Mechanisms of remission in type 2 diabetes mellitus using Roux-en-Y gastric bypass

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Mechanisms of remission in type 2 diabetes mellitus using Roux-en-Y gastric bypass

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Abstract

Introduction

Defects in fat processing, in particular non-esterified fatty acids (NEFA), may hold the link between obesity, hyperlipidaemia, insulin resistance (IR) and type 2 diabetes mellitus (T2DM). The secretion of insulin, more effective at inhibiting lipolysis than glucose production, is inhibited by high NEFA levels. I hypothesised that postprandial hormone alterations following Roux-en-Y gastric bypass (RYGB) improve adipocyte insulin sensitivity through the peripheral effects of incretins.

Methods

Participants with T2DM (n=9) and without T2DM [NDM] (n=10) underwent RYGB for morbid obesity. Fasting and postprandial blood was analysed for glycaemic, lipidaemic and gut hormones changes around surgery. Intra-operatively, adipose tissue biopsies were taken and lipolysis assessed during a 2hr incubation with pre- and post-RYGB plasma and relevant hormonal concentrations.

Results

By post-operative day 4, there were improvements in IR ($p<0.0001$) and reduced fasting insulin levels ($p=0.0017$) despite no reduction in postprandial insulin ($p=0.6714$). Fasting NEFA levels were reduced in NDM ($p<0.05$) but not in the T2DM group ($p=0.0816$), whilst postprandial NEFA (ΔAUC [360min]) reduced in the T2DM group ($p<0.0001$) but not in the NDM group ($p=0.4111$), ANOVA $p<0.05$.

Significant changes in gut hormone levels around RYGB were noted, in fasting: reduced GIP ($p=0.0079$), PYY ($p=0.0066$) and ghrelin levels ($p=0.0005$); post-prandial: increased GLP-1 ($p=0.0018$) and PYY levels ($p<0.0001$); reduced GIP ($p=0.0318$) and ghrelin levels ($p=0.0004$).

Incubation of adipocytes in both fasting and postprandial plasma post-RYGB resulted in increased lipolysis versus pre-RYGB plasma (peripheral $p=0.0235$

and p=0.0205, respectively [n=8]). Insulin and PYY inhibited lipolysis but no effect of GLP-1, GIP and ghrelin on lipolysis was detected.

Conclusions

Although likely that postprandial hormonal alterations improve adipocyte lipolysis through their peripheral effects, it is most likely the global reduction in insulin levels, thereby reducing the anti-lipolytic effect, combined with overall improvements in IR, responsible for these findings and not the peripheral effects of incretins.

Word count: 300

Declaration of contributors

The majority of the work described in this thesis was performed by the author. All collaboration and assistance are described below.

Analysis of plasma for glucose, insulin, gut hormones and lipid profiles was undertaken with the assistance of Tracy Dew and her team in the Department of Biochemistry, King's College Hospital.

The adipocyte experimental techniques including: isolation, incubation, Doles triglyceride extraction and the glycerol assay have been adapted from established techniques learnt from Dr Susan Fried at the Adipocyte Core, Boston Obesity and Nutrition Research Centre, Boston, MA during a two-week training visit 2009.

The adipocyte isolation, incubation and glycerol assay techniques have been optimised with the assistance of Dr Ragai Mitry, Institute of Liver Studies, King's College Hospital.

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Abbreviations

ADA	Adenosine deaminase
Ado	Adenosine
AGB	Adjustable gastric band insertion
AT	Adipose tissue
AUC	Area under curve
BA	Bile acid
BMI	Body mass index
BPD	Biliopancreatic diversion
BSA	Bovine serum albumin
cAMP	cyclic Adenosine monophosphate
CCK	Cholecystokinin
CRP	C-reactive protein
DG	Diacylglycerol
DJG	Duodenal-jejunal bypass
DPPIV	Dipeptidyl peptidase-4
FFA	Free fatty acid
FFAR	Free fatty acid receptor
FPG	Fasting plasma glucose
FPI	Fasting plasma insulin
FSI	Fasting serum insulin
GB	Glycine buffer
GIP	Glucose-dependent insulinotropic polypeptide
GJ	Gastrojejunostomy
GK	Glycerol kinase
GLP-1	Glucagon-like peptide – 1
GPDH	Glycerol-3-phosphate dehydrogenase
HDL-C	HDL-cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HSL	Hormone sensitive lipase
IFG	Impaired fasting glucose
IFSO	International federation of surgical obesity
IGT	Impaired glucose tolerance

IR	Insulin resistance
ISO	Isoproterenol
KRB	Krebs-Ringer bicarbonate solution
LCFA	Long-chain fatty acids
LCTG	Long-chain triglycerides
LDL-C	LDL-cholesterol
LPL	Lipoprotein lipase
MCFA	Medium-chain fatty acids
MCTG	Medium-chain triglycerides
MMT	Mixed meal test
MRI	Magnetic resonance imaging
NDM	Subjects without T2DM
NEFA	Non-esterified fatty acids
NGT	Normal glucose tolerance
ns	non-significant
NTC	No template control
OGTT	Oral glucose tolerance test
PDE	Phosphodiesterase
PIA	N^6-*[R-(-)*-1-methyl-2-phen-ethyl] adenosine
PKA	Protein kinase A
POD	Post-operative day
PYY	Peptide tyrosine tyrosine
RT-PCR	Real-time polymerase chain reaction
RYGB	Roux-en-Y gastric bypass
T2DM	Type 2 diabetes mellitus
TG	Triacylglycerol/triglyceride
VLCD	Very-low calorie diet
VLDL	Very low density lipoprotein
WCC	White cell count
WHO	World health organisation
8-Bromo-cAMP	8-Bromoadenosine 3':5'-cyclic monophosphate sodium
*	$p<0.05$
**	$p<0.01$
***	$p<0.001$

1. Introduction

The world's health is under attack from an epidemic of huge proportions. In 2005, >400 million adults were obese (Body mass index [BMI] >30). This was predicted to increase to >700 million by 2015.[1] In comparison to healthy weight individuals (BMI 22.5-25) morbid obesity (BMI 40-45) reduces medial survival by 8-10 years[2] through obesity-related conditions including cardiovascular disease and Type 2 Diabetes Mellitus (T2DM).[3,4]

The strong underlying pathophysiological link between obesity and T2DM suggests that the prevalence of T2DM is set to increase exponentially. Global projections for the T2DM epidemic suggest a 72% increase in cases between 2003 and 2025.[5]

The development of T2DM occurs when pancreatic islet cells are unable to increase insulin secretion to sufficient levels necessary to compensate for insulin-resistance, and maintain normoglycaemia.[6,7]

Surgical treatment is more effective than medical treatment for T2DM in the morbidly obese[8], Roux-en-Y gastric bypass (RYGB) resulting in remission in 84% of patients[9] and reduction diabetes-related deaths by 92%.[10] Originally it was presumed these changes were due to weight loss but rapid resolution of insulin resistance (IR) and improved insulin secretion occurs prior to discharge from hospital (before major weight loss).[11] This discovery has given hope a tailored surgical procedure and potentially a pharmacological cure to T2DM may be possible.

1.1 Development of the Roux-en-Y Gastric Bypass

Obesity surgery is the only successful long-term management for morbid obesity.[12] Together with advances in complex laparoscopic surgery this has resulted in a surge in demand for this intervention. Over 300,000 bariatric surgical procedures were performed worldwide in 2008, a 135% increase since 2003 (49% were gastric bypass procedures).[13]

The first gastric bypass for morbid obesity was reported by Mason in 1967[14], who had observed that following partial gastrectomy (for peptic ulcer disease), underweight patients had difficulty gaining weight. Accordingly, he designed the operation to combine a 90% antral exclusion with a short loop retro-gastroenterostomy. Minor modifications including the use of the Roux limb formation to reduce bile reflux[15] have resulted in the RYGB which is commonly performed today (+/- addition of a non-adjustable gastric band[16]), see figure 1.1.[17]

Reports of unexpected dramatic changes in T2DM post-RYGB appeared in the literature by 1995.[18] Currently obesity surgery is more effective than medical treatment for T2DM in the morbidly obese.[8] However, there is data to suggest that this effect is transient[12] and so the term “remission of T2DM” rather than the definitive term “cure” is used to describe this phenomenon.

1.1.1. Effects of RYGB upon type 2 diabetes mellitus

Remission of T2DM after weight loss surgery is reliant upon pre-operative β-cell functional capacity with “percentage excess weight lost” and “time with diagnosis of diabetes” being significant predictors of remission.[19] This suggests insulin secretory capacity and therefore insulin secretion to be involved in the remission of T2DM post-bariatric surgery. Although peak postprandial insulin maybe increased, area under the curve (AUC) data reflects an overall reduction in postprandial insulin secretion (not statistically significant difference, possibly underpowered studies for this outcome).[20,21]

Obese subjects may have larger β-cell nuclear diameter than lean subjects and post-RYGB a reduction in β-cell nuclear diameter (and therefore potential reduction in insulin secretion) may not occur.[22] It remains to be shown whether this occurrence is confined to patients with post-RYGB postprandial hyperinsulinaemic hypoglycaemia syndrome (the pathological state investigated in this study), post-RYGB or normal for all patients who have undergone substantial weight loss, irrespective of causation.

The original studies describing remission of T2DM post-RYGB were performed after surgical weight loss has occurred. As such, it was argued that the remission of T2DM was due to a reduction in oral intake and/or weight loss rather than as a result of surgery. This was not the case and studies performed in the early post-operative period have shown that post-RYGB gut hormone changes begin to occur by post-operative day 2[23] however, the inflammatory changes post-surgery may impact upon these results.

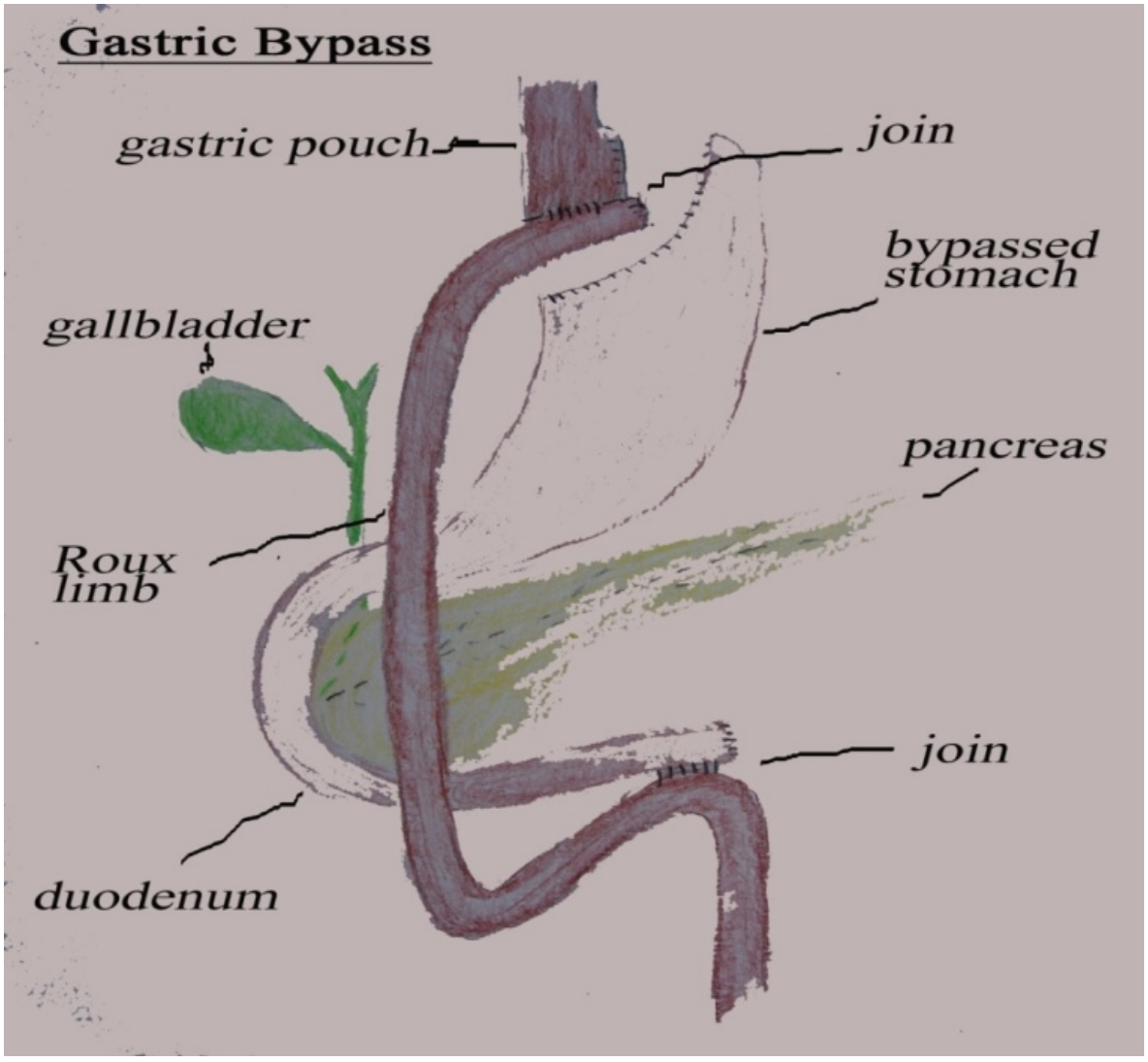

Figure 1.1 Diagrammatic representation of RYGB procedure[17]

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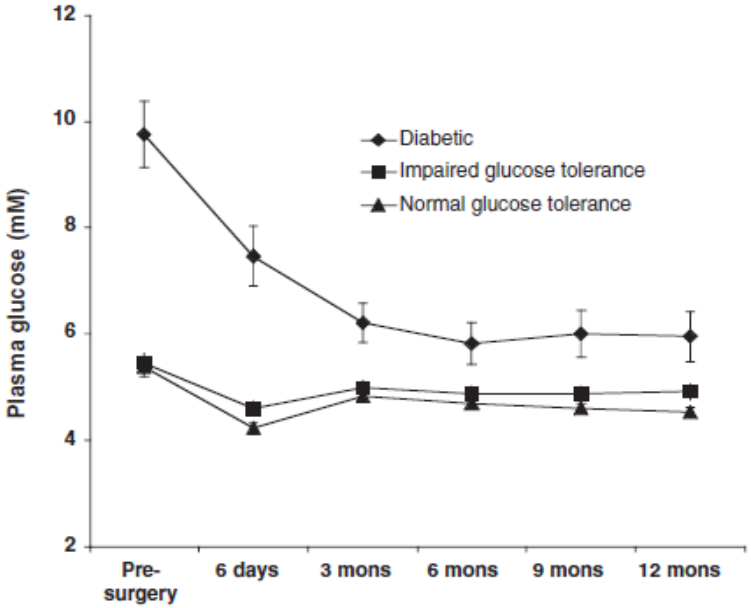

Figure 1.1.1i Fasting Plasma Glucose levels after RYGB[11]

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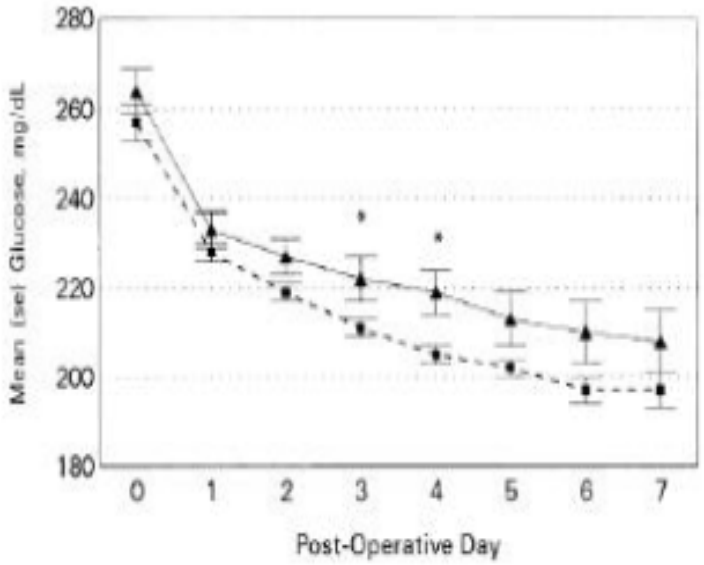


Figure 1.1.1ii Mean capillary glucose in DM patients after coronary artery bypass grafting[24]

American Diabetes Association: Perioperative glycaemic control and the risk of infectious complications in a cohort of adults with diabetes, American Diabetes Association, 1999. Copyright and all rights reserved. Material from this publication has been used with the permission of American Diabetes Association.

It is plausible that the early remission of T2DM is simply due to “fasting for major surgery” as patients after coronary artery bypass surgery (non-gastrointestinal surgery) have also been shown to have dramatic improvements in glucose levels, presumably through these mechanisms, see figures 1.1.1i and 1.1.1ii.[11,24]

1.1.2. Effects of RYGB upon insulin resistance

IR describes the reducing sensitivity of the tissues to insulin.[25] Early dramatic changes in IR have been shown post-RYGB using a homeostatic model assessment of insulin resistance [HOMA-IR] (non-invasive model for IR, see section 2.2.3.2), $p<0.00001$ for differences between pre-op and POD6, figure 1.1.2.[11] This data is supportive of a systemic change in glycaemic control in the body after RYGB. Although caution should always be taken with respect to the very early data, within the first week following major surgery, as these subjects will have fasted for a significant period of time, usually >24hr. Reassuringly these effects persist longer term[11,26], and though it has been postulated that this reflects a reduction in energy intake as diets and result in significantly reduced IR,[27] the same argument could be used in support i.e. as energy intake post-RYGB increases up to a year, no correlation with deterioration in IR is noted. Persistent improvements in HOMA-IR in the first year post-RYGB is supportive of a substantive change in IR after RYGB.[26] These dramatic improvements in IR after weight loss surgery are not confined to RYGB, similar improvements shown after the purely restrictive bariatric procedure, the adjustable gastric band (AGB), irrespective of weight loss.[28] Suggesting a causative link between adipose tissue mass and IR exists.

1.1.3. Proposed mechanisms through which this occurs:

1.1.3.1. Weight loss

Patients with T2DM are pre-disposed to weight gain[29] presumably due to multi-factorial reasons including natural aging, insulin resistance, medications

which predispose to weight gain[30] for example exogenous insulin (which is anti-lipolytic). Patients with T2DM tend to have poor weight loss even with aggressive dietary and medical management rendering this patient group particularly hard to manage. This problem is self-perpetuating with insulin resistance increasing with weight gain. After weight-loss surgery this does not seem to be the case, no difference in weight loss post-AGB when subjects with non-diabetes mellitus (NDM) are compared to T2DM.[31]

If weight loss is achieved in this patient group, obesity-related T2DM has been shown to improve,[32,33] with improvements in insulin secretion being proportional to weight lost.[34] Post-weight loss surgery the link between weight lost and improvements in insulin secretion appears to be altered. Despite similar weight loss after-AGB poorer oral glucose tolerance when compared with post-RYGB.[35] Increases in postprandial glucagon-like peptide-1 (GLP-1) secretion post-RYGB compared with AGB has been postulated as an explanation for these findings.[36]

Changes in gut hormone profiles following bariatric surgery combined with early post-operative improvements in fasting glucose and HOMA-IR data suggest that weight loss is not the sole mechanism of remission of T2DM post-RYGB but certainly is an additional factor.[37] This theory has been put to the test by La Ferrere et al, an elegant study in which T2DM matched for weight loss (diet-induced), revealed 1 month post-RYGB reduced postprandial glucose levels ($p=0.014$) and greater GLP-1 levels ($p<0.001$), not related to weight loss.[38]

1.1.3.2. Restriction of food intake

Reduction in calorie intake is known to improve glycaemic control in obesity-related T2DM more rapidly than weight loss. The converse is also true, after weight loss metabolic control can worsen once calorie intake is increased, without weight gain.[33] In the early post-RYGB period, patients have physical restriction of oral intake which results in substantial reduction in calorie intake. It is therefore plausible that this effect augments T2DM post-operatively.

Very low-calorie diets (VLCDs) of <800kcal/d have shown significant weight loss and ~50% reduction in Fasting Plasma Glucose (FPG) after 2w in obese T2DM groups, maintained for the 6w of treatment.[33] At study completion, weight regain was coupled with increased FPG levels.[39] Another study showed that 10d total fasting with weight loss 5.1% of initial body weight reduced fasting plasma glucose values of T2DM by 64.5% (from 17.2% to 6.1 mmol/L).[40] Complete fasting or prolonged use of VLCDs is not safe and therefore not a long-term management solution for either obesity or obesity-related T2DM however these results are of interest.

"Dumping syndrome" occurs in approximately 15-20% of patients after partial gastrectomy.[41] This syndrome describes postprandial upper gastrointestinal and vagal symptoms resulting from rapid drainage of the stomach contents, exacerbated by high sugar and fatty foods.[42] Post-bariatric surgery dumping syndrome occurs in up to 70% of patients.[43,44] Anecdotally, patients says that the threat of dumping syndrome post-RYGB results in avoidance of high sugar and high fat foods, reinforcing "good" diet choices and improvements in weight loss. To date this has not been formally studied.

Combined with early post-operative nausea, increased physical restriction of the small gastric pouch and potentially the newly created oedematous gastro-jejunostomy, "dumping syndrome" may result in a reduction in caloric intake post-RYGB with increased detection of sugars and fats post-operatively being proposed as a reason for avoidance of particularly "unhealthy" food choices.[45-47]

Gut hormones have been shown to reduce hunger and enhance satiety.[48] Links between sustained postprandial satiety and gut hormone release post-RYGB has been proposed [20] with potential augmentation of central brain responses to food post-RYGB.[49]

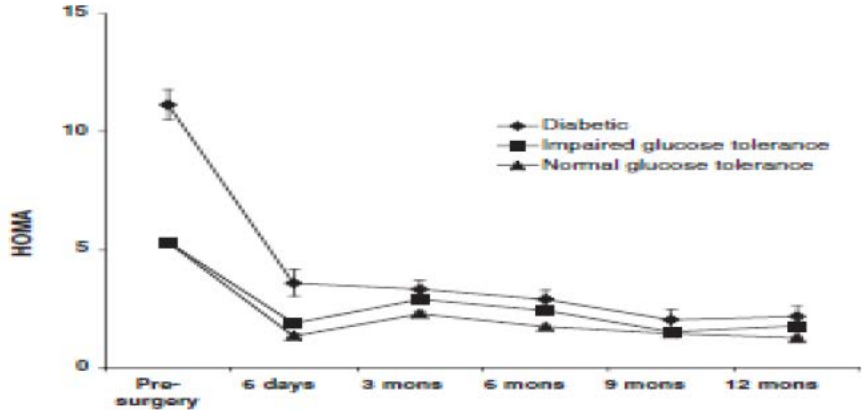


Figure 1.1.2 Time course of HOMA-IR after RYGB. P<0.0000 for the differences between HOMA values before up to 365 days after RYGBP in all patients. P<0.0000 for the differences between HOMA values between the different patient groups.[11]

The possibility of a central augmentation of the satiety response to dietary intake has also been proposed as a mechanism of weight gain in T2DM. Failure of oral glucose to inhibit hypothalamic activity, and therefore potentially satiety, has been shown in patients with T2DM.[50] Differences exist between lean and obese patients brain activation, using fMRI, after over-feeding in response to food images[51] and direct effects of peptide tyrosine tyrosine (PYY) having been shown to activate brain regions related to food reward.[52] However, the effect of obesity surgery on taste reward circuits in the brain is awaited.

1.1.3.3. Re-routing of the gastrointestinal tract

The most tempting proposed mechanisms by which remission of T2DM post-RYGB occurs concentrate upon the re-routing of the gastrointestinal tract, the "foregut" and the "hindgut" hypotheses:

- "foregut" hypothesis – duodenal and proximal jejunal exclusion from dietary nutrients, possibly preventing secretion of signal which promotes IR and T2DM (the "anti-incretin" signal) [53-57]
- "hindgut" hypothesis – expedited delivery of undigested food to the distal intestine, enhancing GLP-1 secretion in the L cells of the distal bowel, improving glucose metabolism [54,58-61]

These theories were tested in a non-obese rodent model of T2DM,[53] where the rats underwent either duodenal-jejunal bypass (DJB) ("foregut") or a gastro-jejunostomy (GJ) ("hindgut"). Oral glucose tolerance was improved in the DJB group but remained unchanged in the GJ group. When the rodents subsequently underwent duodenal exclusion their glucose tolerance improved and conversely, restoration of duodenal passage in DJB rats re-established impaired glucose tolerance, suggesting that the exclusion of the foregut and not the increased speed of transit and/or increased GLP-1 secretion is ultimately responsible for the remission of T2DM post-RYGB.[53] Of interest, the DJB rats had lower fasting plasma non-esterified fatty acid (NEFA) levels, comparable to NDM animals and lower than GJ ($p<0.01$). Early reports in humans of an endoscopically-placed duodenal-jejunal sleeve

is supportive that these effects are transferrable to humans.[62] Rodent studies post-RYGB are suggestive that the duodenal exclusion alters both intestinal structure and glucose transport function with a reduction in glucose absorptive capacity.[63] Human studies are awaited.

Although it is a widely held belief, there are no data to suggest that post-RYGB a reduction in dietary absorption occurs. The anatomical re-routing of the gastrointestinal tract of this procedure results in a delay in contact of the food with the intestinal juices (gastric acid, bile salts and pancreatic enzymes). It is through this rearrangement of the gut that the intentional malabsorption of dietary nutrients is believed to occur. In addition, exclusion of food from the antrum may impair gastrin and subsequent pancreatic enzyme secretion,[64,65] as faecal elastase levels are reduced post-RYGB.[66]

Normal dietary absorption in the intestines is reliant upon alterations in the pH of intra-luminal contents at different levels of the gastrointestinal tract[67] and it is reasonable to presume this will be different post-RYGB, as gastric acid “un-buffered” by foods will pass through the bilio-pancreaticico-duodenal (BPD) limb. Questions remain as to whether gastrin secretion is effected by RYGB and to-date this field remains relatively un-investigated.

Bile secretion and re-absorption post-RYGB may be altered. Bile acids have been linked to GLP-1 secretion from the L cells which can be modified by bile acids via TGR5 signalling pathway[68] in addition to glucose and lipids.[69-71] Plasma bile acids and insulin sensitivity in humans have been linked[72] and plasma bile acids are elevated after bariatric surgery vs obese controls, with a positive correlation between bile acid concentration and peak GLP-1 suggesting further potential mechanisms of remission of T2DM.[73] Serum bile acids are higher in humans with prior gastric bypass, potentially contributing to improved glucose and lipid metabolism.

The impaired breakdown of dietary foods should result in increased viscosity of intra-luminal contents in the post-RYGB gastrointestinal tract however data is currently not available regarding this. Increased viscosity is associated with

slower intestinal absorption and inhibition of glucose transport, a similar mechanism through which increased consumption of soluble fibre is thought to improve postprandial glucose profiles.[74,75]

1.1.3.4. Alteration in dietary fat processing

Initially for T2DM to develop, pancreatic β-cells must be unable to compensate fully for decreased insulin sensitivity.[7] It is believed the β-cell response to changes in insulin sensitivity probably involves NEFA signalling and sensitivity to incretins.[76] The obese and patients with T2DM have increased NEFA levels[77,78] and the siblings of two parents with T2DM have increased postprandial triglyceride (TG) levels and blunted early postprandial lowering of NEFA,[79] suggesting an alteration in dietary fat processing occurs prior to the development of T2DM.

An acute rise in plasma NEFA levels will result in IR within hours in humans.[80] This effect has been shown to be reversible, insulin-mediated glucose uptake and glucose tolerance improve with an acute decrease in NEFA levels after treatment with the antilipolytic agent Acipimox.[81]

Plasma NEFA appearance is related to dietary fat absorption, intravascular TG lipolysis and adipose tissue lipolysis. It is likely insulin is responsible for changes in plasma NEFA levels due to its ability to stimulate LPL-mediated lipolysis of chylomicron and very low density lipoprotein (VLDL) TG in the circulation,[82] suppress intracellular adipose tissue lipolysis[83] and possibly stimulate esterification of NEFA in adipose tissue.[84] Previously it had been postulated that insulin may contribute to adipose tissue uptake of NEFAs generated from intravascular TG lipolysis[83,85] and so reduce NEFA spill-over. This theory is supported by in vivo studies describing NEFA uptake into adipose tissue.[86] However, recently the mechanism of insulin-stimulated clearance of plasma NEFA in humans was shown to be through the reduction of the endogenous appearance rate of NEFA.[87]

Many believe the development of T2DM from insulin resistance is due to impaired postprandial reduction of NEFA by insulin, resulting in increased exposure of non-adipose tissue to NEFA[88], including islet cells. Increased postprandial deposition of dietary fatty acids in liver and skeletal muscle previously being reported in subjects with T2DM[89], supporting the theory that NEFA storage in adipose tissue is impaired in these patients.

In the obese, the release of exogenous NEFA in plasma is altered, possibly due to increased tissue fatty acid uptake aided by the action of lipoprotein lipase (LPL)[90], and preferential uptake of long-chain fatty acids (LCFA) when compared to medium-chain fatty acids (MCFA) by adipocytes thereby potentially increasing fat deposition if greater LCFA is available than MCFA[86].

In turn, NEFA has been shown to directly affect insulin secretion with NEFA receptor expression (GPR40 and 43) by human pancreatic β-cells. Acutely, NEFA stimulates islet cells to secrete insulin[91] but chronic exposure to NEFA is associated with marked impairments in glucose-stimulated insulin secretion and decreased insulin biosynthesis.[92,93]

Alteration in NEFA levels post-RYGB has not been fully established. There is early conflicting data regarding post-operative NEFA levels in rats[53,94] and in humans postprandial NEFA levels were higher than obese and non-obese controls.[95] However, when exogenous fats are defined using a tracer, little spill-over of the lipolysed dietary TG into the plasma NEFA pool occurs in the post-RYGB vs non-obese control. Enhanced fatty acid trapping by peripheral tissues in the post-obese has been postulated as a cause for this finding.[96] Of note, in both studies the post-RYGB groups were not controlled for weight loss and are a skewed population, subject to changes in incretin levels which may contribute to plasma NEFA concentration by directing altering lipolysis. The gastrointestinal tract re-routing of the RYGB results in dietary fat meeting the gastric secretions, bile salts and pancreatic juices later, in the mid-ileum, which may result in consequent alteration in plasma NEFA levels.

Dietary medium-chain and long-chain triacylglycerols are absorbed differently. The majority of the medium-chain triacylglycerols (MCTG) pass into the portal system as MCFA (hydrolysed by gut lipases or directly as MCTG). Long-chain triacylglycerols (LCTG) undergo hydrolysis by mostly pancreatic lipase but are unable to pass into the portal system due to their large size. Instead, LCFAs combine with cholesterol and phospholipids to become chylomicrons, which enter the systemic circulation via the lymphatic system.[97] Most of LCTG absorption occurs in the proximal small bowel, figure 1.1.3.4i. After gastric bypass it is likely MCTG absorption will be greater than LCTG due to anatomical alterations, altering the MCTG:LCTG absorption ratio. However, little is known about MCTG and LCTG absorption in disease and no data available post-gastric bypass.

It is believed the β-cell response to changes in insulin sensitivity probably involves non-esterified fatty acid (NEFA) signalling and sensitivity to incretins[76] which may have a direct effect on adipocyte lipolysis.

1.1.3.5. Alteration in gut transit time

The re-structuring of the gastrointestinal tract in RYGB results in food passing relatively unhindered into distal jejunum; bypassing the antrum, the pylorus, the duodenum and ~70cm proximal jejunum. Logic suggests this will reduce gut transit time thereby reducing dietary absorption and reducing postprandial glucose levels accordingly, this may not be the case. Mixed reports exist with respect to gut transit time post-RYGB.[66,99-101] Possible explanations for the reported delay in gut transit time include changes in intra-luminal content viscosity, increased GLP-1 and other incretin secretion, altering GI motility. Reduction in gut transit time could impact upon dietary fat and sugar absorption however, further data in this field is required. A corroborative study has shown delayed gut transit time post-total gastrectomy and Roux-en-Y esophagojejunostomy, where food rapidly passes into Roux limb with delay in small intestine transit versus controls.[102] Changes in dietary content in the intestinal lumen as a result of the RYGB

may explain this phenomenon with increased consumption of high-fibre diet being shown to delay gut transit time through decreasing bolus propulsion to the rectum and increasing gas production by colonic flora.[103]

A-glucosidase inhibitors are used as a treatment for T2DM because they slow glucose absorption by reducing the rate of enzymatic digestion of starch, delaying release of glucose molecules for absorption, resulting in reduced postprandial glucose and insulin levels.[104] Changes in gut transit time is therefore another potential mechanism through which the RYGB can improve postprandial glucose control.

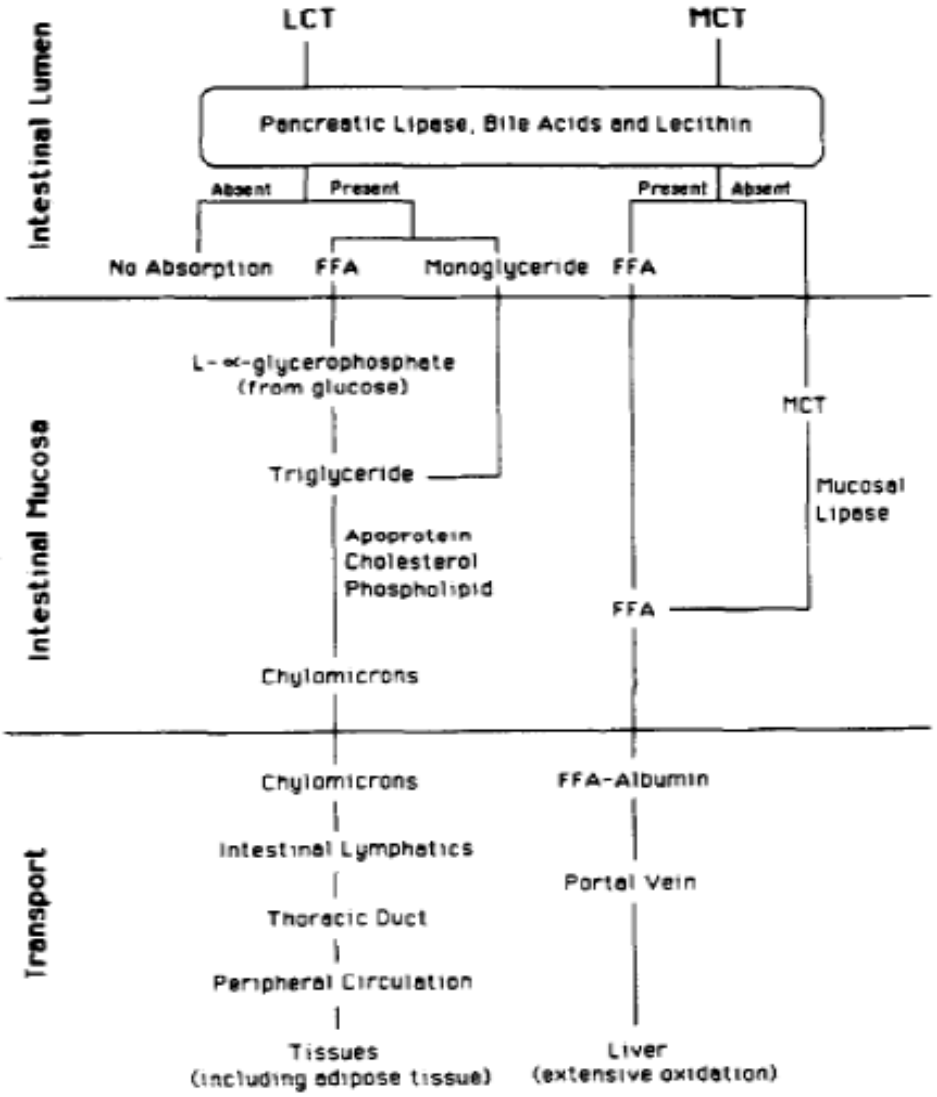


Figure 1.1.3.4 Digestion, absorption and transport of long-chain triglycerides and medium-chain triglycerides[98]

1.1.3.6. Gut hormone secretion

1.1.3.6.1. Incretins

Since the 19th century it has been accepted that humans process oral glucose better than intravenous.[105] It is believed this occurs through the release of peptides known as incretins. An incretin is a peptide which stimulates the internal secretion of the pancreas i.e. insulin secretion.[106]

To be an incretin a substance must meet specific criteria:

1. produced in the GI gastrointestinal tract
2. secretion stimulated by nutrients
3. stimulates insulin secretion in the presence of glucose

(modified from Creutzfeldt[106])

Glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 [the two main incretins] are responsible for 50-60% of postprandial insulin secretion.[107] They are secreted in response to food ingestion, and enhance insulin secretion (the incretin effect) prior to a change in plasma glucose.

The “hindgut” mechanistic theory regarding remission of T2DM post-RYGB suggests that enhanced nutrient delivery to distal intestine enhances stimulation of L cells resulting in increased GLP-1 secretion.[53] Since the GJ operation does not affect glucose homeostasis the hindgut theory does not fully explain these changes.

In T2DM, GIP secretion is normal but the effect of administered GIP on insulin secretion is blunted[108,109] and, GLP-1 secretion, possibly stimulated by GIP via vagus nerve,[110,111] is reduced.[112] IR subjects have been shown to have impaired postprandial GIP and GLP-1 secretion, severity of which is related to degree of IR.[113] Of note in this study pre-operative NDM and T2DM had no differences in peak insulin or AUC insulin postprandially however post-RYGB the T2DM group had an reduction in fasting glucose and insulin. Despite an increased postprandial peak insulin levels, a reduction in AUC insulin (ns) was reported. This hints that remission of T2DM post-RYGB is predominantly due to changes in insulin sensitivity combined with alterations in gut hormone

secretion and improvements in postprandial “tailored” (not increased) insulin secretion.

It should be noted that some of the blunted gut hormone responses in T2DM increase to supra-physiological levels post-RYGB,[21] not merely returning to baseline.

1.1.3.6.1.1. Gastric inhibitory polypeptide

GIP is a peptide which is secreted from the K cells in the duodenal and proximal jejunum[114] in the presence of glucose and fat. But it is the rate of absorption, rather than the luminal content, which is the main stimulus for GIP secretion.[115] In turn, this incretin stimulates insulin secretion in a glucose-dependent manner,[116] responsible for ~ 80% of nutrient-induced enteroinsular pancreatic beta-cell stimulation.[117,1] It can induce β-cell proliferation and enhance resistance to apoptosis.[118]

At the adipocyte, GIP promotes energy storage through fatty acid incorporation,[115,119] stimulates lipolysis and increases NEFA reesterification, similar to insulin, an effect reversed by ANTIGIP.[120]

Increased dietary fat intake has been shown to stimulate GIP secretion more than sugars.[120] The resulting GIP hypersecretion could increase nutrient uptake further in adipocytes[122] thereby increasing mass, linking GIP to obesity. This has been shown in mice, GIP-receptor knockout mice have reduced adipocyte mass and are resistant to diet-induced obesity despite a high-fat diet[123] and GIP knockout animals have increased energy expenditure.[123]

The pathogenesis of T2DM has also been linked to a defective expression of the GIP receptor.[124] Unsurprisingly, in both obesity and T2DM, there is increased fasting and postprandial GIP secretion[115,122,125-127] and the insulinotropic effect of GIP is lost in T2DM,[108] reversible through fasting.[106]

Sadly, the reported effects of RYGB on postprandial GIP secretion are not consistent.[21,36,38,57, 128-131] Likely resulting from many methodological variations discussed in chapter 2.2.5. When paired samples around RYGB surgery are compared, in morbidly obese patients with T2DM the pre-operative blunting of postprandial GIP secretion appears to return to NDM levels post-RYGB.[21]

1.1.3.6.1.2. Glucagon-like peptide -1

GLP-1 is a peptide which is synthesised and secreted from the L cells, which co-secrete PYY[132], predominantly found in the distal ileum and colon.[133,134] They are secreted in a bioactive form as GLP-1 (7-37) and GLP-1 (7-36NH2), which have a short half-life of 1-2min prior to conversion to GLP-1(9-37), GLP-1(9-36NH2) and GLP-1 (7-38) by dipeptidyl peptidase-4 (DPPIV).[136] As DPPIV is on the luminal surface of vascular endothelial cells and circulating in the plasma,[133-135] only approximately 25% of active GLP-1 reaches the portal circulation.[137] It is therefore likely to have paracrine effects via intestinal vagal afferents.[138,139]

GLP-1 can inhibit food intake in mice and rats[140,141] with GLP-1 infusions being shown to enhance fullness and reduce energy intake in healthy weight, obese and T2DM patients.[142-144] Other groups have reported no anorectic effect.[145-147] Supportive of its anorectic role, this effect is reversed both in GLP-1R knockout mice and using a GLP-1R antagonist, exendin(9-39), in intact mice,[148-150] rats[149,150] and humans.[151] Vagotomy can abolish this effect, highlighting its importance in mitigating this neurohumoral response.[140] GLP-1R, a member of the G-protein-coupled receptors[152] are present in the neurons of the nucleus tractus solitarius including the hypothalamus.[153]

GLP-1 is secreted in response to all nutrients, with a preponderance for carbohydrates.[154-160] It has a biphasic postprandial secretory pattern.[134,158,161] The first-phase is within 15min of food ingestion and may be related to neurohumoral activation,[161] small numbers of proximal L-cells.[162] or result from cholecystokinin (CCK) stimulation of GLP-1 secretion, prior to nutrient

stimulation.[158,163] The second-phase is due to nutrient stimulated GLP-1 secretion, metabolite production from gut microflora[164] and bile acid stimulation.[165,166] Although it remains unclear if obesity impacts upon nutrient-stimulated GLP-1 secretion;[159,167,168] in adipose tissue, GLP-1 promotes both lipogenesis and lipolysis dependent upon concentration,[169,170] potentially altering body mass.

GLP-1 (7-37) stimulates insulin release by islet cells through increasing intracellular cyclic monophosphate sodium (cAMP).[171] It is known to increase the activity of several kinases (PI3K, PKB, p44/42 MAPKs, p70s6k), similar to insulin. [136,172-175] It can increase glucose-dependent insulin secretion[171,176-178] This combined with the reduction in plasma glucose levels following insulin secretion suggest that GLP-1 is inherently protective against hypoglycaemia.

In T2DM, there is preservation of the incretin activity of GLP-1 [7-36 amide] but not of synthetic human gastric inhibitory polypeptide.[108] As such GLP-1 analogs (exenatide and largatidine) are now being used as medical treatments for obesity-related T2DM.[179-181]

Over the first 6m post-RYGB the increased postprandial GLP-1 response increases further despite weight loss.[152] The mechanism of this change is unknown. Incretin levels change in patients with T2DM post-RYGB, with increased GLP-1 secretion returning, if not surpassing, obese NDM levels.[21-23] GLP-1 has been noted to reduce B-cell apoptosis and increase B-cell hyperplasia in rats and monkeys, and postulated to cause this effect in humans resulting in the rare phenomenon of postprandial hyperinsulinaemic hypoglycaemia after RYGB.[22,182,183]

1.1.3.6.2. Peptide tyrosine tyrosine

PYY is secreted as PYY (1-36) from the L-cells in the distal ileum and colon, most co-expressing GLP-1.[184,185] It is then converted by DPPIV into its bioactive form, PYY 3-36.[186,187] PYY is inactivated by nonspecific peptidases[186] with a half-life in plasma of approximately 9 min.[184,187]

PYY (1-36) increases eating by binding to neuropeptide NPY1 and Y5 receptors, and can bind to Y2 whereas PYY (3-36) decreases eating through selective binding to only Y2 receptors.[187-189] Corroborated by studies showing, that the eating-inhibitory effect of PYY (3-36) is blocked by Y2 receptor antagonists[190] and that PYY (3-36) cannot inhibit eating in Y2-receptor knockout mice.[191]

Akin to its L-cell counterpart, GLP-1, PYY may be indirectly stimulated via neuroendocrine mechanisms activated in the duodenum in addition to direct cellular contact of nutrients.[187,192] Nutrient stimulated hormonal secretion from the L-cells is specific in that carbohydrates strongly stimulate GLP-1 secretion but are weak for PYY release.[158,184] PYY secretion varies dependent upon the stimuli, in order of increasing stimulation: lipids, protein, glucose.[193,194] Postprandial levels of PYY peak after 1 hour and remaining increased for up to 6 hours.[184,187,191,195]

PYY reduces appetite and food intake in the postprandial period,[196,197] with increasing plasma PYY levels proportional to caloric content in humans.[184] Food intake is reduced when peripheral infusions of PYY (3-36) are given at postprandial levels, in rats, mice and humans.[191,198,199] PYY also slows gastric emptying and delays gastrointestinal transit,[184,200,201] inhibits gallbladder emptying and secretion of gastric acid and pancreatic enzymes.[202]

Morbidly obese patients have reduced plasma PYY concentrations[203] but its effect persists, PYY infusion given to both lean and obese subjects, inhibits 24hr food intake.[191] Postprandial PYY levels are also blunted in the early stage of the development of T2DM, in genetically susceptible individuals, preceding the presence of IR and adiposity.[204] However, as nausea is a common side-effect of the higher dose of these infusions, this may be an additional mechanisms through which PYY suppresses food intake.

Following RYGB an increased postprandial PYY response has been reported when compared with lean and obese controls.[36,205,206] These increases in PYY levels post-RYGB increase within the first 6 months post-operatively. Despite a significant reduction in hunger or satiety post-RYGB no specific effect of an increasing PYY level upon this outcome was reported,[21] possibly due to post-RYGB vagal disruption, Y2 receptors are expressed on vagal fibres and vagotomy attenuates the anorectic effect of peripheral PYY (3-36).[140,207]

In the short term, plasma glucose levels are unaffected by PYY (3-36) but after 4 weeks administration a dose-dependent reduction in HbA1c occurs.[202] This may be due to changes in glucose uptake in muscle and AT rather than insulin release, as PYY (3-36) does not affect glucose metabolism in the fasted state but increases glucose disposal during the hyperinsulinaemic clamp.[208] To date, no correlation between postprandial plasma PYY levels and glycaemic control post-RYGB has been identified.[209]

1.1.3.6.3. Ghrelin

Ghrelin is produced in the gastric fundus and the proximal intestine[210-212] and is known to stimulate food intake in animals and humans through increases in hypothalamic expression of the orexigenic neuropeptide Y.[213,214] Ghrelin infusions have been shown to increase meal size.[215-217] It is increased in fasting and reduced in the postprandial period however presence of food in the stomach alone does not suppress ghrelin.[218-220] These signals appear to originate in the small intestine,[156,219,212,222] are proportional to the amount of calories ingested,[223,224] and may be mediated through changes in plasma glucose and insulin, intestinal osmolarity or enteric neural signalling.[218,225,226] Obese have lower fasting ghrelin levels and reduced postprandial suppression, compared with healthy weight individuals.[227]

RYGB-associated weight loss has shown significant reduction in ghrelin levels vs dietary-associated weight loss.[228] Following RYGB, reduced fasting and postprandial ghrelin[229-233] or no difference in fasting and postprandial levels[23,36,206] have been reported but some groups have shown the

converse.[234,235] Such heterogeneity in these studies could be explained by improvements in insulin resistance and hyperinsulinaemia post-RYGB as both are associated with ghrelin suppression.[236,237] Further implication that ghrelin may feedback upon insulin resistance is its effect upon islet cells, endogenous ghrelin enhancing glucose-induced insulin release from the pancreas however, exogenous ghrelin suppresses insulin secretion.[238] Ghrelin secretagogue receptor 1a antagonists have been shown to enhance insulin secretion in rodents, through currently unknown mechanisms. [239]

1.1.3.6.4. Cholecystokinin

CCK is mainly secreted from the duodenal I-cells in response to intraluminal foods, in particular breakdown products of proteins, oligopeptides and amino acids,[240-243] and long-chain fatty acids.[154,244-246] The plasma concentration rises rapidly and remains elevated for 3-5 hours but it acts via endocrine and paracrine pathways.[247] CCK stimulates exocrine pancreas function, gallbladder contractility assisting bile acid secretion and subsequent fat absorption, slows gastric emptying through stimulation of pyloric contractility,[246] modulates intestinal motility[249,250] and acts via vagal fibres to transmit a sensation of fullness in the dorsomedial hypothalamus.[251] CCK-1 receptor expression being present in both the sub-diaphragmatic vagal nerves and the dorsal vagal complex in the hindbrain.[252,253]

CCK is an important regulator of satiety. Intraperitoneal injections can reduce meal size, in rodents,[254] and intravenous infusions of CCK can reduce food intake[255,256] and induce satiety.[257] These effects are reversed by the type A CCK receptor antagonist loxiglumide.[258] With CCK-1 receptor gene abnormalities are associated with increased meal size, food intake and increased weight.[259-262]

Following RYGB, food bypasses the duodenum and as such one would presume that in the fasting state CCK levels would be unaffected but reduced following meal stimulation. Studies investigating CCK levels around RYGB in the fasting state, including our data above, agree with this theory.[57,263,264]

However controversy exists regarding postprandial CCK levels, some groups reporting increased postprandial CCK levels following mixed meal stimulation,[263-265] whilst others report no significant difference with glucose or protein only meal stimulation.[206,266] As CCK potentiates the secretion and effects of GLP-1, it may diminish gut transit further altering gut hormone changes post-RYGB. [267]

Increased villus height and crypt depth have been shown post-RYGB, in rats, in addition to increased number of villus and crypt goblet cells.[63] Hypertrophy of the roux and common limbs but not the bilio-pancreatico-duodenal (BPD) limb, has also been identified, with increased total and mucosal surface areas of these hypertrophied limbs. Increased enteroendocrine cells are present in the Roux and common limbs including CCK-immunoreactive cells, felt responsible for the increase in relevant circulating hormone levels, due to the increased total numbers of cells.[268] These changes have been reported following jejunoileal bypass[269,270] supportive of gut adaptation following gut diversion surgery.[271] Increased post-operative GLP-2 levels has been linked to this increased mitotic rate and crypt cell proliferation, humans and rats.[272]

1.2. Adipocyte

1.2.1. Structure

Adipocytes are spherical, unilocular structures which have the ability to vary dramatically in size, to accommodate storage of lipids. The fat cell comprises of 90% lipid droplet which is predominantly TG but a small volume of diacylglycerols (DG), phospholipids, NEFA and cholesterol are also present, with the nucleus pressed onto the periphery of the cell.[273] Adipocytes continually modify their structure through fatty acid esterification and lipolysis.

Adipocyte hypertrophy is directly related to insulin resistance in T2DM, irrespectively of total body fat[273] and reduces together with improvements in insulin sensitivity with dietary and exercise-induced weight loss.[274]

1.2.1.1. Regional variations

Significant regional variations in adipocytes exist including differences in adipose tissue (AT) distribution and molecular characteristics: differences in adipocyte size, hormone receptor expression, adipokine secretory profile, expression pattern.[275] Morphological and functional differences have been noted between visceral and peripheral AT.[276]

Visceral adiposity is specifically related to insulin resistance.[277] Differences in AT characteristics from the visceral and subcutaneous depots may help to explain this.[278] Visceral adipocytes express more genes encoding secretory proteins than subcutaneous.[279]

It would stand to reason that as omental AT is more insulin sensitive than subcutaneous AT with respect to lipolysis[278] the increased visceral adiposity associated with IR would be related to this function. Once increased omental/visceral AT volume occurs these cells which are effectively in a perpetual state of lipolysis (increasingly resistant to the effects of insulin) will effectively “flood” the portal system with glycerol and NEFA, resulting in the progression of non-alcoholic fatty liver disease (and impair insulin clearance and action and, increase glucose and VLDL output from the liver).[280,281]

When differences in adipocyte cell size was studied Buren et al[202] and McLaughlin et al[283] found no difference in cell size between NDM and T2DM and insulin sensitive and IR subjects respectively. Questions regarding the most appropriate cell sizing techniques have been raised. Development of fatty liver results in IR in mice without subcutaneous and visceral fat[284] and fatty liver has been noted in lipotrophic patients with IR.[285]

It is known that intra-abdominal fat is more lipolytic than subcutaneous fat and is also less sensitive to the anti-lipolytic effect of insulin.[286] Together with its anatomical proximity to the liver, this potentially could result in greater exposure of the liver than the peripheral tissues to NEFAs. Differences in exposure and the presence of the portal-peripheral NEFA gradient could explain why the liver can be IR at a time when the peripheral tissues are not.[287]

Assessed using magnetic resonance imaging (MRI) scanning, a longitudinal study post-RYGB revealed rapid lipid mobilization from both visceral and peripheral depots post-operatively together with enhanced NEFA flux to the liver. Disconnection between liver fat and insulin sensitivity occurred in the early post-RYGB period.[288]

Fatty liver disease is increased in obesity[289] however it is more likely related to degrees of insulin resistance than obesity.[290,291] Non-alcoholic fatty liver disease being shown to be exaggerated in IR.[289,292,293] Via the portal vein the gut drains the products of dietary absorption (except LCTGs which drain directly into the left subclavian vein via the lymphatic system (see section 1.1.3.4.), and ultimately reaches the omental and peripheral AT without first passing through the liver. The products of omental lipolysis drain directly into the liver via the portal vein.

