), and with weight-change from 12 to 24 months (adjusted odds ratio per kg gained 1.11 (1.03, 1.21), p=0.0103). Likelihood of remission at 24 months was not influenced by baseline BMI (adjusted odds ratio per kg/m^2 0.99 (0.92, 1.06), p=0.7701) or duration of diabetes within the 6-year range included (adjusted odds ratio per year 0.92 (0.76, 1.11), p=0.3949)(Lean et al., 2019).

3.5 Discussion

The premises of both clinical hypotheses being tested in DiRECT trial have been shown to be correct. Type 2 diabetes of less than 6 years duration could be reversed by a low-calorie diet intervention bringing about substantial weight loss, and the remission could be sustained in many for 2 years, all in Primary Care. Weight loss can be reached by many individuals by using a structured weight management programme delivered by routine primary care staff in the community. As per ITT analysis, 24.2 % of the total Intervention group achieved > 15 kg weight loss at 12 months. 45.6 % (45.3 % in Tyneside subgroup respectively) developed remission of type 2 diabetes while being off diabetes treatment at 12 months. The rate of diabetes remission was even higher immediately after weight loss intervention (5 months follow up) at 62.5% as measured in the Tyneside subgroup. This is substantially more in comparison with 22 % remission rate used for the power calculation and considered to be clinically important prior to the start of the study.

The type 2 diabetes remission was linked to the degree of weight loss maintained at 12 months. 86 % of participants with at least 15 kg weight loss and nearly 60 % with ≥ 10 kg were in remission at 12 months study follow up (Lean et al., 2018).

Tyneside subgroup similarly to the whole DiRECT trial illustrated that early type 2 diabetes of up to 6 years' duration can be put into remission. 64 % of those who maintain a weight loss of > 10 kg, and for 70 % with weight loss > 15 kg, were still in

remission at 24 months. The evidence-based structured weight management programme, delivered by routine primary care staff in a community setting, achieved remissions at two years for over a third of those who commenced the intervention, and over 40 % of those with two-year data. The study was well accepted by the people with type 2 diabetes (Taylor et al., 2018c) therefore similar weight management programme, if implemented in clinical practice, could achieve a wider impact on the care of people with type 2 diabetes.

Weight loss maintenance was the main factor behind remission of type 2 diabetes in DiRECT. The Intervention subjects, that relapsed back into type 2 diabetes between 12 and 24 months, regained more weight in comparison to those maintaining their remission. Weight regain was a challenge but was less than in other studies (Lean and Hankey, 2018). The co-primary outcome of > 15 kg weight loss was maintained by only 11.4 % by intention to treat analysis at 2 years which was substantially down from 24 % at 1 year (Lean et al., 2019).

The physiologic changes, demonstrated by the DiRECT study, are associated with the return to normal glucose control in type 2 diabetes. An average decrease in body weight of 15 % was achieved by a structured programme delivered by Primary Care staff. This brought about profound changes in lipid metabolism, irrespective of glucose response. The greatest change was in liver fat content which fell from high levels to normal in the whole Tyneside Intervention group at 12 months. Fall in plasma levels of VLDL1-TG was accompanied by fall in intrapancreatic fat content in Tyneside subgroup. All changes in lipid metabolism and intra-organ lipid remained steady over 12 months if weight loss was maintained. However, only Responders demonstrated early and sustained improvement in beta cell function with striking difference at 12 months and 24 months as discussed in Chapter 6. Crucially, weight loss in early type 2 diabetes brings about similar correction of intra-organ fat content in all.

The DiRECT approach focuses on the necessity for long-term maintenance of weight loss. Variable and extended food reintroduction period was due to largely social reasons and to allow participants to adapt to a new eating habit. Behavioural therapy methods were incorporated into in the weight loss maintenance phase. Intervention participants were

advised to continue their usual daily activities. There was no increase in measured physical activity in either Intervention or Control groups between baseline and 12 months despite the advice to increase physical activity up to 15,000 steps target per day during FR and WLM phases.

The weight changes in DiRECT seen at 12 months were similar to the reported in a Counterweight-Plus study (-9.5kg ITT, n= 91)(Lean et al., 2013). DiRECT weight losses were similar to those achieved in the Counterpoint and Counterbalance studies (Lim et al., 2011a) (Steven et al., 2016a) but were higher than otherwise described in people with type 2 diabetes. Look AHEAD, delivered a in specialist centers, achieved an average weight loss of 8.6 kg (Look, 2014).

Importantly, those in the upper tertile of weight loss showed markedly higher rates of remission than those in the lower tertile, but there was only a minor effect of physical training itself (Lean et al., 2018). There is frequently a confusion about the relative roles of exercise and of weight loss. Both Counterpoint and Counterbalance studies achieved similar weight loss to DiRECT but like Look Ahead these were managed by a specialist research personnel (Steven et al., 2016a). Meta-analysis had demonstrated that a mean weight change of 10 kg at 12 months from Very Low-Calorie Diets Interventions is usually required for remission (Franz et al., 2007). A recent non-randomised study on type 2 diabetes has reported remissions in those who completed an intensive diet programme focussed upon low carbohydrate eating (Hallberg et al., 2018). However, meta-analyses of the controlled trial evidence show no important differences between high and low carbohydrate diets for weight, HDL- and LDL-cholesterol, total cholesterol and blood pressure, as well as HbA1c outcomes (Korsmo-Haugen et al., 2019).

DiRECT study was conducted in routine primary care under real-life conditions, delivered by the trained local nurses or dietitians. Weight loss causes a rapid fall in blood pressure with risk of postural hypotension. Therefore, all antihypertensive and diuretic agents were stopped at the start of the diet in the Intervention group and were only restarted if systolic BP exceeded 140 mmHg. The acute fall in blood pressure on a low energy formula diet is greater than anticipated from reduced salt intake alone (Steven et al., 2016a). 42 % of the Intervention group remained off antihypertensive agents at 12

months, with no blood pressure increase (Lean et al., 2018). The need to take anti-diabetes medications was greatly decreased.
Quality of life improved significantly in the Intervention group with no change in the Control group. The benefits to individuals and the improved physical and psychological wellbeing accompanying substantial weight loss have previously been documented (McCombie et al., 2017, Rehackova et al., 2017).

No study prior to DiRECT has proposed a remission of type 2 diabetes as a primary outcome. The present study is also unusual in being conducted entirely within routine primary care, to provide data which can be readily translated to wider practice.

A high proportion (28 %) of all those eligible volunteered to participate (Taylor et al., 2018c). People with type 2 diabetes as per the recent evidence rank reversal of the disease as their top priority for research (Finer et al., 2017).

The DiRECT strong points are that it is builds upon earlier small studies and has a cluster-randomised study design, managed by a well-established Clinical Trials Unit. The remission rate of 46 % greatly exceeded the level of 22 % considered clinically important. The DiRECT participants had characteristics similar to the general population of people with type 2 diabetes therefore the results are likely to be generalizable (Taylor et al., 2018c).

The study had some limitations. The main limitation is the lack of generalisability of the study. There were only 2 geographical regions involved the study and the participants were predominantly white Scottish or White British and therefore not directly transferrable to other UK ethnic groups. Only 28% of the invited people accepted an invitation to participate in the study. There was also a substantial drop out from the study especially at 12 months follow up. The detailed body composition was not assessed due to the limitations to the data acquisition in the primary care. Control group data were only collected at baseline,12, and 24 months, therefore intercurrent adverse events could not be assessed. The data on physical activity should be viewed with caution as they were based on around half of all participants in each group for whom the data were complete. A considerable media coverage and the publication of the first-year DiRECT results in December 2017 may have attenuated the difference

between the randomised groups. As personally observed, a proportion of the Control group both from Tyneside and Scotland took personal action to lose weight (9 participants in the Control group lost > 10 kg during the second year compared to 2 during the first year). Increased use of SGLT-2 inhibitors may also have contributed to the weight change in Controls. At 12 months no control participants had achieved the co-primary outcome of weight loss greater than 15 kg, but at 24 months it was reached by 3 participants (2.1 %). Despite this the differences in remission and weight loss between groups were still highly significant and clinically important at 24 months. Weight re-gain in the Intervention group contributed to limit the effect size (Lean et al., 2019).

This large trial demonstrated that a professionally supported intensive weight management programme is attractive to many people with early type 2 diabetes. The programme based on the DiRECT intervention, is relatively simple and inexpensive compared with new antidiabetic drugs. The 12-month intervention cost is under half of the average annual UK healthcare cost of a person with type 2 diabetes (Xin et al., 2019). DiRECT study results should influence the future NHS guidelines for the routine primary care of patients with early type 2 diabetes who want to achieve a remission (McCombie et al., 2017).

Follow-up and low intensity support to look at longer term outcomes in DiRECT are currently funded to continue for all participants to a total of 5 years from baseline.

A new UK pilot programme is to be offered on NHS to 5,000 people in selected areas across England (NHS England » Low calorie diets to treat obesity and Type 2 diabetes). This programme is designed to evaluate whether one-to-one, group or IT-delivered tuition is most clinically and cost effective for overall remission of type 2 diabetes. It also aims to build up knowledge and understanding about the use of low-calorie diet interventions in a wider population with early type 2 diabetes and their future impact.

Chapter 4. Effect of weight loss on the liver and evaluation in routine clinical practice

4.1 Introduction

Once considered to be an incidental finding of little clinical importance, non-alcoholic fatty liver disease (NAFLD) now is an established indicator of morbidity and mortality. The overall prevalence of NAFLD in developed countries is thought to range between 20 to 30 percent (Kotronen and Yki-Jarvinen, 2008) and fatty liver now ranks second as cause of liver transplantation (Cholankeril and Ahmed, 2018). In Europe, the prevalence of NAFLD is rising rapidly (Haldar et al., 2019), though detection rates at practice levels are much lower (Alexander et al., 2019). However, in type 2 diabetes the prevalence of NAFLD is at least 70 % (Shibata et al., 2007, Williams et al., 2011). This is an important step in the development of type 2 diabetes (Lim et al., 2011a, Al-Mrabeh et al., 2019, Taylor et al., 2019a) and ALT and gamma glutamyl transpeptidase (GGT) are elevated prior to diagnosis (Ohlson et al., 1988, Sattar et al., 2007, Wannamethee et al., 1995).

There is no practical method of measuring liver fat in Primary Care. Estimation is possible using liver ultrasound (Anstee et al., 2013) but accurate quantification requires magnetic resonance methodology. Type 2 diabetes is now recognised as a potentially reversible metabolic state with a key role of liver fat (Lean et al., 2019). Therefore, quantification in clinical practice is important both for motivation as well as monitoring remission of NAFLD. Liver enzyme measurement at a single time point is not reliable for the detection of NAFLD (Mofrad et al., 2003). However, the change in liver fat content following weight loss is profound and this might be anticipated to be accompanied by change in liver enzymes, as has been suggested by us in a recent algorithm to manage fatty liver in primary care (Sattar et al., 2014).

DiRECT (the Diabetes Remission Clinical Trial) observed the normalisation of liver fat after a low calorie diet intervention in a Primary Care cohort of people with type 2 diabetes (Taylor et al., 2018a). LFT data were collected and the Fatty Liver Index (Bedogni et al., 2006) and Hepatic Steatosis Index (Lee et al., 2010) could be derived. The present study

was conducted to test the hypothesis that widely available serial liver function tests could have a predictive value in indicating change in liver fat content during weight loss.

4.2 Research design and Methods

4.2.1 Participants

Participants in the Tyneside cohort of DiRECT randomised to weight loss were studied (n=64). They were within 6 years of diagnosis of type 2 diabetes, aged 20-65 years, with BMI between 27 and 45 km/m^2 and with HbA1c ≥ 48 mmol/mol within last 12 months if on diet alone or HbA1c ≥ 43 mmol/mol if on treatment with oral hypoglycaemic agents (Taylor et al., 2018a, Leslie et al., 2016).

Non-Diabetic Comparators (NDC, n=25) were recruited to match the Intervention group post weight loss for weight and BMI, age, and gender. The purpose of including non-diabetic comparators was to be able to elicit how close to normal the metabolic parameters of Intervention participants will get post weight loss. Oral Glucose Tolerance Tests were performed on this group at the first visit to confirm normal glucose tolerance and none had known personal or family history of type 2 diabetes. Full data on liver fat and LFT were available on 59 participants at baseline and on 42 participants for paired data at baseline, 12 and 24 months caused by the drop out during the study follow up.

4.2.2 Study protocol

All participants had Liver fat measurement and blood sampling after an overnight fast from 10 pm of the previous day. The morning medications were omitted on the day of the study. Following the baseline visit, the type 2 diabetes group followed 825–853 kcal/d liquid formula diet for 12 weeks.

Antidiabetic and antihypertensive treatments were stopped from the first day of the low-calorie diet. After food reintroduction, participants were reviewed monthly and advised to avoid weight regain during the 2 years follow up phase.

Quantification of liver fat and LFTs was carried out at baseline, then 12 and 24 months of follow up. NDC group was studied on a single occasion. Ethical approval was obtained from the West of Scotland Research Ethics Committee (reference number: 13/WS/0314).

4.2.3 Measurements and Analytical Procedures

Measurement of the liver fat with the help of the MATLAB software (Mathworks, Cambridge, UK) was already described in the Methods chapter (Schneider and Glover, 1991). The fat content was expressed as a percentage of the original signal collected from both water and fat signals. The Image J 1.43 software was used to define the Region-of-Interest (ROI) by utilising the Image J polygon tool (Schneider et al., 2012) and 5 ROIs for the liver were acquired and averaged.
Venous blood was sampled after MRI scanning following the overnight fast from 10 pm of the previous day. Fasting plasma glucose was measured by using the glucose oxidase method (YSI glucose analyser, Yellow Springs Instrument Company, Yellow Springs, OH).

Liver enzymes (ALT, GGT, AST) and HbA1C were measured at the Institute of Cardiovascular and Medical Sciences at Clinical Pathology Laboratory in Glasgow. Fasting plasma insulin was measured by ELISA (Mercodia, Uppsala, Sweden) at the Clinical Pathology Laboratory of Newcastle upon Tyne Trust Hospitals.

Fatty Liver Index (BMI, waist circumference, GGT and triglycerides) (Bedogni et al., 2006, Cuthbertson et al., 2014) was calculated by using MD Calc (https://www.mdcalc.com/fatty-liver-index) and Hepatic Steatosis Index (BMI, AST, ALT, diagnosis of type 2 diabetes) [18] by using MD App (https://www.mdapp.co/hepatic-steatosis-index-hsi-calculator-357/) calculators respectively. BARD score for an assessment of the risk of hepatic fibrosis, based on BMI, ASAT/ALAT ratio, and the

presence/ absence of type 2 diabetes (Harrison et al., 2008), was also calculated by using MD Calc online calculator (https://www.mdcalc.com/bard-score-nafld-fibrosis).

Positive Predictive Values (PPV) and Negative Predictive Values (NPV) were calculated between decrease in ALT, GGT, indexes of fatty liver and normalisation of liver fat post weight loss in diabetes group (n=42) at 12 months.

4.2.4 Statistical analysis

Data were analysed by using IBM SPSS statistical software (www.ibm.com). Normality of the data was examined using histograms and Shapiro-Wilk test. Statistical analysis was performed on 42 type 2 diabetes subjects by comparing them during all time points with baseline and also with NDC (n=25) by using the following tests: for normally distributed data (Pared samples T-test, Independent samples T-test, Pearson correlation test), and for the skewed data (Wilcoxon Rank test, Mann Whitney U test, and Spearman Rank correlation test). Data were presented in mean ± SEM or median with interquartile ranges [25th and 75th centiles] as appropriate. ANOVA (a multilevel regression) was run to predict the changes in liver fat based on the changes in various metabolic parameters post intervention and weight loss (Appendix 1).

4.3 Results

The whole type 2 diabetes group (n=59) at baseline had the following characteristics (mean ± SEM): age 52.5±1.1 years; 50.9 % male; weight 100.4±2.3 kg; BMI 34.9±0.6 kg/m^2. Duration of diabetes since the diagnosis was 3.0±0.2 years. The NDC group was well matched for weight and BMI of DiRECT participants after weight loss (86.6±3.0 kg; 29.7±0.8 kg/m^2) as well as for age and gender (55.8±1.2 years; 52 % male; Table 4.1).

4.3.1 Weight change

In those with diabetes, mean weight loss at 12 months was 11.9±1.2 kg (range 0 - 37 kg). Weight decreased from 98.3±2.5 kg at baseline to 86.4±2.2 kg ($p<0.0001$) at 12 months with 57 % (n=24) achieving weight loss of more than 10 kg. Between 12 and 24 months, weight increased by 4.2±0.6 kg to 90.7±2.4 kg ($p<0.0001$; Table 4.1). 33 % of participants (n=14) had ≥ 10 kg weight loss at 24 months. A Non-Diabetic Control (NDC) (86.6±3.0 kg) matched the Intervention group after weight loss.

57% (n=24, 67 % male) of Intervention subjects achieved weight loss of more than 10 kg at 12 months while 43 % (n=18, 39 % male) lost on average less than 10 kg of weight. At 24 months follow up weight had risen in Intervention group by 4.2±0.6 kg (4.8±0.6 %) to 90.7±2.4 kg ($p<0.0001$) in comparison to 12 months visit (Table 4.1).

4.3.2 Metabolic data

Fasting plasma glucose (FPG) improved from 7.9 [7.1-10.1] at baseline to 5.9 [5.5-7.2] mmol/l ($p<0.0001$) at 12 months while off all anti-diabetic medications (Table 4.1) but was still higher that in NDC (5.1[4.8-5.4] mmol/l; $p<0.0001$). Following weight gain FPG increased at 24 months to 6.5 [5.7-7.9] mmol/l ($p=0.045$). In those who lost > 10kg of weight at 12 months FPG reduced from 8.0 [7.6-10.4] to 5.9 [5.4-6.8] mmol/l ($p<0.0001$). The subjects who lost < 10kg at 12 months had similar but less impressive reduction in FPG from 7.3 [6.8-9.1] to 6.3 [5.9-7.5] mmol/l ($p=0.004$, Table 4.1).

HbA1c decreased from 58.0 [53.3-63.8] mmol/mol (7.5 [7.0-8.0] %) to 42.9 [38.3-53.6] mmol/mol (6.1 [5.7-7.1] %) with $p<0.0001$ at 12 months off all anti-diabetic medications (Table 4.1). At 24 months HbA1c increased to 50.0 [43.0-61.0] mmol/mol (6.7 [6.1-7.7] %). For comparison NDC had HbA1c at 36.0 [32.0-38.0] mmol/mol (5.4 [5.1-5.6] %) and that was still significantly lower than in Intervention group at 12 and at 24 months ($p<0.0001$ for both). In the Intervention subgroup that lost > 10kg of weight HbA1c went into the normal range from 58.0 [54.8-64.0] to 39.5 [37.1-48.4] mol/mol ($p<0.0001$). In the Intervention subgroup that lost < 10kg of weight HbA1c decreased non-significantly from 57.0 [51.3-63.3] to 47.5 [43.4-53.6] mmol/mol ($p=0.055$) at 12 months.

Fasting plasma insulin (FPI) decreased from 75.9 [51.1-113.0] to 30.1 [20.2-54.2] pmol/l at 12 months ($p<0.0001$; Table 4.1) and 44.1 [18.2-59.2] pmol/l at 24 months ($p=0.062$ vs. 12 months). FPI in the total Intervention group at 12 months was still higher ($p=0.021$) than NDC group FPI 16.4 [10.0-37.2] pmol/l. In the > 10 kg weight loss subgroup, there was no statistically significant difference in FPI at 12 months (22.4 [17.6-38.9] pmol/l) versus NDC ($p=0.284$). In the < 10 kg weight loss subgroup, FPI at 12 months (48.2 [30.1-78.2] pmol/l) was higher than that of NDC ($p=0.002$).

Fasting plasma triglyceride (TG) level decreased from 1.72 [1.17-2.22] to 1.19 [0.81-1.60] mmol/l at 12 months ($p<0.0001$) and stayed steady at 24 months at 1.26 [0.91-1.75] mmol/l ($p<0.0001$ vs. baseline). TG became comparable with that of the NDC group (1.10 [0.80-1.50] mmol/l) at 12 and 24 months ($p=0.881$ and $p=0.328$ respectively). For the > 10 kg weight loss subgroup, total TG went down from 1.90 [1.18-2.20] to 0.87 [0.74-1.24] mmol/l at 12 months ($p<0.0001$) and stayed steady at 1.00 [0.84-1.52] mmol/l at 24 months ($p<0.0001$ vs. baseline). On the other hand, for the subjects with < 10 kg weight loss total TG has not changed much being 1.48 [1.21-1.91] mmol/l at 12 months ($p=0.486$) and 1.61 [1.21-1.87] mmol/l at 24 months ($p=0.657$ vs. baseline).

There was no change in total cholesterol (TC) post weight loss at 12 months (Table 4.1). It increased significantly at 24 months vs. baseline from 4.08 [3.53 – 4.90] to 4.56 [3.93-5.56] mmol/l ($p=0.001$). At the same time, TC in NDC (5.30 [4.60-5.80] mmol/l) was significantly higher than in type 2 diabetes group at baseline and 12 months ($p<0.0001$). This could be due to the higher prevalence of statin use in type 2 diabetes group (n=27/42; 64.3 %) vs. that in NDC (n=3/25; 12.0 %).

In the whole Intervention group high density lipoprotein (HDL) have raised post weight loss from 1.07 [0.87-1.18] to 1.15 [0.95-1.42] mmol/l at 12 months ($p=0.002$) and 1.26 [0.91-1.75] mmol/l at 24 months ($p<0.0001$ vs. baseline). At 12 months it became close to NDC (1.50 [1.10-1.70] mmol/l, $p=0.033$). HDL raised similarly at 24 months in the >10kg weight loss group ($p=0.001$ vs. baseline) and in <10kg weight loss group ($p=0.004$ vs. baseline).

	Type 2 diabetes group (n=42)			NDC (n=25)
	Baseline	12 months	24 months	
Weight (kg)	98.3±2.5††	86.4±2.2***	90.7±2.4***	86.6±3.0
BMI (kg/m^2)	34.2±0.7†††	30.2±0.7***	31.6±0.7***	29.7±0.8
HbA1c (mmol/mol)	58.0[53.3 – 63.8]†††	42.9[38.3 – 53.6]***†††	50.0[43.0-61.0]*†††	36.0[32.0 – 38.0]
FPG (mmol/l)	7.9[7.1 – 10.1]†††	5.9[5.5 – 7.2]***†††	6.5[5.7 – 7.9]**†††	5.1[4.8 – 5.4]
FPI (pmol/l)	75.9[51.1 – 113.0]†††	30.1[20.2 – 54.2]***†	44.1[18.2 – 59.2]***††	16.4[10.0 – 37.2]
Liver fat (%)	13.0[7.8 – 23.3]†††	1.8[1.2 – 5.2]***	6.5[3.0 – 9.0]***†††	1.9[1.0 – 4.9]
ALT (units/l)	30.8[22.1 – 38.1]††	15.8[13.7 – 20.3]***	21.6[17.0 – 26.0]**	18.5[15.8 – 26.5]
GGT (units/l)	35.0[24.5 – 51.5]†	22.0[16.3 – 29.5]***	24.0[18.0 – 33.5]***	19.5[15.8 – 41.8]
AST (units/l)	19.3[15.4 – 25.1]	16.4[13.7 – 18.7]***	17.9[15.2 – 19.3]**	Not available
Fasting cholesterol (mmol/l)	4.08[3.53 – 4.90]†††	4.17[3.42 – 5.07]†††	4.56[3.93 – 5.56]**	5.30[4.60 – 5.80]
Total fasting TG (mmol/l)	1.72[1.17 – 2.22]††	1.19[0.81 – 1.60]***	1.26[0.91 – 1.75]***	1.10[0.80 – 1.50]

Table 4.1 Summary of the change in weight and fasting metabolic parameters for type 2 diabetes group at baseline, 12 months, and 24 months and for Non-Diabetic Comparator (NDC) group. Data shown as mean ± SEM or median [IQ range]. Comparisons are shown for baseline to 12 months, and baseline to 24 months (*** p<0.001, ** p<0.01,* p<0.05) and for type 2 diabetes group vs. NDC at each time point (††† p<0.001, †† P<0.01, † p<0.05).

4.3.3 Liver Fat Content

Liver Fat content was grossly elevated at baseline with 13.0 [7.8-23.3] % (upper level of normal 5.56 %). It decreased after weight loss to 1.8 [1.2-5.2] % at 12 months ($p<0.0001$) becoming comparable with NDC (1.9 [1.0-4.9] %, $p=1.000$) (Figure 4.1.). At 24 months liver fat increased to 6.5 [5.7-7.9] % ($p<0.0001$).

The prevalence of the fatty liver at baseline was 86.4 % (n=51/59) and similar in those with paired data after weight loss (83.3 %; n=35/42). After weight loss, the prevalence of fatty liver declined to 21.4 % (n=9/42), similar to NDC (24.0 %; n=6/25). With weight gain by 24 months the prevalence of NAFLD in type 2 diabetes group rose to 54.8 % (n=23/42). NAFLD was remission at 12 months in 26 of all 42 interventions (61.9%) and 26 out of 35 subjects with NAFLD at baseline (74.3%). At 24 months remission of NAFLD was achieved by 14/42 (33.3%) and 14/35 (40%) respectively as subjects gained weight between 12 and 24 months.

In the type 2 diabetes groups with weight losses of >10 kg (n=24) and <10 kg (n=18) at 12months, liver fat decreased from 16.2 [8.6-24.0] to 1.4 [1.1-1.9] % ($p<0.0001$) and from 10.4 [7.6-18.9] to 5.1 [2.1-9.1] % ($p<0.0001$) respectively.

Change in liver fat correlated strongly with change in weight ($r=0.54$; $p<0.0001$), BMI ($r=0.63$; $p<0.0001$), HbA1c ($r=0.61$; $p<0.0001$), and FPG ($r=0.54$; $p<0.0001$) at 12 months follow up. Change in liver fat also correlated positively with change in FPI ($r=0.5$; $p=0.003$) and total fasting TG (0.3; $p=0.028$).

4.3.4 Liver enzymes

In the whole type 2 diabetes group at baseline (n=59) liver fat correlated positively with ALT (r=0.45; p=0.0003) and not with GGT (r=0.23; p=0.079). Alanine aminotransferase (ALT) reduced post weight loss by 38.3 [18.5-59.8] % (30.8 [22.1 – 38.1] U/L at baseline to 15.8 [13.7 – 20.3] U/L (p<0.0001) at 12 months; Table 1). At 24 months ALT was significantly lower than at baseline (21.6 [17.0 – 26.0] U/L; p=0.001). ALT level became comparable at 12 and 24 months (p=0.088 and p=0.308 respectively) to ALT level in NDC (18.5 [15.8 – 26.5] U/L). In > 10 and < 10kg weight loss groups ALT decreased by 48.3 [31.6-60.8] % and 23.3 [13.5-53.7] %, respectively at 12 months.

