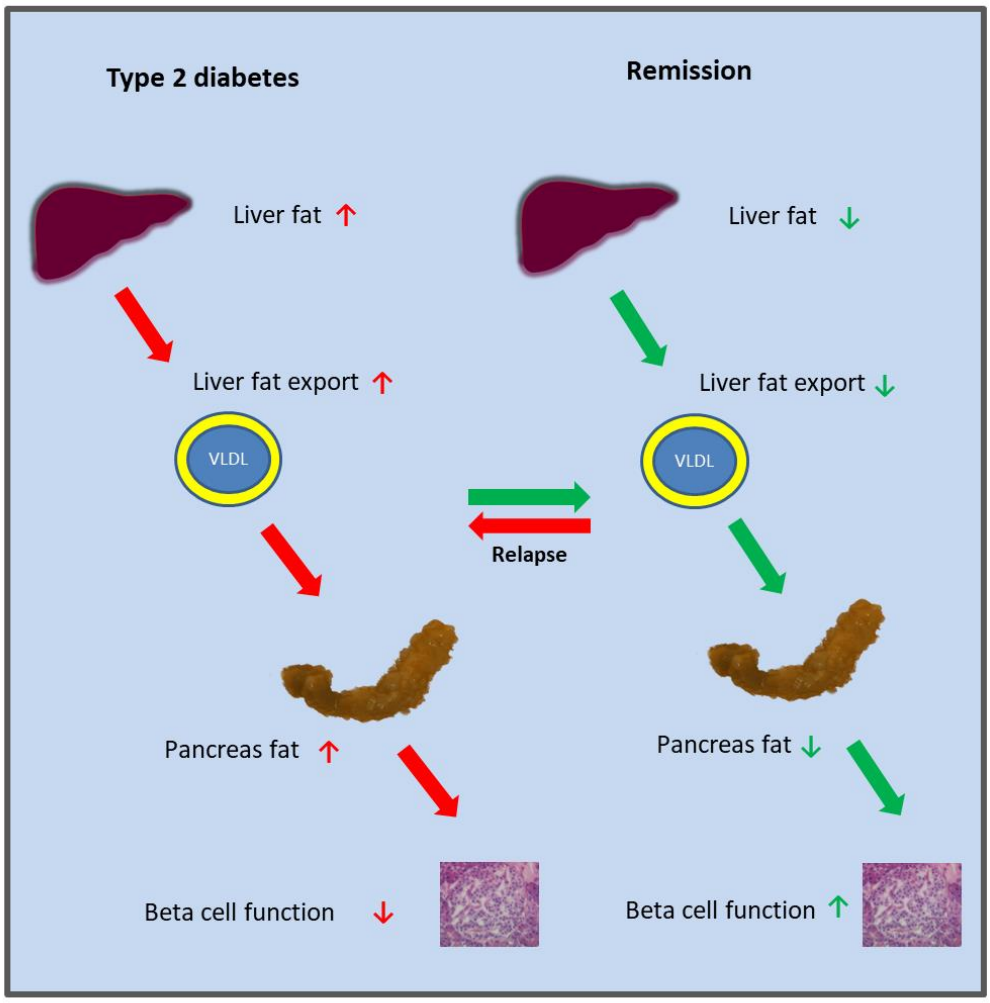

Graphical abstract

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Hepatic Lipoprotein Export and Remission of Human Type 2 Diabetes after Weight Loss

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Running title: Liver fat export and remission of type 2 diabetes

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Summary

The role of hepatic lipoprotein metabolism in diet-induced remission of type 2 diabetes is currently unclear. Here, we determined the contributions of hepatic VLDL1-triglyceride production rate and VLDL1-palmitic acid content to changes in intra-pancreatic fat and return of first phase insulin response in a subgroup of the Diabetes Remission Clinical Trial. Liver fat, VLDL1-triglyceride production and intra-pancreatic fat decreased after weight loss and remained normalized after 24 months of remission. First phase insulin response remained increased only in those maintaining diabetes remission. Compared with those in remission at 24 months, individuals who relapsed after initial remission had a greater rise in the content of VLDL1-triglyceride and VLDL1-palmitic acid, re-accumulated intra-pancreatic fat and lost first phase response by 24 months. Thus, we observed temporal relationships between VLDL1-triglyceride production, hepatic palmitic acid flux, intra-pancreatic fat, and β-cell function. Weight-related disordered fat metabolism appears to drive development and reversal of type 2 diabetes.

Key words

Type 2 diabetes, human, diabetes remission, liver fat, VLDL1- triglycerides, intra-pancreatic fat, palmitic acid, β-cell function, pathophysiology.

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 (Cho et al., 2018; Zheng et al., 2018). The Diabetes Remission Clinical Trial (DiRECT) has demonstrated that substantial weight loss induced by an integrated program 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 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 (Adiels et al., 2008; Donnelly et al., 2005), 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; Flannery et al., 2012) enhances *de novo* lipogenesis, the only other pathway to achieve storage of glucose energy (Petersen et al., 2007; Rabol et al., 2011; Schwarz et al., 2003).

The twin cycle hypothesis was proposed over 10 years ago to explain the etiology of type 2 diabetes and, potentially, mechanisms of reversal to normal (Taylor, 2008). It postulated that long term positive calorie balance would initiate accumulation of liver fat, inducing hepatic insulin resistance, increased hepatic glucose production and hence increased basal plasma insulin levels. This would precipitate a self-reinforcing cycle, as *de novo* lipogenesis is stimulated by insulin. The increased liver fat would promote increased VLDL-TG export. If subcutaneous adipose tissue is unable to accommodate more triglyceride, ectopic fat accumulation is likely in many tissues including the pancreas (Kim et al., 2000; Lotta et al., 2017; Taylor and Holman, 2015). Long-term exposure to saturated fatty acids is harmful to β-cells, and palmitic acid, the main product of *de novo* lipogenesis, is more potent in this respect than other fatty acids (Cnop et al., 2001; Eguchi et al., 2012; Elks, 1993; Jezek et al., 2018; Maedler et al., 2003; Pinnick et al., 2008). Impairment of post-meal insulin secretion would cause prolonged hyperglycemia and hyperinsulinemia and hence further promotion of *de novo* lipogenesis. The process of dedifferentiation is consistent with the impairment of β-cell function in type 2 diabetes (Bensellam et al., 2018; Cinti et al., 2016; Talchai et al., 2012; White et al., 2013). β-cell dedifferentiation can be promoted by metabolic stress induced by high concentration of glucose or fatty acids (Taylor et al., 2019; White et al., 2016). Most people (72%) with BMI greater than 40kg/m^2 do not have type 2 diabetes (Gregg et al., 2007) and remission of type 2 diabetes may be induced by a decrease in weight well within the non-obese range (Steven et al., 2016; Taylor et al., 2018a; Taylor and

Holman, 2015). A genetic basis for susceptibility of beta cells to fat induced dysfunction has been demonstrated (Lee et al., 1994), and it is likely that there may be a range of susceptibility of human beta cells to increased fat exposure.

We have reported a profound fall in liver and in intra-pancreatic fat during weight loss induced reversal of type 2 diabetes, associated with a return of β-cell function for at least 12 months (Lim et al., 2011; Steven et al., 2016; Taylor et al., 2018a). DiRECT was designed to determine what proportion of people with diabetes could be returned to non-diabetic glucose control in routine primary care after weight loss. 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., 2018a). The present study involved a geographically defined sub-group of DiRECT and the design is illustrated in Figure 1. Its main aim was to examine the predictions of the twin cycle hypothesis at the 2 year end point of the study. Secondly, it aimed to quantitate hepatic VLDL1 palmitic acid content, the fatty acid with the most deleterious effect on beta cell function and the major product of *de novo* lipogenesis (Cnop et al., 2001; Eguchi et al., 2012; Elks, 1993; Maedler et al., 2003; Pinnick et al., 2008). Thirdly, it aimed to describe the pathophysiologic processes underlying the recurrence of type 2 diabetes in the group which initially achieved remission but then relapsed back to diabetes. Finally, to determine how closely to normal each parameter was returned, data were compared with those from a non-diabetic control (NDC) 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.

Results and Discussion

Baseline characteristics of responders, non-responders and relapsers

Baseline data are shown in Table 1. Those who did not respond by achieving non-diabetic HbA1c and fasting plasma glucose (<6.5% or <48mmol/mol and <126mg/dl) had longer diabetes duration, lower plasma alanine aminotransferase (ALT), and higher HbA1c at baseline than those who did respond as we previously reported (Taylor et al., 2018a). 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). 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). Total plasma palmitic acid was similar at baseline between responders and non-responders (235.4±11.0 vs. 216.2±15.0 µmol/L, p=0.32) although palmitic acid content of VLDL1-TG at baseline was greater in non-responders compared to responders (67.3±7.4 vs. 50.0±4.6 µmol/L, p=0.009), possibly related to a higher rate of *de novo* lipogenesis in this group.

Neither visceral nor subcutaneous adipose tissue volumes differed between responders and non-responders (284.8 ±12.6 vs. 253.9 ±19.5cm^2, p=0.19 and 317.6±21.1 vs. 313.5±26.9cm^2, p=0.91, respectively, Table 1).

The baseline characteristics observed in the 24 month groups of responders, non-responders, and relapsers were as follows. Non-responders had higher baseline HbA1c (7.9±0.2 vs. 7.4±0.2%, p=0.02), lower ALT (24.4±2.2 vs. 34.1±3.1 U/L, p=0.02) and higher plasma NEFA (0.67±0.05 vs. 0.51±0.02 mmol/l, p=0.015). In non-responders vs. responders, fasting plasma insulin was 66.5±6.4 vs. 107.2±15.7 pmol/l (p=0.094), liver fat was 13.4±2.6 vs. 18.8±2.4% (p=0.143) and first phase insulin was 0.02 [-0.002-0.031] vs. 0.04 [0.023-0.067] nmol/min/m^2 (p=0.053), respectively. The difference in diabetes duration between responders and non-responders seen at baseline was not significant at 24 months (2.9±0.3 vs. 3.6±0.5 years, p=0.19). At baseline, those who subsequently became relapsers were similar to responders who remained in remission except for lower liver fat (12.1±2.0 vs. 18.8±2.4%, p=0.04). Pancreas fat in relapsers was 8.1±0.5% and 8.7±0.4% in the whole group of responders (p=0.40), which, defined as response at 5 months, includes those who subsequently relapsed (baseline pancreas fat for the group who were responders at 24 months was 9.7±0.6% (p=0.07).

In order to evaluate further the impact of baseline metabolic parameters on diabetes remission, a stepwise multiple regression model was used. Remission selected as main outcome and all other metabolic parameters as independent variables, including hepatic VLDL1-TG production, plasma VLDL1-TG concentration, VLDL-TG pool, liver fat, total plasma TG, non-VLDL1-TG were, age, sex, weight, diabetes duration, BMI, FPG, HbA1c, pancreas fat, fasting insulin, total cholesterol, HDL-cholesterol, ALT, SAT, VAT, total plasma palmitic acid and VLDL-TG palmitic acid. Remission of diabetes was associated with plasma VLDL1-TG (p=0.012), VLDL1-TG palmitic acid (p<0.0001), NEFA (p=0.008), ALT (p=0.018), and diabetes duration (p=0.024).

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±1.4kg, 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., 2018a). In those who reverted to diabetes after initial remission (relapsers; n=13), weight gain was 11.3±1.9kg between 5 and 24 months compared with 6.6±1.0kg in those with sustained remission (p=0.036, Table 1, Figure S1A).

HbA1c fell from 7.4±0.2 to 6.0±0.1% (p<0.0001) in those who maintained remission of diabetes. HbA1c was 5.4±0.1% in NDC. There was no significant change in non-responders (7.9±0.2 to 8.1±0.4%, p=0.53), Figure S1C. Likewise, fasting plasma glucose decreased from 150.6±11.8 to 101.5±2.7mg/dl at 24 months (p<0.0001) in responders, but did not change in non-responders (173.8±14.9 to 167.2±19.9 mg/dl, p=0.72), Figure S1B.

In those who had relapsed by 24 months, HbA1c rose from 6.0±0.1% at 5 months to 8.0±0.4% at 24 months (p<0.0001). There was a corresponding increase in fasting plasma glucose over the same period (106.6±3.8 to 145.8±10.27mg/dl, p=0.0005), Figure S1 B, C.