1.2.2. Receptor expression

Adipocytes have multiple hormone receptors on their surface including: adrenaline (B1, 2, 3; alpha), insulin, growth hormone, insulin-like growth factor, cortisol and PPAR.[294] Human adipocytes express receptors for gut hormones: GLP-1;[295] GIP;[123,296] PYY;[297] ghrelin;[298,299] and NEFA: including FFAR2 (GPR43)[300] and GPR 120.[301]

Visceral AT has larger number of adrenergic receptors on the cell surface and higher lipolytic activity.[302] In addition, higher GIPR gene expression levels are found in visceral when compared to subcutaneous adipocytes.[303] Knocking out the gene encoding GLUT4 in adipocytes results in systemic IR in vivo and skeletal muscle IR in vitro, despite normal GLUT4 levels in the skeletal muscle.[304] This would suggest that the adipocyte is of greater importance than skeletal muscle with respect to insulin resistance.

1.2.3. Function

The role of adipose tissue has not been fully elucidated. It is the main energy storage area in the body, storing excess sugars and fats as TG in the lipid droplets and releasing them in the form of NEFA when needed.[305] In addition it is of paramount importance as a physical protector of the vital organs in the body and prevention of heat loss. Adipocytes release multiple cytokines[306] which have pro-inflammatory effects.[307-309] The finding that adipocytes not only respond to but also secrete hormones have identified revealed it to be the largest, and potentially the most important, endocrine organ in the body.[310] In fact, insulin is more efficient at inhibiting lipolysis than glucose production.[311] Alterations in this pathway post-RYGB, through direct effects upon lipolysis could alter plasma NEFA levels and in turn IR.

Depot-related differences in adipose-produced molecules have also been reported including leptin, adiponectin, IL-6 and angiotensinogen.[312,313]

1.3. Lipolysis

1.3.1. Principles of lipolysis

The primary role of the adipocyte is to store triglyceride, in lipid droplets, and release NEFA and glycerol through an enzyme-dependent system known as lipolysis.

Hormone sensitive lipase (HSL) is the rate-limiting enzyme for lipolysis, regulated by reversible phosphorylation. NEFA is released at each step of TG breakdown with glycerol only released when complete hydrolysis of the lipid occurs. Quantification of glycerol concentration is therefore a measure of complete lipolysis breakdown of TG within adipocytes, figure 1.3.1i.

There are many known activators of the lipolytic cascade, summarised in figure 1.3.1.ii.[314] As shown in the diagram, the main known activators of lipolysis are catecholamines. Stimulation of adenylyl cyclase increases intracellular cyclic AMP (cAMP) concentrations which promotes the activation of cAMP-dependent PKA.[314] The PKA phosphorylates HSL and promotes its activation, thereby increasing lipolysis.[315,316] Lipolysis is inhibited by reducing cAMP, which is catalysed to 5'AMP by the enzyme PDE-3.[317]

1.3.2. Effect of weight loss upon lipolysis

Weight loss through diet alone reduces adipocyte lipolysis and fat oxidation however the addition of endurance exercise to diet reverses this effect.[318]

Obese adipocytes have lower basal and stimulated lipolysis when compared to lean.[319] This data is supported by: endurance training in obese AT decreased basal lipolysis, lipolytic effects of β-adrenergic response were increased and the antilipolytic effects of α_2-adrenoceptor and insulin were significantly attenuated.[320]

1.3.3. Regional variations in lipolysis

Adipocytes from visceral and peripheral sites have different characteristics. HSL activity and mRNA expression is higher in subcutaneous than omental fat cells suggestive of increased lipolytic capacity in these cells. Lipolysis rate significantly correlated to HSL activity.[321]

In rodents, there is lower HSL activity and mRNA levels in subcutaneous adipocytes versus visceral,[322] subcutaneous adipocytes have lower expression of the 3 β-adrenoceptor subtypes and HSL.

Human subcutaneous adipocytes have higher LPL activity and are more lipolytic than omental adipocytes however omental adipocytes have a greater response to both adrenergic receptor– and post-receptor-acting agents compared with subcutaneous adipocytes.[323] It is thought that the increased catecholamine-induced lipolysis in visceral fat is due to increase expression of β1- and β2-IRs[302,324] and enhancement of β3-AR function.[325]

Omental adipocytes are more responsive to catecholamines and less sensitive to insulin.[326] Stated another way, omental fat is more lipolytic than subcutaneous fat and less sensitive to the anti-lipolytic effects of insulin.[278,286,327] This could be explained by the reduced insulin binding capacity and reduced activation of insulin receptor signalling events including tyrosine phosphorylation of IR and IRS-1 and PI 3-kinase activation in human visceral adipocytes.[327] A higher glucose uptake rate has also been noted in omental adipocytes and is almost certainly explained by an increased GLUT4 expression.[328]

To date I have found no study regarding the difference between lipolytic effects of incretins from different adipocyte regions.

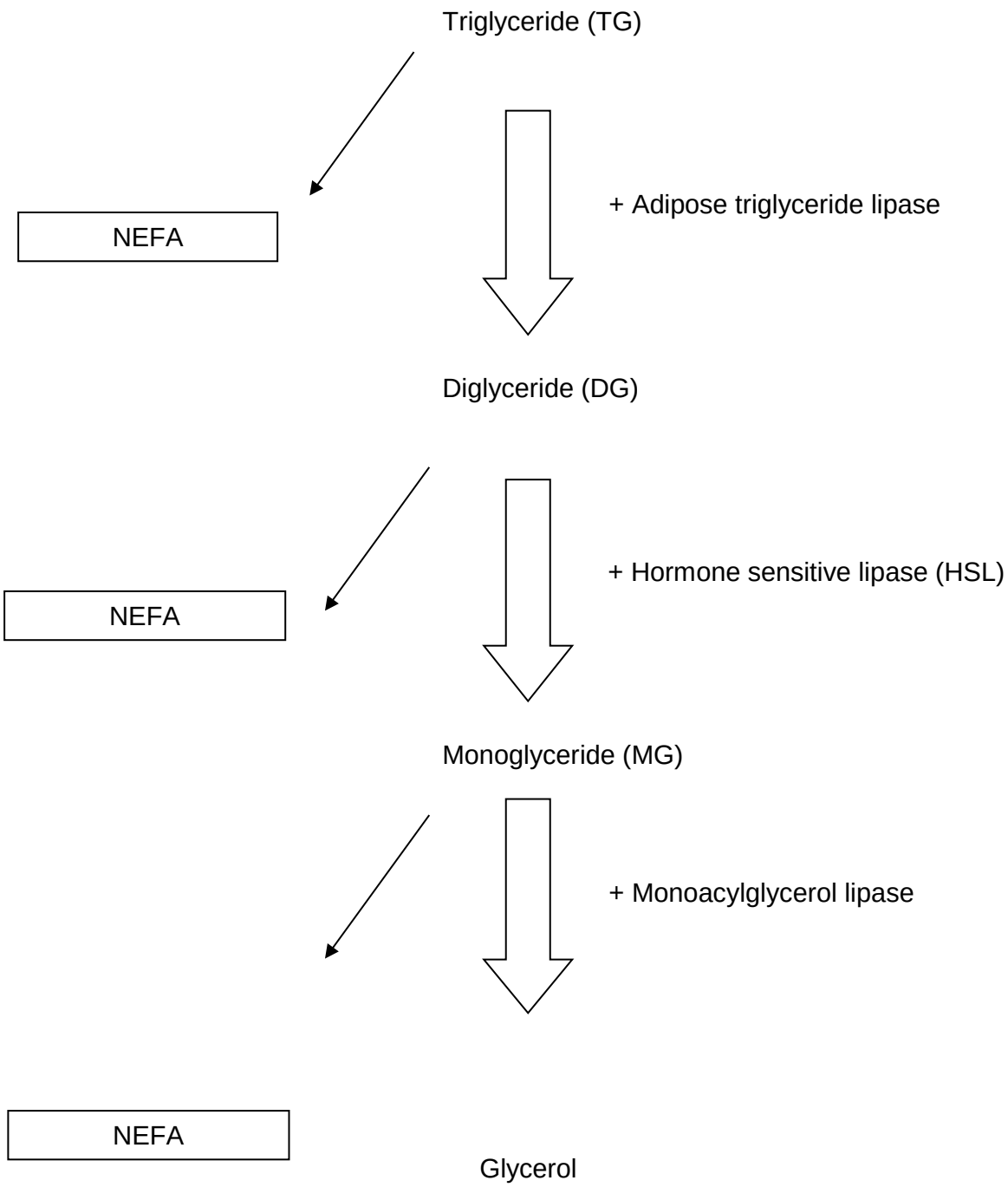


Figure 1.3.1 Principles of lipolysis

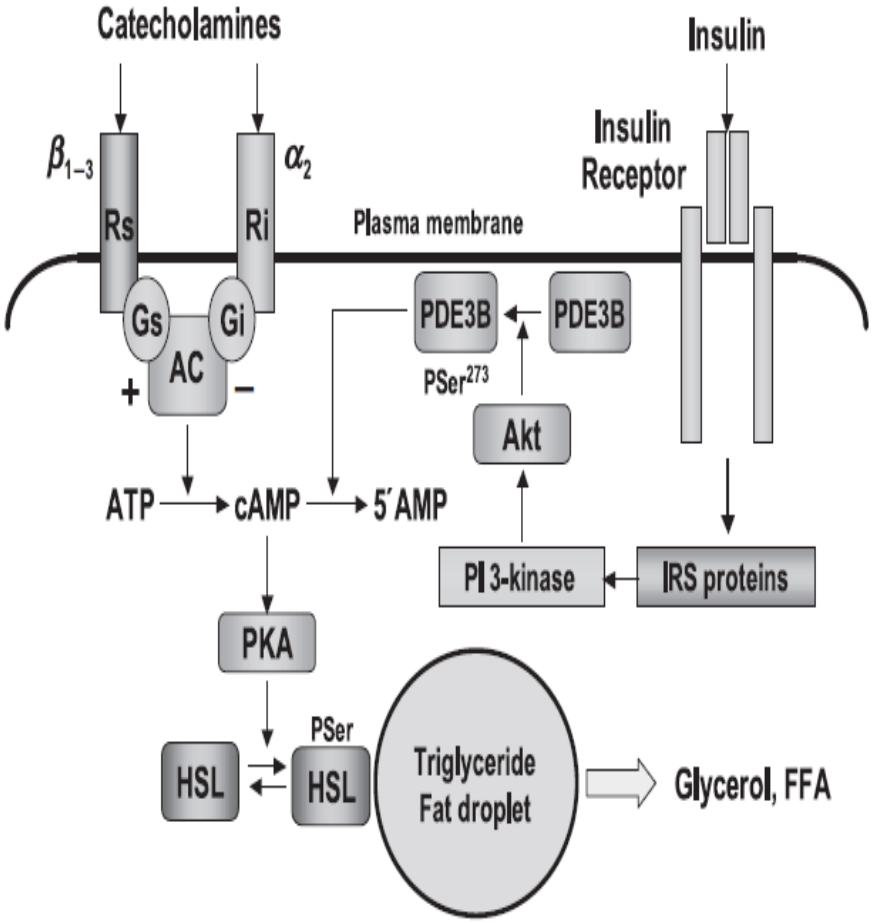


Figure 5 Hormonal regulation of lipolysis in adipocytes. The cascades of signalling events controlling the activity of hormone sensitive lipase (HSL) in adipocytes are represented. Catecholamines, through α- and β-adrenoreceptors, modulate the activity of adenilyl-cyclase (AC), thus increasing the intracellular levels of cAMP. By contrast, insulin decreases cAMP levels by activating the phosphodiesterase 3B (PDE-3B) through the PI 3-kinase/Akt pathway. cAMP is the main agonist of protein kinase A, which phosphorylates and activates the HSL. Rs, adrenoreceptor with stimulatory activity; Ri, adrenoreceptor with inhibitory activity; Gs, G-protein with stimulatory activity; Gi, G-protein with inhibitory activity; PSer, phosphoserine residue; FFA, free fatty acids.

Figure 1.3.2 Hormonal regulation of lipolysis in adipocytes[314]

1.3.4. Hormonal effects upon lipolysis

1.3.4.1. Insulin

The anti-lipolytic effects of insulin are well documented. It is believed that in animals, including humans, the postprandial rise in insulin promotes TG storage in the AT and inhibits lipolysis,[314] optimising storage of reserve energy through this route. However unexpectedly, in vivo fasting reduces systemic sensitivity to antilipolytic effects of insulin[329] in contrast to findings in vitro.[330]

The anti-lipolytic effects of insulin occurs through the phosphorylation and subsequent activation of cAMP hydrolysing enzyme, phosphodiesterase (PDE) -3B[314,331] resulting in decreased activation of protein kinase A (PKA) and HSL and therefore less hydrolysis of TG.[332,333] Inhibition of PDE-3B abolishes the antilipolytic effects of insulin in human AT.[334] PDE-3 stimulation by insulin requires PI 3-kinase in both the rat and humans.[335,336] The mechanisms of insulin-stimulated glucose uptake in adipocytes are summarised in figure 1.3.4.1.[314]

Omental fat has higher expression levels of specific signalling proteins and increased +/- earlier activation of Akt/GSK-3 and ERK signaling pathways in response to insulin.[337] Subcutaneous tissue is more sensitive to these effects than omental AT,[278,286] together with an increase in insulin receptor affinity in the subcutaneous adipocytes despite no difference in receptor number being found. In addition, insulin dissociation rate is increased in omental adipocytes.[278]

1.3.4.2. GIP

GIP reduces NEFA levels and stimulates lipolysis in rat adipocytes.[120] Rodent studies, suggest GIP stimulates lipolysis and increases NEFA reesterification but inhibited isoproterenol (ISO)-stimulated lipolysis by 43% suggesting it to have similar properties to insulin, an effect which was reversed by ANTIGIP.[120]

GIP stimulates fatty acid incorporation into AT, this effect is enhanced in obese vs lean rodents.[338] It stimulates glycerol release through stimulation of adenylyl cyclase and cAMP[339] suggesting it should be lipolytic rather than lipogenic however, in steers GIP infusions increased plasma palmitate levels,[340] effect not repeated in bovine AT in vitro.[120]

A synergistic anti-lipolytic effect of the combination of GIP and insulin was not found in rat adipocytes although postulated.[120] The suggestion that GIP caused increased cellular insulin sensitivity (measured by insulin-stimulated glucose transport) and insulin receptor affinity in rat adipocytes.[341] Other theories that GIP and insulin share similar signalling components (such as PI-3 kinase, glycogen synthase kinase 3B, or PKB) and therefore compete for the action of adipocytes. We known that in 3T3-L1 cells, GIP-stimulated lipolysis was inhibited by insulin (this effect was blocked by the PI3K inhibitor, wortmannin).[339]

Effects of GIP in the presence of insulin, increased LPL activity and TG accumulation through pathways involving increased phosphorylation of PKB and reductions in phosphorylated LKB1 and AMP-activated protein kinase in mouse and cultured human subcutaneous adipocytes.[342]

To date no GIP lipolysis studies appear to have been performed using fresh human adipocytes.

1.3.4.3. GLP-1

GLP-1 is lipogenic at low concentrations and lipolytic at high concentrations.[170] cAMP being implicated as a second messenger for the lipolytic but not the anti-lipolytic effects of GLP-1 upon human adipocytes.[170] In rat liver and skeletal muscle GLP-1 effects glucose metabolism similarly to insulin but does not increase adenylate cyclase activity.[343,344] GLP-1 has been shown to increase basal lipolysis in adipocytes from obese and lean subjects.[345] Its effects upon lipolysis at concentrations equivalent to RYGB physiological levels and in combination with insulin are unknown.

1.3.4.4. PYY

In human subcutaneous adipocytes PYY inhibits lipolysis[346] in a dose-dependent manner, both basal (ADA) and stimulated (isoproterenol- and forskolin-induced lipolysis). This effect was reversed using *Bordetella pertussis* toxin (which blocks the effects on the GTP-binding regulatory proteins in human adipocytes,[347] suggesting that this effect is likely mediated by adenylate cyclase inhibition.[346]

Y1 receptor mediates the antilipolytic effect of NPY and PYY in rodent adipocytes and conversely treatment with Y1 antagonists stopped weight gain through reduction in appetite and adipocyte cell size.[348-350]

1.3.4.5. Ghrelin

Ghrelin has been linked to lipolysis and glycaemic control through several mechanisms.

In adipocytes, ghrelin has been shown to stimulate insulin-induced glucose uptake in adipocytes.[239] In vivo, ghrelin infusion induces lipolysis (as assessed by plasma NEFA levels and interstitial glycerol concentrations using a microdialysis technique) and IR, independently of growth hormone and cortisol[351] and has been reported to reduce the anti-lipolytic effect of hyperinsulinaemia.[351]

The antilipolytic effect of both ghrelin isoforms has been reported in rodents through binding to a specific receptor, distinct from GHS-R1a (more accurately assessed against stimulation[352,353] rather than non-ado basal).

Comparable results were found with cultured human subcutaneous adipocytes, which were lipogenic when incubated with Octanoyl-(OTG) and des-acyl (DSG) ghrelin (partly mediated through the Y1 receptor) and DSG was shown to alter lipolysis (not mediated through Y1 receptors), lipogenesis and leptin secretion.[299]

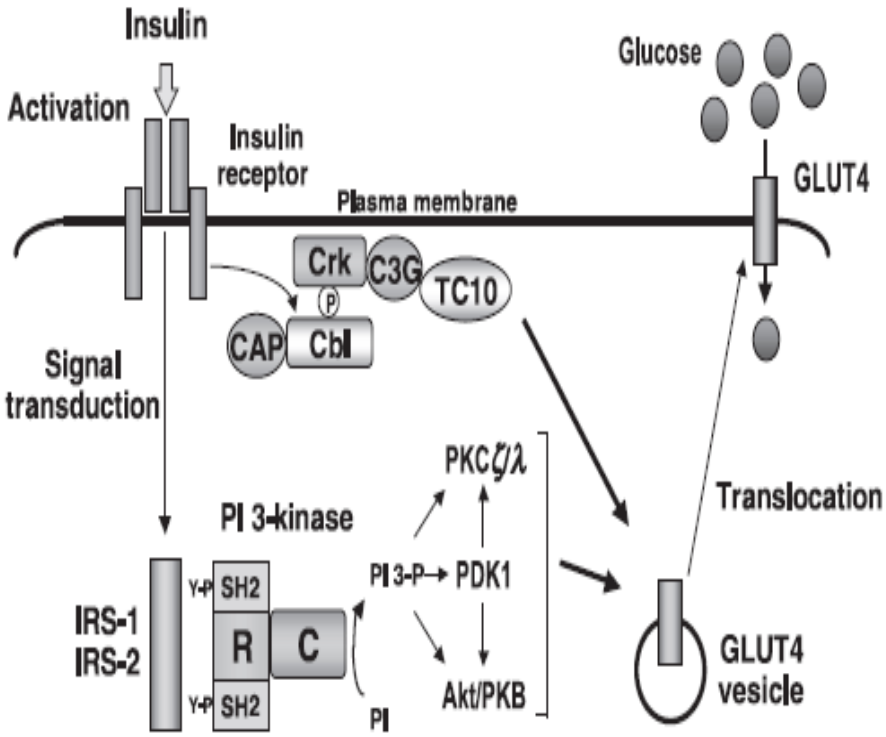


Figure 4 Mechanisms of insulin-stimulated glucose uptake in adipocytes. The intracellular signalling reactions mediating insulin stimulatory effect on glucose transporter translocation and glucose uptake in adipocytes are illustrated. Insulin binding to its transmembrane receptor recruits intracellular docking proteins (the IRS proteins) and activates a cascade of protein-protein interaction events and biochemical reactions (phosphorylation on tyrosine or serine/threonine residues), ultimately leading to the translocation of GLUT4 containing vesicles from intracellular quiescent pools to the plasma membrane, leading to enhanced glucose entry into the cell. PI, phosphatidylinositol; Y-P, phospho-tyrosine residue; SH2, src-homology 2 domain; R, regulatory subunit; C, catalytic subunit.

Figure 1.3.3 Mechanisms of insulin-stimulated glucose uptake in adipocytes[314]

1.4. Hypothesis

The gastric bypass procedure results in remission of T2DM through alterations in postprandial secretion of incretins and insulin combined with improvements in insulin resistance.

I hypothesise that these postprandial hormonal alterations improve adipocyte insulin sensitivity through the peripheral effects of incretins, improving insulin resistance.

2. Materials and methods

2.1 Summary of the study protocol

This is a prospective observational study designed to quantify the lipolytic and anti-lipolytic effects, in visceral and peripheral adipocytes, of the changes which occur in gut hormone levels around RYGB surgery, performed for obesity in people with and without T2DM pre-operatively.

The primary endpoint of this study is a statistical difference in lipolysis rates in human adipocytes between:

a) pre- and post-RYGB plasma
b) gut hormone levels approximating those seen pre- and post-surgery

The study protocol is summarised in figure 2.1.

1. In order to quantify gut hormone response to a set meal challenge using a method which was both reliable and easily reproducible, enabling direct comparison of the same participants before and after surgery, [20] participants underwent a mixed meal test (MMT) with frequent blood sampling on the day prior to their operation and on post-operative day 4
2. At time of RYGB, visceral and peripheral adipose tissue was excised, placed into separate 30ml plastic flasks containing 0.9% saline or PBS buffer and transferred to the laboratory at room temperature immediately. The adipose tissue underwent adipocyte isolation and lipolysis experimentation (sections 2.3.3 and 2.3.4).
3. Following this, all participants underwent routine postoperative bariatric care and follow-up arrangements through the NHS.
4. The study protocols were approved by the King's College Hospital Ethic Committee (LREC no: 08/H0808/83) and King's College Hospital's Research and Development department (08LG13) and all participants gave informed consent prior to enrolment.

2.2 Clinical participation

2.2.1 Recruitment and enrolment

The study had 2 patient groups:

Patients with T2DM (n=9)

Patients without T2DM (NDM) (n=10)

All suitable patients being placed on the waiting list for gastric bypass surgery at King's College Hospital, London were approached regarding interest in participation in this study at this time.

At time of their routine surgical pre-assessment, potential participants in the NDM group underwent formal assessment of T2DM and insulin resistance with fasting blood glucose and insulin levels and an oral glucose tolerance test. Depending upon these results, participants were enrolled.

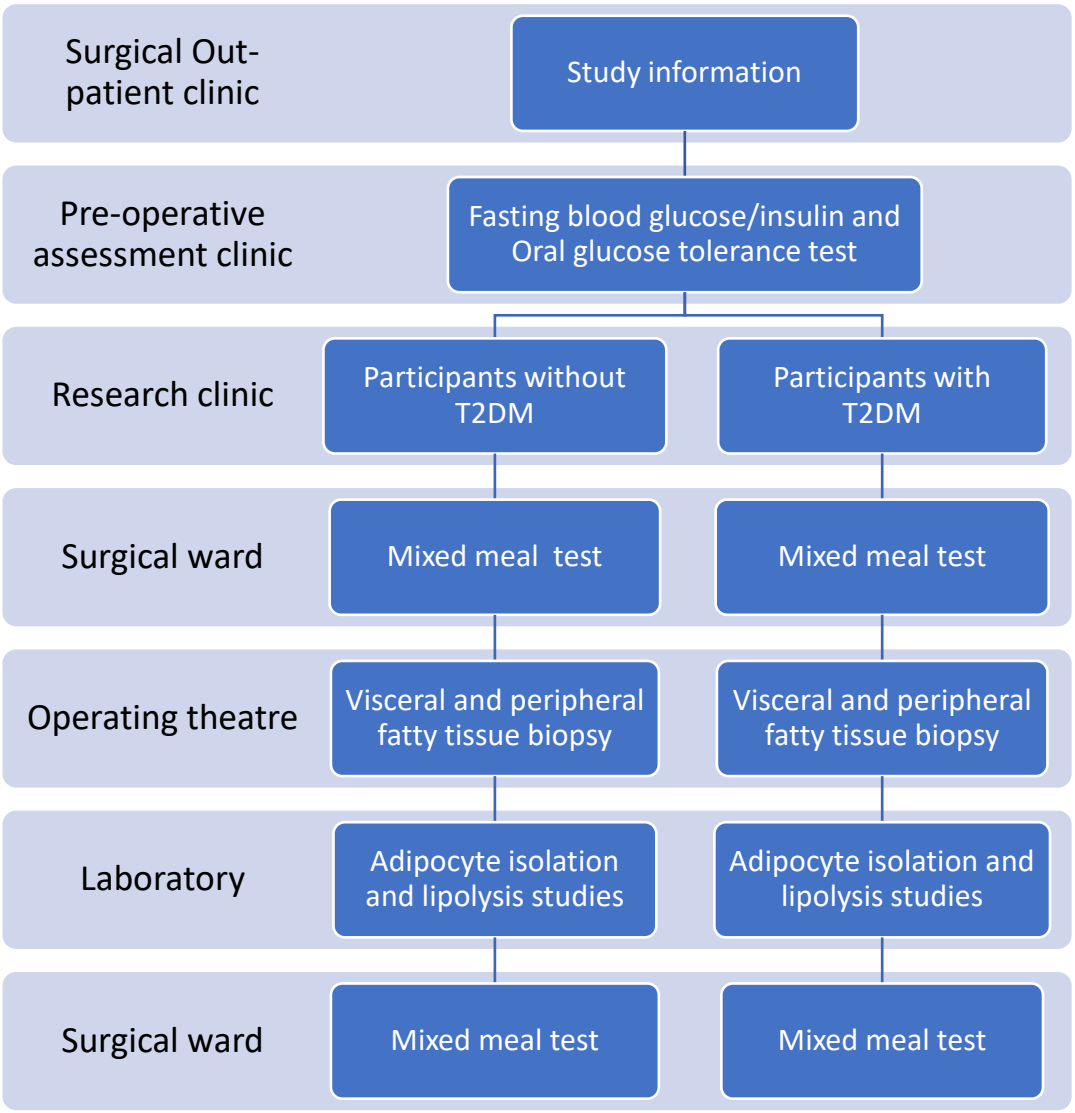


Figure 2.1 Effects of RYGB surgery upon lipolysis study summary diagram

2.2.2 Inclusion and exclusion criteria

Inclusion criteria

- Meeting NICE criteria for bariatric surgery[354]
- 18 – 65 years old
- BMI >35
- Able to provide informed consent
- To understand spoken and written English
- Non-DM group – FBG <6.1 mmol/l and OGTT – 2hr BG <7.8mmol/l
- DM group – FBG >or equal to 7.0 mmol/l and OGTT – 2hr BG > or equal to 11.1mmol/l

Exclusion criteria

- Enrolled in other clinical study involving investigational drug or other surgical intervention
- Unstable diabetic retinopathy
- Stage 3 – 5 renal impairment
- Clinical evidence of cardiac failure
- Myocardial infarction in previous year
- Current angina or heart failure
- Liver function tests > 3 x normal
- Any condition where compliance unlikely e.g. anxiety disorder, inadequate comprehension
- Pregnancy and breastfeeding
- Immunosuppressive drugs inc. steroids
- Coagulopathy (INR>1.5 or plt<50)
- Anaemia (Hb<10)
- Recent history of cancer (<5 years)
- Any disorder of fat storage
- Any contraindication to bariatric surgery

2.2.3 Quantification of T2DM and IR

All NDM participants underwent formal assessment of their glycaemic control with fasting glucose and an oral glucose tolerance test. Fasting glucose and insulin levels were performed for measurement of HOMA-IR[355] on all participants.

Participants were requested to attend for their surgical pre-assessment fasted for 12hr. They received instructions regarding the temporary cessation of medications which could potentially affect their T2DM/IR status assessment in advance, as follows:

1. If you are taking metformin tablets please stop taking these 3 days prior to pre-assessment
2. Do not take any oral tablets for T2DM on the day of pre-assessment
3. Bed-time insulin injections only – do not take this the night before pre-assessment
4. If you have 2 insulin injections a day – take your usual evening insulin with your evening meal no later than 7:30pm the night before pre-assessment
5. If you have 4 insulin injections a day – do not take your bed-time insulin the night before or the morning insulin prior to pre-assessment
6. If you are taking Lantus do not take this after 9am the day before the pre-assessment clinic

As part of national obesity guidelines,[354] patients have had fasting glucose levels checked prior to referral for consideration of bariatric surgery. This was repeated at pre-operative assessment and for the purposes of this study an oral glucose tolerance test was performed in the NDM participants. World Health Organisation (WHO) diagnostic criteria for T2DM were used to define participants' endogenous glycaemic control:[356]

- T2DM: fasting plasma glucose ≥7.0 mmol/l **or** 2hr plasma glucose* ≥ 11.1 mmol/l

- Impaired glucose tolerance (IGT): fasting plasma glucose < 7.0 mmol/l **and** 2hr plasma glucose* ≥ 7.8 and < 11.1 mmol/l
- Impaired fasting glucose (IFG): fasting plasma glucose 6.1 - 6.9 mmol/l **and (if measured)** 2hr plasma glucose* < 7.8 mmol/l
- NDM: fasting plasma glucose ≤ 6.0 mmol/l

* venous plasma glucose 2hr after ingestion of 75g oral glucose load
If 2hr plasma glucose is not measured, status is uncertain as diabetes or IGT cannot be excluded.

All participants in the T2DM underwent repeat T2DM assessment including fasting glucose and insulin levels and an oral glucose tolerance test on post-operative day 7 as per the King's College Hospital bariatric unit protocol.

2.2.3.1 Fasting plasma glucose and insulin levels

Once confirmed that participants had fasted >10 hrs venous blood was collected for glucose analysis (4ml fluoride oxalate tubes) and insulin (6ml plasma tubes). The samples were promptly centrifuged at 3,000 x g for 10min at 4°C. The glucose samples were immediately assayed (see Appendix 2) and the plasma samples were transferred in separate labelled tubes and frozen at -80°C for batch insulin assaying (see Appendix 2).

2.2.3.2 Homeostasis Model Assessment – Insulin Resistance

HOMA-IR is a simple, reproducible index of insulin resistance in both diabetic and non-diabetic subjects. HOMA-IR is also highly correlated with clamp-IR in T2DM subjects of normal (mean BMI 21.4 +/- 2.3) and moderate obesity (BMI 27.2 +/- 2.2).[357]

I used HOMA-1 for this study. HOMA1 is the original HOMA model it is calculated using the following formulae:[355]

- HOMA1-IR = (FPI x FPG) / 22.5

FPI is fasting plasma insulin concentration (mU/L) and FPG is fasting plasma glucose (mmol/l).

This calculation has been superseded with HOMA-2,[358] a programme which is derived from fasting plasma glucose and insulin levels, calculated using a mathematical model which accounts for several variations including hepatic and peripheral glucose resistance, reduction of peripheral glucose-stimulated glucose uptake and renal glucose losses. It can be calculated using the HOMA calculator (version 2.2.2) downloaded from the Diabetes Trials Unit, University of Oxford website (http://www.dtu.ox.ac.uk/homa).[359,360] Unfortunately, this model makes some specific presumptions such as plasma glucose must be between 3 and 25mmol/L and insulin must be between 2. And 57.6 μU/ml. The programmers suggested that any figure out-with these ranges was abnormally high and therefore incorrect. Sadly, this does not allow for patients at the extremes of the insulin range i.e. morbidly obese with profound degrees of insulin resistance. Normally this HOMA-IR calculation would suffice but falls short of being able to analyse my patient cohort.

2.2.3.3 Oral glucose tolerance test

This test was carried out as per WHO guidelines 1999:[361]
The test was preceded by >3 days of normal, unrestricted diet (>150g carbohydrate daily) with normal physical activity.

After fasting for 12hr, plasma glucose and insulin samples were taken. 75g anhydrose glucose in 250-300ml water was then drank over 5min. A repeat venous blood sample was then taken for glucose, 120min after starting the glucose drink.

I ensured the participant was in good health on the day of the study. During the course of the study they sat quietly, remained otherwise fasted and did not smoke.

2.2.4 Participants

Twenty participants were recruited into this study, T2DM (n=10) and NDM (n=10). As summarised in the study attrition diagram, figure 2.2.4, fasting data from 19 participants (10 NDM and 9 T2DM) and post-prandial data from 16 participants (9 NDM and 7 T2DM) was available for analysis.

There were no mortalities in this study. No procedures were converted to the open approach i.e. all RYGB were completed using the laparoscopic approach. Morbidity data was collected together with length of post-operative stay and time in Level II/III care.

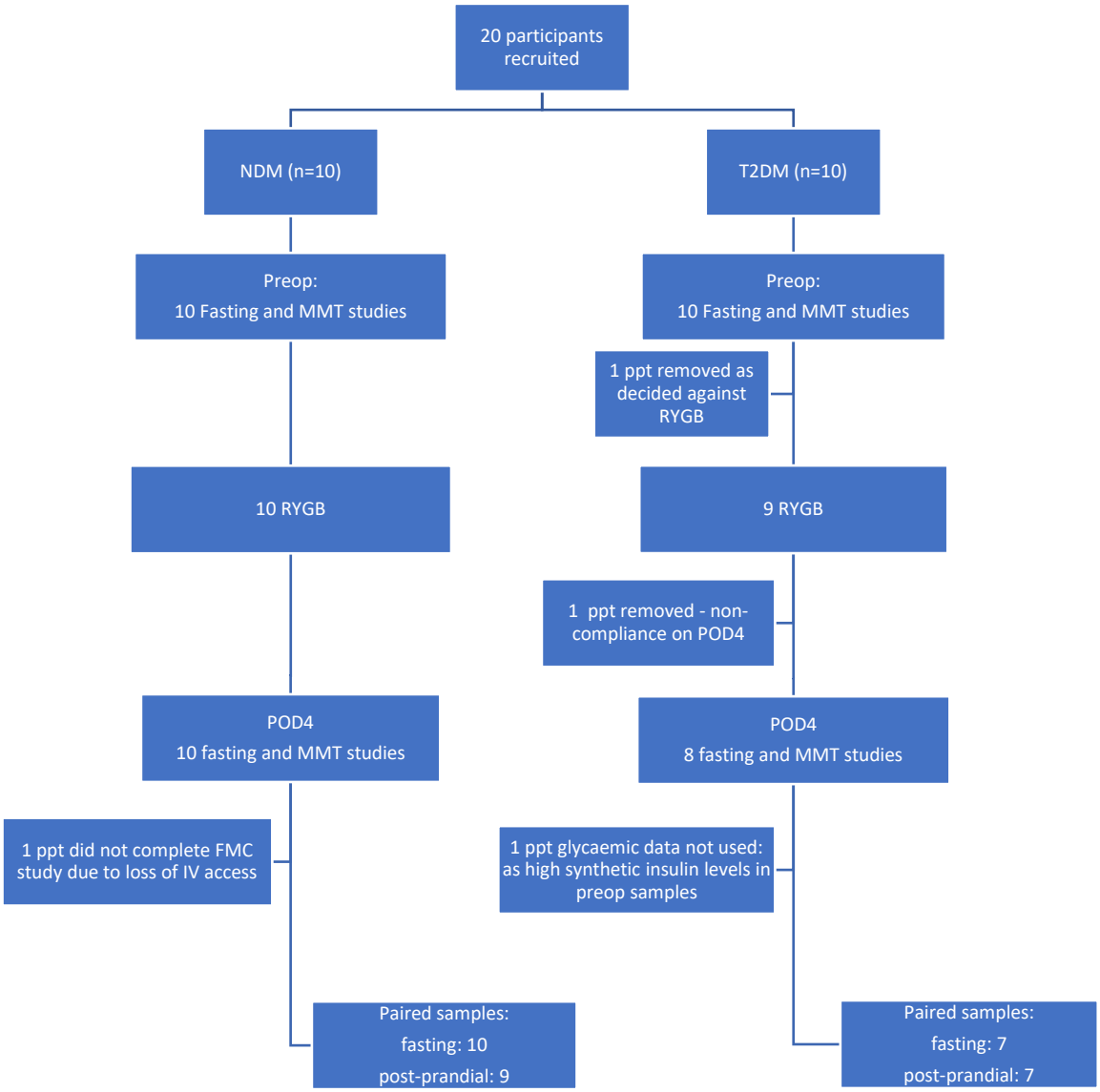


Figure 2.2.4. Effects of RYGB surgery upon lipolysis study attrition diagram

2.2.4.1 Participant characteristics

Participant characteristics are described in table 2.2.4.1. There was no difference in gender or age between the two groups. The participants in the T2DM group had a lower pre-operative weight than their comparators however there no difference in BMI. This may be a reflection of the NICE recommendations for consideration of bariatric surgery at the time of the study:[354]

BMI > 40 with no co-morbidities

BMI ≥ 35 with co-morbidities related to obesity

All participants met the International Federation of Surgical Obesity (IFSO)/NICE recommendations for consideration of bariatric surgery prior to enrolment. Two of the T2DM participants had BMI 35-40.

2.2.4.2 RYGB procedure

Participants underwent laparoscopic RYGB surgery with formation of 30ml gastric pouch and 100-150cm Roux limb (p=0.1402). In one individual the Roux limb was fashioned using the retrogastric retrocolic position rather than antegastric antecolic due to the finding of a “tight” mesentery and subsequent unacceptable increased risk of gastrojejunal anastomotic tension. This slight variation in anastomotic location should not affect the study outcomes. Participants were fasted for three days post-operatively then a normal oral gastrograffin study was confirmed prior to fasting for 10hr for their post-operative day 4 (POD4) MMT study.

As per unit protocol, all T2DM participants were commenced on insulin sliding scale on the night prior to surgery. All other T2DM medications were not recommenced post-operatively prior to discharge from hospital. For participation in the study, the insulin sliding scale was stopped for 24h prior to the POD4 MMT study.

2.2.4.3 Controlling for weight loss

Although a difference in weight between the 4 groups (ANOVA $p<0.05$) was noted, paired analysis detected no difference in either the NDM group ($p=0.3466$) or the T2DM group ($p=0.3666$) from pre-op to POD4, figure 2.2.4.3. The difference detected by ANOVA analysis is perhaps reflective of the difference in weight between the NDM and T2DM pre-operatively.

2.2.4.4 Controlling for post-operative inflammatory response

Plasma white cell count (wcc), serum C-reactive protein (CRP) and serum cortisol were measured pre-operatively and on POD4 in an attempt to quantify systemic inflammatory change which may directly impact upon the outcomes of this study.

There was no difference in the wcc between the four groups ($p=0.2735$). A highly significant difference in CRP levels was noted ($p<0.0001$), increasing post-operatively with no difference in the direction of change between the groups ($p=0.6816$). Fasting cortisol levels were also different between the groups ($p<0.05$), increasing post-operatively with no difference in the direction of change between the groups ($p=0.2271$).

As anticipated following complex major abdominal surgery, these results suggest that a degree of systemic inflammatory response was present on post-operative day 4. This could affect the data. Encouragingly there was no difference in the direction of change between the NDM and T2DM groups, which suggests direct comparison to be possible.

Table 2.2.4.1 Effects of RYGB surgery upon lipolysis study participant demographics

† (n=7)

Demographics	**NDM (n=10)**	**T2DM (n=9)**	**p-value**
Gender (m:f ratio)	2:8	3:6	0.5367
Age (years)	42 +/- 4	48 +/- 3	0.1953
Pre-operative weight (kg)	143.6 +/- 5.55	120.4+/-7.40	0.0211
Pre-operative BMI (kg/m^2)	50.3 +/- 1.7	46.0+/-2.7	0.1796
Pre-operative FPG (mmol/l)	5.35 +/- 0.46	10.16 +/- 5.31†	0.0112
Pre-operative HOMA-IR	7.00 +/- 1.47	16.37 +/- 3.49†	0.0143
Post-operative stay (days)	4 +/- 0.1	5 +/- 1	0.0352

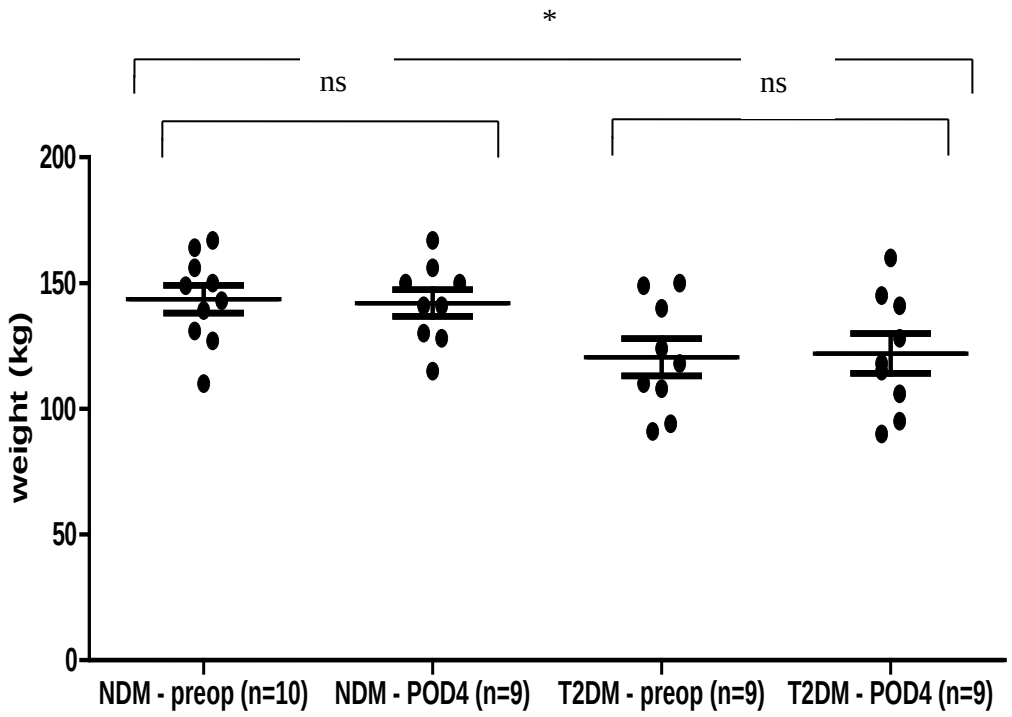


Figure 2.2.4.3 Scatter plot of the effects of RYGB surgery upon weight between pre-operative and post-operative day 4 in participants with and without T2DM

2.2.4.5 Relevant medical history

As summarised in the table 2.2.4.5, the participants had a range of relevant concomitant medical conditions.

2.2.4.5.1 Duration of T2DM

The duration of T2DM was not controlled in this study. Four participants had been diagnosed with the last year, two within 5 years, one within 10 years and two had had T2DM ≥ 10 years.

2.2.4.6 Drug History

Pre-operatively two participants in the NDM group (n=10) and five of the T2DM group (n=9) were taking statin medications. A further participant in the T2DM was taking fenofibrate as they had not tolerated a statin. This was slightly unexpected as lipid reducing medications convey a relative risk reduction of all-cause mortality in T2DM.[362]

One participant in the NDM group (n=10) was taking Metformin tablets pre-operatively (for polycystic ovarian syndrome). Seven participants in the T2DM group (n=9) were taking Metformin tablets pre-operatively, three in combination with a sulfonylurea and one in combination with a thiazolidinedione. Two participants were using a GLP-1 analogue medication pre-operatively, both in combination with insulin injections. Of the four participants who were requiring insulin injections pre-operatively, three of these were taking two oral diabetic medications in addition to this.

2.2.4.7 Length of post-operative stay

The majority of the participants were discharged following participation in the POD4 research activities however the mean post-operative length of stay was longer in the T2DM group (n=9) at 5+/-1 days versus 4 +/- 0.1 days in the NDM group (n=10), p=0.0352. This is comparable with the literature regarding increased length of post-operative stay in T2DM patients.[363]

2.2.4.8 Morbidity and mortality

Bariatric surgery is complex major surgery performed on the morbidly obese patient group. Inherently it has a high expected perioperative morbidity (i.e. within the first 30 days of surgery) with overall complication rates for RYGB between 7 and 14% and a mortality rate 0.5%.[364-366]

Surgical complications are classified as major or minor. For the purposes of this study major complications included: anastomotic leak, pulmonary embolus, internal or ventral hernia or bowel obstruction requiring reoperation, fascial dehiscence, haemorrhage requiring reoperation or more than 1 unit of blood transfusion, myocardial infarction, stroke. Minor complications included wound infection, bleeding requiring one unit or less of blood transfusion, pneumonia, central venous catheter infections.[367] Morbidity data was collected for up to 30 days following RYGB surgery.

The complications encountered during this study are shown in table 2.2.4.8. As per the surgical definitions all our complications were classified as minor and although the pressure sore incidence rate (all grade 1; International NPUAP-EPUAP Pressure ulcer classification system)[368] was thought to be higher than anticipated in this patient group. This may be explained by the prospective data collection.[369] There were no mortalities in this study. The only potential complications which could be attributed to the study participation were wound infection/haematoma and delayed wound healing. There were no severe adverse events or serious unexpected severe adverse events in this study.

Table 2.2.4.5 Relevant medical conditions in participants of the effects of RYGB surgery upon lipolysis study

Concomitant medical conditions	**NDM (n=10)**	**T2DM (n=9)**
Hypertension	3	5
Hyperlipidaemia	2	4
NAFLD	0	1
OSA	3	5
PCOS	1	3
Hypothyroidism	1	0
OA	4	3

Table 2.2.4.8 Morbidities in participants of the effects of RYGB surgery upon lipolysis study

Condition	No. of participants effected	
	NDM	**T2DM**
Respiratory		
- CO_2 retention	1	0
- atelectasis	2	1
- chest infection/pneumonia	0	3
Genito-urinary		
- UTI	1	0
Skin		
- wound infection/haematoma/blistering	2	1
- delayed wound healing	0	1
- pressure sore	1	2
Musculo-skeletal		
- exacerbation of chronic back pain	1	0
- rhabdomyolysis of gluteus maximus bilaterally	0	1
Neurological		
- Frontal headache	1	0
Other		
- transient pyrexia of unknown origin	1	2
- idiopathic palmar oedema	1	0

2.2.5 Quantification of post-prandial gut hormone and metabolite response

2.2.5.1 Mixed meal test

All study subjects participated in two MMT with frequent venous blood sampling, the day prior and on POD4.

MMT summary

1. Confirmation that participants continue to meet the inclusion/exclusion criteria was made
2. Participants were fasted for >10 hours
3. Weight (kg) was recorded
4. A peripheral venous line was inserted using aseptic technique and basal bloods were taken for glucose, insulin, GLP-1, GIP, PYY, Ghrelin, CCK, NEFA, cortisol in pre-prepared, labelled bottles (see section 2.2.5.2)
   [If NDM HOMA>3 – additional 50ml blood taken and stored as plasma in Lithium Heparin tubes with DPPIVi and Trasylol for use in adipocyte experimentation]
5. A 420-kcal mixed meal was consumed over 5 min (150ml Belgium Chocolate ice-cream)
6. Venous bloods were taken for: glucose, insulin, GLP-1, GIP, PYY, Ghrelin, CCK, NEFA at 15, 30, 60, 90, 120, 150, 180, 240, 300 and 360min
   [If NDM HOMA>3 – additional 50ml blood taken and stored as plasma at 60 min time-point]
7. Participants were allowed to drink water after the 120 min samples were taken
8. Once sampled blood was promptly centrifuged at 3,000 x g for 10 min at 4°C, transferred and pipetted for storage in separate pre-prepared and labelled tubes. Samples were stored at -80°C for analysis in batches (see Appendix 2).

9. The intra-venous line was removed if no longer required, after study completion

2.2.5.2 Study bottle preparation

The types of blood sampling tubes, additives and storage recommendations for the study blood samples is shown in table 2.2.5.2.

2.2.5.3 Choice of mixed meal

The incretin response to a mixed fatty and sugar load is greater than to glucose alone both in rats and humans.

The 420kcal fixed meal of 150ml Belgium Chocolate Hagen-Dazs® ice-cream is a well-tolerated choice of meal which results in satiety, reduces hunger and elicits a statistically significant change in glucose, insulin, and incretins when compared to fasting, normal and obese weight participants and participants after bariatric surgery (AGB and RYGB).[20]
[Nutritional information regarding Belgium Chocolate Hagen-Dazs ice-cream, appendix 1].

Table 2.2.5.2 Mixed meal test bottle preparation

Test	Sample type	Conditions
Glucose	Fluoride oxalate	Cold spin & processed immediately
Insulin	Serum	Freeze immediately
NEFA	Serum	Freeze immediately
GLP-1	EDTA & DPPIVi (10ul/ml)	Cold spin and freeze immediately
GIP	EDTA & DPPIVi (10ul/ml)	Cold spin and freeze immediately
CCK	EDTA & Trasylol (100ul)	Cold spin and freeze immediately
PPY	EDTA & Trasylol (100ul)	Cold spin and freeze immediately
Ghrelin	EDTA & Trasylol (100ul)	Cold spin, add 1M HCL (50µl for 450µl plasma)
NDM HOMA>3 **Plasma storage**	Lithium Heparin & DPPIVi (50ul) & Trasylol (150ul)	Cold spin and freeze immediately

2.2.5.4 Principles and description of assays used

The hormonal and metabolite assays were performed at the Biochemistry department in King's College Hospital, London as per their established protocols. All samples were assayed in duplicate.

- Glucose was measured using an automated glucose analyser, the Advia Centaur (Siemens Healthcare Diagnostics, Frimley, UK).
- Insulin was measured using the Advia Centaur (Siemens Healthcare Diagnostics, Frimley, UK). NEFA was measured using an automated analyser, the Prestige 24i (Cosmos Biomedical, Derbyshire, UK).
- Plasma Active GLP-1 (7-36 and 7-37 amide) was measured by ELISA (Linco Research) This assay measures active GLP-1 and detects changes of 2 pM; intra-assay and inter-assay CVs of 6-9% and <1-13%, respectively.
- Total GIP was measured by ELISA (Linco Research, Missouri, USA) with intra-assay and inter-assay CVs of 3-9% and 2-12 %, respectively and detects changes of 8.2pg/ml.
- Total PYY was measured by a previously described radioimmunoassay.[370] The assay detection limit is 10pg/ml and inter-assay and inter-assay CVs were 3-9% and 6-9%, respectively.
- Ghrelin was measured by ELISA (SCETI KK Medical Section, DF Kuasumigaseki Place, Chiyoda-ku, Tokyo, Japan). Intra-assay and inter-assay CVs were 3-9% and 2-12% respectively. CCK was measured by ELISA (Phoenix Pharmaceuticals Inc., CA, USA).
- Total cholesterol, HDL, LDL-cholesterol and triglycerides were measured using an automated analyser, the Advia 2400 (Siemens Medical Solutions Diagnostics Limited, Berkshire, UK), see appendix 2.

2.3 Adipose tissue and adipocytes

2.3.1 Adipose tissue biopsies

2.3.1.1 Extraction technique

At time of RYGB, with the subject under general anaesthetic, the skin was prepared with chlorhexidine or Betadine. Skin incisions were made and 7 laparoscopic ports inserted as per routine practice for laparoscopic RYGB. The subcutaneous adipose tissue biopsies were excised using a scalpel from the 12mm port-sites. 15mmHg CO_2 pneumoperitoneum was then established and a diagnostic laparoscopy performed prior to any GI transection. The visceral adipose tissue biopsy was taken from the greater omentum using either an Endoloop® with Endoshears® or Harmonic® scalpel technique and extracted through the port-site using a bag.

On removal from the participant, AT biopsies were placed into individually labelled 30ml plastic flasks containing either 0.9% Saline or PBS buffer at room temperature immediately.

2.3.1.2 Preparation of AT for isolation

AT biopsy samples were blotted dry, visible stromal tissue and vessels excised then weight recorded. At this time any additional tissue, not required for the purpose of this lipolysis experiments, was snap frozen and stored at -80°C for future research purposes (with ethical permission).

2.3.2 Adipose tissue receptor gene expression analysis

In order to confirm the presence of the relevant gut hormone and fatty acid receptors on human AT, both abdominal subcutaneous and visceral (n=6 for each), I performed gene expression analysis using established techniques from our laboratory. These involved insolation and purification of total RNA, proceeding to reverse transcription and Real-Time Polymerase Chain Reaction (RT-PCR) analysis.

2.3.2.1 Isolation of total RNA

2.3.2.1.1 Reagents

The reagents required for this experiment were:

- Trizol Reagent (Cat No. 15596-026, stored in fridge)
- Chloroform
- Isopropyl alcohol
- 75% ethanol (in distilled water for injection)
- DEPC (diethylpyrocarbonate) water

2.3.2.1.2 Cell lysis

AT samples not required for lipolysis experiments, snap frozen at time of adipocyte isolation, were used in these experiments.

The cells were lysed with 150ul Trizol/1x10^6 cells and the pellet was vortexed. The samples were then incubated for 5min at room temperature to allow the complete dissociation of nucleoprotein complexes.

2.3.2.1.3 Phase separation

I then added 30ul chloroform (200ul per 1ml Trizol reagent used) and shook the tubes vigorously for 15 sec. The tubes were then incubated at room temperature for 2-3min, prior to centrifugation at 12,000 x g for 10min at 2-8˚C. This resulted in separation of the mixture into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase (RNA) in some tubes however some took on a light pink colour in the upper phase. In these tubes, the phase separation was repeated, and a colourless upper phase achieved.