Gamma glutamyl transferase (GGT) decreased by a similar proportion (38.4 [16.0-53.2] % from 35.0 [24.5 – 51.5] U/l to 22.0 [16.3 – 29.5] U/l (p<0.0001) at 12 months; Table 4.1) and kept steady at 24 months (24.0 [18.0 – 33.5] U/L; p<0.0001 vs. baseline). It came close to GGT level in NDC group (19.5 [15.8 – 41.8] U/l) at 12 months (p=0.674) and 24 months (p=0.566). With weight losses of > 10 kg and < 10 kg, GGT reduced by 52.0 [33.1-63.5] % (p<0.0001) and 28.7 [12.3-37.2] % (p=0.003) respectively.

Aspartate aminotransferase (AST) decreased by 20.1 [3.8-39.0] % post weight loss from 19.3 [15.4 – 25.1] U/l to 16.4 [13.7 – 18.7] U/L (p<0.0001) at 12 months and remained steady at 24 months (17.9 [15.2 – 19.3] U/L; p=0.003 vs. baseline). AST reduced post weight loss by 22.8 [12.7-35.9] % in > 10kg weight loss subgroup (p<0.0001) and by 11.9 [-10.5-39.1] % in those who lost < 10 kg (p=0.050).

In the combined type 2 diabetes and NDC group at baseline, liver fat correlated with ALT (r=0.57; p<0.0001) and with GGT (r=0.42; p<0.0001) (Figure 4.2.). Between baseline to 12 months, there were positive correlations between changes in liver fat and ALT (r=0.64; p<0.0001), GGT (r=0.38; p=0.013) and AST (r=0.36; p=0.018) (Figure 4.3).

ANOVA (Appendix 1) was performed to predict the determine interactions between variables potentially affecting change in liver fat post intervention (changes in weight, BMI, ALT, AST, GGT, lipid oxidation, total plasma cholesterol, total plasma triglycerides). The fall in liver fat was independently associated only with fall in weight (p=0.001), BMI (p<0.001), and ALT (p=0.001).

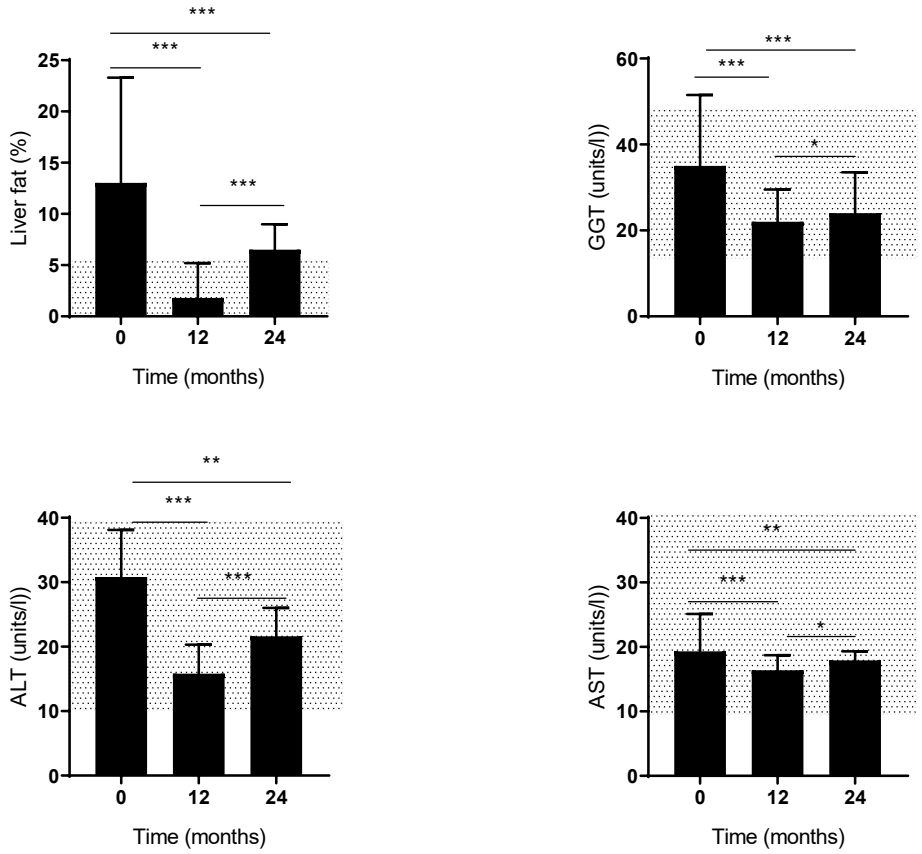

Figure 4.1 Changes in liver fat and liver enzymes in type 2 diabetes group at baseline, 12, and 24 months follow up.

This figure shows liver fat, ALT, GGT, and AST (median with interquartile range) in type 2 diabetes group at baseline, 12, and 24 months follow up (n=42 at each time point). Statistical changes marked with horizontal lines between the time points (*** $p<0.001$, ** $p<0.01$, *$p<0.05$). Shaded area represents normal ranges of the Liver fat, ALT, GGT, and AST respectively.

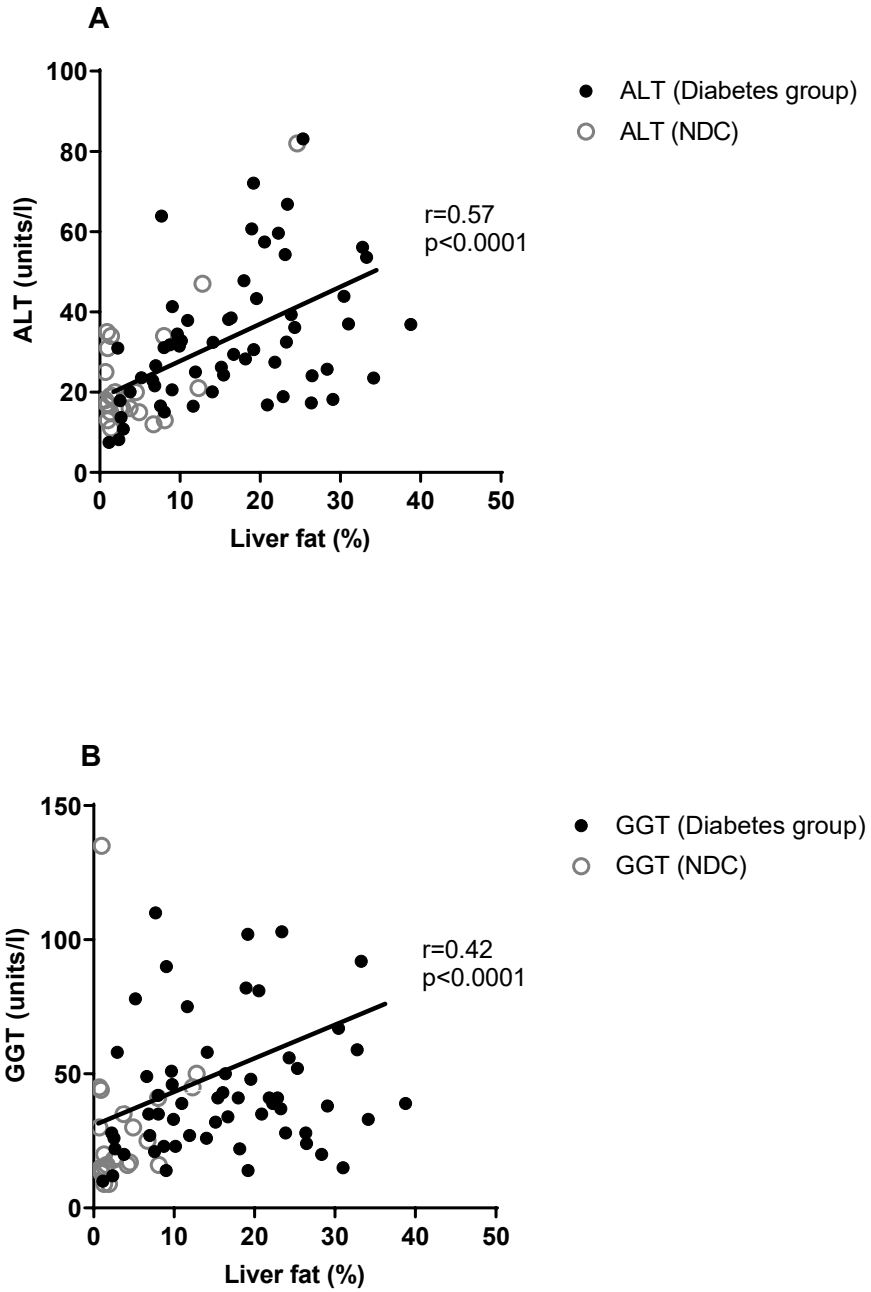

Figure 4.2 Relationship between liver fat and liver enzymes in the whole type 2 diabetes group at baseline and NDC combined.

Graph A shows correlation between ALT (units/l) and Liver fat (%) at baseline.

Graph B shows correlation between GGT (units/l) and Liver fat (%) at baseline.

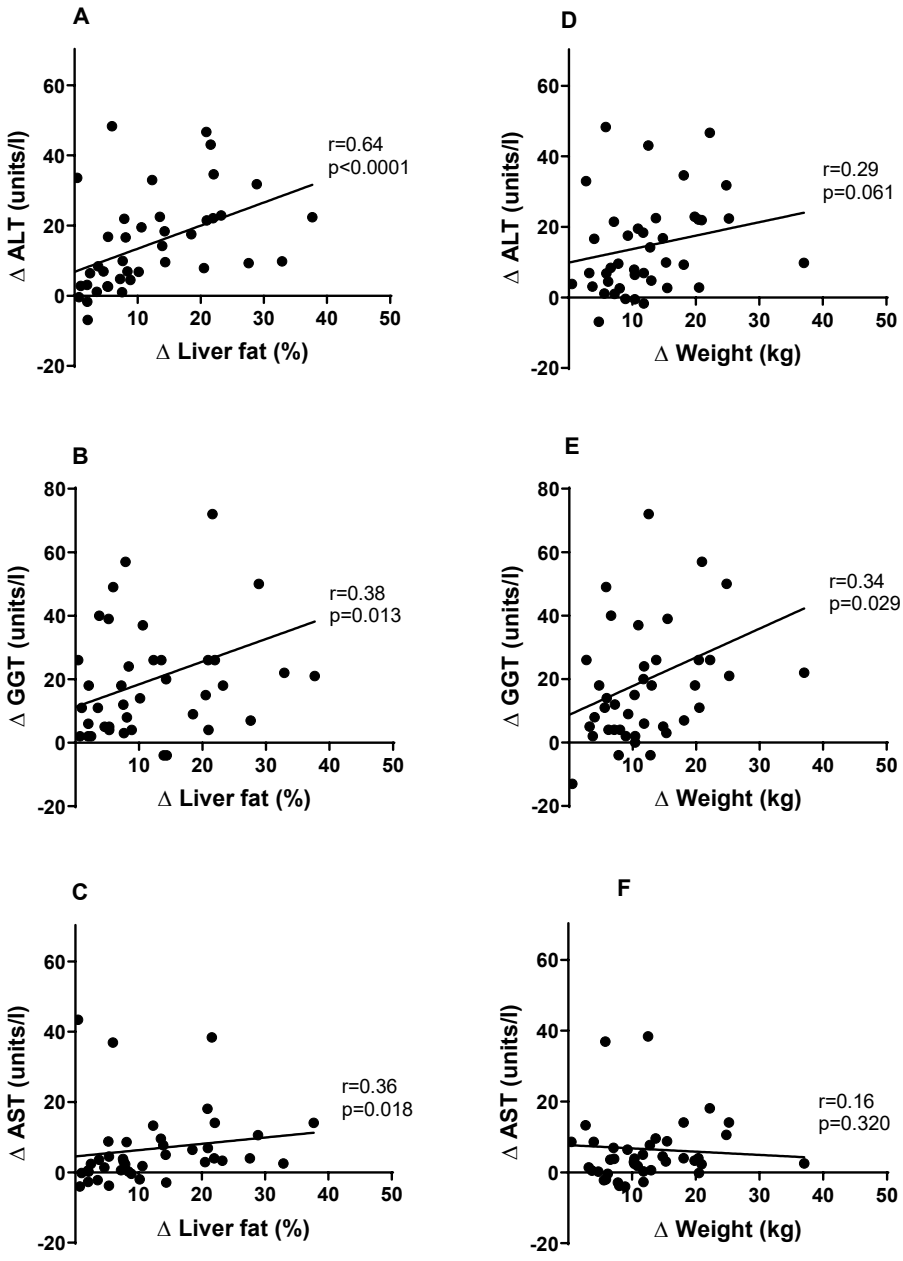

Figure 4.3 Relationship between changes in liver fat, liver enzymes, and weight in type 2 diabetes group.

A, B, C graphs show correlations between changes in liver fat and ALT, GGT, AST respectively in type 2 diabetes group (n=42) post weight loss at 12 months.

D, E, F graphs show correlations between changes in weight and liver enzymes in type 2 diabetes group (n=42) at 12 months.

4.3.5 Indexes of Hepatic Steatosis and Hepatic Fibrosis

The Fatty Liver Index (FLI) decreased post weight loss in type 2 diabetes group from 82.0 [64.5 – 87.0] to 45.0 [27.8 – 68.8] at 12 months ($p<0.0001$), representing change from 'high risk' of NAFLD (FLI≥60) to indeterminate risk of NAFLD (FLI 30 - 60). 32.4 % of participants achieved 'low risk' of NAFLD (FLI <30). At 24 months FLI was 58.5 [28.0 – 76.3], significantly lower than baseline ($p=0.002$). The Hepatic Steatosis Index (HSI) decreased post weight from 43.2±0.8 to 39.7±0.8 at 12 months ($p<0.0001$) and 40.2±0.8 at 24 months.

There was a strong correlation between changes in weight and changes in HSI at 12 months ($r=0.65$; $p<0.0001$). HSI at 12 months correlated strongly with absolute weight ($r=0.58$; $p<0.0001$), liver fat ($r=0.47$; $p=0.002$), FLI ($r=0.56$, $p=0.001$), and BARD ($r=0.64$, $p<0.0001$). There was a strong relationship between liver fat and indexes of hepatic steatosis in type 2 diabetes group at all time points (with FLI $r=0.50$, $p<0.0001$; with HSI $r=0.44$, $p<0.0001$) (Figure 4.4.). FLI also correlated with weight ($r=0.68$; $p<0.0001$), liver fat ($r=0.46$; $p=0.006$), ALT ($r=0.39$; $p=0.022$), and GGT ($r=0.53$; $p=0.001$) at 12 months.

The BARD score of hepatic fibrosis not changed by 12 months (2.7±0.2 vs. 2.8±0.2 at baseline, $p=0.800$) but there was an improvement in BARD score at 24 months (2.4±0.2) in comparison with that at baseline ($p=0.041$) and 12 months ($p=0.032$). BARD at 12 months correlated with weight ($r=032$; $p=0.039$).

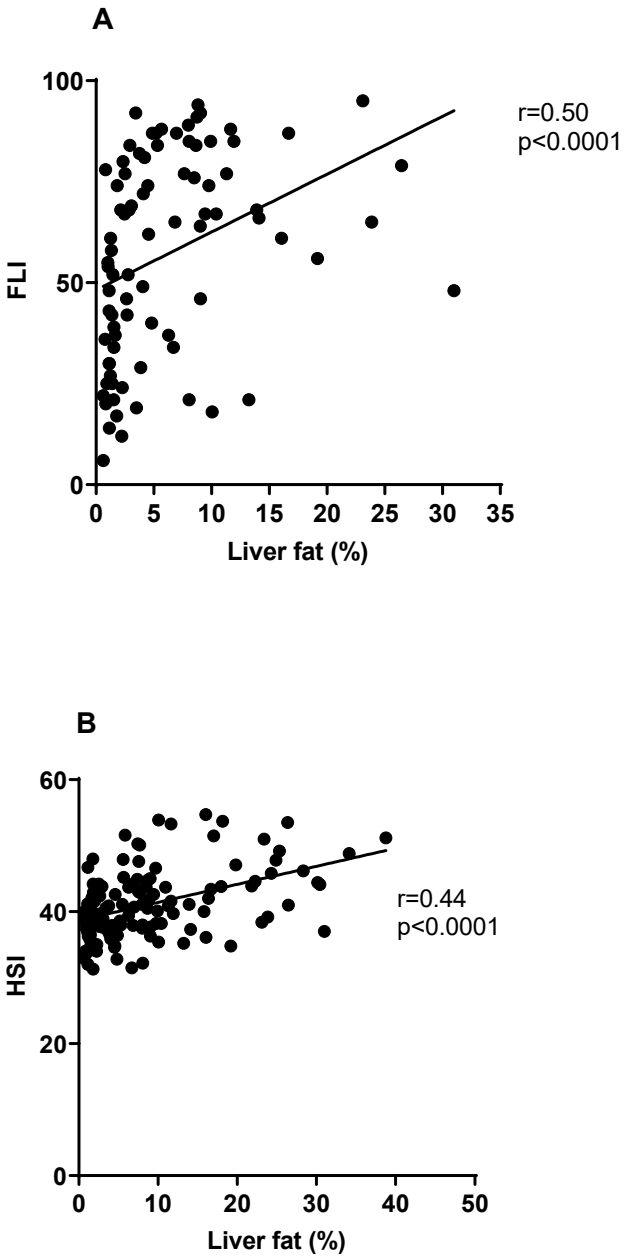

Figure 4.4. Relationship between liver fat and indexes of hepatic steatosis in type 2 diabetes group at all time points.

Graph A shows correlation between Fatty Liver Index (FLI) and Liver fat (%).

Graph B shows correlation between Hepatic Steatosis Index (HSI) and Liver fat (%).

4.3.6 Predictors of change in Liver Fat

Positive predictive value (PPV) for ALT change after dietary intervention at 12 months was 75.0 % and NPV was 16.6 %. For GGT changes post weight loss PPV and Negative Predictive Value (NPV) were 90.0 % and 20.0 %, respectively.

PPV and NPV were also calculated for the changes in ALT and GGT if a threshold level was used. For ALT, PPV was 100% with NPV of 33.3 % if only baseline values of >40 U/l were included. If levels over 25 U/l were included PPV was 91.7% with NPV of 16.7 (Table 4.2). For GGT the optimal threshold for PPV was >35 U/l with PPPV of 92.3 % and NPV of 28.6 %.

PPV for the change in hepatic steatosis index (HSI) detecting the normalisation of liver fat at 12 months for the whole group was 93.3% and NPV was 55.6%.

		PPV (%)	**NPV (%)**
ALT (U/l)	**>40**	**100.0**	**33.3**
	>35	**85.7**	**14.3**
	>30	**90.0**	**10.0**
	>25	**91.7**	**16.7**
GGT (U/l)	**>40**	**87.5**	**28.6**
	>35	**92.3**	**28.6**
	>30	**84.6**	**22.2**
	>25	**85.7**	**14.3**

Table 4.2 Positive predictive values (PPV) and negative predictive values (NPV) between decrease in ALT and GGT and normalisation of liver fat post weight loss in diabetes group (n=42) at 12 months with respect to different baseline cut offs for the named liver enzymes.

4.4 Discussion

Liver fat content decreased into the normal range after weight loss and the prevalence of the fatty liver in type 2 diabetes group became comparable to that of non-diabetic controls and the general population (Browning et al., 2004, Wong et al., 2012). Substantial weight loss induced NAFLD remission as per MRI criteria (liver fat <5.5 %) at 12 months in 26 out of 35 subjects with NAFLD at baseline (74.3 %). NAFLD remission was maintained by 14/35 (40 %) because of some weight re-gain between 12 and 24 months. Plasma levels of ALT and GGT declined at the same time with strong correlations between change in enzymes and change in liver fat at 12 and 24 months after weight loss. There was a significant improvement in indexes of hepatic steatosis (FLI and HSI) after weight loss intervention at 12 months and these also positively correlated with liver fat. It is notable that hepatic fibrosis improved 24 months after diet-induced weight loss as assessed by the BARD score. The positive predictive value for normalization of liver fat post weight loss was 75 % and 90 % for ALT and GGT respectively, although it must be noted that only change in ALT was independently associated with change in liver fat on multivariate analysis. Some individuals started with low normal LFT values, and it was not possible to exclude change in liver fat from such data. If baseline ALT was raised, measurement after weight loss had a positive predictive value of 100 %. Calculated hepatic steatosis index (HSI) gave similar result (PPV of 93.3 %).

Overall, the predictive value of sequential liver enzyme measurement was found to be high for the detection of normalization of liver fat levels. These widely available tests permit estimation of resolution of NAFLD in routine clinical practice.

Neither ALT nor GGT are reliable as a point estimate to confirm or exclude the presence of NAFLD (Mofrad et al., 2003, McPherson et al., 2010). In a Finnish study of the prevalence of the abnormal LFTs and its relationship to clinical findings in type 2 diabetes, 57 % of subjects had at least one abnormal LFT and 27 % had at least two LFT tests (Salmela et al., 1984). GGT is recognised to be associated with type 2 diabetes,

alcohol intake, cigarette smoking, coronary heart disease, BMI, systolic blood pressure, serum triglyceride, heart rate, uric acid, haematocrit and negatively associated with the level of physical activity (Wannamethee et al., 1995). Type 2 diabetic patients exhibit a higher prevalence of elevated ALT (22.9 vs. 5.3 %, $p<0.01$) and GGT (23.7 vs. 10.5 %, $p<0.01$) than those with type 1 diabetes (Salmela et al., 1984), though most with NAFLD do not necessarily have ALT and GGT levels above the "normal" range per se but with levels in the high normal range. This fits with findings in a four-country study of detected NAFLD where average ALT levels were generally in this range, being highest in the UK which uses more imaging for NAFLD diagnosis (Alexander et al., 2019). However, the clinical relevance of the present study is to establish whether sequential change in liver enzymes is indicative of change in liver fat, and the demonstration of clinically utility paves the way for routine use in diabetes care. The data are supported by previous demonstration of sequential change both before diagnosis of type 2 diabetes and during overfeeding studies (Sattar et al., 2007, Sevastianova et al., 2012).

The upper level of ALT which may be regarded as normal has been challenged as this has been based upon the general population including overweight and obese individuals. Acceptance of a normal range for ALT and GGT based upon a population shown not to have NAFLD would pave the way for reassessment of the value of point measurements of these enzymes for diagnosis of NAFLD. In one study, that used MRS for the liver triglycerides quantification, the optimum ALT cut-off to predict NAFLD was determined to be 23 IU/L identified in 94% of people with fatty liver (Martin-Rodriguez et al., 2017). This study showed that ALT could be still a sensitive biomarker of NAFLD if the normal range of ALT is revised and set to a lower level. A further study confirmed this, also recommending a downward revision of ALT cut-off for NAFLD diagnosis (25 IU/L for men and 17 IU/L for women) (Miyake et al., 2012). This would be of great relevance in monitoring of type 2 diabetes and its remission, but also would be useful for general use. It is well established that there is an increased incidence of adverse cardiovascular events in people with NAFLD compared with the general population (Wannamethee et al., 1995, Lee et al., 2006, Schindhelm et al., 2007, Dunn et al., 2008). The early recognition of those at risk of NAFLD and promptly intervention is likely to become more feasible to intervene earlier and reduce the associated cardiovascular mortality.

The complete return of liver fat content to normal after weight loss underscores the relationship of NAFLD with overnutrition (Browning et al., 2004, Wong et al., 2012). The clinical utility of tracking the return to normal using liver function tests or derived indices is of relevance not only to monitoring liver health during remission of type 2 diabetes but also to the wider management of people with presumed or proven NAFLD. Several derived indices were developed to detect fatty liver. The Fatty Liver Index (FLI) (Bedogni et al., 2006) and Hepatic Steatosis Index (HSI) (Lee et al., 2010) are imprecise for diagnosis of NAFLD, but like plasma liver enzymes, both FLI and HSI are useful for sequential demonstration of change after weight loss, especially when baseline levels are raised. However, both require some form of calculation which makes their use less intuitive. Several scores were designed to non-invasively estimate liver fibrosis: Fib-4 score (Sterling et al., 2006), Fibrotest (Imbert-Bismut et al., 2001), Hepacore (Adams et al., 2011), BARD score (Ratziu et al., 2000), NAFLD fibrosis score (NFS) (Angulo et al., 2007). Fib-4 and NFS are considered the most accurate for ruling out advanced liver fibrosis with negative predictive values of more than 90 %. The present study further validates that FLI and HIS are useful for detecting fatty liver disease. The observation of decrease towards normal of the BARD score at 2 years after dietary weight loss is potentially of great importance.

The resolution of NAFLD can be also seen in patients with the large weight loss post bariatric surgery. In one study 85 % of morbid obese patients who lost 25 % of their initial body weight had a resolution of the NAFLD a year after surgery (Lassailly et al., 2015). However, a mean weight loss of 13.5 % after either bariatric surgery or dietary weight loss achieved a maximum decrease in liver fat (Steven et al., 2016c). This is supported by meta-analysis of the bariatric surgery studies (Aguilar-Olivos et al., 2016). With the degree of weight loss achieved and sustained at one year post bariatric surgery, the NAFLD fibrosis score and BARD score both decreased (Nickel et al., 2018).

Limitations of the present study must be considered. Firstly, the study was specifically set up to look at the rates of the remission of early type 2 diabetes following weight loss

rather than an examination of NAFLD in people with type 2 diabetes. Secondly, the exclusion criteria did not specifically exclude those with high alcohol consumption although no participants reported a high alcohol consumption. Thirdly, the intensive nature of DiRECT precluded performance of liver biopsies to confirm a relationship with hepatocyte ballooning, liver inflammation, or fibrosis (Bril et al., 2019). Nonetheless, the change in both fibrosis and steatosis scores were unambiguous. Another limitation is that LFTs were not measured immediately after the weight loss period when maximum change was observed (Lean et al., 2018). However, at 12 months, there was still a correlation between extent of weight loss and decrease in liver fat, which is important to see as at that stage patients were in stable weight phases. Finally, in DiRECT, all other causes of liver disease were not ruled out as it was not in the study design.