Fasting plasma insulin decreased substantially after weight loss (Figure2B), and remained low at 24 months in both responders (107.2±15.7 to 50.8±10.1 pmol/l, p<0.0001) and non-responders (66.5±6.4 to 35.5±6.0 pmol/l, p=0.003).

Effect of weight change on lipid parameters

Liver fat

At baseline, liver fat was elevated in the whole diabetic group compared with NDC (16.0±1.3 vs. 4.4±1.1%, p<0.0001). Immediately 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., 2018a). There was a gradual increase in liver fat from 5 to 24 months in both responders and non-responders (Figure 2A, Table 1). Although there was no significant difference between responders and non-responders, only the non-responder group became significantly different from NDC by 24 months (p=0.011).

Change in liver fat reflected change in body weight between 0-12 months (r=0.46, p=0.001), and 0-24 months (r=0.57, p<0.0001) (Figure S2A). This is in keeping with the personal fat threshold concept which proposes that the capacity to store subcutaneous fat is limited and determined genetically (Lotta et al., 2017; Taylor and Holman, 2015). Total lipodystrophy is an extreme single gene manifestation of this varying capacity for safe storage of fat. An induced increase in adipose tissue capacity has been shown to decrease liver fat levels in mice by transplanting adipose tissue and in humans by use of thiazolidinediones (Kim et al., 2000; Ravikumar et al., 2008). In type 2 diabetes, the association of hepatic steatosis with hepatic insulin resistance is well recognised (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). We previously demonstrated that this occurs within 7 days of commencing a hypocaloric diet (Lim et al., 2011). During weight stability, or at least avoidance of major weight regain, liver fat levels remain normal up to 12 months (Steven et al., 2016; Taylor et al., 2018a). Liver fat level correlated with fasting plasma insulin in the whole intervention group at baseline (r=0.53, p<0.0001), and at 24 months after weight loss (r=0.61, p<0.0001). Although intracellular triglyceride is not directly toxic, it is possibly a reflective of other lipid metabolites including diacylglycerol and ceramides. Diacylglycerol directly causes hepatic insulin resistance by inhibiting IRS-1 signalling via PKCε activation (Samuel et al., 2010). This process has also been shown to be reversed after few days of weight loss in rat model (Perry et al. 2018). Ceramides were also reported to increase insulin resistance and NAFLD in human and animal models (Luukkonen et al., 2016; Petersen and Shulman, 2017; Raichur et al., 2014; Turpin et al., 2014).

In healthy individuals, the fatty acids in hepatic triglyceride derive mainly from lipolysis in adipose tissue (Barrows and Parks, 2006). However, in the presence of muscle insulin

resistance such as seen in type 2 diabetes, the contribution of *de novo* lipogenesis is much greater (Donnelly et al., 2005; Flannery et al., 2012). Following a high carbohydrate meal in the presence of insulin resistance, flux through this pathway increases substantially (Flannery et al., 2012; Lambert et al., 2014; Petersen et al., 2007; Schwarz et al., 2003). This is likely to account for the higher liver fat levels in type 2 diabetes compared with weight-matched controls (Lim et al., 2011; Petersen et al., 2005; Steven et al., 2016). Additionally, in type 2 diabetes, elevated concentrations of glucose and insulin in plasma stimulate transcription factors which activate lipogenesis genes (Kawano and Cohen, 2013).

VLDL1-TG production

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

In responders, there was a 24% decrease in VLDL1-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 NDC (480.7±30.7mg/kg/day, p=0.032 vs. baseline) (Figure 2D, Table 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 than in NDC at 12 and 24 months post-weight loss (649.6±67.0 and 638.2±38.6 vs. NDC: 457.0 ±28.2 mg/kg/day, p=0.003, and p=0.001 respectively, Figure 2D, Table 1). There was a significant difference between responders and non-responders at both 12 and 24 months (Table 1, Figure 2D).

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 NDC (r=0.49, p=0.014). Similarly, there was a positive correlation between change in liver fat and change in VLDL1-TG production from baseline to 5 months in the type 2 diabetes group (r=0.47, p<0.0001) (Figure S3) and separately in responders (r=0.46, p=0.004) and non-responders (r=0.50, p=0.04).

Although the decrease in liver fat was similar in both responders and non-responders, the continuing hyperglycemia in non-responders would be expected to enhance *de novo* lipogenesis and so potentially blunt the response in VLDL1-TG production rate to weight loss.

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 within the whole type 2 diabetes group (to 0.47±0.05 mmol/l; p=0.0008) (Figure 2E). Reflecting the VLDL1-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 was 0.46±0.07 mmol/l in responders and 0.64±0.12 mmol/l in non-responders at 12 months (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) (Table1, Figure 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 NDC (2581± 241 vs. 1581 ± 332 mg, p=0.004) (Figure 2F) and decreased after weight loss (to 1445±179 mg, p=0.0001; and p=0.97 compared with NDC). 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±432 mg; p=0.11). The pool size was stable in responders at 12 and 24 months at 1379±205 mg and 1415±238 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±570mg, p=0.53 and 2109±536mg; p=0.82 at 12 and 24 months, respectively) (Figure 2F). These changes in VLDL1-TG pool size reflect changes in both plasma concentrations and body weight.

Non-VLDL1-TG

Non-VLDL1-TG was higher in type 2 diabetes compared with NDC (1.13±0.08 vs. 0.74±0.08 mmol/l, p=0.002). It fell similarly in responders and non-responders after weight loss, and at all points up to 24 months (1.14±0.13 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 3A). The lack of difference in change in non-VLDL1-TG between responders and non-responders suggests a central role of VLDL1-TG in determining delivery of fatty acids to the pancreas. Non-VLDL1 is likely to reflect TG in remaining 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 NDC (1.86 ±0.1 vs. 1.22±0.1 mmol/l, p=0.0002). Plasma TG concentration decreased after weight loss (to 1.28±0.1 mmol/l, p<0.0001) becoming similar to that of NDC (p=0.996, Figure 2C). As with non-VLDL1-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<0.001, and 1.91±0.25 to 1.24±0.14 mmol/l, p=0.007, respectively) (Figure 3A). The change was maintained to 24 months in responders and non-responders (1.14±0.10 mmol/l, p<0.0001, and 1.34±0.14 mmol/l, p=0.04 vs. baseline respectively). There was a positive correlation between total plasma TG and VLDL1-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 NDC (1.05±0.0.03 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 1).

Non-esterified fatty acids

At baseline fasting plasma NEFA was similar in diabetic and NDC 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 1).

Fasting plasma NEFA remained stable in responders to 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, vs. responders). 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 association of poorer glucose control with plasma NEFA (Karpe et al., 2011) is illustrated by the higher fasting NEFA in non-responders during continued hyperglycemia.

Effect of weight change on palmitic acid in VLDL-TG and total plasma

VLDL1-TG palmitic acid

At baseline, palmitic acid (C16:0) content within the VLDL1 fraction in type 2 diabetes was near double that in NDC (52.0±4.1 vs. 28.4±3.2 µmol /l, p<0.001) (Figure 3B). In responders, the palmitic acid content of 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 NDC at 0, 5, 12 and 24 months (67.3±7.4 µmol /l, p<0.001; 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=0.007, respectively) (Figure 3B, Table1).

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 1), and the difference between non-responders and 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 VLDL1-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).

There is consistent evidence from *in vitro* studies that palmitic acid is more toxic to beta cells than are unsaturated fatty acids (Boslem et al., 2011; Cunha et al., 2008; Eguchi et al., 2012; Lee et al., 1994; Pinnick et al., 2008; Robertson et al., 2004). The pronounced increase in VLDL1-TG palmitic acid levels during the re-emergence of type 2 diabetes in relapsers (Figure 4D) 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 may potentially be of considerable significance (Pinnick et al., 2008). Future observations of VLDL1-palmitic acid levels during the progression from pre-diabetes to type 2 diabetes will be important in providing further evidence for this hypothesis.

Total plasma palmitic acid

At baseline, total plasma palmitic acid concentration in the whole group of type 2 diabetes was not significantly different from NDC (229.3 ±8.9 vs. 206.9 ±7.4 µmol /l, $p=0.06$). Weight loss induced a fall to 192.6 µmol /l ($p<0.0001$), similar to NDC ($p=0.14$, Figure 3B). 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 µmol /l, $p= 0.77$ and 207.4 ±9.6 µmol /l, $p=0.97$ vs. NDC 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. NDC, respectively). Unlike VLDL1-TG palmitic acid concentration, there was no difference in total plasma palmitic acid concentration between responders and non-responders at any time point.

Longitudinal changes in lipid parameters during remission of diabetes

To examine the effect of time and interaction of parameters, repeated measures mixed effect ANOVA was used. All subjects were included into the model, using data for those in remission and for non-responders at each time point. For parametric data, time points (baseline/5 months/12months/24 months) were selected as within subject factors and remission/non-remission of diabetes as between subjects' factors. Post hoc analysis was done using Bonferroni correction. For non-parametric data, Friedman ANOVA was employed with Wilcoxon Signed Rank Test as a follow up post hoc analysis.

Between baseline and 24 months there was a significant change in: VLDL1-TG production, plasma VLDL1-TG, VLDL-TG pool, liver fat, pancreas fat total plasma TG, NEFA, and VLDL1-TG plasma palmitic acid ($p<0.0001$, main ANOVA effect for all). There were no interactions between time and remission. Post hoc analyses showed that the decrease in hepatic VLDL1-TG production was not different between responders and non-responders at baseline and 5

months, but became different at 12 months (p=0.002) and 24 months (p=0.014) due to the subsequent increase in VLDL1-TG production in non-responders. The fall in plasma VLDL1-TG was specific to responders (p=0.002) reflecting the changes between baseline to 5 months (p<0.0001) and baseline to 12 months (p=0.009). Similarly, the VLDL1-TG pool effect was related to responders (p<0.0001) reflecting the changes between baseline and all other time points (p<0.05). The change in liver fat was similar between responders and non-responders between baseline to 5 months and to 12 months (p<0.001). However, there was no change from baseline to 24 months in non-responders (p=0.55). This was consistent with the changes in total plasma TG which remained significant between baseline at 24 months (p=0.004) in responders but not in non-responders (p=0.100).The fall in VLDL1-palmitic acid was specific to the responders (p=0.007). These repeated measure analyses using ANOVA confirm the univariate analyses reported above.

Effect of weight change on visceral (VAT) and subcutaneous (SAT) fat storage

As a consequence of the weight matching, SAT and VAT were similar after weight loss in the diabetic group compared with NDC (SAT: 240.1±16.6 vs 264.3± 19.0cm^2, p=0.34; VAT: 161.0±9.0c vs. 193.9±23.5cm^2 p=0.20). The changes in SAT from baseline were similar in both responders and non-responders (317.6±21.1 to 235.0± 19.8cm^2, p<0.0001, and 313.5±26.9 to 251.6±29.1cm^2, p<0.0001, respectively) and hence unrelated to remission. There was an increase in SAT between 5-24 months in both groups (p<0.001), (Table 1).

Similarly, the changes in VAT from baseline were similar in both responders and non-responders (284.8±12.6 to 162.4±10.0cm^2, p<0.0001, and 253.9± 19.5 to 162.7± 19.5cm^2, p<0.0001). VAT increased between 5-24 months in both groups (p<0.0001 at 24 months vs. 5 months). Again, the similarity in change between responders and non-responders suggests that neither VAT nor SAT volume is directly related to remission. Most but not all studies confirm this observation (Colles et al., 2006; Gastaldelli et al., 2007).

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 (mean change: -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 (mean change:-0.48±0.25 vs. +0.41±0.28%, 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 4E). It should be noted that these data on change in pancreas fat are necessarily paired. The trends are not evident from the group data for each time point shown in Table 1 largely as a result of the 24 month relapsers, who tended to have lower baseline values, being part of the baseline value for the responders.

At baseline, pancreatic fat was 8.7±0.4% in responders and vs. 7.9±0.6% in non-responders, p=0.26; Table 1). However, any true difference 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 (Figure 5B,D,E,F).

In the whole group of type 2 diabetes, the change in intra-pancreatic fat between baseline and 24 months correlated positively with change in plasma VLDL1-TG (r=0.32, p=0.03). There was no correlation between change in intra-pancreatic fat and change in non-VLDL1-TG (r=0.09, p=0.56). The change in intra-pancreatic fat between baseline and 24 months correlated with changes in liver fat in type 2 diabetes (r=0.45, p=0.002). In responders, there was a steadily stronger relationship between VLDL1-TG production rates and intra-pancreatic fat (Figure S2B).