2.3.2.1.4 RNA precipitation

This aqueous phase (the RNA) was then transferred to a new tube however at this time it included approximately 60% of the Trizol reagent used. The RNA was precipitated by mixing with 75ul Isopropyl alcohol (0.5ml per 1ml of Trizol reagent used) and the samples incubated for 10min at room temperature.

2.3.2.1.5 RNA wash

These pellets were centrifuged at 12,000xg for 10min at 2-8˚C and to remove the supernatant, the pellet was washed with 180ul 75% ethanol (1-1.2ml per 1ml Trizol reagent used), vortexed then centrifuged at 7,5000 x g for 5min at 2-8˚C. It was then possible to remove the supernatant and the samples were left to air-dry for 6min (but not completely, as this would decrease its solubility)

2.3.2.1.6 Dissolving the RNA

The pellet was dissolved in ~30ul sterile DEPC-water and incubated for 10min at 55-60˚C, mixed with a brief spin and placed on ice.
At this point the RNA concentration in the samples was checked to ensure the 260/280nm ratio was ≥1.8 prior to storing the lysate at -80˚C.

Normally DNase treatment of the total RNA would be undertaken at this point however the RNA pellets yielded from the above experiments were small volume and it was decided that the potential increased loss of DNA outweighed the minimal benefit of purification.

2.3.2.2 Reverse transcription reaction

The master mix was created first:

Buffer RT	88ul	+2ul
dNTP mix	88ul	+2ul
OligodT primer	44ul	+1ul
RNase inhibitor	11ul	+0.25ul
Omniscript RT	44ul	+1ul

I required 50ng RNA per reaction (therefore 20ul master mix).
2ul cDNA x 3 (triplicate) x 12 genes and as such 72ul was required and 80ul made to allow for pipetting error.

40ul was prepared and aliquoted into 4x 10ul tubes and stored in the -20˚C freezer until use.

2.3.2.3 Qualitative real-time PCR

RT-PCR analysis was performed using Taqman assays for Ins-R, GLP1-R, GIPR, PYY-R (PYYR1), Ghrelin-R (GHS-R1 and NPY1R), GLUT-4 and fatty acid receptors (GPR43, GPR120, FFAR1, FFAR3) from both abdominal subcutaneous and omental human adipose tissue. B-actin was used as the housekeeper gene. Samples were analysed in triplicate.

In the experiment, an endogenous control was used, 18rRNA, and an internal control, no template control (NTC).

The machine was turned on to warm up prior to starting the assay and a new 96-well plate (Abiprism 96-well optical reaction plate, Applied Biosystems 4306737) was used for each experiment.
To minimise error a standardised plate template was used, table 2.3.2.3.

The following mixture was made for each probe:

- 2 x TaqMan Universal Master Mix 80ul
- Sterile dH2O 56ul
- Probe (added last) 8ul

Then each well of the plate had the following mixture added:

- cDNA (or sterile dH2O for NTC) 2ul
- Master mix and probe solution 18ul

The plates were then sealed and centrifuged at 2000rpm for 2min at room temperature.

I had created a template in the Applied Biosystems 7000 Real-Time PCR system for analysis with the wells and relevant probe tasks selected, in advance.
The plates were individually inserted into the machine, 40-50 cycles performed and the data analysed using 7000 System Sequence Detection software v.1.2.3. Applied Biosystems.

2.3.3 Adipocyte isolation

2.3.3.1 Adipocyte isolation technique

The visceral and peripheral AT biopsies were separately minced to approximately 5mg pieces using sharp scissors, to avoid crushing of the cells, and placed in a 1mg/ml Collagenase solution in 50-cc tubes.

Collagenase solution:
Krebs-Ringer Bicarbonate buffer (KRB) – gassed with 95%O2:5%CO2 for 15 min
4%fatty-acid free Bovine Serum Albumin (BSA) (PAA K41-002)
D-glucose (Sigma G7528)
Correct to pH 7.4
200nM Adenosine (Sigma A9251)
Type 1 Collagenase (Sigma C1030)

AT samples were gassed prior to sealing with film and placing in a 37°C water-bath for max. 60 min until the solution had a “soupy” consistency. The solution was then passed through a 210um polypropylene mesh filter (Spectrum Labs 146428) using a cut-off syringe into a 30ml polypropylene flask. The cells were then washed 3 times with BSA buffer to separate the adipocyte fraction from stromal vascular fractions. The volume of fat cell yield was recorded and then diluted with 4% BSA buffer to make an approx. 1/10 solution in a polypropylene Erlenmyer flask. This methodology is described in more detail, see attached paper.[371]

2.3.3.2 Quantification of fat cell diameter

0.1ml fat cell solution was prepared on a microscope slide well, using hydrophobic slide marker (BioGenex Rev. C503). Multiple digital photographs were taken of the images at magnification (x4 & x10). Image J software, a Java-based image processing program developed at the National Institutes

of Health which is in the public domain, http://rsb.info.nih.gov/ij/. It was used to calculate the average diameter of >200 adipocytes.[372]

Using the average cell diameter the mean adipocyte mass was derived as follows:
Adipocyte radius (r) = diameter/2
Mean adipocyte mass = mean adipocyte volume (4/3 Ψr^3) x density (0.915) [assuming that the fat cell is spherical and is composed of mainly TG][373]

2.3.3.3 Quantification of total triglyceride content

The Dole's extraction technique was used to quantify the total triglyceride content in the fat cell solution at time of each lipolysis experiment.[374] I travelled to Prof. Fried's laboratory, Adipocyte Core, Boston Obesity Nutrition Research Center (BONRC) Boston University, USA to train in this and modified the technique to accommodate our laboratory resources:

In advance, I had prepared a standard Dole's solution (800ml Isopropyl alcohol, Sigma I9030; 200ml Heptane, Sigma 246654; 20ml 1N H_2SO_4, Sigma 320501),[357] which was stored in a sealed glass jar, in the locked, flammable liquids cupboard at room temperature for use in these experiments.

At time of each lipolysis experiment, 0.5ml fat cell solution was placed into 4 15cc polyethylene conical centrifuge tubes with caps for Dole's extraction. A combination of Dole's solution, dH20 and Heptane were added to each tube, vortexed well to mix the phases then left to sit in the refrigerator overnight. Then the volume of upper phase was noted (should be 4 ml). 2ml of the upper phase was aliquoted into labelled and weighed 6 tared foil planchettes (2 controls) and left in the fume hood to evaporate overnight. All planchettes were then reweigh to calculate weight of lipid. 2 control planchettes were always used, to ensure the scale was accurate.

In order to calculate mg lipid/ml of fat cells the following calculation was used: divide by volume of upper phase placed in the planchette and multiply by volume of upper phase. Then divide by the ml of fat cells suspension added. e.g. for 2 ml aliquot of 4ml upper phase, lipid weight x 2/0.5ml cells (dilution factor = 4) => mg lipid/ml fat cell suspension

2.3.3.4 Calculation of adipocyte cell number

The adipocyte cell number was extrapolated using the TG volume in the fat cell suspension and the mean cell diameter as per methodology described by Di Giralomo.[375]

As the adipocyte is predominantly composed of TG, the number of adipocytes per ml can be approximated by dividing the total TG in 1ml suspension by mean adipocyte mass.

As discussed earlier, the mean adipocyte mass was calculated as follows: Mean adipocyte mass = mean adipocyte volume ($4/3\ \Psi r^3$) x density (0.915) [assuming that the fat cell is spherical and is composed of mainly TG][373]

Since 50ul volume was used for the glycerol assay, the number of adipocytes/ml was then divided by 20 to give the number of adipocytes per the 50ul.

This allowed the glycerol concentration data to be presented as glycerol release/2hr stimulation (nmol/10^{-5} cells/10^5 adipocytes).

Table 2.3.2.3 RT-PCR standardised plate template

Visceral	**Peripheral**	**Visceral**			**Peripheral**			
18S	18S	GPR43			GPR43			
Ins-R	Ins-R	GPR120			GPR120			
GLP1-R	GLP1-R	FFAR1			FFAR1			
GIPR	GIPR	FFAR3			FFAR3			
GLUT4	GLUT4				18S	Ins-R	GLP1-R	**NTC**
PYY1R	PYYR1				GIPR	GLUT4	PYY1R	
NPY1	NPY1				NPY1	GHSR	GPR43	
GHSR	GHSR				GPR120	FFAR1	FFAR3	

2.3.4 Adipocyte incubation

The AT weight and fat cell yield were recorded for each experiment. The initial adipocyte protocol had included centrifugation of the sample in-between washes. However, it was noted that significant volumes of fat cells were lost during each spin (cell rupture). After discussion with the team at BONRC, modifications were made and subsequently published[371] including:

1. A fine polypropylene tube was inserted through the fat cell layer into the supernatant. The sample was allowed to separate naturally on the bench (~2min). The tube was the used to aspirate the supernatant with minimal disturbance to the fat cells above.
2. I ensured that the Bovine serum albumin (BSA) buffer wash was poured onto the side-wall of the flask, not directly onto the fat cells in order to minimise trauma. The polypropylene tube could then be used to mix the solution prior to re-aspiration, this process was repeated 3 times.

2.3.4.1 Adipocyte incubation technique

1.5ml polypropylene microcentrifuge tubes were pre-labelled and prepared with the freshly prepared volumes with or without pre-requisite experimental solutions as per protocol.

Immediately after the ~1/10 cell solution was prepared, continual fractal mixing of the cell solution was undertaken whilst 0.5ml cell solution was pipetted into each microcentrifuge tube. The filled capped tubes were then placed into a 37°C water-bath for 120min. At the end of this time the samples were placed on ice whilst the infranatant (i.e. without cells) was aspirated into pre-labelled micro-centrifuge tubes. I standardised this process to include a separate aspiration of a fixed volume 200ul sample from these tubes at this time, in preparation for first glycerol assay, however this could be left until a later time.

All samples were stored at -80°C until analysis.

2.3.4.2 Lipolytic experimental conditions

To ensure the results were robust and reproducible a number of internal controls were placed into this experimental protocol as follows.

2.3.4.2.1 Basal

The baseline group of experimental conditions for each experiment are shown in table 2.3.4.2.

2.3.4.2.1.1 Adenosine deaminase

Cell lysis occurs on removing AT from the adenosine rich environment of the body. By including 200nM exogenous adenosine in the buffer this cell lysis is greatly reduced.[376] Adenosine deaminase (ADA) is added to all experimental conditions to remove the adenosine (Ado) [both endogenous and exogenous] and results in rapid elevation in lipolysis. With standard adipocyte experimental conditions 0.5units/ml ADA converted all of the Ado to inosine within 5s, measured using [^{3}H]Ado.[376] This “basal state” was designed for the testing of anti-lipolytic agents by quantifying their inhibition of lipolysis stimulated by ADA.[377]

2.3.4.2.1.2 *N^{6}-[R-(-)*-1-methyl-2-phen-ethyl]adenosine

N^{6}-[R-(-)-1-methyl-2-phen-ethyl]adenosine (PIA) is an adenosine deaminase-resistant Ado receptor agonist which prevents the ADA-induced rise in activity through receptor-mediated inhibition of adenylate cyclase.[376]

As such a fixed concentration of 20nM PIA was used for tests of lipolytic agents. This baseline condition was designed to reveal any stimulation in lipolysis.

2.3.4.3 Stimulated

2.3.4.3.1 Isoproterenol

Isoproterenol (ISO) is a β-adrenergic agonist is a potent stimulator of lipolysis, capable of maximally stimulating lipolysis in the supra-maximal inhibitory condition of PIA, 100nM.[377] As such a basic stimulated control

comparator condition of these experiments included a ADA & PIA20 & ISO condition – maximal lipolytic stimulation.

It was used in the basic experimental run as a comparator for two reasons: to ensure the fat cells in this experiment are capable of stimulation; as an indicator of the maximal stimulation achievable with these cells.

2.3.4.3.2 8-Bromo-cAMP

8-Bromo-cAMP is a stimulator of lipolysis through the non-receptor mitigated cAMP pathway. This substance stimulates lipolysis in a controlled reproducible concentration dependent manner.[378] As such it can be used to partially stimulate a completely inhibited condition (e.g. ADA & PIA) in order to reveal small-order of change stimulations of lipolysis, which may otherwise be masked by the ADA alone or strong inhibitory effect of PIA.

2.3.4.4 Inhibited

2.3.4.3.1 Insulin

Insulin is a potent anti-lipolytic agent which inhibits lipolysis through cAMP-related pathways[379-381] and cAMP-unrelated mechanisms.[382] A basic inhibited control comparator condition of these experiments included dose-response insulin curves (basal and stimulated), to ensure that the fat cell solution was capable of responding in such a manner. Concentrations comparable to human in-vivo plasma insulin concentrations and sub- and supra-physiological levels were used, table 2.3.4.3.

Table 2.3.4.2 Lipolysis experimental control conditions

Tube no	Anti-lipolytic state	Tube no	Lipolytic state	Tube no	Partially stimulated lipolytic state
	Basic (30)				
1-2	**ADA**	11-12	**ADA & PIA_{20}**	15-16	**ADA & PIA_{20} & 1mM 8-Br-cAMP**
3-4	" & Ins_{10}			17-18	" & Ins_{10}
5-6	" & Ins_{100}			19-20	" & Ins_{100}
7-8	" & Ins_{500}			21-22	" & Ins_{500}
9-10	" &Ins_{1000}			23-24	" & Ins_{1000}
		13-14	" & ISO (10^{-6})		
25-26	0' time				
27-28	Doles	29-30	Doles		

2.3.4.5 Gut hormones

The primary objective of this experiment was to quantify the lipolytic response of human adipocytes to gut hormone changes around RYGB. As such the experiment was designed to include: GLP-1, GIP, PYY and ghrelin hormonal conditions at 3 different concentrations, consistent basal and peak levels around RYGB surgery. These levels were calculated from the relevant gut hormone levels in fasting and post-prandial states pre- and post-RYGB in this study, table 2.3.4.4.

Each of these gut hormone conditions were studied with adipocytes both basally (ADA alone) and stimulated (ADA & PIA20 & 1mM 8-Bromo-cAMP - completely inhibited with mild stimulation).

In addition, several different concentrations of GIP were studied with ADA and ADA/PIA20/1mM 8-Bromo-cAMP (for a GIP dose-response curve, because there was no fresh human adipocyte lipolysis data available in literature previously for comparison).

2.3.4.5.1 Incretins +/- insulin

The combined effects of incretins and insulin upon lipolysis were studied using conditions of: 3 concentrations of GIP and GLP-1 combined with 3 concentrations of insulin.

Table 2.3.4.4 Experimental gut hormone concentration calculations

Gut hormone	Mw	Highest and lowest concentrations	Pg/ml	Pmol/l	Ideal experimental range	Conc. in M	Actual exp conc. (M)
GLP-1 (7-36)	3297.63	pM Fasting – 2-5 PP – 10-36	n/a	5 10 50	1 10 50 100	1×10^{-12} 1×10^{-11} 5×10^{-11} 1×10^{-10}	$1x\ 10^{-7}$ $1x\ 10^{-9}$ $1x\ 10^{-12}$
GIP	4983.6	Pg/ml Fasting – 50-95 PP – 273-577	50 100 250 500 1000	10 20 50 100 200	10 100 500	1×10^{-11} $1x\ 10^{-10}$ $5x\ 10^{-10}$	$1x\ 10^{-7}$ $1x\ 10^{-9}$ $1x\ 10^{-12}$
PYY	4310.1	Pg/ml Fasting – 64-107 PP – 91-307	50 100 250 500	12 23 58 116	10 50 100	1×10^{-11} 5×10^{-11} 1×10^{-10}	$1x\ 10^{-7}$ $1x\ 10^{-9}$ $1x\ 10^{-12}$
Ghrelin	3370.2	Pg/ml Fasting – 356-550 PP – 417 - 620	300 400 500 750	89 119 148 223	100 500 1000	1×10^{-10} 5×10^{-10} 1×10^{-9}	$1x\ 10^{-7}$ $1x\ 10^{-9}$ $1x\ 10^{-12}$
Insulin	N/A	mU/L Fasting - 8-35 PP – 75-117	n/a	120 750	1 100 500 1000	$1x10^{-12}$ 1×10^{-10} $5x\ 10^{-10}$ $1x\ 10^{-9}$	$1x\ 10^{-9}$ $5x\ 10^{-10}$ $1x\ 10^{-10}$ $1x\ 10^{-11}$

Conversion of pg/ml to pmol/l; Pg/ml divided by the Mw of substance to convert pg to pmol

2.3.5 Infranatant analysis

2.3.5.1 Glycerol assay

This protocol was modified from the BONRC General Glycerol Assay – unabridged (CH Glycerol Protocol 03/06), adapted for microplate fluorometer.[383]

Reagents needed on the day of experiment:

- Glycerol Kinase (GK): Sigma G0774
- α-Glycerol Phosphate Dehydrogenase (GPDH) Type 1: Sigma G6751
- Hydrazine Hydrate: Aldrich Hydrazine Monohydrate, 98% 207942
- Glycine Buffer (GB) – frozen stock prepared ahead of time
- Imidazole-KCl-KOH – stock prepared ahead of time and stored for 1 month at 4°C
- β-Nicotimamide adenine-dinucleotide (NAD^+): Sigma N6522 minimum 98% from yeast – frozen stock prepared ahead of time
- Ice cold 20% Perchloric acid (PCA): Sigma 77233

The microplate fluorimeter used was Varioskan Flash 4.00.51 with Excitation:350 Emission:466

2.3.5.2 Principles of the glycerol assay

The glycerol assay used is a one-step fluorometric method[384] based upon a reversible reaction, figure 2.3.5.2.

Above pH 8.5, in the presence of excess NAD, the reaction proceeds to the right. To ensure complete conversion of glycerol to dihydroxyacetone the equilibrium can be driven further to the right by the addition of the hydrazine (ketone-trapping) which irreversibly binds to dihydroxyacetone:

Dihydroxyacetone + hydrazine => dihydroxyacetone-hydrazone

The NADH is then measured spectrophometrically or fluorometrically.[383.384]

2.3.5.3 BSA precipitation

Bovine Serum Albumin (BSA) was present in the fat cell suspension (and therefore the adipocyte incubation's infranatant). As it contains lipids, it was essential that the BSA be extracted prior to performing the glycerol assay to minimise contamination.

To control for potential variability with this process, I made the glycerol standards using the same 4% BSA buffer used for the adipocyte isolation in advance (see reagent preparation table). This meant that the glycerol standards had to undergo BSA precipitation at the same time as the samples.

This was performed as follows:

- Samples (including a set of glycerol standards (all concentrations) & plain 4%BSA and KRB buffer control tubes) were thawed and arranged in order. The appropriate volume of ice cold 20%PCA was added to each sample tube and vortexed briefly (for 200ul samples, 30ul of PCA; 1ml samples, 150ul of PCA)
- Samples were then rested on ice for 20min
- To neutralize this extraction Imidazole-KCl-KOH solution was then added. The volume used was usually identical to volume of PCA however a few samples were checked to ensure ~pH7.4-8 was achieved prior to adding this to all samples (if too low, the volume of Imidazole solution was adjusted accordingly by 1ul at a time), amount used was recorded
- Samples were then vortexed for ~20s followed by centrifugation for 10min at 4°C, 13,200rpm

2.3.5.4 Glycerol assay technique

The Reaction Mix was prepared fresh on the day of experiment. Approx. 5ml reaction mix was required for a 1x96-well plate experiment. As such 50ml reaction mix was required for the standard 9 plate experiment.

To make 10ml Reaction mix I combined in order:

- 10ml GB
- 26ul GPDH
- 30.6ul GK
- 115ul Hydrazine Hydrate

A plate diagramme was prepared in advance. This standardised system was used for pipetting of each sample (including standards and KRB blanks) into wells.
The final volume was brought to 50ul using the extracted KRB from the blank i.e. to dilute sample by half, add 25ul sample & 25ul extracted KRB.
These dilutions were determined in some instance to aim for fluorescence values in the mid-range of the standard curve (to improve accuracy of derived glycerol results).
Reverse pipetting was used to avoid air bubbles.

50ul of room temperature reaction mix was added to all wells and plates were mixed on a shaking rotor plate for 5-10min at 200rpm.
The plates were read in the microfluorimeter (excitation: 350 emission: 466). This was the 0' time reading.
3ul NAD^+ was then added to each well. It was imperative that NAD^+ was added directly to the surface of the liquid at the bottom of the well, not stuck to the plastic on the side-wall. The plates were shaken on a rotor plate for 10min @ 200rpm.

The plates were then read on the fluorimeter and results reviewed:

- To ensure readings in samples are within range of standards and dilutions used are appropriate
- If samples were out of range or appeared to give unexpected/abnormal results (do to possible human error), new replicates were prepared with desired dilutions
- Ensure emission of samples has increased from 0'time readings. If not, they may not have received NAD^+ and more can be added at this

time – adding extra NAD cannot effect outcome as 3ul should already be driving the reaction to completion.

Plates were then shaken for a further 20min then re-read at the 30min (total) time point.

Plate readings were cut and paste into a spreadsheet (Excel) and GraphPad PRISM software used for line (curve) fitting to extrapolate glycerol concentrations in each well.

2.3.5.5 Glycerol assay data analysis

Plate reading results transferred from the microfluorimeter were cut and pasted as follows:

Each plate's t=30 and t=0 readings with subsequent "t=30" – "t=0" were organised accordingly. The δ1-3 for the glycerol standard curve were extracted first (Plate 1: rows 1-3, A-H) and arranged next to their known glycerol standard concentrations. These samples were then corrected for 0 and the average corrected glycerol standard values were acquired. Each other result was arranged next to its relevant tube no. and conditions and corrected for blank tube readings.

This data was entered into GraphPad PRISM with glycerol standard results as x-values with their corresponding mean y-values. All triplicate tube data was entered as y-values below this and analyses as both linear and non-linear exponential one-phase decay curves. An R2>0.95 was required in order for the data to be reliable. The graph of the assay was saved with the assay results.

The most accurate results were achieved with non-linear exponential one-phase decay curves as expected. These derived x-values were pasted into the Excel spreadsheet as glycerol (ul/0.5ml) 1-3.

The dilution factor for the wells was entered into the corresponding rows laterally and the derived glycerol values were multiplied by the dilution factor accordingly.

At this time the average of the 0'time triplicates (i.e. volume of glycerol in the 0.5ml cell solution should no incubation be undertaken) was subtracted from all values.

Abnormal triplicate results were removed at this time and mean per tube and condition was calculated.
The glycerol conc. (pmol/50ul) was then multiplied by “factor for 10^5 cells” to give the glycerol (pmol/10^5 adipocytes).
The data was converted to nmol by x1000 and presented as glycerol release (nmol/10^5cells/2hr), 2hr being the duration of the experimental incubation.

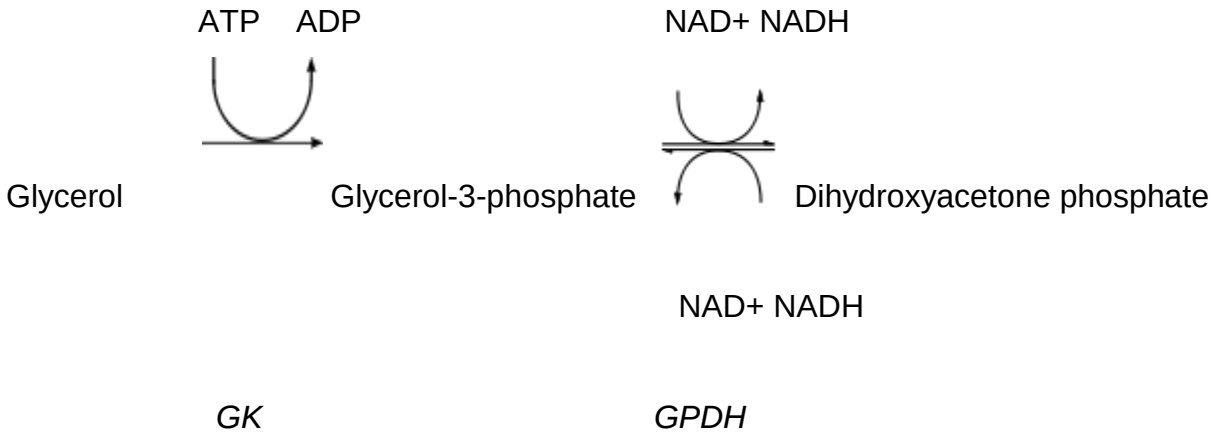


Figure 2.3.5.2 Diagram of the glycerol assay reaction

2.4 Statistical calculations

2.4.1 Sample size calculations

Following the observed differences in incretin levels before and after RYGB surgery in experiment 1 (Table 1[36]), the clinical portion of this study was designed to have power 80% to detect differences in mean GLP-1, GIP, insulin, for which 4 cases are required in each group.

In order to allow for the laboratory portion of the study, following the observed differences in glycerol release at different concentrations of GLP-1 (Figure 2[345]), the laboratory experiments were designed to have power 95% to detect differences in lipolysis rates between pre- and post-RYGB incretin levels of 64 (standard deviation 24), 5 subjects are required. However, in order to detect differences in glycerol release between the visceral and peripheral fatty tissue in these experiments if we follow the observed differences in glycerol release at basal lipolysis from omental and subcutaneous fat (Table 2[278] the lipolytic experiments were designed to have a power 80% to detect differences in glycerol release of 3.6 (standard deviation 3), for which 12 subjects will be required.

In order to calculate the sample size required to quantify the difference in lipolysis from both fatty tissue areas in patients with and without T2DM and IR: following the observed differences in glycerol release in response to basal and insulin responsiveness (Table 2[278]), the experiments were designed to have power 80% to detect differences in lipolysis rates of 4.2 (standard deviation 2.85), for which 8 subjects were required in each group.

For the GIP dose-response sub-study, following the observed differences in glycerol release at different GIP doses in rat adipocytes (Table 1[120]) this sub-study was designed to have power 80% to detect differences of 0.10 (standard deviation 0.09), for which 4 experiments in each condition would be required.

Following the observed differences in GLUT4 receptor difference between subcutaneous and omental adipocytes,[328] we design this study to have power 80% to detect differences in GLP-1 receptor protein expression levels between subcutaneous and omental adipocytes of 200%OD (standard deviation 120%), 6 samples are required per group.

For the laboratory studies:

- Gut hormone gene expression experiments (n=6)
- plasma and gut hormone experiments (n=5)
- visceral and peripheral adipocyte comparison experiments (n=12)
- NDM and T2DM comparison experiments (n=8)
- GIP sub-study experiments (n=4)

Targets for work

- A statistical difference of $p<0.05$ in the lipolytic effects of the pre- and the post-RYGB incretin concentrations
- A statistical difference of $p<0.05$ in the lipolytic effects of incretins upon the fatty tissue from visceral and peripheral regions
- No statistical difference in the lipolytic effect of incretins in patients with and without T2DM

2.4.2 Statistical analysis

Data are expressed as mean +/-sd, unless otherwise specified. Statistical significance was considered if $p<0.05$. Statistical analyses were performed using GraphPad Prism® (GraphPad, San Diego, CA, USA).

2.4.2.1 Participant outcomes

Unpaired t-test was used to detect changes in participant demographics between the NDM and T2DM group.

2.4.2.2 Plasma outcomes

2.4.2.2.1 Changes in plasma hormones and metabolites around RYGB surgery

ANOVA with repeated measures was used to detect hormonal changes over time during the FMC study before and after RYGB in participants with and without T2DM. Paired *t* tests were used to compare data before and after RYGB surgery. The unpaired *t* test was used to compare NDM and T2DM group data. Mann-Whitney test was used to analyse the RYGB effect data.

2.4.2.2.1.1 Fasting

Plasma and serum hormonal and metabolite outcomes were assessed before and after RYGB, in the fasting state. This data was analysed using several different methods. Initially the complete data set (i.e. NDM and T2DM groups) was analysed to identify any potential effects of RYGB surgery using the largest sample size available. Then fasting levels were compared across both groups, pre- and post-RYGB. The effect of RYGB upon fasting hormonal and metabolite outcomes was calculated by subtracting the pre- from post-RYGB levels i.e. delta. The order of effect of RYGB upon fasting levels was compared between the NDM and T2DM groups.

2.4.2.2.1.2 Post-prandial

Hormonal and metabolite response to FMC stimuli was assessed in using several different measures. Crudely, the data was presented as diagrammatic form plotting mean FMC data at each time point. The peak response to food stimuli was analysed. In addition, the time to return to baseline for glucose was assessed. Early phase insulin secretory response was assessed using delta 0-15min insulin levels.[385]

Measurement of the hormonal response to the FMC stimulus was performed using AUC 0-180min for all outcome variables, calculated using the trapezoidal rule (total AUC, tAUC), which is strongly correlated with baseline levels.[386] However as baseline levels are variable post-RYGB, to minimise bias to operative outcome analyses, when meal-stimulated increases were

expected (glucose, insulin, GLP-1, GIP, PYY, CCK) the incremental AUC 0-180min (area above baseline), was calculated, which is strongly correlated with hormone changes to foods.[386] When meal-stimulated responses decrease or no responses were expected (NEFA, Ghrelin), tAUC was used.

2.4.2.2.2 Calculation of the effect of RYGB upon the hormonal and metabolite responses

Comparison of the different hormonal and metabolite responses to RYGB surgery was calculated using a modification of the incretin effect calculation,[387] whereby the difference in AUC post-RYGB was subtracted from AUC pre-RYGB, divided by the AUC pre-RYGB (as the denominator), then multiplied by 100 to give the ratio as a percentage.

$$\frac{\text{(Post-RYGB AUC – Pre-RYGB AUC)}}{\text{Pre-RYGB AUC}} * 100$$

2.4.2.3 Adipocyte outcomes

2.4.2.3.1 Adipocyte cell diameter

Data are expressed as means +/-sem. Differences in adipocyte cell diameter between the adipose tissue depots were directly compared for each participant using paired t-test.

2.4.2.3.2 Lipolysis and anti-lipolysis rates

Data are expressed as means +/-sem. The effects of plasma and different gut hormone conditions were determined by analysis of variance with repeated measures and post-hoc t-tests when main effects of interactions were significant.

2.5 Study management

2.5.1 Study duration

The total duration of the study was expected to be 18 months (with 200 pts undergoing bariatric surgery in our unit in 2010 and estimated 15% expected to agree with a sample size of 45). The expected duration of patient participation was 10 weeks (waiting list for operation 10 weeks).
Study enrolment commenced on 01/04/2009 and participation activities completed by 06/09/2010. The participant duration was exact.

2.5.2 Study involvement

Of the 20 participants recruited into this study, NDM (n=10) and T2DM (n=10), some unanticipated difficulties were encountered:

- Drop-out (5%)
- Declining operation (5%)
- Difficulty obtaining definitive intravenous access for venous sampling resulting in incomplete postprandial data (5%)
- Exogenous insulin contamination, plasma data excluded (5%)

Despite this, we achieved the sample size required in line with the plasma and gut hormone outcomes but not for the adipocyte NDM and T2DM comparator experiment study design. The human adipocyte lipolysis experiment sample size calculations had been derived from the best available data on human changes in lipolysis based upon insulin. As such, I undertook interim analysis following complete recruitment, to ensure statistical significance had been achieved with the primary outcomes prior to study completion.

3. The early effects of RYGB upon glycaemic control and insulin resistance

3.1 Introduction

IR is defined at a cellular level by the requirement for increasing insulin levels to enable glucose transport into cells via the GLUT protein family.[388-390] The IR state progresses towards T2DM when the islet cells are unable to secrete sufficient insulin to prevent hyperglycaemia. T2DM first becomes apparent with postprandial hyperglycaemia. Fasting hyperglycaemia occurs when inadequate insulin secretion results in incomplete suppression of hepatic glucose production and decreased efficiency of liver and peripheral glucose uptake.[76] Islet cells in these individuals contain insulin and maintain the ability to synthesise insulin but can no longer be stimulated to do so.[7]

Hyperglycaemia triggers diabetic tissue damage through repeated acute changes in cellular metabolism and cumulative long-term changes in stable macromolecules, affecting cells which are unable to internally regulate their glucose concentration.[391-392] Particular types of cells affected include retinal capillary endothelial cells, renal glomerular mesangial cells and neurons and Schwann cells in the peripheral nerves.[393] IR increases NEFA flux from adipocytes into arterial endothelial cells.[394] This increases FFA oxidation by the mitochondria in macrovascular endothelial cells, resulting in overproduction of ROS through β-oxidation of fatty acids and oxidation of FFA-derived acetyl CoA by the TCA cycle, generating NADH and FADH2. These are also the same electron donors generated by glucose oxidation in microvascular endothelial cells subjected to hyperglycaemia.[393] It has been suggested that inhibition of FFA release from adipocytes or FFA oxidation in arterial endothelium will prevent the increased production of ROS and its damaging effects.[393]

Gastric surgery improves T2DM[18] and RYGB has become an established treatment for T2DM.[26,395-397] It alters glycaemic control through several mechanisms discussed previously, including: dietary intake, taste, transit time, sugar and fat absorption, bile acid mixing, gut flora, gut hormone alterations, insulin secretion, insulin resistance, fasting and stress effects, alteration in NEFA processing and weight loss (see Section 1.2.3). Longer-term studies have shown that RYGB result in a remission of T2DM rather than a cure, with an increase incidence after 10 years.[12,356]

This study was designed to study the early effects of RYGB surgery upon insulin resistance and glycaemic control.

3.2 Methods

3.2.1 Clinical methods

The methodology of this study is detailed in Chapter 2. Briefly, instructions were given to study participants regarding cessation of oral intake and current medication around the time of the study to minimise their impact upon outcomes. Venous blood was sampled following a 10h fast and at fixed time points after drinking a 420 k-cal mixed meal test (MMT), both before and four days after RYGB. The MMT constituents were (% kcal): 30.1 CHO, 23.3 fat and 5.0 protein (see Appendix 1).

These blood samples were placed in pre-primed sampling tubes (as per Table 2.4.1.1), centrifuged and stored at -20˚C until analysis.

3.2.2 Assays

Automated assays were used to measure glucose and insulin (Advia Centaur, Siemens Healthcare Diagnostics, Frimley, UK).[398] This was performed at the Biochemistry Department of King's College Hospital, London. See section 2.2.5.4 and appendix 2.

3.2.3 Statistical analysis

Outcome variables were plasma glucose and serum insulin concentrations. Assessment of response to MMT stimuli was performed using AUC 0-360' for all outcome variables. As meal-stimulated increases in glucose and insulin were expected, this was calculated as area above baseline (incremental area, iAUC), using the trapezoidal method.[386] ANOVA with repeated measures was used to compare glucose and insulin changes over time during the MMT study before and after RYGB in participants with and without T2DM. Paired *t* tests were used to compare data around RYGB surgery. Comparison of the glucose and insulin responses to RYGB surgery were calculated using a modification of the incretin effect calculation,[387] known as the RYGB effect calculation, see section 2. Mann-Whitney test was used to analyse the RYGB effect data. For the time to return to baseline plasma glucose levels calculations, some assumptions were made. Where 15min

values were lower than the 0min values (presumed due to laboratory error) the 0 time result was taken as the result for the baseline assessment. If the curve did not return to baseline within the 360min test then for the purposes of data analysis it was presumed that the curves returned to baseline by 360 min.

Data are expressed as mean +/- sd. Statistical significance was considered $p<0.05$. Statistical analyses were performed using GraphPad Prism® (GraphPad, San Diego, CA, USA). For further details regarding the statistical analyses see section 2.4.

3.3 Results

Eighteen participants underwent LRYGB in this study. As previously discussed, fasting data was available for analysis for 17 participants (10 NDM and 7 T2DM) and post-prandial data for 16 participants (9 NDM and 7 T2DM). No difference in weight was detected in either group by POD 4.

3.3.1 The early effects of RYGB surgery upon fasting plasma glucose and serum insulin levels

The early effects of RYGB surgery upon fasting plasma glucose and serum insulin and HOMA-IR(1) levels have been summarised, table 3.3.1.

3.3.1.1 Glucose

Analysis of the whole cohort revealed a reduction in fasting plasma glucose (FPG) levels by post-operative day 4 ($p=0.0231$). Analysis of the group data revealed a reduction in FPG levels between the groups ($p=0.0007$), figure 3.3.1.1. This reduction was present in the NDM group ($p=0.0041$) but despite a reduction from 10.16+/-2.01 to 7.66 +/-0.76 mmol/l in the T2DM group it did not reach significance ($p=0.1055$).

There was no difference in the direction of change of FPG levels between the NDM and T2DM groups ($p=0.1219$).

3.3.1.2 Insulin

In the whole cohort, there was a reduction in fasting serum insulin (FSI) levels by POD4 ($p<0.0001$). Analysis of the group data revealed a reduction in FSI levels between the groups ($p=0.0017$), with a reduction in both groups: NDM ($p=0.0034$) and T2DM group ($p=0.0166$), figure 3.3.1.2.

There was no difference in the direction of change of FSI levels between the NDM and T2DM groups was detected ($p=0.8095$).

3.3.1.3 HOMA-IR(1)

Analysis of the whole cohort showed a reduction in HOMA-IR by POD4 ($p=0.0002$). Analysis of the group data revealed a difference in HOMA-IR

between the groups ($p<0.0001$), with a reduction in both the NDM ($p=0.0022$) and the T2DM group ($p=0.0074$), figure 3.3.1.3i.

There was a difference in the direction of change of HOMA-IR(1) levels between the NDM and T2DM groups ($p=0.0286$), figure 3.3.1.3ii.

Table 3.3.1 Early effects of RYGB surgery upon fasting glucose, insulin and HOMA-IR(1) levels

	Combined pre-op (n=17)	**Combined post-op (n=17)**	**NDM pre-op (n=10)**	**NDM post-op (n=10)**	**T2DM pre-op (n=7)**	**T2DM post-op (n=7)**	**ANOVA**
Glucose (mmol/L)	7.329 +/- 4.078	5.894 +/- 2.000 *	5.350 +/- 0.4577	4.660 +/- 0.5719 **	10.16 +/- 5.307	7.657 +/- 2.004 ns	***
Serum insulin (mU/L)	31.73 +/- 20.19	9.609 +/- 4.480 ****	29.52 +/- 20.40	8.283 +/- 3.720 **	34.89 +/- 21.06	11.50 +/- 5.063 *	**
HOMA-IR(1)	10.86 +/- 8.177	2.591 +/- 1.735 ***	6.996 +/- 4.656	1.729 +/- 0.8135 **	16.37 +/- 9.243	3.823 +/- 2.006 **	****

Data are presented as mean and sd. Data analysis: paired t-test and ANOVA for analysis across the four groups.

*= p<0.05; ** = p<0.01; ***=p<0.001; ****=p<0.0001; ns = non-sign

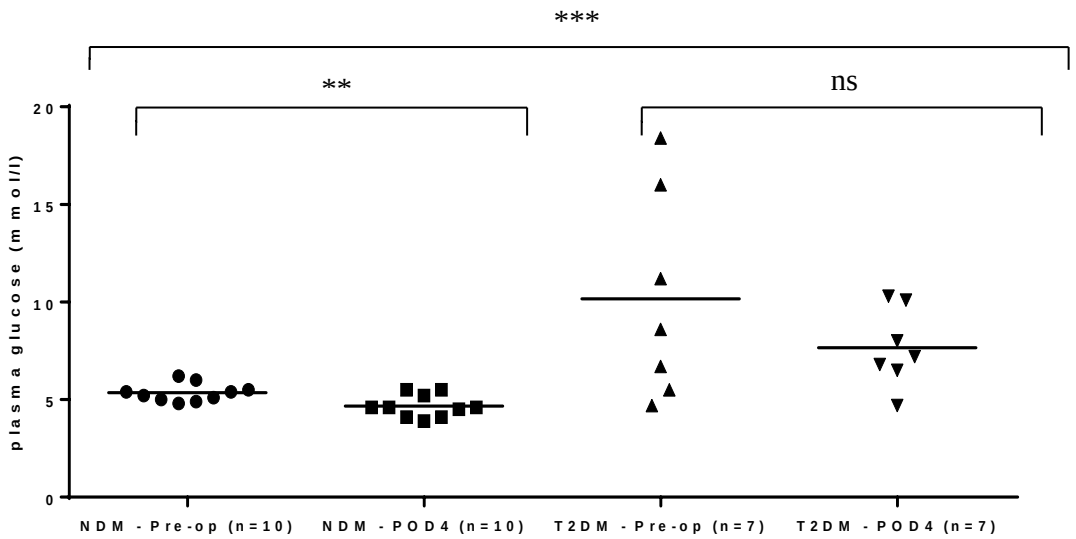


Figure 3.3.1.1. Scatter plot of the early effect of RYGB surgery upon fasting plasma glucose in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; ns = non-significant.

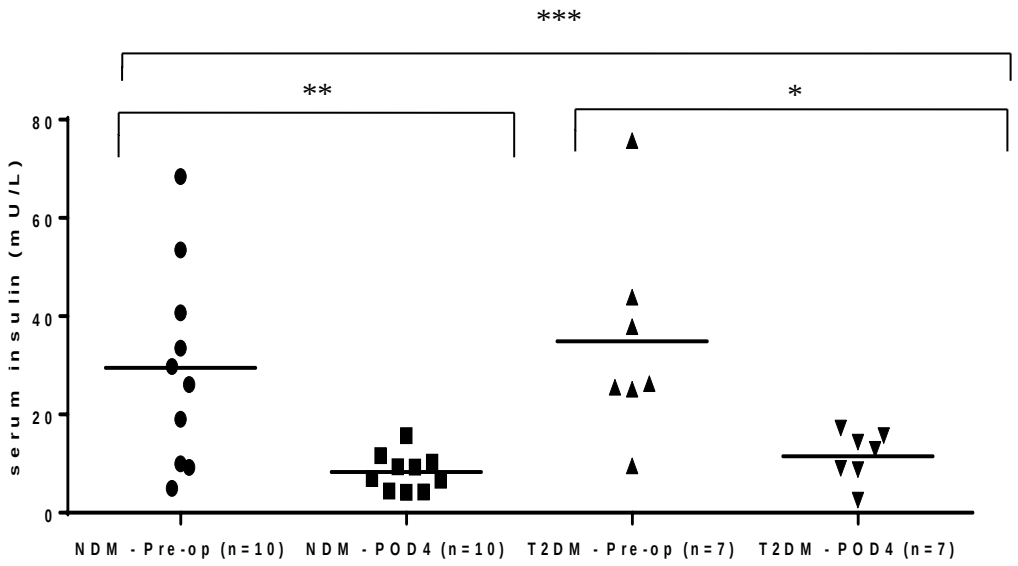


Figure 3.3.1.2 Scatter plot of the early effect of RYGB surgery upon fasting serum insulin in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; ns = non-significant.

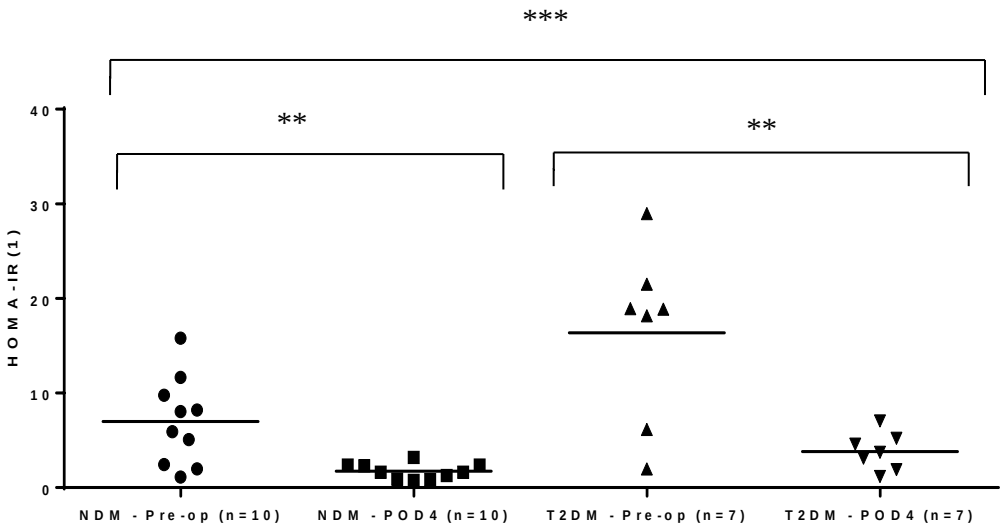


Figure 3.3.1.3i Scatter plot of the early effects of RYGB surgery upon HOMA-IR(1) in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; ns = non-significant.

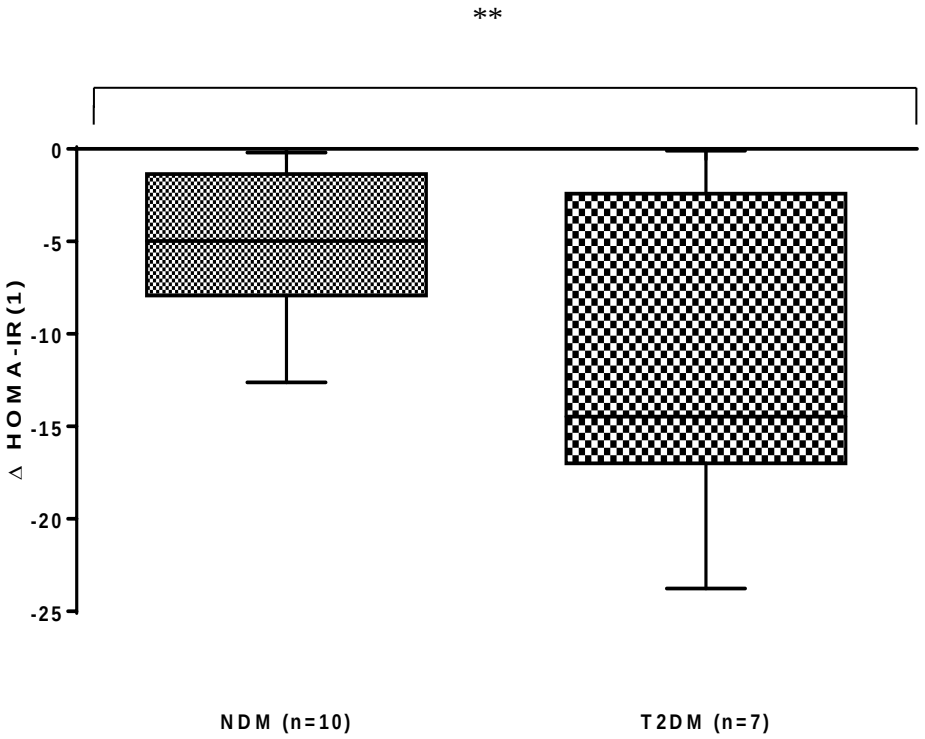


Figure 3.3.1.3ii. Box plot of delta HOMA-IR(1) pre-and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Unpaired t-test was used for comparison, **=p<0.01.

3.3.2 The early effect of RYGB surgery upon post-prandial plasma glucose and serum insulin levels

3.3.2.1 Glucose

The post-prandial effect of RYGB surgery upon glucose levels are shown in figure 3.3.2.1i.

The peak post-prandial glucose levels were different between the groups ($p=0.0008$) being higher in the T2DM group both before and after surgery, $p=0.0146$ and $p<0.0001$ respectively. However, there was no difference around RYGB surgery in either group, NDM $p=0.6287$ and T2DM $p=0.4297$. The post-prandial glucose excursion was different between the groups ($p=0.0019$) but no difference around RYGB surgery was detected in either the NDM ($p=0.4627$) or the T2DM group ($p=0.9422$), figure 3.3.2.1ii. There was no difference in the RYGB effect upon post-prandial glucose levels between the two groups ($p=0.3955$).

A significant difference in time to return to baseline glucose levels existed between the 4 groups, $p=0.0185$, being quicker before RYGB surgery in both groups, NDM ($p=0.0060$) and T2DM group ($p=0.1193$), figure 3.3.2.1iii. There was no difference in the direction of change of time for postprandial plasma glucose levels to return to baseline around RYGB surgery, $p=0.1357$. This supports the theory that the prolongation of the time to return to baseline is more likely to be as a result of surgery than T2DM status.

3.3.2.2 Insulin

The post-prandial effect of RYGB surgery upon insulin levels are shown in figure
3.3.2.2i.

There was no difference in peak post-prandial insulin levels between the groups ($p=0.1965$). Delta 0-15min post-prandial insulin levels is a surrogate indicator of the first phase response of oral stimulation to insulin secretion.

There was no difference in delta 0–15min insulin levels between the groups (p=0.2862), supportive of the theory that improvements in post-prandial glycaemic control following RYGB surgery are not predominantly due to improvements in insulin secretion, figure 3.3.2.2ii.

There was no difference in the postprandial insulin secretion around RYGB surgery between the groups (p=0.6714), figure 3.3.2.2iii. There was no difference in the effect of RYGB surgery upon post-prandial insulin secretion between the two groups (p=0.7292).

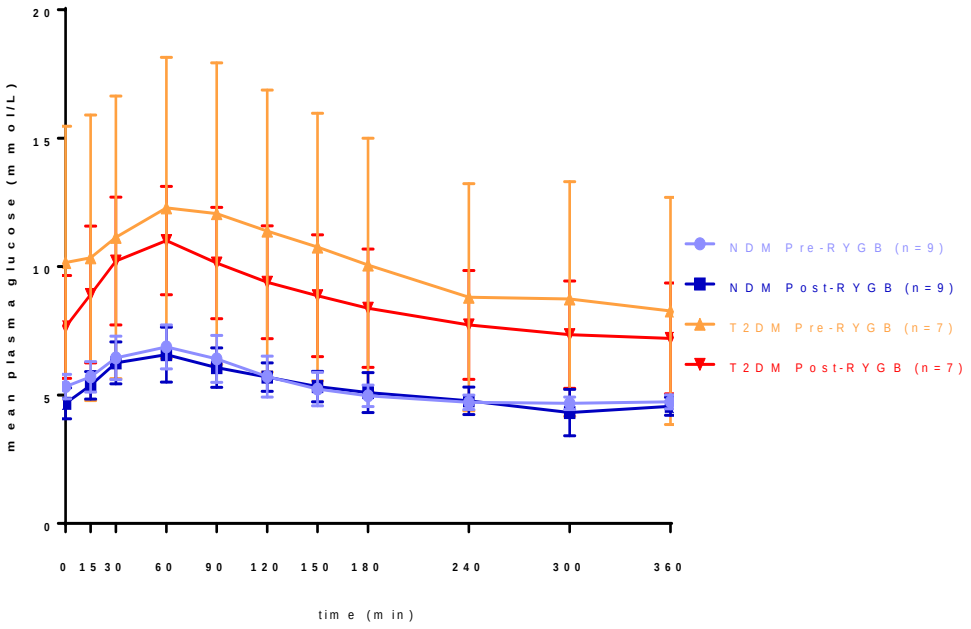


Figure 3.3.2.1i Graph of the post-prandial plasma glucose levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

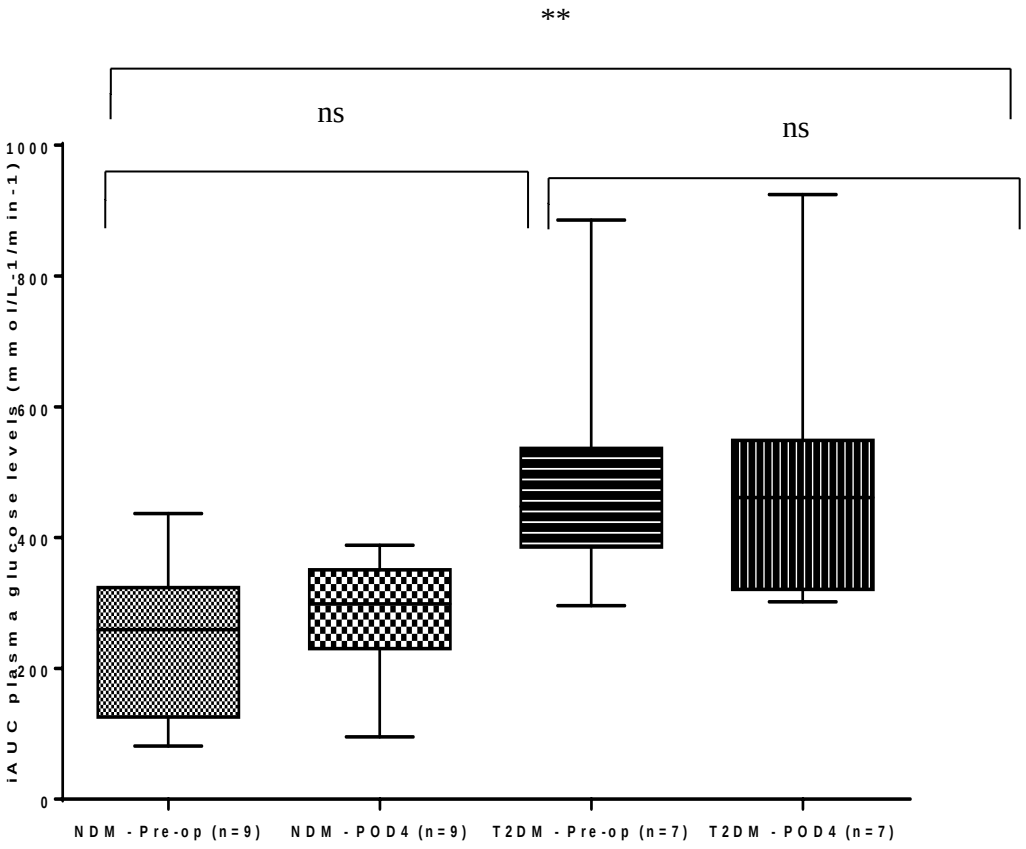


Figure 3.3.2.1ii Box plot of iAUC post-prandial plasma glucose levels after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Paired t-test was used for pre- and post-operative and ANOVA for all group comparison. **=p<0.01; ns= non-significant.