In summary, weight loss produced profound decrease in liver fat content which is raised in type 2 diabetes. Following weight loss, decreases in ALT and GGT levels generally reflect change in liver fat content. When baseline levels are raised, these tests have a high positive predictive value for normalisation of liver fat content. Both change in ALT and GGT within the 'normal' range as well as FLI and HSI correlated with change in liver fat content, although it must be noted that in multivariate analysis change in ALT but not GGT was independently associated with decrease in liver fat. The data emphasize the need to establish the upper limits of normal which may reflect the levels of a healthy, non-obese population. Currently, high values in range accepted as normal appear to be associated with adverse outcomes. One of the most important implications of the data described in this chapter is the recognition of the value of sequential monitoring following weight loss of liver enzymes and calculation of indices which can provide useful indication change in liver fat in day-to-day clinical practice. The overall postulation of the hypothesis under test was confirmed.

Chapter 5. Effect of weight loss on lipid metabolism

5.1 Introduction

The prevalence of type 2 diabetes continues to rise despite efforts to control this disease globally. Over the past 40 years, pharmaceutical agents have proven relatively ineffective in controlling the epidemic or in avoiding complications of diabetes (Zheng et al., 2018, Cho et al., 2018). The Diabetes Remission Clinical Trial (DiRECT) has demonstrated that weight loss induced by calorie restriction can achieve long-term remission of diabetes (Lean et al., 2018, Lean et al., 2019). These findings have led to important changes in European and US clinical guidelines to treat type 2 diabetes (Davies et al., 2018). Clarification of the underlying pathophysiologic mechanisms that explain remission is critical to understanding type 2 diabetes. The twin cycle hypothesis was proposed over 10 years ago to explain the ethiology of type 2 diabetes development and the mechanisms of reversal to normal (Taylor, 2008). Its predictions have been confirmed in several studies, all identifying the importance of accumulation of excess fat within the liver and pancreas (Lim et al., 2011b, Steven et al., 2016b, Taylor et al., 2018b).

The association between type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) is well recognized, and lipid metabolites have been shown to compromise hepatic insulin sensitivity and control of glucose production (Birkenfeld and Shulman, 2014, Perry et al., 2018). Hepatic VLDL-TG production is raised in NAFLD (Adiels et al., 2008).

In health, about 80% of fatty acid substrate for VLDL-TG export in the fasting state derives from adipose tissue lipolysis (Donnelly et al., 2005, Adiels et al., 2008), compared with less than 4% from lipogenesis (Barrows and Parks, 2006). However, when liver fat levels are raised the contribution of de novo lipogenesis to VLDL-TG is considerably greater (Donnelly et al., 2005, Lambert et al., 2014). Insulin resistance in muscle and failure of storage of meal-derived glucose as glycogen in people with type 2 diabetes

(Carey et al., 2003) enhances de novo lipogenesis specifically as it is the only other pathway to achieve storage of the energy in glucose.

If subcutaneous adipose tissue is unable to accommodate more triglyceride when hepatic triglyceride is increased, ectopic fat accumulation is likely in many tissues including the pancreas (Taylor and Holman, 2015b, Lotta et al., 2017) and long-term exposure to saturated fatty acids is harmful to β-cells (Pinnick et al., 2008, Eguchi et al., 2012, Jezek et al., 2018). Loss of β-cell specialist function through dedifferentiation is now widely accepted to explain the impairment of β-cell function in type 2 diabetes (Talchai et al., 2012, White et al., 2013, Cinti et al., 2016, Bensellam et al., 2018). β-cell dedifferentiation can be promoted by metabolic stress induced by high concentration of glucose or fatty acids (White et al., 2016b, Taylor et al., 2019b).

A profound fall in liver fat and in intra-pancreatic fat during weight loss induced reversal of type 2 diabetes, was associated with a return of beta cell function for at least six months (Lim et al., 2011b, Steven et al., 2016b). DiRECT was designed to determine what proportion of people with diabetes could be returned to non-diabetic glucose control in routine primary care. The study also aimed to identify the underlying pathophysiologic changes associated with remission. At 24 months, 36 % of the intervention cohort were in sustained remission (Lean et al., 2019). The metabolic changes during the first year of remission have been reported (Taylor et al., 2018b). The present study tested the hypothesis that weight loss would produce a clinically significant lowering of hepatic export of VLDL-TG. The design is illustrated in Figure 1. In a geographically defined sub-group of DiRECT, we examined the effect of weight loss intervention on hepatic VLDL-TG export, plasma VLDL-TG levels, intra-pancreatic fat content, and restoration of β-cell function for up to 24 months after weight loss. As palmitic acid has the greatest deleterious effect of all fatty acids on beta cell function and is the predominant fatty acid synthesised by de novo lipogenesis (Cnop et al., 2001, Eguchi et al., 2012, Pinnick et al., 2008, Elks, 1993b, Maedler et al., 2003), VLDL-TG palmitic acid content was also quantified. Additionally, the 24-month data permit observation of the changes, which

underlie the development of type 2 diabetes in the group initially achieving remission but who relapsed back to diabetes. All data were compared with those from a non-diabetic comparator group selected to match the type 2 diabetes group after weight loss. For the first time, we are able to report the underlying physiologic changes during a full cycle of disease reversal and re-emergence.

5.2 Study design

For this study, data were evaluated on those subjects with complete lipoprotein data both at baseline and post weight loss, who were recruited in the Tyneside Intervention subgroup of DiRECT. Studies were also conducted on 25 non-diabetic comparators (NDC), selected to match the intervention group for weight in the post-weight loss state (Figure 5.1), and studied at baseline only.

56 people with early type 2 diabetes were recruited in the Tyneside cohort of DiRECT by their general practices (n=56, 26F/30M, (mean± SD): age 53.1±7.6 years, weight 100.9±17.3 kg, BMI 35.2±4.6 kg/m^2, diabetes duration 3.1±1.7 years, HbA1c 7.5±1.0 %). Inclusion criteria were diabetes duration of <6 years, age between 20-65 years, HbA1c $\geq$ 6.5 % ($\geq$ 6.1 % if anti-diabetes agents are used), and BMI of 27-45 kg/m^2.
All NDC underwent an oral glucose tolerance test to demonstrate normality (n=25, 12F/13M, (mean± SD): age 55.8±6.0 years, weight 86.6±14.9kg, BMI 29.7±3.8 kg/m^2, HbA1c 5.4±0.3 %). Stepped Insulin Secretion Test with Arginine stimulation (SISTA) was used to define beta cell function in response to intravenous glucose challenge (Lim et al., 2011b, Toschi et al., 2002, Zhyzhneuskaya et al., 2020) as described in Chapter 7 of this thesis.

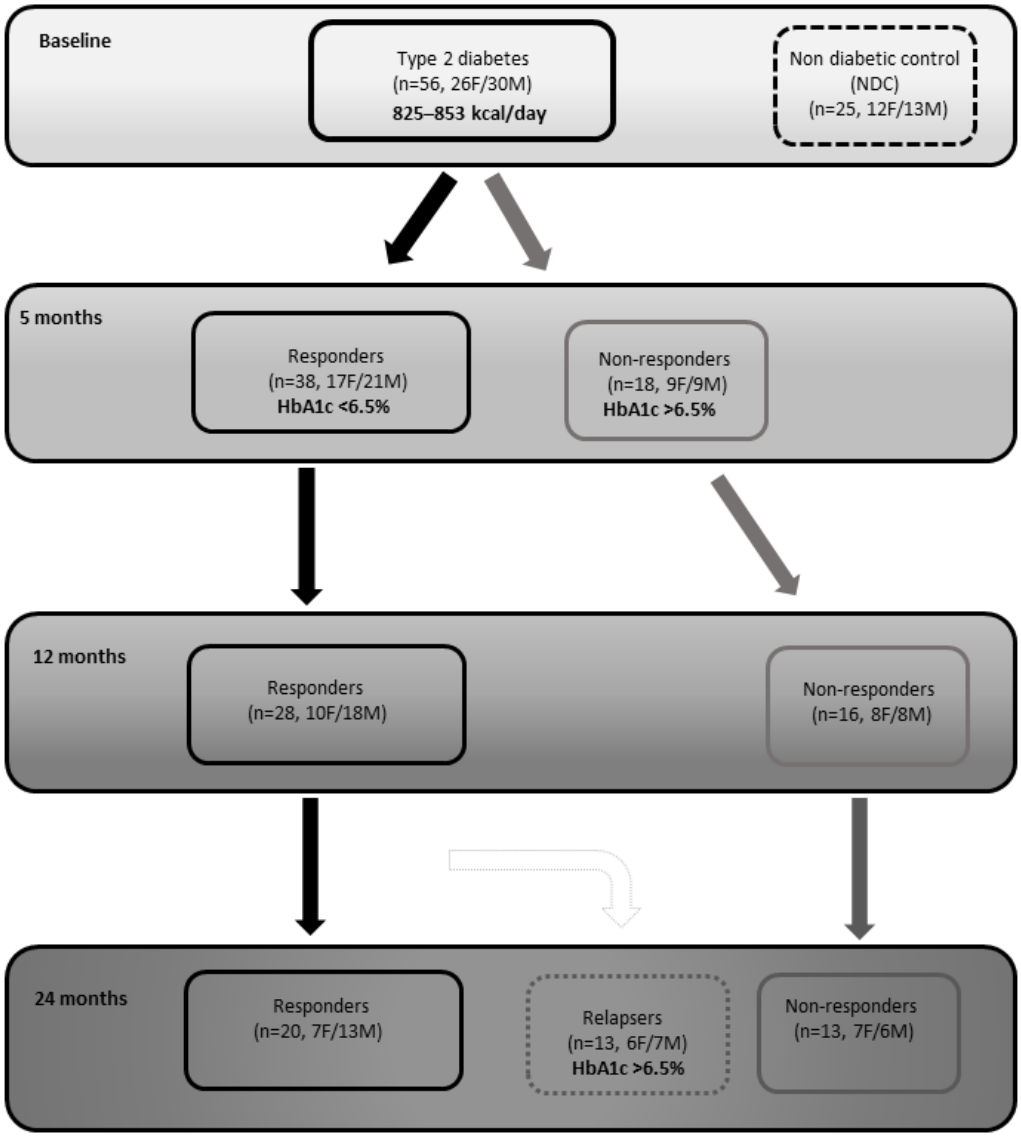

Figure 5.1 Illustrative diagram of the study design.

56 Participants were randomized to receive a low-calorie diet (825-853kcal/day) for 3-6 months, followed by stepped food reintroduction (SFR) and weight maintenance up to 24 months. After weight loss and reintroduction of weight maintenance diet (5 months on average), participants were classified as responders or non-responders based on HbA1c <48 mmol/mol (<6.5%) and blood glucose <7 mmol/l off any anti-diabetes agents. Detailed metabolic tests were carried out at baseline, 5 months, 12 months, and 24 months. A group of nondiabetic controls (NDC) matching for the type 2 diabetes group was selected and studied at one single occasion (BMI was selected to match after weight loss). A group who initially reversed diabetes but who lost remission by 24 months (HbA1c >48 mmol/mol (>6.5%) and blood glucose >7 mmol/l) was studied separately (Al-Mrabeh et al., 2019).

5.2.1 Intralipid infusion and lipoprotein separation

This was performed on the first day of the study as described in the previous study (Al-Shayji et al., 2007). Antecubital veins of both arms were cannulated with large intravenous cannula (18g Green). Blood was withdrawn at baseline followed by a bolus of 20 % Intralipid (Fresenius Kabi Ltd, UK) at (0.1 g/kg body mass) through one cannula within 60 seconds followed immediately by a continuous infusion of 10 % Intralipid at 0.1 g/kg/hr by using infusion pump (Arcomed Infusion Ltd, UK). At 75 minutes, cannula was removed, and breakfast was given to the participant. During infusion, blood samples were taken at 5, 15, 30, 45, 60, and 75 minutes. After two steps of low-speed centrifugation at 4°C, to remove blood cells then chylomicrons plus Intralipid particles (Scientific Laboratory Supplies Ltd, UK), plasma samples were ready for lipoprotein separation.

VLDL1 (Sf 60-400) was isolated from plasma by cumulative ultracentrifugation density gradient technique as reported by (Lindgren et al., 1972) with some modification. Density solutions are prepared from stock solutions at density 1.006g/ml (0.195 M NaCl/0.001 M NaOH/0.001 % Na2EDTA), and d 1.182 g/ml (2.44 M NaBr /0.195 M NaCl/0.001M NaOH/0.001 % Na2EDTA/ (Sigma-Aldrich, UK, Alfa Aesar, USA, and VWR International Ltd, UK). The density of the prepared solutions was measured using analytical balance (Ohaus, Switzerland) and adjusted with de-ioinised water.

2 ml of plasma was adjusted to 1.118 g/ml by adding 0.341 g of NaCl (Sigma-Aldrich, UK), then carefully layered over a 0.5 ml of 1.182 g/ml density solution in an ultracentrifuge tube pre-coated with polyvinyl alcohol (SETON SCIENTIFIC, INC, USA) using a multichannel peristaltic pump (Joyfay International, US). A density gradient was formed by layering 1ml of 1.0988 g/ml, 1ml 1.0860 g/ml, 2 ml d 1.0790 g/ml, 2 ml 1.0722 g/ml, 2 ml d 1.0641 g/ml, and 2 ml 1.0588 g/ml. Centrifugation was carried out in a Beckman SW40 rotor at 23 °C in a Beckman L7-80 ultracentrifuge at 278,000 g for 98 minutes with deceleration (Beckman Coulter, Inc, USA). VLDL1 fractions was removed from the top of the tube using a finely drawn glass Pasteur pipette (VWR International Ltd, UK), and stored at 4°C until TG was measured then the VLDL1 fraction was stored at -40 °C.

5.2.2 Intra organ and abdominal fat quantification

Magnetic Resonance (MR) was used for quantification of pancreatic and hepatic fat as previously was described (Al-Mrabeh et al., 2017, Al-Mrabeh et al., 2016). This was carried out at baseline, following return to isocaloric eating after weight loss, at 12 months, and 24 months. Liver fat content was measured by selecting homogenous regions of interest on five image slices of liver (Lim et al., 2011b). Intra-pancreatic fat content was quantified using the MR-opsy method optimized to exclude interlobular adipose tissue areas (Al-Mrabeh et al., 2017).

Three-point Dixon MRI was also acquired at the level of the L4-L5 intervertebral space to estimate subcutaneous and visceral fat (SAT/VAT). Thresholding and watershed analysis using ImageJ were applied to calculate VAT and SAT from the proton density fat fraction map (Al-Mrabeh et al., 2017). Analyses of pancreas fat and abdominal fat were carried out by single observers in a blinded manner (Pancreas fat: AAM; abdominal fat: AJ).

5.2.3 Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared from the VLDL1 fraction and total plasma following direct transesterification using 14 % Boron Trichloride-Methanol solution (Sigma Aldrich, UK), and based on published procedures (Lepage and Roy, 1986, McEneny et al., 2000). Briefly, 200 µl of VLDL1 or plasma was transferred to 10 ml Pyrex glass culture tubes (Sigma-Aldrich, UK), and spiked with 5 µl of Nonadecanoic acid, analytical standard (Sigma-Aldrich, UK). Afterwards, 2ml of 14 % Boron Trichloride-Methanol (Sigma-Aldrich, UK) was added and the tubes were properly capped and

vortexed for 1minute (Vortex-Genie 2, Scientific Industries Inc, USA), and then the mixture was incubated at 65°C for 2 hours.
Tubes were kept at room temperature for 5 min, then 1 ml of 40 -60°C analytical grade, petroleum spirit (VWR International Ltd, UK), and 1 ml of de-ioinised water were added to each tube followed by vigorous vortexing for 1 minute. Then samples were centrifuged at 3000 RCF for 5 minutes at room temperature (Mistral 3000i, MSE, UK). 0.8 ml of the upper petroleum spirit layer was transferred to a clean Pyrex glass tube, then gentle stream of O2 free nitrogen (BOC Ltd, UK) was applied under incubation in Thermoblock at 45°C for around 5 minutes until compete dryness. FAME were reconstituted in 50 µl (100 µl for plasma samples) and transferred to MS glass vial (Sigma-Aldrich, UK).

For identification and quantification of fatty acids, the Thermo "Voyager" single quadruple mass spectrometer attached to Thermo "Trace" gas chromatograph (Thermo Scientific, Germany) equipped with SLB-IL60 Capillary GC Column (L × I.D. 30 m × 0.25 mm, df 0.20 µm, Sigma-Aldrich, UK). Helium (BOC Ltd, UK) was used as a carrier at 1.2 ml/min, and oven temperature was programmed to start at 170°C ramping at 10 °C/min until 225°C then hold for 2 min. Injector and source temperature were 250°C and 300°C, respectively.

1 µl of the sample was injected in the split mode (1:20), and data were acquired in full mode scan using version 1.3 of Xcalibur software (Thermo Scientific, Germany). FAMEs were identified based on spectral information and retention time compared with known peaks from the Supelco 37 Component FAME Mix (Sigma-Aldrich, UK)(Al-Mrabeh et al., 2019, White et al., 2016a).

5.2.4 Quantification and statistical analysis

Analyses were conducted on all subjects with paired data both before and after weight loss. Paired data were analysed as presented in the Figures (and data on all subjects at

each time point in Table 5.1). Data are presented as mean ± SEM or median (IQ range) based on data distribution. Student paired or two-sample t test was used as appropriate for parametric data and Mann Whitney U test for nonparametric data. Stepwise logistic regression analysis was carried out using baseline to 24 months changes in all studied metabolic parameters. P value <0.05 was considered as significant. People who withdrew from the study were automatically excluded from the analysis due the paired nature of data analysis.

This study was designed to compare change in parameters between responders and non-responders, assuming a 60 % emission at 5 months and 25 % loss during the follow up visits. It was powered on the most stringent variable (change in pancreas fat) in responders compared with non-responders. The calculated sample size was achieved by randomising a greater proportion of general practices to Intervention in the Tyneside region. As there was 69 % remission of diabetes after weight loss, 64 % at 12 months, and 61 % at 24 months, the above assumptions for statistical analysis were satisfied(Al-Mrabeh et al., 2019).

5.3 Results

5.3.1 Effect of weight change on glucose control and fasting insulin

At 24 months, weight loss was not significantly different in Responders and Non-responders (-10.5±1.5 vs. -8.4±1.4 kg, p=0.33) and 20/33 people (61 %) remained in remission. Remission rates had been 40/58 (69 %) at 5 months and 29/45 (64 %) at 12 months (Taylor et al., 2018b). In those who reverted to diabetes after initial remission (relapsers; n=13), weight gain was 11.3±1.9 kg between 5 and 24 months compared with 6.6±1.0 kg in those with sustained remission (p=0.036).

HbA1c fell from 57.4±2.2 mmol/mol to 42.1±1.1 mmol/mol (p<0.0001) in those who maintained in remission of diabetes. HbA1c was 35.5±1.1 mmol/mol in non-diabetes comparators. There was no significant change in Non-responders (62.8±2.2 to 65.0±4.4 mmol/mol (p=0.53). Likewise, fasting plasma glucose decreased from 8.4±0.7 mmol/l to

5.6±0.2 mmol/l at 24 months (p<0.0001) in Responders but did not change in Non-responders (9.7±0.8 to 9.3±1.1 mmol/l, p=0.72). In those who had relapsed by 24 months, HbA1c rose from 42.16.0±1.1 mmol/mol at 5 months to 63.9±4.4 mmol/mol at 24 months, (p<0.0001). There was a corresponding increase in fasting plasma glucose over the same period (5.9±0.2 to 8.1±0.6 mmol/l, p=0.003).

At baseline fasting plasma insulin was over three-fold elevated in type 2 diabetes compared with NDC (97.7±7.6 vs. 27.4±4.8 pmol/l, p<0.0001). This decreased substantially after weight loss (Figure 5.2.B) and remained low at 24 months in both Responders and Non-responders (107.2±15.7 to 50.8±10.1, and 66.5±6.4 to 35.5±6.0 pmol/l, p<0.0001, respectively).

5.3.2 Effect of weight change on lipid variables

Liver fat

At baseline, liver fat was elevated in the whole diabetic group compared with non-diabetes comparators (16.0±1.6 vs. 4.4±1.1 %, p<0.0001). Straight after weight loss, levels normalised similarly in Responders and Non-responders (3.4±0.7 vs. 2.6±0.5 %, p=0.69, (Taylor et al., 2018b). There was a gradual increase in liver fat at 12 and 24 months in both Responders and Non-responders (Figure 5.2A, Table 5.1), but remained close to normal at 24 months (p=0.28 and 0.05 vs. non-diabetic comparators). Change in liver fat reflected change in body weight between 0-12 months (r=0.45, p=0.003), and 0-24 months (r=0.59, p<0.0001) (Figure 5.3A).

	Baseline	***5 months***	***12 months***	***24 months***	***NDC***
Responders Non-responders Relapsers	*(n=38)* *(n=18)* *(n=13)*	*(n=38)* *(n=18)* *(n=13)*	*(n=28)* *(n=16)* *(n=13)*	*(n=20)* *(n=13)* *(n=13)*	*(n=25)*
Weight (kg) Responders Non-responders Relapsers	 99.3±4.3 100.1±4.4 100.5±4.0	 82.2±3.2*** 88.7±4.4*** 84.6±3.4	 84.7±3.6*** 91.9±4.9*** 90.2±4.0**	 88.8±4.0*** 90.3±4.0*** 95.9±4.4*#	 86.6±3.0
Liver fat (%) Responders Non-responders Relapsers	 18.8±2.4 14.5±2.6 12.1±2.0	 3.0±0.9*** 2.6±0.5*** 2.1±0.5***	 3.2±0.8*** 5.3±1.8*** 4.7±1.9**	 6.6±1.6***## 8.7±1.8## 8.3±1.4*##	 4.4±1.1
VLDL1-TG PR. (mg/kg/day) Responders Non-responders Relapsers	 563.5±32.5 581.1±52.1 457.6±39.9	 388.6±37.5** 521.8±41.9 406.1±42.2	 427.0±25.6** 649.6±67.0 506.5±39.6	 480.7±30.7*# 638.2±38.6 561.3±37.3#	 457.0±28.2
VLDL1-TG pool (mg) Responders Non-responders Relapsers	 2488±267 2775±505 2690±484	 1245.4±162** 1866±342 1328 ±272 *	 1379±205* 2234±570 1677±296	 1415±238* 2109±563 3014±668#	 1581±332
Plasma VLDL1-TG (mmol/l) Responders Non-responders Relapsers	 0.71±0.07 0.73±0.11 0.77±0.14	 0.43±0.06** 0.55±0.12 0.46±0.10	 0.46±0.07 0.64±0.12 0.55±0.10	 0.44±0.07 0.66±0.15 0.88±0.16#	 0.48±0.09
Total plasma TG (mmol/l) Responders Non-responders Relapsers	 1.84±0.13 1.91±0.25 1.78±0.19	 1.30±0.13*** 1.24±0.14*** 1.28±0.14*	 1.26±0.12** 1.21±0.12** 1.41±0.13	 1.14±0.10*** 1.34±0.14* 1.68±0.23#	 1.2±0.1
HDL Cholesterol (mmol/l) Responders Non-responders Relapsers	 1.08±0.06 1.00±0.05 1.07±0.07	 - - -	 1.23±0.08* 1.12±0.06* 1.22±0.10*	 1.43±0.12** 1.22±0.08* 1.14±0.09	 1.42±0.07
NEFA (mmol/l) Responders Non-responders Relapsers	 0.57±0.03 0.66±0.04 0.65±0.08	 0.54±0.03 0.59±0.05 0.58±0.06	 0.51±0.03 0.61±0.04 0.60±0.06	 0.55±0.03 0.76±0.03 0.63±0.07	 0.57±0.04
VLDL1 C16:0 (µmol/L) Responders Non-responders Relapsers	 45.0±4.6 67.3±7.4 47.4±8.3	 33.5±4.2** 50.7.9* 33.4±8.5	 33.9±4.7* 53.3±6.3 50.0±7.7##	 31.6±5.4* 62.0±9.7 74.1.2±8.1*##	 28.4±3.2
Pancreas fat (%) Responders Non-responders Relapsers	 9.7±0.6 7.9±0.6 8.1±0.5	 8.5±0.6*** 7.1±0.5** 7.1±0.5**	 8.0±0.4*** 6.9±0.5* 7.7±0.6	 8.0±0.6*** 7.0±0.3 8.0±0.6#	 6.2±0.4
SAT (cm^2) Responders Non-responders Relapsers	 320.2±20.7 313.5±26.9 332.9±38.6	 233.3±19.3*** 251.6±29.1*** 244.0±34.4***	 231.2±19.1***## 296.6±37.4**### 280.9±34.7*#	 254.4±27.6**### 294.3±35.2**## 310.95±29.7##	 264.3± 19

VAT (cm^2)					
Responders	281.9 ±12.7	159.5±10.2***	175.3±14.0***##	207.4±20.8***###	
Non-responders	253.9±19.5	162.7±19.5***	180.0±23.6**#	184.0±16.7*###	193.9±23.5
Relapsers	275.7±17.9	154.0±13.2***	187.9±19.9***#	245.5±20.8*###	

Table 5.1 Changes in main metabolic markers over 24 months of study follow up.

Statistics analyses were carried out on paired data between baseline and other time points. Baseline data paired with 5 months were presented; baseline data paired with 12 and 24 months were used in the main body text, but not presented in the table. 24 months relapsers are shown separately as a group for each time point even though they contribute to the data on responders at 0, 5, and 12 months. Therefore, the numbers do not add to the total (* p<0.05 vs. Baseline, ** p<0.01 vs. baseline, *** p<0.001 vs. baseline. # p<0.05 vs. 5 months, ## p<0.01 vs. 5 months, ### p<0.001 vs. 5 months)(Al-Mrabeh et al., 2019).

VLDL1-TG production

At baseline, VLDL1-TG production rate was higher in the whole diabetic group compared with non-diabetic comparators (556.2±25.5 vs. 457.0 ±28.2 mg/kg/day, p=0.01). This has been observed in both non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes and the difference is particularly marked for plasma levels of VLDL1-TG (Adiels et al., 2008). After weight loss, production rates decreased to 448.4±22.9 mg/kg/day (p<0.0001), similar to non-diabetic comparators (457.0 ±28.2 mg/kg/day; p=0.81). This is consistent with our earlier observations (Steven et al., 2016b).

