Effect of Bariatric Surgery on Small Bowel Physiological Changes Pertaining to Absorption of Nutrients & Bile Acid Metabolism

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Abstract

Background: The dogma that persisted for many years was that malabsorption plays a significant role in contributing to the weight loss following bariatric surgery. The aim was to assess for evidence of malabsorption after bariatric procedures; adjustable gastric banding (AGB), Roux-en-Y gastric bypass (RYGB) and biliopancreatic diversion-duodenal switch (BPD-DS).

Methods: This cross sectional study recruited participants into four groups: obese controls (n=7), AGB (n=6), RYGB (n=7) and BPD-DS (n=5). Biochemical tests were used to assess; **(i) entire gut:** sulphasalazine test for oro-caecal transit time (OCTT) & plasma citrulline for functional enterocyte mass. **(ii) foregut:** faecal elastase-1 (FE1) for exocrine pancreatic function. **(iii) mid-gut:** Lactulose:rhamnose (L:R) ratio for gut permeability, faecal calprotectin (FCp) for gut inflammation & plasma and urine bile acids (BAs) for BA metabolism. **(iv) hindgut:** Faecal fat (Ffat) excretion for fat malabsorption.

Results: (i) entire gut: There was no difference in OCTT ($p=0.935$) or functional enterocyte mass ($p=0.819$). **(ii) foregut:** FE1 was lower in the RYGB vs. the control group ($p=0.002$), with no difference between other groups (all $p>0.05$). **(iii) mid-gut:** L:R ratio was higher in the BPD-DS vs. the control ($p=0.012$), AGB ($p=0.016$) and RYGB ($p=0.012$), with no difference between other groups. FCp was higher in the RYGB vs. the control ($p=0.016$), with no difference between other groups. The fasting plasma and urine BAs were elevated in BPD-DS vs. the control, ABG and RYGB (all $p<0.05$). **(iv)**

hindgut: The BPD-DS had higher Ffat excretion vs. the control (p=0.038), AGB (p=0.046) and RYGB (p=0.024). The RYGB had higher Ffat excretion vs. the control (p=0.033). There was no difference between the RYGB and AGB (p=0.808).

Conclusion: There was no evidence to support the notion that RYGB causes severe malabsorption of fats or sugars. However, BPD-DS does cause fat malabsorption.

Declaration of Originality

This thesis was presented in part and won the British Journal of Surgery (BJS) prize paper session at the Association of Upper Gastrointestinal Surgeons of the UK and Ireland, Belfast 2011.

This thesis was published (in part) in abstract form;
Absorption of nutrients after bariatric surgery. *British Journal of Surgery* 2011; **98**(Supp 7): 2.

The candidate and the supervisor obtained ethical approval for this study.

The candidate was helped by a surgical research fellow Miss K Carswell in the recruitment of participants and sample collection during the sulphasalzine/sulphapyridine test and small bowel permeability test.

All the analytical work and statistical analysis in this thesis were carried out by the candidate.

"I Royce P Vincent, declare that the work presented in this thesis is my own and that it is the one upon which I expect to be examined."

TABLE OF CONTENTS

Abstract 02
Declaration of Originality 04
Table of contents 05
Acknowledgements 08
List of Figures and Tables 09
Abbreviations 11

Chapters:

1. Introduction 14
1.1. Obesity epidemic 15
1.2. Bariatric surgery 15
1.2.1. Adjustable Gastric Banding 19
1.2.2. Roux-en-Y Gastric Bypass 21
1.2.3. Biliopancreatic Diversion-Duodenal Switch 23
1.3. Bile Acids 26
1.3.1. Bile acid synthesis 26
1.3.2. Bile acids as a signalling molecule and glucose homeostasis 29
1.3.2.1. Membrane receptors: TRG5 signalling 30
1.3.2.2. Nuclear receptors: FXR signalling 31
1.4. Glucagon like peptide-1 (GLP-1) 38
1.4.1. GLP-1 and incertin effect 38
1.4.2. GLP-1 and Bile Acids 39
1.4.3. GLP-1 and Bariatric surgery 40
1.4.4. Bariatric Surgery and type 2 diabetes mellitus 40
1.5. Malabsorption after gastrointestinal surgery 42
1.6. Scientific justification & Rationale 46
1.7. Hypothesis 47
1.8. Research questions and Aims 47
1.9. Assessment of malabsorption & adaptation 48
1.9.1. Entire gut 48
1.9.1.1. Oro-caecal transit time 48
1.9.1.2. Functional enterocyte mass 49
1.9.2. Foregut 50

1.9.2.1. Exocrine pancretic function 50
1.9.3. Mid-gut 51
1.9.3.1. Samll bowel permeability 51
1.9.3.2. Gut inflammation 52
1.9.3.3. Bile acid metabolism 53
1.9.4. Hindgut 54
1.9.4.1. Faecal fat excretion 54
2. Methods & Materials 58
2.1. Recruitment 59
2.1.1. Protocol 59
2.1.2. Identification of participants 60
2.1.3. Inclusion criteria 61
2.1.4. Exclusion criteria 61
2.1.5. Ethics 61
2.1.6. Power calculation 60
2.2. Assessment of malabsorption 62
2.2.1. Entire gut 62
2.2.1.1. Oro-caecal transit time 62
2.2.1.2. Functional enterocyte mass 63
2.2.2. Foregut 64
2.2.2.1. Exocrine pancreatic function 64
2.2.3. Mid-gut 66
2.2.3.1. Samll bowel permeability 66
2.2.3.2. Gut inflammation 67
2.2.3.3. Bile acid metabolism 69
2.2.3.3.1. Bile acids by LC-MS/MS 69
2.2.3.3.2. Reagents 70
2.2.3.3.3. Plasma bile acids 70
2.2.3.3.4. Urine bile acids 71
2.2.4. Hindgut 74
2.2.4.1. Faecal fat excretion 74
2.3. Statistical analysis 75
3. Results 76
3.1. Entire gut 82
3.2. Foregut 86
3.3. Mid-gut 88

3.4. Hindgut 95
4. Discussion 101
4.1. Entire gut 104
4.2. Foregut 108
4.3. Mid-gut 111
4.4. Hindgut 117
5. Conclusion 123
6. Bibliography 126

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List of Figures & Tables

Figures

Fig 1: Normal anatomy of the gastrointestinal tract.

Fig 2a: Schematic representation of the anatomical alteration following adjustable gastric banding.

Fig 2b: Schematic representation of the anatomical alteration following Roux-en-Y gastric bypass.

Fig 2c: Schematic representation of the anatomical alteration following biliopancreatic diversion-duodenal switch.

Fig 3: (A) Structure of selected bile acids (BA). (B) Farnesoid X receptor α (FXRα) target genes involved in the enterohepatic recycling and detoxification of BA.

Fig 4: Overview of the Effects of Bile Acid Activation of TGR5 and farnesoid X receptor α (FXRα) in the gastrointestinal tract.

Fig 5: Schematic overview of the functions of bile acids (BA) in the regulation of BA, energy, glucose and lipid metabolism via farnesoid X receptor-α (FXRα) and TGR5 mediated signalling pathways.

Fig 6: A chromatogram of a 1 µmol/L bile acid standard mixture showing all the fractions measured.

Fig 7: Flowchart demonstrating participant recruitment and sample analysis.

Fig 8: Box and Whisker plots of the sulphapyridine concentration between the four groups.

Fig 9: Box and Whisker plots of citrulline concentration between the four groups.

Fig 10: Box and Whisker plots of faecal elastase-1 (FE1) between the four groups.

Fig 11: Box and Whisker plots of lactulose:rhamnose (L:R) ratio between the four groups.

Fig 12: Box and Whisker plots of faecal calprotectin (FCp) concentration between the four groups.

Fig 13a: Box and Whisker plots of the total plasma bile acid concentration between the four groups.

Fig 13b: Box and Whisker plots of the total urine bile acids concentration between the four groups.

Fig 14: Box and Whisker plots of faecal fat excretion between the four groups.

Fig 15: Spearman correlation between FCp (faecal calprotectin) and FE1 (faecal elastase-1) in all the groups.

Fig 16a: Spearman's correlation between weight loss in % and faecal fat (Ffat) excretion in all the groups.

Fig 16b: Spearman's correlation between rate of weight loss (kg/month) and faecal fat (Ffat) excretion in all the groups.

Tables

Table 1: Bile acid fractions with retention times.

Table 2: Demographics of the four groups.

Table 3: All the biochemical investigations done on urine, blood and faecal samples in the four groups.

Abbreviations

ABTS - 2, 2'-Azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium

ADP - Adenosine di-phosphate

AGB - Adjustable gastric band

ASBT - Apical Sodium Bile Acid Transporter

ANOVA - Analysis of Variance

ATP - Adenosine tri-phosphate

BA - Bile Acid

BMI - Body Mass Index

BPD-DS - Biliopancreatic diversion-duodenal switch

CA - Cholic acid

cAMP - Cyclic adenosine monophosphate

CCK - Cholecystokinin

CDC - Chenodeoxycholic acid

CFU - Colony forming units

CoA - Coenzyme A

CREB - cAMP response element-binding protein

DC - Deoxycholic acid

ELISA - Enzyme-linked immunosorbent assay

FCp - Faecal calprotectin

FE1 - Faecal elastase-1

Ffat - Faecal fat

FGF-19 - Fibroblast growth factor 19

FXR - Farnesoid X receptor

GCA	-	Glycocholic acid
GCDC	-	Glycochenodeoxycholic acid
GC-MS	-	Gas chromatography mass spectrometry
GDC	-	Glycodeoxycholic acid
GIP	-	Gastric inhibitory peptide
GLC	-	Glycolithocholic acid
GLP-1	-	Glucagon-like peptide-1
GLP-2	-	Glucagon-like peptide-2
GUDC	-	Glycoursodeoxycholic acid
HCL	-	Hydrochloric acid
HNF4a	-	Hepatocyte nuclear factor 4a
HOMA	-	Homeostasis model assessment
HPLC	-	High pressure liquid chromatography
IQR	-	Inter quartile range
JIB	-	Jejunoileal bypass
LC	-	Lithocholic acid
LC-MS/MS	-	Liquid chromatography-Tandem mass spectrometry
LRH1	-	Liver receptor homolog 1
MAPK	-	Mitogen-activated protein kinase
MRM	-	Multiple reaction monitoring
NICE	-	National Institute for Clinical Excellence
OCTT	-	Oro-caecal transit time
PBA	-	Plasma bile acid
PBS	-	Phosphate-buffered saline
PIU	-	Programmed Investigation Unit

PKA	-	Protein kinase A
PYY	-	Peptide tyrosine-tyrosine
QC	-	Quality control
REC	-	Research Ethics Committee
RPM	-	Resolutions per minute
RYGB	-	Roux-en-Y gastric bypass
SBPT	-	Small bowel permeability test
SBS	-	Short bowel syndrome
SeHCAT	-	Selenium-75-homochloric acid taurine
SHP	-	Short heterodimer protein
SIBO	-	Small intestinal bacterial overgrowth
SREBP1c	-	Sterol regulatory binding element 1c
T2DM	-	Type 2 diabetes mellitus
TCA	-	Taurocholic acid
TCDC	-	Taurochenodeoxycholic acid
TDC	-	Taurodeoxycholic acid
TLC	-	Taurolithocholic acid
TMAH	-	Tetramethyl ammonium hydroxide
TUDC	-	Tauroursodeoxycholic acid
UBA	-	Urine bile acid
UDC	-	Ursodeoxycholic acid
WHO	-	World Health Organisation

Chapter One

Introduction

1. Introduction

1.1. Obesity epidemic

Obesity is currently the largest nutrition related condition affecting the developed and increasingly developing countries. Its prevalence over the past 30 years has increased worldwide by nearly 75% such that the World Health Organisation (WHO) has classified obesity as an epidemic (WHO, 2000). In 2008, 1.5 billion adults (20 years and older) were overweight of these over 200 million men and nearly 300 million women were obese (WHO, 2011). Furthermore, 65% of the world's population lives in countries where overweight and obesity kills more people than underweight (WHO, 2011). Obesity is not limited to the adult population with nearly 43 million children under the age of five classified as overweight in 2010 (WHO, 2011). Obesity is globally responsible for a huge economic burden to world health services due to mortality and its associated co-morbidities including type 2 diabetes mellitus (T2DM), sleep apnoea, hypertension and hyperlipidaemia. The exact mechanisms that contribute to obesity have not yet been fully elucidated. Evidence implicates multiple factors such as poor diet, sedentary lifestyle, environmental factors, genetics and disturbed energy balance (James, 2008).