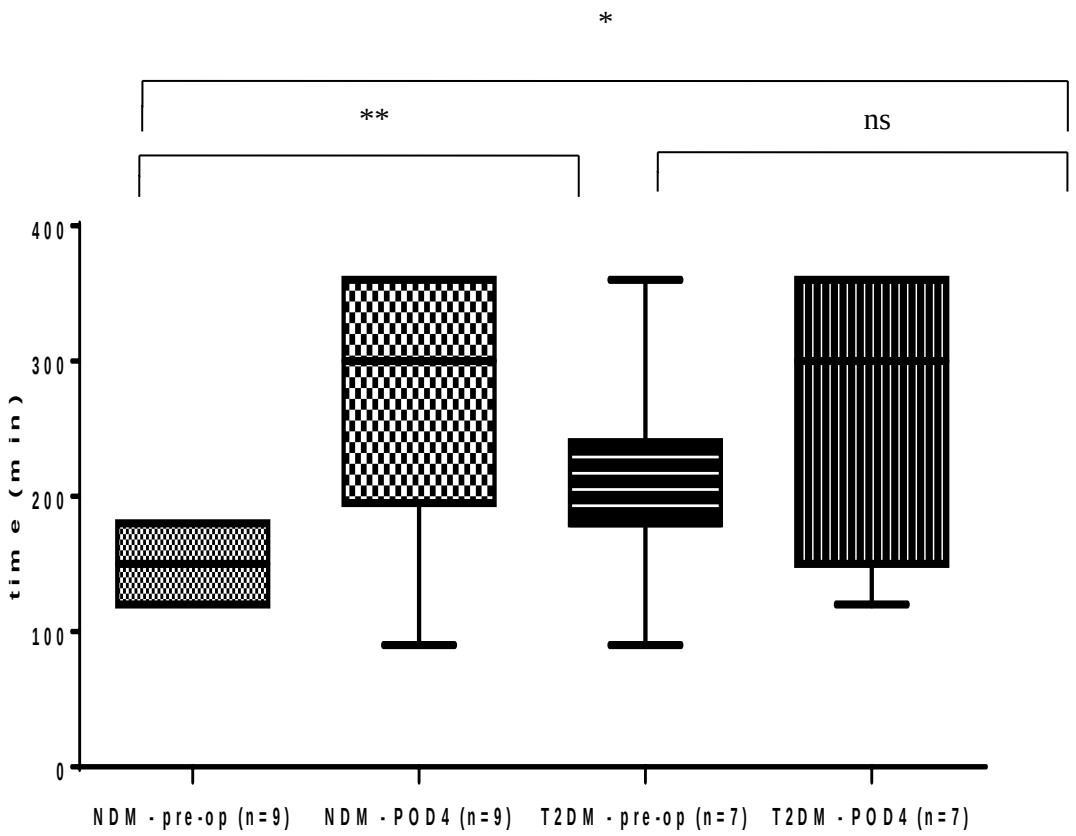


Figure 3.3.2.1iii Box plot of time for plasma glucose levels to return to baseline following mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Paired t-test was used for pre- and post-operative and ANOVA for all group comparison. *= p<0.05; **=p<0.01; ns= non-significant.

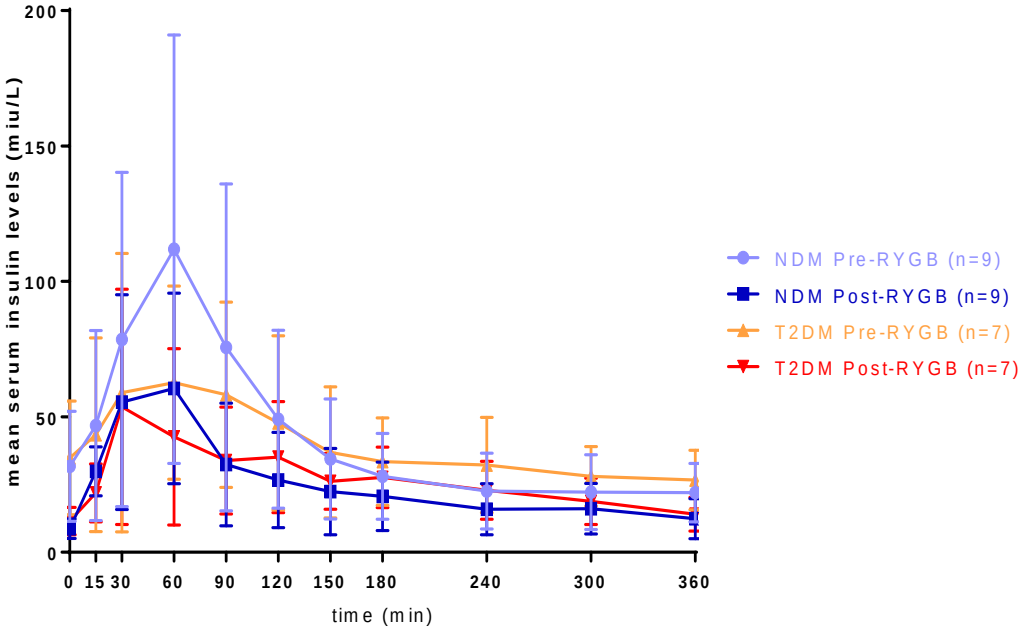


Figure 3.3.2.2i Graph of the post-prandial serum insulin levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM].
Data is plotted as mean with bars representing sd.

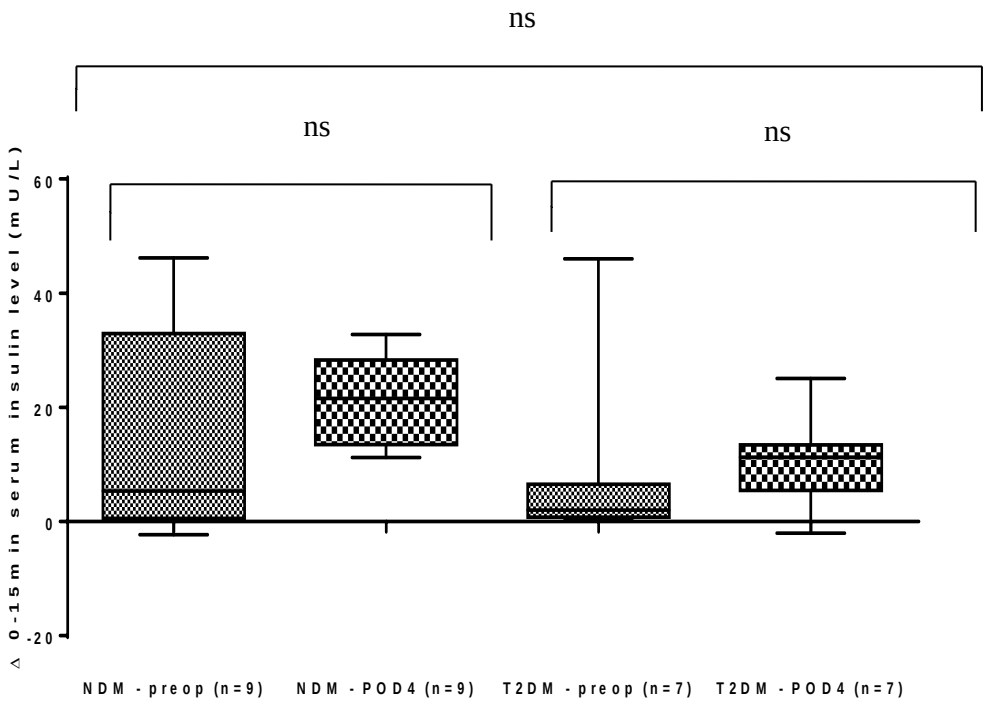


Figure 3.3.2.2ii Box plot delta 0-15min post-prandial serum insulin levels after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Paired t-test and ANOVA were used for analysis, ns= non-signficant.

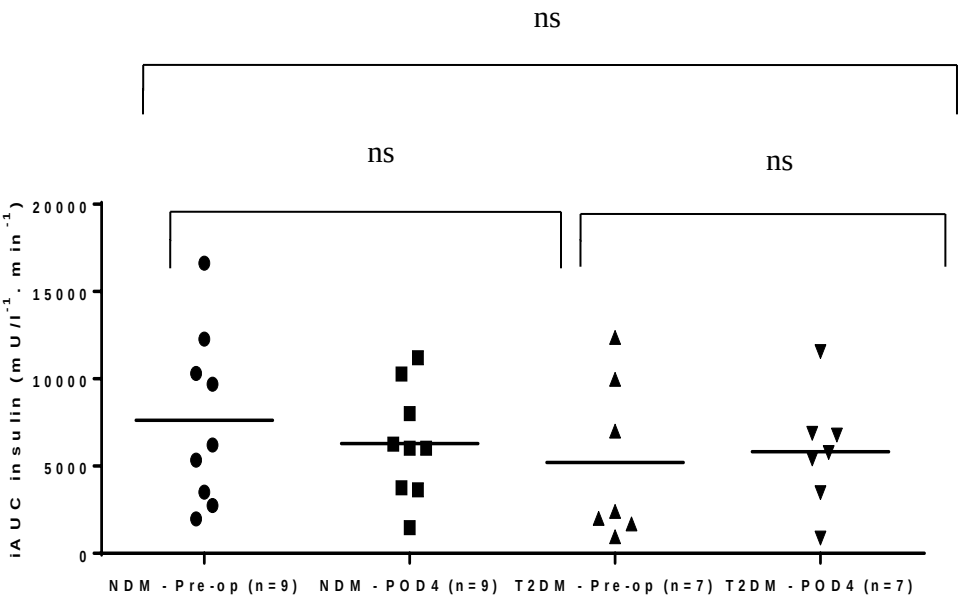


Figure 3.3.2.2iii Scatter plot of iAUC post-prandial serum insulin levels after a mixed meal test, pre- and post-operative day 4 around RYGB in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups; ns= non-significant.

3.4 Discussion

Obesity and T2DM are associated with insulin resistance.[399] As IR develops, insulin release increases to accommodate for the reduced efficiency of insulin action.[6,400,401] When the B-cells are unable to fully compensate for this, T2DM (that is hyperglycaemia) develops.[7]

This process is closely linked with elevated NEFA levels, present in obesity and T2DM.[77,78,402] Increased NEFA levels can induce IR, impair insulin secretion and reduce insulin biosynthesis.[76,92,93,403] This is likely to occur because intracellular NEFA competes with glucose substrate oxidation, augmenting pyruvate dehydrogenase, phosphofructokinase and hexokinase II activity.[404] In turn, fatty acid metabolite concentration increases, reducing activation of PI(3)K[405] and diminishing events downstream of insulin-receptor signalling.[76]

RYGB results in remission of T2DM and most patients are able to discontinue diabetes-related medications.[9,12,18] There is considerable variability in the criteria and methods employed to diagnose T2DM remission following weight loss surgery. Standardisation of clinical and physiological outcomes after surgery for T2DM have not been established.[406]

In this chapter, I have looked at the impact of RYGB on fasted and post-prandial glucose and insulin levels in both diabetic and non-diabetic states. The WHO diagnostic criteria for T2DM are: fasting plasma glucose ≥7.0 mmol/l **or** 2hr plasma glucose* ≥ 11.1 mmol/l; IGT: fasting plasma glucose < 7.0 mmol/l **and** 2hr plasma glucose* ≥ 7.8 and < 11.1 mmol/l; IFG: fasting plasma glucose 6.1 - 6.9 mmol/l **and (if measured)** 2hr plasma glucose* < 7.8 mmol/l; NDM: fasting plasma glucose ≤ 6.0 mmol/l. * venous plasma glucose 2hr after ingestion of 75g oral glucose load.[356] As such, in order to confirm remission of T2DM, FPG and 2h oral glucose tolerance test (OGTT) measurements are appropriate.[385,407] Impairment in insulin secretion is more relevant in IFG, while deterioration in insulin sensitivity is more important to

IGT.[408] However there are concerns regarding the validity of the 2h OGTT post-RYGB.[409]

Although the effect of RYGB upon transit time remains controversial,[66,101,410-412] it does not appear to result in sugar malabsorption.[66] Irrespective of this, faster enteric absorption occurs,[413] supported by higher post-prandial peak glucose concentration[414] and a quicker return to baseline level.[233]

The OGTT study assesses the ability of the subject to optimally process dietary glucose. It reflects the first phase of insulin secretion, inhibiting hepatic glucose production with a poor or inappropriate first phase of insulin secretion being associated with unsuppressed postprandial glucose production, leading to subsequent higher postprandial glycaemia.[415] As such, an abnormal first phase insulin secretion is associated with increased risk of T2DM,[416-418] making the OGTT the most effective test for the early diagnosis of T2DM.[419]
Impaired β-cell function can alter post-prandial plasma glucose excursion, with the time to return to baseline being an important measurement.[420] A 2hr OGTT can establish normal glucose tolerance (NGT), IGT or diagnose previously unrecognised T2DM. It can also establish whether an IFG subject has normal 2hr PG. This is an important finding, as the majority of IFG subjects with NGT have a better prognosis, suggesting their B-cell function is more efficient or better preserved.[421,422] Although 1hr PG has a better predictive power to diagnose future diabetes than either FPG or 2hPG,[422] its variability is greater. Hence 2h plasma glucose is more reproducible and accurate as a standard diagnostic test.

In this study a MMT (with 30.1% CHO) rather than 75g glucose load was used. The time to return to baseline plasma glucose was longer post-RYGB. Although this finding conflicts with other studies,[233] it does question the validity of 2h OGTT as an accurate method of assessing glucose tolerance post-RYGB. This issue of standardisation and accuracy is compounded by variation in the constituents of the MMT. Some studies used 50g glucose load, instead of 75g, citing concerns regarding dumping syndrome for this

variation.[21,38,264] The reduction of the glucose load, in an attempt to avoid dumping symptoms in this patient group, is unnecessary and has augmented the confusion surrounding remission of T2DM post-RYGB.[423] MMT is the optimal method of assessing post-prandial gut hormone secretion and should not be used for T2DM assessment.[385] All T2DM participants in this study underwent formal T2DM assessment on POD7 using FPG and 2hr OGTT with 75g glucose load for clinical management purposes (mean FPG 8.73+/-4.92 mmol/L, 2hOGTT 12.78+/-4.32 mmol/L (n=6); four patients' T2DM status persisted as FPG≥7.0 mmol/l and two patients had IGT but, would have been classified as NDM without OGTT). Further studies using the standard 75g glucose load around RYGB in patients with and without T2DM are required to validate this test.

A less sensitive measure of glycaemic control is HbA1c. A value ≥ 6.5% is diagnostic of diabetes while 5.7-6.4% should be considered high risk for future development.[424] A high HbA1c value suggests the presence of fasting hyperglycaemia,[425] whereas whilst borderline levels are associated with only post-prandial hyperglycaemia. There is no clear target HbA1c level after RYGB to define T2DM remission.[385,407,426] A 2hOGTT should also be performed.[421] Medically-treated T2DM patients undergoing targeted treatment to reduce their HbA1c have been found to have higher mortality rates.[427] Although of interest, the clinical relevance of HbA1c in this context is still unclear.

Despite the lack of standardisation in diagnosis of T2DM after RYGB, a number of comparative studies have shown that bariatric surgery (RYGB or sleeve gastrectomy) leads to improved glycaemic control and cardiovascular risk factors, compared with best medical therapy alone.[428-430] Factors predicting remission of T2DM following bariatric surgery include greater weight loss and shorter history of diabetes following restrictive surgery,[19] lower BMI and fasting C-peptide levels post-RYGB.[431]

Relapse of T2DM does occur.[12,432] Risk of relapse is increased by weight regain[433] and reduced in patients with a shorter pre-surgery diabetes

duration.[434] Patients should be counselled accordingly. They should be told clearly that remission of T2DM following RYGB may not be permanent, but bariatric surgery is associated with fewer micro- and macrovascular complications than non-operative management.[434]

The most widely reported assessment of T2DM around RYGB in FPG (see table).[18,21,26,38,385,396,407,426] FPG is an important measurement in the diagnosis of T2DM,[356] and both FPG and fasting serum insulin (FSI) reflect insulin resistance.[360] RYGB reduces FPG and FSI in both the short- and long-term.[435] I report similar findings by POD4 in this study.

This study shows improved insulin sensitivity by POD4, assessed using HOMA-IR, in participants with and without T2DM. This reflects improved hepatic insulin sensitivity.[436] These findings are consistent with other studies, both within the first week post-RYGB[385] and over a longer timeframe.[11,20,95,385] Other groups have corroborated these results using hyperglycaemic clamp studies.[437] When combined with isotope tracer studies, these investigations show that reduction in hepatic glucose production rather than improvements in peripheral insulin sensitivity (predominantly skeletal muscle) are responsible for remission of T2DM.[438-440] The results of hyperinsulinaemic-euglycaemic clamp studies with tracer technique have also reached similar conclusions, showing an improvement in hepatic IR and also improved AT IR, although beta cell function is largely unchanged early post-RYGB.[441]

Caloric restriction alone could explain these findings,[27,442-445] as comparable results are reported following sleeve gastrectomy.[446] Taken together with studies showing improvements post-AGB[28,385] and improvement in IR post-RYGB disproportionate to diet at equivalent weight loss in T2DM,[447] it is likely that other factors are responsible. Longer-term improvements in IR are associated with weight loss and improvements in peripheral insulin sensitivity are detected latterly.[439,441] Changes in plasma TG levels (Chapter 4), and altered lipid processing are also implicated.[88,448] A reduction in hepatic but

not muscle lipid content has been reported post-RYGB in rodents,[449] suggesting that reduced lipotoxicity may have an effect.

The rate of hepatic glucose production and hepatic insulin sensitivity is inversely proportional to intrahepatic lipid content[450-453] and both reduced energy intake and moderate weight loss can affect this.[454-456] Conversely, high NEFA levels lead to insulin resistance[88,402] and their reduction post-RYGB may improve IR and insulin secretion.[76]

Post-prandial glucose excursions are a reflection of total carbohydrate intake, glucose absorption, glucose processing and insulin secretion.[457,458] Following RYGB, dietary sugar intake is reduced,[459] although there are no changes in monosaccharide absorption.[66] Studies are not consistent regarding potential alteration in transit time post-RYGB.[66,101,410-412] In addition, the early post-operative dietary restrictions have been shown to improve glucose tolerance levels in T2DM, potentially affecting these results.[27,442]

As anticipated, peak glucose levels were higher in participants with T2DM than NDM, with no difference in the effect of RYGB between the two groups. There were higher iAUC glucose levels in T2DM subjects, but no difference in post-prandial glucose excursions were detected in either group around RYGB surgery. Other authors have reported a reduction in post-prandial glucose levels after RYGB surgery.[21] Diet-equivalent weight loss does not lower postprandial glucose levels to the same extent in patients with T2DM.[38] This disparity could be due to variations in meal challenge and data analysis (tAUC/iAUC). In addition, there was no difference between the two groups, supporting the assertion that there is no improvement in insulin secretion early post-RYGB.

Post-prandial insulin levels are a reflection of glucose absorption rate,[460] beta-cell response and reserve,[461-464] and incretin responses.[465,466] Enhanced insulin response and exaggerated incretin secretion alone may improve the response of beta-cells to changes in plasma glucose,[21,437,439,467] with improvements occurring early post-RYGB.[468,469]

This study reports no difference in post-prandial insulin secretion around RYGB surgery using peak insulin, iAUC insulin or the first phase insulin response, approximated using Δ0-15min insulin, corroborating other studies.[266] This differs from current dogma regarding gut hormone stimulation of insulin secretion post-RYGB. Theories of remission of T2DM post-RYGB hinge on improvements in IR and increased incretin secretion, due to the response of the distal small bowel to nutrients. This results in increased incretin stimulation (the hindgut theory),[54,59-61] and subsequent insulin secretion. Increased post-prandial incretin secretion following RYGB has been shown[20,21] and may be a potential mediator of improved insulin secretion.[21] Other studies have reported increased insulin production post-RYGB (Δ0-15m),[385] which is different to this study.

Although an increased incretin effect upon post-prandial insulin has been shown one month post-RYGB in T2DM patients,[21] the same study reported lower postprandial insulin levels post-RYGB. There was no difference in tAUC insulin one month after RYGB,[21,38] corroborating my results. Hyperglycaemic clamp studies in RYGB patients have been used to assess insulin secretion.[470] They have shown no change in the first phase insulin concentration after surgery and that the second phase insulin concentration is reduced by 40% at one and four weeks post-RYGB.[428,437,471]

These data are not easy to understand. Insulin secretion is contingent upon insulin sensitivity and the requirement to impr ove intra-cellular glucose transport. Therefore, as insulin resistance has decreased by POD4, a larger volume of insulin secretion is no longer required to improve hyperglycaemia. Although much interest surrounds the reversal of the blunted incretin effect of T2DM following RYGB as the potential mediator of T2DM remission,[21] it is most likely the improvements in insulin resistance, rather than increased incretin and tailored insulin secretion which are predominantly responsible for remission of T2DM after RYGB.

There are other potential hypotheses to explain the remission of T2DM post RYGB. Reduction in oral intake of sugar either due to alteration in preferences,[472] changes in brain-reward response,[473,474] dumping avoidance[475] or simply reduction in caloric intake can all contribute to improved beta cell function and hepatic insulin sensitivity.[476]

Gut realignment is also very important. The entero-insular axis is designed to stimulate islets to secrete insulin in response to gut stimuli, via endocrine transmission, neuro-transmission and substrate stimulation.[477] In T2DM, there is a reduced GIP response to oral glucose,[478] which is not reversed by increased exogenous GIP.[176] The development of glucose intolerance has been linked to a defect in GIP signalling pathways, reducing the expression of GIPR and attenuating the effect of GIP, in T2DM.[479,480] Post-RYGB, nutrients reach the distal gut causing increased post-prandial GLP-1 and GIP levels[20,21,23,385] which could be a potential mediator of improved insulin secretion (the hindgut theory).[481] Although these changes occur prior to and independent of weight loss,[38,385] diet-induced weight loss can increase GLP-1 levels.[167]

Bypassing the proximal intestine directly ameliorates T2DM, independent of the effects on food intake, body weight, malabsorption, or nutrient delivery to the hindgut.[53] This was evident in diabetic patients undergoing gastrectomy and duodenal exclusion for oncological or emergency indications.[18,54,55,482-484] When these operations are performed in NDM subjects, impaired glucose tolerance can still occur[482,485-487] suggesting it disrupts the physiologic entero-insular axis, suggesting that some degree of duodenal-jejunal dysfunction may be associated with T2DM.[53]

Additional support for the foregut theory are that studies involving a duodenal-jejunal plastic sleeve insertion, in effect creating a duodenal bypass, have shown improvements in glucose tolerance in patients with T2DM.[62,488-490] These findings were not independent of GLP-1 changes,[489] which supports the hypothesis that reduced caloric intake is not the primary mechanism of diabetes control.[53] Although RYGB alters fasting and post-

prandial gut hormone changes prior to weight loss,[20,23,57] this is not the dominant mechanism of remission of T2DM, rather that improvements in IR predominate.

Pancreatic β-cell function improves early after RYGB.[468,469] Changes in NEFA levels may be responsible for this. An acute negative energy balance study showed that improvements in hepatic insulin sensitivity and increased in beta cell function were associated with decreased pancreatic and liver TG concentrations, by releasing the beta cells from the chronic inhibitory effects of excess fatty acid exposure.[476]

Insulin resistance in adipose tissue is characterised by excess lipolysis, increased NEFA levels. This occurs despite the presence of hyperinsulinaemia, with impaired suppression of plasma NEFA levels.[402,491-493] In healthy NDM subjects, elevated plasma NEFA causes hepatic and skeletal muscle IR.[402,491-495] This may explain why pathologically increased rates of lipid turnover precede the development of T2DM in subjects with a family history of T2DM[496-498] or non-diabetic obesity.[499,500]

Chronic exposure to NEFA is associated with marked impairments in glucose-stimulated insulin secretion and decreased insulin biosynthesis.[92,93,501-505] Acute increases in NEFA levels can contribute to insulin resistance and impair compensatory β-cell response. This is consistent with the changes seen in obesity-related T2DM,[403] with prolonged elevation in NEFA impairing β-cell function.[506]

NEFA changes after RYGB are discussed in more detail in Chapter 4 and 5. The increased NEFA levels early post-RYGB are consistent with improved lipolysis arising from enhanced adipocyte insulin sensitivity in presence of large volume of fat mass.[95] This effect is reduced after weight loss.[507] Differences in fasting and post-prandial NEFA and TG following RYGB have been reported, possibly due to low inhibition of lipolysis due to lower insulin levels.[95]

Ultimately, the main aim for treatment of T2DM is reduction of microvascular and macrovascular complications and mortality. Intensive treatment of diabetes can decrease the development and/or progression of microvascular and macrovascular complications associated with the disease.[29,508] Although therapy was focussed on lowering HbA1c levels,[509] persistent elevation in postprandial blood glucose levels remains the primary pathophysiological problem.[510] Isolated reduction in HbA1c is associated with increased mortality. The goal of treatment should be prevention of complications, using a multi-faceted approach including hypertension control, lipid reduction and glycaemic control.[427]

Reassuringly end organ damage associated with diabetes can be halted or reversed. For example, after pancreas transplantation, diabetic neuropathy can be halted,[511] diabetic nephropathy reversed[512] but diabetic retinopathy is neither reversed nor progression halted.[513] Bariatric surgery leads to a reduction in all-cause mortality, and cardiovascular deaths, including a reduction in cardiovascular events in the pre-surgery T2DM group.[514-516] RYGB is associated with cessation of the development of microvascular complications such as nephropathy[517] and reduced micro- and macrovascular complications of T2DM.[434,517]

Obesity is associated with a chronic inflammatory state. There is extensive literature supporting this, including the finding of increased CRP in obese individuals.[519] The finding of microparticles in obese subjects may promote the expression of tissue factor-mediated athero-thrombotic vascular injury.[520-522] The reduction in T2DM-related end-organ damage might be linked to a reduction in microparticles after bariatric surgery in T2DM patients, with reduced inflammation.[523] Reduction in microparticle concentrations (endothelial and tissue factor) occurs after bariatric surgery in patients with T2DM and is associated with falling HbA1c levels.[523]

Although the majority of T2DM patients undergoing RYGB develop improved glycaemic control, substantial aberrations in B-cell function and/or insulin sensitivity remain.[447,524] Gut adaptation post-RYGB leads to increased GLP-2

and mucosal crypt cell proliferation, which may limit any malabsorptive effect of RYGB over the long-term, accounting for eventual weight regain.[272] The chance of relapse of T2DM, along with the potential for progression of end-organ damage, means that diabetic patients should continue to be followed up and managed actively, irrespective of the degree of T2DM remission or improvement.

The strengths of this study are that it is a prospective paired analysis, in participants with a similar degree of weight loss and includes separate sub-group analysis of subjects with and without T2DM. This study also has limitations. The sample size was small with gender and ethnic diversity. Subjects were in a negative energy balance in the early post-operative period and these results may not fully reflect what happens once a stable weight is reached. Both physical activity and pre-operative diet were not strictly controlled which can also introduce further biases.[525]

In summary, RYGB significantly improves glycaemic control and can result in improvements in micro- and macrovascular complications of T2DM. Remission of T2DM, as defined by 2h OGTT, occurs before any significant weight loss. In the first week after RYGB, there is a reduction in both fasting glucose and insulin levels, indicating that IR improves quickly after surgery. However, there was no change in postprandial glucose or insulin levels after RYGB. There was still a prolongation in the time required for glucose levels to return to baseline. Taken together with the wide variation in published methodology used to assess diabetic status after RYGB, highlights that our understanding of the true prevalence of T2DM remission is still unclear. Further multi-centre large scale studies using a standardised framework of diagnostic tests for T2DM are required.

3.5 Conclusion

By postoperative day 4, there is no effect upon postprandial glucose control in subjects with T2DM after RYGB. However, surgery improves insulin resistance and tailors insulin secretion.

The diagnosis of T2DM relies upon FPG and 2h OGTT results. Although currently there is no evidence to support the augmentation of the diagnostic criteria of T2DM in the post-RYGB cohort further investigation is likely to prove otherwise.

4. The effect of RYGB surgery upon gut hormones

4.1 Introduction

It is widely believed that gut hormone changes around RYGB surgery result in, or are at least a major contributor to, remission of T2DM. These gut hormones elicit their effects through taste,[526] hunger and satiety,[20] food intake and choice,[527] gut transit time,[528] gut microflora symbiosis,[529,530] and bile acid circulation.[73,166,530,531] More recently, it has been recognised that their effect upon insulin secretion and subsequent augmentation of glycaemic control and fat cell breakdown may be at least as important.

Incretins are gut peptides which stimulate insulin secretion in response to orally-ingested nutrients, in the presence of glucose.[106] GLP-1 and GIP are the main incretins, responsible for 50-60% of post-prandial insulin secretion.[107] RYGB surgery alters incretin responses. The hindgut theory suggests that expedited delivery of undigested food to the distal intestine exaggerates GLP-1 secretion from the intestinal L cells, improving glycaemic control.[54,59-61] However, animal models suggest duodenal exclusion is required in addition to gastro-jejunostomy to improve glucose tolerance, the foregut theory.[53]

Although gut hormone responses to food ingestion around RYGB have been widely studied, there is a paucity of data regarding them in patients without T2DM. This prospective study was designed to quantify the gut hormone response to food stimuli before and after RYGB, prior to weight loss, and compare these effects in participants with and without T2DM.

4.2 Methods

4.2.1 Clinical methods

The methodology of this study is detailed in chapter 2. In summary, venous blood was sampled following a 10hr fast and at fixed time points after drinking a 420 k-cal mixed meal test (MMT), constituents % kcal from carbohydrate, fat and protein were 30.1, 23.3 and 5.0 respectively (see appendix 1), both before and four days following RYGB surgery.
These blood samples were placed in pre-primed sampling tubes (as per table 2.4.1.1), centrifuged and stored at -20˚C until analysis.

4.2.2 Assays

All hormonal and metabolites assays were assayed in duplicate, this was performed at the Biochemistry department of Kings College Hospital, London. ELISA was used to measure active GLP-1 (7-36 and 7-37 amide) and total GIP (Linco Research, Missouri, USA), Ghrelin (SCETI KK Medical Section, DF Kuasumigaseki Place, Chiyoda-ku, Tokyo, Japan) and CCK (Phoenix Pharmaceuticals Inc., CA, USA). Total PYY was measured by Radioimmunoassay (RIA) (Linco Research, Missouri, USA), see section 2.2.5.4 and appendix 2.

4.2.3 Statistical analysis

Outcome variables were plasma GLP-1, GIP, PYY, Ghrelin and CCK concentrations. Assessment of hormonal responses to the MMT stimuli was performed using AUC 0-180' for all outcome variables, calculated as total area (tAUC) and the area above baseline (incremental area, iAUC), using the trapezoidal method.[386] iAUC was used when meal-stimulated increases were expected (GLP-1, GIP, PYY, CCK) and tAUC when meal-stimulated responses decrease or no responses were expected (ghrelin). ANOVA with repeated measures was used to compare hormonal changes over time during the MMT study before and after RYGB in participants with and without T2DM.

Paired *t* tests were used to compare data around RYGB surgery. Comparison of the different gut hormone responses to RYGB surgery (the RYGB effect) were calculated using a modification of the incretin effect calculation,[387] whereby the difference in AUC post-RYGB was subtracted from AUC pre-RYGB, divided by the AUC pre-RYGB (as the denominator) the multiplied by 100 to give the ratio as a percentage, using iAUC or tAUC as per above.

$$\frac{\text{(Post-RYGB AUC – Pre-RYGB AUC)}}{\text{Pre-RYGB AUC}} * 100$$

Mann-Whitney test was used to analyse the RYGB effect data. Data are expressed as mean +/-sd. Statistical significance was considered $p<0.05$. Statistical analyses were performed using GraphPad Prism® (GraphPad, San Diego, CA, USA). For further details regarding the statistical analyses see section 2.4.

4.3 Results

4.3.1 The early effects of RYGB surgery upon fasting plasma gut hormone levels

Participants were grouped according to T2DM status: NDM (n=10) and T2DM (n=9). Participant characteristics were similar between the groups, see section 2.2.4 for further details.

The early effects of RYGB surgery upon fasting plasma gut hormone levels have been summarised, table 4.3.1.

4.3.1.1 GLP-1

The total participant group data were analysed first, revealing no difference in fasting plasma GLP-1 levels by post-operative day (POD) 4 (p=0.6300). Analysis of the group data revealed no difference in fasting GLP-1 levels between the groups (p=0.1659), figure 4.3.1.1. There was no difference in the direction of change of fasting GLP-1 levels between the NDM and T2DM groups (p=0.6860).

4.3.1.2 GIP

The total participant group data revealed a difference in fasting plasma GIP levels by POD 4 (p=0.0079) with a reduction from mean 82.74 +/- 38.44 to 55.86 +/- 20.52 pg/ml.

There was a significant difference in fasting GIP levels between the groups (p=0.0416) but this did not reach significance in either group: NDM mean 74.74 +/- 40.64 to 49.64 +/- 15.63 (p=0.0558) and T2DM mean 94.17 +/- 34.68 to 64.76 +/- 24.48 (p=0.0978), figure 4.3.1.2. There was no difference in the direction of change of fasting GIP levels between the NDM and T2DM groups (p=0.7107).

Table 4.3.1 Early effects of RYGB surgery upon fasting plasma gut hormone levels

Plasma gut hormones	**Combined pre-op (n=17)**	**Combined post-op (n=17)**	**NDM pre-op (n=10)**	**NDM post-op (n=10)**	**T2DM pre-op (n=7)**	**T2DM post-op (n=7)**	**ANOVA**
GLP-1 (pM)	3.780 +/- 3.187	3.472 +/- 2.038 ns	4.822 +/- 3.777	4.050 +/- 2.450 ns	2.291 +/- 1.136	2.647 +/- 0.851 ns	ns
GIP (pg/ml)	82.74 +/- 38.44	55.86 +/- 20.52 **	74.74 +/- 40.64	49.64 +/- 15.63 ns	94.17 +/- 34.68	64.76 +/- 24.48 ns	*
PYY (pg/ml)	85.74 +/- 28.40	78.20 +/- 34.61 ns	71.05 +/- 16.62	63.79 +/- 10.82 ns	106.7 +/- 29.38	98.79 +/- 46.67 ns	**
Ghrelin (pg/ml)	541.3 +/- 96.0	386.6 +/- 92.97 ***	550.2 +/- 98.31	407.7 +/- 108.0 **	528.6 +/- 98.79	356.4 +/- 61.25 **	***
CCK (ng/ml)	4.149 +/- 11.93	1.120 +/- 1.892 ns	6.273 +/- 14.97	1.460 +/- 2.356 ns	0.589 +/- 0.318	0.553 +/- 0.355 ns [+]	ns

[+] n=6 for this category. Data analysis: paired t-test and ANOVA for analysis across the four groups.

*= p<0.05; ** = p<0.01; ***=p<0.001; ns = non-significant

Using unpaired t-test both pre- and post-operative fasting gut hormone levels were analysed for differences between the NDM and T2DM groups. A difference was detected both pre- and post-operatively with PPY levels, p=0.0059 and 0.0352 respectively. No differences were detected with the other gut hormones.

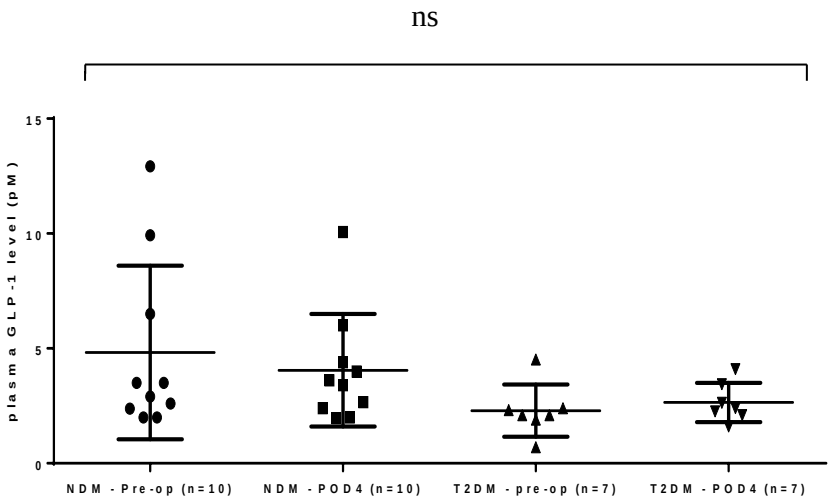


Figure 4.3.1.1 Scatter plot of the early effect of RYGB surgery upon fasting plasma GLP-1 levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for comparison; ns = non-significant.

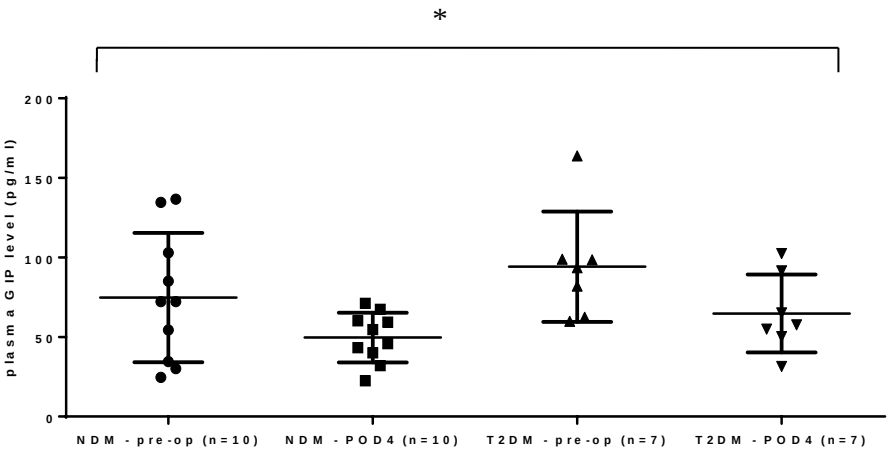


Figure 4.3.1.2 Scatter plot of the early effect of RYGB surgery upon fasting plasma GIP levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for comparison; *= $p<0.05$.

4.3.1.3 PYY

The total participant group data showed no difference in fasting plasma PYY levels by POD 4 (p=0.2498). However, the analysis of the group data revealed a significant difference in fasting PYY levels between the groups (p=0.0066) with a reduction in fasting plasma PYY levels following RYGB which did not reach significance in either group individually: NDM from mean 71.05 +/- 16.62 to 63.79 +/- 10.82 (p=0.1521) and T2DM from 106.7 +/- 29.38 to 98.79 +/- 46.67 (p=0.6051), figure 4.3.1.3. There was no difference in the direction of change of fasting PYY levels between the NDM and T2DM groups (p=0.7212).

4.3.1.4 Ghrelin

The total participant group data showed a difference in fasting plasma ghrelin levels by POD4, p<0.0001 with a reduction from mean 541.3 +/- 96.0 to 386.6 +/- 92.97 pg/ml. The group data revealed a significant difference in fasting ghrelin levels between the groups (p=0.0005) with a reduction in both groups: NDM group from 550.2 +/- 98.31 to 407.7 +/- 108.0 (p=0.0039) and in the T2DM group from 528.6 +/- 98.79 to 356.4 +/- 61.25 pg/ml (p=0.0032), figure 4.3.1.4. No difference in the direction of change in fasting plasma ghrelin levels between the NDM and T2DM groups was detected (p=0.4575).

4.3.1.5 CCK

The total participant group data showed no difference in fasting plasma CCK levels by POD 4 (p=0.3167). Analysis of the group data revealed no difference in fasting CCK levels between the groups (p=0.4333), figure 4.3.1.5. No difference in the direction of change of fasting CCK levels between the NDM and T2DM groups (p=0.9634) was detected.

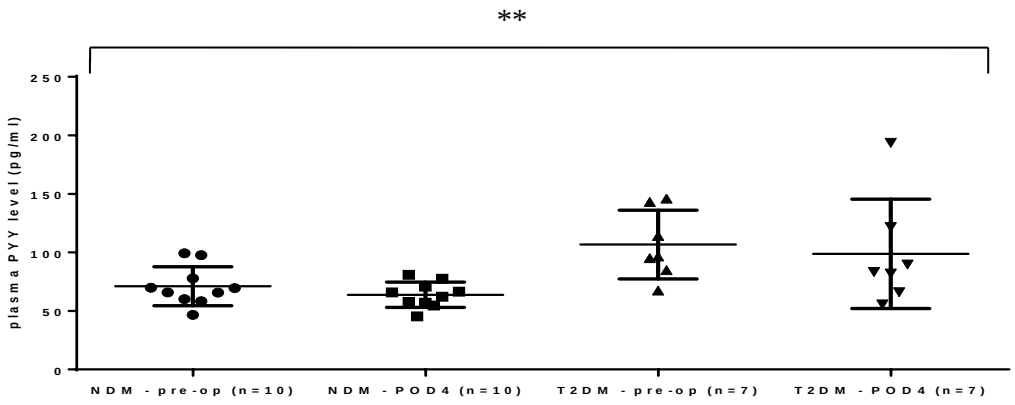


Figure 4.3.1.3 Scatter plot of the early effect of RYGB surgery upon fasting plasma PYY levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for comparison; **= $p<0.01$.

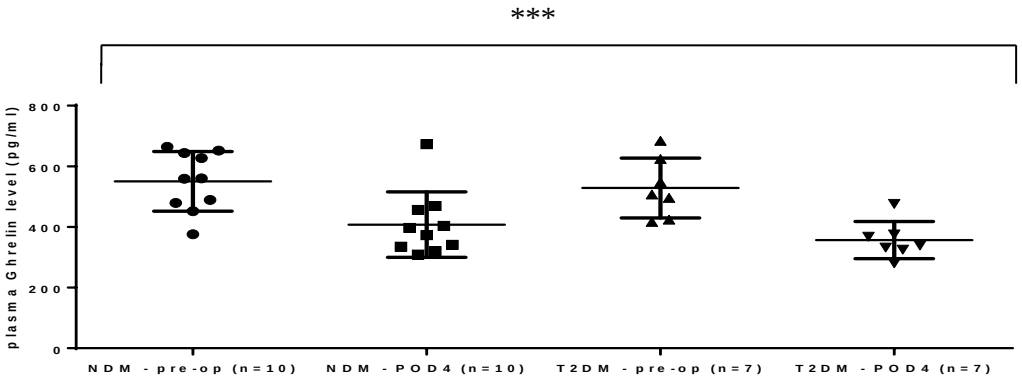


Figure 4.3.1.4 Scatter plot of the early effect of RYGB surgery upon fasting plasma ghrelin levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for comparison; ***= $p<0.001$.

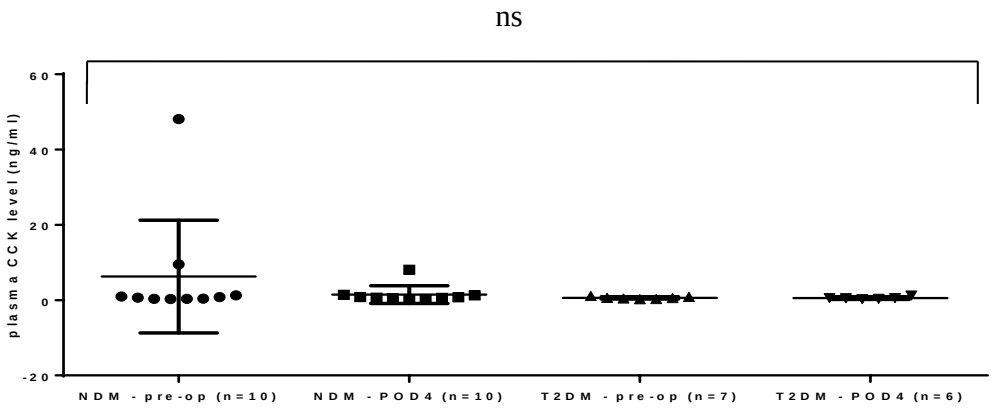


Figure 4.3.1.5 Scatter plot of the early effect of RYGB surgery upon fasting plasma CCK levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for comparison; ns = non-significant.

4.3.2 The early effect of RYGB surgery upon post-prandial plasma gut hormone levels

4.3.2.1 GLP-1

The post-prandial effect of RYGB surgery upon GLP-1 levels are shown in figure 4.3.2.1i.

The peak post-prandial GLP-1 levels were different between the groups (p=0.0018) being higher than pre-operatively in the both the NDM (p=0.0271) and T2DM groups (p=0.0290).

The post-prandial GLP-1 excursion was significantly different following RYGB (p=0.0004), being higher than pre-RYGB in both the NDM (p=0.0227) and T2DM groups (p=0.0161), figure 4.3.2.1ii.

There was no difference in the RYGB effect upon post-prandial GLP-1 secretion between the two groups (p=0.5197).

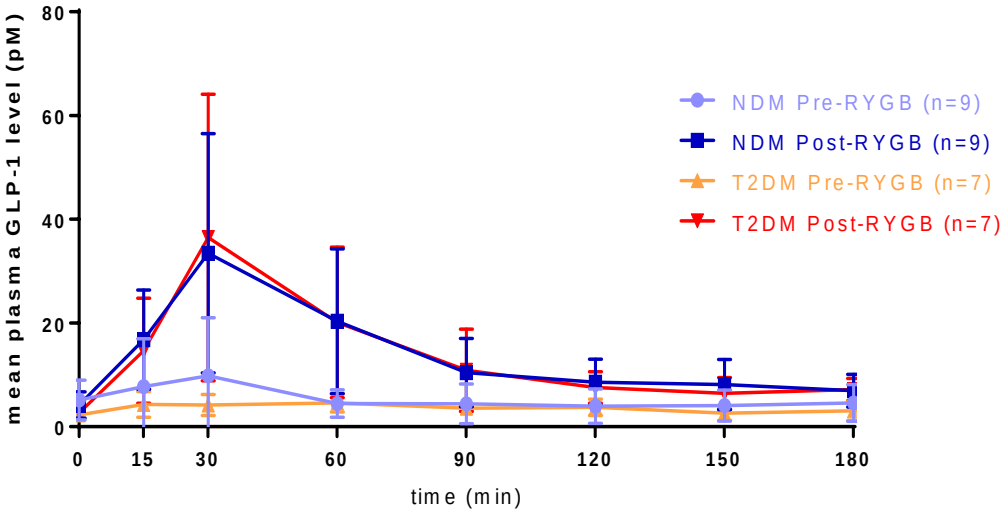


Figure 4.3.2.1i Graph of the post-prandial plasma GLP-1 levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

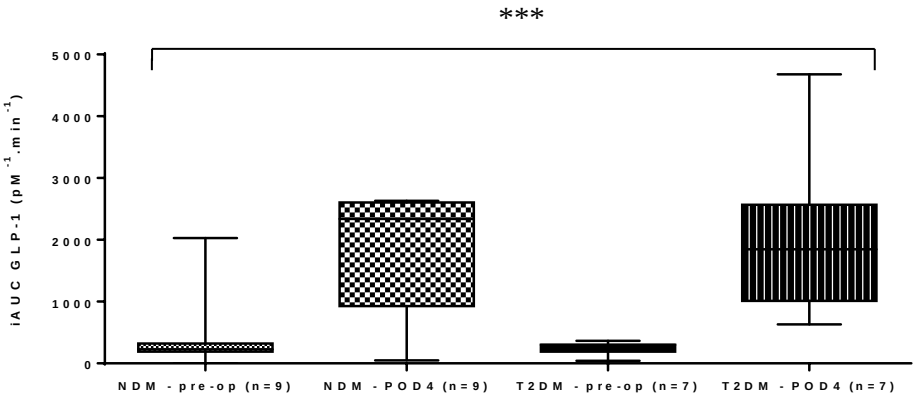


Figure 4.3.2.1ii Box plot of post-prandial response of GLP-1 (iAUC) after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. ANOVA was used for comparison, ***=p<0.001.

4.3.2.2 GIP

The post-prandial effect of RYGB surgery upon GIP levels are shown in figure 4.3.2.2i.

The peak post-prandial GIP levels around RYGB surgery were different between the groups, p=0.0006. Following RYGB surgery the post-prandial peak of GIP levels was lower than pre- in both groups.

The post-prandial GIP excursion was significantly different following RYGB (p=0.0318), being lower than pre-RYGB in the NDM (p=0.0475) but not reaching significance in the T2DM group (p=0.0819), figure 4.3.2.2ii.

Despite this, there was no difference in RYGB effect upon post-prandial GIP secretion between the two groups (p=0.7292).

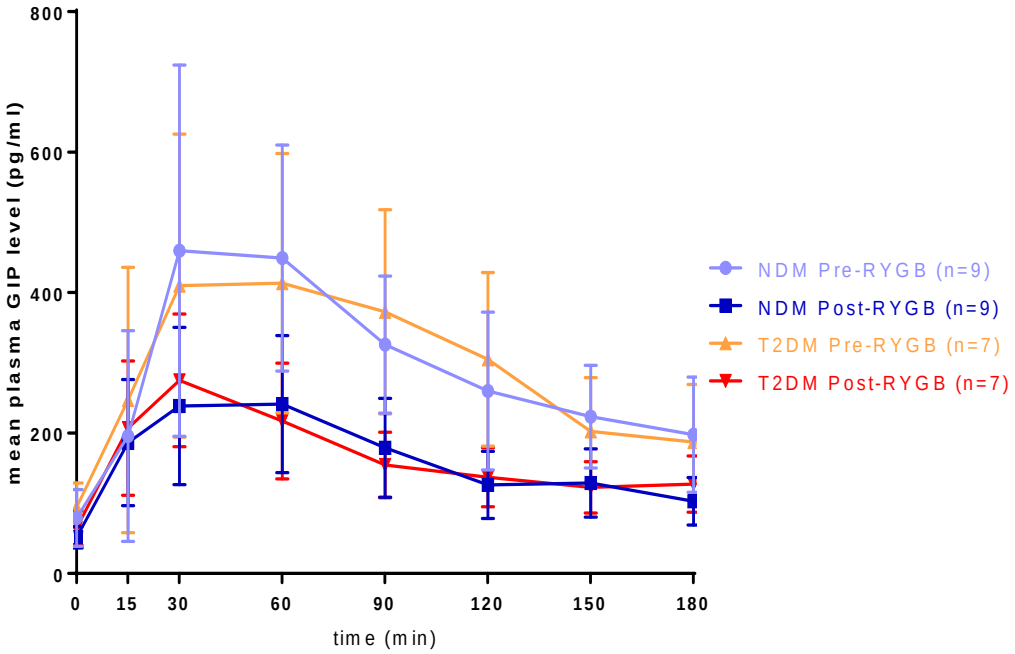


Figure 4.3.2.2i Graph of the post-prandial plasma GIP levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

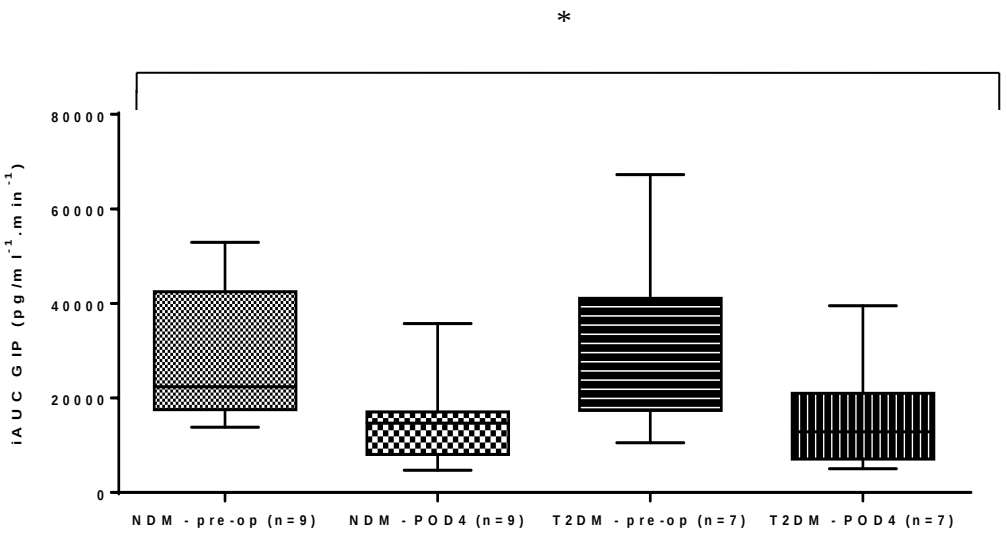


Figure 4.3.2.2ii Box plot of post-prandial GIP response (iAUC) after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. ANOVA was used for comparison; *=$p<0.05$.