In Responders, there was a 24 % decrease in VLDL-TG production (544.4±28.7 to 413.6±25.8 mg/kg/day; p<0.0001) at 5 months, remaining decreased to 24 months and similar to the non-diabetic comparators (480.7±30.7mg/kg/day, p=0.032 vs. baseline) (Figure 5. 2D, Table 5.1). The modest decrease in Non-responders was not significantly different from that in responders (p=0.24), although non-significant compared with baseline (10 %: 581.1 to 521.8 mg/kg/day; p=0.28). It remained higher at 12 and 24 month post-weight loss than in non-diabetic comparators (649.6±67.0 and 638.2±38.6

vs. non-diabetic comparators: 457.0 ±28.2 mg/kg/day, p=0.003, and p=0.001 respectively, Figure 5.2D, Table 5.1).

At baseline, there was a positive correlation between liver fat and VLDL1-TG production both in type 2 diabetes (r=0.36, p=0.007) and in non-diabetic comparators (r=0.49, p=0.014). There was also a positive correlation between changes in liver fat and VLDL1-TG production from baseline to 5 months in the type 2 diabetes group (r=0.47, p<0.0001) and in Responders (r=0.46, p=0.004) and Non-responders (r=0.50, p=0.04), separately (Figure 5.3).

Although the decrease in liver fat was similar in both Responders and Non-responders, the continuing hyperglycaemia in non-responders would be expected to enhance de novo lipogenesis and so potentially blunt the response in VLDL-TG production rate to weight loss.

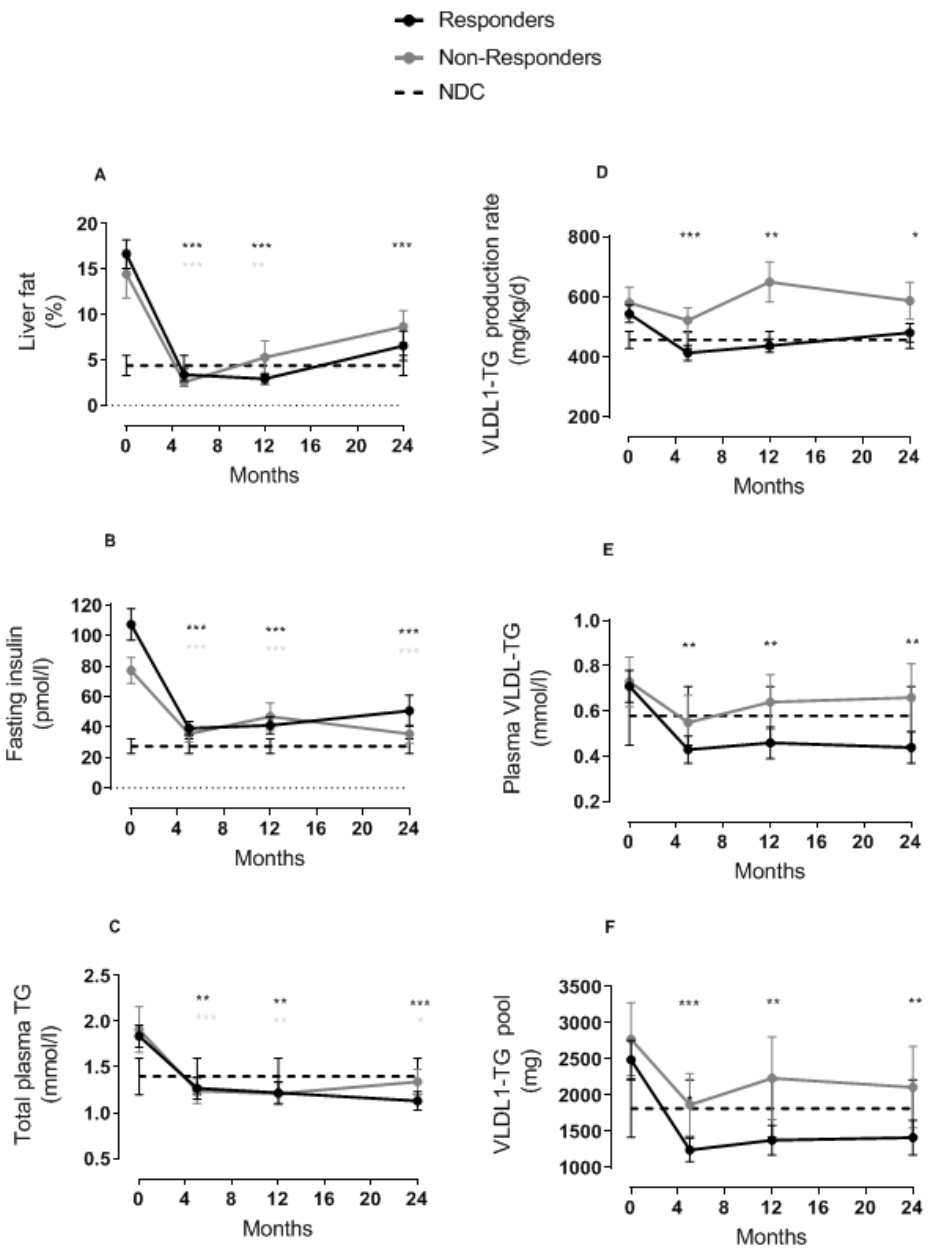

Figure 5.2 Changes in Hepatic VLDL1-TG metabolism over 24 months before and after intervention.

Liver fat (A), fasting plasma insulin (B), total plasma TG (C), hepatic VLDL1-TG production (D), fasting plasma VLDL1-TG (E), and VLDL1-TG pool (F) at baseline, post weight loss (5 months), 12 months, and 24 months. Responders are presented as a solid black line, non-responders as a solid grey line, and NDC (measured on one occasion) as a dotted black line (data are presented as means ± SEM. * $p<0.05$ vs. baseline, ** $p < 0.01$ versus baseline, *** $p < 0.0001$ versus baseline)(Al-Mrabeh et al., 2019).

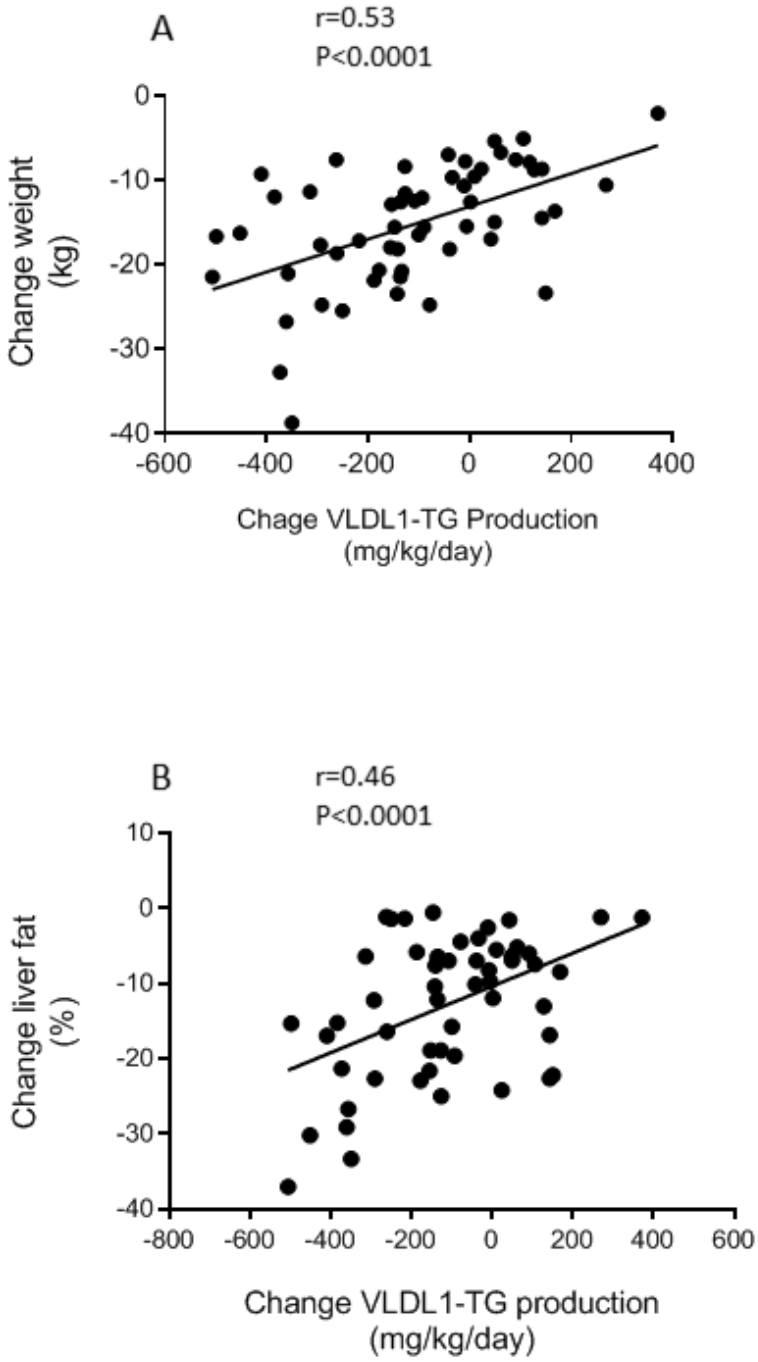

Figure 5.3 Relationships between changes in weight liver fat and hepatic VLDL1-TG export.

Correlations between change in VLDL1-TG production and changes in weight (A), and liver fat (B) between baselines to 5 months in the whole type 2 diabetes group (Al-Mrabeh et al., 2019).

Plasma VLDL1-TG and pool size

At baseline, fasting plasma VLDL1-TG concentration was higher in type 2 diabetes compared with NDC (0.72±0.06 vs. 0.48±0.09 mmol/l, p=0.012). This decreased immediately after weight loss (to 0.47±0.05 mmol/l; p=0.0008) (Figure 5.2E, Table 5.1). Reflecting the VLDL-TG production rate, baseline fasting plasma VLDL1-TG concentrations were similar in Responders and Non-responders (0.71±0.07 vs. 0.73±0.11 mmol/l). The weight loss induced decrease was not significantly different between groups but declined significantly after weight loss only in Responders (39 %; to 0.43±0.06 mmol/l; p=0.003). In Non-responders, there was a smaller decline (25 %; to 0.55±0.12 mmol/l; p=0.12).

Plasma VLDL1-TG concentration remained non-significantly lower in Responders than Non-responders at 12 months (0.46±0.09 vs. 0.64±0.12 mmol/l, p=0.15) and this persisted to 24 months (Responders: 0.44±0.07 mmol/l, Non-responders: 0.66 ±0.15 mmol/l, p=0.24) (Figure 5.2E).

At baseline, VLDL1-TG production correlated with fasting plasma VLDL1-TG (r=0.34, p=0.01) and after weight loss this correlation became stronger (r=0.72, p<0.0001). A similar pattern was observed in Responders (r=0.25, p=0.13, to r=0.70, p<0.0001) and in Non-responders (r=0.53, p=0.04, to r=0.89, p<0.0001).

The VLDL1-TG pool size was larger in type 2 diabetes compared with non-diabetic comparators (2581± 241 vs. 1581 ± 332mg, p=0.004) (Figure 5.2F) and decreased after weight loss (to 1445±179 mg, p<0.0001; p=0.97 compared with non-diabetic comparators). Baseline VLDL1-TG pool sizes were similar in Responders and Non-responders (2488±267 vs. 2775±505 mg) and decreased significantly only in Responders (1245±162 mg; p=0.0002; Non-responders 1866±342; p=0.11). The pool size was stable in Responders at 12 and 24 months at 1379±205 mg and 1415±1064 mg, respectively. In Responders, VLDL1-TG pool size remained significantly different from baseline (p=0.001), whereas it increased in Non-responders and returned to near baseline values (2234±570 mg, p=0.53 and 2110±536 mg; p=0.82 at 12 and 24 months respectively)

(Figure 5.2F). These changes in VLDL1-TG pool size reflect changes in both plasma concentrations and body weight. The elevated VLDL- pool size in type 2 diabetes compared with non-diabetic comparators raises the possibility of saturation of lipoprotein lipase activity due to high rates of substrate delivery. The stronger correlation between VLDL1-TG production and fasting TG after weight loss supports the concept of saturation of lipoprotein lipase activity under the baseline condition of elevated secretion of VLDL (Brunzell et al., 1973, Merkel et al., 2002).

Non-VLDL1-TG

Non-VLDL1-TG was higher in type 2 diabetes compared with non-diabetic comparators (1.13±0.08 vs. 0.74±0.08 mmol/l, p=0.002) and, after weight loss, fell similarly in Responders and Non-responders at all points up to 24 months (1.14±0.08 to 0.69±0.08 mmol/l, p=0.01 and 1.0±0.12 to 0.68±0.14 mmol/l, p=0.04, respectively) (Figure 5.4A, Table 5.1). The lack of difference in change in non-VLDL1-TG between Responders and Non-responders underscores the likely central role of VLDL1-TG in determining delivery of fatty acids to the pancreas. Non-VLDL1 predominantly reflects TG derived from chylomicrons as the TG content of LDL and HDL is minor.

Total plasma TG

At baseline, total plasma TG was higher in type 2 diabetes compared with non-diabetes comparators (1.86 ±0.1 vs. 1.22±0.1 mmol/l, p=0.0002). Plasma TG concentration decreased immediately after weight loss (to 1.28±0.1 mmol/l, p<0.0001) becoming similar to that of non-diabetes comparators (p=0.996, Figure 5.2C). As with non-VLDL-TG, the weight loss induced decrease was similar in Responders and Non-responders (1.84±0.13 to 1.30±0.13 mmol/l, p=0.0004, and 1.91±0.25 to 1.24±0.14 mmol/l, p=0.007, respectively) (Figure 5.4A). The change was maintained to 24 months in Responders and Non-responders (1.14±0.10 mmol/l, p<0.0001 vs. baseline, and 1.32±0.14 mmol/l, p=0.04 vs. baseline, respectively). There was a positive correlation between total plasma TG and VLDL-TG within the whole type 2 diabetes group at baseline (r=0.80, p<0.0001),

and this was maintained after weight loss (r=0.67, p<0.0001). However, the diabetes-related effects on plasma total TG appeared to be driven entirely by VLDL1-TG.

HDL Cholesterol

At baseline, HDL cholesterol concentration was low in type 2 diabetes compared with non-diabetes comparators (1.05±0.0.06 vs. 1.42±0.07mmol/L, p=0.0001). In Responders, HDL cholesterol increased steadily to 24 months (1.12±0.07 to 1.43±0.12mmol/L, p=0.001) becoming similar to NDC (p=0.96). HDL cholesterol increased also in non-responders between baseline and 24 months (1.0±0.06 to 1.22±0.08mmol/L, p=0.03) but remained non-significantly below the NDC level (p=0.07) (Table 5.1).

Non-esterified fatty acids

At baseline fasting plasma non-esterified fatty acids (NEFA) were similar in diabetic and non-diabetic participants (0.60±0.03 vs. 0.57±0.03 mmol/l, p=0.43) and there was no change after weight loss (0.60±0.03 to 0.56±0.03 mmol/l, p=0.22). This lack of effect was seen in both Responders (0.57±0.03 to 0.54±0.03 mmol/l, p=0.54) and Non-responders (0.66±0.04 to 0.59±0.05 mmol/l, p=0.29) (Table 5.1). However, Non-responders had significantly higher NEFA at baseline compared with Responders (0.66±0.04 vs. 0.54±0.03 mmol/l; p=0.04). Fasting plasma NEFA remained stable in Responders by 24 months (0.55±0.03 mmol/l) and the difference between groups increased (Non-responders 0.76± 0.05 mmol/l; p=0.002). Although fasting plasma NEFA may be slightly raised in type 2 diabetes, the individuals studied in DiRECT had short duration diabetes (< 6 years since diagnosis) and were relatively well controlled. The effect of poorer glucose control upon plasma NEFA (Karpe et al., 2011) is illustrated by the difference between Responder and the Non-responder groups during continued hyperglycaemia.

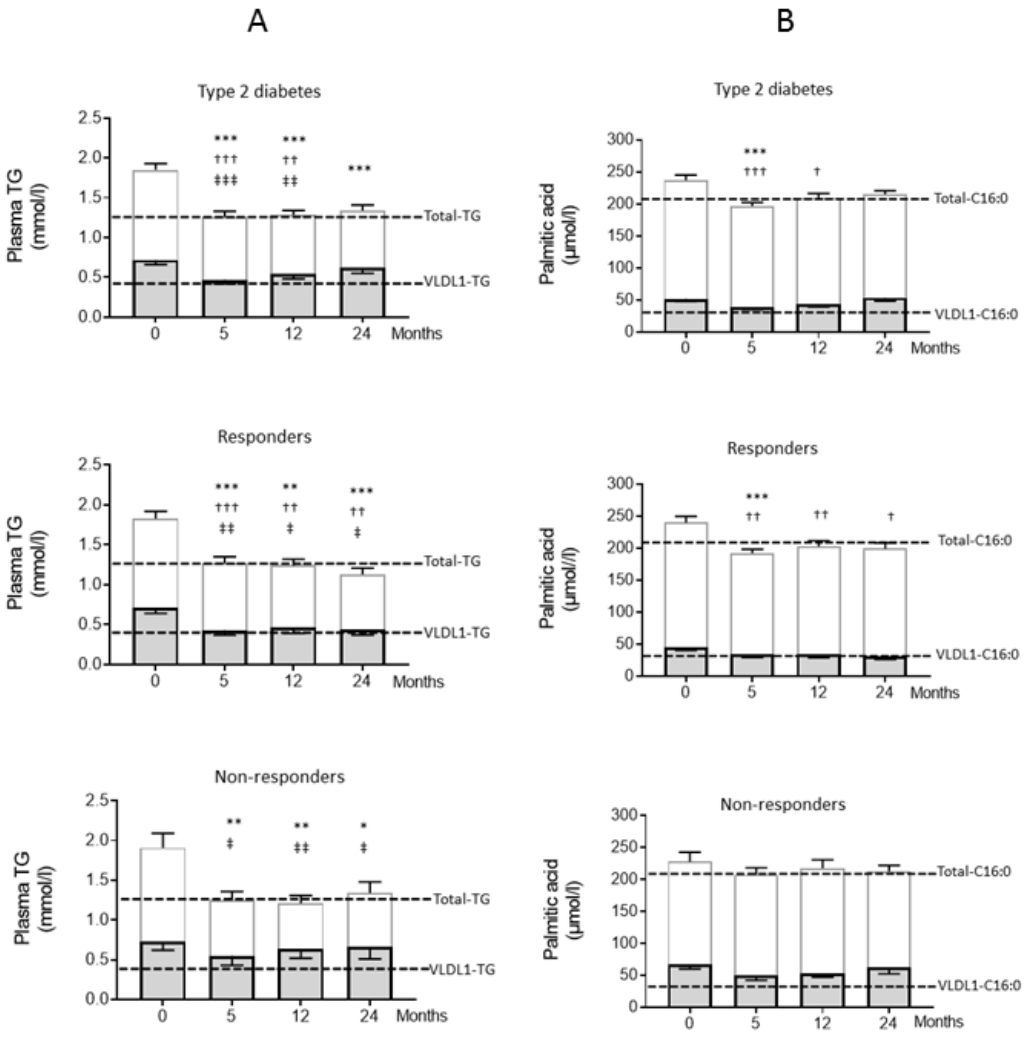

Figure 5.4 Changes in plasma triglycerides and palmitic acid flux over 24 months after initiation of intervention.

Panel A: Change in plasma VLDL-TG (grey), non-VLDL-TG (white), and total TG (sum of both) within the whole type 2 diabetes group, responders, and non-responders. Plasma levels of VLDL-TG and total TG in NDC (measured on one occasion) are represented by dotted lines. **Panel B**: Change in plasma VLDL-palmitic acid (grey), non-VLDL- palmitic acid (white), and total palmitic acid (sum of both) within the whole type 2 diabetes group, responders, and non-responders. Plasma levels of VLDL- palmitic acid and total palmitic acid in NDC (measured on one occasion) are represented by dotted lines. Data are presented as means ± SEM. *p<0.05 vs baseline (Total TG/ Total palmitic acid), **p<0.01 vs baseline (Total TG/ Total palmitic acid), ***p<0.001 vs baseline (Total TG/ Total palmitic acid). †p<0.05 vs baseline (VLDL-TG/ VLDL- palmitic acid), ††p<0.01 vs baseline (VLDL-TG/ VLDL- palmitic acid), †††p<0.001 vs baseline (VLDL-TG/ VLDL- palmitic acid). ‡p<0.05 vs baseline (Non-VLDL-TG), ‡‡p<0.01 vs baseline (Non-VLDL-TG), ‡‡‡p<0.001 vs baseline (Non-VLDL-TG)(Al-Mrabeh et al., 2019).

Substrate oxidation

Indirect calorimetry was carried out in the fasting state after 30 minutes of supine rest using a Quark ventilated hood calorimeter (COSMED, Italy). Substrate oxidation was derived by using standard equations (Frayn, 1983). Indirect calorimetry was performed on the Tyneside Intervention group. Data was available for 58 subjects at baseline and immediately after weight loss at 5 months, and for 45 subjects at 12 months (40 and 29 responders, 18 and 16 non-responders respectively). Indirect calorimetry was also performed on 25 Non-diabetic comparator group subjects on a single occasion.

The return to non-diabetic glucose control was accompanied by an increase in basal glucose oxidation rates and a fall in basal lipid oxidation rates. These changes were not fully developed immediately after weight loss, but maximal at 12 months. The glucose oxidation rates in Responders increased from 1.27±0.12 at baseline to 1.44±0.14 mg/kg/min at 5 months after weight loss, and to 2.00±0.19 mg/kg/min at 12 months during weight maintenance phase ($p<0.0001$ between baseline and 12 months). This was reflected in greater increase in responders between baseline and 12 months compared with non-responders (0.75 ±0.18 vs. -0.11±0.23 mg/kg/min; $p=0.006$). The glucose oxidation in non-diabetic comparators was 1.90±0.75 mg/kg/min.

Lipid oxidation rates decreased in Responders from 0.96±0.05 to 0.87±0.06 ($p<0.28$) at 5 months and to 0.64±0.09 mg/kg/min; $p<0.0001$) at 12 months versus 0.84±0.08, 0.89±0.11, and 0.83±0.07 respectively in Non-responders, with a greater decrease than in Non-responders over the same period (-0.32±0.08 vs. -0.05±0.09 mg/kg/min; $p<0.04$). Overall, there were no significant changes in substrate oxidation rates in Non-responders. For comparison, the lipid oxidation in non-diabetic comparator subjects was 0.57±0.38 mg/kg/min.

Resting energy expenditure (REE) decreased significantly in both Responders and Non-responders. In Responders it went up from 1,996.5±57.0 kcal/day to 1,647.1±48.1 kcal/day at 5 months ($p<0.0001$) and to 1,6996.4±67.3 kcal/day ($p<0.0001$) at 12 months. For Non-responders REE went down from 1,981.7±108.0 to 1,641.1±89.5 at 5

months (p<0.01 vs. baseline) and to 1,733.2±109.5 at 12 months (p<0.01 vs. baseline). REE in Non-diabetic comparators was 1,647.7±360.0 kcal/day.

5.3.3 Effect of weight change on palmitic acid

VLDL1-TG palmitic acid

At baseline, palmitic acid (C16:0) content within the VLDL1 fraction in type 2 diabetes was near double that in non-diabetes comparators (52.0±4.1 vs. 28.4±3.2 µmol /l, p<0.0001) (Figure 5.4B). In Responders, the palmitic acid content in the VLDL1 fraction decreased significantly after weight loss (45.0±4.6 to 33.5±4.2µmol /l, p=0.006), becoming similar to NDC (p=0.34) with no further changes to 12 months (33.9 ±4.7 µmol /l, p=0.34 vs. NDC) or 24 months (31.6±5.4 µmol /l, p=0.62 vs. NDC). In Non-responders, the decrease in palmitic acid was modest (67.3±7.4 to 50.1±7.9 µmol /l, p=0.05). It was significantly higher than non-diabetes comparators at 0, 5, 12 and 24 months (67.3±7.4 µmol /l, p<0.0001; 50.1±7.9 µmol /l, p=0.02; 53.3±6.3 µmol /l, p=0.002, and 62.0±9.7 µmol /l, p=007, respectively) (Figure 5.4B, Table 5.1). Non-responders had higher palmitic acid level at baseline compared with Responders (67.3±7.4 vs. 45.0±4.6 µmol /l, p=0.02, Table 5.1) and the difference between Responders and Non-responders remained significant at 12 and 24 months (53.3±6.3 vs. 33.9±4.7 µmol/l, p=0.02, and 62.0±9.7 vs. 31.6±5.4 µmol/l, p=0.02, respectively).

As expected for the major saturated fatty acid component of VLDL-TG, there was a strong correlation between fasting VLDL1-TG content and palmitic acid content at baseline and at all time points in Responders (r=0.86, p<0.0001; r=0.85, p<0.0001, r=0.94, p<0.0001; and r=0.78, p<0.001, respectively), and in Non-responders (r=0.82, p<0.0001; r=0.80, p<0.0001; r=0.84, p<0.0001, and r=0.96, p<0.0001, respectively).

Total plasma palmitic acid

At baseline, total plasma palmitic acid concentration in the Tyneside Intervention group was not significantly different from non-diabetic comparators (229.3 ±8.9 vs. 206.9 ±7.4 µmol /l, p=0.06). A reduction in plasma palmitic acid to 192.6 µmol /l post weight loss (p<0.0001), remained similar to non-diabetes comparators (p=0.14, Figure 5.4B). This fall was significant in Responders (235.4±11.0 to 190.5 ±6.8 µmol /l, p<0.0001), but not in Non-responders (216.2 ±15.0 to 197.0 ±12.2 µmol /l, p=0.26). In Responders, total plasma palmitic acid concentration was stable at both 12 and 24 months (203.4 ±9.8, p= 0.77 and 207.4 ±9.6 µmol /l, p=0.97 vs. non-diabetes comparators respectively). Total plasma palmitic acid concentration also remained stable in Non-responders at 12 and 24 months (209.2 ±15.1 µmol /l, p=0.90, and 209.1±10.8 µmol /l, p=0.87, vs. non-diabetic comparators, respectively).

In vitro studies demonstrated that palmitic acid is more toxic to beta cells than are unsaturated fatty acids (Lee et al., 1994a, Robertson et al., 2004, Cunha et al., 2008, Pinnick et al., 2008, Boslem et al., 2011, Eguchi et al., 2012). The pronounced increase in VLDL-TG palmitic acid levels during the relapse of type 2 diabetes observed in some participants (Figure 5.5D) is, to our knowledge, the first *in vivo* human data which supports this potential mechanism. Given the specific effect of palmitic acid in bringing about beta cell de-differentiation this is potentially of considerable significance (Pinnick et al., 2008). Future observations of VLDL-palmitic acid levels during the progression from pre-diabetes to type 2 diabetes will be important in providing evidence for this hypothesis.