First phase insulin response was minimal in type 2 diabetes at baseline compared with NDC (0.03[0.002-0.058] vs. 0.25 (0.226 - 0.429) nmol/min/m^2, p<0.0001). At 5 months in responders, this increased by 0.076 [0.023-0.109] nmol/min/m^2 compared with no change -0.002 [-0.019-0.013] nmol/min/m^2 in non-responders (p=0.0001, median change in responders vs. non-responders). The improvement (median change) from baseline in first phase insulin response was maintained in responders at 12 and 24 months (0.082 [0.022-0.155] and 0.082 [0.060-0.130] nmol/min/m^2, respectively), and there was no improvement in non-responders (-0.003 [-0.017-0.023] and -0.002 [-0.028-0.027] nmol/min/m^2, respectively) (Figure 4F). 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 liver and pancreas correlated with restoration of 1st phase insulin secretion within the whole intervention group at 12 months (Figure 5C/6F).

Since the Twin Cycle Hypothesis was proposed (Taylor, 2008), data from animal and human *in vitro* studies demonstrated that saturated fatty acids induce cellular stress and inhibit β-cell function (Boslem et al., 2011; Pinnick et al., 2010; Pinnick et al., 2008). Our present data are consistent with the original hypothesis, and further demonstrate that reversal of type 2 diabetes is associated with decreased plasma load of palmitic acid, potentially the most beta cell-toxic saturated fatty acid, based on *in vitro /ex vivo* models. Although cell death or apoptosis has been suggested to explain β-cell failure in type 2 diabetes (Butler et al., 2003; Huang et al., 2007), de-differentiation under the lipid-induced endoplasmic reticulum stress is most consistent with our *in vivo* observations in humans (Biden et al., 2014; Guo et al., 2010; Pinnick et al., 2010; Talchai et al., 2012; White et al., 2016). We demonstrate that this stress to β-cell is reversible, at least in the majority of people within the early years after diagnosis both in the present data and in our previously published studies (Lim et al., 2011; Steven et al., 2016). Our current data are in keeping with the concept that lipotoxicity may be 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; Bensellam et al., 2018; Taylor et al., 2018a; White et al., 2016). Lipid metabolism is regulated by autophagy and calorie restriction enhances this cellular process (Longo and Mattson, 2014; Singh et al., 2009). Recent studies reported abnormalities of autophagy in beta cells under conditions of high lipid, and removing this metabolic stress protects the

beta cell (Ji et al., 2019; Zummo et al., 2017). It is therefore possible that restoration of normal autophagy by decreasing exposure of the beta cell to palmitic acid contributes to beta cell re-differentiation.

Recently, markers of β-cell de-differentiation have been reported in human studies and such studies may open new windows to understand why, and how, de-differentiation occurs (Cinti et al., 2016; Diedisheim et al., 2018).

Change in metabolic and clinical parameters during relapse into diabetes

Those participants who achieved remission immediately after weight loss but subsequently relapsed to re-develop type 2 diabetes are of particular interest. They regained more weight between 5 months and 24 months than in those who remained in remission (11.3 ±1.9 vs.6.6±1.0kg, p= 0.036, Table 1). Liver fat increase from 2.1±0.5% at 5 months to 8.3±1.4% at 24 months (p=0.001, Figure 6C). 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±668mg, p=0.014) (Figure 4 & Figure 6). In addition, 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 6E), and this was associated with increased intra-pancreatic fat content (7.1±0.5 to 8.0±0.6%, p=0.03, Figure 6G). The rise in liver fat correlated with intra-pancreatic fat content (r=0.64, p=0.018).

In contrast, those who remained in remission exhibited a modest increase in VLDL1-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 mg/kg/day, p=0.62). There was no significant change in fasting plasma VLDL1-TG (0.38±0.07 to 0.44±0.07 mmol/l, p=0.37), VLDL1-TG pool size (1021 ±185 to 1415 ±238 mg, p=0.24), or intra-pancreatic fat (8.5±0.6 to 8.0±0.6%, p=0.07), (Figure 4 & Figure 6).

In those who relapsed between 5 and 24 months, intra-pancreatic fat had decreased immediately after weight loss from 8.1±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 (33.4±8.5 to 74.1±8.1 µmol/L, p=0.006). Restriction of carbohydrate intake can decrease *de novo* lipogenesis by 80% (Mardinoglu et al., 2018), but *de novo* lipogenesis produces mainly palmitic acid. It is therefore likely that *de novo* lipogenesis was more active during relapse, explaining the higher level of VLDL1-TG palmitic acid compared with NDC (p<0.001), (Figure 4D).

First phase insulin response decreased between 5 and 24 months in relapsers (0.096[0.054-0.122] to 0.038 [0.027- 0.052] nmol/min/m^2, p=0.012). In contrast, those who remained in remission at 24 months maintained 1st phase insulin secretion (0.125[0.065-0.166] compared with 0.113[0.072-0.183] nmol/min/m^2 at 5 months, p=0.99 (Figure 4F & Figure 6H).

To examine the effect of time and of lipid parameters during relapse between 5-24 months, repeated measure mixed effect ANOVA was used. Data for responders and relapsers were included in the model. For parametric data, time points (5 months/24 months) were

selected as within subject factors and remission/relapse of diabetes as between subjects factors. Post hoc analysis was done using Bonferroni correction. For non-parametric data, Friedman repeated measures ANOVA was employed with Wilcoxon Signed Rank Test as a follow up post hoc analysis.

Based on the main ANOVA effect, there was a significant increase between 5 to 24 months in lipid parameters: VLDL1-TG production ($p<0.0001$), plasma VLDL1-TG, ($p=0.022$), VLDL1-TG pool ($p=0.007$), liver fat ($p<0.0001$) total TG ($p=0.004$), non-VLDL1-TG ($p=0.004$), total plasma palmitic acid ($p<0.0001$), and VLDL-TG palmitic acid ($p<0.0001$). Post hoc analyses showed that the change in liver fat and VLDL1-TG production rate was not shown to differ between responders and relapsers. However, the effects were significant in relapsers for: plasma VLDL1-TG ($p=0.025$), VLDL1-TG pool ($p=0.006$), total plasma TG ($p=0.023$), non-VLDL1-TG ($p=0.023$), and VLDL1-TG palmitic acid ($p=0.008$). This was associated with a significant decrease in 1st phase insulin response ($p=0.019$), and an increase in intrapancreatic fat content within the relapsers ($p=0.003$).

The extent of weight loss determines change in subcutaneous and visceral fat content. In relapsers, the decrease 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±13.2cm^2 respectively, $p<0.0001$ for both) was lost at 24 months. SAT increased and became non-significantly different from baseline at 24 months (310.95±29.7 cm^2, $p=0.19$, Table 1). For those who remained in remission, the change in VAT between baseline to 24 months was greater compared with the relapsers (-85.0±14.9 vs. -30±12.4cm^2, $p=0.008$). Taken together, these data suggest that SAT storage capacity may have reached maximum limit in relapsers, causing overflow of fat to VAT, liver and pancreas.

Conclusion

Firstly, the present data demonstrate that remission of type 2 diabetes is closely associated with decrease in liver-derived VLDL1-TG. During 2 years of remission of type 2 diabetes, hepatic VLDL1-TG production rates initially decline and then remain stable and normal. Plasma VLDL1-TG concentrations reflect this pattern of change, as does intra-pancreatic fat content. The findings are consistent with the twin cycle hypothesis. In those who did not achieve remission, the initial weight loss brought about similar, although more modest, changes in VLDL1-TG production rate, plasma concentrations of VLDL1-TG and intra-pancreatic fat. It was notable that palmitic acid content of VLDL1-TG 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., 2018a; Taylor and Holman, 2015).

Secondly, we were able to observe the process of re-development of type 2 diabetes in a subgroup of participants who had achieved remission initially but then regained excessive weight. This weight regain was associated with increased hepatic VLDL1-TG production, increased plasma VLDL1-TG and re-accumulation of intra-pancreatic fat. The palmitic acid content of VLDL1-TG increased markedly during the re-development of type 2 diabetes in

relapsers. The specific enrichment of palmitic acid in VLDL1-TG is may be related to increased *de novo* lipogenesis and potentially contributes to beta cell dysfunction.

Thirdly, the findings provide information on the potential pathophysiology underlying both the etiology and remission of type 2 diabetes. By definition, only individuals with beta cells susceptible to develop type 2 diabetes were included in DiRECT. In these individuals, excess fatty acid exposure appears to promote loss of specialised endocrine function (Cinti et al., 2016; Pinnick et al., 2010; Talchai et al., 2012). The observations are in keeping with de-differentiation of the β-cell as a likely mechanism bringing about reversible failure in early type 2 diabetes (Cinti et al., 2016; Talchai et al., 2012; Taylor et al., 2019; White et al., 2016), with hepatic VLDL1-TG being the "upstream" deliverer of the excess fat which could be causal for beta cell dysfunction and development of diabetes.

Fourthly, hepatic VLDL1-TG export rates in responders decreased with weight loss and remained close to those of the matched non-diabetic control group. Re-development of diabetes was associated with a return to baseline levels.

The temporal associations we report in human type 2 diabetes now require to be tested in appropriate animal models to determine causal relationships.

Limitations of the study

Firstly, the present study has investigated the association between VLDL1-TG and change in glucose homeostasis, but chylomicrons were not investigated directly. Secondly, direct measurement of *de novo* lipogenesis after weight loss induced reversal would be of interest as in type 2 diabetes, a much higher proportion of VLDL-TG derives from *de novo* lipogenesis compared with non-diabetic controls (Barrows and Parks, 2006; Lambert et al., 2014; Mardinoglu et al., 2018; Petersen et al., 2007; Schwarz et al., 2003) . The abnormally high rate would be expected to return to normal during reversal of diabetes (Taylor, 2008) and warrants quantification. Thirdly, since the risk allele of the *PNPLA3* gene confers susceptibility to hepatic steatosis but not hepatic insulin resistance, targeted studies of different genotypes would be illuminating (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). Fourthly, the present study establishes associations and does not prove causal relationships, although the coherent temporal patterns we report in both remission and reversal of type 2 diabetes suggest causality. Finally, the 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 particularly so for people from south Asia, where type 2 diabetes occurs at lower BMI (Ntuk et al., 2014).

Responders Non-responders Relapsers	***Baseline*** *(n=38)* *(n=18)* *(n=13)*	***5 months*** *(n=38)* *(n=18)* *(n=13)*	***12 months*** *(n=28)* *(n=15)* *(n=13)*	***24 months*** *(n=20)* *(n=13)* *(n=13)*	***NDC*** *(n=25)*
Weight (kg) Responders Non-responders Relapsers	 100.3±2.7[h] 102.1±4.4[g] 100.5±4.0[g]	 84.2±2.2[c] 88.7±4.4[c] 84.6±3.4[c]	 85.5±3.1[c] 91.9±4.9[b] 90.2±4.0[b]	 88.8±4.0[c] 90.3±4.0[b] 95.9±4.4[a,d]	 86.6±3.0
Liver fat (%) Responders Non-responders Relapsers	 16.7±1.6[i] 14.5±2.6[i] 12.1±2.0[i]	 3.4±0.7[c] 2.6±0.5[c] 2.1±0.5[c]	 2.9±0.6[c] 5.3±1.8[c] 4.7±1.8[b]	 6.6±1.6[c,e] 8.7±1.8[e,g] 8.3±1.4[a,e,g]	 4.4±1.1
VLDL1-TG PR. (mg/kg/day) Responders Non-responders Relapsers	 544.4±28.7[g] 581.1±52.1[g] 452.2±42.9	 413.6±25.8[c] 521.8±41.9[j] 406.1±42.2	 437.5±22.4[b] 649.6±67.0[h,k] 506.5±39.6	 480.7±30.7[a] 638.2±38.6[h,k] 561.3±37.3[d,g]	 457.0±28.2
VLDL1-TG pool (mg) Responders Non-responders Relapsers	 2488±267[h] 2775±505[g] 2580±512[g]	 1245±162[c] 1866±432 1328 ±272[a]	 1379±205[c] 2234±570 1677±296	 1415±238[c] 2109±563 3014±668[d,h,k]	 1581±332
Plasma VLDL1-TG (mmol/l) Responders Non-responders Relapsers	 0.71±0.07[g] 0.73±0.11[g] 0.75±0.15	 0.43±0.06[c] 0.55±0.12 0.46±0.10	 0.46±0.07[b] 0.64±0.12 0.55±0.10	 0.44±0.07[b] 0.66±0.15[d] 0.88±0.16[d,h,j]	 0.48±0.09
Total plasma TG (mmol/l) Responders Non-responders Relapsers	 1.84±0.13[i] 1.91±0.25[h] 1.79±0.21[g]	 1.30±0.13[c] 1.24±0.14[c] 1.31±0.15[a]	 1.26±0.12[b] 1.21±0.12[b] 1.45±0.14	 1.14±0.10[c] 1.34±0.14[a] 1.75±0.24[d,g,j]	 1.22±0.13
HDL Cholesterol (mmol/l) Responders Non-responders Relapsers	 1.08±0.06[h] 1.00±0.05[h] 1.07±0.07[h]	 - - -	 1.23±0.08[a] 1.12±0.06[g] 1.22±0.10[a]	 1.43±0.12[b] 1.22±0.08[a] 1.14±0.09[g]	 1.42±0.07
NEFA (mmol/l) Responders Non-responders Relapsers	 0.57±0.03 0.66±0.04[j] 0.67±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±0.05[h,k] 0.63±0.07	 0.57±0.04
VLDL1 C16:0 (µmol/L) Responders Non-responders Relapsers	 45.0±4.6[g] 67.3±7.4[ij] 47.4±8.3[g]	 33.5±4.2[b] 50.1±7.9[g] 33.4±8.5	 33.9±4.7[a] 53.3±6.3[h,j] 50.0±7.7[g]	 31.6±5.4[a] 62.0±9.7[h,j] 74.1±8.1[a,e,i,l]	 28.4±3.2
Pancreas fat (%)					