4.3.2.3 PYY

The post-prandial effect of RYGB surgery upon PYY levels is depicted in figure 4.3.2.3i.

The peak post-prandial PYY levels were different between the groups, $p<0.0001$, being higher than pre-operatively in both the NDM ($p=0.0011$) and T2DM groups ($p=0.0154$).

The post-prandial PYY excursion was significantly different following RYGB ($p<0.0001$). Post-operatively there was an increase in post-prandial PYY levels in both the NDM ($p=0.0015$) and T2DM groups ($p=0.0063$), figure 4.3.2.3ii.

There was no difference in the effect of RYGB surgery upon post-prandial PYY secretion between the two groups ($p=0.8054$).

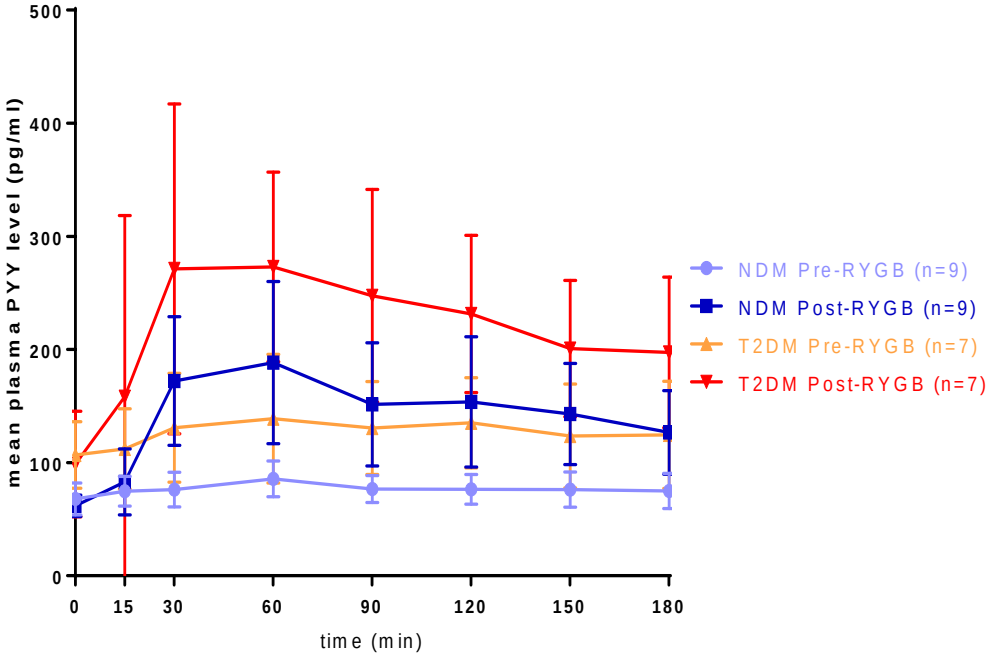


Figure 4.3.2.3i Graph of post-prandial plasma PYY levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

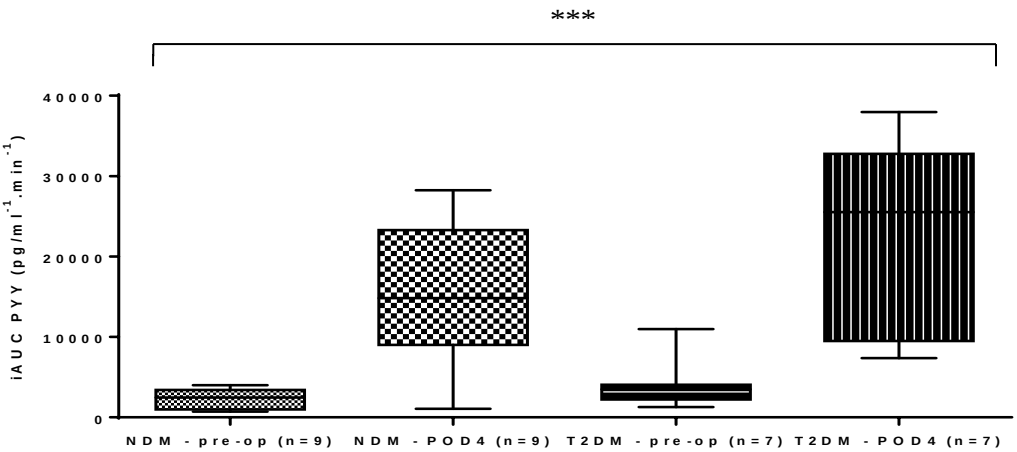


Figure 4.3.2.3ii Box plot of post-prandial PYY response (iAUC) after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. ANOVA was used for comparison; ***=p<0.001.

4.3.2.4Ghrelin

The post-prandial effect of RYGB surgery upon plasma ghrelin levels is depicted in figure 4.3.2.4i.

The peak post-prandial ghrelin levels were different between the groups, p=0.0029. Following RYGB surgery, there was a reduction in the post-prandial peak ghrelin levels in both the NDM (p=0.0407) and T2DM groups (p=0.0126).

Post-RYGB post-prandial ghrelin levels were reduced, p=0.0004. With a reduction in both the NDM (p=0.0017) and T2DM groups (p=0.0024), figure 4.3.2.4ii. However, the data was suggestive of an absence of any acute response but a change in the baseline level which was confirmed by analysis using iAUC, ANOVA p=0.5312.

There was no difference in the RYGB effect upon post-prandial ghrelin secretion between the two groups (p=0.8054).

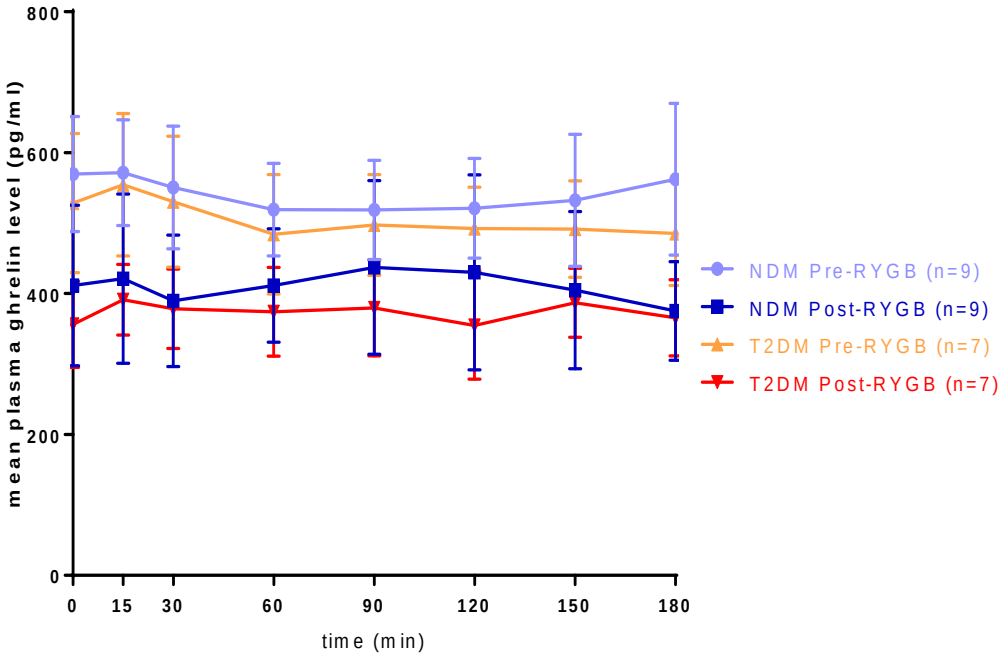


Figure 4.3.2.4i Graph of the post-prandial plasma ghrelin levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

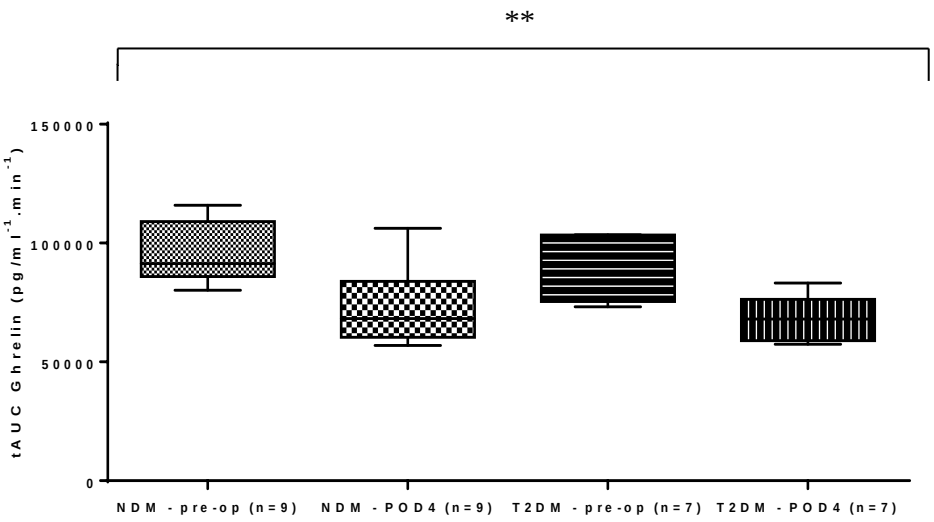


Figure 4.3.2.4ii Box plot of post-prandial ghrelin response (tAUC) after a mixed meal test, pre- and post-operative day 4 around RYGB in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. ANOVA was used for comparison; **=p<0.01.

4.3.2.5CCK

The post-prandial effect of RYGB surgery upon plasma CCK levels is depicted in the figure 4.3.2.5i.

There was no difference in the peak post-prandial CCK levels around RYGB surgery between the groups (p=0.3326).

Following RYGB surgery, the post-prandial CCK excursions were not significantly different (p=0.4920). There was one extreme outlier in the NDM pre-operative group but even if this data was excluded, there was no significant difference (p=0.6399), figure 4.3.2.5ii.

There was no difference in the effect of RYGB surgery upon post-prandial CCK secretion between the two groups (p=0.9391).

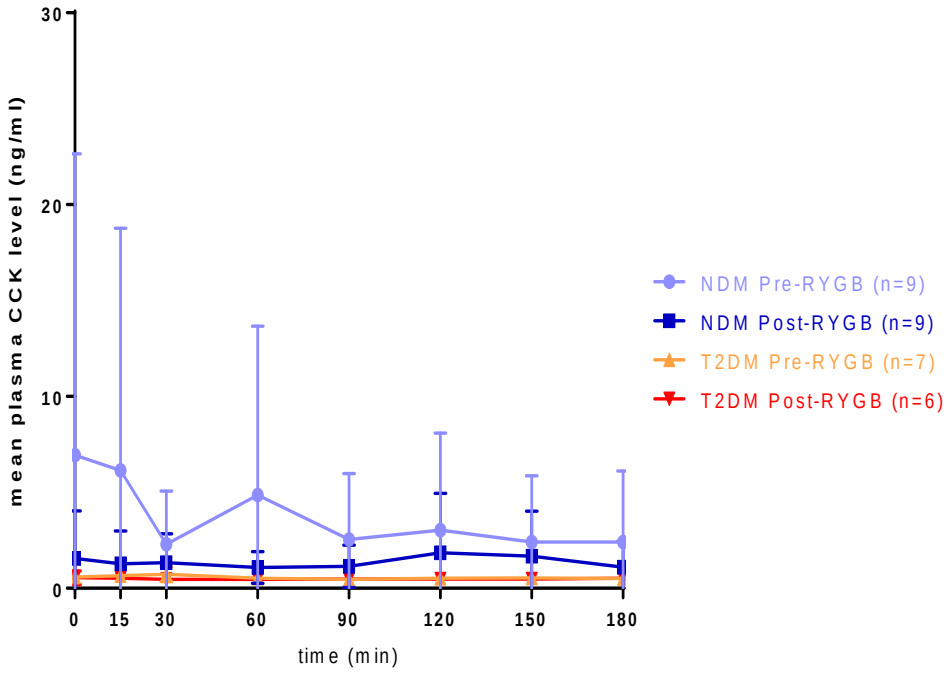


Figure 4.3.2.5i Graph of post-prandial plasma CCK levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

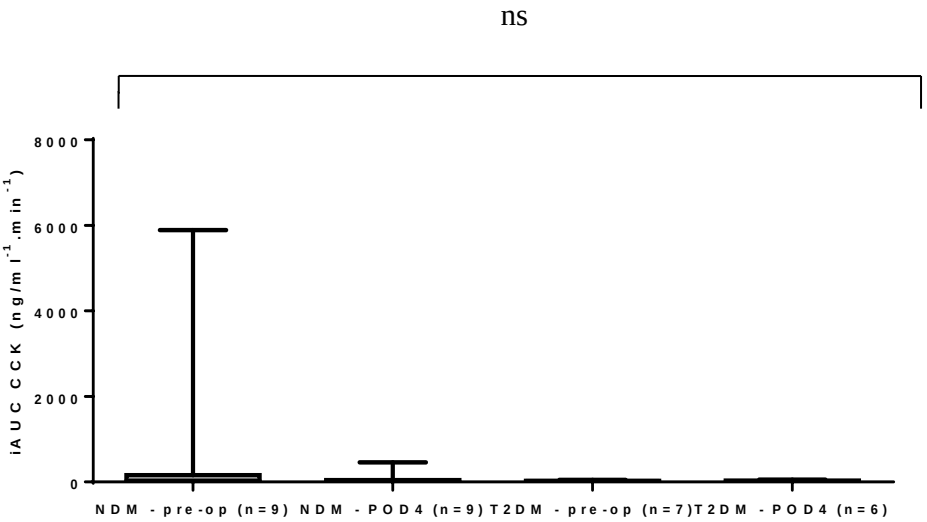


Figure 4.3.2.5ii Box plot of post-prandial CCK response (iAUC) after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. ANOVA was used for comparison; ns= non-significant.

4.4 Discussion

Gut hormones play a vital part in the relationship between organisms, their energy intake, utilisation and storage. Although it was originally believed that the RYGB procedure caused weight loss through restriction of gastric size and malabsorption,[14] significant alterations in the food transit route through the gut results in changes in gut hormone secretion. This creates an opportunity to learn more about the pivotal role these hormones play, with an in vivo model. In this chapter, I have looked at the impact of T2DM on these responses finding an impact of RYGB on fasted concentrations of GIP, PYY and ghrelin in both diabetic and non-diabetic states and likewise an impact of surgery on GLP-1 and PYY responses to meal ingestion in both states.

GLP-1 is secreted from L-cells, which are distributed mostly in the distal ileum and colon.[133,134] Similar to other studies,[20,57,131] we found no difference in fasting GLP-1 between the four situations tested. PYY is also secreted by L-cells. After RYGB surgery there is an exaggerated postprandial response in GLP-1, in both NDM and T2DM groups. Direct stimulation of the L-cells in the distal bowel, which normally receive only later products of digestion, with nutrient rich foods explains this finding, consistent with other studies.[20,21,36,131,410] It is this effect of the RYGB which is widely purported to be a main contributor to the remission of T2DM, reversible with GLP-1 antagonists.,[36,128,532-534] Indeed there is a concern that the high GLP-1 responses may contribute to post-prandial hyperinsulinaemic hypoglycaemia.[128] Despite this, as shown in one previous study,[524] the effect of RYGB surgery upon post-prandial GLP-1 secretion was not different between the NDM and T2DM groups.

GIP is a major incretin, responsible for the majority of nutrient-induced enteroinsular pancreatic beta-cell stimulation.[117] It is secreted from the K cells in the duodenum and proximal jejunum[114] in the presence of glucose and fat, dietary fat stimulating secretion more than sugars.[121] In contrast to GLP-1, GIP's stimulation is more reliant upon rate of nutrient absorption than luminal

content.[115] Increased GIP secretion in both fasting and post-prandial states has been reported in T2DM[115,122,125-127] but, in T2DM the insulinotropic effect of GIP is lost,[108] reversible by fasting.[106] The impact of RYGB is controversial.

In this study fasting GIP levels were reduced post-RYGB in both my groups, consistent with Clements et al,[129] others reporting no change,[21,38] and some showing a reduction in T2DM but not NDM groups.[57] My study may have been underpowered to detect a difference between the NDM and T2DM groups, but importantly, no difference in the direction of change following RYGB, between the two groups was detected.

Post-RYGB food by-passes the duodenum and proximal jejunum and the absorption of fats is reduced.[66] This may explain why post-prandial GIP levels are reduced post-operatively in both groups, confirming a previous report.[131] Some groups have reported no difference in post-prandial GIP levels around RYGB surgery however differing meal stimuli could explain this disparity.[21,264] I found no difference in RYGB effect for postprandial GIP secretion between the NDM and T2DM groups.

PYY secretion in the distal ileum and colonic L-cells[184,185] is vagally mediated in response to neuroendocrine mechanisms in the duodenum or through direct nutrient stimuli.[187,192] As anticipated, there was no difference in fasting PYY levels following RYGB. Post-RYGB the duodenal stimuli route may be disrupted but the increased direct stimuli of nutrient rich food in the distal bowel explain the increase in post-prandial PYY levels in this and supporting studies.[20,23,36,206,524,535]

Postprandial PYY levels are blunted in the early stage of the development of T2DM.[204] This study confirmed a difference in PYY levels between the NDM and T2DM groups both pre- and post-operatively. However, no significant difference in the RYGB effect upon post-prandial PYY was noted between the NDM and T2DM groups. This may be because very little post-prandial effect was seen in either group in the pre-RYGB state as obesity alone blunts

the fasting and PYY response to a meal-stimulus.[198] RYGB resulted in exaggerated basal concentrations and postprandial responses in both groups however a non-obese surgical group would be required to investigate this further.

Ghrelin stimulates food intake through stimulation of hunger in the hypothalamus.[213,214] Concentrations increasing in fasting and are suppressed post-prandially,[218-220] once nutrients reach the small bowel.[156,219,221,222] Suppression of ghrelin in the post-prandial period is reliant upon active absorption of nutrients in the small bowel.[221] In obesity and insulin-resistance, both fasting and post-prandial suppression of ghrelin are reduced,[227,236,237] and increased concentrations post-RYGB may be secondary to improvements in insulin resistance and a reduction in hyperinsulinaemia, increasing further as weight loss occurs. However current literature suggests a reduction or no difference in fasting and postprandial ghrelin levels following surgery.[23,36] In agreement with this, we showed a reduction in fasting ghrelin levels by POD4 with no difference in effect between the NDM and T2DM groups.

I report a reduction in post-prandial ghrelin levels following a mixed meal after RYGB, perhaps related to the rapid transit of food to the jejunum,[221] which supports other reports.[233] Further analyses of this data is more suggestive that the change in baseline ghrelin post-RYGB, rather than a change in the acute response, is more likely responsible for this finding. There was no difference in the effect when controlled for T2DM. This may be due to improvements in insulin sensitivity in both groups. Further studies are required to delineate this further.

CCK is predominantly secreted from the duodenal I-cells in response to intra-luminal foods.[240-243,263] As food bypasses the duodenum following RYGB surgery, one would expect to see CCK levels diminish post-operatively. My data are consistent with other studies investigating CCK levels around RYGB, showing no difference in the fasting state.[57,264] However, conflicting results have been published including reports that show no significant

difference in post-prandial CCK levels with glucose- or protein-only meal stimulation[206,266] and increased post-prandial CCK levels following mixed meal stimulation.[263-265] My study used a mixed meal stimulus, yet showed no difference in post-prandial CCK levels post RYGB. This may be because my study was undertaken on POD4, early after surgery, before there has been time for gut adaptation, which has been linked to increase in CCK levels.[268,272]

The large number of reported variations in gut hormone changes around RYGB surgery lead to confusion. Many factors may contribute to this. There is no global consensus regarding optimal gastric pouch size, orientation and Roux limb length. Post-surgical vagal nerve disruption is variable. Timing of post-operative assessment, may have an impact upon stress response, gut adaptation and weight loss. Different gut hormone responses have been shown in obesity, T2DM and IR states. Many studies do not control for these variables. Results may be affected by the different types of test meals used to measure the gut hormone response.[264]

There is also much variation in the quality and suitability of the data analyses across the literature. Paired statistical analyses are preferable with post-prandial assessment i.e. iAUC or tAUC for increasing and reducing postprandial responses respectively.[386] Variation in timing and number of measurements to facilitate AUC analysis also exist. Heterogeneity between studies may be reduced by introduction of agreed standardised methodology in the in vivo assessment and reporting of gut hormone responses.

Timing of investigation is key. Remission of T2DM occurs within days of RYGB surgery, prior to weight loss.[18] Previous studies into the early effects of RYGB surgery upon gut hormones confirmed that changes occurred up to 2 days post-operatively, coinciding with the improvements in glycaemic control.[20,23] Post-operative assessments performed on day 4, to control for the effect of weight loss upon the outcomes (ns). This study is part of a larger project, designed to delineate the ***early*** effects of plasma and gut hormone changes following RYGB surgery upon lipolysis in human

adipocytes, controlling for T2DM and weight loss. The sample size was calculated to assess this primary outcome and as such may not have appropriate power to detect real differences in peptide responses between the NDM and T2DM groups. Nevertheless, participants served as their own controls and this increases the validity of the findings.

The prolonged post-operative fasting period may have an important confounding impact on these findings. GIP and ghrelin levels are reduced following fasting[536,537] but no difference in PYY and CCK have been shown.[538,539] The effect of fasting upon GLP-1 remains unreported. Although laparoscopic surgery in general may have little effect on gut transit and absorption in the early post-operative period,[540] the RYGB procedure may increase transit times.[66,410]

This study has shown that RYGB exerts specific effects on each of the various gut hormones studied. There is no evidence to support a concept of global suppression of the gut hormone response by POD4 following RYGB. Animal and human studies evaluating the in vivo effect of targeted inhibition of specific hormones would further illuminate our understanding of the effect of gut hormones on glycaemic and lipidaemic control and may give rise to novel treatments of T2DM.

Many complex explanations for the gut hormone changes around RYGB surgery have been proposed. The predominant reason is that re-routing of the food transit through the gut changes absorption, rate and volume, and direct and indirect stimulation of these hormones accordingly. Gut hormones are secreted (GLP-1, PYY, GIP, CCK) or inhibited (ghrelin) in the post-prandial period to impact food desire, intake, utilisation and storage. Post-RYGB, foods reach the distal bowel early, resulting in increased GLP-1 and PYY stimulation together with ghrelin suppression. The reduction in GIP secretion resulting from reduced duodenal food transit. Other mechanisms have a smaller impact.

4.5 Conclusion

RYGB surgery significantly changes post-prandial gut hormone secretion, independent of T2DM.

5. The effect of RYGB surgery upon plasma lipids

5.1 Introduction

The post-operative alterations in glycaemic control, including remission or improvement in T2DM, are increasingly being considered as the primary goal for RYGB, rather than weight loss per se.[8,12,14,15,18,396,541] The changes in glucose metabolism are being widely investigated, with numerous mechanisms under consideration.[396,542-544] Conversely, there is little interest in the improvements in hyperlipidaemia after RYGB.

Dyslipidaemia of obesity comprises: hypercholesterolaemia, hypertriglyceridaemia, lower HDL-cholesterol (HDL-C), normal to increased LDL-cholesterol (LDL-C) levels in the blood.[545] Hyperlipidaemia is a risk factor for cardiac disease, independent of obesity. Increased total cholesterol and LDL-C is associated with atherosclerosis, plaque formation and rupture.[546-548] Reduction of LDL-C with statins slows the rate of atherosclerotic disease progression and reduces major vascular events.[549-552] Changes in HDL-C are an independent predictor of changes in atheroma burden.[553] The association between obesity and hyperlipidaemia is most likely a consequence of increased IR. Approximately 40% of morbidly obese patients undergoing bariatric surgery have hyperlipidaemia.[554] Despite the increased risk of atherosclerosis,[555] only 27% of morbidly obese patients are taking statins before weight loss surgery.[556]

The aim of this prospective study was to assess the early effect of RYGB surgery upon plasma lipid levels and post-prandial lipid fluxes, before weight loss occurs, in participants with and without T2DM. In addition, a systematic review and meta-analysis of the published literature was undertaken to assess the longer-term effects of RYGB upon plasma lipid levels.

5.2 Methods

5.2.1 Prospective study into the effects of RYGB upon fasting plasma lipids in the first post-operative week

The methodology of this study is detailed in chapter 2. In summary, venous blood was sampled following a 10hr fast and at fixed time points after drinking a 420 k-cal MMT, before and four days following RYGB surgery. Constituents of the MMT were CHO 30.1, fat 23.3 and protein 5.0 %kcal. In a sub-group of participants, fasting samples were also collected one week post-operatively. The participants' past medical and drug history was recorded. Lipid-lowering medications were not discontinued.

The blood samples were centrifuged and processed fresh for total cholesterol, LDL- and HDL-cholesterol and TG as per hospital assay protocols (see appendix 2). For the NEFA assay, samples were snap frozen at 4°C then analysed in batches.

Outcome variables were fasting plasma total cholesterol, LDL-C, HDL-C, triglycerides and NEFA concentrations and post-prandial NEFA concentrations. The data is reported as mean +/-sd. Assessment of NEFA responses to the MMT stimuli was performed using AUC 0-360min, calculated as total area (tAUC) using the trapezoidal method.[386] Inter-group analysis was performed using ANOVA. Paired and unpaired t-tests were used for intra-group analyses. Results were considered statistically significant if $p<0.05$. Statistical analyses were performed using GraphPad Prism® (GraphPad, San Diego, CA, USA). For further details regarding the statistical analyses see section 2.4.

5.2.2 Systematic review and meta-analysis of the effects of RYGB upon plasma lipids

5.2.2.1 Search strategy

A systematic review of the published medical literature was undertaken, using the electronic databases: Ovid, Medline (January 1946-March 2012), Pubmed (January 1960 to March 2012) and Embase (January 1980-March 2012). The databases were searched using the following search terms in various combinations: “obesity surgery”, “bariatric surgery”, “gastric bypass”, "Roux-en-Y gastric bypass", “cholesterol”, “lipids", "triglycerides" and “NEFA”. Two reviewers (KC and AB) screened all retrieved articles and their reference lists to identify studies that fitted the selection criteria and independently assess their fit with the inclusion criteria. Any disagreement about inclusion between the two researchers was resolved by discussion.

5.2.2.2 Literature screening

Study selection was accomplished through 2 levels of study screening. At level 1 screening, abstracts were reviewed for the following exclusion criteria: publication of abstracts only, comments, reviews and editorials, animal or in vitro studies, languages other than English, not relevant. Full articles were then obtained for all studies accepted at level 1 and for any citations for which a determination could not be made from the abstract. For level 2 screening, inclusion required that the studies included patients with plasma lipid levels both before and after Roux-en-Y gastric bypass surgery for morbid obesity; aged>18 years; n=10 or more.

5.2.2.3 Data extraction and analysis

All papers meeting level 2 screening were included in data extraction. The extracted articles could be of any design, published from 1960-March 2012 incorporating patients who had paired plasma lipid levels (any or all of: total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides or NEFA) before and after RYGB.

Characteristics of included studies are presented in *Table 1*. Data extracted were: number of cases; patients' age and gender; BMI, plasma lipid levels (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, NEFA) and time point when the outcome was measured. *Kin relationships*, defined as multiple publications describing the same or overlapping series of patients, were identified and entered into the database only once to avoid double-counting patients.

5.2.2.4 Definitions

Where the data included a range of time points >12m, they were excluded. If the data were pooled for a shorter time range e.g. 6-12m they were entered at the latest time point i.e. 12m.[557,558]

5.2.2.5 Statistical analysis

Analyses were performed only on the data from the studies in the data extraction subset. Study, patient, and plasma lipid data were summarised using descriptive statistics (simple counts and means+/- s.d.). On review of the literature, the majority of the data was presented as mg/dl therefore where data was presented as mmol/l this was converted to mg/dl prior to analysis. Where data was presented as mean +/- sem in order to minimise data loss this was converted to mean +/-sd assuming normal distribution data using the calculation $sd = se \times \sqrt{n}$

Meta-analyses of efficacy outcomes were calculated within Review Manager 5 using a random-effects model (RevMan. Review Manager [computer program]. Version 5. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration. 2008.

5.3 Results

5.3.1 The early effect of RYGB upon fasting plasma lipids

Participants were grouped according to T2DM status: 10 NDM and 9 T2DM. Participant characteristics were similar between the groups, see section 2.2.4 for further details.

The early effect of RYGB upon fasting plasma lipid levels has been summarised in table 5.3.1.

The plasma lipid molecules are discussed individually in the subsequent sub-chapters.

5.3.1.1 Total cholesterol

In the whole cohort, there was a reduction in fasting total cholesterol levels from baseline by POD4 ($p<0.0001$). In both groups, there was a reduction in the fasting total cholesterol levels (NDM $p<0.0001$; T2DM $p=0.0035$, see figure 5.3.1.1). There was no difference in the direction of change of fasting total cholesterol levels between the NDM and T2DM groups ($p=0.1383$).

5.3.1.2 LDL-cholesterol

Analysis of the whole cohort showed a reduction in plasma LDL-C levels by POD4 ($p<0.0001$). Analysis of the group data revealed a reduction in the fasting LDL-C levels in the NDM group ($p<0.0001$) but this reduction was not statistically significant in the T2DM group ($p=0.0993$, see figure 5.3.1.2). This may reflect a sample size error for this outcome, as there was no difference in the direction of change of fasting LDL-C levels between the NDM and T2DM groups ($p=0.9710$).

5.3.1.3 HDL-cholesterol

Plasma HDL-C levels by POD4 were reduced ($p<0.0001$) in the whole cohort. There was also a significant reduction in both groups (NDM group $p<0.01$; T2DM group $p<0.05$; see figure 5.3.1.3.). There was no difference in the direction of change of fasting HDL-C levels between the NDM and T2DM groups ($p=0.9860$).

5.3.1.4 Triglycerides

Plasma triglyceride levels fell by POD4 (whole cohort $p<0.05$). Analysis of the group data showed a reduction in the fasting plasma triglycerides in the T2DM group ($p<0.05$) but not in the NDM group ($p=0.3616$, see figure 5.3.1.4i). There was a difference in the direction of change of fasting triglyceride levels between the NDM and T2DM groups ($p=0.0163$), figure 5.3.1.4ii.

5.3.1.5 NEFA

5.3.1.5.1 Fasting

Although analysis of the cohort as a whole showed that fasting serum NEFA levels fell significantly by POD4 ($p<0.01$), the falls in each group were not statistically significant ($p=0.0875$), see figure 5.3.1.5.1. There was no difference in the direction of change of fasting serum NEFA levels between the NDM and T2DM groups ($p=0.8106$).

Table 5.3.1 Early effects of RYGB upon fasting plasma lipid levels

Plasma lipids	**Combined pre-op (n=19)**	**Combined post-op (n=18)**	**NDM pre-op (n=10)**	**NDM POD4 (n=10)**	**T2DM pre-op (n=9)**	**T2DM POD4 (n=8)**
Total cholesterol						
- mmol/l	5.13+/-1.18	3.91+/-0.66***	4.83+/-0.86	3.85+/-0.60***	5.46+/-1.45	3.98+/-0.75**
- mg/dl	193.5+/-45.37	149.4+/-25.42	186.5+/-33.07	148.6+/-23.26	203.5+/-60.42	150.6+/-30.16
LDL-cholesterol	n=16				n=6	
- mmol/l	2.96+/-0.84	2.22+/-0.55	2.98+/-0.79	2.27+/-0.58***	2.92+/-1.01	2.15+/-0.55
-mg/dl	96.27+/-52.17	81.21+/-28.53	115.2+/-30.49	87.78+/-22.26	75.19+/-64.25	73.90+/-34.07
HDL-cholesterol	n=18				n=8	
- mmol/l	1.14+/-0.28	0.88+/-0.22	1.23+/-0.32	1.00+/-0.20**	1.03+/-0.16	0.74+/-0.14**
- mg/dl	41.66+/-14.48	32.31+/-11.31	47.49+/-12.48	38.61+/-7.722	35.18+/-14.37	25.31+/-10.77
Triglycerides						
- mmol/l	2.91+/-2.80	1.81+/-0.75*	1.43+/-0.57	1.34+/-0.37	4.56+/-3.39	2.39+/-0.70*
- mg/dl	259.2+/-258.6	154.6+/-64.36	126.5+/-50.07	118.6+/-32.37	448.8+/-321.4	206.1+/-64.97
NEFA		n=17				n=7
- mmol/l	0.71+/-0.30	0.51+/-0.30	0.62+/-0.27	0.45+/-0.30	0.81+/-0.31	0.58+/-0.30
- mg/dl	20.33+/-8.57	12.98+/-9.25	17.69+/-7.70	12.97+/-8.54	23.27+/-8.94	12.98+/-10.50

Data analysis: paired t-test and ANOVA for analysis across the four groups.

*= $p<0.05$; ** = $p<0.01$; ***=$p<0.001$; ns = non-significant

5.3.1.5.2 Post-prandial

The post-prandial effect of RYGB upon serum NEFA levels are shown in figure 5.3.1.5.2i.

The post-prandial NEFA excursion was significantly different following RYGB ($p<0.05$), being lower than pre-RYGB in the T2DM group ($p<0.0001$) but not in the NDM group ($p=0.4111$), figure 5.3.1.5.2ii.

There was a difference in the effect of RYGB upon the direction of change of the post-prandial NEFA levels between the two groups ($p<0.01$). There was a normalisation of the difference in post-prandial NEFA excursions between the NDM and T2DM groups pre-operatively and post-operatively ($p=0.0320$ and $p=0.9830$) respectively, figure 5.3.1.5.2iii.

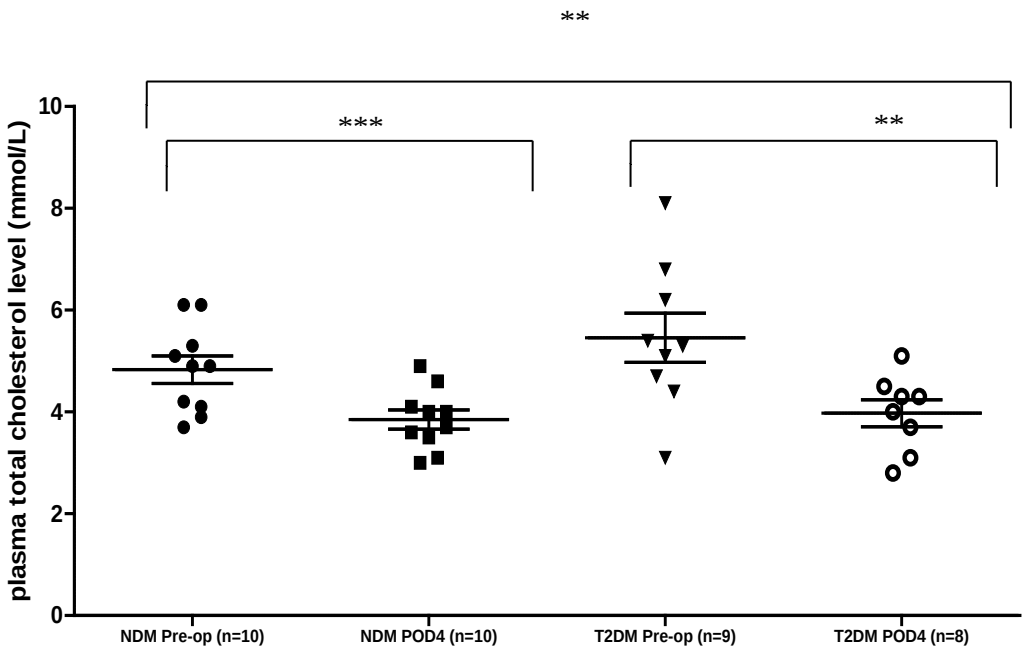


Figure 5.3.1.1 Scatter plot of the early effect of RYGB surgery upon fasting plasma total cholesterol levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= p<0.05; **= p<0.01; ***= p<0.001; ****= p<0.0001; ns = non-significant.

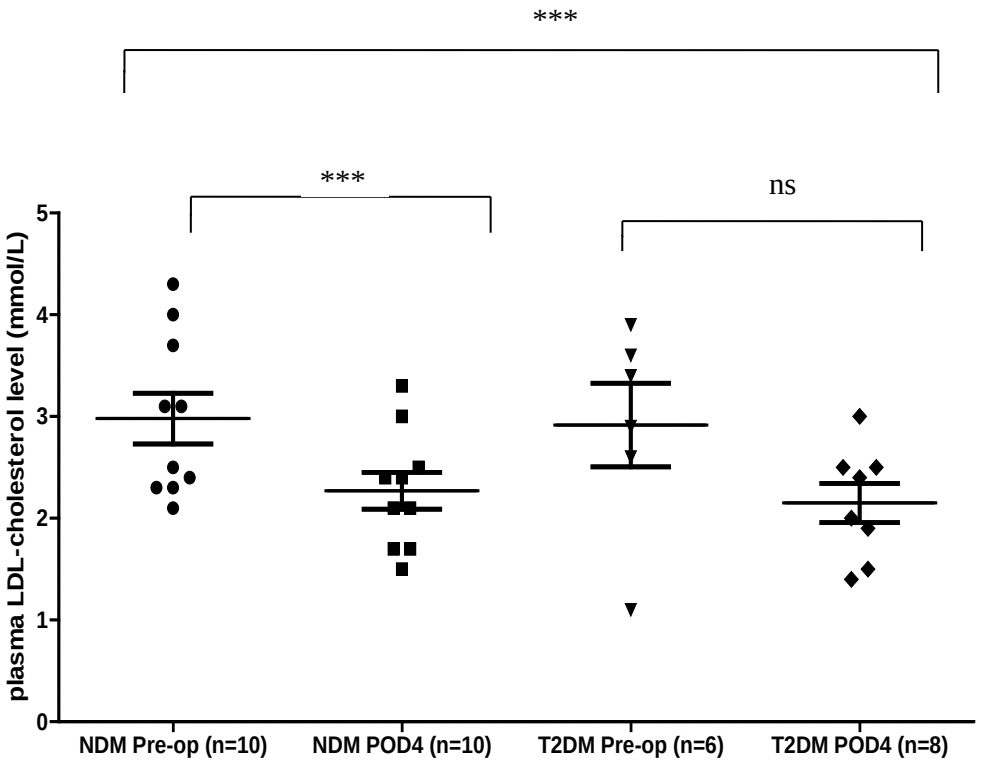


Figure 5.3.1.2 Scatter plot of the early effect of RYGB surgery upon fasting plasma LDL-cholesterol levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; ns = non-significant.

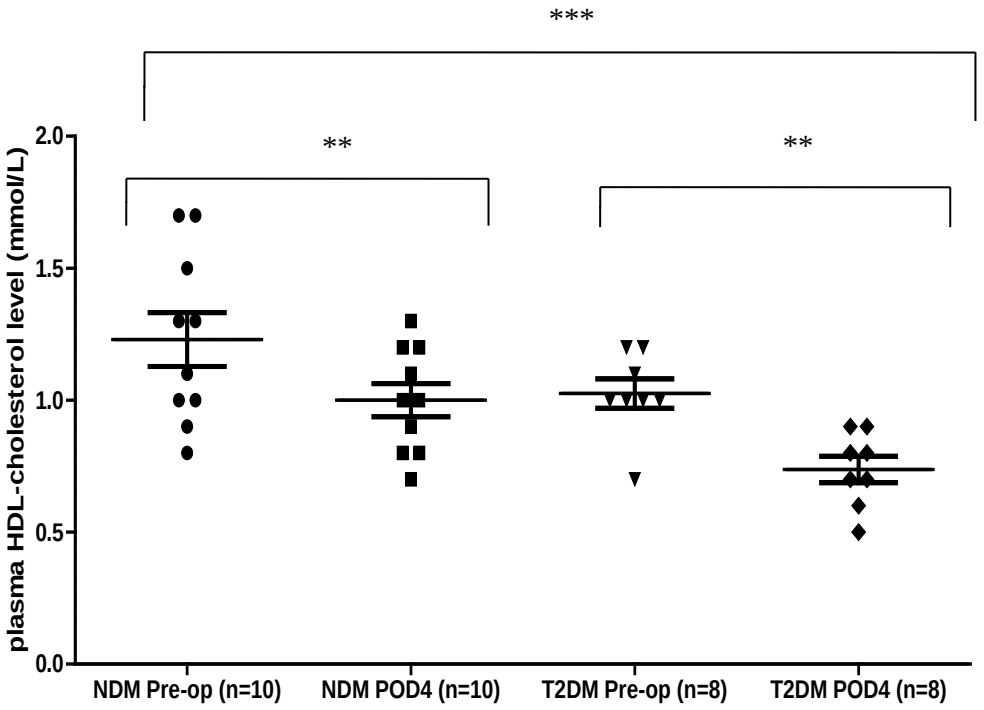


Figure 5.3.1.3 Scatter plot of the early effect of RYGB surgery upon fasting plasma HDL-cholesterol levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= p<0.05; **= p<0.01; ***= p<0.001; ****= p<0.0001; ns = non-significant.

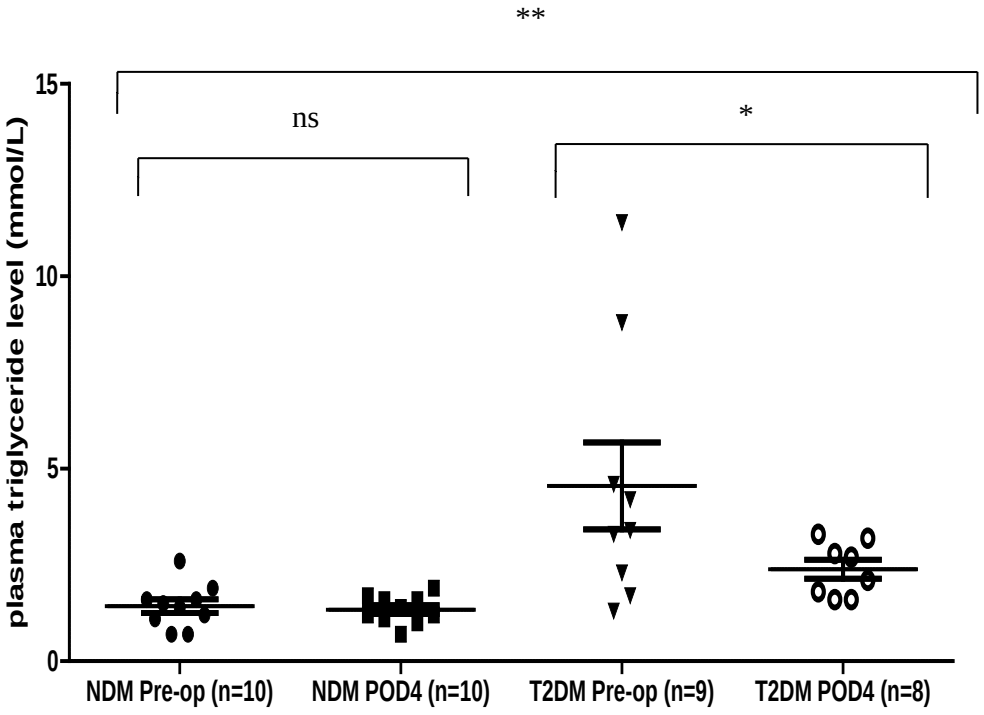


Figure 5.3.1.4i Scatter plot of the early effect of RYGB surgery upon fasting plasma triglyceride levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= p<0.05; **= p<0.01; ***= p<0.001; ****= p<0.0001; ns = non-significant.

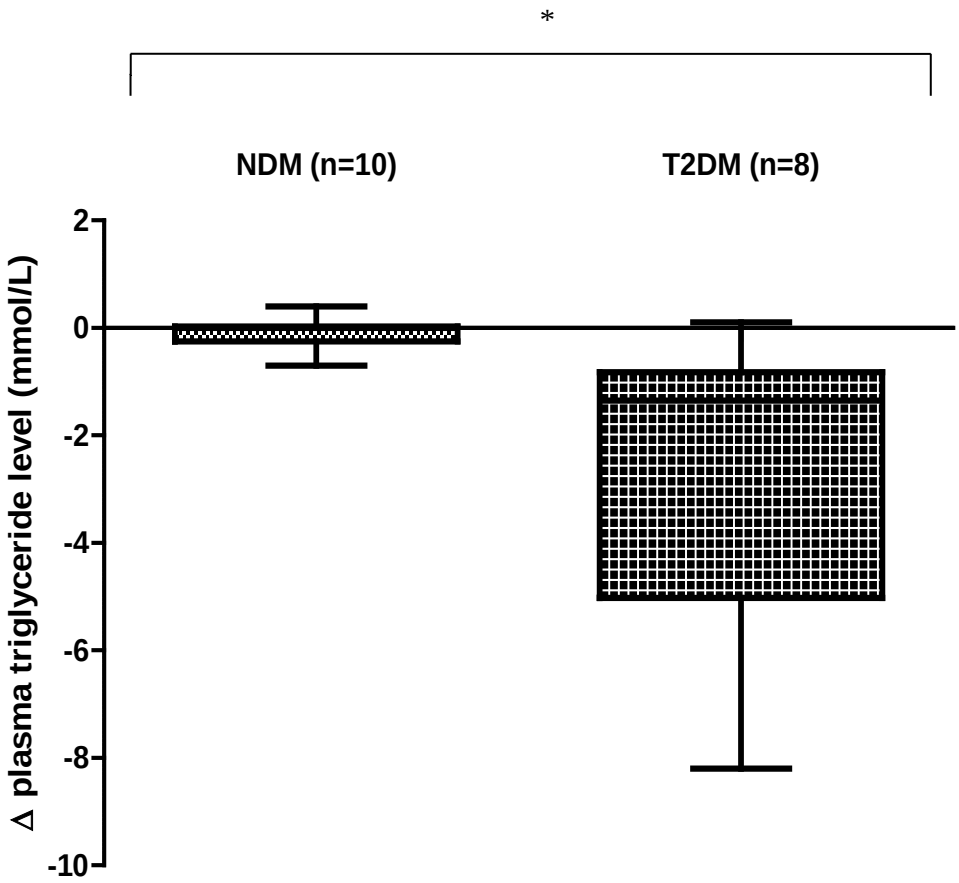


Figure 5.3.1.4ii Box plot of the early effect of RYGB surgery (pre- and post-operative day 4) upon Δ fasting plasma triglyceride levels in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Unpaired t-test was used for comparison, *=$p<0.05$.

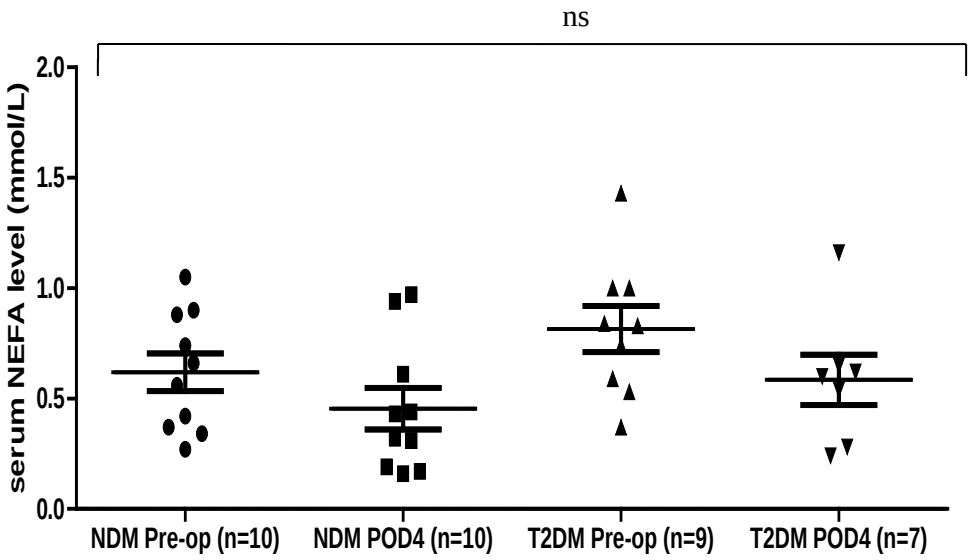


Figure 5.3.1.5.1 Scatter plot of the early effect of RYGB surgery upon fasting serum NEFA levels in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). ANOVA was used for analysis, ns = non-significant.

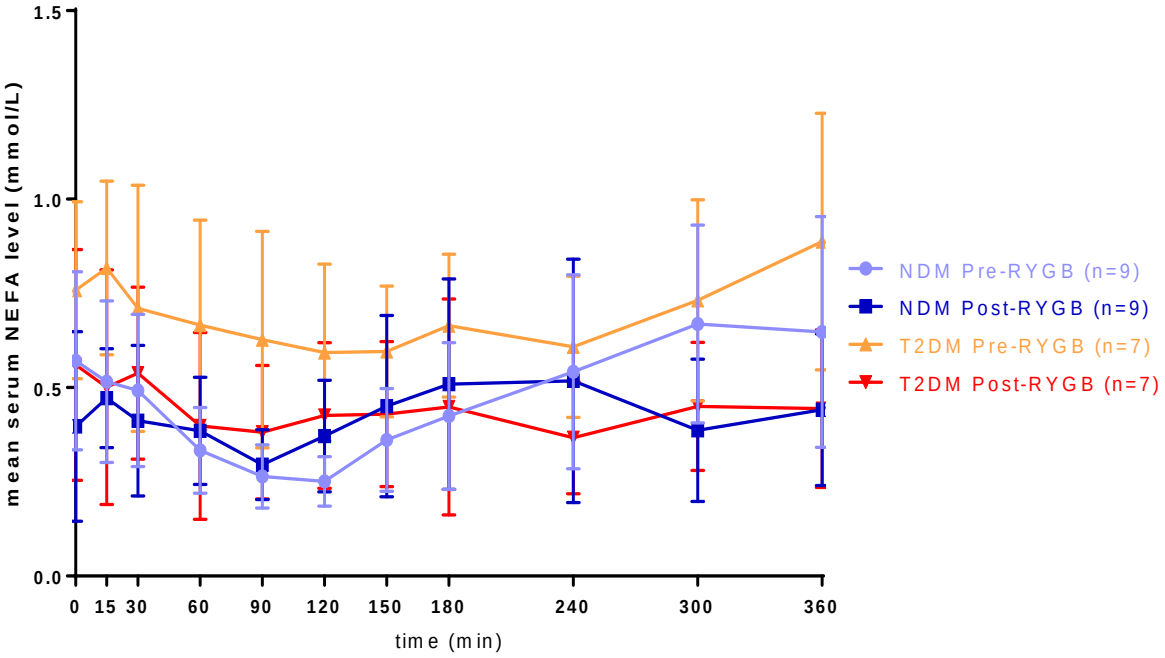


Figure 5.3.1.5.2i Graph of the post-prandial serum NEFA levels at set time points after a mixed meal test, pre- and post-operative day 4 around RYGB surgery in participants with [T2DM] and without T2DM [NDM]. Data is plotted as mean with bars representing sd.

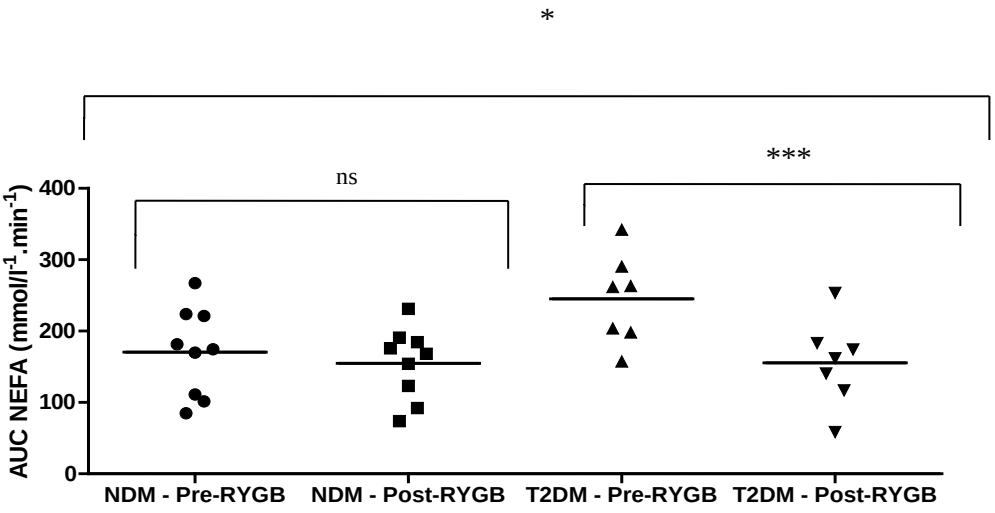


Figure 5.3.1.5.2ii Scatter plot of the early effect of RYGB surgery upon post-prandial NEFA excursions, without baseline correction, in participants with [T2DM] and without T2DM [NDM]. Pre-operatively (pre-op) and POD4 (post-operative day 4). Paired t-test was used to compare the pre- and post-operative data and ANOVA for all groups. *= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$; ns = non-significant.