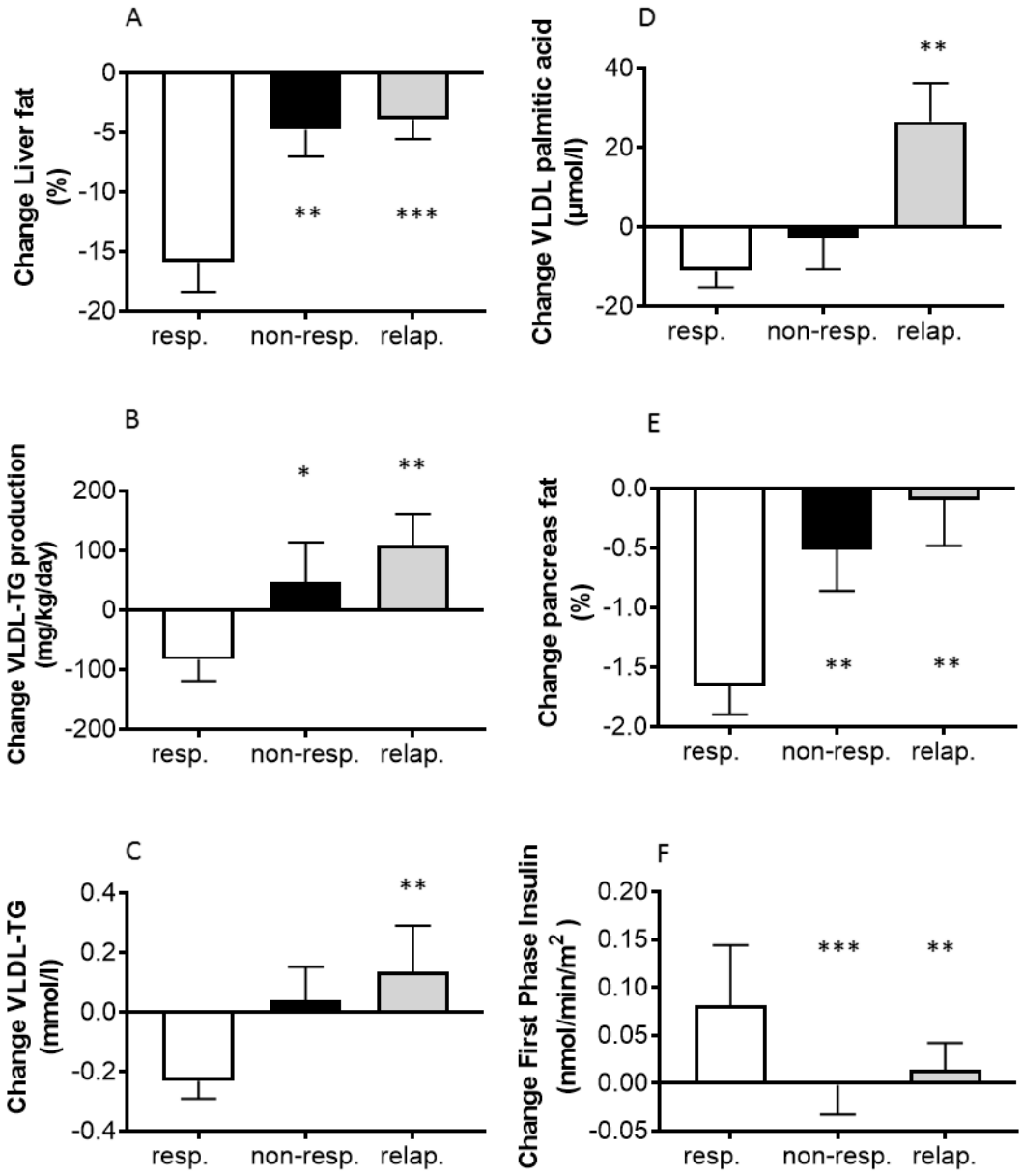

Figure 5.5 Changes in lipid measures and beta cell function between baseline and 24 months in responders, non-responders, and relapsers.

Change in liver fat (A), hepatic VLDL1-TG production (B), fasting plasma VLDL1-TG (C), VLDL1-palmitic acid (D), intra-pancreatic fat (E), and beta cell function (F) between baseline and 24 months in responders (white), non-responders(black), and relapsers (grey)(data are presented as mean ±SEM except for first phase insulin (Median with IQ range). *p<0.05 vs. responders, **p<0.01 vs. responders, **p<0.01 vs. responders)(Al-Mrabeh et al., 2019)

5.3.4 Effect of weight change on visceral and subcutaneous fat storage

Subcutaneous fat (SAT) and visceral fat (VAT) after weight loss in the Intervention group were close those of non-diabetes comparators (238.7 vs 264.3± 19.0cm^2; VAT: 160.4±9.1cm^2 vs. 193.9±23.5cm^2 p=0.19) (Table 5.1). SAT changed similarly from baseline in both Responders and Non-responders (320.2±20.7 to 233.3± 19.3cm^2, p<0.0001, and 313.5±26.9 to 251.6cm^2, p<0.0001, respectively) and therefore was not attributed to remission. SAT increased between 5 and 24 months in both groups (p<0.0001 at 24 vs. 5 months) (Table 5.1).

The changes in VAT from baseline were also same in both Responders and Non-responders (281.9±12.7 to 159.5±10.2 cm^2, p<0.0001, and 253.9± 19.5 to 162.7± 19.5cm^2, p<0.0001). VAT increased between 5 and 24 months in both groups (p<0.0001 at 24 months vs. 5 months). The similarity in VAT and SAT response post Intervention between Responders and Non-responders suggests that neither Visceral nor Subcutaneous fat accumulation is not related to remission of type 2 diabetes. Majority of studies confirm this study observation (Gastaldelli et al., 2007, Colles et al., 2006).

5.3.5 Pancreas fat and beta cell function

Intra-pancreatic fat was higher in type 2 diabetes at the baseline compared with NDC (8.5±0.3 vs. 6.2±0.4%, p<0.0001). Weight loss brought about similar change in intra-pancreatic fat in Responders and Non-responders (-0.91±0.17% vs. -0.78±0.23, p=0.65). However, intra-pancreatic fat continued to fall between 5-24 months in Responders but not in the Non-responders (-0.48±0.25 vs. +0.41±0.35, p=0.03). At 24 months, pancreatic fat had decreased by 1.65±0.24% in Responders, compared with 0.51±0.35% in the Non-responders (p=0.013) (Figure 5.5.E).

Pancreatic fat was non-significantly higher at baseline in Responders than Non-responders (8.7±0.4 vs. 7.9±0.6%, p=0.26, Table 5.1). However, this could be secondary to the higher fasting insulin concentration in Responders that would drive de novo

lipogenesis in the liver, thereby elevating liver fat level, hepatic-TG export and intra-pancreatic fat.

In the whole group of intervention participants, the change in intra-pancreatic fat between baseline and 5 months correlated positively with change in total plasma TG (r=0.29, p=0.04) and change in plasma VLDL-TG (r=0.30, p=0.04, respectively). There was no correlation between change in intra-pancreatic fat and change in non-VLDL1-TG. The change in intra-pancreatic fat between baseline and 24 months correlated with changes in liver fat in all intervention participants (r=0.45, p=0.002). In Responders, there was a steadily closer relationship between VLDL1-TG production rates and intra-pancreatic fat (Figure 5.6.B).

First phase insulin response remained sub-normal in Responders however it was sufficient to maintain non-diabetic blood glucose control. Fat removal from the pancreas correlated with restoration of first phase insulin secretion within the whole intervention group at 12 months (Figure 5.8D).

The present series of studies were initiated to test the twin cycle hypothesis which postulated that increased VLDL-TG supply increased pancreatic fat content and decreased beta cell function, leading to type 2 diabetes (Taylor, 2008). Since then, data from animal and human *in vitro* studies demonstrated that saturated fatty acids induce cellular stress and inhibit beta cell function (Pinnick et al., 2008, Pinnick et al., 2010, Boslem et al., 2011). Present data are consistent with the original hypothesis. It also demonstrated that reversal of type 2 diabetes is associated with reduction in plasma palmitic acid which is the most toxic saturated fatty acid to beta cells, based on data from in vitro /ex vivo models. Although apoptosis has been suggested to explain beta cell failure in type 2 diabetes (Butler et al., 2003a, Huang et al., 2007), de-differentiation under the lipid-induced endoplasmic reticulum stress is most consistent with *in vivo* observations in humans (Talchai et al., 2012, Biden et al., 2014, White et al., 2016b, Guo et al., 2010, Pinnick et al., 2010). It was demonstrated that the fat induced stress to beta cells is potentially reversible in majority of people with early type 2 diabetes both in the present data and in previously published studies (Lim et al., 2011b, Steven et al., 2016b, Taylor et al., 2018b). Current data support the concept that lipotoxicity is the initiating

factor in beta cell de-differentiation, although it is likely that increased glucose exposure will act synergistically once type 2 diabetes is established (Accili et al., 2016, White et al., 2016b, Bensellam et al., 2018). Lipid metabolism is regulated by autophagy and calorie restriction enhances this cellular process (Singh et al., 2009, Longo and Mattson, 2014). Recent studies reported abnormalities of autophagy in beta cell under conditions of high lipid, and removing this metabolic stress protects the beta cell (Zummo et al., 2017, Ji et al., 2019). Therefore, it is likely that restoration of normal autophagy by decreasing exposure of the beta cells to palmitic acid contributes to beta cell re-differentiation.

Recently, markers of beta cell de-differentiation have been reported in human studies which open new opportunities to understand better the process of de-differentiation (Cinti et al., 2016, Diedisheim et al., 2018).

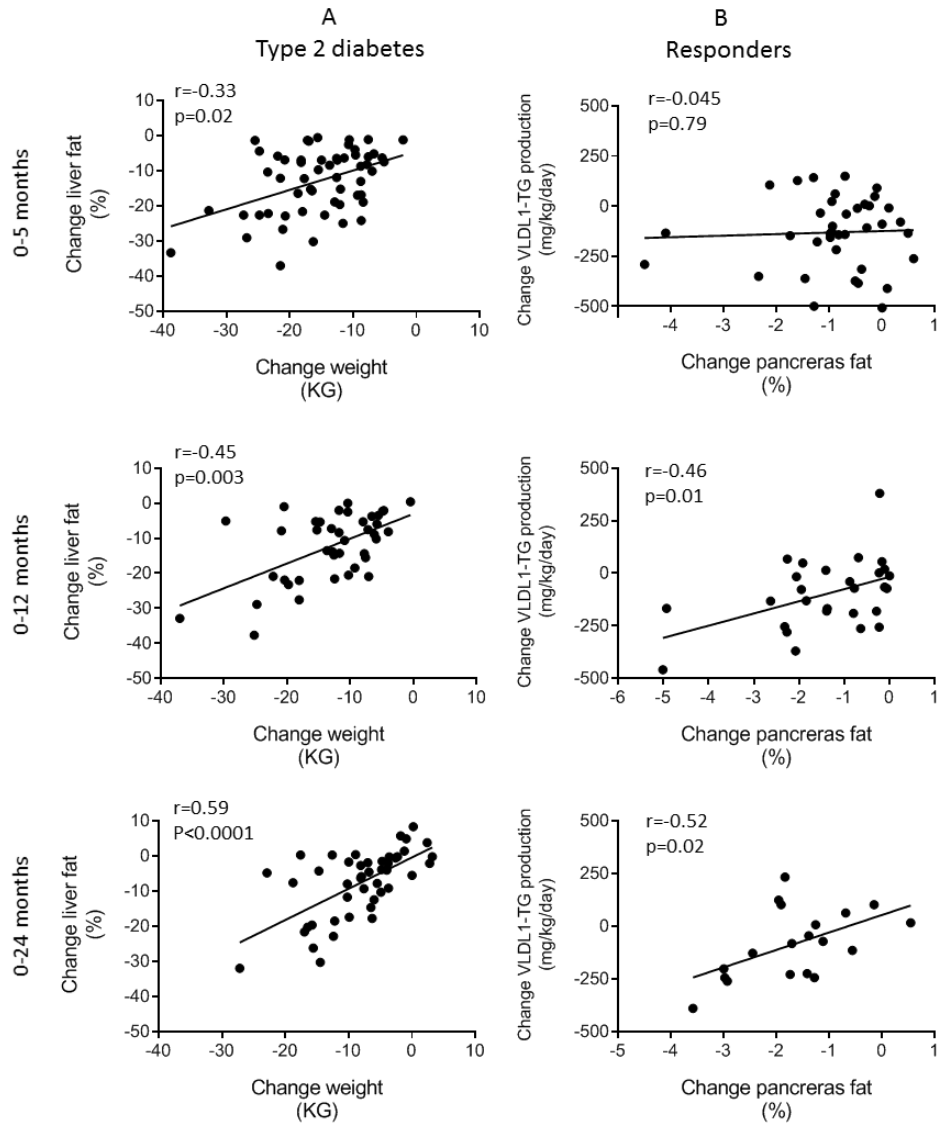

Figure 5.6 Effect of weight loss on hepatic VLDL-TG export and intra-pancreatic fat content.

Panel A: Correlation between the change in weight and change in liver fat between baseline, 5 months, 12 months, and 24 months in the whole type 2 diabetes group.
Panel B: Correlation between the change in pancreas fat and change in hepatic VLDL-TG export between baseline, 5 months, 12 months, and 24 months in the Responder group (Al-Mrabeh et al., 2019).

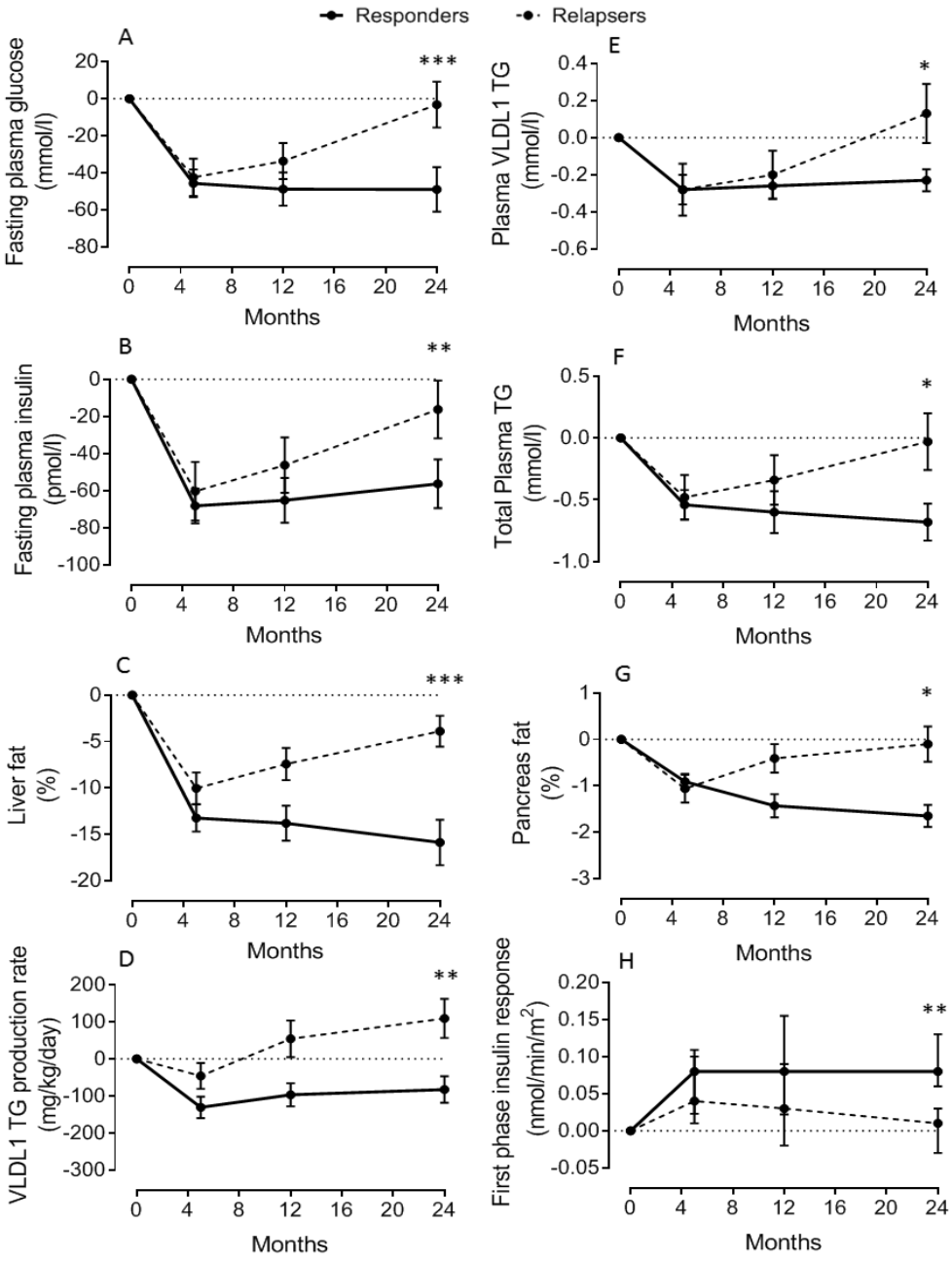

Figure 5.7 Metabolic markers associated with sustainability of remission.

Change from baseline in fasting plasma glucose (A), fasting plasma insulin (B), liver fat (C), hepatic VLDL1-TG production (D), and fasting plasma VLDL1-TG (E), total plasma TG (F), intra-pancreatic fat (G), and beta cell function (H) at 5 months, 12 months, and 24 months. Responders are presented as a solid black line and relapsers as a dotted line. Paired data between baseline and each time point are presented. Data are presented as Mean ±SEM except for first phase insulin (Median with IQ range)(Al-Mrabeh et al., 2019)*p<0.05 vs. 5 months in those who lost remission (relapsers), ** p<0.01 vs. 5 months in relapsers, ***p<0.001 vs. 5 months in relapsers.

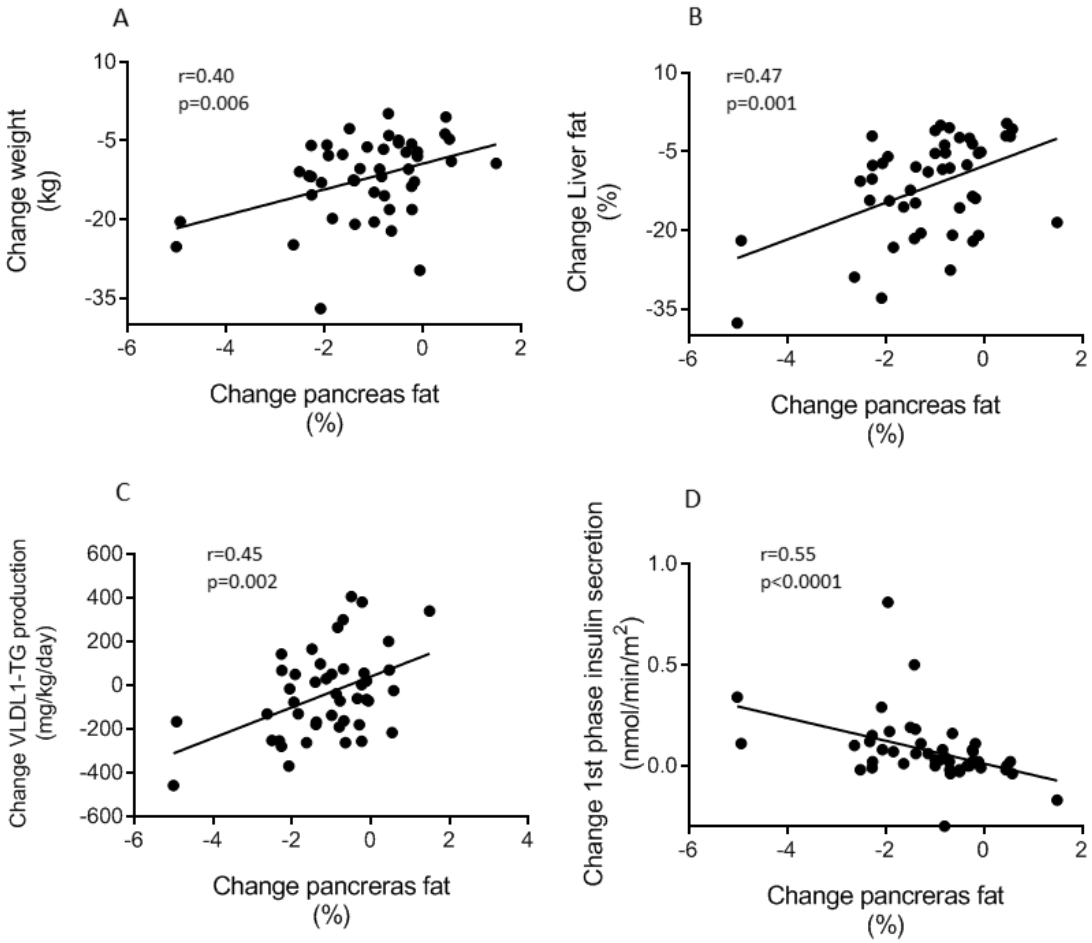

Figure 5.8 Effect of weight loss induced changes in hepatic and intra-pancreatic fat on beta cell function.

Correlations between change in intra-pancreatic fat and changes in weight (A), liver fat (B), VLDL1-TG production(C), and beta cell function(D) between baseline and 12 months within the whole type 2 diabetes group (Al-Mrabeh et al., 2019)

5.3.6 Change in metabolic and clinical parameters following diabetes relapse

The participants who achieved remission immediately after weight loss but subsequently relapsed into type 2 diabetes were of particular interest. Those subjects regained more weight between 5 months and 24 months than ones who remained in remission (11.3 ±1.9 vs. 6.6±1.0 kg, p= 0.036, Table 5.1). The relapsers also increased hepatic VLDL1-TG production (406.1±42.2 to 561.3±37.3 mg/kg/day, p=0.005) and VLDL1-TG pool size (1328±272 to 3014±668 mg, p=0.014) (Figure 5.5 and Figure 5.7). In addition, in relapsers, fasting plasma VLDL1-TG increased by almost two-fold between 5 and 24 months (0.46±0.10 to 0.88±0.16 mmol/l, p=0.02, Figure 5.7E), and this was associated with increased intra-pancreatic fat content (7.1±0.5 to 8.0±0.4 %, p=0.03, Figure 5.7G). The rise in liver fat correlated with intra-pancreatic fat content (r=0.64, p=0.018) in relapsers.

In contrast, those who remained in remission exhibited a modest increase in VLDL-TG production (388.6±37.5 to 480.7±30.7 mg/kg/day, p=0.02) remaining similar to NDC (457.0±28.2). There was no significant change in fasting plasma VLDL1-TG (0.39±0.07 to 0.44±0.07, p=0.46), VLDL1-TG pool size (1021 ±185 to 1415 ±238, p=0.24), or intra-pancreatic fat (8.5±0.6 to 8.0±0.6 %, p=0.068), (Figure 5.5 & Figure 5.7).

In those who relapsed between 5 and 24 months, intra-pancreatic fat had decreased immediately after weight loss from 8.2±0.5 to 7.1±0.5 % (p=0.004) but increased between 5-24 months to 8.0±0.5 % (p=0.03). Similarly, VLDL1-TG palmitic acid content increased between 5-24 months (32.4±7.4 to 67.4±8.3 mg/l, p=0.006). Restriction of carbohydrate intake can decrease de novo lipogenesis by 80 % (Mardinoglu et al., 2018) and as palmitic acid is synthesized, it is likely that de novo lipogenesis was more active during relapse, explaining the higher level of VLDL-TG palmitic acid compared with NDC (p=0.001), (Figure 5.5D).

In contrast to those remained in remission, first phase insulin response decreased in relapsers at 24 months (Figure 5.5F and Figure 5.7H).

The extent of weight loss determines change in subcutaneous and visceral fat content. In relapsers, the change in both SAT and VAT between baseline and 5 months (332.9±38.6 to 244.0±34.4 cm^2, and 275.7±17.9 to 154.0±13.2 cm^2, $p<0.0001$, respectively) was not sustained at 24 months when SAT was not significantly different from baseline (310.95±29.7 cm^2, $p=0.19$, Table 5.1). For those who remained in remission, VAT remained lower compared with the relapsers (-85.0±14.9 vs. -30±12.4cm^2, $p=0.008$). Combined together, these data suggest that SAT storage capacity had be maximised in this group, causing overflow of fat to VAT, liver and pancreas (Taylor and Holman, 2015b).

Multivariate logistic regression was used to evaluate the impact of changes in metabolic parameters on diabetes remission. A panel of metabolic markers including major lipid markers (liver fat, VLDL-TG production, VLDL-TG pool, plasma VLDL-TG, total plasma TG, plasma NEFA, HDL cholesterol, pancreas fat, and SAT/VAT, weight, BMI, fasting plasma glucose, HbA1c, ALT, AST, GGT, and total cholesterol) were included in the regression model. Remission of type 2 diabetes was associated with decrease in liver VLDL1-TG production ($p=0.035$) between baseline and 24 months (Appendix 2). None of the other factors predicted remission, supporting the concept of primacy of triglyceride supply to the islets in determining the presence of type 2 diabetes in susceptible people.

5.4 Discussion

The present data demonstrate that remission of type 2 diabetes is linked to a decrease in liver-derived VLDL-TG and not with a change in non-VLDL-TG. During 2 years of remission of type 2 diabetes, liver VLDL-TG production rates initially declined and then remained stable and normal. Plasma VLDL-TG concentrations reflect this pattern of change, as does intra-pancreatic fat content. In those who did not achieve remission, the initial weight loss resulted in similar but more modest changes in VLDL-TG production

rate, plasma concentrations of VLDL-TG and intra-pancreatic fat. Notably, plasma palmitic acid concentration remained significantly higher in Non-responders compared with Responders and this could be a factor in preventing recovery of beta cell function (Taylor et al., 2018b).

The relapse of type 2 diabetes was observed in a subgroup of participants who had achieved remission initially but then regained weight. This weight regain was associated with increased liver VLDL1-TG production, increased plasma VLDL1-TG and re-accumulation of intra-pancreatic fat. The VLDL1-TG content of palmitic acid, known to be particularly inhibitory of beta cell function, increased markedly during the re-development of type 2 diabetes in those who relapsed. The specific enrichment of palmitic acid in VLDL1-TG is likely to be a consequence of increased *de novo* lipogenesis.