1.2. Bariatric surgery

The term Bariatric is derived from the Greek words: baros (weight) and iatreia (medical treatment). Bariatric surgery was first introduced in the 1950s; however, it was in the last two decades that safe and successful management of surgery had progressed (Jones, 2004).

At present, bariatric surgery has the best long term results in terms of significant and sustained weight loss in the morbidly obese population when compared to lifestyle changes and pharmacotherapy (Sjöström *et al*, 2007). A large cohort study showed that surgical intervention leads to considerable improvements in the management of obesity related co-morbidities and heath related quality of life when compared to patients treated with diet alone (Sjöström *et al*, 2004).

The National Institute for Clinical Excellence (NICE) 2006 guidance 43 states that surgical treatment is beneficial for people with obesity if they have a Body Mass Index (BMI) equal or more than 40kg/m^2 or between 35kg/m^2 and 40kg/m^2 if they have a coexisting disease that could be improved by weight loss such as, T2DM, sleep apnoea and high blood pressure (NICE, 2006). Given the significant benefits of bariatric surgery the number of procedures done in the UK and worldwide have considerably increased.

Bariatric surgical procedures are traditionally divided into three categories based on the effect they have on food intake and absorption; limiting food intake (restriction) or affecting nutrient absorption by delivering food lower down in the intestine (malabsorption; defined as a state arising from abnormality in absorption of food nutrients across the gastrointestinal tract); combination of both (restriction and malabsorption) (Kim *et al*, 2003). The most commonly performed procedures worldwide are adjustable gastric banding (AGB), Roux-en-Y gastric bypass (RYGB) and biliopancreatic

diversion-duodenal switch (BPD-DS). These surgical procedures are based on altering the normal anatomy of the gastrointestinal tract (Figure 1).

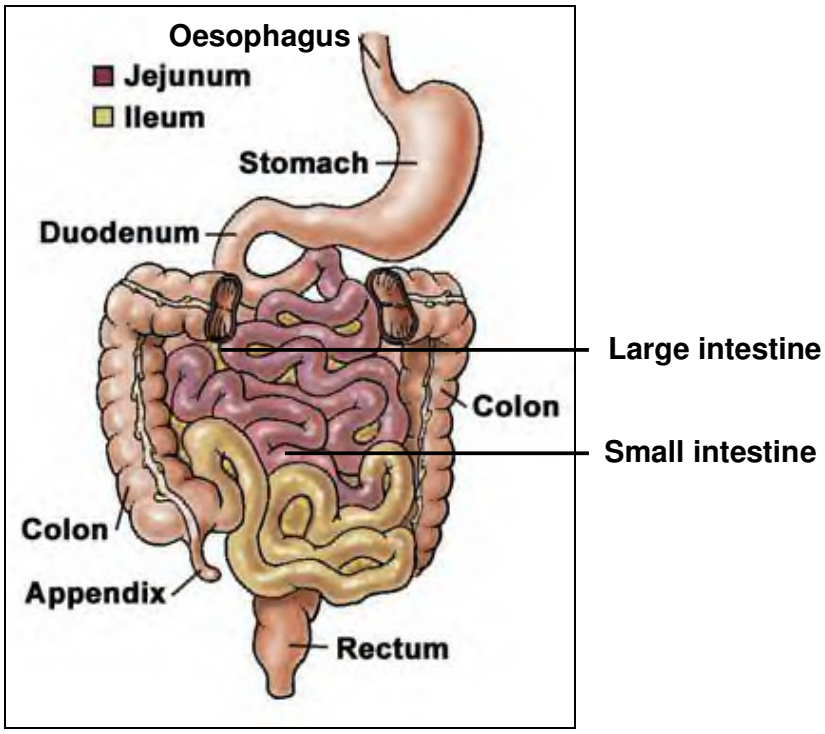


Figure 1: Normal anatomy of the gastrointestinal tract. Sciencerevolution.net [online]. [Accessed 21 January].

Available at: <http://sciencerevolution.net/SmBowelAnat.bmp>

1.2.1. Adjustable Gastric Banding (AGB)

In the UK, one of the most commonly performed, bariatric procedure is AGB. An adjustable band is placed around the upper potion of the stomach to create a pouch about 15-30mL^3 in size (Figure 2a). Retention of a small meal above the band has traditionally been the mode of action attributed to the AGB. The restriction exerted by the band has been shown to reduce hunger (desire to eat) and increase satiety (feeling of fullness) (Dixon *et al*, 2005). The pressure applied by the band creates a sensation of fullness and delays passage of solid food through the upper part of the stomach even with very small potions, causing individuals to chew thoroughly and eat slowly (Kerrigan, 2008). Furthermore, decreased total energy intake, due to sustained behavioral adaptations, has been observed, resulting in weight loss (Burton & Brown, 2011). Although following AGB, patients are unable to consume foods of a coarser texture, this does not entirely explain weight loss, whereas induction of satiety appears critical to achieving weight loss (Burton & Brown, 2011). The AGB consistently presents an intra-luminal resistance just beneath the oesophageal–gastric junction. This leads to delayed transit of semi-solids into the infra-band stomach, with flow across the AGB mediated by repeated esophageal peristalsis (Burton & Brown, 2011). These findings suggest that retention of food in a restricted proximal pouch is not the mode of action of AGB hence; it should not be classified as a restrictive procedure. It appears that the AGB activates the peripheral satiety mechanism (probably via the vagus nerve) without physically restricting the meal size. The precise mechanism of weight loss following AGB remains to be delineated (Burton & Brown, 2011).

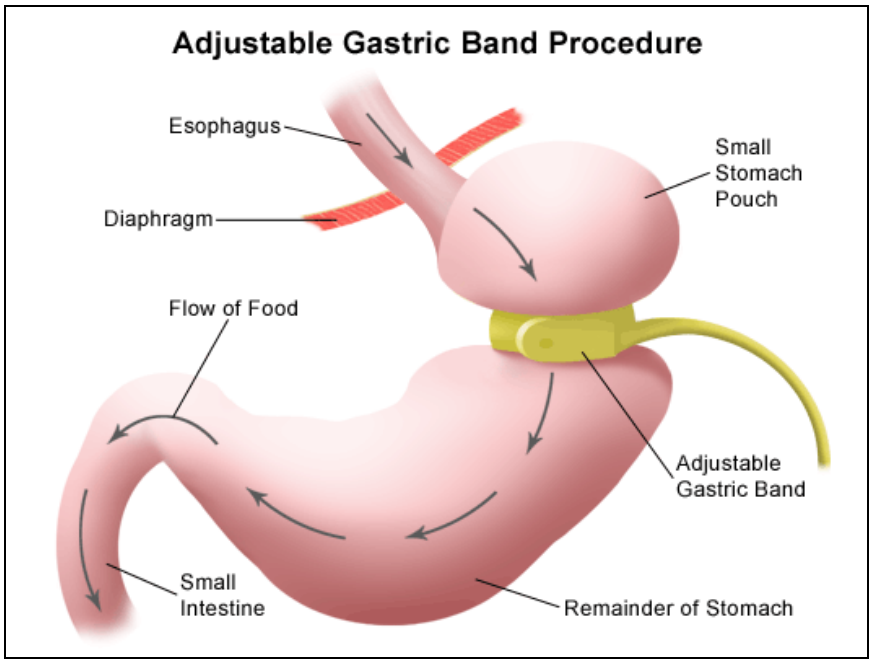


Figure 2a: Schematic representation of the anatomical alteration following adjustable gastric banding. An adjustable gastric band is placed around the upper potion of the stomach to create a pouch about 15-30mL^3 in size. [Accessed 21 January 2010].

Available at:

<http://www.upgradeyourbody.com/images/stories/organs/stomach-gastric-banding.jpg>

1.2.2. Roux-en-Y Gastric Bypass (RYGB)

RYGB is a popular procedure where the size of the stomach is reduced to 15-30mL^3 pouch and a potion of the upper small intestine is bypassed. Further dissection of the jejunum (second part of small intestine) approximately 100 cm distal to the pylorus (inferior part of the stomach) is made. The distal segment of the transsected jejunum is joined to the gastric pouch (Roux limb / alimentary limb), while the proximal end of the transsected jejunum is joined to the jejunum approximately 75-100 cm from the joint of gastric pouch and distal segment of the jejunum (biliopancreatic limb) (Fig. 2b). The inferior part of the stomach and duodenum (first part of the small intestine) still secrete gastric secretions and digestive enzymes (hydrochloric acid, pancreatic enzymes, biliary salts etc) to aid in digestion and absorption within the common channel (Fobi, 2004).

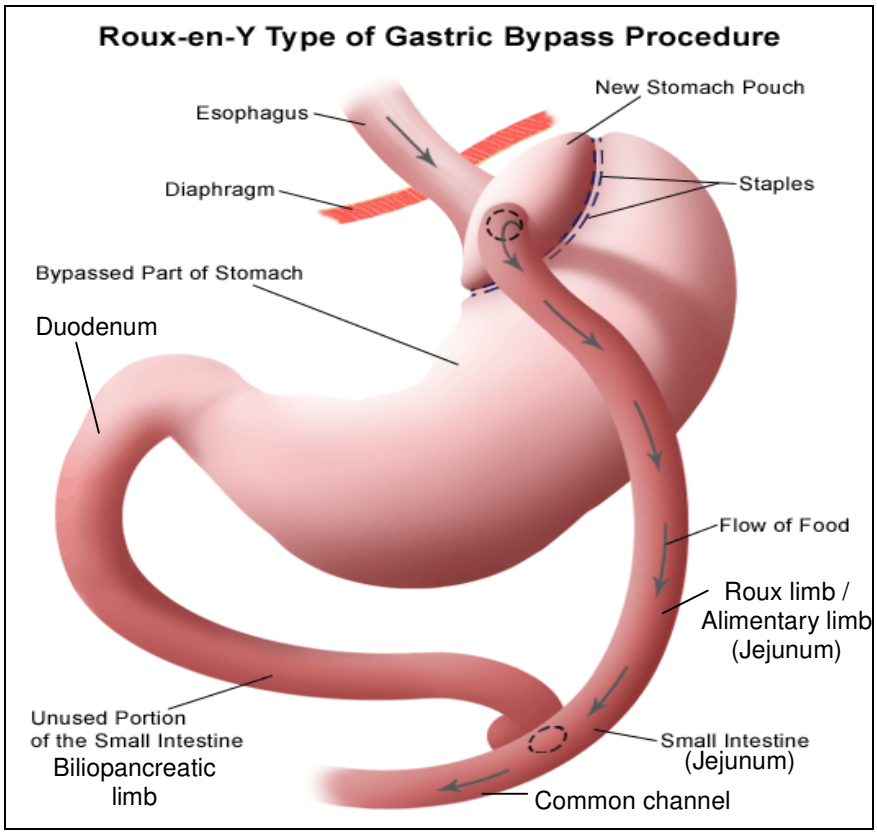


Figure 2b: Schematic representation of the anatomical alteration following Roux-en-Y gastric bypass. The size of the stomach is reduced to a 15-30mL[3] pouch and the remainder of the stomach, the duodenum and proximal jejunum are bypassed. Two limbs are fashioned in a 'Y' shape. The Roux/altimetry limb (approximately 150 cm) and the biliopancreatic limb (75-100 cm). Both the limbs are joined distally to form the common channel. [Accessed 21 January 2010].