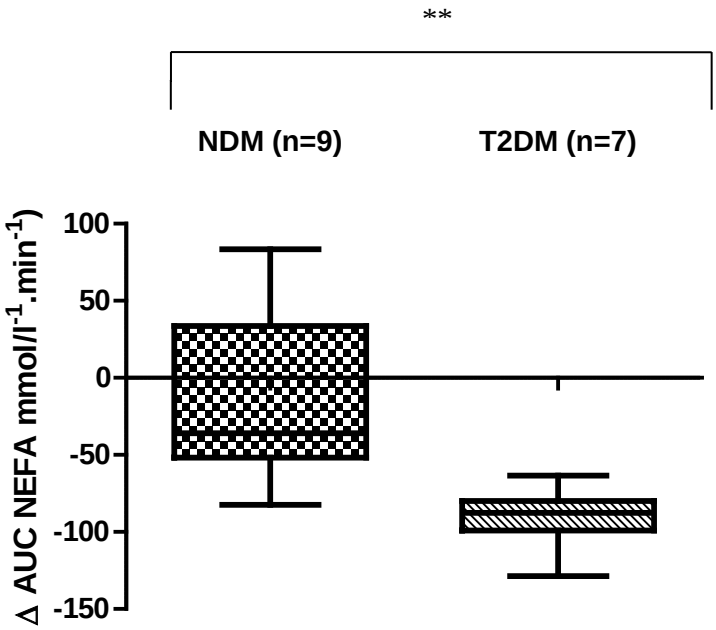


Figure 5.3.1.5.2iii Box plot of the early effect of RYGB surgery (pre- and post-operative day 4) upon post-prandial ΔAUC NEFA, following a mixed meal test, in participants with [T2DM] and without T2DM [NDM]. Box plots showing median levels, boxes show interquartile ranges, and bars represent highest and lowest values. Unpaired t-test was used for comparison; **=p<0.01.

5.3.2 Systematic review of the effects of RYGB upon plasma lipids

5.3.2.1 Data retrieval

The original search yielded 2442 manuscripts for screening. Of these, 1976 were excluded after review of the abstracts. Of the remaining 466, 75 met the inclusion criteria (for which there were 7 kin relationships), figure 5.3.2.1.

5.3.2.2 Description of studies identified

Over 90% of the included papers were published after 1999, with 39 being published from 2010 onwards. 27 studies originated from North America, 28 from Europe, 13 from South America and 2 from other continents. The included papers comprised 5 randomised controlled trials, 42 non-randomised trials or studies and 23 uncontrolled case series. The majority of the papers originated from single centres (65/70). Continuous outcomes time points >2 were only available in 29% of the manuscripts, the majority reporting ≤2, table 5.3.2.2.

5.3.2.3 Patient characteristics

Paired data is available for 7815 subjects who underwent RYGB for morbid obesity, with a female preponderance at 81% female (n=6079). The subjects were aged 42+/-4.597 years (n=4145) and baseline BMI was 47.88+/-3.511 kg/m^2 (n=2331).

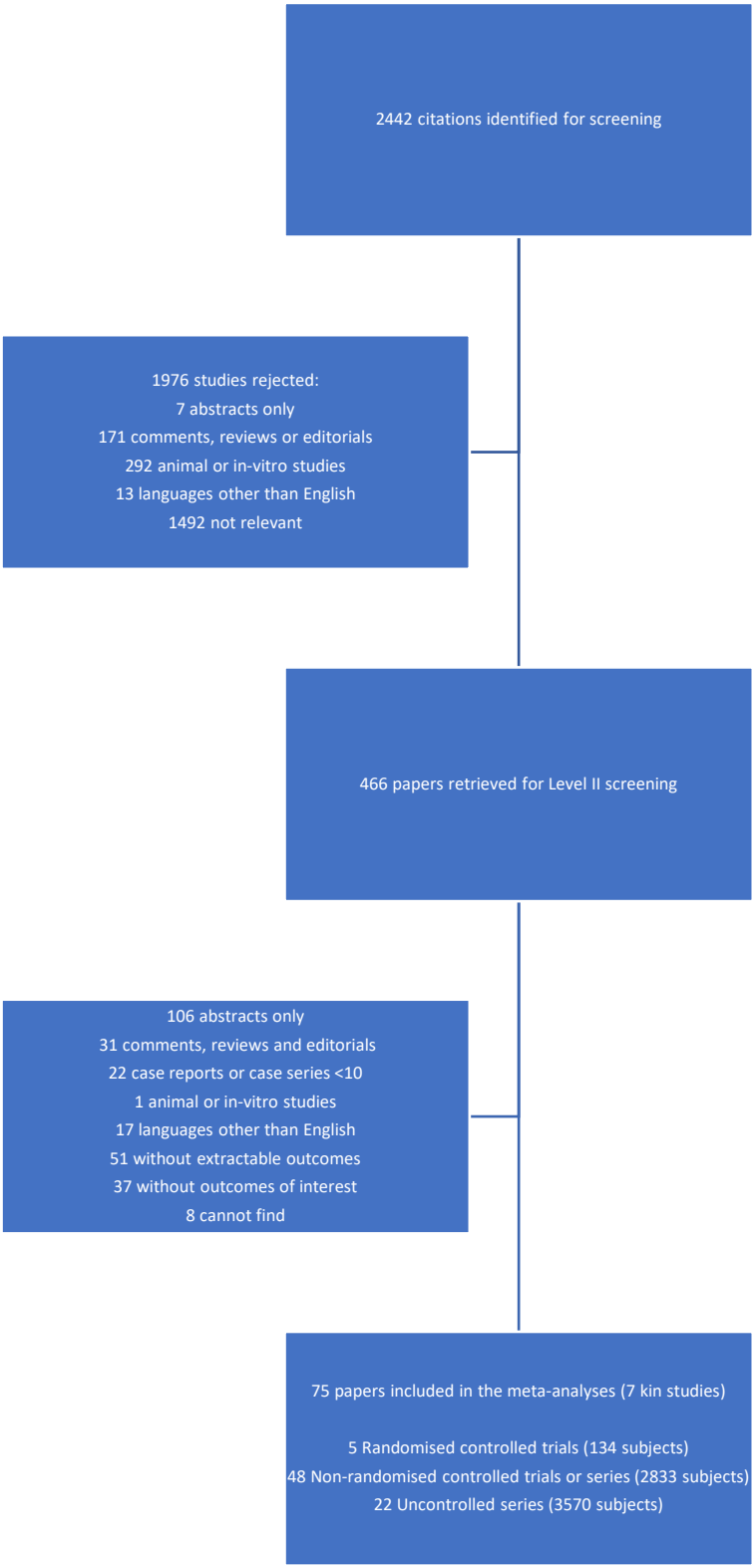


Figure 5.3.2.1 A systematic review of the effects of RYGB surgery upon plasma lipid levels - study attrition diagram

Table 5.3.2.2 Study characteristics for included publications in the systematic review of the effects of RYGB surgery upon plasma lipids levels

	No. of studies	**No. of RYGB patients***
Publication year		
- 1980 - 1989	3	235
- 1990 - 1999	2	69
- 2000 - 2009	28	2377
- 2010 - onwards	39	3856
Study location		
- Europe	28	1407
- North America	27	3744
- South America	13	1302
- Other	2	84
Study design		
- Randomised controlled trial	5	134
- Nonrandomised controlled trial or series	42	2833
- Uncontrolled case series	23	3570
Institutional setting		
- Single	65	5964
- Multicentre	5	573
Continuous outcomes		
time point	50	50145
- ≤ 2	20	1523
- > 2		

* without omentectomy

5.3.2.4 The longer-term effects of RYGB surgery upon plasma lipids

5.3.2.4.1 Total cholesterol

Total cholesterol levels were assessed in 63 studies, figure 5.3.2.4.1i. After RYGB, the SMD (standard mean difference) in plasma total cholesterol levels was -0.91 (95% CI -1.11 to -0.2), p<0.00001. Heterogeneity among the studies was high (I^2 = 97%, p<0.00001). Subgroup analyses revealed a SMD in total cholesterol levels of 0.9 by one month post-operatively (p<0.00001) which was maintained through all time points, see figure 5.3.2.4.1ii. There is relative symmetry of the data as depicted in the funnel plot which would support the lack of publication bias for this outcome, appendix 3.

5.3.2.4.2 LDL-cholesterol

LDL-cholesterol levels were reported in 48 studies, figure 5.3.2.4.2i. Following RYGB, plasma LDL-C levels were reduced (SMD -1.33, 95% CI -1.63 to -1.02, p<0.00001). Heterogeneity among the studies was high (I^2 =98%, p<0.00001). Subgroup analyses revealed a SMD in LDL-C by one month post-operatively (SMD -0.92, 95% -1.31 to -0.52, p<0.00001) which was maintained through all time points, see figure 5.3.2.4.2ii. There is relative asymmetry of the data as depicted in the funnel plot which would normally raise concern regarding publication bias for this outcome. However, these data have come from publications commenting upon all plasma lipid changes rather than LDL-cholesterol specific changes. It is unlikely to be a publication bias and more likely to be a true representation of an interesting effect of RYGB upon LDL-cholesterol, appendix 3.

5.3.2.4.3 HDL-cholesterol

HDL-cholesterol levels were reported in 47 studies, figure 5.3.2.4.3i. Following RYGB, plasma HDL-C levels increased (SMD 0.51, 95% CI 0.29 to 0.74, p<0.00001). Heterogeneity among the studies was high (I^2=98%,

p<0.00001). Subgroup analyses revealed an increase in HDL-C by one year post-operatively (SMD 1.10, 95%CI 0.57 to 1.63, p<0.0001), figure 5.3.2.4.3ii. There is relative symmetry of the data as depicted in the funnel plot, which would support the lack of publication bias for this outcome, appendix 3.

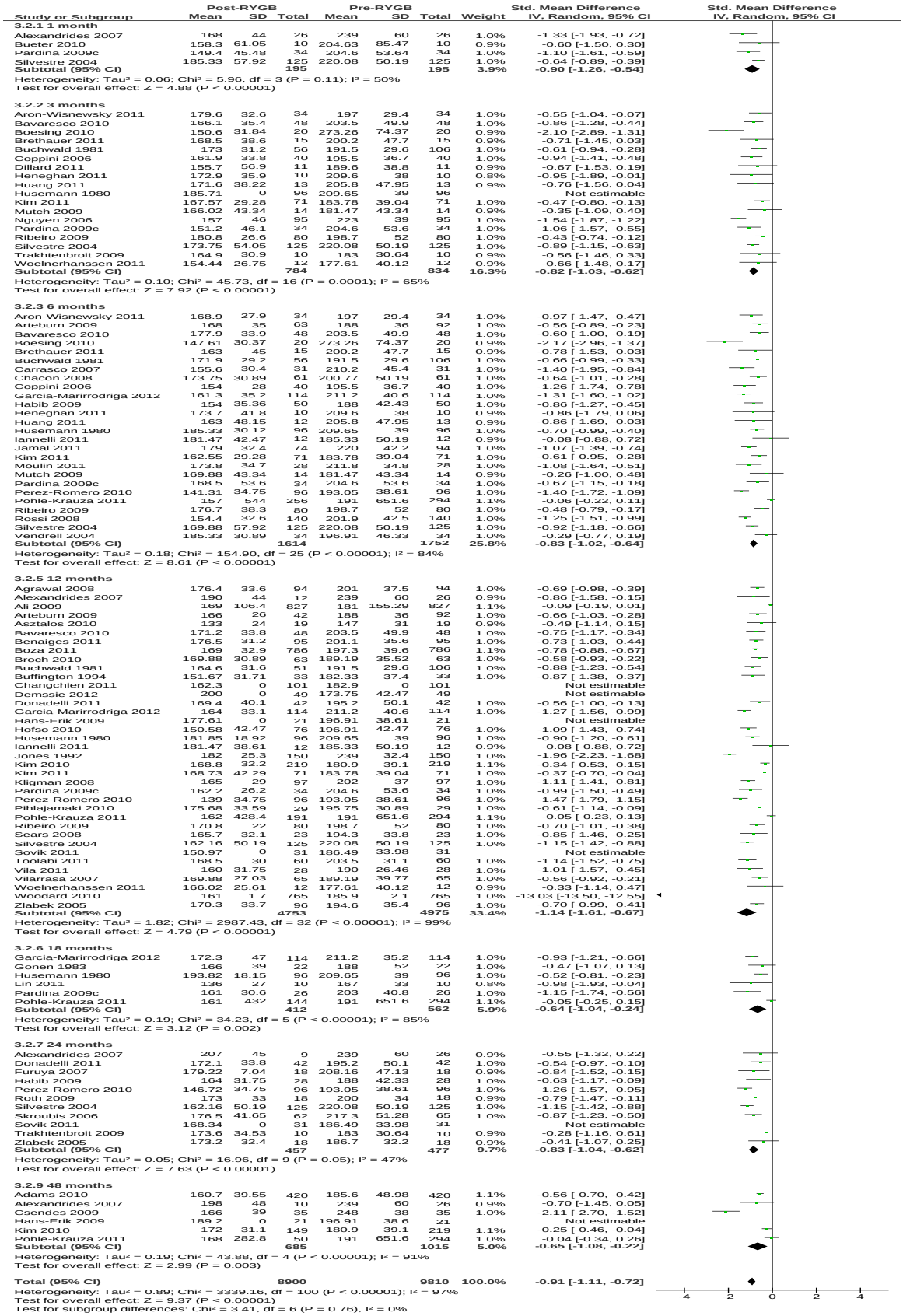


Figure 5.3.2.4.1i Forrest plot of the effects of RYGB surgery upon total cholesterol levels including all subgroups. Standard mean difference (with 95% confidence intervals)

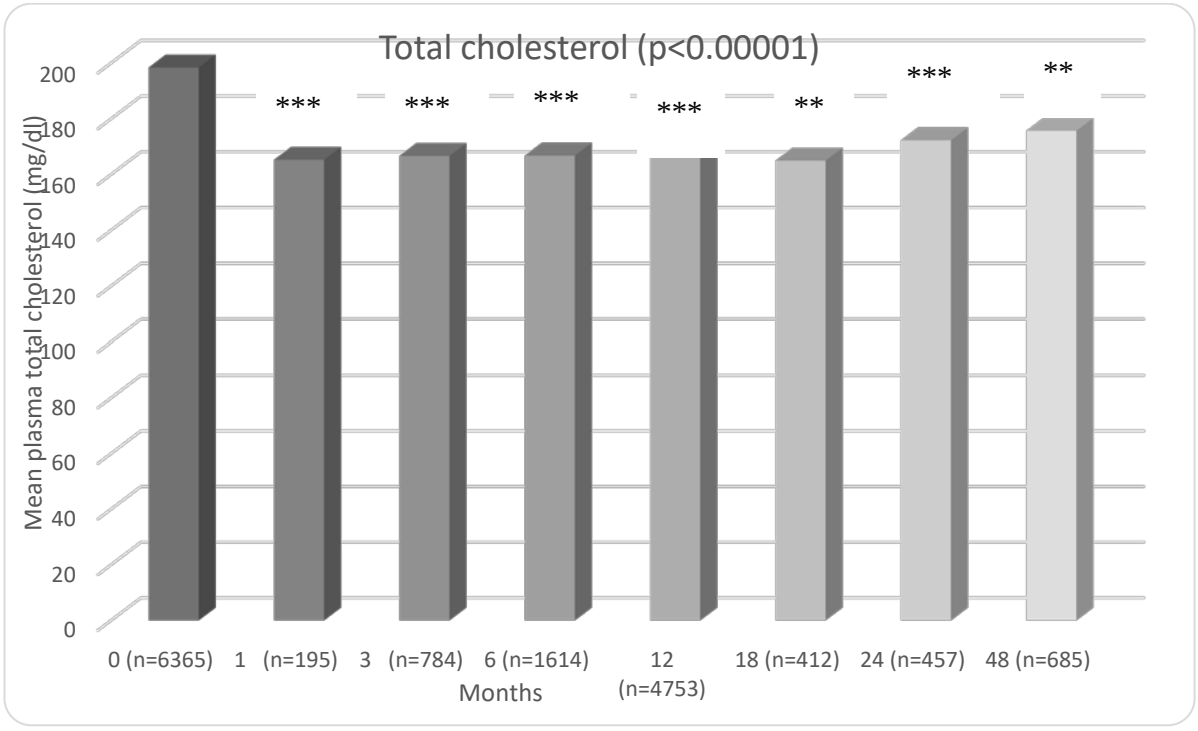


Figure 5.3.2.4.1ii Graph of the mean plasma total cholesterol levels at all time points following RYGB surgery (meta-analysis data). Unpaired t-test was used for analysis; **= p<0.01; ***= p<0.001.

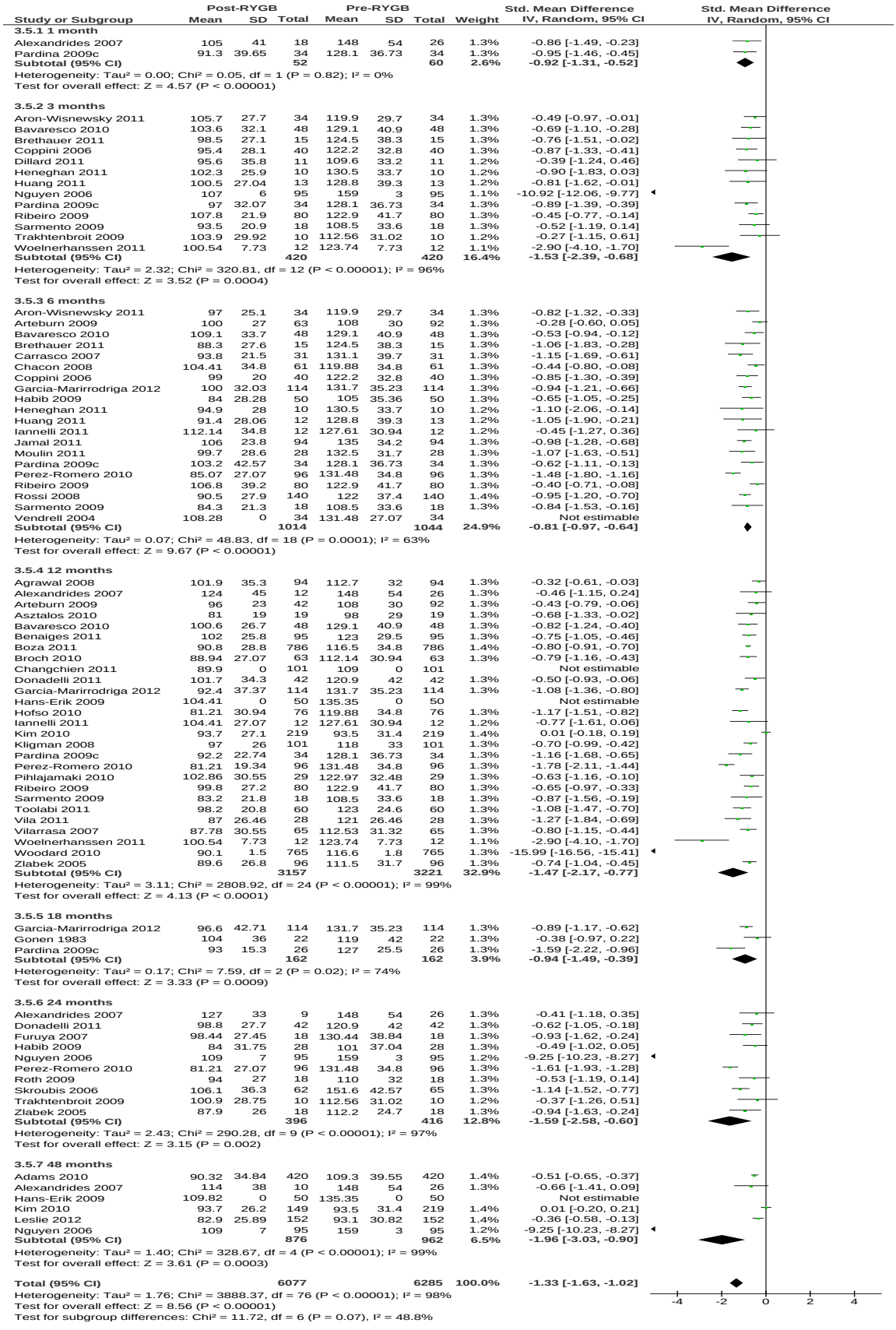


Figure 5.3.2.4.2i Forrest plot of the effects of RYGB surgery upon plasma LDL-cholesterol levels including all subgroups. Standard mean difference (with 95% confidence intervals)

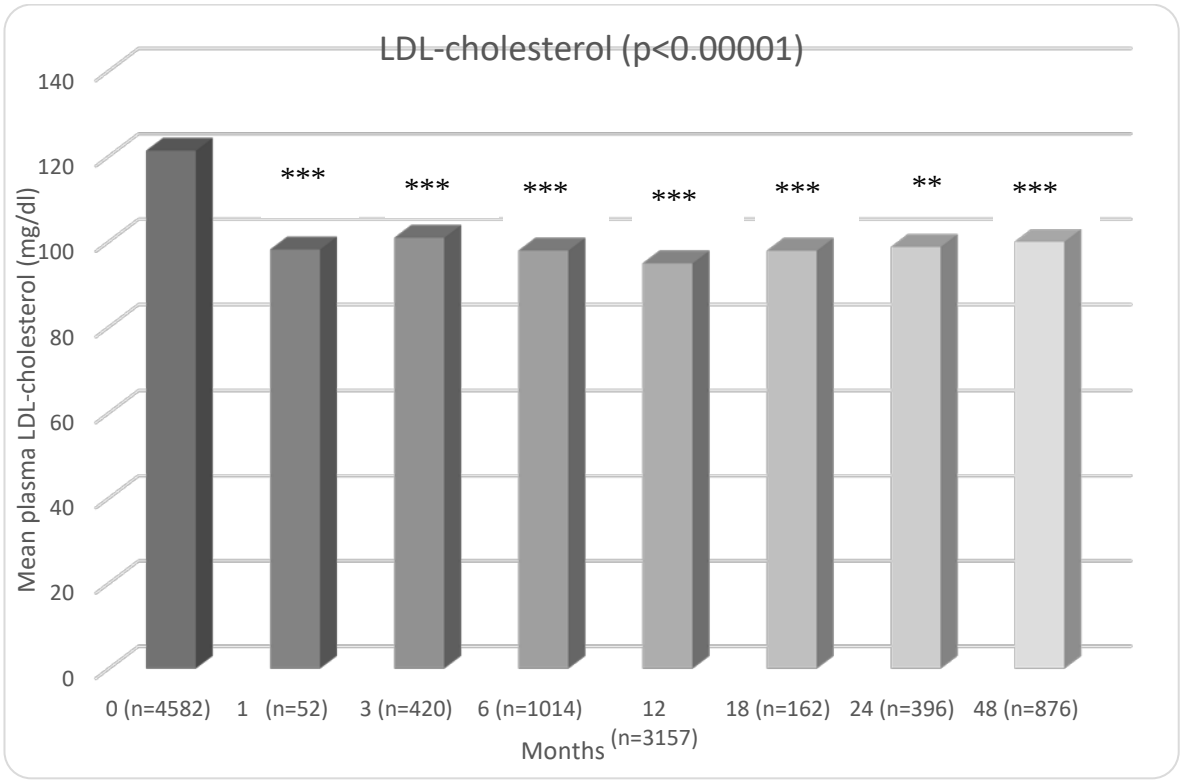


Figure 5.3.2.4.2ii Graph of the mean plasma LDL-cholesterol levels at all time points following RYGB surgery (meta-analysis data). Unpaired t-test was used for analysis; **= p<0.01; ***= p<0.001.

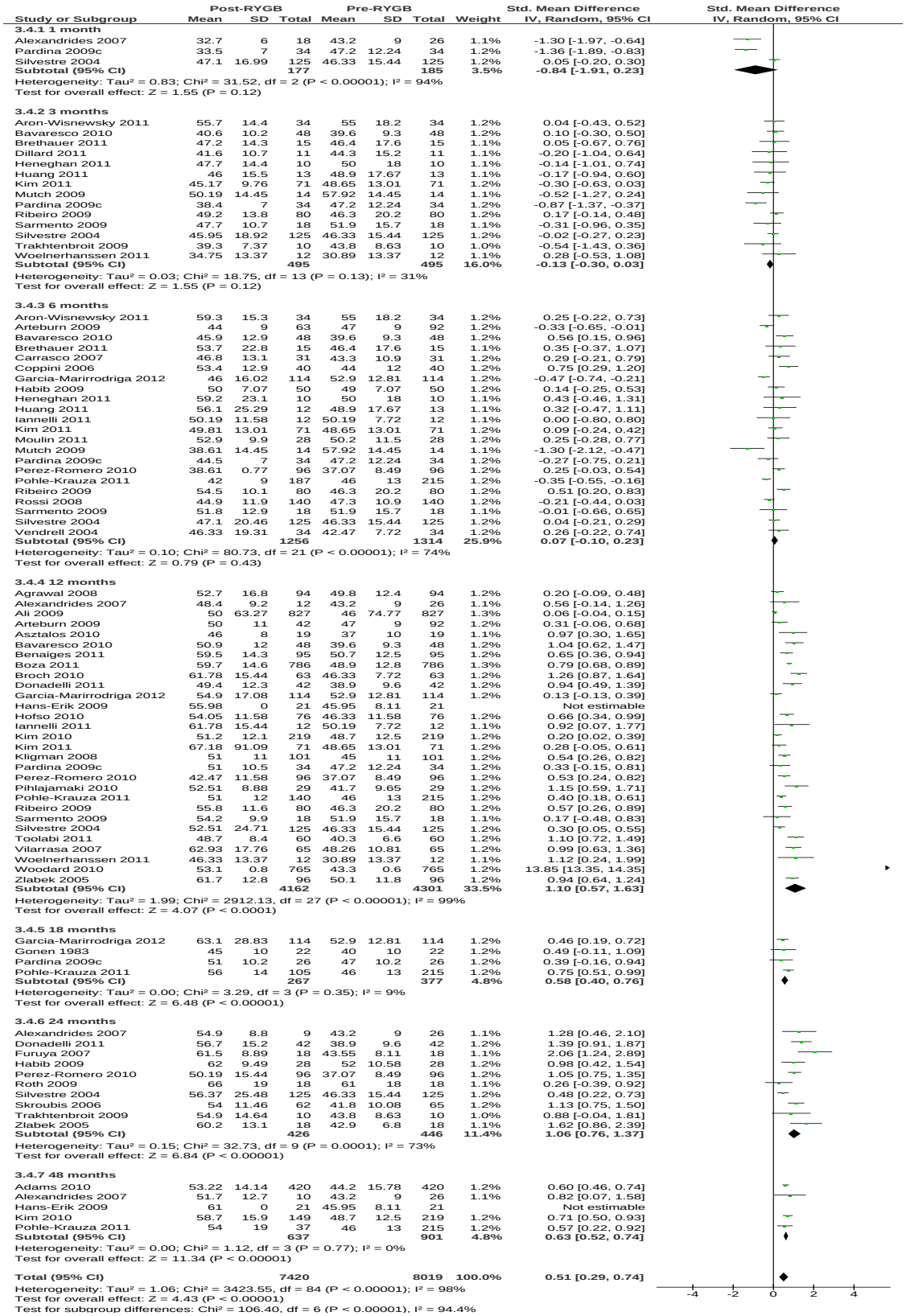


Figure 5.3.2.4.3i Forrest plot of the effect of RYGB surgery upon plasma HDL-cholesterol levels including all subgroups. Standard mean difference (with 95% confidence intervals)

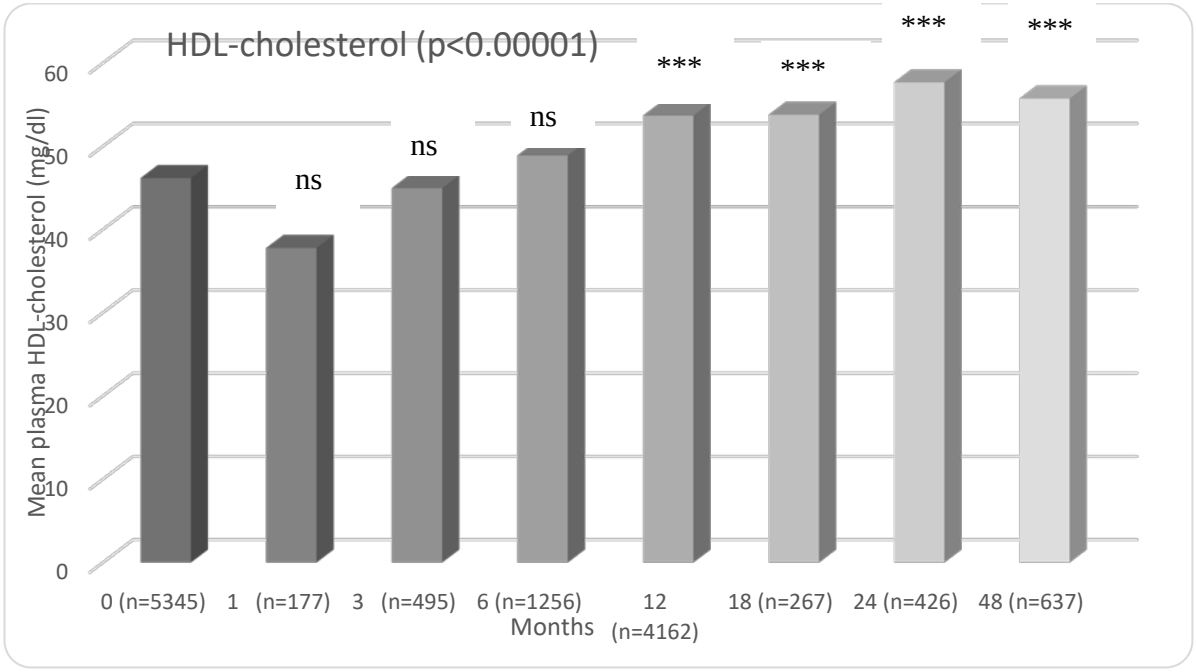


Figure 5.3.2.4.3ii Graph of the mean plasma HDL-cholesterol levels at all time points following RYGB surgery (meta-analysis data). Unpaired t-test was used for analysis; **= p<0.01; ***= p<0.001; ns= non-significant.

5.3.2.4.4 Triglycerides

Plasma TG levels were reported in 55 studies, figure 5.3.2.4.4i. Following RYGB, plasma TG levels were reduced (SMD -0.94, 95% CI -1.15 to -0.73, p<0.00001). Heterogeneity among the studies was high (I^2=97%, p<0.00001). Subgroup analyses revealed a reduction in TG levels by three months post-operatively (SMD -0.57, 95%CI -0.76 to -0.37, p<0.00001) which reduced further at subsequent time points, see figure 5.3.2.4.4ii. There is relative symmetry of the data as depicted in the funnel plot which would support the lack of publication bias for this outcome, appendix 3.

5.3.2.4.5 NEFA

Plasma NEFA levels were reported in 9 studies, figure 5.3.2.4.5i. Following RYGB surgery, plasma NEFA levels were reduced (SMD -0.2, 95% CI -0.36 to -0.04, p=0.01). Heterogeneity of the studies was I^2 = 84%, p<0.00001. One month post-RYGB, there is a 3-fold increase in mean plasma NEFA levels however by three months this has returned to comparably pre-operative levels. At 6 and 12 months post-RYGB a significant reduction in plasma NEFA levels was noted but this was not maintained. There is no data available in the literature for plasma NEFA levels at 48 months post-RYGB, figure 5.3.2.4.5ii. There is relative symmetry of the data, which would support the lack of publication bias for this outcome, appendix 3.

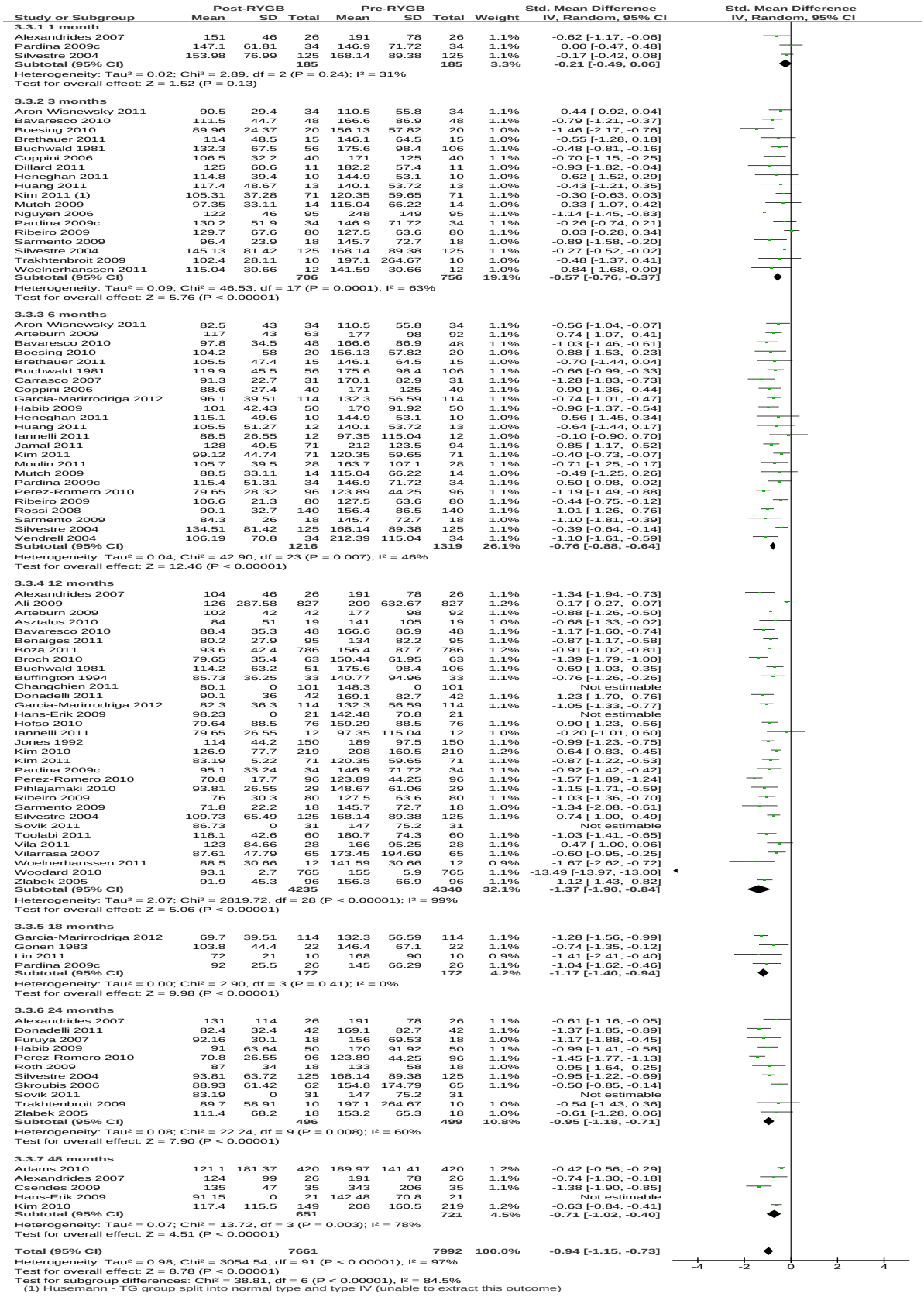


Figure 5.3.2.4.4i Forrest plot of the effect of RYGB surgery upon plasma triglyceride levels including all subgroups Standard mean difference (with 95% confidence intervals)

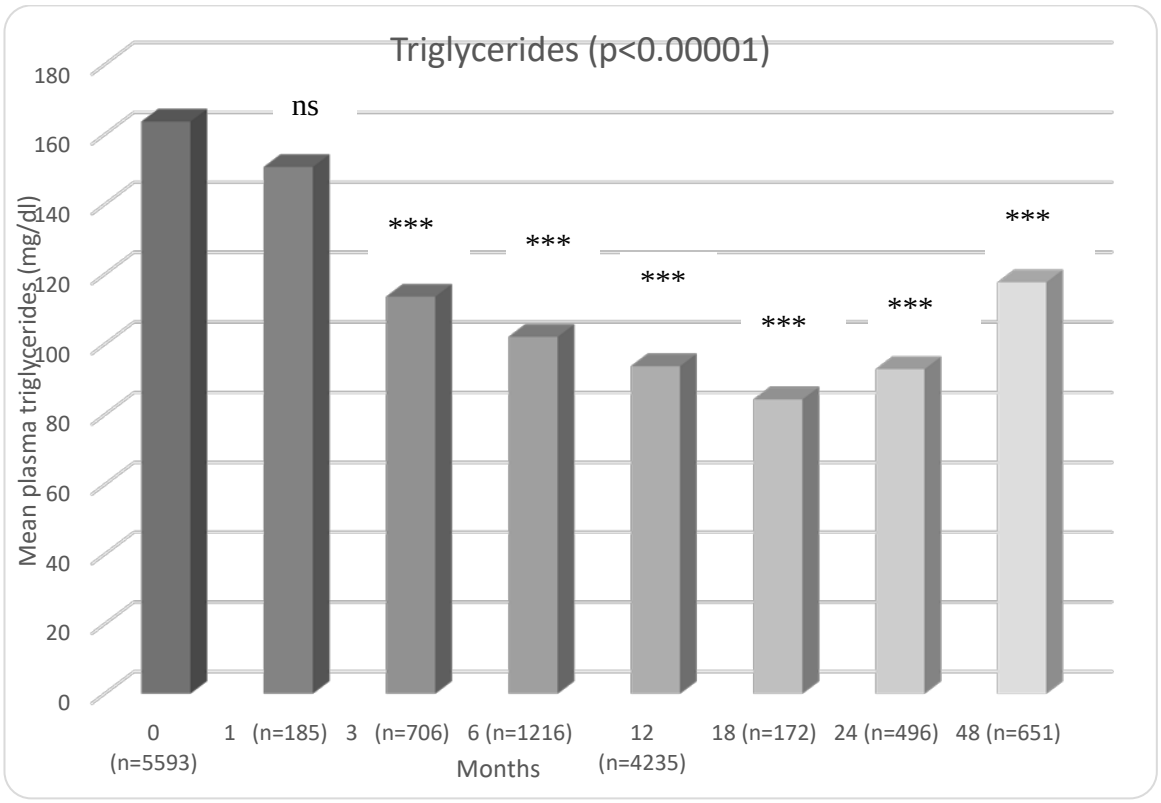


Figure 5.3.2.4.4ii Graph of the mean plasma triglyceride levels at all time points following RYGB surgery (meta-analysis data). Unpaired t-test was used for analysis; ***= p<0.001; ns= non-significant.

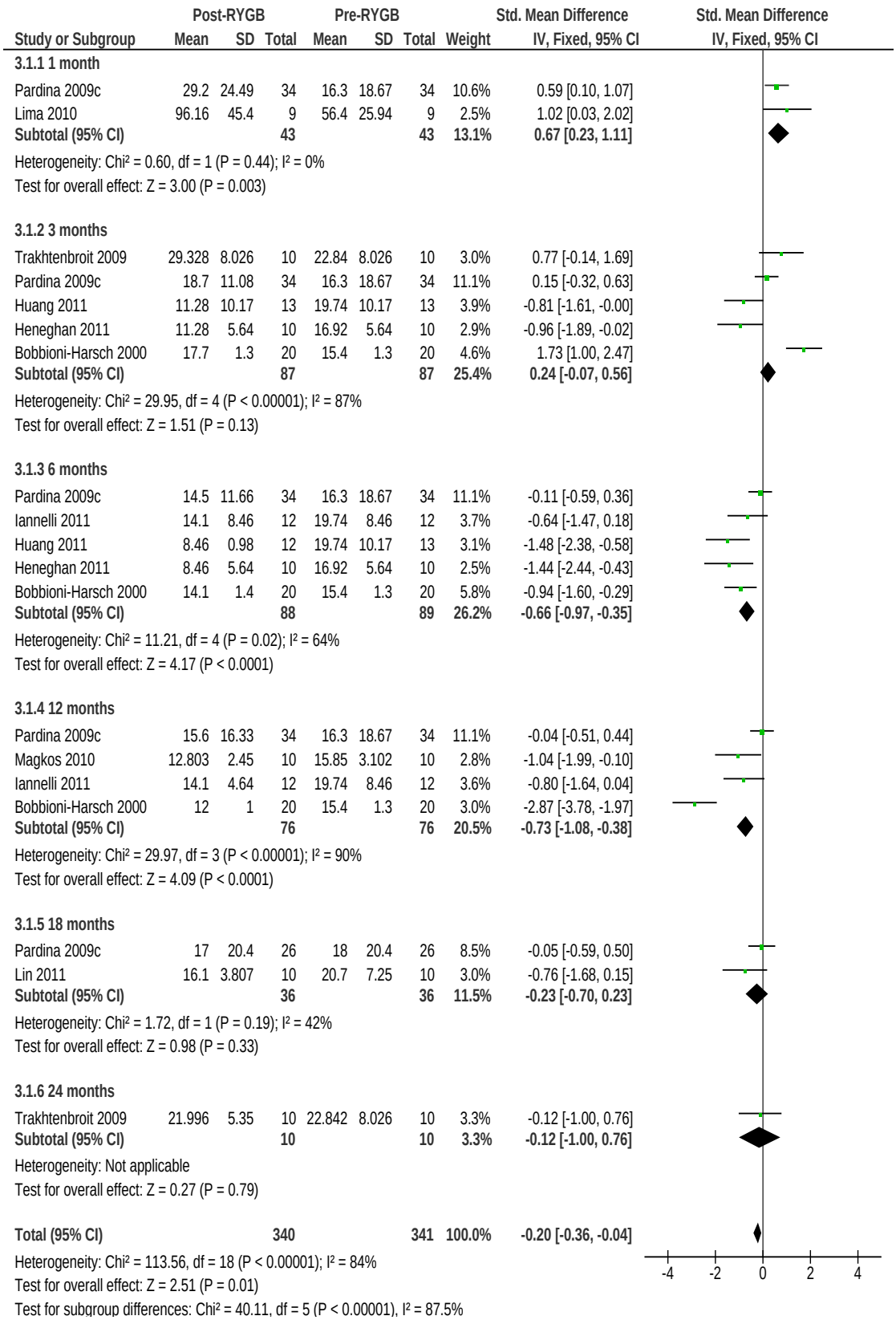


Figure 5.3.2.4.5i Forrest plot of the effect of RYGB surgery upon plasma NEFA levels using a fixed effects model including all subgroups. Standard mean difference (with 95% confidence intervals)

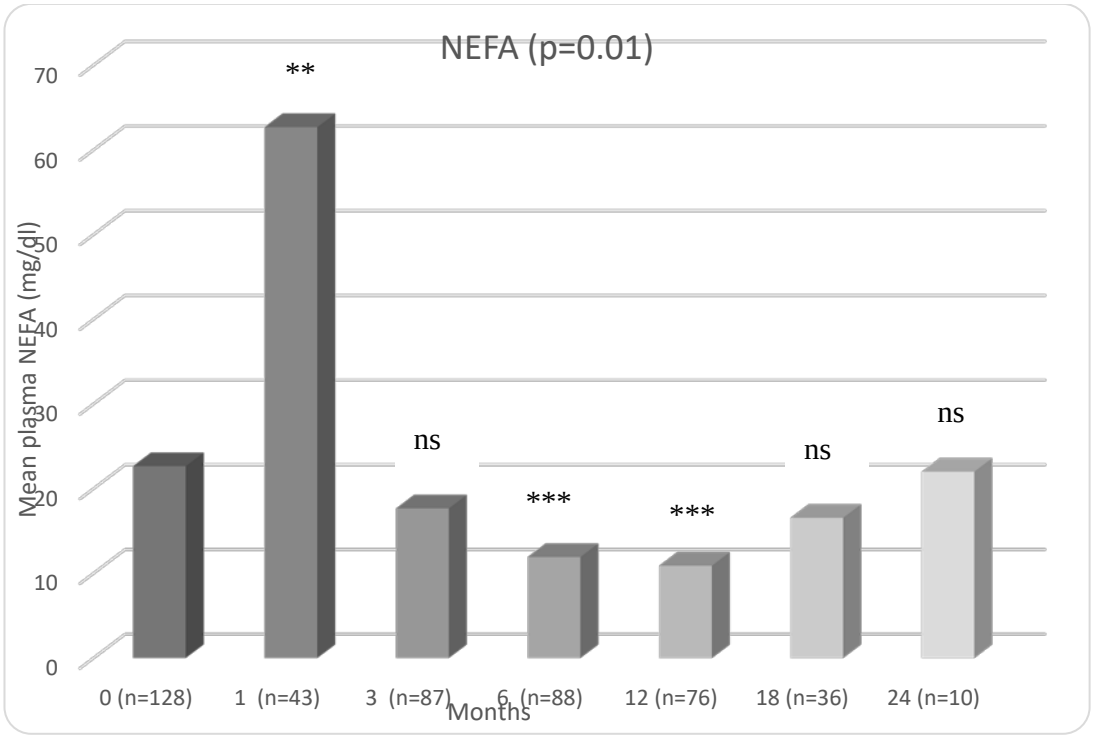


Figure 5.3.2.4.5ii Graph of the mean plasma NEFA levels at all time points following RYGB surgery (meta-analysis data). Unpaired t-test was used for analysis; **= p<0.01; ***= p<0.001; ns= non-significant.

5.4 Discussion

The effects of weight loss and subsequent reduction in adiposity upon plasma lipids is uncertain.[559-536] Following bariatric surgery there is a reduction in cardiovascular mortality.[564,565] This has been attributed to a reduction in plasma lipids, through as yet undetermined mechanisms. In non-obese patients, rising plasma insulin levels are normally associated with reduced lipolysis.[311] This study presents novel data showing that this association appears to uncouple, as adiposity increases towards morbid obesity, whilst IR persists.[566] This study group included ten participants with TC > 5 mmol/l[567] (n=19); and ten participants with TG ≥ 1.7mmol/l[568] (n=19). Rising plasma insulin levels are normally associated with reduced lipolysis.[311] This association appears to uncouple, as adiposity increases towards morbid obesity, whilst IR persists.[566]

In this chapter, I have looked at the impact of T2DM on fasting plasma lipid levels and post-prandial NEFA changes. I demonstrate that RYGB has an early impact on fasting concentrations of total cholesterol, LDL-C, HDL-C and NEFA in both diabetic and non-diabetic states. RYGB also has an effect on fasting TG levels and post-prandial NEFA excursions in the T2DM group alone. The meta-analyses show that clinically important improvements in plasma lipids following RYGB occur within one month of surgery, prior to substantial weight loss. Total cholesterol and LDL-C levels are reduced within one month, supporting the assertion that these changes are a direct consequence of the surgery rather than weight loss, *per se*. These effects persist throughout follow-up. In contrast, NEFA levels increase at one month then return to pre-operative levels by three months post-operatively. HDL-C levels increase after 12 months and the post-operative reduction in plasma TG levels occurs later, reaching significance after three months. The curve is similar to that of weight loss after RYGB,[12] although matching weight loss data was not available in all papers to allow a linear correlation analysis. One hypothesis is that compensatory mechanisms maintain plasma TG levels initially post-operatively, despite the reduction in fat and calorie intake after

RYGB. A number of plausible endogenous and exogenous mechanisms exist through which RYGB may reduce plasma lipid levels directly.

5.4.1 Exogenous

5.4.1.1 Food intake

Exogenous lipid sources are altered following RYGB. There is a global reduction in food intake,[45,46,566] which has been linked to early satiety[569] and a loss of appetite, associated with post-prandial increased PYY and GLP-1 levels.[20,23] There is reduced preference for high-fat foods.[570-572] This persists in the long-term, despite a slowly increasing caloric intake, after an initial post-surgery reduction.[571,573] These changes in food preference may be compounded by behavioural modifications to avoid dumping syndrome.[574,575] This virtuous circle of positive reinforcement leads to long-term changes in dietary choices, with exclusion of calorie-dense foods and smaller portion-sizes.[44,576]

5.4.1.2 Gut realignment

RYGB surgery creates a small gastric pouch, separated from the distal stomach and anastomosed to a jejunal loop using a Roux-en-Y configuration.[14,15] This allows food to bypass the distal stomach, diminishing gastric acid and pancreatic enzyme secretion.[472] These effects are compounded by a delay in food mixing with gastrointestinal secretions, reducing emulsification and lipid absorption in the ileum. Intestinal cholesterol absorption is reduced at one year post-RYGB,[577] corroborated by higher faecal fat levels in RYGB patients compared to obese controls in some studies.[66,572,578] Reduced lipid absorption may be due to diminishing enterocyte contact time. This may occur because of alterations in intra-luminal viscosity, as higher fibre reduces bolus propulsion,[579] and postprandial gut hormone transit effects.[103]

5.4.1.3 Bile acids

Bile acids (BAs) are synthesised from cholesterol in the liver and excreted into bile.[528] As lipids no longer traverse the duodenum post-RYGB, a reduction in BA secretion may be anticipated.[580] Conversely fasting and postprandial BAs are increased post-RYGB, [73,581-583] possibly due to increased hepatic production of BAs. This finding suggests that production and conjugation of BA is upregulated[528] in response to less cholesterol being absorbed.[577]

5.4.1.4 Gut microflora

Exogenous changes in diet, gut realignment and altered luminal pH may all modify gut microflora.[531,584,585] Changes in gut microbiota have been linked with alterations in gut hormones[586] and BA fractions post-RYGB.[529,530] Various species of intestinal microbiota have also been shown to promote fatty acid absorption. Gut microbiota presence increases fat storage in AT in mice[587-589] and can cause significant alterations in secondary lipid metabolites in serum, liver, and AT.[590,591] Weight loss and fat mass reduction occurs when post-RYGB gut microbiota are transferred into non-operated germ-free mice.[592]

5.4.1.5 Dietary lipid absorption and re-absorption

Cholesterol synthesis is increased in obesity, which is associated with reduced HDL-C.[593] Low levels of HDL-C are associated with increased cardiovascular events[594] and increased HDL-C levels have been shown to reduce atherosclerosis.[595,596] Weight loss reduces cholesterol synthesis.[597] The delayed increase in HDL-C described in this meta-analysis, occurring one year post-RYGB is compatible with the benefit being secondary to weight loss, perhaps through altered cholesterol absorption and reduced synthesis.

Statins (HMG-CoA reductase inhibitors) may have an impact on this mechanism, by inhibiting cholesterol synthesis in the liver. This leads activation of LDL receptors and increased hepatic uptake of HDL from the circulation. Administration of statins is associated with a mean reduction in total cholesterol, LDL-C, TG by 20%, 28% and 13% respectively, with an

increase in HDL-C by 5%.[598] In long-term cohort studies of patients treated with statins, there is a reduction in relative risk for major cardiovascular events by ~1% for every 1% reduction in LDL-C, in a linear relationship. [599-602] At present, no studies demonstrate that modification of HDL-C has a significant effect on.[552,603] The data presented here show that RYGB has an important early effect. Mean total cholesterol, LDL-C and TG is 16%, 21% and 36% respectively with an increase in HDL-C by 11%, which is comparable to that of statin therapy.

Plasma triglyceride levels are increased in obese compared to lean humans.[604] Alteration in dietary fat processing may occur prior to the development of T2DM, as siblings of two parents with T2DM have increased postprandial TG levels and blunted early postprandial lowering of NEFA.[594] Post-RYGB, this effect may be reversed as this study demonstrates. There is a reduction in plasma TG levels post-RYGB in the T2DM but not in the NDM group, together with a difference in the direction of change between the two groups following surgery.

5.4.2 Endogenous

RYGB results in rapid lipid mobilisation from visceral and subcutaneous adipose depots as weight loss occurs.[79] In the post-prandial period, raised insulin levels enhance TG storage in adipose tissue and inhibit lipolysis.[288] In addition, insulin has the ability to suppress the endogenous appearance rate of NEFA,[314,605] intracellular adipose tissue lipolysis[87] and possibly stimulate esterification of NEFA in adipose tissue.[83] Chronic elevation of NEFA levels is associated with IR, leading to accumulation of lipids in insulin-responsive non-adipose tissues.[84] Changes in post-prandial insulin and other gut hormone levels post-RYGB may impact upon plasma lipid flux through lipolytic mechanisms.[170,345,346,606,607] The importance of this mechanism is still in question. Hyperinsulinaemic-euglycemic clamp studies demonstrate that hepatic insulin sensitivity, rather than peripheral, improves following RYGB.[120]

The explanation for the initial, and temporary, rise in NEFA levels by 1 month post-RYGB is not clear. Fasting NEFA are almost entirely produced from hydrolysis of TG within the adipocyte[440] and are normally higher in the fasting state due to the anti-lipolytic effect of insulin.[77,608,609] Thus fasting NEFA levels reflect endogenous flow. The increase seen at one month post-RYGB may reflect increased lipolysis. Once insulin sensitivity improves in the presence of a large adipose tissue volume, one might have expected to see fasting NEFA levels rise as weight loss occurred after surgery. However, these expected changes in post-RYGB NEFA levels are not seen. It may be due to a down-regulating feedback response, occurring one to three months after surgery. This feedback response may be gut hormone-mediated at a cellular or tissue level. Prospective long-term cohort studies are required to confirm this finding. At present, the significance of the findings of this meta-analysis are still uncertain given the small sample sizes and variabilities in NEFA standardisation amongst the included studies.[610,611]

Similarly, the very early effect of RYGB upon post-prandial NEFA levels just four days post-surgery are remarkable and merit further attention. Although no difference in the postprandial NEFA in the NDM group, there was a reduction in the T2DM group. There is a difference in NEFA spillover rates between NDM and T2DM patients.[83,612] This study suggests that RYGB may reverse this. Further investigation is required as the data presented here may have been affected by fasting and NEFA levels are known to increase in stress states.[613] At present there is no consensus on the impact of fasting on NEFA levels.[614-619]

5.4.3 Limitations

There was significant clinical and statistical heterogeneity amongst the included studies. Comprehensive evaluation of the heterogeneity observed was not possible as some of the studies did not fully report data on the population characteristics, including a lack of data regarding cardiovascular risk profiles. To minimise confounding factors, only papers with paired data were included. Some potentially relevant studies were excluded from the

analysis because of incomplete reporting on the outcome measure of plasma lipid levels, either pre- or post-operatively.