Overall, these findings provide evidence for the pathophysiologic basis underlying both the aetiology and remission of type 2 diabetes. In individuals with susceptible beta cells, excess fatty acid exposure appears to promote loss of specialised endocrine function ((Talchai et al., 2012, Cinti et al., 2016, Clark and Matera, 2010). The observations are in keeping with de-differentiation of the beta cell as the most likely mechanism bringing about reversible failure in early type 2 diabetes (Talchai et al., 2012, Cinti et al., 2016, White et al., 2016b, Taylor et al., 2019b), with hepatic VLDL1-TG being the “upstream” deliverer of the toxic products which appear to be causal for beta cell dysfunction and development of diabetes.

One of the limitations of this study that it investigated the effect of VLDL-TG lipoprotein on change in glucose homeostasis, but chylomicrons were not investigated directly. Also, the direct measurement of *de novo* lipogenesis after weight loss induced reversal would be of great interest. This could support the data extrapolation from other studies showing that 26 % of post prandial *de novo* lipogenesis derives from glucose in the diabetic state (Donnelly et al., 2005, Barrows and Parks, 2006, Lambert et al., 2014, Taylor, 2008) but this would be expected to return to normal during reversal of diabetes. As the risk allele of the *PNPLA3* gene confers susceptibility to liver steatosis but not liver insulin resistance, targeted studies of different genotypes would throw light on this subject (Dongiovanni et al., 2013). It would be useful to determine whether those who

re-accumulated liver fat carry the risk allele, and whether their lipid profile is different. A recent study reported difference in lipidomics between metabolically induced and *PNPLA3*-related NAFLD (Luukkonen et al., 2016). Finally, this study recruited people with white ethnicity, reflecting the population in the Tyneside area of England. However, it will be important to investigate whether the same metabolic changes lead to type 2 diabetes reversal in other ethnicities. This is very important for south Asians in whom type 2 diabetes occurs at lower body BMI (Ntuk et al., 2014) .

Chapter 6. Effect of weight loss on Beta cell function

6.1 Introduction

Type 2 diabetes has long been known to be associated with decreased functional beta cell capacity. This has appeared to be progressive, resulting in requirement for insulin therapy in 50 % of people within 10 years of diagnosis (1995a). The early observation of decreased number of islets able to be isolated from the pancreas of people with type 2 diabetes (Deng et al., 2004) was followed by histological studies which reported 24-65 % decrease in beta cell number compared with weight matched non-diabetic subjects (Butler et al., 2003b, Rahier et al., 2008). However, the functional deficit has been reported to be greater than expected, with 50 to 97 % decrease (Gastaldelli et al., 2004, Ferrannini et al., 2005, Jensen et al., 2002). Consistent with this, a 50 % hemipancreatectomy does not bring about type 2 diabetes in most people (Kendall et al., 1990).

The decrease in overall beta cell function has been conventionally ascribed to beta cell death or apoptosis (Butler et al., 2003b, Marchetti et al., 2007, Shimabukuro et al., 1998). This has been challenged by recent studies which have identified loss of beta cell insulin secretory function due to suppression of relevant genes (Pinnick et al., 2010, Cinti et al., 2016, Talchai et al., 2012, Weir and Bonner-Weir, 2013). This de-differentiation is potentially reversible. Previous shorter term studies have shown that type 2 diabetes can be returned to non-diabetic glucose control following dietary weight loss (Lim et al., 2011a, Steven et al., 2016a, Taylor et al., 2018a, White et al., 2016a).

DiRECT (the Diabetes Remission Clinical Trial) reported return to non-diabetic glucose control in over one third of a large Primary Care population of people with type 2 diabetes at two years using a simple, effective dietary method (Taylor et al., 2018a, Lean et al., 2018, Lean et al., 2019). There are few *in vivo* studies assessing functional beta cell capacity in type 2 diabetes, and none after reversal of type 2 diabetes (Weir and Bonner-Weir, 2013, Pipeleers et al., 2008). We now report upon functional beta cell capacity as assessed by SISTA technique in a geographically defined cohort of DiRECT participants.

This was quantified together with first phase insulin secretion before and up to 24 months after substantial weight loss in type 2 diabetes, comparing those who achieved HbA1c < 48 mmol/mol and fasting plasma glucose < 7.0 mmol/l) with those who remained in the diabetic range. For comparison, an additional group of Non-Diabetic Controls were studied.

The aims of this study were to look at the pathophysiological differences between Responders and Non-responders to the low-calorie diet intervention in people with duration type 2 diabetes of less than 6 years. The seminal question is now how closely back to normal the functional beta cell capacity returns over two years of remission of type 2 diabetes. This has not been previously 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 Study protocol

Weight loss and supportive weight maintenance was managed by Primary Care nurses with specific training on an integrated, structured, weight management programme (Counterweight-Plus)(Lean et al., 2018). All metabolic tests were performed at the Newcastle Magnetic Resonance Centre.

In order to interpret the beta cell function data, the Intervention group was divided into Responders who achieved non-diabetic glucose control (HbA1c < 48 mmol/mol and fasting plasma glucose < 7.0 mmol/l) off all anti-diabetic agents and Non-Responders who did not fit these current criteria (Lean et al., 2018, Lean et al., 2019, Taylor and Barnes, 2018). The Consort diagram (Figure 6.1) shows that the criteria were applied at the post-weight loss visit with Responders and Non-Responders being analysed separately. At 5 months, 40 of the 64 Intervention subjects achieved non-diabetic plasma glucose control after the weight loss intervention and 39 Responders underwent

the SISTA (Figure 6.1). One subject declined all the SISTA tests after baseline and counted as withdrawn. At 12 and 24 months, insulin secretion data were available on 28 and 20 Responders and 16 and 12 Non-Responders respectively. To allow clear description of time course, those who achieved non-diabetic levels of HbA1c and fasting plasma glucose but subsequently relapsed were not added to the defined Non-Responder group. In the diabetic Control group data were available on 23 participants at baseline and 5 months, 20 at 12 months, and 19 at 24 months. The Non-Diabetic Control group (n=25) was studied on one occasion. Ethical approval was obtained from the West of Scotland Research Ethics Committee (reference number: 13/WS/0314).

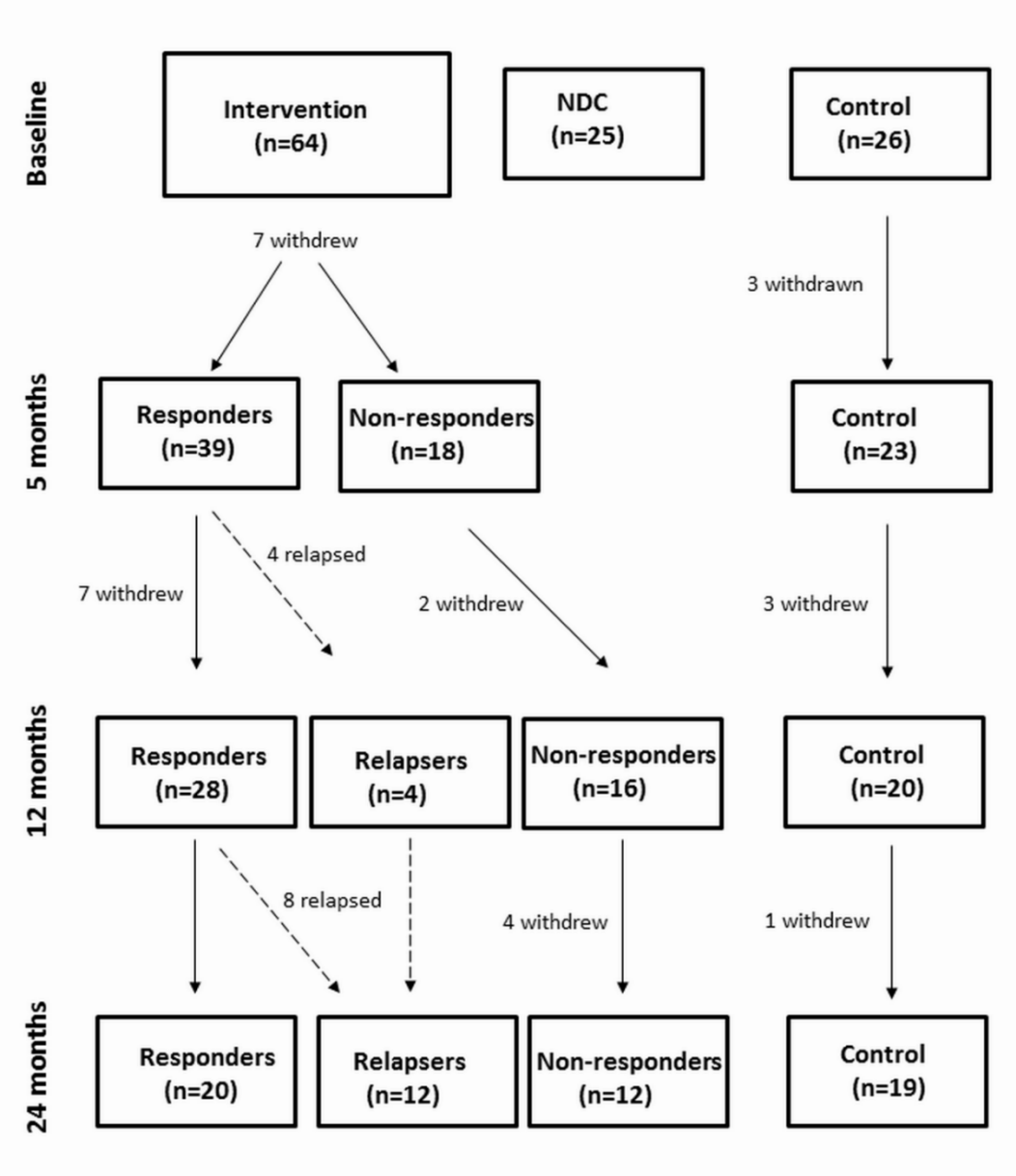

Figure 6.1 Consort diagram of the DiRECT participants underwent SISTA.

6.2.2 Stepped Insulin Secretion Test with Arginine (SISTA)

After an overnight fast of at least 10 hours, participants were transported to the Magnetic Resonance Centre. Only water was allowed to maintain hydration. Any diabetes medications were withdrawn on the evening prior to the test. The functional capacity of the beta cells was assessed using the Stepped Insulin Secretion Test with Arginine (SISTA) (Toschi et al., 2002), as modified by Lim and colleagues (Lim et al., 2011a). SISTA test was already described in the Methods chapter of the thesis and the schematic and details of performing the SISTA are shown in Figure 6.2. First phase insulin response was assessed by choosing the highest value of c-peptide in between 4 to 10 minutes post infusion of the first dextrose bolus at time 0 of the test. The maximal capacity for insulin response was assessed by choosing the highest value of c-peptide post Arginine bolus (given at 60 minutes of the test) around 62 or 64 minutes of the SISTA test.

Insulin secretion rates were estimated by deconvolution from C-peptide concentrations. The ISEC computer program was used which applies a regularisation method of deconvolution giving an output of insulin secretion rate in pmol/min/m^2 of body surface area (Lim et al., 2011a, Hovorka et al., 1996). This took account of gender, age, diabetes status, height, weight, body surface area and BMI. Data are reported on the assessed functional beta cell capacity (maximal rates of insulin secretion under the test conditions) and first phase response. Data are also reported for the insulin response to the second rapid increment in plasma glucose ('second step response') although the *in vivo* relevance of this is not yet established.

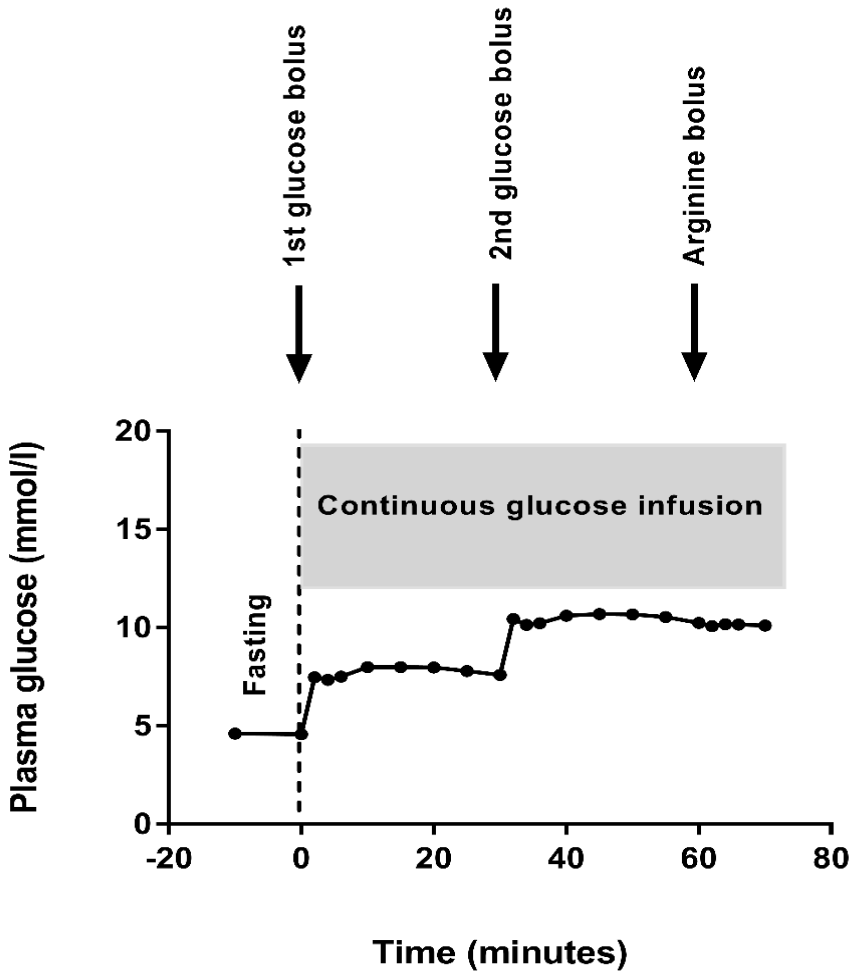

Figure 6.2 Schematic details of Stepped Insulin Secretion Test with Arginine (SISTA).

6.2.3 Analytical Procedures

Plasma glucose was measured by the glucose oxidase method (YSI glucose analyser, Yellow Springs Instrument Company, Yellow Springs, OH) and serum insulin and C-peptide by ELISA (Mercodia, Uppsala, Sweden). HbA1c was measured centrally at the Institute of Cardiovascular and Medical Sciences at Clinical Pathology Laboratory in Glasgow. Total fasting plasma triglyceride (TG) was quantified (Roche Diagnostics, West Sussex, U.K.) at the Department of Clinical Biochemistry, Newcastle upon Tyne Hospital National Health Service Foundation Trust.

6.2.4 Statistical analysis

Data were analysed by using IBM SPSS statistical software (www.ibm.com) using Independent Samples T test, Mann Whitney U test, and Spearman Rank correlation test as appropriate. The primary comparison between Responder and Non-Responder groups was the insulin secretion data. These were skewed in both groups and analysed non-parametrically. Data are presented as median and interquartile range (25th and 75th centiles) or mean ± SD as appropriate.

6.3 Results

6.3.1 Baseline comparison of Responders and Non-Responders

At baseline, both Responders and Non-Responders had similar weight (100.4±16.6 vs 102.1±18.8 kg). Fasting plasma insulin (FPI) in Responders was 86.4 [55.5-145.5] pmol/l and 71.4 [51.5-100.6] pmol/l in Non-Responders (p=0.094). There were no significant differences between insulin secretion rates in Responders and Non-Responders at baseline although median rates of both maximal insulin secretion (581 [480-811] vs. 451 [296-691] pmol/min/m^2; p=0.081) and first phase (42 [4-67] vs. 23 [10-36] pmol/min/m^2; p=0.299) were higher in Responders. Responders and Non-Responders differed

significantly in HbA1c (57.5±10.6 mmol/mol vs.62.5±8.8 mmol/mol); p=0.041) and duration of diabetes (2.7±1.6 years vs. 3.8±1.6 years; p=0.026) respectively.

6.3.2 Changes in weight and glucose control

Responders and Non-Responders lost weight similarly at 5 months (Table 6.1). Weight increased in Responders by 3.2±4.2 kg between 5 and 12 months and by 6.6±4.3 kg between 5 and 24 months. By design, weight of the non-diabetic comparators was similar to both study groups at 5 months (86.6±14.9 vs. 84.0±13.4 kg and 88.7±18.8 kg respectively) and remained similar at both 12 months (86.6±14.9 vs. 85.5±16.2 kg; p=0.845 and 92.5±18.3 kg; p=0.552 respectively) and 24 months (88.8±17.7 kg; p=0.648 and 90.3±14.6 kg; p=0.649 respectively). Those who failed to maintain remission between 5 and 24 months were characterized by more weight regain (11.3±6.7 kg vs. 6.6±4.3 kg; p=0.036). There was no change in weight in the Control group from baseline.

Hba1c remained in the non-diabetic range in Responders (n=20) at 24 months having remained steady although significantly higher than in NDC (41.7±3.5 mmol/mol vs. 35.2±3.4 mmol/mol; p<0.0001, Table 1). At 24 months mean Hba1c in Responders remained stable at 41.7±3.5 mmol/mol; p<0.0001 vs. NDC and higher in Non-Responders (65.3±14.4 mmol/mol; p<0.0001) despite their use of anti-diabetes medications. Controls and Non-Responders showed no change in HbA1c from baseline to 24 months. Fasting plasma glucose level exhibited comparable changes to HbA1c in all groups (Table 6.1).

		Baseline	5 months	12 months	24 months
		Resp=39 Non-Resp=18 Control=26 -	Resp=39 Non-Resp=18 Control=23 NDC=25	Resp=28 Non-Resp=16 Control=20 -	Resp=20 Non-Resp=12 Control=19 -
Weight (kg)	Resp Non-Resp Control NDC	100.4±16.6 102.1±18.8 96.7±11.8 -	84.0±13.4 p<0.0001 88.7±18.8 p=0.008 95.8±12.8 86.6±14.9	85.5±16.2 p<0.0001 92.5±18.3 p=0.050 95.2±14.4 -	88.8±17.7 p=0.016 90.3±14.6 p=0.028 95.0±13.9 -
Weight loss (%)	Resp Non-Resp Control NDC	- - - -	16.0±6.1 p<0.0001 13.2±6.1 p=0.008 1.1±3.1 -	14.3±6.9 p<0.0001 9.4±5.2 p=0.050 0.8±4.4 -	10.5±6.1 p=0.016 8.3±4.5 p=0.028 1.7±5.5 -
HbA1c (%)	Resp Non-Resp Control NDC	7.4±1.0 7.9±0.8 *p=0.041 7.3±1.0 -	5.9±0.4 p<0.0001 8.0±1.7 *p<0.0001 7.8±1.5 5.4±0.3	5.8±0.3 p<0.0001 7.6±0.7 *p<0.0001 8.5±2.2 -	6.0±0.3 p<0.0001 8.1±1.3 *p<0.0001 7.2±1.0 -
FPG (mmol/l)	Resp Non-Resp Control NDC	8.3±2.4 9.3±2.8 8.3±2.0 -	5.7±0.8 p<0.0001 8.8±2.6 *p<0.0001 8.4±2.3 5.1±0.4	5.6±0.6 p<0.0001 8.5±1.8 *p<0.0001 8.5±2.2 -	5.6±0.7 p<0.0001 9.3±4.0 *p<0.0001 8.0±2.0 -
FPI (pmol/l)	Resp Non-Resp Control NDC	86.4[55.5-145.5] 71.4[51.5-100.6] 70.1[44.7-122.5] -	32.6[19.6-53.3] p<0.0001 33.8[20.7-43.3] p<0.0001 58.4[36.2-83.0] 16.4[10.0-37.2]	28.9[17.6-65.7] p<0.0001 32.7[23.7-61.7] p=0.006 65.5[39.9-76.9] -	43.5[18.4-61.6]p<0.0001 34.4[16.5-49.5]p=0.001 44.7[31.3-64.5] p=0.050 -
Fasting C-peptide (nmol/l)	Resp Non-Resp Control NDC	0.99±0.32 0.84±0.28 0.92±0.41 -	0.59±0.23 p<0.0001 0.61±0.20 p=0.003 0.87±0.34 0.53±0.32	0.59±0.21 p<0.0001 0.65±0.24 p=0.042 0.87±0.41 -	0.65±0.25 p<0.0001 0.59±0.23 p=0.022 0.78±0.22 -

Total fasting TG (mmol/l)	Resp	1.71[1.15-2.29]	1.05[0.73-1.53] p<0.0001	1.09[0.75-1.62] p<0.0001	1.00[0.79-1.46] p<0.0001
	Non-Resp	1.57[1.25-1.99]	1.15[0.90-1.40] p=0.009	1.24[0.92-1.47] p=0.048	1.29[0.95-1.53]
	Control	1.06[0.81-1.58]	1.20[1.00-1.50]	1.21[1.00-1.91]	1.05[0.89-1.63]
	NDC	-	1.10[0.80-1.50]	-	-

Table 6.1 Summary of weight change and fasting metabolic parameters for Responder, Non-Responder, Diabetic Control and Non-Diabetic Comparator (NDC) groups at baseline, 5, 12, and 24 months. Data for the NDC group are shown in the 5 months column as this group was recruited to have weight equivalent to the Intervention group after weight loss. Data shown as mean ± SD or median [IQ range]. Comparisons are shown for baseline to 5 months, baseline to 12 months, and baseline to 24 months (p value) and for Responders vs. Non-Responders at each time point (*p value). N.B. The percentage of weight loss is shown for each group at each time point vs. the same number of participants at the baseline.

6.3.3 Fasting plasma insulin and C-peptide

Fasting plasma insulin (FPI) decreased to similar levels in Responders and Non-Responders at 5 months (32.6 [19.6-53.3] vs. 33.8 [20.7-43.3] pmol/l respectively) and stayed similar at 12 months (Table 6.1.). The newly achieved level in both groups was similar to that of NDC (16.4 [10.0-37.2] pmol/l). At 24 months FPI stayed significantly lower than baseline in Responders (43.5 [18.4-61.6] pmol/l; p<0.0001) as well as in Non-Responders (34.4 [16.5-49.5] pmol/l; p=0.001). FPI in Controls did not change significantly from baseline to 24 months (70.1 [44.7-122.5] to 44.7 [31.3-64.5] pmol/l; p=0.050). In all groups, fasting C-peptide exhibited a similar pattern of change to FPI (Table 6.1). Fasting C-peptide in Responders decreased from baseline (baseline 0.99±0.32 nmol/l, 5 months 0.59±0.23 nmol/l, 12 months 0.59±0.21 nmol/l, 24 months 0.65±0.25 nmol/l; p<0.0001 for each vs. baseline). Following similar weight loss in the

Non-Responder group reduction in C-peptide was also observed after weight loss (Table 6.1).

6.3.4 Assessment of functional beta cell capacity

The changes in plasma glucose for Responders vs. Non-Responders are shown in Figure 6.3. (A, B, C & D). The intended rapid increase of 2.8 mmol/l was achieved in all groups for each of the 2 steps of the SISTA, followed in each case by stable hyperglycaemia. The profile of insulin secretion rate at baseline, 5 months and 12 months is shown for Responders (Figure 6.3., panels E, F, G, &H) and Non-Responders (panels I, J, K, &L).

For Responders, maximal rates of insulin secretion in response to arginine bolus during hyperglycaemia were 581 [480-811] pmol/min/m^2 at baseline and 736 [542-998] pmol/min/m^2 at 5 months. These two points were not significantly different (p=0.160), but an almost linear increase was observed across 12 months following weight loss such that rates increased to 942 [565-1240] pmol/min/m^2 (p=0.028 compared with baseline). This improvement was maintained at 24 months (936 [635-1435] pmol/min/m^2; p=0.023 vs. baseline) (Figure 6.4.A). The maximal rate of insulin secretion for non-diabetic comparators was 1016 [857-1509] pmol/min/m^2, comparable to that for the Responders at 12 and 24 months (p=0.064 and p=0.244 respectively) (Figure 6.4.A). Non-Responders showed no change in median maximal insulin responses (baseline 451 [296-691], 5 months 491 [388-629], 12 months 485 [387-568], and 24 months 452 [347-616] pmol/min/m^2). The difference between Responders and Non-Responders was significant by 5 months (p=0.002) and remained so at 12 months (p=0.001) and 24 months (p=0.002). The maximal insulin response in the diabetic Control group remained unchanged over 2 years follow up (546 [453-844], 567 [472-867], 559 [429-835], and 488 [378-720] pmol/min/m^2 respectively) (Figure 6.4.A and Figure 6.5.). Maximal insulin response to arginine in the Intervention group at 12 months correlated with first phase insulin response (r=0.6; p<0.0001), FPG and HbA1c (r=-0.4; p=0.003 for both). There was no correlation between maximal insulin response and total fasting TG at 12 months.