Available at: <http://www.pabariatricsurgery.com/procedures/laprygb.html>

1.2.3. Biliopancreatic Diversion-Duodenal Switch (BPD-DS)

BPD-DS is a procedure that replaced the earlier jejunoileal bypass (JIB) surgery, which was abandoned due to its severe nutritional complications. BPD-DS involves dissection of the stomach to form a sleeve gastrectomy (approximately 100mL^3). A further two transsections of the small bowel are made; first at the level of the duodenum; second dissects the jejunum from the ileum (third part of small intestine). The proximal end of the transsection at the level of the duodenum (pylorus) is then joined to the distal end of the transsection at the level of the jejunum/ileum (alimentary limb). The distal end of the transsection at the level of the duodenum is closed while the proximal end of the transsection at the level of the jejunum/ileum is joined to the ileum 100 cm proximal to the caecum (Fig. 2c) hence, diverting food to the terminal segment of the small intestine. This forms a common channel that allows for mixture of nutrients with digestive enzymes in the ileum, the length of which (typically, 50-100 cm) determines the degree of absorption (Fobi, 2004; Brolin, 2002). The distal end of the transsection at the level of duodenum to the anastamosis 100 cm from the caecum forms the biliopancreatic limb. Weight loss achieved through this procedure is greater than other Bariatric procedures (Cummings *et al*, 2004).

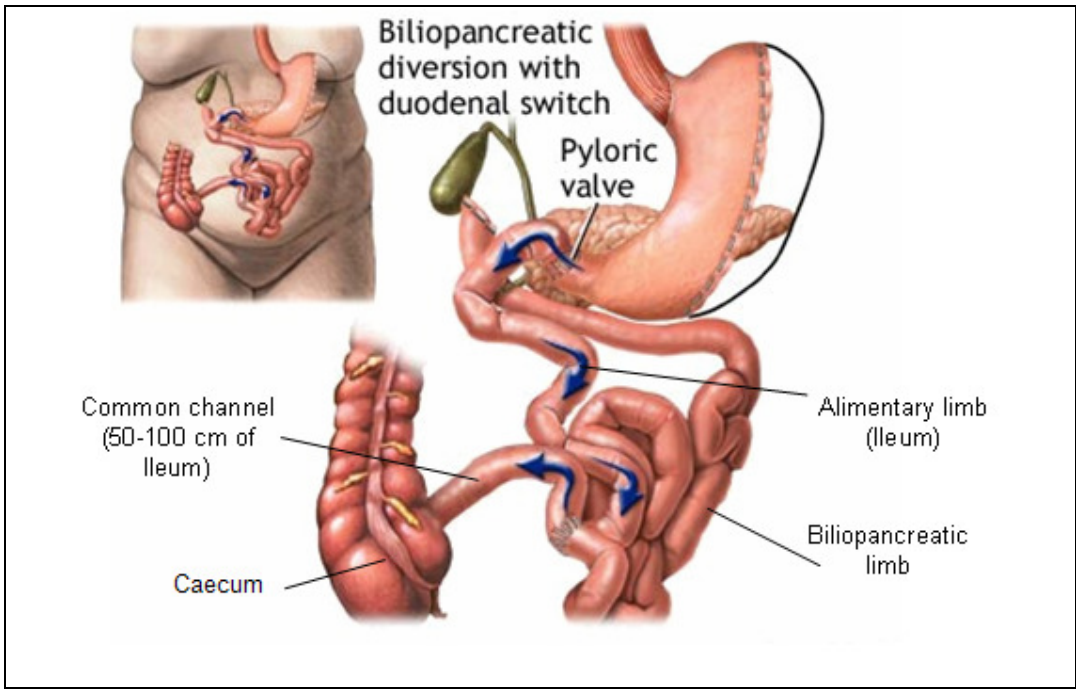


Figure 2c: Schematic representation of the anatomical alteration following biliopancreatic diversion-duodenal switch. A vertical sleeve gastrectomy is performed (stomach size is reduced to approximately 100mL^3). A further two transsections of the small bowel are made; first at the level of the duodenum; second dissects the jejunum from the ileum. The proximal end of the transsection at the level of the duodenum (pylorus) is then joined to the distal end of the transsection at the level of the jejunum/ileum (alimentary limb). The distal end of the transsection at the level of the duodenum is closed while the proximal end of the transsection at the level of the jejunum/ileum is joined to the ileum 100 cm proximal to the caecum. This forms the biliopancreatic limb and the common channel (75-100 cm). [Accessed 21 January 2010]. Available at: <http://medicalimages.allrefer.com/large/biliopancreatic-diversion-with-duodenal-switch.jpg >

Previously the commonly held dogma was that AGB worked through restriction of food intake, BPD-DS worked through calorie malabsorption and RYGB was a hybrid between restriction and calorie malabsorption. However, these simplistic concepts are being challenged as there is limited data to support their role in weight loss (Pilkington *et al*, 1976; Prachand & Alverdy, 2009).

Thus, the mechanisms responsible for the remarkable effect of bariatric surgery on weight loss remain controversial. Some of the mechanisms that have been proposed in recent years are as follows;

Malabsorption (Odstrcil *et al*, 2010)

The effect of gut hormones on hunger (the desire to eat) and satiety (the feeling of fullness) (Vincent *et al*, 2008)

Changes in taste preference (Pournaras & le Roux, 2009)

Changes in dietary intake and eating behaviour (Sarwer *et al*, 2011)

The effect of gut hormones on energy homeostasis (Ionut & Bergman, 2011)

Changes in gut microbiota (Li *et al*, 2011)

The effect of bile acids (BA) on energy homeostasis (Patti *et al*, 2009)

1.3. Bile Acids

BAs are 24-carbon compounds derived from cholesterol. Their molecular structure is based on a saturated tetracyclic hydrocarbon perhydrocyclopentanophenanthrene system common to a number of compound classes, such as steroid hormones. Along with cholesterol and phospholipids, BAs are one of the major constituents of bile (Houten *et al*, 2006). The amphipathic, detergent-like properties of BAs account for their role in the solubilisation of dietary lipids, aiding in their digestion and absorption in the ileum (Houten *et al*, 2006).

1.3.1. Bile Acid Synthesis

Initially cholesterol is converted into the primary BAs cholic acid (CA) and chenodeoxycholic acid (CDC) by two pathways (Russell, 2003). The classical or neutral pathway accounts for over 75% of BA synthesis in the hepatocytes, whilst the alternative or acidic pathway accounts for 6% of BA synthesis. In the classical pathway, cholesterol 7α-hydroxylase (encoded by CYP7A1) is the first and rate-limiting step, being subject to complex modes of regulation (Staels & Fonseca, 2009). In the alternative pathway, hydroxylation of oxysterol precursors is performed by sterol 27-hydroxylase with subsequent hydroxylation at position 7 by CYP7B1. The ratio of CA to CDC formation is determined by the relative rate of interaction of intermediates with CYP8B1; hydroxylation by this enzyme leads to formation of the more hydrophilic CA (Pandak *et al*, 2001). Majority of the synthesised primary BAs are conjugated to either glycine (75%) or taurine (25%) by the enzymes BA coenzyme A (CoA) synthetase and BA-CoA amino acid-acetyltransferase (Monte *et al*,

2009). This conjugation process renders BA into even more effective detergents due to their enhanced amphipathic nature (Pircher *et al*, 2003, Thomas *et al*, 2008). The primary BAs are stored in the gallbladder and released via the common bile duct into the duodenum, on ingestion of fat and protein rich food, which stimulate the release of cholecystokinin (CCK) which in turn causes gallbladder contraction and relaxation of the sphincter of Oddi (Ganong & Ganong, 1995). Anaerobic bacteria residing in the colon are responsible for the formation of the secondary unconjugated BA; deoxycholic acid (DC), lithocholic acid (LC) and ursodeoxycholic acid (UDC) by deconjugation, oxidation and 7α-hydroxylation of primary BA (Thomas *et al*, 2008). In the terminal ileum, approximately 95% of BAs are reabsorbed both by passive diffusion and active transport using the apical BA transporter and delivered to the liver via the enterohepatic circulation, where they can be conjugated with glycine or taurine (Lefebvre *et al*, 2009) , the remaining 5% is lost in the faeces or excreted in the urine (Ganong & Ganong, 1995). These processes are finely regulated, by BAs themselves, to assure the maintenance of a BA pool of defined size and composition (Prawitt & Staels, 2010). Plasma fibroblast growth factor-19 (FGF19) produced by enterocytes in the ileum in response to BA absorption also regulates hepatic BA synthesis by inhibiting CYP7A1 (Walters *et al*, 2009). BA structure and its metabolism are summarised in Figure 3.

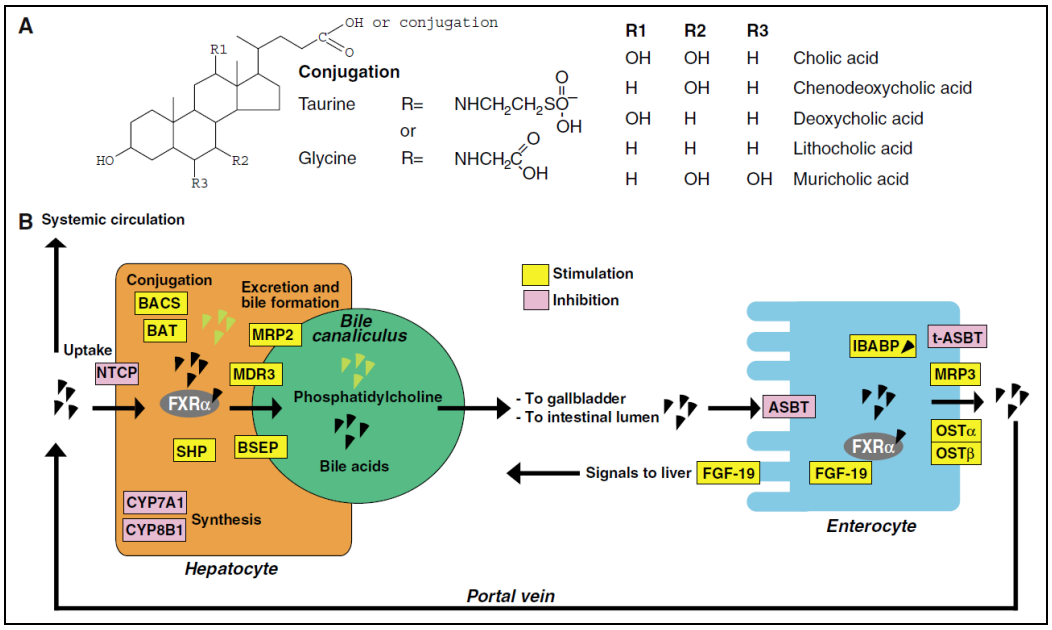


Figure 3: (A) Structure of selected bile acids (BA). (B) Farnesoid X receptor α (FXRα) target genes involved in the enterohepatic recycling and detoxification of BA. Genes whose expression is directly induced by BA and FXRα are in yellow rectangles; those in pink rectangles are inhibited by BA. Abbreviations; BACS, BA-CoA synthetase; BAT, BA-CoA: amino acid N-acetyltransferase; BSEP, bile salt export pump; MDR3, multidrug resistance 3 p-glycoprotein; MRP2, multidrug resistance-associated protein 2; IBABP, ileal BA-binding protein; CYP8B1, sterol 12α-hydroxylase; NTCP, sodium taurocholate cotransporting polypeptide; ASBT, apical sodium-dependent bile salt transporter (Houton *et al*, 2006)

1.3.2. Bile acid as a signalling molecule and glucose homeostasis

In addition to their function in lipid digestion, BAs are increasingly studied for their metabolic regulator roles. By triggering different signalling pathways, BAs are involved in the metabolism of BA as well as lipid, and glucose homeostasis (Prawitt & Staels, 2010). Furthermore, modulation of BA metabolism by intestinal sequestration such as cholestyramine leads to improved glycemic control in T2DM, indicating a strong link between BA and glucose homeostasis (Garg & Grundy, 1994).