Post-operatively the participants were fasted for 4 days according to the standard management protocol for King's College Hospital, London. The rationale for the timing of this evaluation has been discussed previously, see Methods. These results could be confounded by surgical stress, inflammation or fasting. Similar studies including patients in the first few days after surgery suggest that that these factors may not be important.[11,20,620] All study participants were fasted for 10 hours prior to study activities, as TG levels vary with fasting although there is little difference between fasting and non-fasting levels of total cholesterol and HDL-C.[385] Elevated postprandial TG levels persist for several hours.[621] As a consequence, reference values for lipid levels are given in the fasting state to establish cardiovascular risk.[622,623] Non-fasting TG levels may be a more accurate predictor of cardiovascular risk compared to fasting TG.[624,625] Post-prandial TG levels were not assessed in either study. It was not possible to control for fasting in the study design for the systematic review. Evaluation of the impact of RYGB upon postprandial TG would be pertinent, as multiple studies postulate that cellular changes in dietary fat processing is intimately connected with cardiovascular health.

Variable changes in physical activity following bariatric surgery between studies may be another important confounding variable. Exercise is associated with plasma TG reduction and increased HDL-C. Total cholesterol and LDL-C levels are not altered unless dietary fat intake is reduced and/or body weight lost.[626] The current literature shows conflicting outcomes, with some studies showing physical activity did not change post-RYGB[627] and others reporting improved weight loss with increased physical activity post-RYGB.[628,629]

A wide variety of lipid-lowering medication regimens were employed in the included studies, and RYGB may have exerted varying effects on the pharmacokinetics of these drugs.[630]

5.5 Conclusion

RYGB surgery reverses the dyslipidaemia of obesity. Although the meta-analysis shows that improvements in TG and HDL-C levels are seen only after weight loss has occurred, long term changes in total cholesterol and LDL-C start to occur immediately after RYGB. This implicates weight-independent mechanisms.

This chapter provides evidence that RYGB surgery leads to a healthier lipid profile. Treatment with lipid reducing medications is known to convey a relative risk reduction of all-cause mortality in T2DM, including fatal and non-fatal myocardial infarcations.[631] Additional research is needed to determine whether there is any additional cardio-protective effect of statins in patients with or without T2DM, following RYGB surgery.

6. The lipolytic effects of plasma and gut hormone changes around RYGB surgery

6.1 Introduction

Lipolysis is the release of NEFA and glycerol from the adipocyte through an enzyme-dependent system. Insulin has been shown to effect lipolysis, in humans, by impairing the clearance of plasma NEFA, through the reduction of endogenous appearance rate of NEFA.[87] The morbidly obese have lower basal and stimulated lipolysis but a larger AT volume than lean subjects,[319] as such increased plasma NEFA levels occurs. It is likely that as adipocyte insulin resistance progresses, the suppression of endogenous NEFA is impaired, compounding this further.[76-79,88-90,491-493]

The remission of T2DM post-RYGB occurs prior to weight loss, as IR improves. These improvements have been linked to changes in NEFA levels[81,95,507] potentially altering dietary fat cell processing, NEFA re-esterification and improving islet cell function.[76]

The adipocyte plays an important role in IR.[276] Regional adipocyte variability has been established in: adipocyte size; hormone receptor expression; adipokine secretory profile; expression pattern.[275,278,279] Accordingly, adipocytes from different AT depots respond differently to hormones e.g. insulin,[276,278,280,281,286] linking visceral adiposity to IR.[277] Following RYGB, as weight loss occurs, this disparity is evident; there is a reduction in visceral before peripheral AT mass, the former possessing a larger number of adrenergic receptors on the cell surface and higher lipolytic activity.[288,302]

Human adipocytes have multiple hormone receptors on their surface which may alter fat cell processing. These include receptors for GLP-1,[295] GIP,[123,296] PYY,[297] insulin,[327] ghrelin.[298,299] Little is known regarding the impact of changes in gut hormone levels upon weight loss, lipolysis and IR

however, synthetic treatment of T2DM with GLP-1 is associated with weight loss,[632] improvements in insulin sensitivity and β-cell function, these effects were not independent of a reduction in fasting and post-prandial NEFA levels.[633]

This study was designed to assess the effect of fasting and postprandial plasma and gut hormone changes around RYGB surgery upon lipolysis in human adipocytes from both peripheral and visceral adipose tissue depots.

6.2 Methods

6.2.1 Human adipose tissue handling

The methodology of this study is detailed in chapter 2. In summary, at time of RYGB surgery, AT was excised from both the subcutaneous and omental regions. These samples were placed in separate 30ml plastic flasks containing PBS buffer and transferred to the laboratory at room temperature for experimentation.

AT from 8 females and 2 males (mean age 41.9+/-10.7 yr and BMI 47+/-6.75 kg/m^2) were used. Any surplus AT was snap frozen in liquid nitrogen, and stored at -80ºC for use in RT-PCR analysis.

6.2.2 Gene expression

To confirm the presence of the relevant gut hormone and fatty acid receptors on human AT, both abdominal subcutaneous and omental (n=6), I performed gene expression analysis using established techniques from our laboratory. Total RNA was extracted using TRIzol (Invitrogen) and reverse transcribed using a High capacity cDNA reverse transcription kit (Applied Biosystems). qPCR was performed on an ABI PRISM 7900HT sequence detection system (Applied Biosystems) with Taqman probes (Applied Biosystems): GLP-1R, GIPR, PYY (PYYR1), ghrelin (GHS-R1 and NPY1R), insulin (Ins-R), GLUT-4 and fatty acid receptors (GPR43, GPR120, FFAR1, FFAR3). 18S rRNA was used as a reference gene and B-actin was used as housekeeper gene. Samples were analysed in triplicate. Relative expression levels were calculated using 7000 System sequence detection software v.1.2.3. (Applied Biosystems). Relative expression compared with visceral AT were presented.

6.2.3 Lipolysis and anti-lipolysis experiments

The adipocytes were isolated, cell number extrapolated through quantification of the fat cell diameter and the total triglyceride content of the fat cell solutions calculated. Adipocytes from both visceral and peripheral depots were incubated with relevant lipolytic and anti-lipolytic experimental conditions, basal (with ADA) and stimulated (with ADA & PIA_{20} & 1mM 8-Br-

cAMP), for 120min. These conditions included fasting and post-prandial plasma from participants before and POD4 around RYGB surgery and insulin, GLP-1, GIP PYY and ghrelin hormonal conditions consistent with basal and peak levels around RYGB surgery. In addition, the combined effects of incretins and insulin upon lipolysis were studied.

After 120min, the infranatant was aspirated and all samples were stored at -80°C until analysis. Glycerol accumulation in the infranatant was measured as an indicator of lipolysis. ADA and PIA were used to standardise any potential variations in adenosine levels. Basal lipolytic rates were defined as ADA (0.5U/ml) and stimulated lipolysis were measured with isoproterenol (10^{-6}M) and 8-bromo-cAMP (1mM). The acute insulin anti-lipolysis (0,10,100,500,1000 pM) was measured against 8-bromo-cAMP (1mM).
For the plasma experiments, in order to detect small order lipolytic effects, lipolysis was almost completely inhibited with ADA and PIA (basal state) and added 8-Bromo-cAMP (1mM) in the stimulated state. Both the basal and stimulated control samples had Lithium Heparin, Trasylol and DPPIVi added to control for the plasma experimental conditions. All calculations are presented as a % change from the control conditions.

6.2.4 Assays

The samples underwent BSA precipitation prior to performing the glycerol assay in triplicate using a one-step fluorometric method.[384]

6.2.5 Statistical analysis

Data are expressed as mean +/- sem. Difference between the AT depots were directly compared for each participant using paired t-test. Glycerol release was calculated using non-linear exponential one-phase decay curves using GraphPad PRISM. The effects of plasma and different gut hormone conditions were determined by analysis of variance with repeated measures, KW test and post-hoc t-test when main effects or interactions were significant ($p<0.05$). For further details, see section 2.4.

6.3 Results

6.3.1 Gene expression of gut hormone and fatty acid receptors in human adipose tissue from visceral and peripheral depots

Gene expression of gut hormone receptors for insulin (INS-R), GLP-1 (GLP1-R), GIP (GIP-R), PYY (PYY1R) and ghrelin (NPY1R and GHSR) and GLUT4 receptors were detected in human adipose tissue from both visceral and peripheral depots (n=12).
In addition, gene expression of fatty acid receptors (GPR43, GPR120, FFAR1, FFAR3) were detected in human adipose tissue from both visceral and peripheral depots (n=12).
These experiments showed increased gene expression for both the gut hormone and fatty acid receptors in peripheral adipose tissue relative to visceral with potential disparities in T2DM, see figure 6.3.1.

6.3.2 Lipolytic and anti-lipolytic effects of plasma and gut hormones in human adipocytes from visceral and peripheral depots

Lipolysis experiments were performed on human adipocytes, visceral and peripheral, from ten participants, five with T2DM.
A difference in cell diameter was detected between the groups, $p<0.0001$ but no difference in cell size was detected between the either visceral or peripheral tissue depots, $p=0.2123$. There was a difference in the adipocyte cell size between the NDM and T2DM groups in both the peripheral and visceral depots, $p<0.0001$ for both. There is a difference between visceral and peripheral cell size in the NDM participants ($p=0.0132$) but not in the T2DM participants ($p=0.9531$), see figure 6.3.2.

6.3.2.1 Plasma

Adipocytes responded differently to plasma taken around RYGB in both the basal and stimulated states, p=0.0335 and p=0.0347 respectively. On closer analysis, fasting plasma pre- and post-RYGB had a difference in the lipolytic effect in peripheral adipocytes, p=0.0235 (n=8), in the basal state, not detected when stimulated, p=0.4287 (n=7), but not shown in visceral adipocytes (p>0.05 in both states).

When the adipocytes were stimulated with post-prandial plasma an increase in lipolysis was revealed following RYGB surgery in peripheral adipocytes (p=0.0205, n=8, in basal state and p=0.0012, n=7, in stimulated state). This effect was not detected in the visceral adipocytes in the basal state, p=0.1957 (n=7) but was detected when stimulated, p=0.0071 (n=7), figure 6.3.2.1.

No difference was detected in the effect of RYGB on meal suppression of lipolysis, p=0.6016.

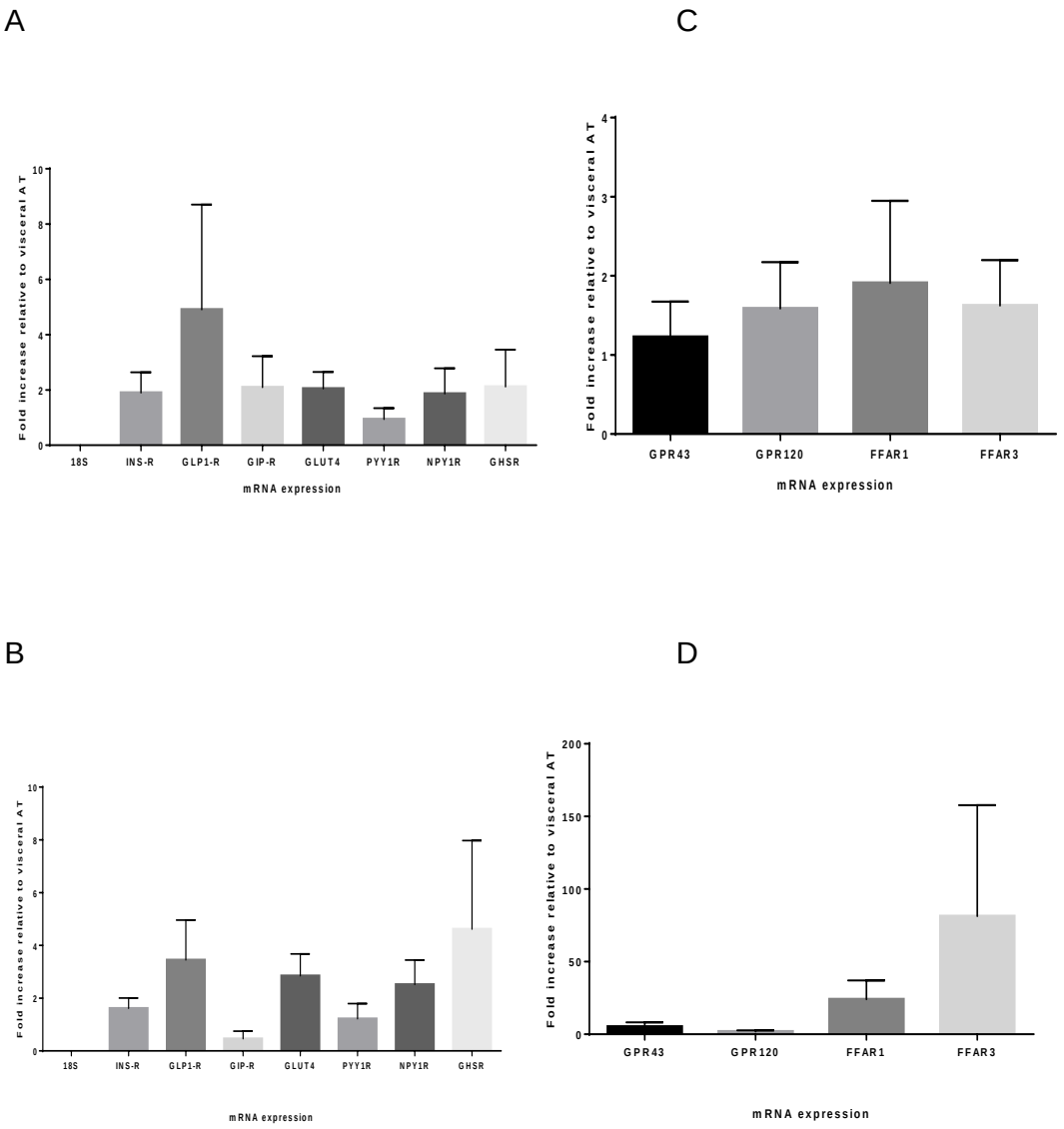


Figure 6.3.1 Relative gene expression levels in human adipose tissue from visceral and peripheral depots. A – gut hormone receptors and GLUT4 in NDM; B – gut hormone receptors and GLUT4 in T2DM; C – fatty acid receptors in NDM; D – fatty acid receptors in T2DM. Total RNA was isolated, and mRNA expression levels were measured by RT-qPCR, n=6 in each group.

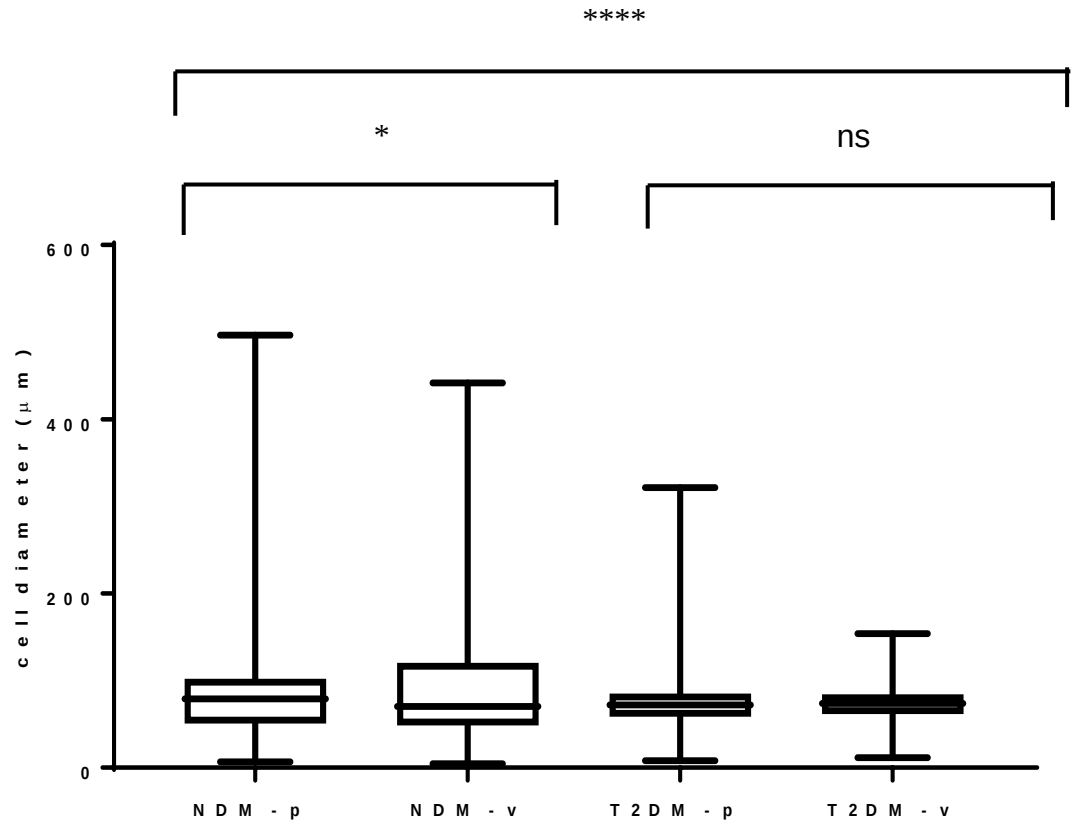


Figure 6.3.2 Box plot showing the median levels of human adipocyte cell diameter (μm) from peripheral (p) and visceral (v) depots in participants with (T2DM n=5) and without T2DM (NDM, n=5). Boxes show interquartile ranges, and bars represent highest and lowest values. * p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001, with paired t-test for comparison between the two groups and ANOVA.

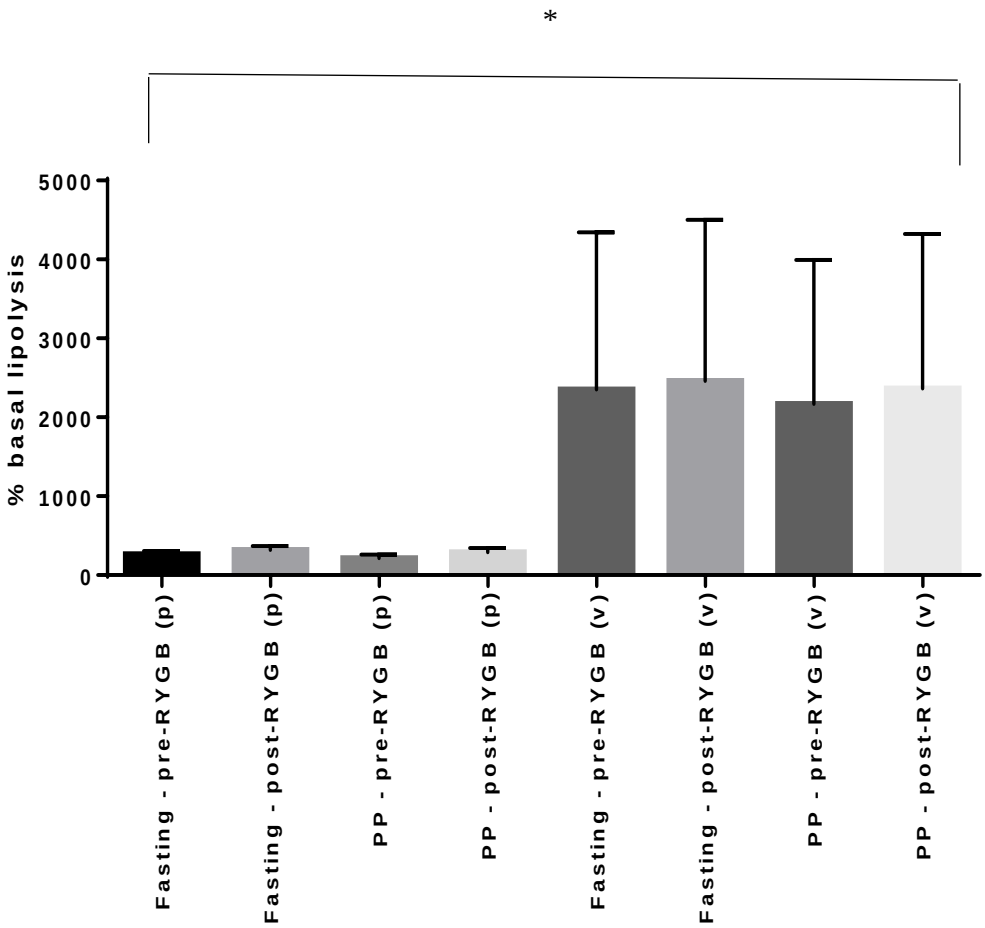


Figure 6.3.2.1 Graph of the mean and sem of the effect of fasting and post-prandial plasma taken around RYGB surgery upon basal lipolysis (%) in human adipocytes (visceral and peripheral). (p) = peripheral, n=8; (v) = visceral, n=7.* p<0.05, **p<0.01; *** p<0.001.

6.3.2.2 Gut hormones

6.3.2.2.1 Insulin

Insulin suppresses lipolysis in human adipocytes in both basal and stimulated conditions, $p<0.0001$. Increasing insulin concentrations suppresses lipolysis in a dose-dependent manner in human adipocytes from both peripheral and visceral depots, in both basal and stimulated conditions (all $p<0.001$), figure 6.3.2.2.1.

6.3.2.2.2 GLP-1

In this study, GLP-1 had no effect upon lipolysis in human adipocytes from either peripheral or visceral depots, in both basal and stimulated conditions (all $p>0.05$), figure 6.3.2.2.2.

6.3.2.2.3 GIP

In this study, GIP had no effect upon lipolysis in human adipocytes from either peripheral or visceral depots, in both basal and stimulated conditions (all $p>0.05$), figure 6.3.2.2.3.

6.3.2.2.4 PYY

PYY had a lipolytic effect on human adipocytes from both peripheral and visceral depots in basal state, both $p<0.0001$, but no significant difference was detected in stimulated state, $p>0.05$. On closer analysis, PYY was lipolytic at 10^{-7} concentration ($p<0.0001$) in adipocytes from both peripheral and visceral depots in basal state but not at 10^{-9} or 10^{-12} M, $p>0.5$, figure 6.3.2.2.4.

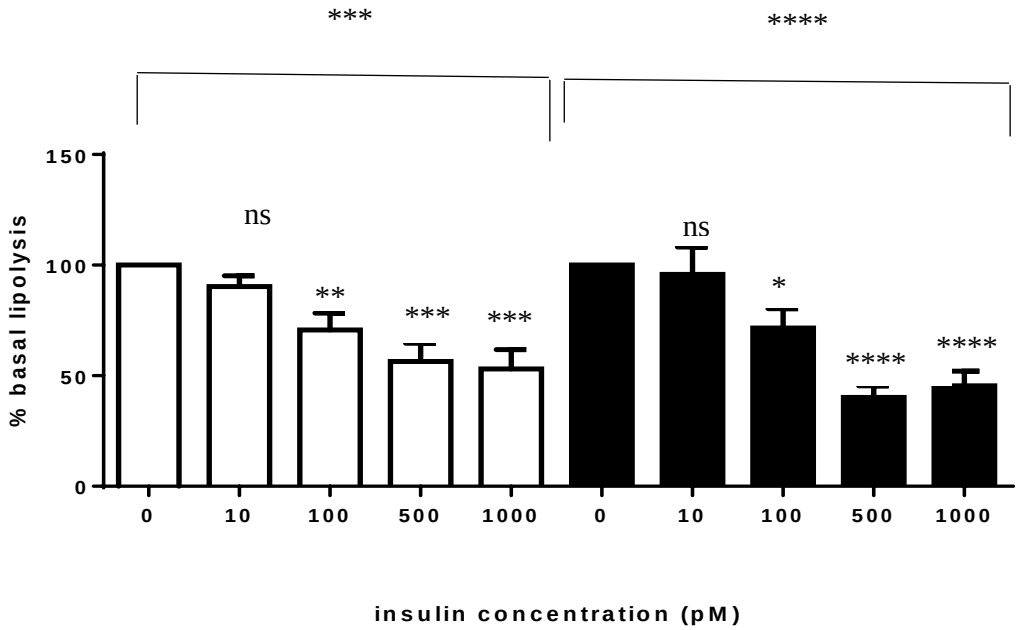
6.3.2.2.5 Ghrelin

In this study, ghrelin had no effect upon lipolysis in human adipocytes from either peripheral or visceral depots, in both basal and stimulated conditions (all $>p>0.05$), figure 6.3.2.2.5.

6.3.2.2.6 Combined Insulin and incretins

When adipocytes are subjected to varying degrees of insulin and GLP-1 or GIP, in stimulated state, although a significant difference is detected between the groups (both peripheral and visceral depots, $p<0.0001$), no difference was detected between when the same insulin concentration data was analysed with different GLP-1 or GIP concentrations (all conditions $p>0.05$), figures 6.3.2.2.6.1 and 6.3.2.2.6.2 respectively.

A



B

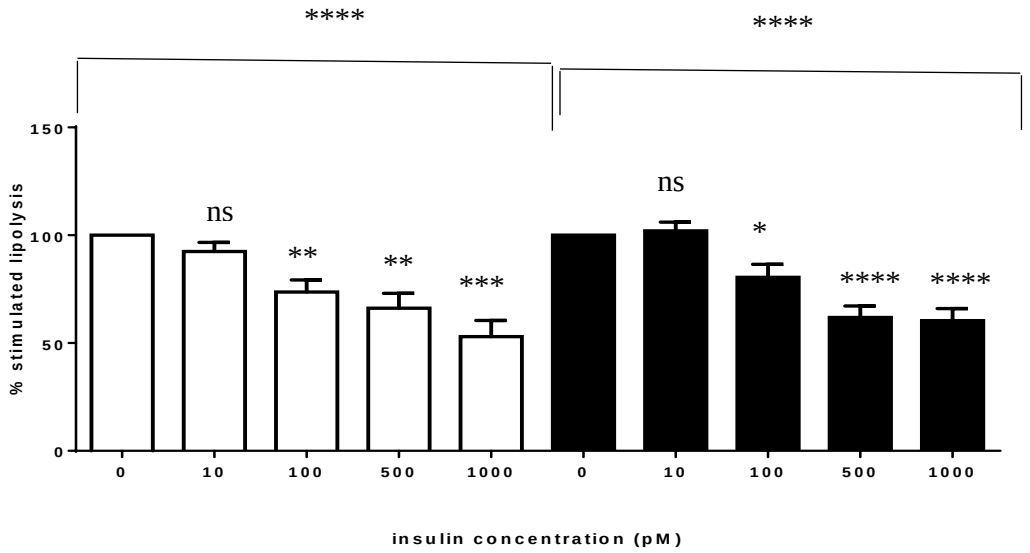
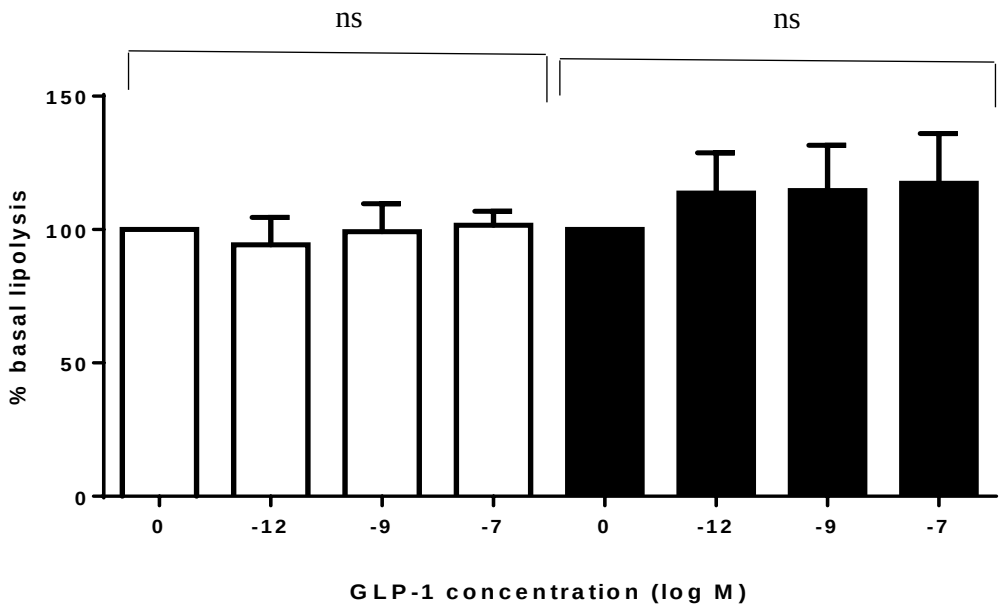


Figure 6.3.2.2.1 Box plot of the anti-lipolytic effect of insulin upon human adipocytes from both peripheral and visceral depots.

Effects were insulin were measured against 8-bromo-cAMP-stimulated lipolysis at 0,10,100,500 and 1000 pM. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 (n=9 peripheral, n=10 visceral). Data is presented as mean +/- SEM. Graph A is basal state and B is stimulated state. Clear box is peripheral adipocytes and shaded box is visceral adipocytes.

A



B

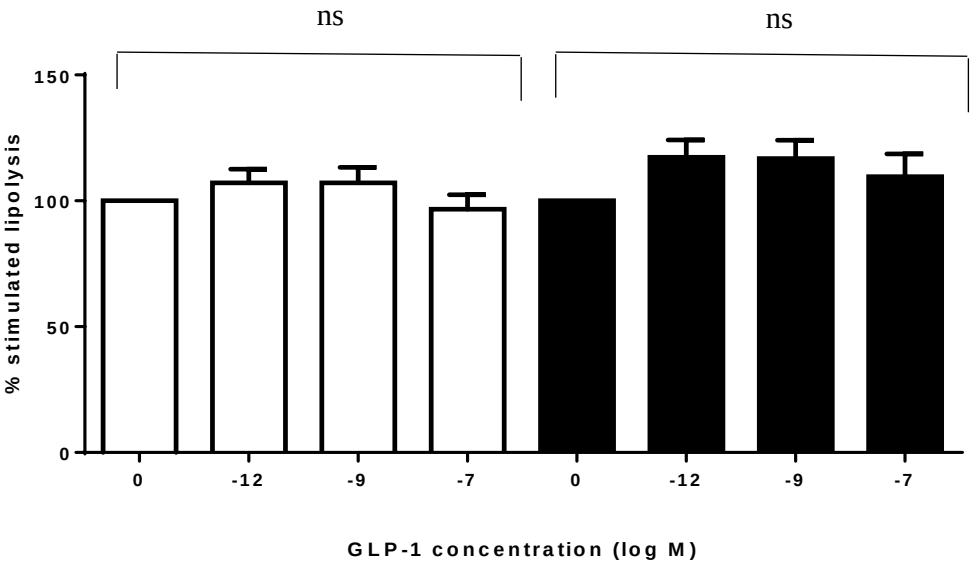
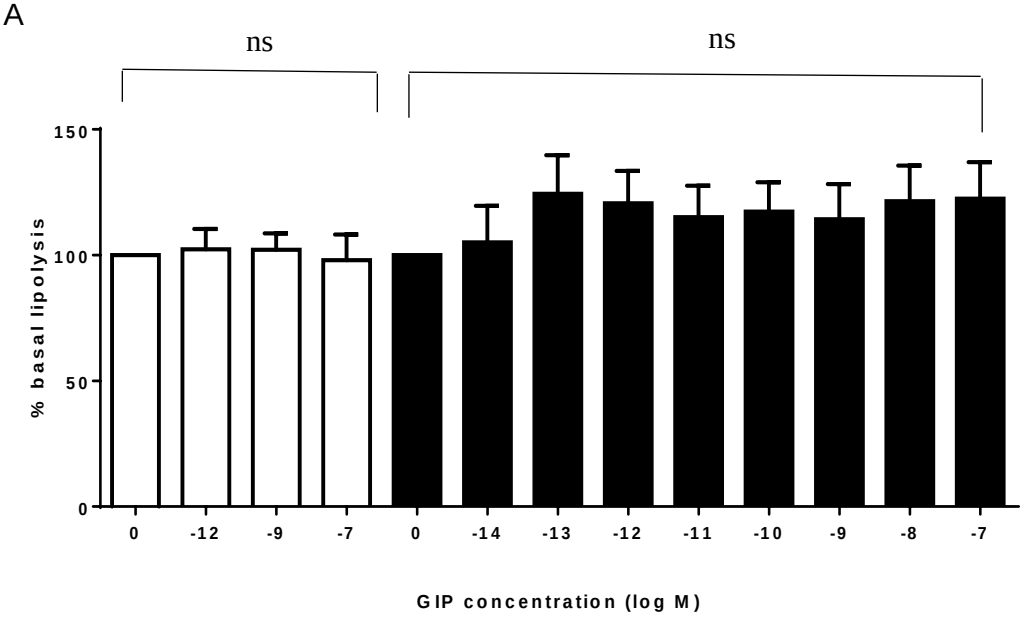


Figure 6.3.2.2.2 Box plot of the effect of GLP-1 upon lipolysis in human adipocytes from both peripheral and visceral depots.

Effects were measured against 8-bromo-cAMP-stimulated lipolysis at 0, -12, -9, -7 logM. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 (n=9 peripheral, n=10 visceral). Data is presented as mean +/- SEM.
Graph A is basal state and B is stimulated state. Clear box is peripheral adipocytes and shaded box is visceral adipocytes.

A



B

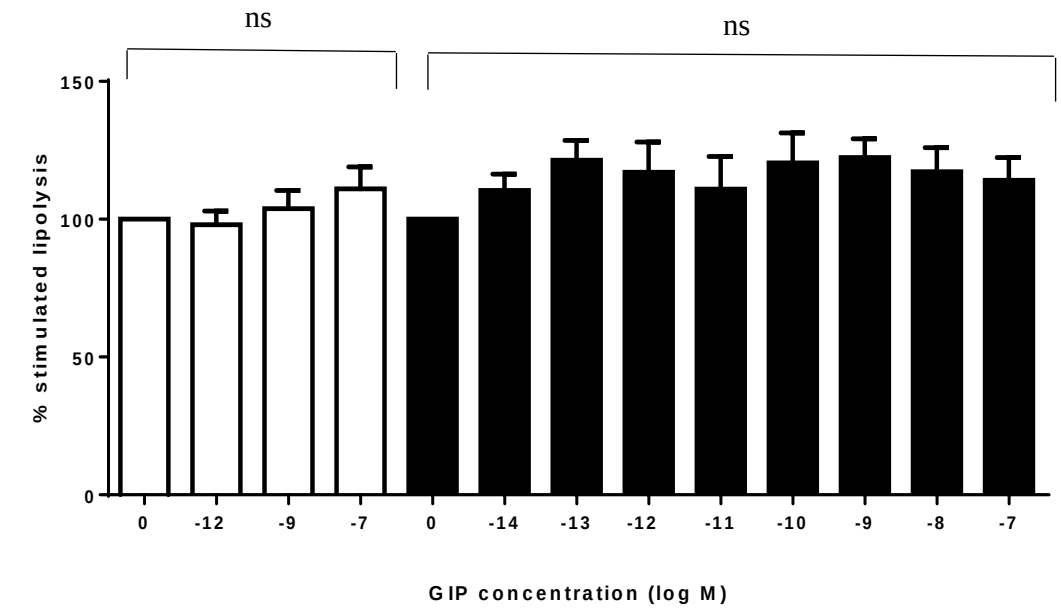


Figure 6.3.2.2.3 Box plot of the effect of GIP upon lipolysis in human adipocytes from both peripheral and visceral depots.

Effects were measured against 8-bromo-cAMP-stimulated lipolysis at 0, -14 - -7 log M. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 (n=9 peripheral, n=10 visceral). Data is presented as mean +/- SEM.
Graph A is basal state and B is stimulated state. Clear box is peripheral adipocytes and shaded box is visceral adipocytes.

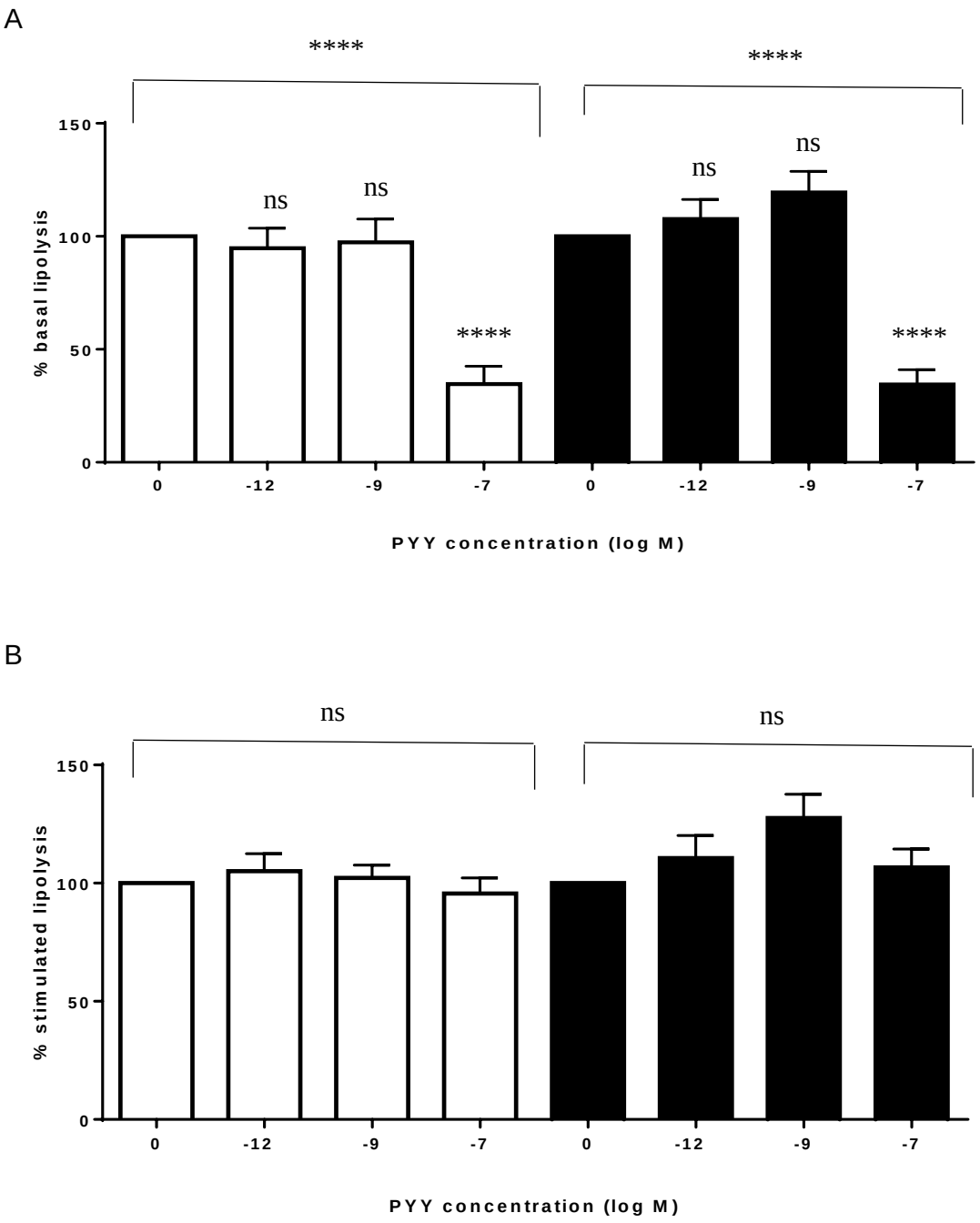
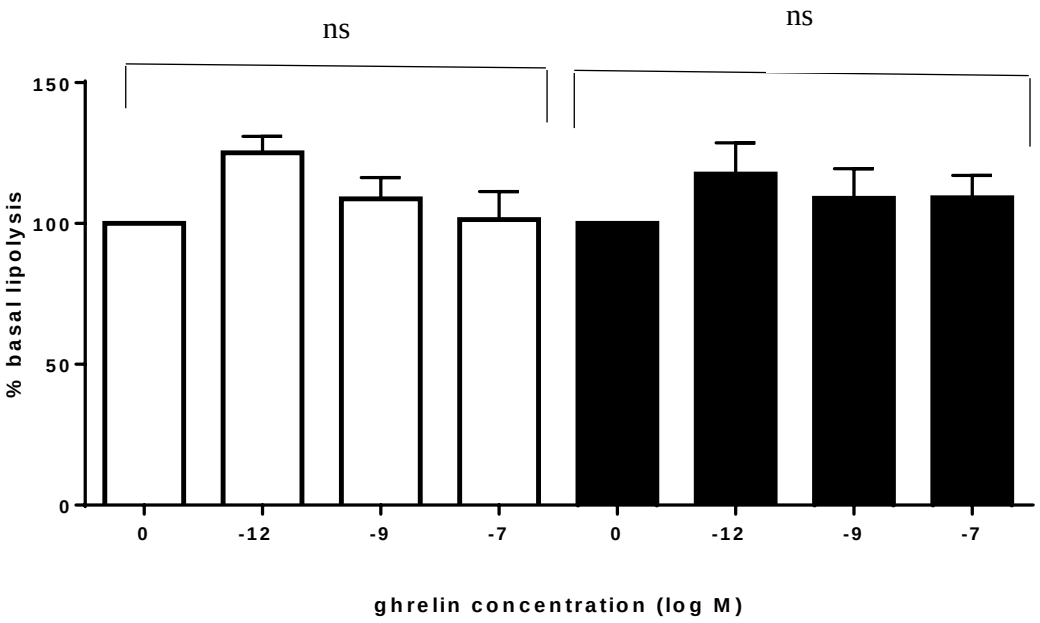


Figure 6.3.2.2.4 Box plot of the effect of PYY upon lipolysis in human adipocytes from both peripheral and visceral depots.

Effects were measured against 8-bromo-cAMP-stimulated lipolysis at 0, -12, -9, -7 log M. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 (n=9 peripheral, n=10 visceral). Data is presented as mean +/- SEM.
Graph A is basal state and B is stimulated state. Clear box is peripheral adipocytes and shaded box is visceral adipocytes.

A



B

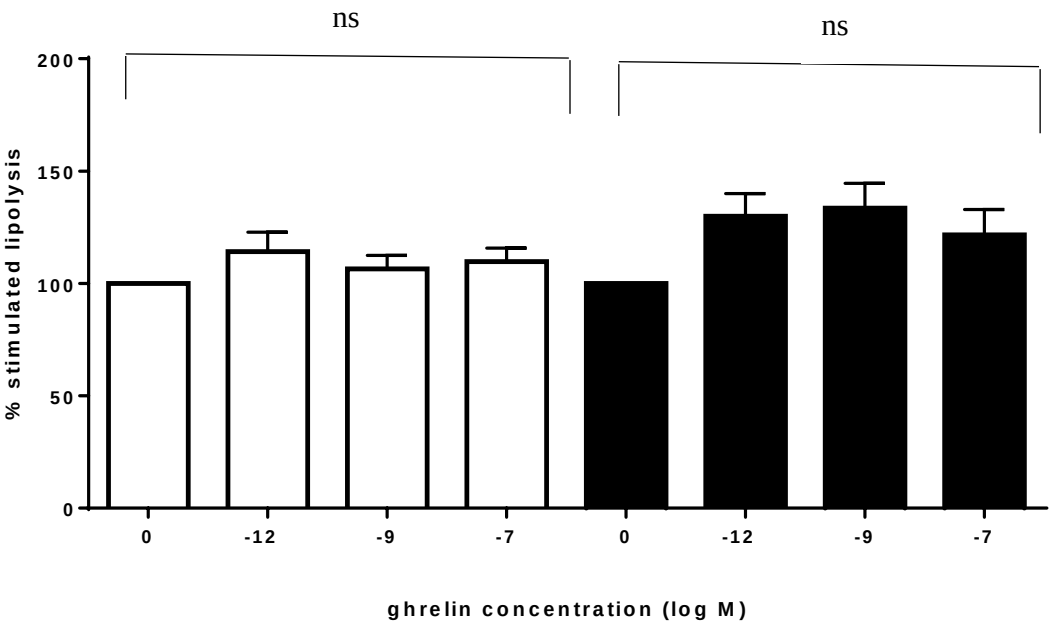


Figure 6.3.2.2.5 Box plot of the effect of ghrelin upon lipolysis in human adipocytes from both peripheral and visceral depots.

Effects were measured against 8-bromo-cAMP-stimulated lipolysis at 0, -12, -9, -7 log M. *p<0.05, **p<0.01, ***p<0.001, ****p≤0.0001 (n=9 peripheral, n=10 visceral). Data is presented as mean +/- SEM.
Graph A is basal state and B is stimulated state. Clear box is peripheral adipocytes and shaded box is visceral adipocytes.

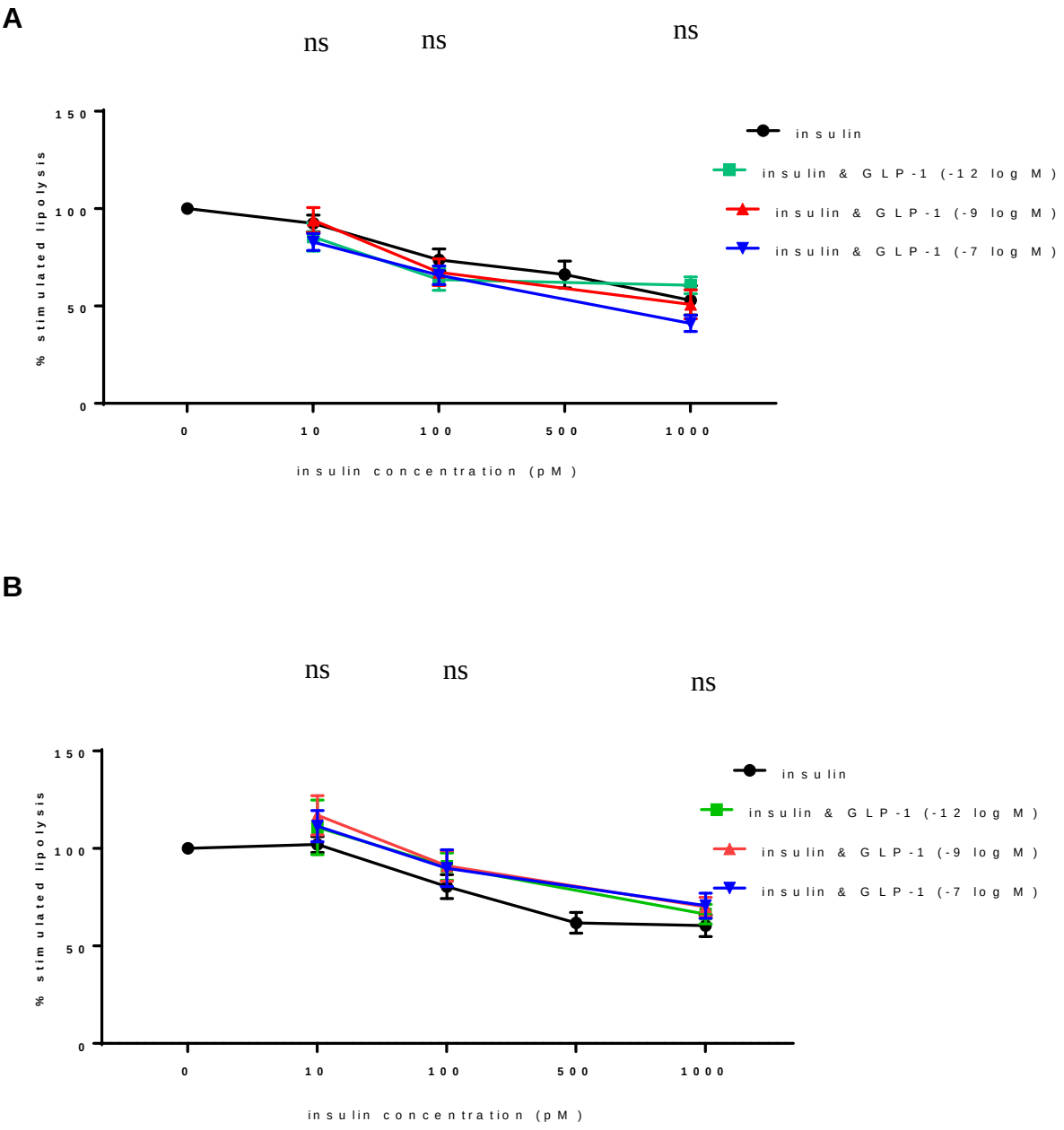
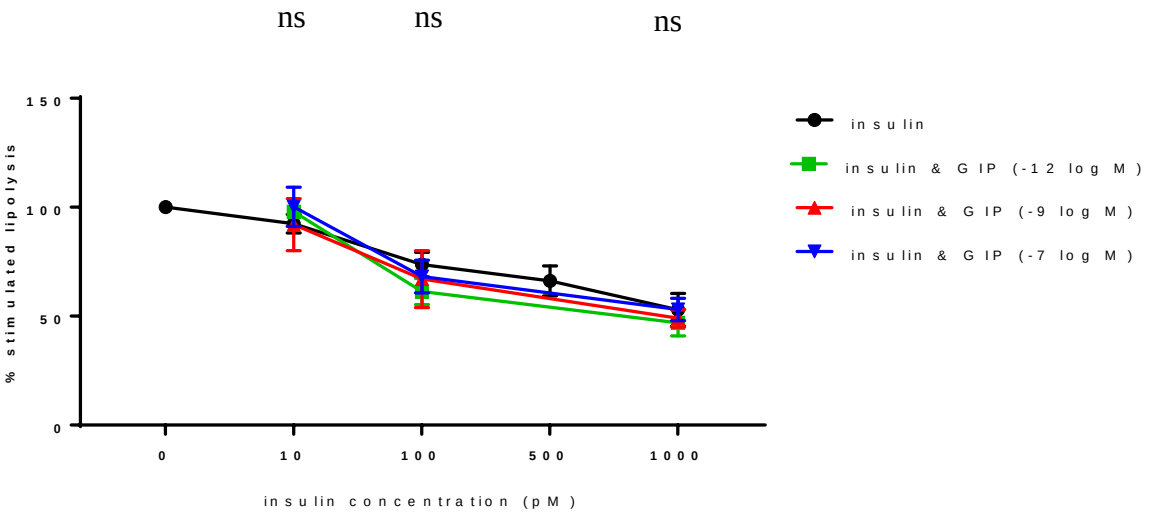


Figure 6.3.2.2.6.1 Graph of the antilipolytic effects of insulin in combination with GLP-1 at varying concentrations upon human adipocytes from both peripheral and visceral depots
The combined antilipolytic effects of insulin (10, 100, 1000 pM) and GLP-1 (-12, -9, -7 log M) were measured against 8-bromo-cAMP-stimulated lipolysis. Data is presented as mean +/- SEM. ns = p>0.5.
Graph A is peripheral adipocytes, n=9 except for the insulin 1000pM & GLP-1 -12 logM condition, n=3.
Graph B is visceral adipocytes, n=10 except for the insulin & GLP-1 -9 and -7 log M conditions, n=9.

A



B

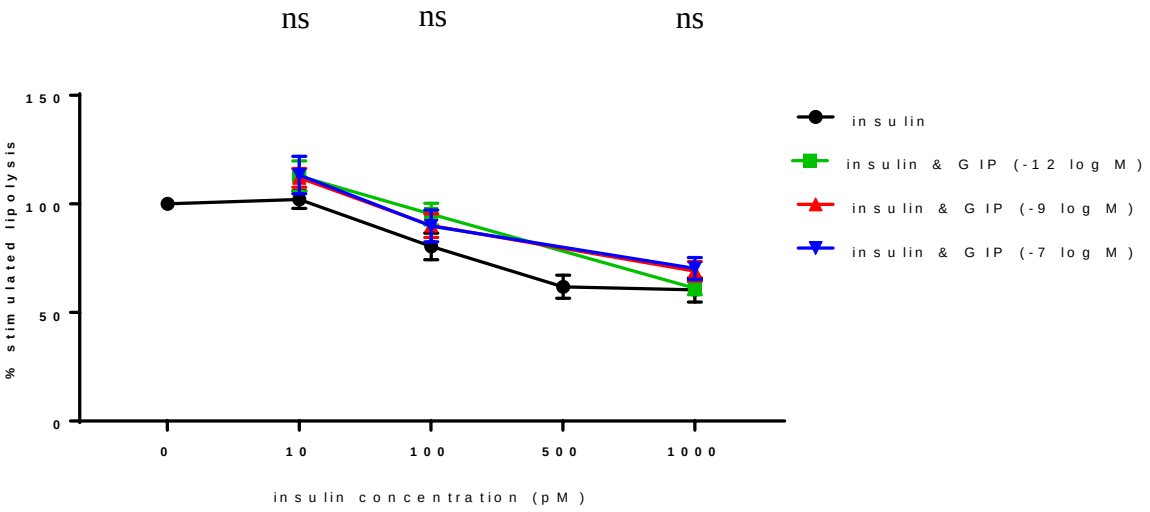


Figure 6.3.2.2.6.2 Graph of the antilipolytic effects of insulin in combination with GIP at varying concentrations upon human adipocytes from both peripheral and visceral depots
The combined antilipolytic effects of insulin (10, 100, 1000 pM) and GIP (-12, -9, -7 log M) were measured against 8-bromo-cAMP-stimulated lipolysis. Data is presented as mean +/- SEM. ns = p>0.5.
Graph A is peripheral adipocytes, n=9 except for the insulin & GIP -12 and insulin & GIP -9 logM conditions, n=7.
Graph B is visceral adipocytes, n=10.