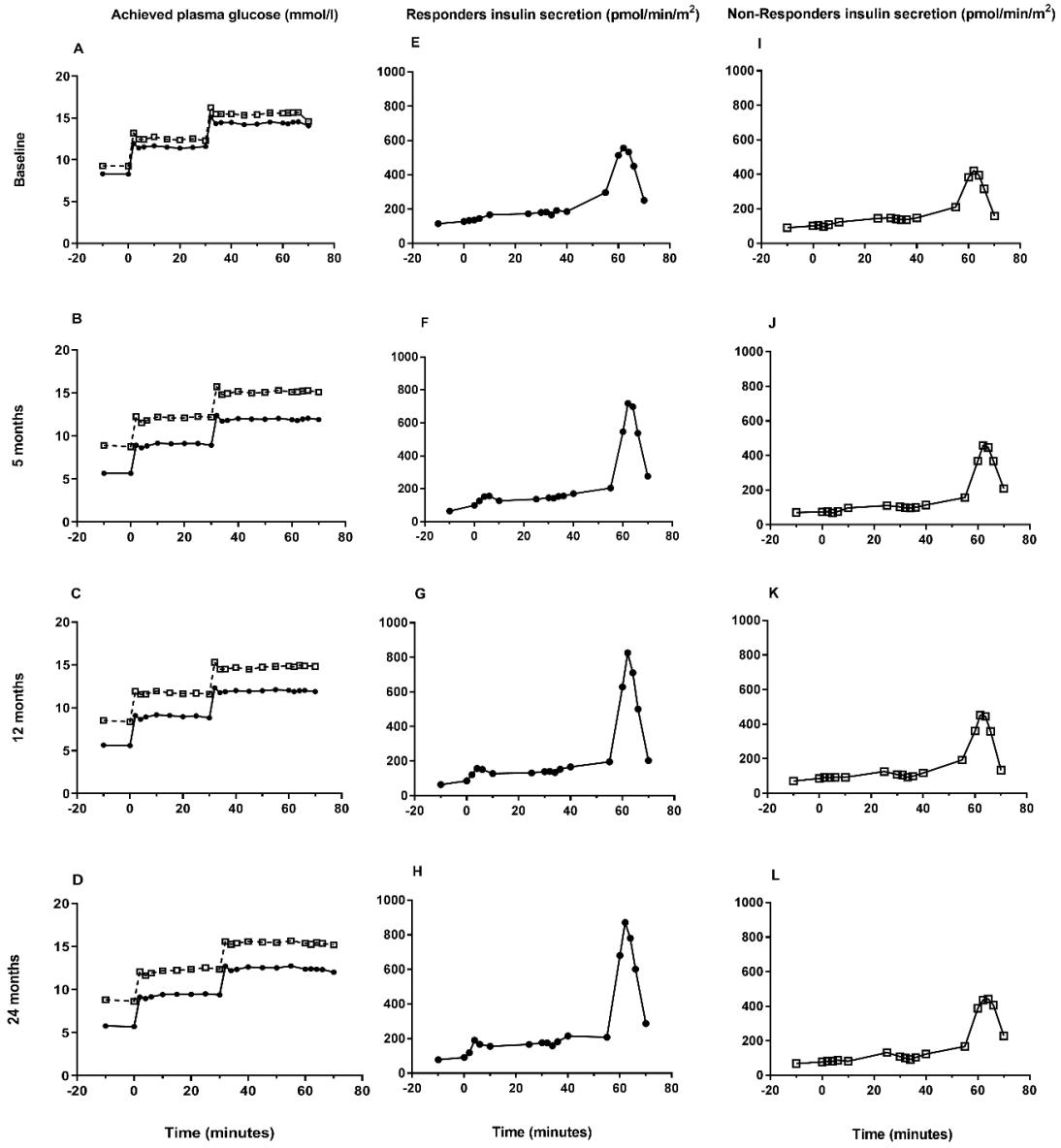

Figure 6.3 Stepped insulin secretion test in Intervention group (Responders vs. Non-Responders).

A, B, C, D: mean plasma glucose during SISTA in Responders —●— and Non-Responders - -□- - at baseline, 5 months, 12 months, and 24 months respectively.

E, F, G, H: median insulin secretion rates (ISR) in Responders at baseline, 5 months, 12 months, and 24 months respectively.

I, J, K, L: median insulin secretion rates (ISR) in Non-Responders at baseline, 5 months,12 months, and 24 months respectively.

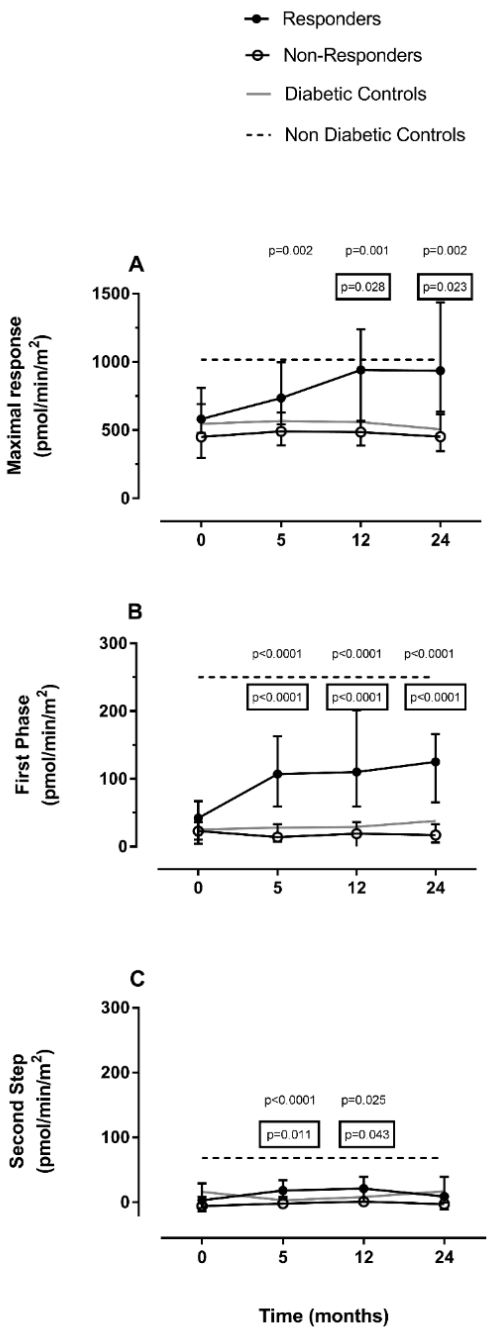

Figure 6.4 Beta cell response during SISTA.

Comparison of the median maximal insulin response (A), median first phase insulin response (B), and median second step insulin secretion (C) in Responders, Non-Responders, non-diabetic comparators (NDC) at baseline, 5, 12, and 24 months (p value in a box for Responders at each time point vs. baseline; p value without a box for Responders vs. Non-Responders at each time point).

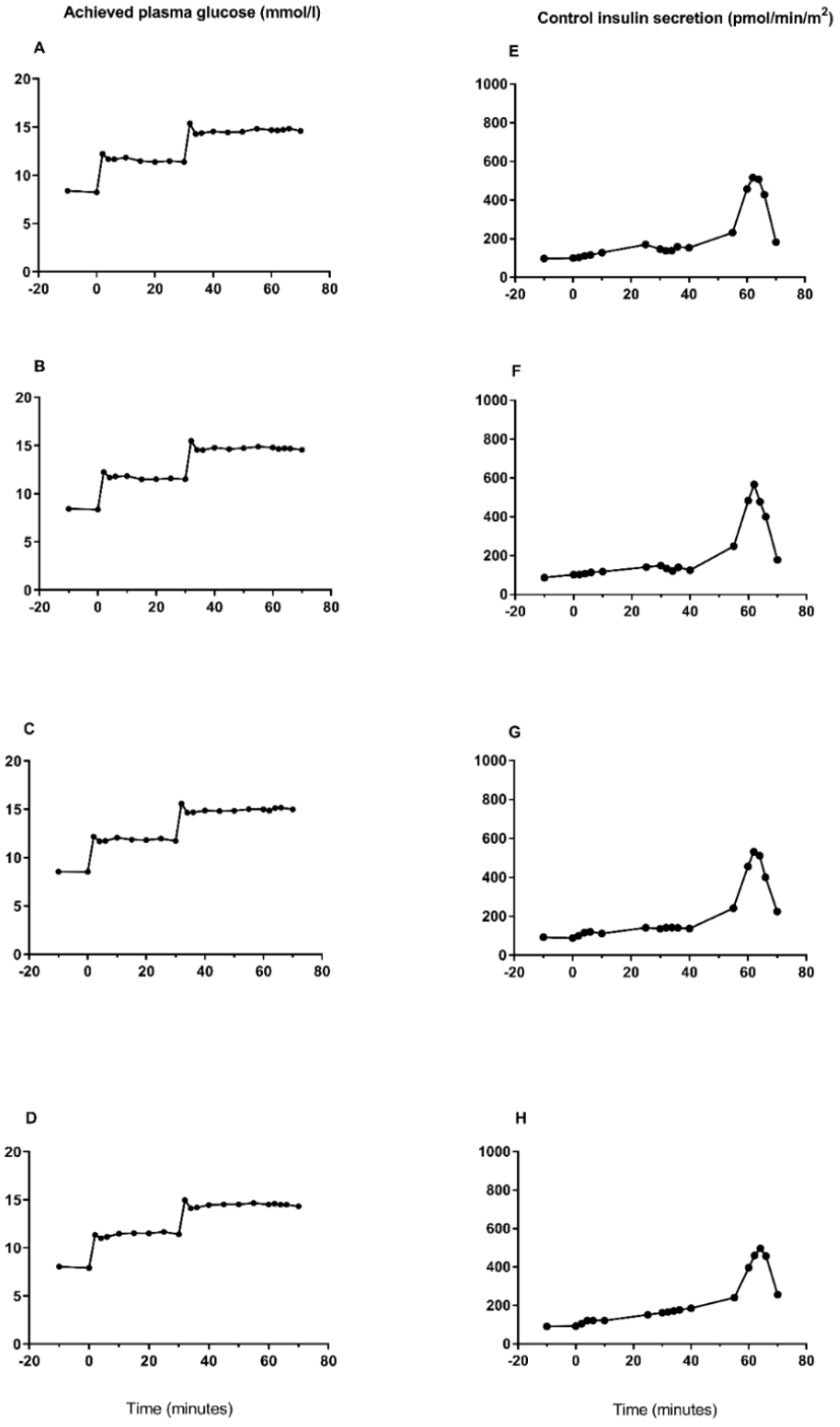

Figure 6.5 Stepped insulin secretion test in Control group.

A, B, C, D: mean plasma glucose during SISTA in Control group at baseline, 5 months, 12 months, and 24 months respectively.

E, F, G, H: median insulin secretion rates (ISR) in Control group at baseline, 5 months, 12 months, and 24 months respectively.

6.3.5 First phase insulin secretion

First phase insulin secretion increased in Responders from 42 [4 - 67] pmol/min/m^2 at baseline to 107 [59 - 163] pmol/min/m^2 ($p<0.0001$) at 5 months, remaining constant at 12 months (110 [59 -2 01] pmol/min/m^2; $p<0.0001$). At 24 month this was maintained in Responders (125 [65 - 166] pmol/min/m^2; $p<0.0001$) (Figure 6.4.B). The first phase response remained substantially lower than in the non-diabetic comparators (250 [226 - 429] pmol/min/m^2; $p<0.0001$) compared with Responders at each time point after weight loss. There was no change in Non-Responders from baseline (23 [10 - 36] pmol/min/m^2) either after weight loss at 5 months (14 [7 - 33] pmol/min/m^2; $p=0.864$), at 12 months (19 [-7 - 36] pmol/min/m^2; $p=0.746$) (Figure 2B), or at 24 months (17 [6 - 33] pmol/min/m^2; $p=1.000$). The first phase insulin response of the diabetic Control group remained low and unchanged (25 [-5 - 61], 28 [3 - 43], 29 [-11 - 60] and 35 [4-5] pmol/min/m^2 respectively (Figure 6.4.B and Figure 6.5.).

The median first phase insulin response was associated with ambient fasting plasma glucose, being 89 [59-188] pmol/min/m^2 with FPG ≤ 6.0mmol/l, 70 [45-81] with fasting plasma glucose 6.1 - 6.9 mmol/l, and 35 [-3-45] pmol/min/m^2 with FPG ≥ 7mmol/l. The relationship between first phase insulin response and fasting plasma glucose is shown in Figure 6.6. First phase insulin response correlated significantly in Intervention group with fasting plasma glucose ($r=-0.9$; $p<0.0001$), HbA1c ($r=-0.8$; $p<0.0001$), maximal insulin secretion ($r=0.6$; $p<0.0001$) and second step insulin response ($r=0.4$; $p=0.022$) at 12 months. There was no significant correlation with total fasting TG ($r=0.3$; $p=0.098$). In the diabetic Control group first phase insulin response did not change during the study (Figure 6.5.).

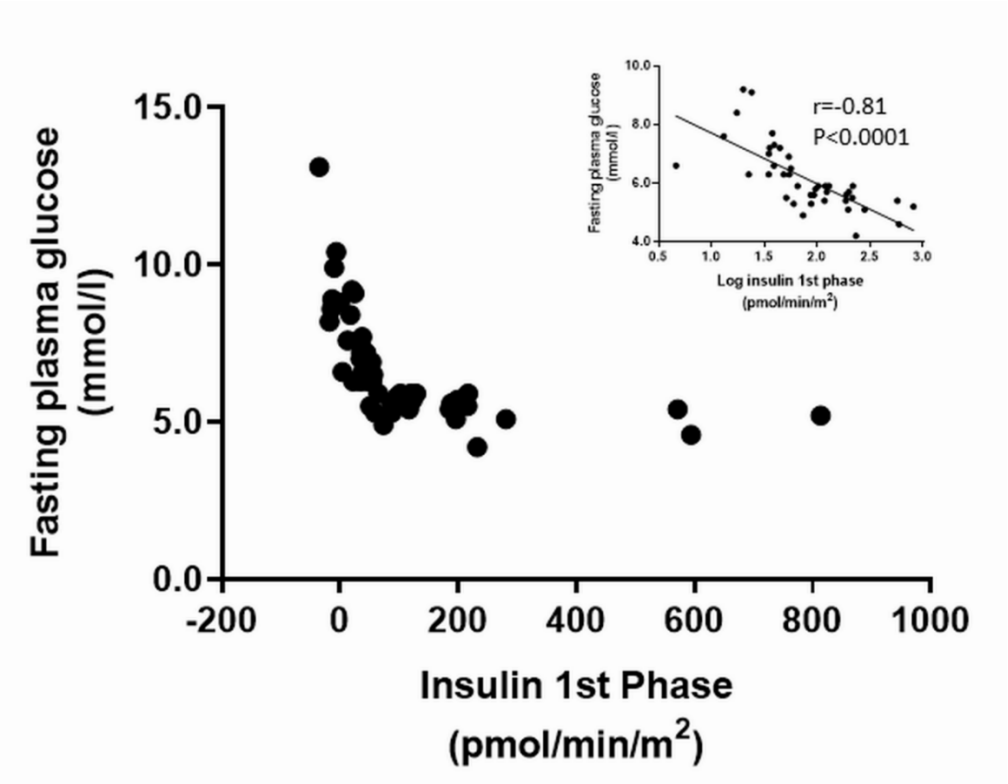

Figure 6.6 Relationship between fasting plasma insulin and first phase insulin secretion.

Fasting plasma glucose and first phase insulin secretion in the whole intervention group at 12 months (n=48: Responders=28, Non-Responders=16, Relapsers=4) were plotted. The inset graph shows the log transformed data (7 individuals omitted due to negative first phase insulin secretion values unable to be logged).

6.3.6 Second step insulin response

There was a small but significant increase in median second step insulin response in Responders 3 [-9 - 29] pmol/min/m^2 to 18 [8 - 34] pmol/min/m^2 (p=0.011) at 5 months (Figure 6.4.C), remaining steady to 12 months (21 [1 - 39] pmol/min/m^2; p=0.043), and with no significant change by 24 months (9 [-1-39] pmol/min/m^2; p=0.256) vs. baseline. Median second step did not change significantly either in Non-Responders (baseline -6 [-14 - 8], 5 months -2 [-8 - 6], 12 months 1 [-1 - 7], 24 months -3 [-11 - 9] pmol/min/m^2) or in Controls (16 [-2 - 33], 3 [-13 -15], 8 [-7 - 50], and 17 [1 - 23] pmol/min/m^2 respectively). There was a significant difference between second step in Responders and Non-Responders at 5 months (p<0.0001) and at 12 months (p=0.025) but not at 24 months (p=0.070). The median second step insulin response in non-diabetic comparator group was 68 [42 - 135] pmol/min/m^2, greater than that of the Responders at every time point (p<0.0001).

6.3.7 Pancreatic fat

Intra-pancreatic fat was higher in type 2 diabetes at the baseline compared with non-diabetes comparators (8.5±0.3 vs. 6.2±0.4 %, p<0.0001). Weight loss brought about similar change in intra-pancreatic fat in Responders and Non-responders (-0.91±0.1 7 % vs. -0.78±0.23, p=0.65). However, intra-pancreatic fat continued to fall between 5-24 months in Responders but not in the Non-responders (-0.48±0.25 vs. +0.41±0.35, p=0.03). At 24 months, pancreatic fat had decreased by 1.65±0.24 % in Responders, compared with 0.51±0.35 % in the Non-responders (p=0.013) (Figure 5.5E). There was no change in pancreas fat in the control group.

Pancreatic fat was non-significantly higher at baseline in Responders than Non-responders (8.7±0.4 vs. 7.9±0.6 %, p=0.26, Table 5.1). However, this could be secondary

to the higher fasting insulin concentration in Responders that would drive de novo lipogenesis in the liver, thereby elevating liver fat level, hepatic-TG export and intra-pancreatic fat.

In the whole group of intervention participants, the change in intra-pancreatic fat between baseline and 5 months correlated positively with change in total plasma TG ($r=0.29$, $p=0.04$) and change in plasma VLDL-TG ($r=0.30$, $p=0.04$, respectively). There was no correlation between change in intra-pancreatic fat and change in non-VLDL1-TG. The change in intra-pancreatic fat between baseline and 24 months correlated with changes in liver fat in all intervention participants ($r=0.45$, $p=0.002$). In Responders, there was a steadily closer relationship between VLDL1-TG production rates and intra-pancreatic fat (Figure 5.6B).

The improvement from baseline in first phase insulin response was maintained in Responders at 12 and 24 months), and there was no improvement in Non-responders as mentioned before in this thesis. Although first phase insulin response remained sub-normal in Responders it was sufficient to maintain non-diabetic blood glucose control as reflected by HbA1c. Fat removal from the pancreas correlated with restoration of 1st phase insulin secretion within the whole intervention group at 12 months (Figure 5.8D).

6.4 Discussion

The gradual increase in assessed functional beta cell capacity was assessed over the 24-months was observed following weight loss-induced reversal of type 2 diabetes up to 6 years duration. The first phase insulin response followed a distinct time course, with those returning to non-diabetic plasma glucose control exhibiting an early increase followed by stability from 5 to 24 months. The first phase insulin response improved significantly but remained around half of that of the non-diabetic comparators.

However, in contrast the assessed maximum beta cell capacity returned completely to normal, gradually over 12 months. It remained steady thereafter.

Studies of rodent beta cells *in vitro* suggest a relatively rapid resumption of insulin secretory function after removal of a metabolic stress (Pinnick et al., 2010). However, these studies typically involve cells from young animals and exposure to metabolic stress for a relatively short time. In human type 2 diabetes, exposure to excess lipid supply has been present for years or decades, affecting beta cells during middle age or more advanced years. This may account for the prolonged phase of recovery reported here following weight loss and restoration of glucose control in type 2 diabetes. Given the recognised heterogeneity of beta cells (Bonner-Weir and Aguayo-Mazzucato, 2016), it is possible that some cells are at a more advanced stage of de-differentiation and the slow return to normal functional beta cell capacity reflects different rates of re-differentiation (Weir et al., 2013a). It is of considerable interest that this process continues for at least 24 months after commencing negative calorie balance, in sharp contrast to the previously reported inevitable steady decline in beta cell number or function during maintained or increased body weight (1995a, Butler et al., 2003b, Rahier et al., 2008). No comparable observations have been made in previous studies, and the pathophysiologic mechanism underlying this requires investigation. GLP-1 responsiveness was not examined in this study and dietary weight loss has previously been shown not to change this after return to non-diabetic glucose control (Lingvay et al., 2013, Steven et al., 2016c)

The first phase insulin response did not completely normalise, although the degree of recovery was compatible with maintaining of non-diabetic blood glucose control. Not all beta cells are required to achieve a normal first phase insulin response, which can be achieved by rapid degranulation of some proportion of the whole. More detailed studies are required to determine the effect of an apparently adequate but sub-normal response during the post-prandial period. During 8 weeks of negative calorie balance in the Counterpoint study, a gradually improving first phase insulin response over 8 weeks in step with a gradually decreasing pancreas fat content was originally observed (Lim et al., 2011a). The extent of recovery appeared to be within the non-diabetic range but all the participants were in the first 4 years after diagnosis, had previously been treated

with diet or metformin only and were studied during continued low calorie diet which was more restricted (approximately 700 kcal/day). The present data extend these findings, demonstrating that the recovery during the first few months is maximal in a group with duration of type 2 diabetes up to 6 years and previously treated with any number of anti-diabetes agents but not insulin (Taylor et al., 2018a). Subjects with pre-diabetes and first-degree relatives of people with type 2 diabetes have a subnormal first phase response sufficient to permit overall control of plasma glucose prior to onset of type 2 diabetes (Elbein et al., 1999, Lundgren et al., 1990) so it is possible that we may have observed return to the premorbid levels in those achieving remission.

Metabolic stress on the beta cells can be produced by exposure to excess glucose or excess fat (Pinnick et al., 2010, Boucher et al., 2004, Nolan and Prentki, 2008). In human type 2 diabetes it is almost certain that both contribute, although initiation of the metabolic stress during normoglycemia likely to be via fat. Although the early sharp decrease in fasting plasma glucose levels will enhance the first phase response, this typically happens within hours with rapid recovery (Ferner et al., 1986, Boland et al., 2019). This was evident within 7 days of commencing a very low-calorie diet (Lim et al., 2011a) although the subsequent return to near normal first phase insulin response to a glucose stimulus was observed to develop steadily over 8 weeks, in step with the gradual decrease to normal of the excess lipid exposure of the beta cells. In contrast, there was no change in muscle insulin sensitivity over 2 months following remission in Counterpoint (Lim et al., 2011a). These observations were extended in the Counterbalance study which demonstrated continued normal hepatic insulin sensitivity but minor improvement only in muscle insulin sensitivity by 6 months following weight loss (Steven et al., 2016a). The conditions of the insulin secretion test used in this study permit assessment of beta cell function largely independent of tissue insulin sensitivity. As an indication of the whole-body insulin sensitivity, fasting plasma insulin, and hence its inverse, was not different between Responders and Non-responders at any time point (Table 1).

The present studies were conducted on people with less than 6 years duration of diagnosed type 2 diabetes at the time of recruitment. The actual duration of diabetes will have been longer, and many participants reported delay in seeking medical advice

after symptom onset. However, this will be common to all primary care populations with type 2 diabetes and time from diagnosis remains the practical yardstick. The protocol for DiRECT was informed by the Counterpoint study and the early results of the Counterbalance study which showed no return of first phase insulin secretion beyond 11 years of diagnosis (Lim et al., 2011a, Steven et al., 2016a). Even within 6 years of diagnosis of type 2 diabetes, it is apparent that there are some individuals who are susceptible to a more rapid loss of beta cell function in response to the metabolic stress. At 12 months, Responders had a significantly lower duration of diabetes than Non-Responders (Taylor et al., 2018a). This durability over time to withstand the beta cell stress induced by the combination of high glucose and high fat exposure, suggests that exploration of the genetic basis of this beta cell behaviour is required. The majority of discovered genes associated with type 2 diabetes code for beta cell processes (Taneera et al., 2012, Xin et al., 2016) and this information on phenotypic heterogeneity between individuals offers a route to linking specific genes with durability under metabolic stress. An early indication of this phenomenon was provided by Unger's demonstration of complete resistance to fat induced stress of islets isolated from ZDF rats not predisposed to develop diabetes upon high fat feeding (Lee et al., 1994b). It is possible that novel therapeutic targets may be identified to protect the beta cells of susceptible individuals, guided by genotyping.

Attempts to study beta cell mass have evolved from post-mortem histological studies (Butler et al., 2003b, Rahier et al., 2008, Rahier et al., 1983), through techniques of fresh pancreas slice histology (Marciniak et al., 2014) and incubation, to imaging of beta cells *in vivo* (Chen et al., 2017). Although conceptually attractive, the latter lack precision and at present there is no practical method of quantifying it *in vivo*. In contrast, the mass of beta cells which are functional can be assessed indirectly by metabolic tests (Nano et al., 2016, Meier et al., 2012). An arginine bolus during hyperglycemia elicits a large spike in insulin secretion dependent upon functional beta cell mass. There is a tight correlation between the response seen at different levels of plasma glucose in type 2 diabetic and non-diabetic groups (Fritsche et al., 2000, Ward et al., 1984). Although a true maximal response may be obtained at 25 mmol/l plasma glucose, the relative differences would be expected to remain. The SISTA test utilizes this defined response to permit an

assessment of the functional beta cell capacity *in vivo*, and observation of the time course of recovery of function.

Re-differentiation is a potential mechanism for beta cell function recovery after a reduction in islet fat content over the time course observed *in vivo* in humans therefore de-differentiation could be explained as a "hideaway" metabolic state till the insult to beta cells is removed. (White et al., 2016a). However this window of opportunity for restoration is limited given the failure to rescue beta cell function in subjects with long standing diabetes (Steven et al., 2015). Non-responders to low-calorie diet weight loss interventions typically have longer duration of type 2 diabetes making it likely that the duration of metabolic stress underlies non-response in the face of an adequate weight loss. There is a wide range of durations of type 2 diabetes in both Responders and Non-responders both in DiRECT study (duration of diabetes for Tyneside Responders 2.7±1.6 years vs. 3.8±1.6 years for Non-responders, p=0.026) and in Counterbalance (mean duration for responders 3.8±3.3 years and for Non-responders 9.8±5.3 years, p=0.007)(Steven et al., 2016a). This is likely to reflect a considerable variation in beta cells' resilience to metabolic stress in different individuals.

In summary, the functional beta cell capacity as assessed in this study returned completely to normal over 12 months in those who maintained weight loss-induced reversal of type 2 diabetes. First phase insulin response improved more rapidly but did not return to normal. Provided weight regain was minimised, both functional beta cell capacity and first phase insulin response remained stable at least up to 2 years with no evidence of any time-dependent decrease in beta cell function.

Chapter 7. General discussion

7.1 New knowledge on an effective weight loss strategy

These studies based on the DiRECT trial have demonstrated that type 2 diabetes of less than 6 years duration (3.0±1.7) can be reversed to normal in primary care by substantial weight loss. The disease process, previously believed to be chronic and inevitably progressive, can be stopped in its tracks. In this work the underlying reasons for this, namely the pathophysiological determinants of reversal of type 2 diabetes were further explored and elucidated.

Remission of type 2 diabetes was achieved by 62.5 % of the Tyneside subgroup immediately after the weight loss intervention. Remission is defined as HbA1c <48mmol/mol off all anti-diabetes medications (Nagi et al. 2019). Mean weight loss at 5 months was 15.3±7.3 kg and weight loss ≥ 15 kg was achieved by 50 % of the subjects. Some weight regain occurred by 12 months diabetes when remission was maintained in 45.3 % of participants. This was on a par with the total DiRECT cohort outcomes with remission rate in the whole group being 45.6 % at 12 months. Also this finding was in line with previously published study where diabetes remission was sustained in 40 % of the subjects at 6 months post very low calorie diet intervention (Steven et al., 2016a).