Metabolomic studies have identified that BA form part of the axis regulating insulin sensitivity in response to a meal. In patients who had received an oral glucose load, plasma glycochenodeoxycholic acid (GCDC), taurochenodeoxycholic acid (TCDC) and glycocholic acid (GCA) rapidly increased in contrast to those subjects who received water (Shaham *et al*, 2008, Zhao *et al*, 2009). A further non-targeted study identified that 22 metabolites of BA synthesis were associated with insulin resistance (Lucio *et al*, 2010). However, whether the modulation of BA metabolism also positively affects glycemic control in T2DM patients has not yet been addressed.

BAs exert differential regulatory functions via nuclear and membrane receptors as well as via receptor-independent signalling pathways. Two major BA regulated signalling mechanisms have received the most attention in the literature; BAs are ligands firstly for the G-protein coupled receptor TGR5 (or 'membrane bile-acid receptor') and secondly for nuclear hormone receptors such as farnesoid X receptor α (FXRα) (Prawitt & Staels, 2010).

1.3.2.1. Membrane receptors: TRG5 signalling

TGR5 (also known as Gpbar1, M-Bar, and BG37) is a G protein-coupled membrane receptor activated by certain BAs (Prawitt & Staels, 2010). They are mainly expressed in gallbladder, ileum, colon, brown and white adipose tissue as well as to a lesser extent, in skeletal muscle and liver, TGR5 is activated by nanomolar concentrations of LC and taurine-conjugated lithocholic acid (TLC) and micromolar concentrations of CA, DC, and CDC (Prawitt & Staels, 2010). When BA binds to TGR5, the GaS subunit is released and activates the release of cyclic adenosine monophosphate (cAMP). This leads to a consecutive activation of protein kinase A (PKA) and the transcription factor cAMP response element-binding protein (CREB) (Prawitt & Staels, 2010). Furthermore TGR5 can also activate the mitogen-activated protein kinase (MAPK) pathway. Importantly BA can exert TGR5-mediated control on glucose homeostasis by affecting the incretin system (discussed in chapter 1.4.1.) (Prawitt & Staels, 2010).

BAs have been attributed a regulatory function in energy metabolism that is mediated by TGR5. CA supplementation to a high-fat diet increased energy expenditure in mice and subsequently alleviated obesity and insulin resistance (Prawitt & Staels, 2010). However, TGR5-deficient mice and TGR5-overexpressing mice do not display a difference in weight gain. Thus, the importance of the TGR5 axis for the regulation of body weight remains unknown (Prawitt & Staels, 2010).

1.3.2.2. Nuclear receptors: FXRα signalling

BA activates nuclear receptors, transcription factors regulating expression of target genes by binding to specific response elements in their promoter. The best-characterized nuclear receptor mediating the regulatory actions of BA is the FXRα (Prawitt & Staels, 2010) which displays high expression levels in liver and intestine and lower expression in adipose tissue, pancreas and the adrenal glands. The most effective activator of FXRα is CDCA (Prawitt & Staels, 2010).

Two distinct mechanisms have been shown by which FXRα mediates BA signalling. In the liver, FXRα activation induces the expression of the orphan nuclear receptor short heterodimer protein (SHP), which subsequently inhibits a number of transcription signal transduction pathways such as the liver receptor homolog 1 (LRH1) induction of CYP7A1, hepatocyte nuclear factor 4a (HNF4a) activation of gluconeogenic genes and sterol regulatory binding element 1c (SREBP1c) induction of lipogenesis (Prawitt & Staels, 2010). The second mechanism implies the induction of the FGF19 in the intestine (Prawitt & Staels, 2010).

The first observation to link FXRα, to glucose metabolism was based on dysregulated hepatic FXRα expression is in rodent models with diabetes mellitus (Prawitt & Staels, 2010). FXRα expression is repressed by insulin and this effect is reversed in the presence of glucose. FXRα-deficiency also results in altered kinetics of intestinal glucose absorption and hepatic glucose production (Prawitt & Staels, 2010).

Pharmacological activation of FXRα in diabetic rodent models attenuate insulin resistance. Furthermore administration of human FGF19 reversed weight gain and insulin resistance in mouse models of genetic and diet-induced obesity, due to an increase in metabolic rate and hepatic fatty acid oxidation (Prawitt & Staels, 2010). A recent study showed that plasma CDC, CA and DC concentrations were negatively associated with insulin sensitivity assessed by both homeostasis model assessment (HOMA) and the gold-standard hyperinsulinemic-euglycemic clamp method, in healthy volunteers, obese and T2DM (Cariou *et al*, 2011). The potential of metabolic regulation by BAs via the intestinal FXRα–FGF19 axis is appealing, but requires further studies.

BA signalling via the TGR5 and FXRα receptors in the gastrointestinal tract are summarised on Figure 4.

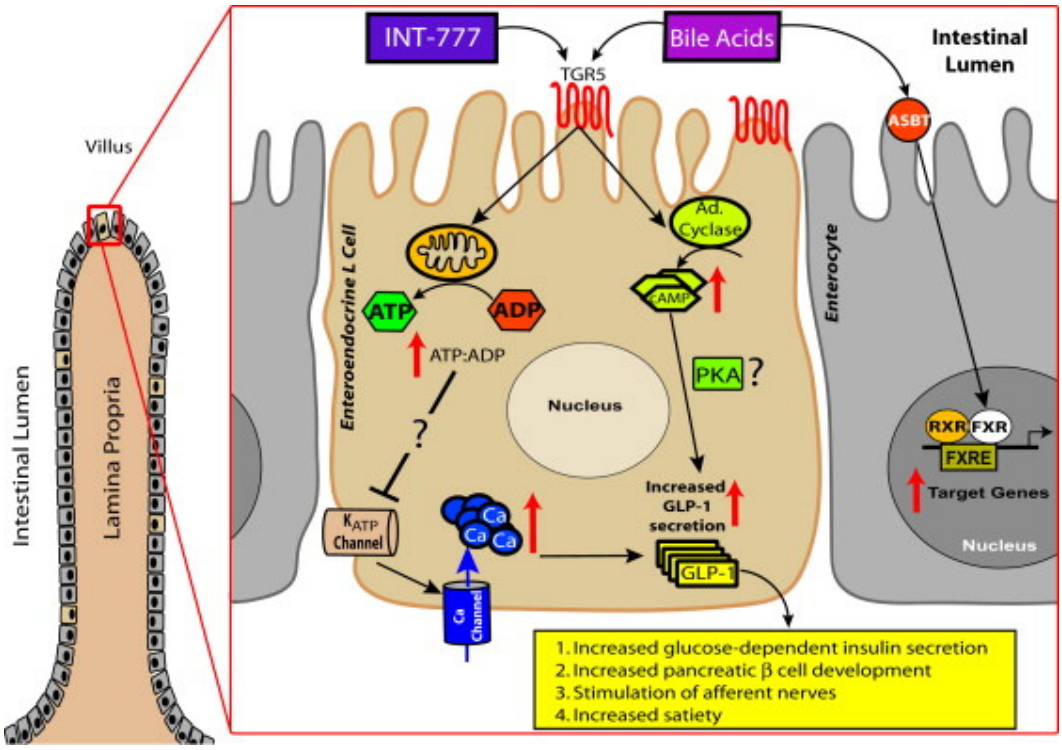


Figure 4: Overview of the Effects of Bile Acid Activation of TGR5 and farnesoid X receptor α (FXRα) in the gastrointestinal tract. Bile acids (BA) present in the intestine activate both TGR5 on the surface of enteroendocrine L cells and the nuclear receptor FXRα in enterocytes. Ninety-five percent of the BA entering the intestinal lumen from the gall bladder are subsequently reabsorbed via the apical sodium-dependent bile acid transporter (ASBT), leading to activation of the FXR:RXR heterodimer bound to the FXR response element (FXRE) and induction of target genes. BA in the intestinal lumen also activate TGR5 on the surface of enteroendocrine L cells, leading to an increase in oxidative phosphorylation and the ratio of ATP:ADP. The increased concentrations of ATP are thought to inhibit the ATP-sensitive potassium channel, leading to a depolarization of the cell membrane and opening of calcium channels. TGR5 activation also activates adenylate cyclase (Ad. Cyclase), thus increasing cellular cAMP concentrations.

Increases in intracellular calcium and cAMP both lead to increased secretion of GLP-1 from L cells. Secreted GLP-1 has a number of effects. It augments glucose-dependent insulin release from β cells, it promotes β cell development, and it stimulates afferent nerves. GLP-1 can also induce satiety. [Accessed 10 May 2012]. Available at: <http://www.sciencedirect.com/science/article/pii/S1550413109002344>

Current evidence suggests that BA seem to regulate glucose homeostasis by three mechanisms, intestinal absorption, hepatic glucose production and insulin sensitivity (Figure 5).

Secondary BAs, LC and DC have been shown to induce gut hormone secretion [peptide tyrosine-tyrosine (PYY) and glucagon-like peptide 1 (GLP-1), a potent incretin] from intestinal enteroendocrine L cells in vitro (Prawitt & Staels, 2010). Another study showed that glucose tolerance is reduced by TGR5-deficiency and improved by TGR5 over-expression in high-fat diet-fed mice via increased GLP1 and insulin secretion (Thomas *et al*, 2009). Two other recent investigations found an increase in GLP1 secretion from the intestine of sequestrant-treated diabetic rats, which might mediate the increased responsiveness towards insulin (Prawitt *et al*, 2011).

A study which evaluated BA kinetics more systematically in T2DM demonstrated no difference in the size of the total BA pool however; the contribution of specific BA species to the pool was altered in T2DM. Where the pool of the secondary BA, DC and the DC input rate were elevated and the primary BA, CDC pool decreased (Prawitt *et al*, 2011). Furthermore, the primary BA, CA pool was unchanged, even though the CA synthesis rate was elevated in T2DM. Another study used a metabolomic approach to show lower CA and elevated DC concentrations in controlled T2DM compared to controls (Prawitt *et al*, 2011). Hence, CA might be increasingly converted into DC in the gut of patients with T2DM. Thus, collectively current literature evidence

suggests that changes in BA homeostasis are either a cause or a consequence of the metabolic disturbances observed during T2DM.

BAs have also been suggested to play a role in appetite regulation. Obese individuals have an attenuated post prandial BA response along with attenuated satiety hormone response (PYY and GLP-1) (Glicksman *et al*, 2010). Thus, the manipulations of the BA pool itself either by BA substitution, intestinal sequestration, or BA receptors such as FXRα and TGR5 present promising strategies to develop therapeutics for T2DM and obesity.