Responders	8.7±0.4[i]	7.8±0.4[c,h]	7.7±0.4[c,g]	8.0±0.6[c,g]	6.2±0.4
Non-responders	7.9±0.6[g]	7.1±0.5[b]	6.9±0.5[a]	7.0±0.3	
Relapsers	8.1±0.5[h]	7.1±0.5[b]	7.7±0.6	8.0±0.6[d,g]	
SAT (cm^2)					
Responders	317.6±21.1	235.0±19.8[c]	231.2±19.1[c,e]	254.4±27.6[b,f]	
Non-responders	313.5±26.9	251.6±29.1[c]	296.6±37.4[b,f]	294.3±35.2[a,e]	264.3± 19.0
Relapsers	332.9±38.6	244.0±34.4[c]	280.9±34.7[a,d]	310.9±29.7[e]	
VAT (cm^2)					
Responders	284.8 ±12.7[h]	162.4±10.0[c]	175.3±14.0[c ,d]	207.4±20.8[c,f]	
Non-responders	253.9±19.5	162.7±19.5[c]	180.0±23.6[b,d]	184.0±16.7[a,f]	193.9±23.5
Relapsers	275.7±17.9[h]	154.0±13.2[c]	187.9±19.9[c,d]	245.5±20.8[a,f]	

Table 1: Overview of main metabolic markers over 2 years

Data were presented as mean +/- SEM or median and interquartile range for 1st phase insulin secretion. All data for each time point are shown and statistical analyses were carried out on paired data between time points. Baseline data paired with 5 months were presented (except for HDL cholesterol for which 5 months data are not available, baseline data were paired with 12 months).

24 month relapsers are shown separately as a group for each time point although by definition the individuals contribute to the data on responders at 0, 5, and 12 months. Therefore, the numbers do not add to the total. Data on responders and non-responders up to 12 months have previously been reported (Taylor et al 2018) except for palmitic acid, SAT, and VAT.

[a] $p<0.05$ vs. baseline, [b] $p<0.01$ vs. baseline, [c] $p<0.001$ vs. baseline.
[d] $p<0.05$ vs. 5 months, [e] $p<0.01$ vs. 5 months, [f] $p<0.001$ vs. 5 months.
[g] $p<0.05$ vs. NDC, [h] $p < 0.01$ vs. NDC, [i] $p < 0.001$ vs. NDC
[j] $p<0.05$ vs. responders, [k] $p < 0.01$ vs. responders, [l] $p < 0.001$ vs. responders

Legends to Figures

Figure 1. Illustrative diagram of the study design

Participants were randomized to receive a low calorie diet (825-853kcal/day) for 3-6 months, followed by stepped food reintroduction 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 <6.5% and blood glucose <126mg/dl 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 (age, sex, and BMI after weight loss) was selected and studied at one single occasion. A group who initially reversed diabetes then lost remission by 24 months (HbA1c >6.5% and blood glucose >126mg/dl) was studied separately.

At baseline, there were 57 intervention participants with 56 participants who had VLDL data paired with 5 months. The additional person declined to give blood for metabolic tests (VLDL1-TG production/insulin secretion) after baseline, but continued in the study for clinical, fasting blood, and MR data. At 12 months, there were eight participants (6 responders/2 non-responders) who left the study for personal reasons (moving out of area and change in personal circumstances), and a further one without VLDL data. There were also four participants who redeveloped diabetes after initial remission (relapsers). By 24 months, other three participants (non-responders) had left the study (for personal reasons), and one participant did not undergo the VLDL1-TG production and insulin secretion test (included for clinical and MR data only). There were further eight participants who had relapsed into diabetes plus the subject who declined to carry out VLDL1-TG production test after baseline. The non-diabetic controls (NDC) were studied at one single occasion.

Figure 2. Changes in Hepatic VLDL1-TG metabolism over 2 years after weight loss

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 gray line, and NDC (measured on one occasion) as a dotted line. Weight loss itself brought about no significant differences between responders and non-responders at any time point. Data up to 12 months for responders and non-responders have previously been reported (Taylor et al., 2018a).
Data are presented as means ± SEM.

Responders vs. baseline: * p<0.05, **p < 0.01, ***p < 0.001
Non-responders vs. baseline: † p<0.05 , ††p < 0.01, †††p < 0.001
Responders vs. 5 months: ‡ p<0.05, ‡‡p < 0.01
Non-responders vs. 5 months # p<0.05, ##p < 0.01 Responders vs. NDC: ǂ p<0.05, ǂǂ p<0.01, ǂǂǂ p<0.001
Non-responders vs. NDC: + p<0.05, ++ p<0.01, +++ p<0.001
Responders vs. non-responders: ¥¥ p<0.001

Figure 3. Changes in plasma triglycerides and palmitic acid flux over 2 years after weight loss

Panel A: Change in plasma VLDL1-TG (gray), non-VLDL1-TG (stippled), and total TG (sum of both) within the whole type 2 diabetes group, responders, and non-responders. Data on NDC (measured on one occasion) are represented by dotted lines: for total TG (upper line) and for VLDL-TG (lower line). In responders, there is a fall in both VLDL1-TG and total TG which is maintained for 24 months, whereas in non-responders only a fall in total TG is seen.
Panel B: Change in plasma VLDL1-palmitic acid (gray), non-VLDL- palmitic acid (stippled), and total palmitic acid (sum of both) within the whole type 2 diabetes group, responders, and non-responders. Data on NDC (measured on one occasion) are represented by dotted lines: for total plasma palmitic acid (upper line) and for VLDL-TG palmitic acid (lower line)VLDL1-TG palmitic acid concentration falls only in responders with no significant change in total plasma palmitic acid in either group. Data were available on 48/56 subjects at baseline and 5 months, 39/48 at 12 months and 35/45 at 24 months.
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/ VLDL1- palmitic acid), ††p<0.01 vs baseline (VLDL1-TG/ VLDL- palmitic acid), †††p<0.001 vs baseline (VLDL-TG/ VLDL1- palmitic acid). ‡p<0.05 vs baseline (Non-VLDL1-TG), ‡‡p<0.01 vs baseline (Non-VLDL1-TG), ‡‡‡p<0.001 vs baseline (Non-VLDL1-TG).

Figure 4: 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 (gray). Relapsers show significant increase in liver fat, VLDL1-TG production rate, VLDL-TG plasma concentration and VLDL1-TG palmitic acid with return to baseline levels of pancreas fat and first phase insulin secretion. 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≤0.001 vs. responders.

Fig.5 Effect of weight loss induced changes in hepatic and intra-pancreatic fat on beta cell function

Correlations between change in intra-pancreatic fat and weight (A), change in fasting insulin and liver fat content (B), change in liver fat and beta cell function (C), change in liver fat and intra-pancreatic fat (D), change in VLDL1-TG production and intra-pancreatic fat (E), and change in intra-pancreatic fat and beta cell function (F) between baseline and 12 months within the whole type 2 diabetes group (n=46(A/B/C/D/F), and n=45(E)). The change in liver fat correlates with change in fasting plasma insulin, first phase insulin secretion and pancreas fat. The change in pancreas fat also correlates with change in VLDL1-TG production and change in first phase insulin response.

Figure 6. 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 (responders n=38; relapsers n=13), 12 months (n=28/n=13 respectively), and 24 months (n=20/n=13 respectively). Those who relapse into type 2 diabetes show a return towards baseline levels in the postulated underlying pathophysiological parameters. Responders are presented as a solid black line and relapsers as a dashed line. The dotted line is the gridline at y value=0. 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).

*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.

STAR Methods

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals Peptides, and Recombinant Proteins		
Sodium Chloride (NaCl)	Sigma-Aldrich, UK	Cat No: S9888
Sodium Bromide (NaBr)	Alfa Aesar, USA	Cat No: 14037
Sodium Hydroxide (NaOH)	VWR International Ltd, UK	Cat No: 102525P
Na2 EDTA	VWR International Ltd, UK	Cat No: 100935V
Sodium Chloride (0.9%)	Fresenius Kabi Ltd, UK	Freeflex
Intralipid 20%	Fresenius Kabi Ltd, UK	Intralipid 20%
Intralipid 10%	Fresenius Kabi Ltd, UK	Intralipid 10%
20% Dextrose	Fresenius Kabi Ltd, UK	20% Dextrose
Infusion pump	Arcomed Infusion Ltd, UK	VP7000 PVC
Nonadecanoic Acid	Sigma-Aldrich, UK	Cat No: 72332
Supelco 37 Component FAME Mix	Sigma-Aldrich, UK	Cat No: CRM47885
Boron Trifluoride-Methanol (14%)	Sigma-Aldrich, UK	Cat No: B1252
Toluene Anhydrous (99.8%)	Sigma-Aldrich, UK	Cat No: 244511
Ethyl Acetate Anhydrous (99.8%)	Sigma-Aldrich, UK	Cat No: 270989
Aceton (≥99.5%)	Fisher Scientific, Germany	Cat No: A/0560/17
Petroleum spirit AnalaR(40 - 60 °C)	VWR International Ltd, UK	Cat No: 23835.294
Heptacosafluorotributylamine	Sigma-Aldrich, UK	Cat No: 77299
Clear Glass Vial(2ml)	Sigma-Aldrich, UK	Cat No: 29009-U
PTFE/RUBB Screw Cap (9mm)	Sigma-Aldrich, UK	Cat No: 29315-U
SLB-IL60 Capillary GC Column	Sigma-Aldrich, UK	Cat No: 29505-U
Clear Glass Insert (0.25 ml)	Sigma-Aldrich, UK	Cat No: 29436-U
Pyrex glass culture tubes(10ml)	Sigma-Aldrich, UK	Cat No: Z653586
Helium Grade A	BOC Ltd, UK	Cat No: 101720-L
Nitrogen (O2 free)	BOC Ltd, UK	Cat No: 44-W
Ultracentrifuge	Beckman Coulter, Inc, USA	Model L7-80

SW 40 Ti Rotor	Beckman Coulter, Inc, USA	PN: 331302
Ultracentrifuge tubes	SETON SCIENTIFIC, INC, USA	Cat No:7031W
Peristaltic Pump	Joyfay International, US	BT100M
Glass Pasteur Pipettes	VWR International Ltd, UK	Cat No: 612-1701
Analytical balance	Ohaus, Switzerland	DV215CD
Vortex-Genie 2	Scientific Industries Inc, UK	SI-0236
Critical Chemical Assays		
Triglyceride	Roche Diagnostics, UK	Cat No: 05171407190
Insulin ELISA kit	Mercodia AB, Sweden	10-1128-01
C-peptide ELISA kit	Mercodia AB, Sweden	10-1136-01
HbA1c	Tosoh Bioscience, UK	HPLC-923G8
NEFA enzymatic calorimetric kit	BMG labtech, Germany	FLUOstar Omega microplate reader
Software and Algorithms		
3T Philips Achieva scanner	Philips, Netherlands	SN: 17497
Six-channel cardiac array	Philips, Netherlands	PN: 453567009711
Three-point Dixon acquisition	Philips, Netherlands	mDixon
Balanced Turbo Field Echo acquisition	Philips, Netherlands	BTFE
Glucose analyser	Yellow Springs Inc, USA	YSI 2300 STAT Plus
GC-MS system	Thermo Scientific, Germany	Voyager
Xcalibur	Thermo Scientific, Germany	Version 1.3
MATLAB	MathWorks, UK	Version R2013a
ImageJ	National Institutes of Health, USA	Version 1.50
Minitab	Minitab Inc., USA	Version 17
MRIcro	University of South Carolina, USA	Version 1.40
GraphPad Prism	GraphPad Software Inc, USA	Version 8.01
Deposited Data		
Raw data	Mendeley Dataset	doi:10.17632/8z6jfmvdtf.1
Others		

Liquid formula diet (825–853 kcal/day)	Cambridge Weight Plan Ltd., UK	N/A

Lead Contact and Materials Availability

The Lead Author is Roy Taylor (roy.taylor@ncl.ac.uk) who is the main point of contact for responding to material and resource requests. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Author. Raw data belonging to this study are deposited at Mendeley (doi in KEY RESOURCES TABLE above). This study did not generate new unique reagents. Materials availability including research papers cited in the STAR Methods section will be provided by Roy Taylor.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This mechanistic study was part of a cluster-randomised controlled design of the Diabetes Remission Clinical Trial (DiRECT) registered under clinical trial number ISRCTN03267836 (Leslie et al., 2016). Ethical approval for DiRECT was obtained from the West of Scotland Ethics Committee, and all participants provided written informed consent.