In contrast to previous assumptions that type 2 diabetes was a disease with complex and heterogenous causes, the data present incontrovertible evidence that decrease in total fat content of the body brings about return to non-diabetic HbA1c. There was a clear dose response effect of weight loss. Remission occurred in 86 % of participants with at least 15 kg weight loss and nearly 60 % with ≥10 kg were in remission at 12 months study follow up (Lean et al., 2018). At 24 months, 64 % of those who maintain a weight loss of >10 kg, and 70% with weight loss > 15 kg were in remission. To underscore this, those who relapsed into type 2 diabetes between 12 and 24 months, regained more weight in comparison to those maintaining remission. Weight regain which occurred mostly in weight maintenance phase, presented a challenge even though lower than in the other studies (Lean and Hankey, 2018). The emphasis on weight maintenance after gradual food reintroduction was a crucial element of the success of DiRECT study.

The extended period of food reintroduction allowed participants to adapt to new eating habits and was one of the elements that allowed to minimise weight regain in intervention subjects. Overall, 31.3 % of Tyneside Intervention participants and 35.6 % in the whole intervention DiRECT group were still in remission at 24 months, and in view of widespread medical views on the great difficult of achieving weight loss is a remarkable achievement.

Previous weight loss studies did not result in comparable to DiRECT weight loss. Despite very large expenditure on staff and resources, Look AHEAD achieved an average weight loss of only 8.6 kg (Look, 2014). The Counterbalance study was the only study which achieved similar weight loss to DiRECT but was small study and was managed in a research setting (Steven et al., 2016a).

The DiRECT low calorie diet intervention was done in the routine community settings and was very well accepted by the public (Taylor et al., 2018c). Therefore, there is a high expectation, that similar weight management programme rolled out in the community can achieve a wider impact on the care of the people with early type 2 diabetes. However, careful consideration of the views of participants is required.

7.2 The impact of the rapid weight loss programme on participants

Previous weight loss and weight maintenance studies showed up a number of barriers for a compliance with meal replacements: hunger, lack of variety of flavours, restriction of social activities despite the regular support from healthcare providers and social support and general satisfaction with the outcomes (Astbury et al., 2020, Rehackova et al., 2017, Kleine et al., 2019, Thom et al., 2021).

Therefore, exploring and understanding the experiences of DiRECT study participants was important to help in optimisation of the dietary intervention. This knowledge of participants' individual perceptions and behavioural change allows implementation of the study results into healthcare. Participants were allocated by practice, in a cluster-randomised design, to routine care, or to a behavioural intervention (Counterweight-Plus)(Leslie et al., 2016). The DiRECT patients' experiences seemed to follow a similar process of adaptation to change, consisting of uncertainty and expectations, learning and overcoming difficulties, and acceptance of continuous effort and establishment of routines during all phases of weight loss intervention and weight maintenance.

The DiRECT Intervention participants were hoping that the intervention would help them make to lose weight, put their type 2 diabetes into remission, allow discontinuation of their diabetes and antihypertensive medications for good, and to improve their wellbeing including mobility and energy levels. These factors encouraged commitment to the study despite the uncertainty of the outcomes and potential difficulties with the diet and a lifestyle.

"It was the potential outcome of the weight loss and diabetes potentially going into remission".

Learning new behaviours and overcoming initial difficulties with total dietary replacement adherence were the main challenges at the start of Intervention. The most common obstacles were hunger and fatigue during first few weeks.

"Initially the first few days were terrible. I felt miserable. And then as promised, after about day four I stopped feeling hungry, my headache went away, and I felt good".

"The hunger sort of came and went. There are days I don't think about food at all, and there are days I get really hungry".

The strategies to facilitate low calorie diet adherence identified in this study were: drinking a lot of liquids, distraction, modifying the flavours of the shakes or adding

vegetables, and planning dietary deviations. If hunger persisted, participants were trying to distract themselves by doing some work or exercise, watching television or engaging in hobbies. Boredom with the lack of variety of flavours in the middle of dietary intervention was encountered as major obstacle to maintaining motivation. Weight loss had slowed down in some participants. Holidays and social events were requiring additional planning.

"I actually found it very difficult to go back on to just having the shakes and soups. I had obviously come out of that state where you are not hungry, so what I found is that having taken some food, I was then hungry. If I had not gone on holiday, I think I would have managed to actually get down to the 13 stone, which is what we were looking for".

The fast weight reduction and improvement in blood glucose levels were rewarding despite the challenges experienced during the early stages and provided continuous motivation for the rest of the study. Most of the Intervention participants were perceiving their weight loss as a compliment.

Food reintroduction was described as a steep learning curve by some participants because they had to learn about calories and portion sizes. However, during weight loss maintenance participants became more comfortable once the new 'normal' eating pattern was established. Continuous behavioural support from health care professionals in DiRECT was vital to the participants and the study.

"The regular check-ups and the fact that you're being monitored and the fact it's part of a clinical trial, you can't just give in because you're letting other people down as well as yourself. That keeps you honest and it makes it a lot easier. I would say my chances of being where I am just now without that help would be as much as half".

Similar to DiRECT in DROPLET study participants also emphasized that in their positive experience of the dietary intervention one of the fundamental roles was played by the councillors (Astbury et al., 2020). Provision of ongoing support both in terms of encouragement and support is hugely important and should be regarded as a prime factor to be built into future healthcare delivery of type 2 diabetes remission.

Quality of life improved significantly in the Intervention group with no change in the Control group (Lean et al., 2018). The improvement in physical and psychological wellbeing after substantial weight loss, described in this study, have been reported in previous studies (McCombie et al., 2017, Rehackova et al., 2017).

7.3 Redefining the relationship between the liver and type 2 diabetes

The Twin cycle hypothesis postulated the primary role of the liver in initiating the type 2 diabetes process (Taylor, 2008). If excess triglyceride was not exported by the liver, the pancreas would not accumulate excess fat. This excess liver triglyceride production is closely related to liver fat content. As fatty liver disease is associated with rises in liver enzymes, it may be thought that routine liver function tests might reflect liver fat levels. However, measurement at a single time point is a very poor guide to liver fat levels (Mofrad et al., 2003). In one study 57% of subjects with type 2 diabetes had at least one abnormal LFT and 27% had at least two LFT tests (Salmela et al., 1984). Abnormal liver function tests are prevalent in type 2 diabetic patients in comparison with type the ones with type 1 diabetes (Salmela et al., 1984), however most individuals with NAFLD do not necessarily have ALT and GGT levels in the high normal range (Alexander et al., 2019). Sequential measurement may provide more useful clinical information, and indeed sequential change in liver function tests provides a measurement of change in liver fat both over the years before diagnosis of type 2 diabetes and during overfeeding studies (Sattar et al., 2007, Sevastianova et al., 2012).

In DiRECT, plasma levels of ALT and GGT declined at the same time with strong correlations between change in enzymes and change in liver fat at 12 and 24 months after weight loss. There was a significant improvement in indexes of hepatic steatosis (Fatty Liver Index and Hepatic Steatosis Index) after weight loss intervention at 12 months and these also positively correlated with liver fat. Notably hepatic fibrosis improved 24 months after diet-induced weight loss as assessed by the BARD score. The positive predictive value for normalization of liver fat post weight loss was high for both ALT and AST at 75% and 90% respectively. Some individuals started with low normal LFT values, and it was not possible to exclude change in liver fat from such data. If baseline ALT was raised, measurement after weight loss had a positive predictive value of 100%. Calculated hepatic steatosis index (HSI) gave similar result (PPV of 93.3%).

Overall, the predictive value of sequential liver enzyme measurement was found to be high for the detection of normalization of liver fat levels. These widely available tests permit estimation of resolution of NAFLD in routine clinical practice and this could be useful in the encouragement and informing of patients undergoing remission in Primary Care.

The association of hepatic steatosis with hepatic insulin resistance is well recognised in type 2 diabetes (Roden, 2006, Shulman, 2014, Yki-Jarvinen, 2014). Weight loss reverses the metabolic abnormalities associated with fatty liver and improves hepatic insulin sensitivity (Petersen et al., 2005). Liver fat content decreased into the normal range after weight loss and the prevalence of the fatty liver in type 2 diabetes group became comparable to that of non-diabetic controls and the general population (Browning et al., 2004, Wong et al., 2012). Similar findings in relation to weight loss and NAFLD prevalence were observed in this study. With stable weight and avoidance of major weight regain, liver fat levels remain normal up to 12 months (Steven et al., 2016b, Taylor et al., 2018b) which was confirmed in DiRECT study Tyneside subgroup. Substantial weight loss induced NAFLD remission as per MRI criteria (liver fat <5.5 %) at 12 months in 74.3 % of intervention subjects with NAFLD at baseline. NAFLD remission was maintained by 40 % of subjects with fatty liver at baseline as there was weight regain between 12 and 24 months of study.

It was demonstrated in this study that ALT is a sensitive NAFLD biomarker especially when the normal range of ALT is revised and set to a lower level. This could be used in monitoring of type 2 diabetes and NAFLD, and their remissions. This is also generally useful keeping in mind the increased incidence of cardiovascular mortality in people with NAFLD compared with the general population (Wannamethee et al., 1995, Lee et al., 2006, Schindhelm et al., 2007, Dunn et al., 2008) therefore the early recognition of NAFLD and promptly intervention is so important.

The resolution of NAFLD can be also seen in patients with the large weight loss post bariatric surgery. In one study 85 % of morbid obese patients who lost 25 % of their initial body weight had a resolution of the NAFLD a year after surgery (Lassailly et al., 2015). A mean weight loss of 13.5 % after either bariatric surgery or dietary weight loss achieved a maximum decrease in liver fat (Steven et al., 2016c) which is supported by meta-analysis of the bariatric surgery studies (Aguilar-Olivos et al., 2016). With the degree of weight loss achieved and sustained at one year post bariatric surgery, liver function tests are informative with the NAFLD fibrosis score and BARD score both decreasing (Nickel et al., 2018).

The present study further validated that Fatty Liver Index and Hepatic Steatosis Index are useful for detecting fatty liver disease. The observation of decrease towards normal of the BARD score at 2 years after dietary weight loss is potentially of great importance.

Liver VLDL export is the mechanism behind the reduction in liver fat content apart from trough increasing liver fat oxidation. In DiRECT baseline VLDL1-TG production rate was higher in the whole diabetic group compared with non-diabetic comparators. This has been observed in both type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) and the difference is particularly marked for plasma levels of VLDL1-triglyceride (Adiels et al., 2008). After weight loss, production rates decreased and became comparable with non-diabetic comparators. This is consistent with previous observations (Steven et al., 2016b). The Twin Cycle hypothesis, that increased VLDL-TG export from the liver could be a source of inhibitory metabolites affecting the pancreas in people with type 2 diabetes, was postulated back in 2008 (Taylor, 2008). DiRECT study data demonstrated

that remission of type 2 diabetes is associated with a decrease in liver-derived VLDL-TG and not with a change in non-VLDL-TG.

7.4 Insight into the likely aetiology of beta cell failure

UKPDS data demonstrated that beta cell function had already declined to approximately 50% of normal at the time of type 2 diabetes diagnosis and continued to decline despite pharmacological treatment (1995a, Rudenski et al., 1988). Death or apoptosis was suggested to be responsible for the apparent fifty percent decrease in beta cell number and for its continuous loss on the basis of the past histological studies (Butler et al., 2010, Rahier et al., 2008).

In the present study, not only did the previously reported inevitable decline in beta function stop, but also the functional beta cell capacity (maximum insulin response to glucose and arginine) gradually increased over a 24-month period following weight loss intervention in people with type 2 diabetes. The first phase insulin response increased during the weight loss period and stabilised from 5 to 24 months. Unlike the functional beta cell capacity, first phase insulin response improved significantly but remained around half of that of the non-diabetic comparators. The degree of return towards normal first phase response was related to the degree of glycaemic control achieved by those remaining in the 'pre-diabetes' range of HbA1c having lower responses than those achieving normal HbA1c.

After removal of a metabolic stress, the insulin secretory function demonstrated a relatively quick awakening in rodent beta cells *in vitro* (Pinnick et al., 2010), although the cells were taken from young animals . Exposure to excess lipid supply in human type 2 diabetes has been present for many years, presenting mostly in middle aged individuals. This may explain the gradual recovery after weight loss and restoration of glucose control in type 2 diabetes. It appears most likely that loss of specialist function under the

chronic endoplasmic reticulum stress of excess energy supply explains the beta cell changes in type 2 diabetes, a process known as 'de-differentiation'. A slow return to normal functional beta cell capacity is consistent with expects rates of re-differentiation (Weir et al., 2013a).

This study further tested the Twin Cycle hypothesis which theorized that a rise in VLDL TG supply underlay the increase in pancreatic fat content and subsequent decrease in beta cell function, paving the way to the development of type 2 diabetes (Taylor, 2008). Previous data from animal and human *in vitro* studies demonstrated that saturated fatty acids induce a cellular stress and inhibit beta cell function (Pinnick et al., 2008, Pinnick et al., 2010, Boslem et al., 2011). These data are consistent with the hypothesis. The type 2 diabetes reversal is associated with decreased plasma palmitic acid as per DiRECT study data which is the most beta cell toxic saturated fatty acid. De-differentiation under the lipid-induced endoplasmic reticulum stress is most consistent with human *in vivo* observations (Talchai et al., 2012, Biden et al., 2014, White et al., 2016b, Guo et al., 2010, Pinnick et al., 2010). The fat induced stress to beta cells is reversible in most people with early type 2 diabetes in present data and in previously published studies (Lim et al., 2011b, Steven et al., 2016b, Taylor et al., 2018b). The lipotoxicity is the initiating factor in beta cell de-differentiation, however the increased glucose exposure acts on par with lipotoxicity when type 2 diabetes is already established (Accili et al., 2016, White et al., 2016b, Bensellam et al., 2018). Lipid metabolism is regulated by autophagy therefore a calorie restriction enhances this process (Singh et al., 2009, Longo and Mattson, 2014). Abnormalities of autophagy were reported in beta cells under high lipid conditions, so taking away this metabolic stress is protective for beta cells (Zummo et al., 2017, Ji et al., 2019). This suggests that normalisation of autophagy by reducing the beta cell exposure to palmitic acid may also contribute to beta cell re-differentiation.

Beta cell de-differentiation markers were reported in human studies and support the overall concept as well as being helpful in understanding how the de-differentiation occurs (Cinti et al., 2016, Diedisheim et al., 2018).

People with pre-diabetes and first-degree relatives of people with type 2 diabetes have a subnormal first phase response sufficient to permit overall control of plasma glucose

prior to onset of type 2 diabetes (Elbein et al., 1999, Lundgren et al., 1990). It is possible that we may have observed return to the premorbid levels in those achieving remission in this study.

The beta cell studies in DiRECT were conducted on people with less than 6 years duration of type 2 diabetes at recruitment. The actual duration of diabetes may have been much longer as many participants reported delay in getting medical attention after the onset of diabetes symptom which is so common to primary care populations. The Counterbalance study which showed no return of first phase insulin secretion beyond 11 years of diagnosis (Steven et al., 2016a). Even within 6 years of diagnosis of type 2 diabetes, there would some people who are susceptible to a more rapid loss of beta cell function in response to the metabolic stress. At 12 months, Responders had a significantly lower duration of diabetes than Non-Responders (Taylor et al., 2018a). This durability over time to withstand the beta cell stress induced by the combination of high glucose and high fat exposure, suggests that exploration of the genetic basis of this beta cell behaviour is required. The majority of discovered genes associated with type 2 diabetes code for beta cell processes (Taneera et al., 2012, Xin et al., 2016) and this information on phenotypic heterogeneity between individuals offers a route to linking specific genes with durability under metabolic stress. Unger et al demonstrated a complete resistance to fat induced stress of islets isolated from ZDF rats which did not lead to developing diabetes after high fat feeding (Lee et al., 1994b). It is possible that novel therapeutic targets may be identified to protect the beta cells of susceptible individuals, guided by genotyping.

Overall, functional beta cell capacity and first phase insulin response with minimum weight regain remained stable at least up to 2 years with no evidence of any time-dependent decrease in beta cell function.

7.5 Implications for future management of type 2 diabetes

Demonstration of the basic pathogenesis of type 2 diabetes and demonstration of how this dangerous disease state can be reversed to normal leads to a reconsideration of how routine management should be approached. Critically, this has been shown to be relevant to everyday management by primary care staff in a community setting. The therapeutic approach has been shown to be well accepted by most people (Taylor et al., 2018c). Therefore, similar weight management programmes, if implemented in clinical practice, have the potential to achieve a wider impact on the care of people with type 2 diabetes in UK and elsewhere. Quality of life improved significantly in DiRECT intervention group with no change in controls. The benefits to individuals and the improved physical and psychological wellbeing accompanying substantial weight loss have been previously documented (McCombie et al., 2017, Rehackova et al., 2017). Importantly, 10-year cardiovascular risk (calculated as QRISK) was shown in the Counterbalance study to fall from 15% to the 6% (level of non-diabetic matched controls 6 months after achieving remission (Melhem et al., 2021). The DiRECT study has inspired a lot of people with early type 2 diabetes to take the initiative and go on low calorie diets to achieve diabetes remission. The additional benefits of being able to go off antidiabetic and antihypertensive medications, to improve blood pressure and lipid profile, and simply looking younger and being fitter must be also very attractive to the type 2 diabetic population.

It is important for healthcare systems that interventions for common conditions are not too expensive to be afforded. The programme based on the DiRECT intervention, is relatively simple and not expensive. The 12-month intervention cost is under half of the average annual UK healthcare cost of a person with type 2 diabetes (Xin et al., 2019).

Certainly, further work is required to optimise the weight maintenance phase. Specific long-term use dietary interventions supported by evidence are the low-fat diet, restricted carbohydrate diet, Mediterranean diet and intermittent energy restriction.

A low-fat diet (< 30 % total energy from fat) has long been widely advised. The idea became popularised by an epidemiological association between different countries of high fat intake with cardiovascular death (Keys, 1953). However, a head-to-head comparison of low fat diet with an energy restricted diet showed no significant difference in weight loss (Jeffery et al., 1995), unlike a combination of the low-fat plus low energy diet versus low-fat diet alone (Schlundt et al., 1993, Pascale et al., 1995).

Moderate carbohydrate restriction is simple to implement, particularly in the context of family eating. A restricted carbohydrate diet brings about an increase in the proportion of calories from fat, conflicting with long-held beliefs about the risks of higher fat diets. However, the practical outcome has been shown to be beneficial for both weight management and improvement in cardiovascular risk factors (Bazzano et al., 2014). The macronutrient composition of diet, for equivalent weight loss, does not affect liver fat content nor any other aspect of fat distribution (de Souza et al., 2012). These points have been incorporated into evidence-based nutrition guidelines (Dyson et al., 2011).

The Mediterranean diet were reported to be advantageous in terms of weight control and cardiovascular health (Estruch et al., 2016, Garcia-Fernandez et al., 2014, Martinez-Gonzalez and Martin-Calvo, 2016), with a decreased diabetes incidence independent of weight (Salas-Salvado et al., 2011). A combination of Mediterranean with carbohydrate restriction may be particularly beneficial (Esposito et al., 2014).

Time-limited approaches to eating (such as alternate day or intermittent fasting) appear to be acceptable to some individuals as an alternative to daily calorie restriction. This is as effective as calorie restriction for weight loss and maintenance for up to 12 months (Davis et al., 2016). The proportion of people losing more than 5% in weight has been reported to be higher with intermittent energy reduction (60-65%) compared to standard daily energy restriction (37%) (Harvie et al., 2013). Using the 5:2 approach in type 2 diabetes achieves comparable reductions in weight and HbA1c to calorie restriction with no adverse effects on exercise levels or appetite (Carter et al., 2016, Harvie and Howell, 2016).

Omission of breakfast is another possible dietary intervention (Brown et al., 2013) but this approach of not eating before lunch time is not suitable for everyone. Studies have

shown a major energy advantage of this eating pattern with no disbenefit in terms of eating later in the day (Clayton et al., 2016, Kealey, 2016).

There is most unlikely to be one 'best' way of eating that applies to everyone. Factors such as individual preferences and cultural influences underlie this general point. Eating is a social activity and family patterns of eating are important to take account of in planning advice for any one person. Overall, long term energy intake can be minimised by using an approach suited to the individual.

However, it important to consider that not everyone will be willing to undertake any significant dietary intervention. In Tyneside subgroup of DiRECT, which I had been supervising during metabolic studies, quite a few subjects in the control arm told me that they would not be willing to undergo such an intervention and that they are happy being randomised to the control group. In those people other weight loss interventions should be entertained. Use of purely dietary restraint has been vital in demonstrating both pathophysiology and potential economic benefits. However, pharmacological modification of appetite is increasingly feasible. In an addition to Orlistat, Saxenda (Liraglutide 3 mg) has been approved by NICE as a treatment for people with obesity in UK on 9th December 2020. It also has a potential for people with type 2 diabetes and obesity to achieve a substantial weight loss which could lead to the diabetes remission.

For those who are unable or unwilling to change their food habits, bariatric surgery could be a good solution. The overall effects of surgery – including involuntary restriction of food intake, rapid weight loss, post-prandial hypoglycaemia, risk of surgical complications - must be discussed with the individual and spouse/partner. For those people unable to restrain their intake of food, bariatric surgery can confer overall benefit. Randomised studies comparing outcomes are not informative, as individuals most suited to surgery are not the same people as those most suited to an effective dietary approach. The multicentre Swedish Obese Subjects study (SOS) is important as an observational non-randomised study comparing different types of bariatric surgeries with medical weight loss treatment (Sjostrom et al., 2004). The remission was 3 times greater and the risk of type 2 diabetes development was more than 3 times lower for the bariatric surgery group at 10 years of follow up (Sjostrom et al., 2004).

Bariatric surgery is very successful in achieving sustained major weight loss (Buchwald et al., 2009, Dixon et al., 2008) and it until recently has been the only intervention available on offer to patients irrespective of their degree of weight loss motivation which can achieve the necessary degree of weight loss.

Weight loss achieved by bariatric surgery decreases mortality in people with type 2 diabetes by up to 92% (Adams et al., 2007). International Diabetes Organisation had issued a treatment algorithm for bariatric surgery for type 2 diabetes, supporting use in people with BMI 35-39.9 kg/m^2 when hyperglycaemia is inadequately controlled by lifestyle and optimal medical therapy and in those with BMI ≥ 40 kg/m^2 (Rubino et al., 2016). Nonetheless, it should be considered whether an individual has exhausted the available dietary methods of weight loss shown to be effective before referring for surgery.

Follow-up and low intensity support to look at longer term outcomes in DiRECT are currently funded to continue for all DiRECT Intervention arm participants to a total of 5 years from baseline. A UK pilot programme based on the DiRECT trial was launched following the study outcomes to offer a low calorie diet intervention funded by NHS to 5,000 people in selected areas across England (NHS England » Low calorie diets to treat obesity and Type 2 diabetes). This programme has been designed to identify the most cost-effective way of delivering remission of type 2 diabetes to a wider population. The outcomes of this pilot programme will be important to guide the mechanism of delivering this important health care intervention to the many for whom it is appropriate both in the UK and worldwide.

In summary, the results which I present should influence application of Low-Calorie Diet, careful food reintroduction and supportive follow up to be considered as first line in the routine primary care of patients with early type 2 diabetes who want to achieve a remission. Given that remission is more likely to be achievable in the earliest phase of type 2 diabetes (Steven et al., 2016a, Sjostrom et al., 2014), the diagnosis of type 2 diabetes should be considered to be a medical emergency requiring immediate counselling and planning of therapy.

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Appendix

1. **Multivariate logistic regression analysis to evaluate the impact of the changes in metabolic parameters on liver fat change post DiRECT intervention at 12 months.**

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	3028.718	8	378.590	14.641	<.001[b]
	Residual	853.321	33	25.858		
	Total	3882.039	41			

a. Dependent Variable: LFchange

b. Predictors: (Constant), TGchange, ASTchange, FOchange, TCchange, GGTchange, BMIchange, ALTchange, WTchange

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	-2.099	1.735		-1.210	.235	-5.628	1.430
	WTchange	-1.805	.502	-1.413	-3.597	.001	-2.826	-.784
	BMIchange	7.538	1.418	1.957	5.317	<.001	4.654	10.422
	ASTchange	-.209	.136	-.227	-1.539	.133	-.484	.067
	ALTchange	.384	.111	.538	3.470	.001	.159	.609
	GGTchange	-.010	.050	-.021	-.192	.849	-.110	.091
	FOchange	.022	1.810	.001	.012	.990	-3.661	3.704
	TCchange	.003	.814	.000	.004	.997	-1.653	1.659
	TGchange	1.026	1.684	.079	.609	.546	-2.401	4.454

a. Dependent Variable: LFchange

2. Multivariate logistic regression evaluating the impact of changes in metabolic parameters on diabetes remission in DiRECT.

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	7.235	18	.402	3.167	.006[b]
	Residual	2.665	21	.127		
	Total	9.900	39			

a. Dependent Variable: Remission

b. Predictors: (Constant), SATchange, GGTchange, TGpoolchange, NEFAfast, TGPRchange, TCchange, VATchange, ASTchange, FPGchange, TTGchange, HDLchange, PFchange, LFchange, Wtchange, Hba1cchange, ALTchange, VLDLTGchange, BMIchange

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95.0% Confidence Interval for B	
		B	Std. Error	Beta			Lower Bound	Upper Bound
1	(Constant)	1.490	.132		11.297	<.001	1.216	1.764
	BMIchange	.294	.212	1.280	1.384	.181	-.148	.736
	FPGchange	.020	.041	.114	.486	.632	-.065	.105
	PFchange	-.043	.072	-.113	-.591	.561	-.193	.107
	TGPRchange	-.001	.000	-.242	-1.473	.156	-.001	.000
	TGpoolchange	.000	.000	1.461	1.916	.069	.000	.001
	LFchange	-.018	.013	-.341	-1.337	.196	-.045	.010
	NEFAfast	.013	.358	.005	.036	.972	-.732	.758
	TCchange	-.110	.079	-.208	-1.385	.181	-.274	.055
	TTGchange	.065	.126	.092	.513	.613	-.198	.327
	VLDLTGchange	-2.070	.917	-1.755	-2.257	.035	-3.977	-.163
	HDLchange	.116	.323	.062	.359	.723	-.556	.789
	Hba1cchange	-.015	.007	-.493	-2.039	.054	-.031	.000
	ASTchange	.013	.013	.284	1.060	.301	-.013	.040
	ALTchange	-.005	.010	-.133	-.472	.642	-.026	.016
	GGTchange	.005	.004	.240	1.184	.249	-.004	.014
	Wtchange	-.066	.067	-.875	-.985	.336	-.207	.074
	VATchange	-.002	.001	-.298	-1.818	.083	-.005	.000
	SATchange	.001	.002	.091	.525	.605	-.003	.005

a. Dependent Variable: Remission