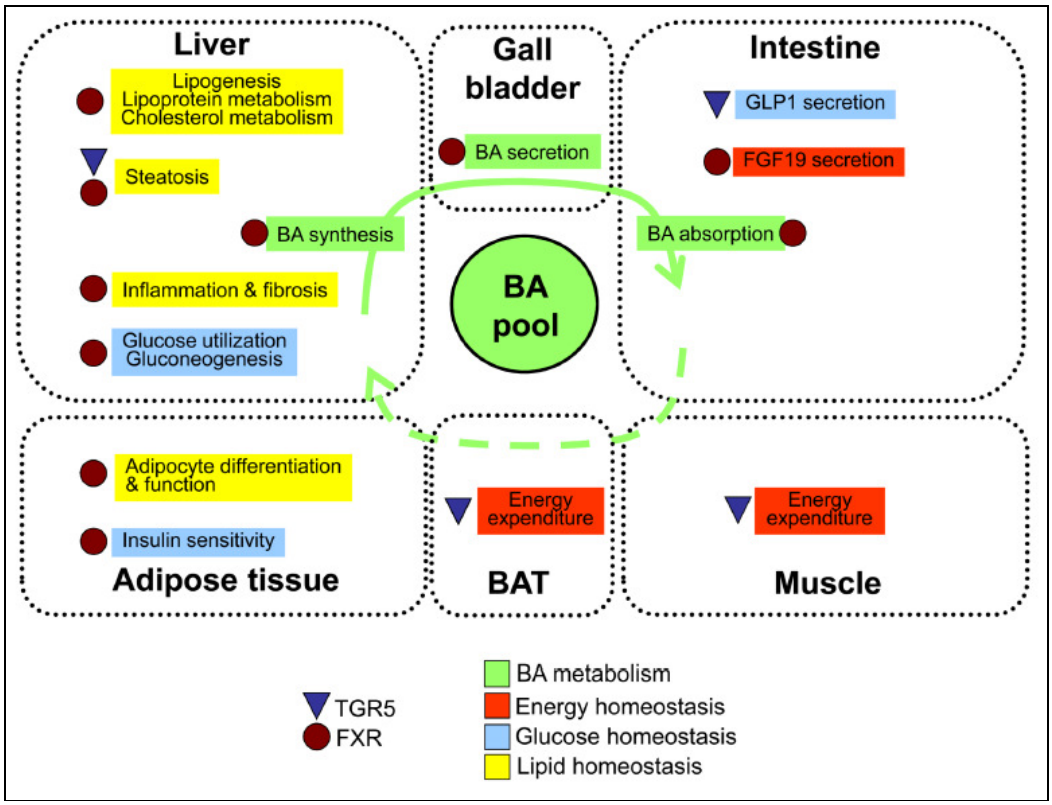


Figure 5: Schematic overview of the functions of bile acids (BA) in the regulation of BA, energy, glucose and lipid metabolism via farnesoid X receptor-α (FXRα) and TGR5 mediated signalling pathways. Abbreviations; GLP1, glucagon-like peptide 1; FGF, fibroblast growth factor; BAT, brown adipose tissue (Prawitt *et al*, 2011).

1.4. Glucagon like peptide-1 (GLP-1)

GLP-1 is a neuropeptide hormone produced by post-translational processing of the preproglucagon gene in the central nervous system and the gastrointestinal tract (Vincent *et al*, 2008). This preproglucagon is secreted in the gastrointestinal tract by the same enteroendocrine L cells that secrete PYY (Vincent *et al*, 2008). GLP-1 secretion is stimulated by nutrients in the intestinal lumen (Vincent *et al*, 2008) and BAs (Adrian *et al*, 1993). GLP-1 acts through the GLP-1 receptor, a member of 'Family B' of the G-protein-coupled receptors (Vincent *et al*, 2008). These receptors have been identified in neurons of the nucleus tractus solitarius, extending to regions of the hypothalamus that are important for the regulation of food intake (Vincent *et al*, 2008). It has been reported that both peripheral and central GLP-1 administration activates neurons in the arcuate nucleus, the hypothalamic paraventricular nucleus, nucleus tractus solitarius and area postrema (Vincent *et al*, 2008).

1.4.1. GLP-1 and incretin effect

GLP-1 and gastric inhibitory polypeptide (GIP) are incretin (gastrointestinal hormones that cause an increase in the amount of insulin released from the β-cells of the islets of Langerhans after eating, even before blood glucose concentrations become elevated) peptides, with GLP-1 the more potent of the two (Vincent *et al*, 2008). GLP-1 enhances glucose-dependent insulin secretion, up-regulates the expression of pancreatic β-cell genes, promoting β-cell neogenesis and inhibiting apoptosis (Vincent *et al*, 2008) as well as suppresses gastric acid secretion and gut emptying. Newly introduced GLP-1

based therapies, that mimic GLP-1 (exenatide) or slow its clearance [dipeptidyl peptidase IV (DPP-IV) inhibitors], have proven to be highly successful agents for the treatment of T2DM (Drucker *et al*, 2006).

It had been debated whether reduced postprandial GLP-1 concentrations may contribute to the pathophysiology of T2DM, augmenting the exaggerated postprandial glucose concentrations which occur in these patients (Meier & Nauck, 2008; Toft-Nielsen *et al*, 2001). However, reduced postprandial concentrations in patients with T2DM have not been universally observed (Fukase *et al*, 1995; Orskov *et al*, 1991; Theodorakis *et al*, 2006), thus any potential impairments in GLP-1 secretion in subjects with T2DM may be a consequence, rather than a cause, of T2DM.

1.4.2. GLP-1 and Bile Acids

TGR5-overexpressing mice fed a high-fat diet do not develop glucose intolerance and have elevated GLP1 concentrations resulting in increased insulin release in response to an oral glucose load (Prawitt & Staels, 2010). The proposed mechanism involves a TGR5-mediated increase of the adenosine triphosphate (ATP) to adenosine diphosphate (ADP) ratio that subsequently leads to increased intracellular calcium concentrations that trigger GLP-1 secretion (Prawitt & Staels, 2010). Pharmacological activation of TGR5 by the semi-synthetic TGR5 agonist 6-ethyl-methyl CA40 or by oleanolic acid also has shown to improve diet-induced obesity and insulin resistance (Prawitt & Staels, 2010). Glucagon immunoreactivity has been shown to increase following infusion of BA (Namba *et al*, 1983) and GLP-1

concentrations were found to increase in portal effluent following the luminal perfusion of BA into perfused rat colon preparations (Plaisancie *et al*, 1995). Furthermore recently, intra-rectal infusion of TCA has also been shown to increase GLP-1 and PYY concentrations in obese rats T2DM (Adrian *et al*, 2010).

1.4.3. GLP-1 and Bariatric surgery

Number of studies has shown an increase in the satiety hormones GLP-1 and PYY in the distal small intestine following bariatric surgical procedures which lead to increased satiety and reduced food intake (Vincent & le Roux, 2008; Kokkinos *et al*, 2010). Compared with lean and obese controls, following RYGB, patients have increased postprandial plasma PYY and GLP-1 consistent with increased satiety (Vincent & le Roux, 2008). BPD-DS has also been shown to result in intestinal hypertrophy and elevated concentration of PYY and GLP-1 in rats (Borg *et al*, 2007). Furthermore exogenous PYY reduces food intake and blockade of endogenous PYY and GLP-1 increases food intake (Fenske *et al* 2011). Thus exaggerated postprandial PYY and GLP-1 response after RYGB, may contribute to significant and sustained weight loss after this procedure.

1.4.4. Bariatric Surgery and type 2 diabetes mellitus

At present the most effective treatment for T2DM is metabolic surgery (Pournaras *et al*, 2010). A meta-analysis reported improved glycemic control and remission of T2DM in 84% of patients following RYGB and 48% following AGB, but the change in the American Diabetes Association criteria has

reduced the 'remission' rate to approximately 41% (Pournaras *et al*, 2010; Pournaras *et al,* 2012). Two hypotheses have been proposed to explain this rapid resolution of T2DM following bariatric surgery; the 'hindgut hypothesis' and the 'foregut hypothesis'. The former is based upon the ability of rapid delivery of ingested nutrients to the distal part of the small intestine to enhance the secretion of incretin GLP-1, improving glucose metabolism; whereas the latter advocates the glucose-lowering effect to depend on exclusion of the duodenum and proximal jejunum from the ingested nutrients thus preventing them form eliciting a 'diabetogenic signal' (Knop, 2009).

However, data comparing RYGB with AGB in the same centre are limited, and definitions of remission are inconsistent between studies. Interestingly the improved glycemic control after AGB is seen after weight loss; however, after RYGB this improvement occurs well before weight loss (Pournaras *et al*, 2010). The mechanisms proposed based on these observations are firstly; the exclusion of the duodenum and proximal jejunum which may reduce insulin resistance and secondly the exaggerated gut hormone (incretin) responses from the distal small bowel to nutrients (Pournaras *et al*, 2010). Another mechanism may involve the direct access of BAs to L-cell in the intestine and that enhanced circulating BA concentrations may contribute to the rapid remission of T2DM after RYGB (Nakatani *et al*, 2009; Patti *et al*, 2009). At the other extreme there have been case reports of hyperinsulinemic hypoglycaemia following RYGB, due to inappropriately increased insulin secretion (Meier *et al*, 2006; Service *et al*, 2005).

1.5. Malabsorption after gastrointestinal surgery

Over the years a number of gastrointestinal operations have been developed and modified for optimum benefit whist reducing the associated complications which also include post operative nutritional status. Some of the common surgical procedures as well as bariatric procedures which have been studied for evidence of malabsorption are discussed below. Malabsorption constitutes the pathological interference with the normal physiological sequence of digestion (intra-luminal process), absorption (mucosal process) and transport (post-mucosal events) of nutrients (Bai, 1998).

Faecal fat (Ffat) excretion rises in nearly all patients after total and anterior selective vagotomy and pyloroplasty (procedure in which the pylorus valve at the lower portion of the stomach is cut and re-sutured, relaxing and widening its muscular opening) (Wastell & Ellis, 1996). This is associated with diminished stool colour due to the dilution of faecal pigments. Bowel frequency is usually increased by total and anterior selective vagotomy and pyloroplasty, but in a few cases there may be episodic increases of frequency or no change at all (Wastell & Ellis, 1996). In Billroth I (Billroth's operation I) procedure the pylorus is removed and the distal stomach is anastomosed directly to the duodenum. Steatorrhea is rare after this procedure (Butler, 1969) with Ffat excretion in the normal range (Orr, 1964).

JIB was the first gastrointestinal operation performed specifically to treat severe obesity (Buchwald *et al*, 1987). This was based on the observation that massive small bowel resection led to significant weight loss, and was

theoretically designed to radically alter the absorption of nutrients (Prachand & Alverdy, 2009). By late 1980s JIB was abandoned due to associated complications including massive diarrhoea, electrolyte disturbance, osteomalacia and liver failure (Requarth *et al*, 1995). These complications were attributed to a "blind loop" syndrome due to direct mucosal injury, alterations in intestinal permeability to bacteria and their toxins, circulation and deposition of immune complexes (Wands *et al*, 1976). Newer bariatric procedures theoretically avoid the complications of a "blind loop" by ensuring luminal flow is preserved, hence minimising bacterial over growth and associated malabsorption.