The sub-study was designed to investigate the metabolic changes during weight loss and remission of diabetes. This is important in order to understand the underlying mechanisms of type 2 diabetes reversal and remission. Full details of the participant's characteristics of DiRECT, and major baseline characteristics of this mechanistic study were previously published (Taylor et al., 2018a; Taylor et al., 2018b). In this study, we report data on 56 subjects with lipoprotein data, who were recruited in the intervention arm of DiRECT. Studies were conducted on 25 non-diabetic controls (NDC), selected to match the intervention group for weight, age and sex in the post-weight loss state (Figure 1), and studied at baseline only.

People with early type 2 diabetes were recruited in the Tyneside cohort of DiRECT by their general practices (n=57, 26F/31M, (mean± SD): age 53.2±7.5 years, weight 101.0±17.2kg, BMI 35.2±4.6 kg/m^2, diabetes duration 3.0±1.7 years, HbA1c 7.5±0.9%). 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 27-45kg/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%).

Weight loss in DiRECT was induced using a liquid formula diet (825–853 kcal/d), as the first phase of an integrated, structured, weight management programme (Counterweight-Plus).

This was continued for 3-5 months, followed by a 2-6 week food reintroduction phase, then ongoing weight maintenance support up to 24 months.

Studies were carried out at baseline then at 5 months, 12 months, and 24 months. The same metabolic studies were carried out on the NDC group at a single time point.

After weight loss, participants were classified as responders or non-responders based on both HbA1c <6.5% and blood glucose <126mg/dl off any anti-diabetes medication. At 12 and 24 months these criteria were applied to identify those who had relapsed into the diabetic state and responders who were in sustained remission. Metabolic studies were carried out after overnight fasting over 2 days for each time point, with attention to minimising stress or physical activities prior to and during each study.

METHOD DETAILS

Intralipid infusion and lipoprotein separation

This was performed on the first day of the study as previously described (Al-Shayji et al., 2007). In brief, antecubital veins both arms of the patient were cannulated with venflon Cannula (18g Green). Blood was withdrawn at baseline, then a bolus of 20% Intralipid (Fresenius Kabi Ltd, UK) at (0.1g/kg body mass) was injected through one cannula within 1 min followed immediately by a continuous infusion of 10% Intralipid at 0.1g/kg/hr using infusion pump (Arcomed Infusion Ltd, UK). At 75 min, cannulae were removed, and breakfast was given to the participant. During infusion, blood samples were taken at 5, 15, 30, 45, 60, and 75 min.
After two step 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 (0.195M NaCl/0.001M NaOH/0.001% Na2EDTA), and d 1.182g/ml (2.44 M NaBr /0.195M NaCl/0.001M NaOH/0.001% Na2EDTA/(Sigma-Aldrich, UK, Alfa Aesar, USA, and VWR International Ltd, UK). The density of the prepared solutions were measured using analytical balance (Ohaus, Switzerland) and adjusted with de-ioinised water.

2ml of plasma was adjusted to 1.118g/ml by adding 0.341g of NaCl (Sigma-Aldrich, UK), then carefully layered over a 0.5ml of 1.182g/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 1.0988g/ml, 1ml of 1.0860g/ml, 2 ml of 1.0790g/ml, 2 ml of 1.0722g/ml, 2 ml of 1.0641 g/ml, and 2 ml of 1.0588 g/ml. Centrifugation was carried out using SW40 rotor in L7-80 ultracentrifuge at 278,000g for 98 min with deceleration at 23 °C (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.

VLDL1-TG production rate was measured from the slope of plasma increment in VLDL1-TG concentration over 0-75 min during the Intralipid infusion test.

Insulin secretion and β-Cell Function

Stepped Insulin Secretion Test with Arginine stimulation (SISTA) was used to define β-Cell function in response to intravenous glucose challenge (Lim et al., 2011; Toschi et al., 2002). This was carried out on the second day of the study. After an overnight fast a bolus of 20% Glucose was given at time 0. This was calculated using the formula: 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). The bolus was followed by 20% Glucose infusion to clamp plasma glucose, achieving a square wave step increase in plasma glucose level: +2.8 mmol/l. The glucose infusion rate was commenced using the formula: infusion rate (ml of 20% Dextrose/min) = 1mg/kg/min x body weight (kg) / 200 (mg/mL). Plasma glucose concentration was measured every 5 minutes and the glucose infusion rate was varied according to standard glucose clamp methodology for 30 minutes. The bolus was repeated at 30 minutes and plasma glucose was then clamped at +5.6 mmol/l above fasting level for the rest of the test. At 60 minutes a bolus of 5 g of Arginine was given intravenously to elicit a maximal insulin response under the condition of the test. Blood samples for determination of C-peptide concentrations were obtained every 2 min for the first 10 min of each step, then every 5 min. Insulin secretion rates were calculated using a deconvolution method, modelling C-peptide kinetics, and first phase insulin response was defined as the maximum rate of secretion within the first 6 minutes of the test (Lim et al., 2011).

Intraorgan and abdominal fat quantification

Magnetic Resonance (MR) was used for quantification of pancreatic and hepatic fat as previously described (Al-Mrabeh et al., 2016; Al-Mrabeh et al., 2017). This was carried out at baseline, following return to isocaloric eating after weight loss, at 12 months, and 24 months. MR data were acquired using a 3T Philips Achieva scanner with six-channel cardiac array (Philips, Netherlands) by the three-point Dixon method, with gradient-echo scans during one breath hold. Hepatic fat content was measured by selecting homogenous regions of interest on five image slices of liver(Lim et al., 2011). 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 vertebral space to estimate subcutaneous and visceral fat (SAT/VAT). Thresholding and watershed analysis using ImageJ were applied to calculate VAT and SAT areas at L4-L5 from the proton density fat fraction map (Al-Mrabeh et al., 2017; Steven et al., 2016). Analyses of pancreas fat and abdominal fat were carried out by single observers in a blinded manner (pancreas fat: AAM; abdominal fat: AJ).

Fatty acid analysis

Fatty acid methyl esters (FAME) were prepared from the VLDL1 fraction and total plasma following direct transesterification based on published procedures (Lepage and Roy, 1986; McEneny et al., 2000). Briefly, 200µl of VLDL1 or plasma was transferred to 10 ml Pyrex glass culture tubes (Sigma-Aldrich, UK), and spiked with 5µl of Nonadecanoic acid (0.5 mg/ml), analytical standard (Sigma-Aldrich, UK). Afterwards, 2ml of 14% Boron trifluoride-Methanol (Sigma-Aldrich, UK) was added and the tubes were properly capped and vortexed for 1min (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, then1 ml of 40 -60°C analytical grade, petroleum spirit (VWR International Ltd, UK), and 1ml of de-ioinised water were added to each tube followed by vigorous vortexing for 1min. Samples were then centrifuged at 3000 RCF for 5 min 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 min until compete dryness. FAME were reconstituted in 50µl (100µl 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 2.5 °C/min until 225°C then hold for 2 min. Injector and source temperature were 250°C and 200°C, respectively.

1µl of the sample was injected in the split mode (1:20), and mass spectrometry data were acquired in full scan mode 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).

Analytical Procedures

Glucose was measured by the oxidase method (Yellow Springs, USA). HbA1c was quantified using HPLC (Tosoh Bioscience, UK). HDL cholesterol and total TG were analysed by standard methods at the Institute of Cardiovascular and Medical Sciences, University of Glasgow.
C-peptide, insulin, glucose, NEFA, VLDL1-TG, and other metabolites were analysed at Clinical Pathology Accreditation Laboratory (Newcastle upon Tyne Hospital NHS Foundation Trust, UK) using standard kits as described in the Key Resources Table.

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 1). Responders who subsequently relapsed were analysed as a separate group and not added to Non-responders at each time point. Data are presented as mean±SEM or median (IQ range) for insulin secretion. Student paired or two-sample t test were used as appropriate for parametric data and Mann Whitney U test or Wilcoxon Rank test for nonparametric data. Multivariate analyses were additionally conducted. For longitudinal changes over time, repeated measures ANOVA and Friedman ANOVA were employed. Post hoc analyses were carried out using Bonferroni correction and Wilcoxon test as appropriate. Stepwise multiple regression models were used for prediction of the effect of baseline lipid parameters on remission and for prediction of the effect of change in lipid parameters on relapse (5-24 months).

Statistical analyses were performed using Minitab 17 (Minitab, USA) and SPSS version 25 (IBM, USA), and a 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% remission 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.

DATA AND CODE AVAILABILITY

The original data using for all Figures in the paper is available in Mendeley Data (doi:10.17632/8z6jfmvdtf.1). The study did not generate any unique code.

AUTHOR CONTRIBUTIONS

Conceptualization, R.T. and A.A.-M; Methodology, A.A.-M., K.G.H., and C.P.; Investigation and Validation, S.Z., C.P., A.C.B., S.M., A.J., and A.A.-M.; Writing – Original Draft, A.A.-M; Writing – Review & Editing, R.T., A.A.-M., N.S., G.L., J.C.M., and M.E.J.L.; Visualization, A.A.-M. and R.T.; Data Curation and Formal Analysis, A.A.-M., and R.T. Funding Acquisition, R.T., M.E.J.L., N.S., K.G.H., and J.C.M.; Software and Resources, B.S.A., K.G.H. and G.L; Supervision, R.T., A.A.-M., N.S., J.C.M., and M.E.J.L.