6.4 Discussion

The dramatic improvements in IR and tailoring of insulin secretion following RYGB surgery in the morbidly obese, occurs before weight loss.[395] As previously discussed in earlier chapters, raised insulin levels in the post-prandial period enhance TG storage in AT, inhibit lipolysis,[288,319] and suppress the endogenous appearance rate of NEFA.[83,314] Paradoxically, increased NEFA levels can induce IR, impair insulin secretion and reduce insulin biosynthesis.[77,92,93,403] In turn, increasing lipolysis and driving NEFA levels higher as adipocyte insulin sensitivity deteriorates.[288,491-493] Increased rates of lipid turnover precede the development of T2DM in subjects with a family history of T2DM[496-498] or non-diabetic obesity,[499,500] linking this with causation.

Changes in post-prandial insulin and other gut hormone levels post-RYGB can impact upon plasma lipid flux through lipolytic mechanisms.[170,345,346,606] As a reduction in NEFA levels using the antilipolytic agent Acipimox has been shown to reverse IR,[81] changes in the NEFA/insulin relationship around RYGB may support initial improvements seen in IR.[11,20,95] The importance of this mechanism is still under debate as clamp studies demonstrate that hepatic insulin sensitivity, rather than peripheral, improves following RYGB.[120]

In this chapter, I have established the presence of relevant gut hormone and fatty acid receptors gene expression on human AT from peripheral and visceral depots. In addition, I studied the impact of fasting and postprandial plasma and relevant gut hormone changes around RYGB upon lipolysis in human adipocytes from both peripheral and visceral AT depots.

6.4.1 Relevant gene expression

Adipocytes have multiple hormone receptors on their surface including adrenaline (β1, 2, 3; α), insulin, growth hormone, insulin-like growth factor, GK-R (cortisol), PPAR.[294] In addition the receptors for several gut hormones have been detected in human adipocytes: insulin,[327] GLP-1,[295] GIP,[123,296,634] PYY,[297] ghrelin,[298,299] FFAR2 (GPR43)[300] and GPR120.[301] This study corroborates these findings, confirming gene expression of gut hormone receptors for insulin, GLP-1, GIP, PYY and ghrelin in human adipose tissue from both visceral and peripheral AT depots. Gene expression of GLUT4 receptors and fatty acid receptors were also detected suggestive that they may play a role in fat cell processing.

Variation in hormone receptors between the AT depots supports the concept that adipocytes offer differing roles relating to site. The metabolically more active visceral adipocytes[635] having larger number of adrenergic receptors on the cell surface,[302] higher GIPR gene expression levels[303] and higher lipolytic activity.[302] GIPR gene expression has also been shown to be reduced in peripheral AT in obese-IR, resistant to moderate weight reduction, questioning the role of hyperinsulinaemia in regulating GIPR gene expression.[303] Conversely, increased gene expression for both gut hormone and fatty acid receptors in peripheral AT relative to visceral was shown in this study, highlighting the need to perform quantitative gene expression and immunohistochemistry studies to further investigate these disparities.

6.4.2 The effect of RYGB surgery upon lipolysis

Rapid lipid mobilisation occurs post-RYGB, with a reduction in visceral[636] but to a lesser extent subcutaneous AT depots. This results in elevations of NEFA due to enhanced lipid turnover.[288] As visceral is more metabolically active than subcutaneous fat,[637] this will explain the AT depot disparity. This study corroborates this effect, combined with a significant variability in the

plasma visceral experiments, unfortunately highlighting an under-powering in this group, when studying these cells in further stimulated conditions. Although insulin is the most likely mediator of these effects, the ability of insulin to induce antilipolysis and stimulate NEFA re-esterfication is reduced in visceral adipocytes compared to peripheral, due to a reduction in insulin receptor autophosphorylation and signal transduction through an IRS-1 associated PI 3-kinase pathway.[327]

In agreement with my findings, other groups have noted initial increases in the basal lipolytic rate post-RYGB[441,638,639] but, this reduced at six[640] and twelve months.[441] This interestingly supports an augmentation of process however the short-term following RYGB is such a dynamic phase that there are many contributing factors to investigate e.g. weight loss, increased energy intake, hormone receptor upregulation, gut microbiota change, to name a few.

Mixed reports regarding the insulin effect upon lipolysis post-RYGB exist.[638,641] In vivo clamp studies suggest that RYGB does not impact upon tissue-specific insulin resistance in the early post-RYGB period.[439,441] In contrast, others groups have found an increase in lipolysis short term post-RYGB together with improvements in insulin stimulated anti-lipolysis and increased AT mitochondrial respiratory capacity through increases in the phosphorylation system ratio.[642] In addition, basal lipolytic rate per fat mass is unchanged following diet-induced weight loss but increased in parallel to a decrease in fasting insulin concentration with surgically induced weight loss post-RYGB.[642]

Camastra et al.[441] found that although early post-RYGB, tissue sensitivity to insulin is little changed, the sharp fall in insulin levels induced by the energy intake deficit reduces inhibition of lipolysis, whereby fatty substrates flood the circulation and force lipid oxidation and weight loss ensues. They postulated that these effects were due to the energy intake deficit reducing the plasma insulin levels and subsequent reduction in anti-lipolytic effect[643] but I would argue that as shown in chapter 3, paired post-prandial insulin levels are

reduced post-RYGB irrespective of energy intake, most likely due to impact upon intestinal absorption, transit and gut flora changes.[66] Whilst extreme caution should be used attempting to relate the in vitro data of this study with in vivo metabolic states, the study showed no difference in the effect of early RYGB plasma on meal suppression of lipolysis, on a larger fat mass model and dynamic effects in vivo. Supportive that this effect may not be due to post-prandial dietary metabolites and/or gut hormone changes.

That being said, NEFA are increasingly considered as extracellular signalling molecules, no longer merely nutrients and metabolic substrates. Many studies reporting the effects of fatty acid upon glucose-stimulated insulin release with free fatty acid receptors (FFARs) and their potential role as drug targets for T2DM.[644-653] Their effect upon lipolysis remains to be elucidated and repeat experiments with delayed time point sampling, to include higher fatty acid flux in the plasma and artificial manipulation studies would be of use.

6.4.3 The effect of gut hormones upon lipolysis

The anti-lipolytic effects of insulin are well documented, and supported by this study. As the antilipolytic effect of insulin occurs at a lower concentration than is required to stimulate glucose metabolism,[654-656] many believe that this is its primary role. The post-prandial rise in insulin promotes TG storage in the AT and inhibits lipolysis,[314] and increases the rate of re-synthesis of TG from NEFA i.e. re-esterification,[327] optimising energy storage. Adipocytes have marked increase in insulin sensitivity in the post-prandial period.[657] This effect is present in obese subjects in the fasting state but unlike healthy weight subjects, no further increase in insulin sensitivity is demonstrated in the postprandial state,[657] hinting that in obesity the adipocytes have defaulted to a storage/post-prandial state even when fasted. Despite this, in vivo, fasting reduces systemic sensitivity to antilipolytic effects of insulin[329] in contrast to findings in vitro.[330] Given the alterations in energy intake,

intestinal absorption, gut microbiota and transit following RYGB, this mechanism could contribute to the early increases in lipolysis post-RYGB.

Regional differences in the influence of obesity upon insulin binding of human adipocytes has been reported.[657-660] The antilipolytic and re-esterification effects of insulin are reduced in visceral compared to peripheral adipocytes, presumed due to reduced insulin receptor autophosphorylation and signal transduction through an IRS-1 associated PI 3-kinase pathway adipocytes.[327] This results in increased delivery of NEFA to the liver by the visceral fat depot and is believed to be an important pathophysiological factor contributing to several of the metabolic complications in obesity.[661-663]

The effect of GLP-1 on lipolysis remains controversial. Synthetic GLP-1 treatment improves post-prandial lipidaemia and reduces plasma NEFA levels in concert with insulin secretion. It was proposed that this was a reflection of the antilipolytic effect of insulin.[664] Nevertheless, in its own right groups purport GLP-1 to be lipogenic at low concentrations and lipolytic at high concentrations,[170] and lipolytic in other studies.[345] This data suggests a tendency to increase lipolysis but it did not reach statistical significance. No synergistic effect was detected when GLP-1 was combined with insulin.

GIP has been shown to impair the insulin sensitivity of glucose uptake in human adipocytes,[665] an effect which may be reduced post-RYGB as GIP levels are reduced, see chapter 4.[57,129] In vitro, GIP stimulates lipolysis in both human[339] and rodent adipocytes, reversible with ANTIGIP, and can increase NEFA reesterification.[120] Although this study suggested increased lipolysis with GIP stimulation, it failed to reach statistical significance. This effect is inhibited by insulin in rodents,[347] and in agreement with this study, a synergistic anti-lipolytic effect of this combination with insulin was not detected.[120]

PYY reduced basal lipolysis at high concentrations, partially corroborating previous studies but, we were not able to reproduce the inhibition of stimulated lipolysis, using 8-bromo-cAMP, previously reported with

isoproterenol- and forskolin-induced lipolysis.[346] In that instance, this effect was reversed using *Bordetella pertussis* toxin, which blocks the effects on the GTP-binding regulatory proteins in human adipocytes,[347] suggesting that this effect is likely mediated by adenylate cyclase inhibition.[346] Post-RYGB, fasting PYY levels remain unchanged but elevated in the post-prandial period. The anti-lipolytic effect was only noted at high concentrations, as such an in-vivo effect cannot be presumed.

Ghrelin can stimulate insulin-induced glucose uptake in adipocytes.[239] In vivo, Ghrelin infusion induces lipolysis (as assessed by plasma NEFA levels and interstitial glycerol concentrations using a microdialysis technique) and IR.[351] Perhaps more accurately, it reduces the anti-lipolytic effect of hyperinsulinaemia, in peripheral AT.[351] This antilipolytic effect occurs by binding to a specific receptor, distinct from GHS-R1a, rodent model.[352,353] Although our data did not reach statistical significance, at lower concentrations ghrelin appeared to increase lipolysis. The corollary has also been shown using cultured human peripheral adipocytes, which were lipogenic when incubated with Octanoyl-(OTG) and des-acyl (DSG) ghrelin. DSG was shown to alter lipolysis, lipogenesis and leptin secretion.[299] Most studies, ours included, have found a reduction or no difference in fasting and postprandial ghrelin levels post-RYGB.[23,36] As such it is unlikely that it has a dominant effect upon lipolysis around this period.

6.4.4 Strengths and limitations

This prospective study is unique as it is the first in vitro study designed to investigate the link between lipolysis and plasma and gut hormone changes particular to RYGB, using human adipocytes. As differences exist in the lipolytic effects of hormones upon adipocytes from AT depots,[327,666] both visceral and peripheral were assessed.

This study has limitations. Sample size calculations were designed to reach significance of 0.8. Despite this, the small number of cases in the time frame allowed for recruitment, availability and cost influenced the recruitment of subjects with diversity in both gender and ethnicity. Although gender should not effect lipolysis outcomes,[666] ethnicity may.[667] Nevertheless, participants served as their own controls for the gene expression levels and to standardise the lipolysis results for analysis, increasing the validity of the findings.

Subjects were in a negative energy balance in the early post-operative period and these results may not fully reflect what happens once a stable weight is reached. Both physical activity and pre-operative diet were not strictly controlled which can also introduce further bias. High- and low-fat diets have been shown to effect ISO-mediated stimulation of lipolysis in murine adipocytes.[668] To minimise these confounding factors, paired gut hormone and plasma data was used and the AT samples were taken at the start of the surgical procedure to minimise the surgical stress and inflammatory insult.

It should also be noted that this series of experiments were performed to allow assessment of both lipolytic and anti-lipolytic effects as the effect of plasma around RYGB upon adipocytes was unknown. The basal activities in these studies, as well as the magnitude of stimulation achieved under experimental conditions described here, may not reflect those activities in vivo. The conditions are designed to permit comparisons of adipocyte behaviour. The so-called basal activities measure in the present studies are most likely artificially low and are designed to permit viewing of maximal stimulated activities.[668]

The basal experimental conditions studied here were performed without PIA. As there were no previous studies into the effect of RYGB plasma upon lipolysis and as such the lipolytic or anti-lipolytic effects of this plasma upon human adipocytes was unknown. It was thought that a fully inhibited basal condition (ADA and PIA) may have masked an anti-lipolytic response and

was therefore not used. Following this study, further studies can be tailored accordingly.

These findings would support further studies controlling for gender, ethnicity and multiple time point post-RYGB. Assessing not only plasma changes, gut adaptation but adipocyte adaptive response over time.

This study achieved the primary objective of assessing the effect of fasting and post-prandial plasma and gut hormone changes around RYGB surgery upon human lipolysis. The increased basal lipolysis rate post-RYGB together with, or due to, improvements in insulin sensitivity, a reduced basal insulin and PYY levels will support the weight loss process. This initially will increase NEFA levels, due to increased fat mass, but reduces latterly,[507] as adiposity and lipotoxicity are reversed. Sadly, this raises more questions than it answers as the pathways through which this occurs and the role of NEFA, as a passive or more likely active participant in lipolysis, remain to be elucidated.

6.5 Conclusion

Lipolysis is increased post-RYGB in both peripheral and visceral AT. This study shows that factors in the plasma following surgery may be responsible, if not contribute, to changes in lipid flow seen post-operatively. Gut hormone changes around RYGB, in particular insulin and PYY, rather than GLP-1 and GIP, are of interest with respect to lipolysis. The effects of NEFA themselves upon this process should be determined.

7. Conclusions

The closely intertwined relationship of the diet and subsequent disease manifestations such as obesity and T2DM, highlight the role with which both the dietary metabolites and the gut hormones play. The finding that RYGB surgery results in remission of T2DM and hyperlipidaemia, prior to weight loss, unhinges this link temporarily. This offers a unique opportunity to study this in more detail in a human model.

Dramatic improvements in insulin sensitivity globally following RYGB surgery improve core processes at the islet, hepatocyte and adipocyte level. This coupled with increased incretin secretion and changes in exogenous and endogenous lipid flow exacerbate these effects.

Although it is likely that post-prandial hormonal alterations improve adipocyte lipolysis through their peripheral effects, it is most likely the global reduction in insulin levels, thereby reducing the anti-lipolytic effect, and overall improvements in IR are responsible for these findings, not the peripheral effects of incretins. The role of fatty acid metabolites in this process remains of interest.

8. Future work

- Formal assessment of the effects of RYGB surgery upon fasting plasma glucose and 2h OGTT (currently used for diagnosis and T2DM) – ideally assessed as a prospective study into the effect of RYGB surgery upon fasting and 2h OGTT in participants with and without T2DM at several time points up to 1yr, with an obese NDM control group
- RYGB surgery reverses the dyslipidaemia of obesity. As such there maybe no benefit from the addition of a statin treatment to their long-term cardiovascular risk – ideally assessed using a randomised controlled trial of continuing statin versus stopping statin treatment following RYGB surgery
- Quantification of the effect of RYGB upon dietary fat absorption using radiolabelled LCTG and MCTG assessed around RYGB surgery (ethical and R&D approval obtained)
- Quantification RT-PCR of gut hormone and free fatty acid receptors on human adipocytes from different AT depots and the impact of RYGB surgery upon this
- Immunohistochemistry analysis of gut hormone and free fatty acid receptors on human adipocytes from different AT depots and the impact of RYGB surgery upon this
- The lipolytic effect of plasma around RYGB surgery taken at later time points i.e. whether the changes seen in this experiment persist long-term or attenuate, consistent with weight loss plateauing
- The effect of plasma and gut hormone changes around RYGB surgery upon adipocyte re-esterification
- The effect of differing NEFA levels upon lipolysis and glucose uptake in human adipocytes

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10. Appendices

- Appendix 1 – Nutritional information for Belgium chocolate Haagen-Dazs ice-cream
- Appendix 2 – King's College Hospital relevant biochemical assay protocols
- Appendix 3 – Funnel plots of the meta-analysis of the effects of RYGB upon plasma lipids

Appendix 1 – nutritional information for Belgium Chocolate Haagen-Dazs ice-cream

Belgium Chocolate ice-cream

	Per 100ml serving
ENERGY	1155kJ / 277kcal
Protein	3.9g
Carbohydrate	23.8g
of which sugars	19.6g
Fat	18.4g
of which saturates	11.1g
Fibre	1.7g
Sodium	0.06g

Ingredients

Fresh cream (29%), skimmed milk, sugar solution (sugar, water), dark Belgian Chocolate (13%) (cocoa mass, sugar, emulsifier: soya lecithin, natural flavouring: vanilla), chocolate chunks with vegetable oil (10%) (chocolate [sugar, cocoa mass, cocoa butter, emulsifier : soya lecithin, natural flavouring : vanilla], cottonseed oil, coconut oil), egg yolk, cocoa powder, sugar, natural flavouring: vanilla.

Contains milk, egg and soya ingredients.

Information obtained from website: www.haagen-dazs.co.uk (23/01/10)

Appendix 2 - King's College Hospital relevant biochemical assay protocols

Glucose

Glucose reagent supplied by Bayer Diagnostics Europe Ltd, Bayer House, Strawberry Hill, Newbery, Berks. RG14 1JA

Method

The Bayer Advia method for the measurement of glucose uses an endpoint enzymatic reaction. Glucose is converted to gluconic acid and hydrogen peroxide using the enzyme glucose oxidase. In the presence of peroxidase, the hydrogen peroxide then reacts with 4-aminophenazone and phenol to produce a red quinoneimine dye. Absorbance is measured at 596/605 nm.

Technical

Testing of the precision of the assay has been performed prior to this study it the following way.

Each sample was assayed 2 times per run, 2 runs per day, for at least 10 days. Precision estimates computed by the manufacturer according to CLSI document EP05-A2, Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline.

	Within-Run		**Total**	
Level (mg/L)	**SD**	**CV (%)**	**SD**	**CV (%)**
4.4	0.02	0.6	0.07	1.6
16.3	0.08	0.5	0.19	1.2

Standardization

The ADVIA glucose oxidase method is traceable to the CDC Reference Method, which uses reference materials from the National Institute of Standards and Technology (NIST), via patient sample correlation and verified with NIST Reference serum. Assigned values of Bayer Chemistry Calibrator, Bayer Assayed Chemistry Controls, and ADVIA Chemistry Urine Controls are traceable to this standardization.

INSULIN CENTAUR (IRI)

The Advia Centaur Insulin assay is a two-site sandwich immunoassay using direct chemiluminescent technology which uses constant amounts of two antibodies. The first antibody, the Lite Reagent, is a monoclonal mouse anti-insulin antibody labelled with acridinium ester. The second antibody , in the solid phase, is a monoclonal, mouse anti-insulin antibody, which is covalently coupled to paramagnetic particles.
A direct relationship exists between the amount of insulin present in the patient sample and the amount of relative light units (RLU) detected by the system.

The system automatically performs the following steps:
1. Dispenses 25 uL of sample into a cuvette
2. Dispenses 50 uL of Lite Reagent and incubates for 5 minutes at 37°C
3. Dispenses 250 uL of solid phase and incubates for 2.5 minutes at 37°C
4. Separated, aspirates and washes the cuvettes with reagent water
5. Dispenses 300 uL each of acid reagent and base reagent to initiate the chemiluminescent reaction.

Technical Data

Dilutions

Samples with Insulin concentrations greater than 300 mU/L must be diluted and retested.

High dose Hook Effect

Patient samples with high insulin concentrations can cause a paradoxical decrease in the RLU. In this assay, patient samples with insulin concentrations as high as 3000 mU/L will assay greater than 300 mU/L.

Heterophilic antibodies

Heterophilic antibodies in human serum can react with reagent immunoglobulins, interfering with in vitro immunoassays . Patients routinely

exposed to animals or animal serum products can be prone to this interference and anomalous results may be observed.

Interferences

Specimens that are….	Demonstrate < 6% change in results up to
Haemolysed	125 mg/dL
Lipaemic	1000 mg/dL
Icteric	20 mg/dL
Proteinemic	12 g/dL

Specificity

The cross-reactivity of the Adivia Centaur was determined by spiking serum samples with the following compounds at the indicated concentrations. These compounds did not have a significant effect on the insulin measurement:

Substance	Amount added	Mean % recovery
Proinsulin	1 ug/mL	100.8
C-peptide	500 ng/mL	95.1
Gastrin-1	1 ug/mL	96.6
Glucagon	1 ug/mL	100.2
Secretin	1 ug/mL	101.6

Sensitivity and assay range

The Advia Centaur Insulin assay measures insulin concentrations up to 300 mU/L with a minimum detectable concentration of 0.5 mU/L.

Dilution Recovery

Five human serum samples in the range of 129.7 to 237.8 mU/L of insulin were serially diluted 1:2, 1:4 and 1:8 with insulin diluent and assayed as shown below:

Sample	Dilution	Observed (mU/L)	Expected (mU/L)	Recovery %

1	-	129.7		
	1:2	55.8	64.9	86.0
	1:4	21.4	32.4	84.6
	1:8	13.9	16.2	85.8
	Mean			58.5
2	-	192.7	96.4	
	1:2	105.5	48.2	109.4
	1:4	47.0	24.1	97.5
	1:8	24.1		100.0
	Mean			102.3
3	-	237.8	118.9	
	1:2	127.4	59.4	107.1
	1:4	56.1	29.7	94.4
	1:8	29.2		98.3
	Mean			99.9

4	-	229.9		
	1:2	114.9	114.9	100.0
	1:4	51.3	57.5	89.3
	1:8	25.2	28.7	87.8
	Mean			92.4
5	-	154.0		
	1:2	74.2	77.0	96.4
	1:4	33.6	38.5	87.2
	1:8	18.2	19.3	94.3
	Mean			92.6
Mean				94.6

Spiking recovery

Sample	Amount added	Observed	Expected	Recovery %
1	-	2.9		
	25.7	30.3	28.6	105.9
2	-	2.6		
	51.4	58.4	53.9	108.1
3	-	4.8		
	25.7	33.2	30.5	108.9
4	-	4.3		
	51.4	61.3	55.7	110.1
5	-	8.1		
	25.7	35.1	33.8	103.8
6	-	7.2		
	51.4	66.7	58.6	113.8
Mean				**108.4**

Precision

Three samples were assayed twice in 3 runs, on 3 systems (n=72 for each sample) over a period of 4 days. The following results were obtained:

Mean Insulin mU/L	Within run % CV	Run-to-run % CV	Total % CV
14.68	4.6	5.9	7.5
45.72	3.2	2.6	6.5
124.51	3.3	4.8	6.3

Glucagon-like-peptide-1 (active)

Method

Linco Research. 6 Research Park Dr. St Charles, Missouri 63304 USA.

This kit is for non-radioactive quantification of biologically active forms of Glucagon-Like Peptide-1 [i.e. GLP-1 (7-36 amide) and GLP-1 (7-37)] in plasma and other biological media. It is highly specific for the immunologic measurement of active GLP-1 and will not detect other forms of GLP-1 (e.g. 1-36 amide, 1-37, 9-36 amide or 9-37). The GLP-1 sequence is highly conserved between the species, with no sequence variation occurring in all mammals.

This assay is based, sequentially, on: 1) capture of active GLP-1 from the sample by a monoclonal antibody, immobilised in the wells of a microwell plate, that binds specifically to the N-terminal region of active GLP-1 molecule, 2) washing to remove unbound materials, 3) binding of an anti-GLP-alkaline phosphatise detection conjugate to the immobilised GLP-1, 4) washing off unbound conjugate, and 5) quantification of bound detection conjugate by adding MUP (methyl umbelliferyl phosphate) which in the presence of alkaline phosphatise forms the fluorescent product of umbelliferone. Since the amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1.

TECHNICAL DATA

Intra-assay precision

	Level 1	Level 2	Level 3	Level 4	Level 5
N	8	8	8	8	8
Mean (pg/mL)	4	8	12	28	76
CV%	8	7	6	7	9

Inter-assay precision

	Level 1	Level 2	Level 3	Level 4	Level 5

N	8	8	8	8	8
Mean (pg/mL)	4	8	12	28	76
CV%	13	12	7	7	<1

Sensitivity

The minimal detectable GLP-1 concentration is 2 pM

Human Gastric Inhibitory Polypeptide (GIP) (total)

Method

Linco Research. 6 Research Park Dr. St Charles, Missouri 63304 USA.

This kit is for non-radioactive quantification of human GIP in human serum, plasma, tissue extract and cell culture samples. This kit has 100% cross reactivity to human GIP (1-42) and GIP (3-42).

This assay is a Sandwich ELISA based, sequentially, on: 1) capture of human GIP molecules
from samples to the wells of a microtiter plate coated by a pre-titered amount of anti-GIP
monoclonal antibodies, 2) wash away of unbound materials from samples, 3) binding of a second biotinylated anti-GIP polyclonal antibody to the captured molecules, 4) wash away of unbound materials from samples, 5) incubation of streptavidin-Horseradish peroxidase conjugate to bind to the immobilized biotinylated antibodies, 6) wash away of free enzyme conjugates, and 7) quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5' tetramethylbenzidine. The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured human GIP in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of human GIP.

TECHNICAL DATA

Intra-assay precision

	Level 1	Level 2	Level 3	Level 4
N	6	6	6	6
Mean (pg/mL)	279	185	21	15
CV%	3.0	8.8	7.3	6.7

Inter-assay precision

	Level 1	Level 2	Level 3	Level 4

N	6	6	6	6
Mean (pg/mL)	166	134	50	26
CV%	11.8	2.3	3.3	6.1

Sensitivity

The minimal detectable GIP concentration is 8.2 pg/mL

Peptide YY (PYY) (total)

Explanation of the test

Peptide YY (P-YY), a novel 36 amino-acid amidated hormone is a component of the complex neuroendocrine control process. This gut hormone (fragment 3-36) when infused into subjects has been shown to reduce food intake in normal weight and obese individuals. PYY infusion also reduced the plasma levels of the hunger-promoting hormone ghrelin. PYY levels have been shown to drop pre-meal and then increase post prandially. In circulation, PYY exists at least in two molecular forms: 1-36 and 3- 36.

Method

Linco Research. 6 Research Park Dr. St Charles, Missouri 63304 USA.

Millipore's PYY (Total) Radioimmunoassay (RIA) Kit utilizes an antibody, which recognizes both the 1-36 and 3-36 forms of Human PYY. Sensitivity of 10 pg/mL can easily be achieved when using a 100µl serum or plasma sample in a two-day, disequilibrium assay (400 µl Total Volume).

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 40%-50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labelled form of the

antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The Millipore PYY (Total) assay utilizes 125I-labeled PYY and a PYY antiserum to determine the level of total PYY in serum, plasma or tissue culture media by the double antibody/PEG technique.

Technical Data

Sample requirements

Samples should be processed as quickly as possible and kept on ice to retard the breakdown of PYY. Treatment of the blood with Aprotinin is recommended at a final concentration of 500 KIU/mL of blood.

A maximum of 100 µL per assay tube of serum of plasma should be used. Tissue culture and other media may also be used. Care must be taken when using heparin as an anticoagulant, since excess will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.
For longer storage, specimens should be aliquoted and stored at ≤-20 ^{o}C. Multiple freeze/thaw cycles should be avoided.

Intra-assay precision

Sample no.	Mean pg/mL	%CV
1	82.7	9.4
2	111.1	2.9
3	542.6	3.6

Inter-assay precision

Sample no.	Mean pg/mL	%CV
1	82.7	8.5
2	111.1	7.1
3	542.6	5.5

Sensitivity

The lowest level of PYY that can be detected by this assay is 10 pg/mL when using a 100µL sample size.

High Dose Hook

Reference Range

Interpretation of Results

Performance

The following parameters of assay performance are expressed as Mean + Standard Deviation.

ED80 = 36 ± 5
ED50 = 103 ± 12
ED20 = 300 ± 38

Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

PYY RIA Crossreactivity

PYY 1-36 human 100%
PYY 3-36 human 100%
[Pro34] PYY 100%
[Leu31, Pro34] PYY 100%
Rat/Porcine PYY 1-36 <0.1%
Rat/Porcine PYY 3-36 <0.1%
HPPP <0.1%
NPY <0.1%
Human Leptin *
Glucagon *
Human Ghrelin *
Human Insulin *
GLP-1 *
*-Not detectable

Precision

Within and Between Assay Variation

Sample No	Mean pg/mL	Within %CV	Between %CV
1	82.7	9.4	8.5
2	111.1	2.9	7.1
3	542.6	3.6	5.5

Within and between assay variations were performed on three human plasma samples containing varying concentrations of Human PYY. Data (mean and %CV) shown are from one assay with eight duplicate determinations of each plasma sample for intra-assay precision. For inter-assay precision, data are generated using eight separate assays run for the three samples in duplicate.

Recovery

Spike and Recovery of PYY in Human Plasma

Sample No	PYY added pg/mL	% recovery
1	40	111
2	320	96
3	1280	83

Varying concentrations of Human PYY were added to three different human plasma samples and the PYY content was determined by RIA. Mean of the observed levels from duplicate determinations in one assay are shown. Percent recovery was calculated as the observed over expected multiplied by 100.

Linearity

Effect of Plasma Dilution

Sample No	Volume sampled (uL)	Observed pg/mL	Expected pg/mL	% expected
1	100	161	161	100

	75	162		101
	50	170		105
	25	180		112
2	100	156	156	100
	75	167		107
	50	169		7108
	25	179		115
3	100	199	199	100
	75	220		111
	50	217		109
	25	246		124
4	100	124	124	100
	75	125		101
	50	133		107
	25	155		125

Aliquots of pooled Human Plasma containing varying concentrations of PYY were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2, and 4 representing 100_l, 75_l, 50_l, and 25_l respectively, were applied in calculating observed concentrations.

Human Active Ghrelin

Method

SCETI K.K. Medical Section, DF Kuasumigaseki Place, 3-6-9 Kuasumigaseki, Chiyoda-ku, Tokyo, Japan.

This kit is for non-radioactive quantification of human active Ghrelin human plasma samples.

Ghrelin a novel growth hormone releasing peptide is an acylated peptide that stimulates the release of growth hormone from the pituitary gland. It was isolated from rat stomach and the structure was determined as a peptide consisting of 28 amino acids by Dr Kenji Kankawa. The Ser3 residue of ghrelin is modified by n-octanoic acid, a modification necessary for hormone activity.
This active ghrelin Elisa kit measures the active form of ghrelin based on the principle of 2 site sandwich enzyme linked immunosorbent assay. It can detect not only octanoylated human ghrelin but also octanoylated rat/mouse ghrelin. This kit is manufactured using the high specific antibody pairs generated by Dr Kangawa

TECHNICAL DATA

Intra-assay precision

	Level 1	Level 2	Level 3	Level 4
N	6	6	6	6
Mean (pg/mL)	279	185	21	15
CV%	3.0	8.8	7.3	6.7

Inter-assay precision

	Level 1	Level 2	Level 3	Level 4
N	6	6	6	6

Mean (pg/mL)	166	134	50	26
CV%	11.8	2.3	3.3	6.1

Sensitivity

The minimal detectable GIP concentration is 8.2 pg/mL

Cholecystokinin Octapeptide (CCK)

Method

Phoenix Pharmaceuticals, Inc. 330 Beach Road, Burlingame, California 94010.

The immunoplate in this kit is pre-coated with secondary antibody and the non-specific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the primary antibody (peptide antibody) whose Fab fragment will be competitively bound by both biotinylated peptide and peptide standard or targeted peptide in the samples. The biotinylated peptide interacts with streptavadin-horseradish peroxidise (SA-HRP) which catalyses the substrate solution. The intensity of the yellow colour is directly proportional to the amount of biotinylated peptide-SA-HRP complex but inversely proportional to the amount of peptide in standard solutions or samples. A standard curve of know concentration can be established accordingly. The unknown concentration in samples can be determined by extrapolation to this standard curve.

TECHNICAL DATA

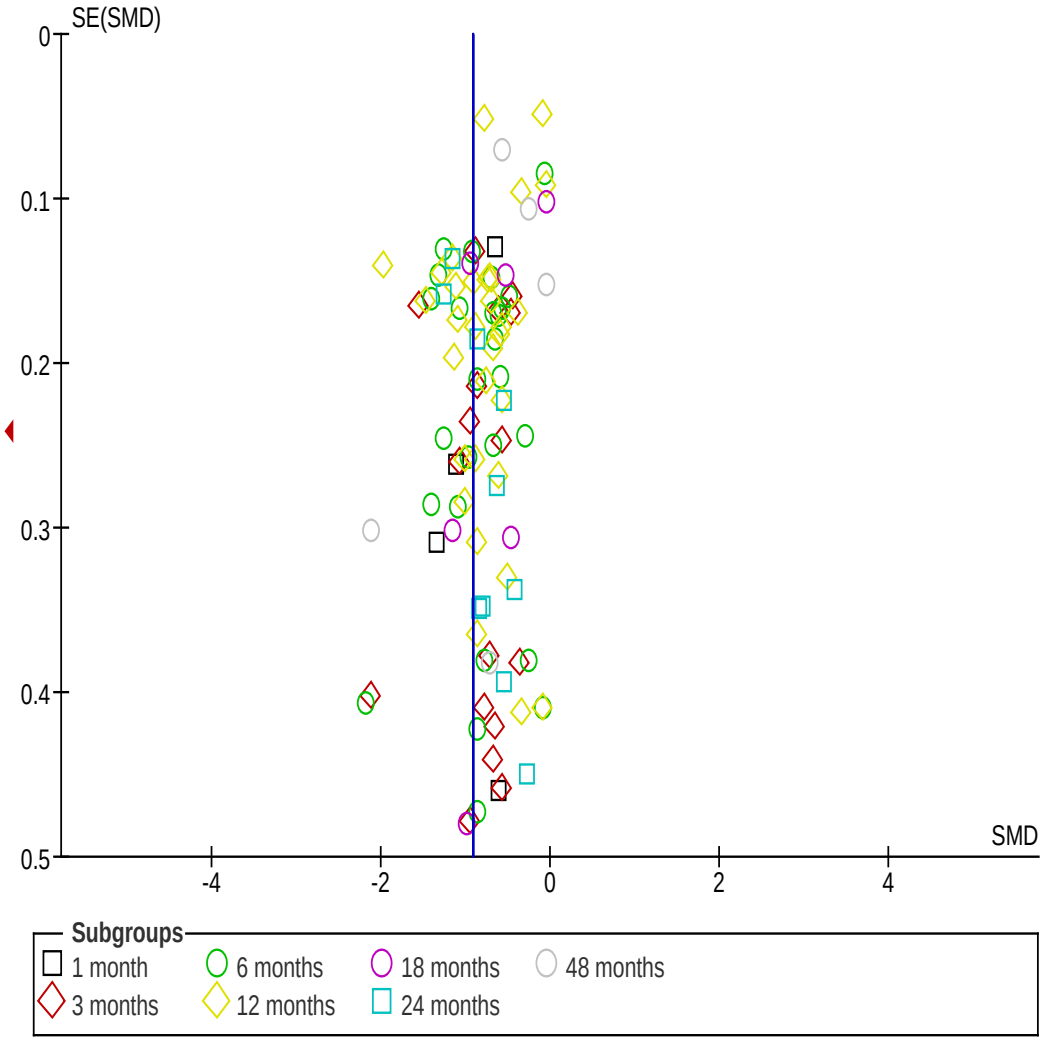
Sensitivity

The minimal detectable CCK concentration is 0.06 ng/mL

Appendix 3 – Funnel plots of the meta-analysis of the effects of RYGB surgery upon plasma lipids

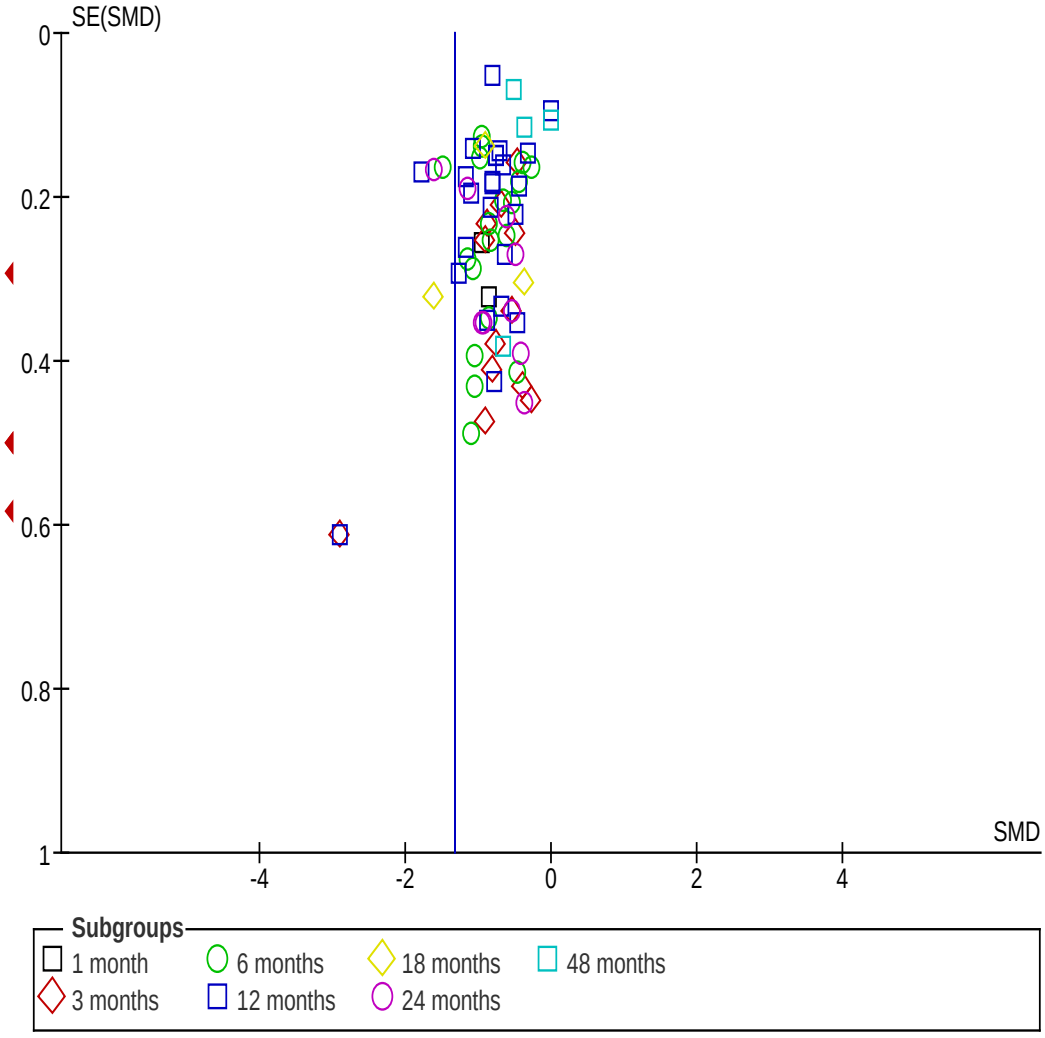
Total cholesterol

Funnel plot of total cholesterol changes including all subgroups (random effects model)



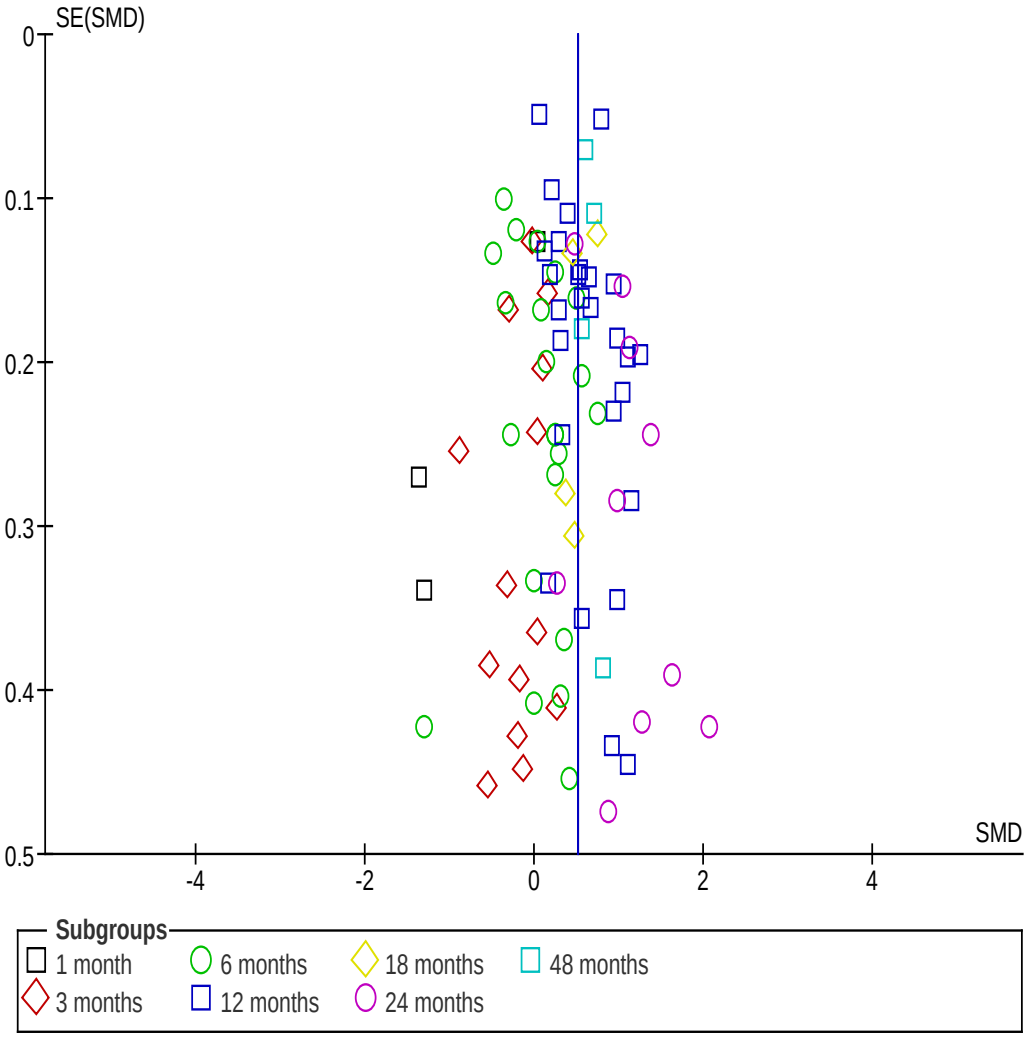
LDL-cholesterol

Funnel plot of LDL-cholesterol changes after RYGB including all subgroups (random effects model)



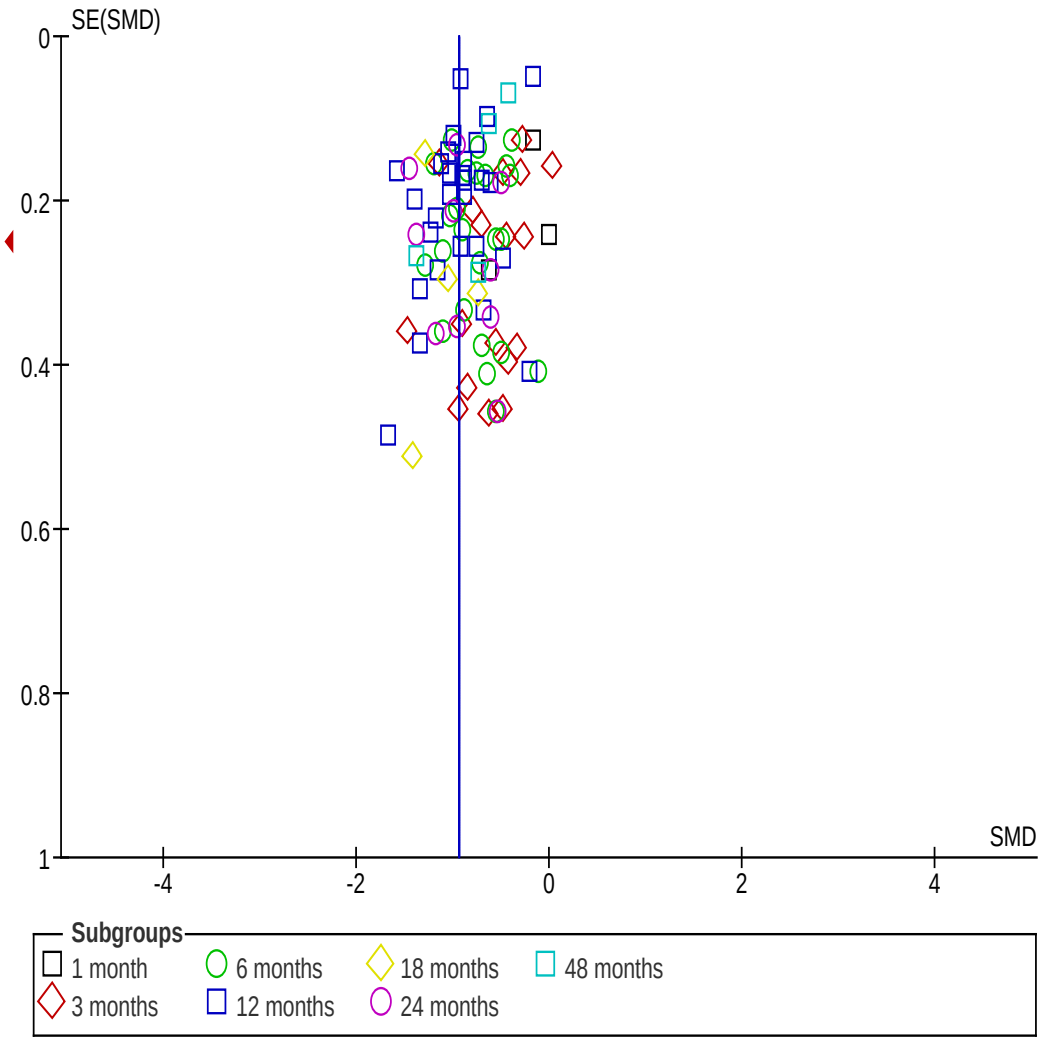
HDL-cholesterol

Funnel plot of HDL-cholesterol including all subgroups (random effects model)



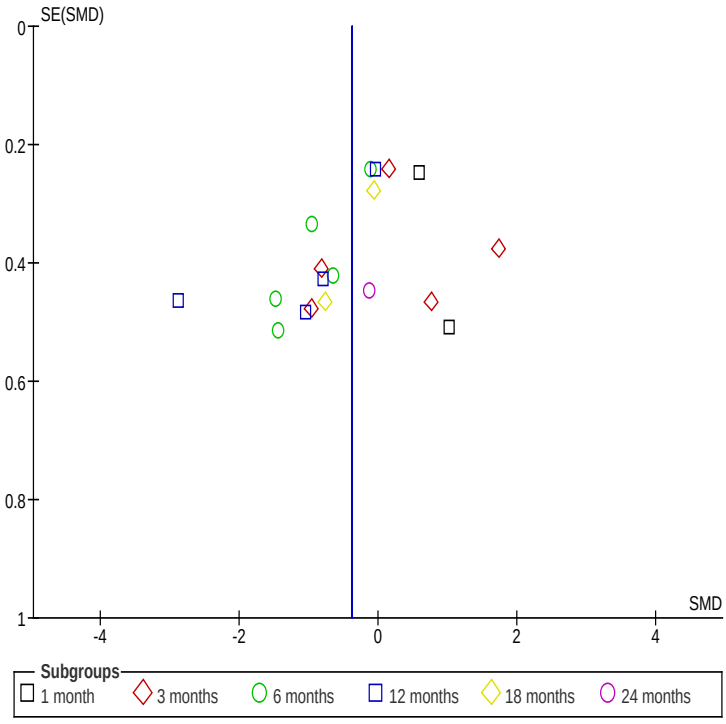
Triglycerides

Funnel plot of plasma triglyceride changes including all subgroups (random effects model)



NEFA

Funnel plot of the effect of RYGB upon plasma NEFA levels including all subgroups (random effects model)



11. Papers, presentations and awards

Book chapter

- Carswell KA, Lee MJ, Fried SK. Isolation and culture of human adipocytes and adipose tissue. Human Cell Culture Protocols, Methods in Molecular Biology Vol. 806 3rd edition (Ed. Mitry RR, Hughes RD), Humana Press 2012 (ISBN 978-1-61779-366-0)[371] (see supplementary material)

Papers

- Carswell KA, Belgaumkar AP, Amiel SA, Patel AG. A systematic review and meta-analysis of the effect of gastric bypass surgery on plasma lipid levels. Obesity Surgery 2016; 26:843-855 (see supplementary material)
- Carswell KA, Vincent RP, Belgaumkar AP, Sherwood RA, Amiel SA, Patel AG, le Roux CW. The effect of bariatric surgery on intestinal absorption and transit time. Obesity Surgery 2014; 24:796-805[66] (see supplementary material)

Abstracts

- Carswell KA, Belgaumkar A, Amiel SA, Patel AG. The systematic review and meta-analysis of plasma lipid levels around gastric bypass surgery. Obesity Surgery 2015
- Carswell KA, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel SA, Patel AG. Mechanism of remission of type 2 diabetes mellitus following gastric bypass surgery. BJS online 2011; 98 (S7):10
- Carswell KA, Vincent R, Belgaumkar A, Amiel SA, Patel AG, Le Roux CW. Absorption of nutrients after bariatric surgery. BJS online 2011; 98 (S7):2 and Obesity Surgery 2012
- Carswell KA, Belguamkar A, Dew T, Amiel S, Patel AG. Obesity surgery can impact upon lipid-induced insulin resistance. Obesity Surgery 2011; 21:1072

- Carswell KA, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel S, Patel AG. The lipolytic effects of gastric bypass surgery in patients with and without type 2 diabetes mellitus. Obesity Surgery 2011; 21: 1098
- Carswell K; Belgaumkar A; Patel AG. Systematic review of post-prandial hypoglycaemia after gastric bypass. Obesity Surgery 2009; 19: 972

Presentations

Oral presentations

- Carswell K, Belgaumkar A, Mitry R, Dew T, le Roux C, Amiel SA, Patel AG. The effects of RYGB surgery upon glycaemic and lipidaemic control. Rank Prize Funds, Grasmere, UK 19-22/10/15
- Carswell KA, Belgaumkar A, Amiel SA, Patel AG. The systematic review and meta-analysis of plasma lipid levels around gastric bypass surgery. IFSO, Vienna, Austria 2015
- Carswell KA, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel SA, Patel AG. Mechanism of remission of type 2 diabetes mellitus following gastric bypass surgery. AUGIS 2011.
- Carswell K, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel SA, Patel AG. The lipolytic effects of gastric bypass surgery in patients with and without type 2 diabetes mellitus. Rank Prize Funds, Grasmere, UK 09/03/11
- Carswell KA, Vincent R, Belgaumkar A, Amiel SA, Patel AG, Le Roux CW. Absorption of nutrients after bariatric surgery. AUGIS 2011 and IFSO - European Chapter, Barcelona 2012
- Carswell K; Belgaumkar A; Patel AG. Systematic review of post-prandial hypoglycaemia after gastric bypass. IFSO, Paris 2009

Poster presentations

- Carswell KA, Belgaumkar A, Mitry R, Dew T, le Roux CW, Amiel SA, Patel AG. Mechanism of remission of Type 2 Diabetes Mellitus following gastric bypass surgery. Alfred Benzon Symposium - adipose tissue in health and disease, Copenhagen Denmark 2012

- Carswell KA, Belguamkar A, Dew T, Amiel S, Patel AG. Obesity surgery can impact upon lipid-induced insulin resistance. IFSO, Hamburg, Germany 2011.
- Carswell KA, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel S, Patel AG. The lipolytic effects of gastric bypass surgery in patients with and without type 2 diabetes mellitus. IFSO Hamburg, Germany 2011.

Awards

- British Journal of Surgery best paper prize – AUGIS Belfast 2011
  Carswell KA, Vincent R, Belgaumkar A, Amiel SA, Patel AG, Le Roux CW. Absorption of nutrients after bariatric surgery

- International Federation of Surgical Obesity poster prize competition - 1st prize, Hamburg 2011
  Carswell KA, Belgaumkar A, Mitry R, Dew T, Le Roux C, Amiel S, Patel AG. The lipolytic effects of gastric bypass surgery in patients with and without type 2 diabetes mellitus