Anatomically, the small bowel measures five to nine meters in length (Prachand & Alverdy, 2009) and is divided into the duodenum, the jejunum, which constitutes the proximal 40% of the small bowel, and the ileum. The small bowel has an absorptive surface area of about 200m^2, with the jejunum accounting for nearly two-third (Taguchi & Suita, 2002). Furthermore in comparison to the ileum, the jejunum is greater in diameter, has a thicker wall and longer villi (Prachand & Alverdy, 2009). Given the wide variation in the length of small bowel between individuals, it is technically very difficult to estimate the length encountered during surgery. Given the 500-fold difference in the relative contribution to surface area of the microscopic features (villi and microvilli) compared with the macroscopic features (length and width) and the significant differences between jejunal and ileal morphology, limb length measurements during surgery are an inaccurate estimation of small bowel surface area (Prachand & Alverdy, 2009). This is evident in studies that

involved large number of patients post RYGB with varying limb lengths found that longer limb lengths do not seem to provide substantial additional malabsorption (Choban & Flancbaum, 2002; MacLean *et al*, 2001).

A study which prospectively followed 257 consecutive patients who had RYGB reported that only 5% had symptomatic steatorrhea and only 4% were at risk of protein or calorie malnutrition (Nelson *et al*, 2006). However, the postoperative vitamin supplementation recommended would be considered insufficient for malabsorptive procedures. Furthermore, there were no efforts to identify preoperative nutritional deficiencies (Prachand & Alverdy, 2009). Sugerman *et al* (1997), showed a high rate of malnutrition in patients with a common limb of 50cm compared to 150cm following distal gastric bypass. However, in another study, measurements of calories lost in faeces showed that the malabsorption could not account for the weight loss following JIB (Pilkington *et al*, 1976). Also, in the post JIB study the degree of malabsorption noted was not decreased two years post surgery, when weight loss had plateaued (Pilkington *et al*, 1976).

Hence, a clinical need exists to give patients more accurate information on calorie malabsorption for either the RYGB or BPD-DS procedures. Previous studies have shown that after RYGB and BPD-DS in rodents, significant gut hypertrophy takes place thus reducing the chances of calorie malabsorption (le Roux e*t al*, 2010; Borg *et al*, 2007). Thus, there is considerable need for a study to be done in humans. Recent evidence suggests that alterations in the neurohormonal control mechanisms for appetite, satiety and energy

homeostasis play a significant role in weight loss. The weight loss following surgery can be explained by reduced food intake which may be associated with subsequent increase in gut hormones. PYY and GLP-1 have been shown to modulate appetite regulation by reducing hunger and increasing satiety (le Roux *et al*, 2006a; le Roux *et al*, 2006b). Another contributor to weight loss may be the change in taste perception and food preferences (aversion to fatty and sugary food) noted after these procedures which further challenges the malabsorption theory (Sugerman *et al*, 1987; Kenler *et al*, 1999; Olbers *et al*, 2006). The relative contribution of each of these mechanisms to surgical weight loss has not been fully elucidated, but may differ from individual to individual.

1.6. Scientific justification & Rationale

The average weight loss in clinical practice after two years for AGB is 20%, for RYGB, 30% and for BPD-DS, 40% (Cummings *et al*, 2004). However, there is a group of patients who lose much less weight because they are under the misimpression that most of the food they eat will not be absorbed. While another group of obese patients who would benefit from bariatric surgery, is put off by the notion that they may have significant malabsorption. Hence, the concept of “surgical malabsorption” needs to be evaluated as the prevalence of obesity and bariatric procedures done to combat it continue to rise exponentially.

As far as we know no study has systematically assessed post bariatric surgery patients for evidence of malabsorption using multiple biochemical markers.

1.7. Hypothesis

Can the significant and sustained weigh loss following bariatric surgery be attributed to malabsorption?

The hypothesis was tested using biochemical investigations that assessed the different sections of the gut; (i) entire gut (mouth to caecum), (ii) foregut (oesophagus to first two sections of the duodenum), (iii) mid-gut (lower duodenum, to the first two-thirds of the transverse colon) and (iv) hindgut (last third of the transverse colon, to the upper part of the anal canal) [Figure 1].

1.8. Research questions and Aims

(i). Assessment of the Entire gut

- Is there a change in oro-caecal transit time after bariatric surgery?
- Is there a change in functional enterocyte mass after bariatric surgery?

(ii). Assessment of the Foregut

- Is there a change in exocrine pancreatic function after bariatric surgery?

(iii). Assessment of the Mid-gut

- Is there a change in gut permeability after bariatric surgery?
- Is there evidence of gut inflammation after bariatric surgery?
- Is there a change bile acid metabolism after bariatric surgery?

(iv). Assessment of the Hindgut

- Is there a change in faecal fat excretion after bariatric surgery?

1.9. Assessment of malabsorption & adaptation

Malabsorption constitutes the pathological interference with the normal physiological sequence of digestion (intra-luminal process), absorption (mucosal process) and transport (post-mucosal events) of nutrients (Bai, 1998). Intestinal malabsorption can be due to; mucosal damage (enteropathy), congenital or acquired reduction in absorptive surface, defects of specific hydrolysis, defects of ion transport, pancreatic insufficiency or impaired enterohepatic circulation (Walker-Smith *et al*, 2002). There is no specific test for malabsorption. However, as for most medical conditions, investigation is guided by symptoms and signs.

In this study the assessment of malabsorption and adaptation was divided into four sections as follows; entire gut, foregut, mid-gut and hind gut.

1.9.1. Entire gut

1.9.1.1. Oro-caecal transit time

Oro-caecal transit time (OCTT) helps assess gut motility abnormalities in clinical practice. Methods used for investigating upper gastrointestinal transit include non-invasive tests such as isotopic scintigraphy and the lactulose hydrogen breath tests as well as invasive test such as intubation (Saniforth, 1989). Intubation is not suitable for routine clinical use and has been shown to inhibit gastric emptying and to speed up small bowel transit (Read *et al*, 1983). Isotopic methods involve exposure to radiation and the lactulose used in the breath test accelerates gut transit time and gives a non-physiologic measurement (Saniforth, 1989). Other drawbacks with the lactulose test are

that 25% of population cannot hydrolyse the sugar and small intestinal bacterial overgrowth (SIBO) can cause early hydrogen peaks which are interpreted as rapid transit times (Hung *et al*, 2006). An alternative method for the assessment of gut transit time was initially described by Kennedy *et al* (1979), which used the principle of the azoreductase producing gut microorganisms of the large bowel to split the azo link releasing, 5-aminosalicylic acid and sulphapyridine from the orally administered sulphasalazine. The sulphapyridine is immediately absorbed hence; the time taken for its appearance in the blood provides a measure of the gut transit time (Kellow *et al*, 1986). Importantly there is a highly significant correlation between transit times determined with plasma sulfapyridine and isotopic scintigraphy (Kellow *et al*, 1986). The sulfapyridine test is also a more accurate measurement of transit time compared with the lactulose hydrogen breath test (Saniforth, 1989). A decrease in OCTT in an absorption study (i.e. intestinal hurry/rapid transit) is in keeping with malabsorption.

1.9.1.2. Functional enterocyte mass

Short bowel syndrome (SBS) occurs when there is insufficient length of the small intestine to maintain adequate nutrition and or hydration status without supplemental support (Cisler & Buchman, 2005). Citrulline which is a non protein amino acid is a component of the hepatic pathway that conveys protein nitrogen into the urea cycle (Rabier & Kamoun, 1995). It is synthesised exclusively in small intestinal enterocytes from glutamine and glutamine-related compounds and then metabolised into arginine by the kidney (Jianfeng *et al*, 2005). Citrulline concentration is known to be a marker of

residual small intestinal length in patients with SBS and consequently of intestinal failure (Crenn *et al*, 2000). In recent years citrulline has been established as a candidate marker for small bowel functional enterocytes mass (Crenn *et al*, 2000). Importantly citrulline concentration is not affected by liver diseases, protein energy malnutrition or inflammatory diseases of the bowel (Weber & Reiser, 1982; Papadia *et al*, 2007). A decrease in citrulline in an absorption study (i.e. decreased functional enterocyte mass) is in keeping with malabsorption.

1.9.2. Foregut

1.9.2.1. Exocrine pancreatic function

Recognition of pancreatic exocrine insufficiency is highly relevant to avoid malnutrition-related morbidity and mortality (Domínguez-Muñoz, 2007). For evaluation and follow-up of exocrine pancreatic function, several tests (direct and indirect) are available. The gold standard test secretin-cholecystokinin test (direct) requires duodenal intubation, is time consuming, expensive and lacks standardisation. Pancreatic elastase-1 is a specific protease synthesised by the acinar cells of the pancreas (Dominici & Franzini, 2002). Measurement of elastase-1 in the faeces (faecal elastase-1) is considered specific for human pancreatic elastase so that exogenous enzyme supplements do not affect the test result (Stein *et al*, 1996; Dominguez-Munoz *et al*, 1995). Elastase-1 concentration in faeces is about five times higher than in pancreatic juice, highlighting its stability during intestinal transport (Katschinski *et al*, 1997). Furthermore it is hardly influenced by motility disorders or mucosal defects (Dominguez-Munoz *et al*, 1995). Hence, faecal

elastase-1 (FE1) test is sensitive in detecting severe and moderate exocrine pancreatic insufficiency and specific in differentiating pancreatic from non-pancreatic causes in patients with steatorrhea (Symersky *et al*, 2004). Studies have revealed that FE1 correlates well with other direct and indirect tests of exocrine pancreatic function (Kampanis *et al*, 2009) hence; its use has been supported by the British Society of Gastroenterology Guidelines (Thomas *et al*, 2003). A decrease in FE1 in an absorption study (i.e. pancreatic exocrine insufficiency) is in keeping with malabsorption.

1.9.3. Mid-gut

1.9.3.1. Small bowel permeability

In a clinical context the assessment of absorption is of value when seeking an explanation for weight loss and malnutrition. The investigation of intestinal permeability provides a useful indication of the integrity of the intestinal mucosa for diagnostic screening (Johnston *et al*, 2001). Intestinal permeability is assessed non-invasively *in vivo* by measuring urinary excretion of orally administered substances. The substances should preferably not be naturally present in urine, be completely excreted in the urine following oral administration and be easily and accurately measurable (Tibble & Bjarnason, 2001). The test utilises oligosaccharides (non-hydrolysed disaccharide - lactulose) and a monosaccharide (L-rhamnose or D-xylose) as test substances for the non-invasive assessment of intestinal permeability (Menzies, 1974). The differential urinary excretion principle overcomes most, if not all the problems associated with the use of a single test substance, where urinary excretion is dependent on a number of pre and post-mucosal

factors as well as intestinal permeability (Tibble & Bjarnason, 2001). As the pre- and post-mucosal determinants of renal excretion affects all sugars to a similar extent the differential five hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables hence, the urinary excretion ratio becomes a specific measure of intestinal permeability. An increase in lactulose:rhamnose ratio in an absorption study (i.e. increased small bowel permeability) is in keeping with malabsorption.

1.9.3.2. Gut inflammation

Inflammation of the bowel mucosa is associated with malabsorption (Shah & Hanauer, 2007). Calprotectin is an abundant neutrophil protein found in both plasma and stool that is significantly elevated in infections and inflammatory conditions, including inflammatory bowel disease (Konikoff & Denson, 2006). It is a calcium-binding protein that exhibits antibacterial and antifungal activity (Steinbakk *et al*, 1990). It is also resistant to bacterial degradation in the gut and is stable in stool for up to one week at room temperature (Roseth *et al*, 1992). Faecal calprotectin (FCp) measurement correlates well with the more difficult and more expensive measurement of ^{111}Indium-labelled leucocyte excretion (Roseth *et al*, 1999). Importantly FCp correlates well with the definitive test for inflammatory bowel disease; histological inflammation detected on biopsy and has been shown to successfully predict relapses in inflammatory bowel disease (Konikoff & Denson, 2006). An increase in FCp in an absorption study (i.e. gut inflammation) is in keeping with malabsorption.