DECLARATION OF INTERESTS

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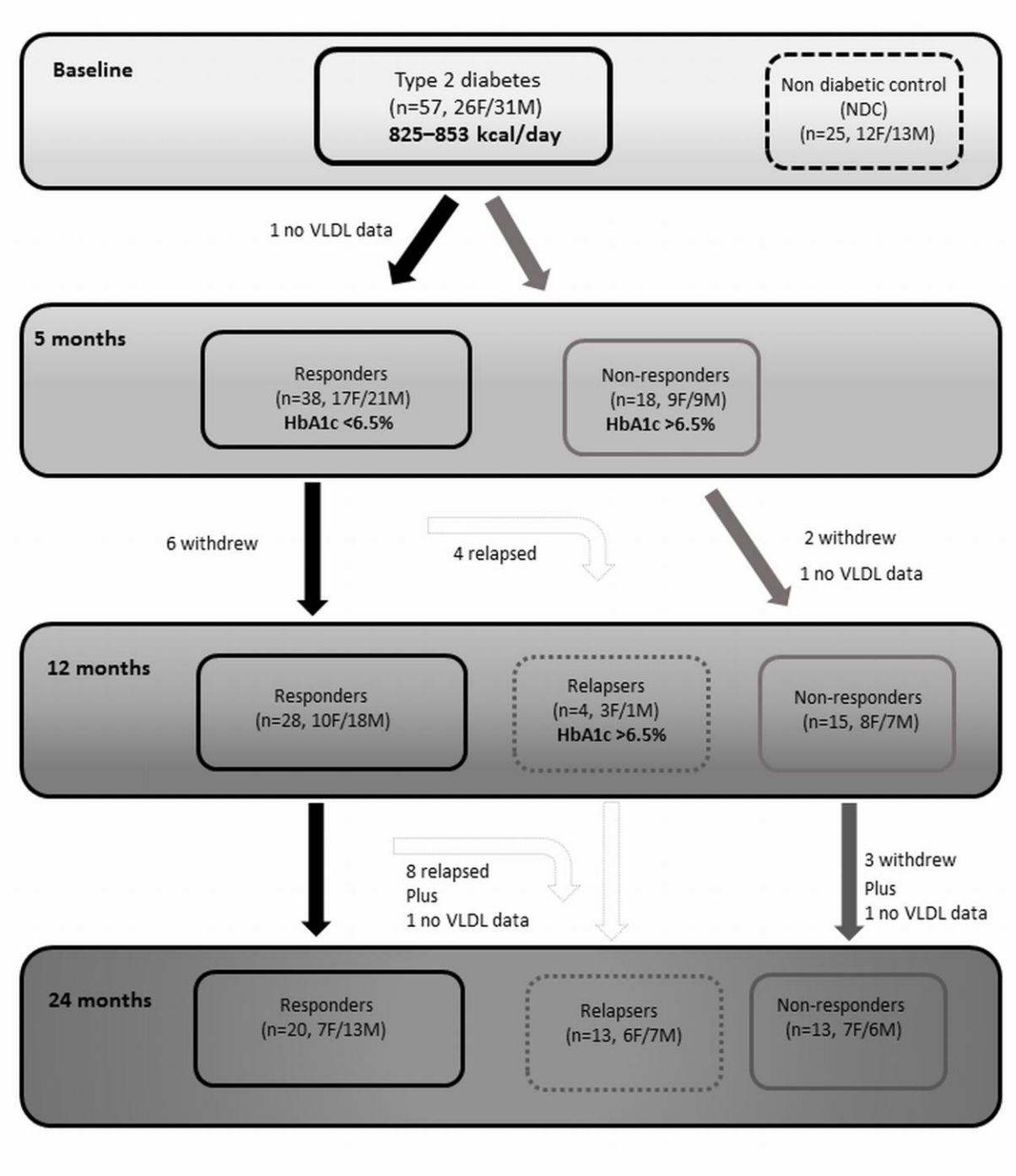

Baseline
Type 2 diabetes
(n=57, 26F/31M)
825–853 kcal/day
Non diabetic control
(NDC)
(n=25, 12F/13M)
1 no VLDL data
5 months
Responders
(n=38, 17F/21M)
HbA1c <6.5%
Non-responders
(n=18, 9F/9M)
HbA1c >6.5%
6 withdrew
4 relapsed
2 withdrew
1 no VLDL data
12 months
Responders
(n=28, 10F/18M)
Relapsers
(n=4, 3F/1M)
HbA1c >6.5%
Non-responders
(n=15, 8F/7M)
8 relapsed
Plus
1 no VLDL data
3 withdrew
Plus
1 no VLDL data
24 months
Responders
(n=20, 7F/13M)
Relapsers
(n=13, 6F/7M)
Non-responders
(n=13, 7F/6M)

Figure 1

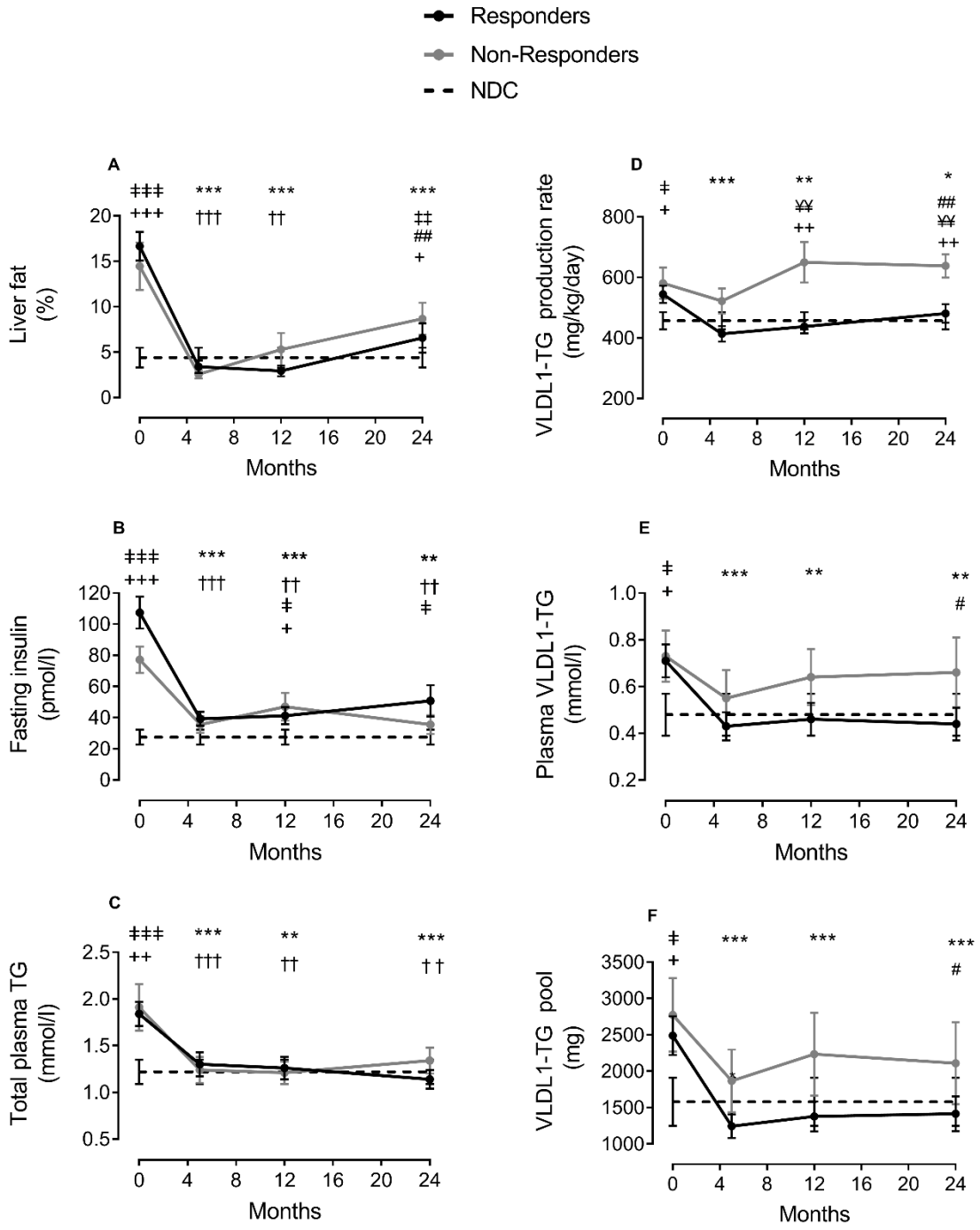

Responders
Non-Responders
NDC
A
Liver fat (%)
Months
B
Fasting insulin (pmol/l)
Months
C
Total plasma TG (mmol/l)
Months
D
VLDL1-TG production rate (mg/kg/day)
Months
E
Plasma VLDL1-TG (mmol/l)
Months
F
VLDL1-TG pool (mg)
Months

Figure 2

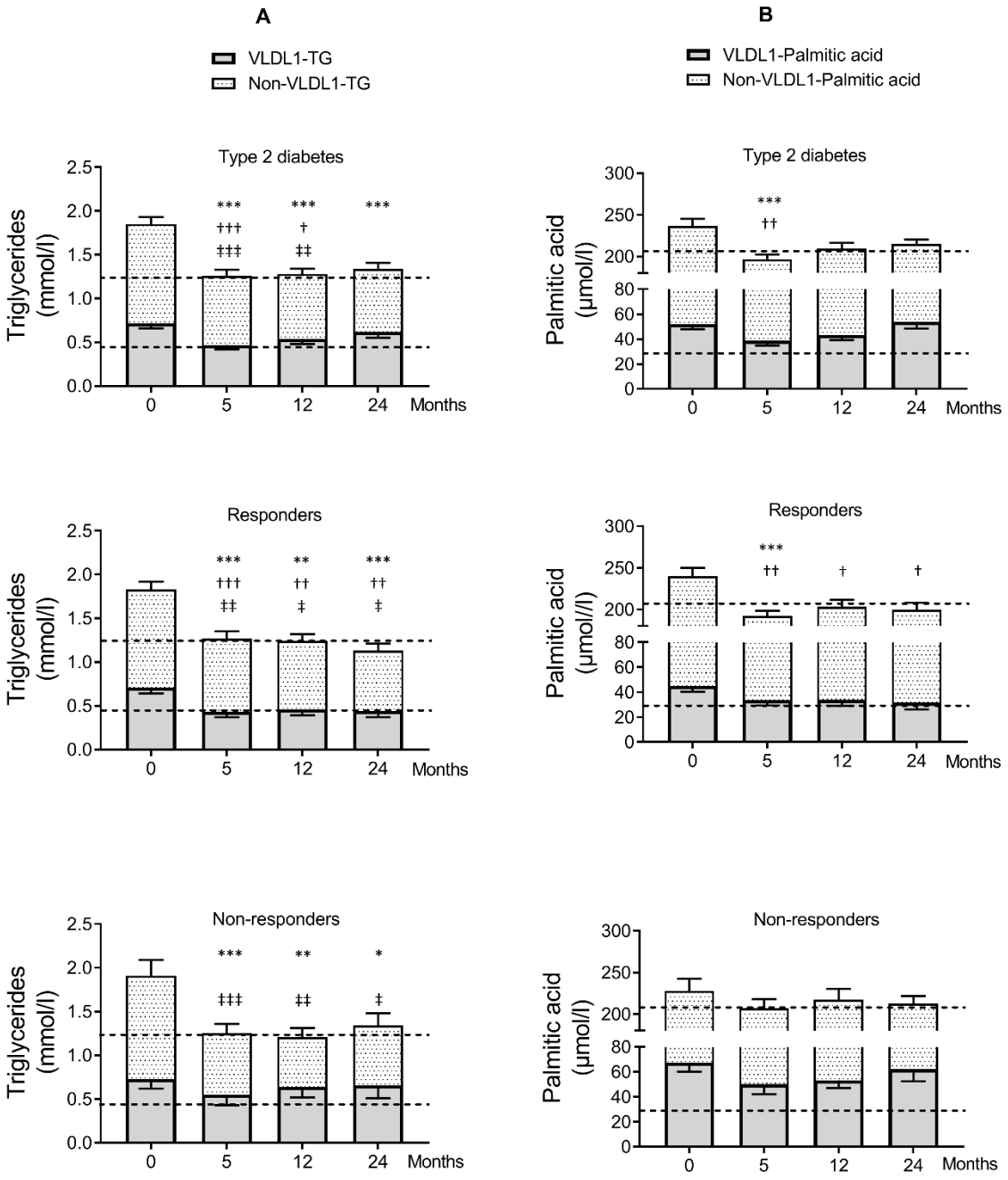

A
VLDL1-TG
Non-VLDL1-TG
Type 2 diabetes
Triglycerides (mmol/l)
Months
Responders
Non-responders
B
VLDL1-Palmitic acid
Non-VLDL1-Palmitic acid
Palmitic acid (µmol/l)

Figure 3

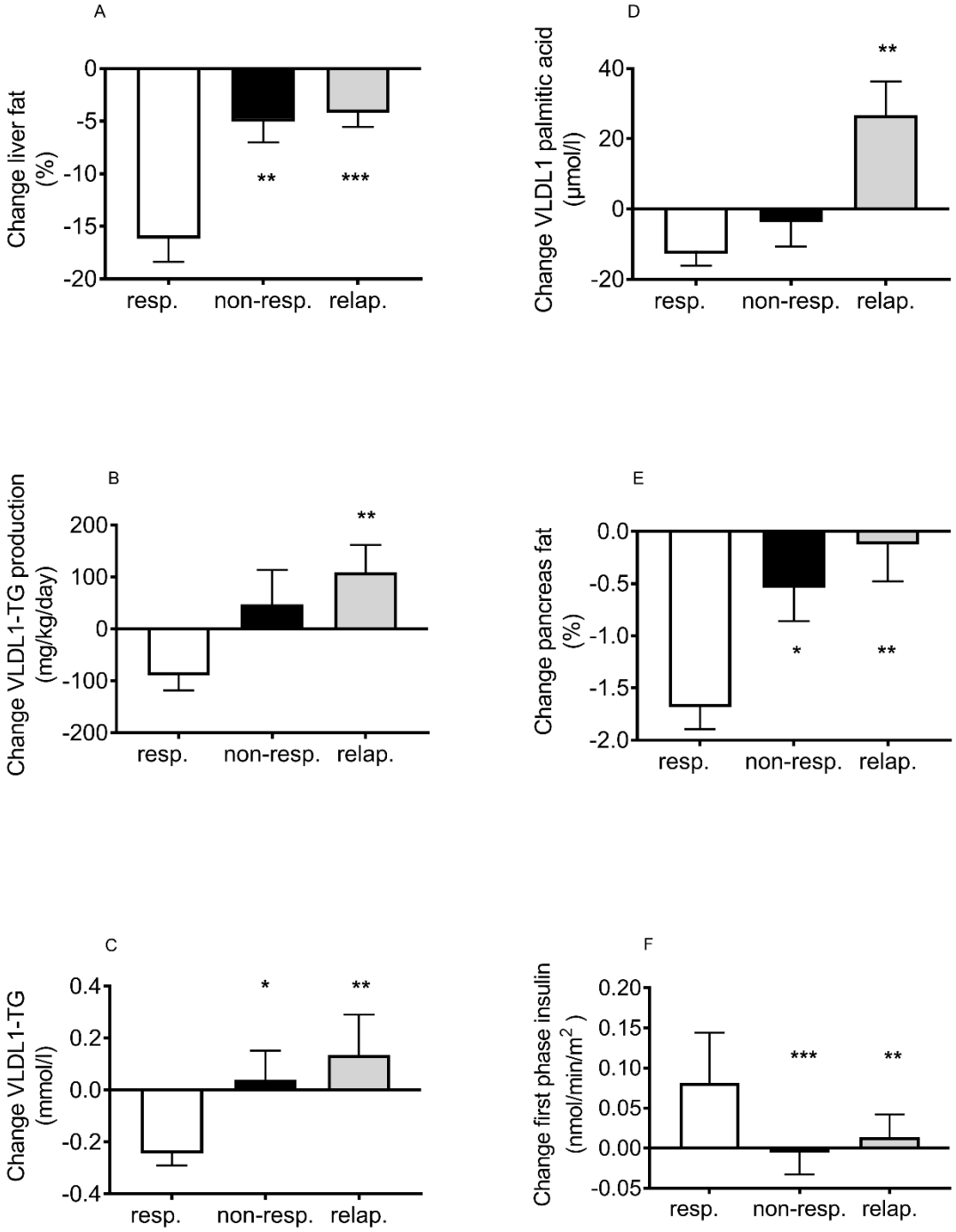

A
Change liver fat (%)
0
-5
-10
-15
-20
**
***
resp.
non-resp.
relap.
D
Change VLDL1 palmitic acid (µmol/l)
40
20
0
-20
**
resp.
non-resp.
relap.
B
Change VLDL1-TG production (mg/kg/day)
200
100
0
-100
-200
**
resp.
non-resp.
relap.
E
Change pancreas fat (%)
0.0
-0.5
-1.0
-1.5
-2.0
*
**
resp.
non-resp.
relap.
C
Change VLDL1-TG (mmol/l)
0.4
0.2
0.0
-0.2
-0.4
*
**
resp.
non-resp.
relap.
F
Change first phase insulin (nmol/min/m2)
0.20
0.15
0.10
0.05
0.00
-0.05
***
**
resp.
non-resp.
relap.

Figure 4

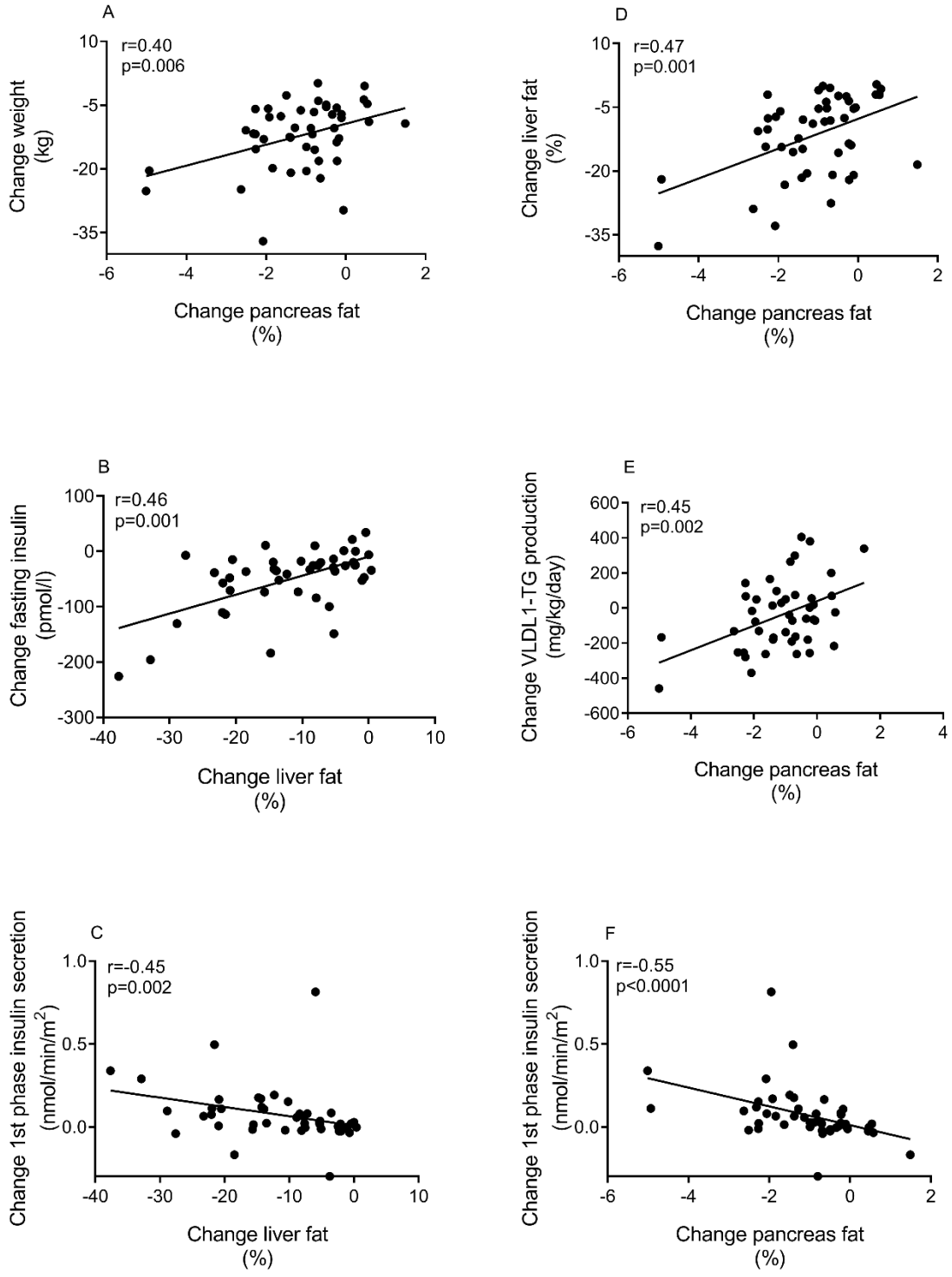

A
r=0.40
p=0.006
Change weight (kg)
Change pancreas fat (%)
D
r=0.47
p=0.001
Change liver fat (%)
Change pancreas fat (%)
B
r=0.46
p=0.001
Change fasting insulin (pmol/l)
Change liver fat (%)
E
r=0.45
p=0.002
Change VLDL1-TG production (mg/kg/day)
Change pancreas fat (%)
C
r=-0.45
p=0.002
Change 1st phase insulin secretion (nmol/min/m2)
Change liver fat (%)
F
r=-0.55
p<0.0001
Change 1st phase insulin secretion (nmol/min/m2)
Change pancreas fat (%)

Figure 5

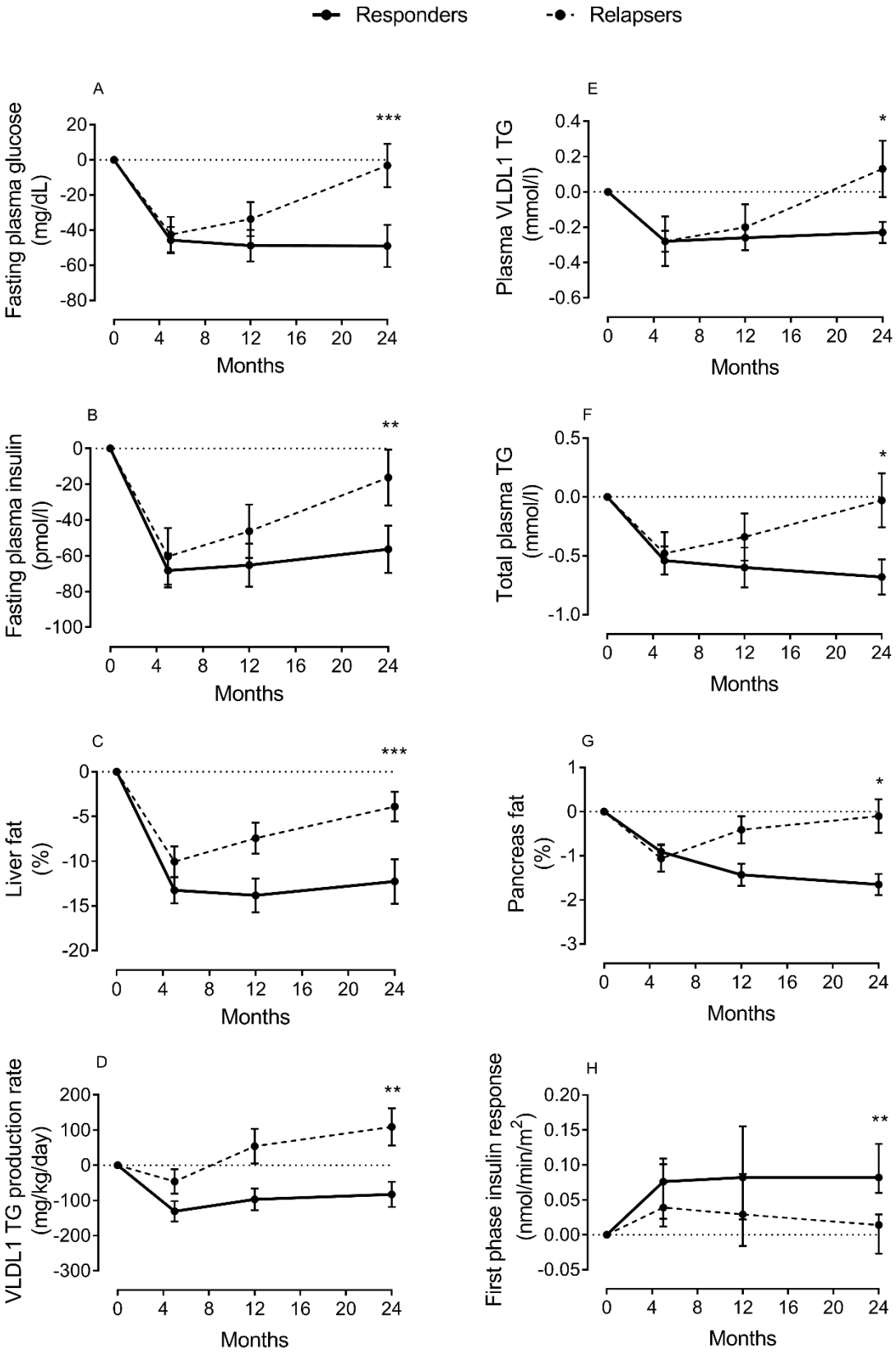
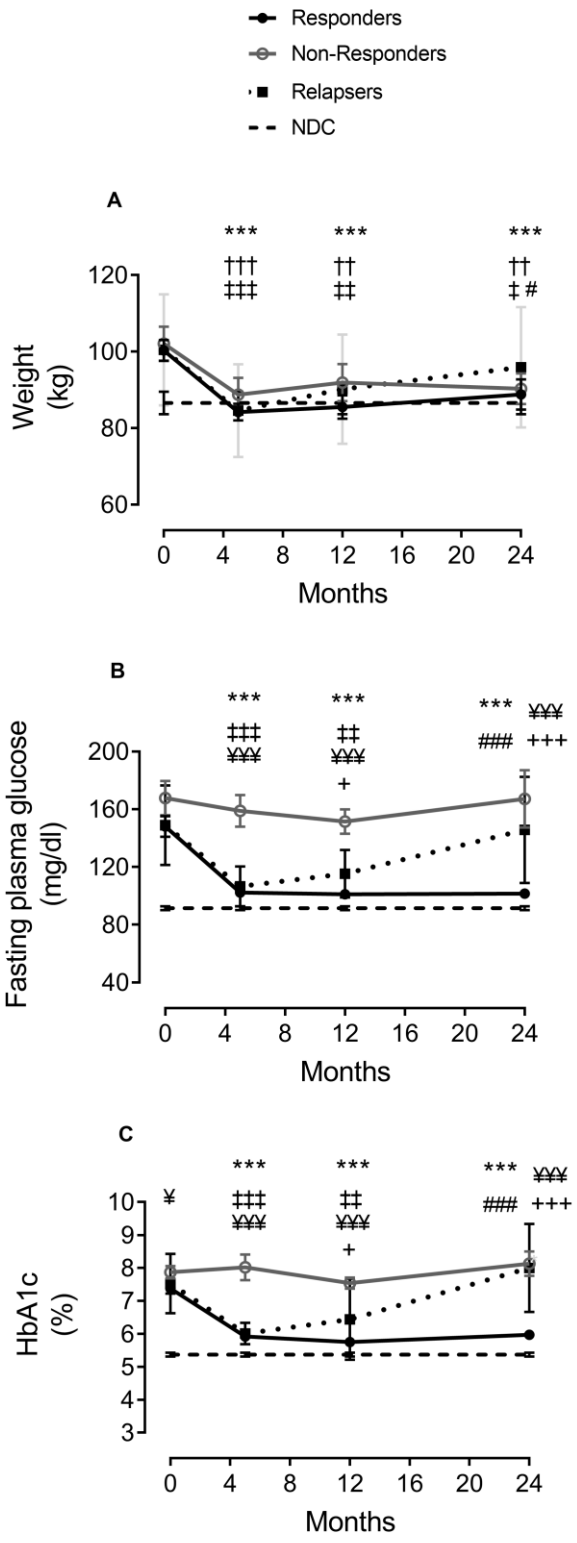