1.9.3.3. Bile acid metabolism

The assessment of alterations in BA metabolism using currently available methods can be challenging. Number of tests has been used in research settings and a few in clinical setting. Most of these tests are based around the diagnosis of diarrhoea secondary to BA malabsorption (Johnston *et al*, 2011). The tests; stool BA assay by gas-chromatography mass spectrometry (GC-MS), selenium-75-homochloric acid taurine (SeHCAT), FGF-19, stool 14 C tauocholate excretion and serum 7α-hydroxy-4-cholesten-3-one, have their advantages in terms of sensitivity and specificity but also number of drawbacks, such as limited availability, radioactivity, indirect measurement and lack of standardisation (Johnston *et al*, 2011). This study utilised an in-house liquid-chromatography tandem mass spectrometry (LC-MS/MS) method adapted from previously described methods (Zhang *et al*, 1993, Tagliacozzi et al, 2003, Taylor, 2006) to measure plasma and urine fractionated BAs. A decrease in BA in an absorption study (i.e. insufficient digestion and absorption of fats and fat-soluble vitamins in the small intestine) is in keeping with malabsorption.

1.9.4. Hindgut

1.9.4.1. Faecal fat excretion

Ffat are derived from four sources; unabsorbed dietary residue, cellular debris from the intestinal wall, gut micro-organisms and excretions of the body into the intestinal lumen. It is composed of triglycerides, diglycerides, monoglycerides and free fatty acids, as well as sterols and phospholipids (Ramakrishna, 2009). In cases of malabsorption, the bulk of Ffat is

unabsorbed ingested fat. The clinically significant content in Ffat is the triglycerides and the digestion products of triglycerides (free fatty acids, monoglycerides and diglycerides), since on average out of the 50-100 g/day of fat that is ingested daily is almost totally composed of triglycerides (Ramakrishna, 2009).

Measurement of Ffat in the laboratory has been accomplished by a variety of methods including chemical (chemical titration and gas chromatography), physical (gravimetric methods, near infrared spectrometry) and microscopy (Ramakrishna, 2009). The classic method used to measure Ffat quantitatively was the chemical titration method described by van de Kamer in 1949. In the titration method by converting all forms of long chain fat to free fatty acids, quantitatively estimates all Ffat (Ramakrishna, 2009). The method does not estimate fecal sterols because they cannot be saponified or converted into fatty acid salts. The van de Kamer method estimates fat malabsorption reasonably accurately when the diet contains mainly long chain triglycerides however, when the diet contains significant amounts of medium chain fat such as in patients with chronic pancreatitis or other conditions causing fat malabsorption receiving medium chain triglycerides as therapy, it is known to underestimate Ffat (Ramakrishna, 2009).

Another method is by visualisation of fat globules in the feces using Sudan III or IV stain (Drummey *et al*, 1961). This method depends on the hydrophobicity of lipid in a water based solution and the affinity for fat to bind the stain, but this method is not standardised (Hill, 2001). The gravimetric

methods utilize the physical properties of lipid to partition into different solvents in order to measure Ffat (Jeejeebhoy *et al*, 1970). It measures neutral fat (triglycerides, either long chain or medium chain) along with fecal sterols, phospholipids and BAs. The Jeejeebhoy method usually gives a higher Ffat value than the van de Kamer method particularly when the patient has been ingesting medium chain triglycerides (Ramakrishna, 2009). Another methods used for Ffat measurement are the near infrared spectroscopy and steatocrit. Near infrared spectroscopy of stool utilizes the physical properties of fat for quantitation and correlates moderately with the van de Kamer and Jeejeebhoy methods (Benini *et al*, 1989). However, it is unsuitable for quantitating Ffat in stool samples containing >75% water content (Korpi-Steiner *et al*, 2009) furthermore, the technique requires the use of a fairly expensive instrument and is not in routine use. The steatocrit was introduced in 1981 as a simple method to estimate stool fat in infants (Phuapradit *et al*, 1981), even though it is a simple test for measuring Ffat in chronic pancreatitis, the scientific basis behind the use of the test, and its limitations, it is not clinically useful in diagnosing steatorrhea (Ramakrishna, 2009). The standard fat meal is an indirect screening test for abnormal fat absorption (Penfold & Keynes, 1971) but, suffers from the drawbacks of similar tolerance tests. The plasma concentration after the ingestion of a test substance is dependent both on the rate of absorption and the rate of removal of the substances from the plasma. While a tolerance test may be useful in many patients, it is likely to give misleading results in some (Penfold & Keynes, 1971).

Measurement of Ffat excretion over 72 hours is considered to be the gold standard for detection of disorders of fat digestion and absorption (Thomas *et al*, 2003). Even thought it suffers from the difficulties of obtaining accurate collections and involves the handling of large quantities of faeces, to date, alternatives such as semi-quantitative or qualitative fat determinations have proven to be inferior (Lust *et al*, 2006) and lipid tolerance tests have little place in the estimation of fat absorption (Teh *et al*, 1983). 14C-glycerol trioleate fat absorption test with a sensitivity of 85% and a specificity of 93% may be an alternative (Turner *et al*, 1987).

In the investigation of chronic diarrhoea guidelines by the British Society of Gastroenterology, the use of 72 hour Ffat quantification was discouraged (Thomas *et al*, 2003) and clinical biochemistry laboratories have suggested that 72 hour Ffat estimations are unreliable, have limited clinical value and should no longer be available (Hill, 2001). However, a survey of members of the British Society of Gastroenterology in 1998 (Duncan & Hill 1998) and the Australian gastroenterologists in 2006 indicated that the majority considered the test to be useful in clinical practice (Lust *et al*, 2006). An increase in Ffat in an absorption study (i.e. steatorrhoea) is in keeping with malabsorption.

Chapter Two

Materials & Methods

2. Materials and Methods

2.1. Recruitment

This was a cross sectional study consisting of four groups. All subjects were recruited from a London teaching hospital. Three groups consisted of those who had Bariatric surgery (AGB, RYGB or BPD-DS) and a control group consisting of those who did not have obesity surgery but with a BMI > 30kg/m^2. All participants in the control group were recruited from the pre-bariatric surgery assessment clinic.

2.1.1. Protocol

All participants underwent the same investigations which consisted of:

1. Day 1 faecal sample collection at home

2. Day 2 faecal sample collection at home

3. Day 3 faecal sample collection at home

4. Day 3 fasting from 22:00

5. Day 4 participants brought in faecal sample collection pots to hospital. Faeces were measured for faecal elastase-1 and faecal calprotectin to indicate pancreatic insufficiency and gut inflammation respectively as well as for 3 day faecal fat excretion.

6. a. Day 4 (09:00) admitted to the Programmed Investigation Unit (PIU)

6. b. Day 4, fasting EDTA-plasma sample collected from a venous canula for the measurement of citrulline as an estimate of the functional enterocyte mass.

6. c. Day 4, participants took one tablet of Sulphasalazine at 09:00 and blood was collected (EDTA-plasma) at times 0, 60, 90, 120, 150, 180, 210 and 240

min to measure sulphapyridine appearance in plasma. Sulphasalazine is converted to sulphapyridine in the colon before absorption.

6. d. Day 4, Small bowel permeability test; participants drank 100mL of fluid containing D-xylose, L-rhamnose and lactulose. Urine was collected over 5 hours to measure ratio of lactulose: L-rhamnose which would indicate small bowel permeability.

6. e. Day 4, participants were discharged from the PIU at 14:00.

7. Bile acids were measured on the baseline (fasting) plasma and urine samples.

2.1.2. Identification of participants

Participants were identified by referring clinicians in the bariatric surgery and obesity service from referral and clinic letters. Once suitable potential participants were identified, they were approached by telephone and if interested in participating, a copy of the information sheet was sent by post allowing them at least 24 hours to consider this prior to their clinic appointment. In the outpatient clinic, the individuals interested in participating were approached and provided the opportunity to discuss participation further and answer any questions they had. We have found that such an approach to potential research participants is well received and importantly, it reduces the inconvenience to the participant. If still keen to participate in the study, written consent was obtained.

2.1.3. Inclusion criteria

Patients aged between 18 and 70 years, who had AGB, RYGB, BPD-DS or a BMI >30kg/m^2 but not undergone bariatric surgery. Subjects in the control group have been chosen with a BMI >30kg/m^2 as this is comparable with the BMI of the patients who already had bariatric surgery.

2.1.4. Exclusion criteria

Ages <18 or >70 years, pregnancy, substance abuse, more than three alcoholic drinks per day, psychiatric illness, those on weight loss medication, recent participation in other studies or chronic medical conditions that would make it unsafe to perform the standard investigations involved in the study.

2.1.5. Ethics

Ethical approval was grated by the King's College Hospital NHS Foundation Trust Research Ethics Committee and the Research and Development Committee (REC reference number 06/Q0703/209).

2.1.6. Power calculation

Twelve participants in each group are required if the mean faecal fat content in the control or AGB group is 18 mmol/24h and in the RYGB or BPD-DS group 72 mmol/24h with a standard deviation of 36 mmol/24h. At a significance level of 0.05 this would give a power of 95%. A faecal fat content of less than 18 mmol/24 h is considered as negative for malabsorption and the value of 72 mmol/24 h was obtained form a pilot study that we conducted on faecal fat content in patients who had clinical malabsorption.

2.2. Assessment of Malabsorption & adaptation

2.2.1. Entire gut

2.2.1.1. Oro-caecal transit time (Sulphapyridine by HPLC)

Plasma sulphapyridine was measured using reverse-phase high pressure liquid-chromatography (HPLC) (Dionex U3000). The method was adapted in-house from the analytical method first described by Chungi *et al* (1989). EDTA-plasma samples were collected after an overnight fast and every 30 minutes for 240 minutes following the ingestion of sulphasalazine capsule (1 gram). All samples were stored at -20℃ until analysis.

The method involved adding 100µL of plasma with 100µL methanol for protein precipitation. Then 800µL of internal standard (benzamide 20 µg/mL methanol) was added to the tubes. They were then votex mixed and centrifuged at 10000 resolutions per minute (RMP) for 5 minutes. The supernatant was transferred in to fresh tubes and evaporated to dryness under compressed air at 60℃ on a heat block. The dried samples were then reconstituted with 500µL of mobile phase. The mobile phase consisted of 20% methanol in 5.0 mmol/L phosphate buffer (pH 6.0), with 0.5 mmol/L tetrabutylammonium chloride as an ion-pairing agent (5mL in 1L phosphate buffer). Chromatography was accomplished with a reversed-phase ODs C-18 column (5µm; 4.6 x 250 mm). An injection volume of 30µL was set at a flow rate of 1.7 mL/min. The total run time for each sample was 15 minutes. Sulphapyridine and benzamide were detected using an ultraviolet detector at 254 nm. Limit of detection for sulphapyridine was 50ng/mL. The transit time was estimated by the initial detection of sulphapyridine in the timed samples.

The normal reference range for oro-caecal transit time is 210 to 300 min (Sharpstone *et al*, 1999).

2.2.1.2. Functional enterocyte mass (Citrulline by HPLC)

The method used to quantitate plasma citrulline was a reverse-phase HPLC (Dionex U3000). EDTA-plasma samples collected after an overnight fast were used. All samples were stored at -20℃ until analysis.