Responders
Relapsers
A
Fasting plasma glucose (mg/dL)
***
Months
B
Fasting plasma insulin (pmol/l)
**
Months
C
Liver fat (%)
***
Months
D
VLDL1 TG production rate (mg/kg/day)
**
Months
E
Plasma VLDL1 TG (mmol/l)
*
Months
F
Total plasma TG (mmol/l)
*
Months
G
Pancreas fat (%)
*
Months
H
First phase insulin response (nmol/min/m2)
**
Months

Figure 6

Supplementary data



Supplementary Figure 1 related to Table 1: Weight change and glucose homeostasis over 24 months

Body weight (A), fasting blood glucose (B), and HbA1c (C) at 0, 5, 12, and 24 months following intervention.

Responders are represented as a solid black line, non-responders (solid grey line), relapsers (dotted black line), and non-diabetic control (NDC: measured on one occasion, horizontal black dashed line).

Data are presented as means ± SEM.

* p<0.05 vs. baseline, **p < 0.01 versus baseline, ***p < 0.0001 versus baseline (responders).

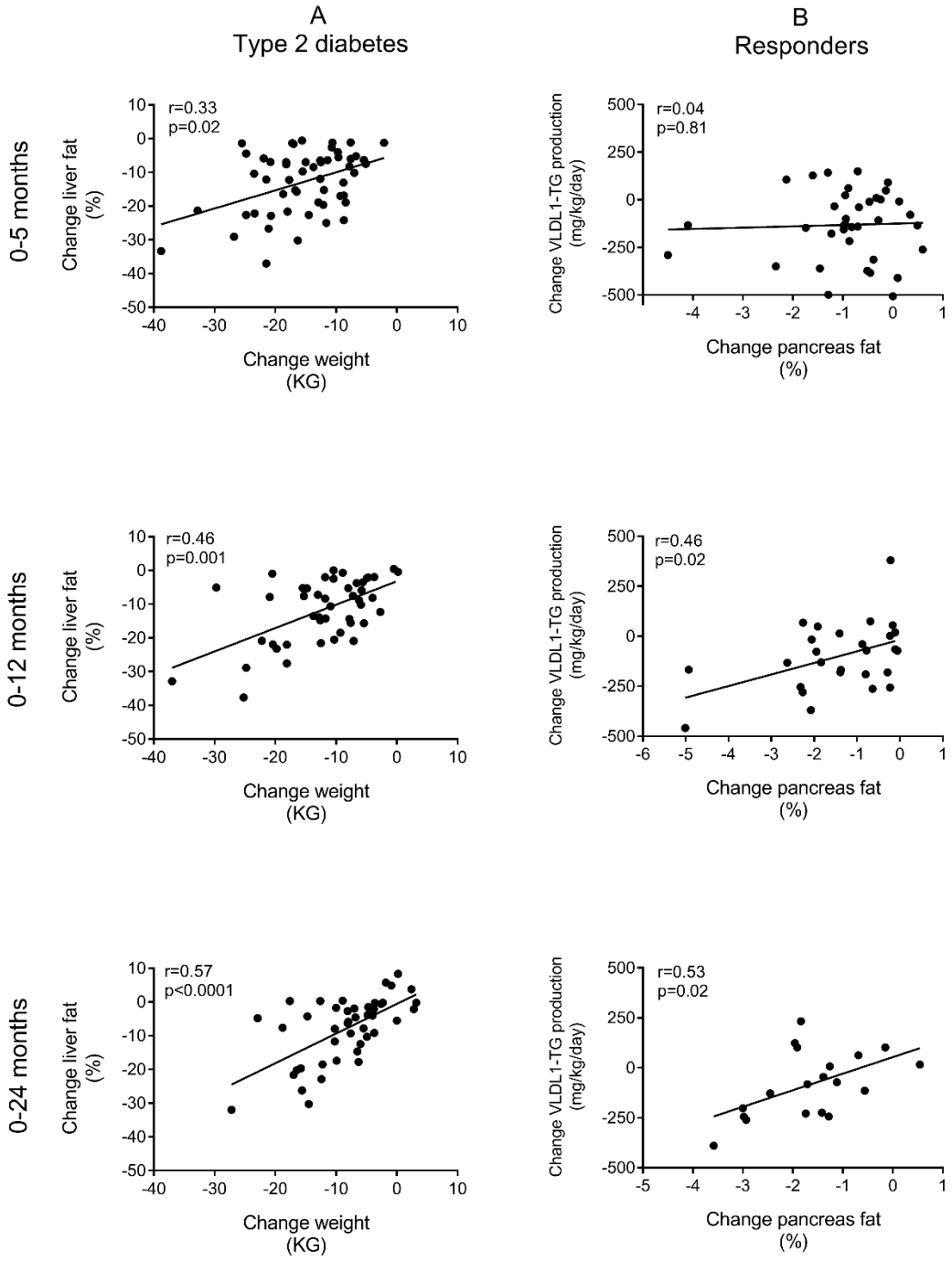
† p<0.05 vs. baseline, ††p < 0.01 versus baseline, †††p < 0.0001 versus baseline (non-responders).

‡ p<0.05 vs. baseline, ‡‡p < 0.01 versus baseline, ‡‡‡p < 0.0001 versus baseline (relapsers).

# p<0.05 vs. 5 months, ## p<0.01 vs. 5 months, ### p<0.001 vs. 5 months (relapsers).

¥ p<0.05 and ¥¥¥ p<0.0001: responders vs. non-responders
+p<0.05 and +++p<0.0001: responders vs. relapsers

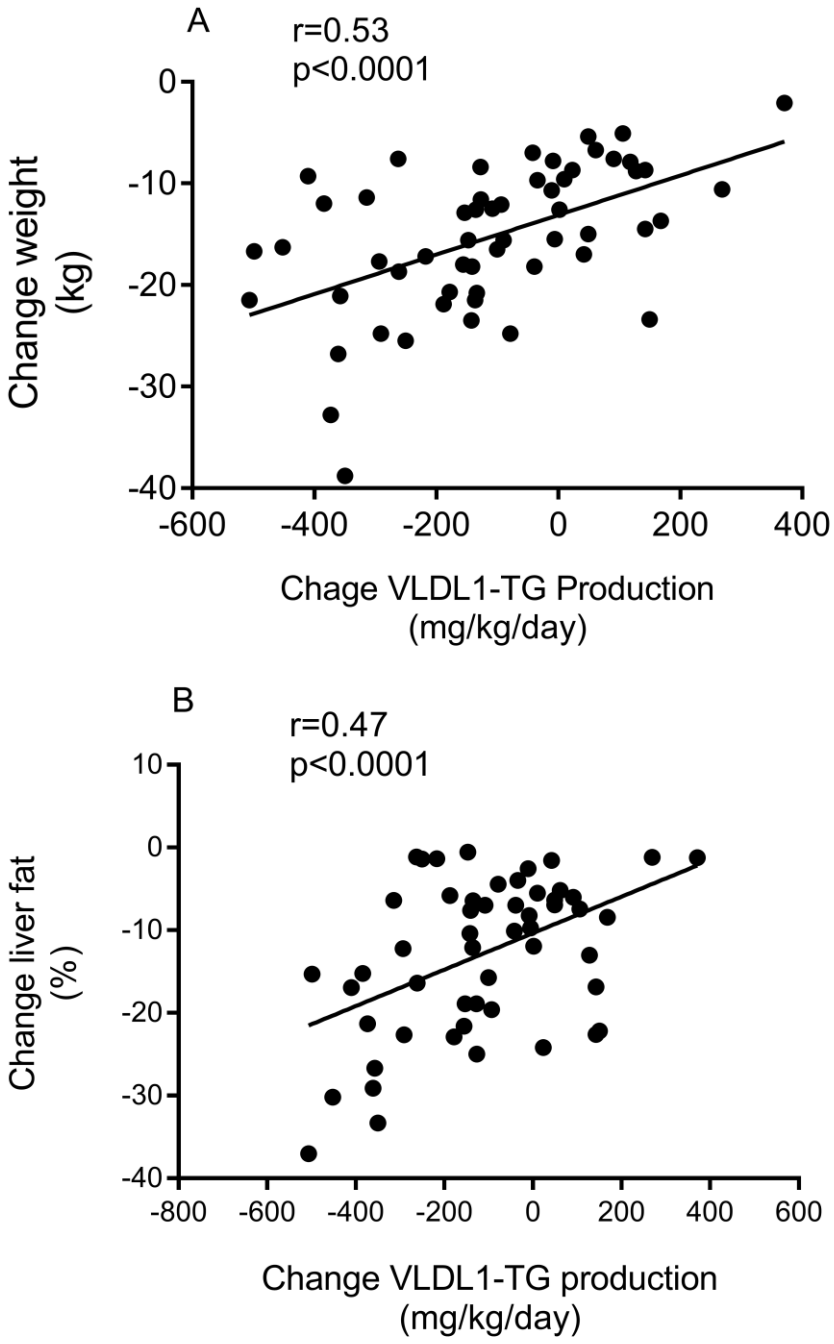
For clarity, statistical significance for comparisons with NDC is not indicated on the Figure, and p values compared with NDC were as follows: Baseline weight p<0.05 compared with all other groups, and both FPG and HbA1c p<0.0001; For responders at 5, 12, and 24 months, both FPG and HbA1c p<0.05 at each time point. For non-responders at 5, 12, and 24 months both FPG and HbA1c p<0.0001. For relapsers at 12 and 24 months both FPG and HbA1c p<0.0001.



Supplementary Figure 2 related to Figure 2: Effect of weight loss on hepatic VLDL1-TG export and intra-pancreatic fat content

Panel A: Correlation between the change in weight and change in liver fat between 0-5 months, 0-12months, and 0-24months in the whole type 2 diabetes group (n=54, n=46, and n=44, respectively). The correlation became stronger during the 24 months.

Panel B: Correlation between the change in change in hepatic VLDL-TG export and pancreas fat between 0-5 months, 0-12months, and 0-24months in the responder group (n=37, n=27, and n=19, respectively). Change in pancreas fat is slow and incomplete by 5 months and the relationship became evident by 12 months.



Supplementary Figure 3 related to Figure 5: 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 (n=56(A), and n=54(B)).