100µL of water was pipetted into a micro-centrifuge tube, to check for system peaks. Quality control (QC) and patient samples were thawed. 100µL of QC and patient samples were pipetted into respective micro-centrifuge tubes. This was followed by 50µL sulphosalicylic acid precipitating reagent into to all tubes whilst vortexing at the same time to ensure that samples were cleaned up or deproteinised by total precipitation. The mixture was left for 30 minutes at 4℃. Tubes were then centrifuged at 12500 RPM for 10 minutes and 50µL of supernatant was transferred into fresh micro-centrifuge tubes. This was followed by adding 200µL acetonitrile and vortex mixed. Then 40µL of phenylisothiocyanate was added to form phenylthiocyanate derivatives in the presence of a coupling solvent and vortex mixed. This was allowed to react for 20 minutes at room temperature. The micro-centrifuge tubes were then evaporated to dryness on the Genevac EZ-2 Personal Evaporator at 30℃, on HPLC fraction for 1 hour. Finally the dry tubes were reconstituted by adding 200µL sodium acetate buffer and vortex mixed. This was then centrifuged and 180µL of the supernatant was pipetted into labeled HPLC vials. These derivatives were separated using a gradient elution system (RP-HPLC

column) and detected using an ultraviolet detector at 254 nm. Results were calculated using the HPLC software. The normal reference range for citrulline is 30-50 µmol/L (Crenn *et al*, 2008).

2.2.2. Foregut

2.2.2.1. Exocrine pancreatic function (Faecal elastase-1 by ELISA)

FE1 was measured by sandwich Enzyme Linked Immuno-Sorbent Assay (Pancreatic Elastase-1 Stool Test; ScheBo® Biotech, Giessen, Germany) with two monoclonal antibodies highly specific for human pancreatic FE1. From the three day faecal collection about 100mg of faeces was transferred into blue capped universal tubes and stored at -20℃ until analysis.

Faecal samples were thawed overnight and individually extracted using the E1 Quick-Prep™ (Schebo®) sample preparation system in a fume cupboard. The tubes of the E1 Quick-Prep™ sample preparation system contain ready to use extraction buffer. Following extraction the faecal samples were analysed using the Schebo Elastase kit. 50µL of blank, standards (1 to 4), QC (1 & 2) and patients' samples were pipetted into the corresponding wells of the ELISA plate in duplicates. This was incubated for 30 minutes at room temperature (FE1 from both the samples and standards binds to the antibody and is immobilised on the plate) and followed with three wash cycles. Then 50µL monoclonal anti-elastase-1-biotin and peroxidase (POD)-Streptavidin was added and incubated (monoclonal anti-elastase-1-biotin and peroxidase (POD)-Streptavidin binds to FE1) for 15 minutes in the dark at room temperature and followed up with three wash cycles. Colour reaction; 100µL

of substrate solution was then added and incubated for 15 minutes in the dark at room temperature. The reaction was stopped by adding a further 100µL of stop solution per well. The peroxidase oxidizes ABTS (2, 2'-Azino-bis-(3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt), which turns dark green. Finally, the concentration of oxidized ABTS was determined photometrically at 405nm between five and 30 minutes after adding the stop solution. The final concentrations were obtained by using the ELISA software.

The detection limit of the assay was 15 µg/g of faeces. Any sample with a concentration >500 µg/g of faeces was reported as 500 µg/g. Sample types unsuitable for FE1 analysis include the following: grossly contaminated with blood, lots of fibrous matter, hard stools, mucous samples, and watery stool samples. The normal reference range for FE1 is ≥200 µg/g faeces (Duncan & Hill, 2007).

2.2.3. Mid-gut

2.2.3.1. Small bowel permeability (Reducing sugars by LC-MS/MS)

This method involved separating the urinary sugars by HPLC (Jasco™ LC-2000 series) using an amino (NH2) column in hydrophilic interaction liquid chromatography (HILIC) mode. The sugars were then quantitated by electrospray tandem mass-spectrometry (MS/MS) (API 3200™ by Applied Biosystems, Cheshire, UK). The mechanisms behind HILIC are not fully understood but the general consensus is that they involve partitioning of the solute between the eluent and a water enriched layer that forms on the particles within the column. Secondary interactions are thought to occur between the NH2 groups on the column and the hydroxyl groups on the sugars.

A baseline urine sample (pre administration of sugar load - five grams lactulose, 500mg D-xylose and one gram L-rhamnose) and a five hour urine collection following administration of sugar load were collected in bottles containing a preservative (10mL of 10% Thymol in Iso-Propanol was added to each 1L urine collection bottle and 250µl to each universal collection pot). Aliquots of baseline and five hour urines were stored at -20℃ until analysis.

SBPT run required water blank, calibration standards (1 to 5) and QC (1 to 3). Frozen standards, QC material and patient samples were placed on the roller mixer until they defrosted. 1.5mL micro-centrifuge tubes were labelled for each blank, calibration standard, QC and patient samples. 975µL of internal standard diluent was aliquoted into each micro-centrifuge tube and 25µL of

blank, calibration standard, QC and samples into the corresponding tubes. They were then votexed thoroughly for 30 seconds and centrifuged at 12000 RPM for three minutes. Finally supernatants were aliquoted into labelled HPLC vials and loaded onto the auto-sampler of the LC-MS/MS.

The internal standard or diluent was made by diluting 10mg of 5-13C D-Xylose into 50mL of deionised H_2O in a 250mL volumetric flask. Once the sugar had dissolved slowly, acetonitrile was added and swirled, to the final volume. The internal standard has been found to be stable for at least three months when stored at 4℃. A 1 in 40 dilution of a sample containing 0.1mg% of Lactulose gave a signal to noise ratio of 10:1. As this was the lowest sensitivity peak, this was deemed to be the lowest limit of detection for this assay. Chromatograms were checked and peak heights adjusted individually for lactulose, D-xylose and L-rhamnose. The data was then analysed using Excel spreadsheet (Microsoft®) and final values calculated based on five hour urine volumes. The normal reference range ranges are; Lactulose <0.9%, D-xylose 20 - 55%, L-Rhamnose 6.7 - 21%, Lactulose: L-Rhamnose ratio <0.05 (Sharpstone *et al*, 1999).

2.2.3.2. Gut inflammation (Faecal calprotectin by ELISA)

The calprotectin assay was done by quantitative ELISA (PhiCal® Calprotectin ELISA Kit, Immundiagnostik, Bensheim, Germany) using monoclonal antibodies for the detection of calprotectin in the faeces. From the three day faecal collection about 100mg of faeces was transferred into blue capped universal tubes and stored at -20℃ until analysis.

Initially faecal samples were thawed overnight and weighed out individually (weight requirement was >50g but <100g) in a fume cupboard in 5mL screw cap tubes. From the weight of the sample the volume of dilution extraction solution needed was calculated using a spreadsheet. After pipetting the extraction solutions, tubes were mixed vigorously for 30 seconds using a vortex mixer and homogenised for a further 25 minutes on a shaker. 1mL of the fluid was then transferred into Eppendorf tubes and centrifuged for 20 minutes (12000 RPM at room temperature). Then 0.5mL of the supernatant was transferred to a new Eppendorf tubes. Calprotectin concentrations were then detected by ELISA.

20µL of sample extract was diluted with 980µL working dilution solution (1:50) and votex mixed. 100µL of blank, calibrator (1 to 5), QC (1 & 2) and diluted samples were added to respective wells in duplicates. This was incubated at room temperature for 45 minutes and washed (three cycles). Then 100µL conjugate was added and incubated in the dark at room temperature for a further 45min. Plate was washed (three cycles) and 100µL of substrate was added to each well. This was followed by the final incubation step in the dark for 30 minutes. Plate was read at 405nm and results calculated using the software. Duplicate coefficient of variation values were less than 15%. The detection limit of the assay was 10 µg/g of faeces. Any sample with an initial concentration >600 µg/g of faeces were diluted (to a maximum of 1 in 8) in incubation buffer to ensure that the calprotectin concentration falls within the

linear portion of the standard curve. The normal reference range quoted by the manufacture was ≤60 µg/g of faeces.

2.2.3.3. Bile acid metabolism

2.2.3.3.1. Bile acids by LC-MS/MS

BA fractions were analysed using an in-house method using a HPLC system (Jasco^{TM}) with three PU-pumps, MX-2080-32 solvent mixing module, AS-2059 auto-sampler and CO-2067 column oven (Tokyo, Japan). The HPLC system was connected to an API 3200^{TM} triple quadrupole MS (Applied Biosystems) operated with an electrospray ionisation source. Chromatography was performed using an Acentis fused-core C18 analytical column (150x4.6 mm, particle size 2.7 µm, Sigma-Aldrich, Poole, UK) incubated at 40℃. Mobile phases comprised (A) methanol or (B) deionised H2O each containing 5 mM ammonium acetate (w/v) and 0.012% formic acid (v/v). Negative ion mass spectra of the fractionated BAs were recorded in multiple reactions monitoring (MRM) mode. Data were acquired using provided software and quantitated using peak area analysis corrected by comparison to the respective internal standard. The method was linear between 0.1 and 10 µmol/L for all BAs and their conjugates with coefficients of variation (CV%) of 3.6-8.0% at the lower limit of quantitation (0.1 µmol/L). The inter-assay CV was 1.5-6.8%. A chromatogram of the BA fractions are shown and listed with their corresponding retention times in Figure 6 and Table 1 respectively.

2.2.3.3.2. Reagents

All solvents and chemicals used were of analytical grade. Sodium salts of DC, GDC, TDC, CDC, GCDC, TCDC, UDC, TUDC, LC, GLC, TLC, CA, GCA and TC were sourced from Sigma-Aldrich. GUDC was purchased from Merck (Feltham, UK). The deuterated (d) internal standards deoxycholic-2,2,4,4-d4 acid (d4-DC) and glycodeoxycholic-2,2,4,4-d4 acid (d4-GDC) were obtained from QMX Laboratories (Essex, UK), whilst taurodeoxycholic-2,2,4,4-d4 acid (d4-TDC) had been synthesised in house previously according to an existing protocol (Zhang *et al*, 1993). Helix pomatia sulphatase enzyme, sodium acetate, sodium ascorbate, formic acid and phosphate-buffered saline (PBS) were from Sigma-Aldrich. Methanol, acetonitrile and ammonium acetate were purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland).

2.2.3.3.3. Plasma Bile Acids

Extraction of BA from EDTA-plasma was performed as described previously Tagliacozzi *et al*, 2003). To 250µL of sample 800µL of protein precipitating solution (acetonitrile containing each of the internal standards; a 2,2,4,4,d4-DC for each of the BA chemistries; unconjugated, glycine- and taurine-conjugated) was added and vortex-mixed. Then samples were centrifuged at 13000 RPM for 10 minutes, after which 900µL of the supernatant was transferred into fresh microtubes. The supernatant was then blown to dryness using compressed air in a 60℃ heated block. The residue was re-constituted in 250µL of a 50:50 (v/v) mix of mobile phases A and B. The re-constituted solution was transferred to HPLC vials and 10µL injected onto the column.

2.2.3.3.4. Urine Bile Acids

Enzymatic hydrolysis of glucuronide and sulphate BA conjugates was carried out in the urine samples using an established method used for urine steroid profiling (Taylor, 2006). Samples were Sep-Pak® (C18 column) extracted, blown to dryness using compressed nitrogen in a 60℃ heated block and dissolved in 2.5mL of 0.5M sodium acetate containing 10 mg/mL of Helix pomatia powder and 20 mg/mL of sodium ascorbate. This was then incubated for 72 hours in a water bath at 37℃. Freed BAs were then re-extracted on Sep-Pak® columns, eluted and dried. Once purified, they were treated the same way as the plasma samples described in chapter 2.2.3.3.3.

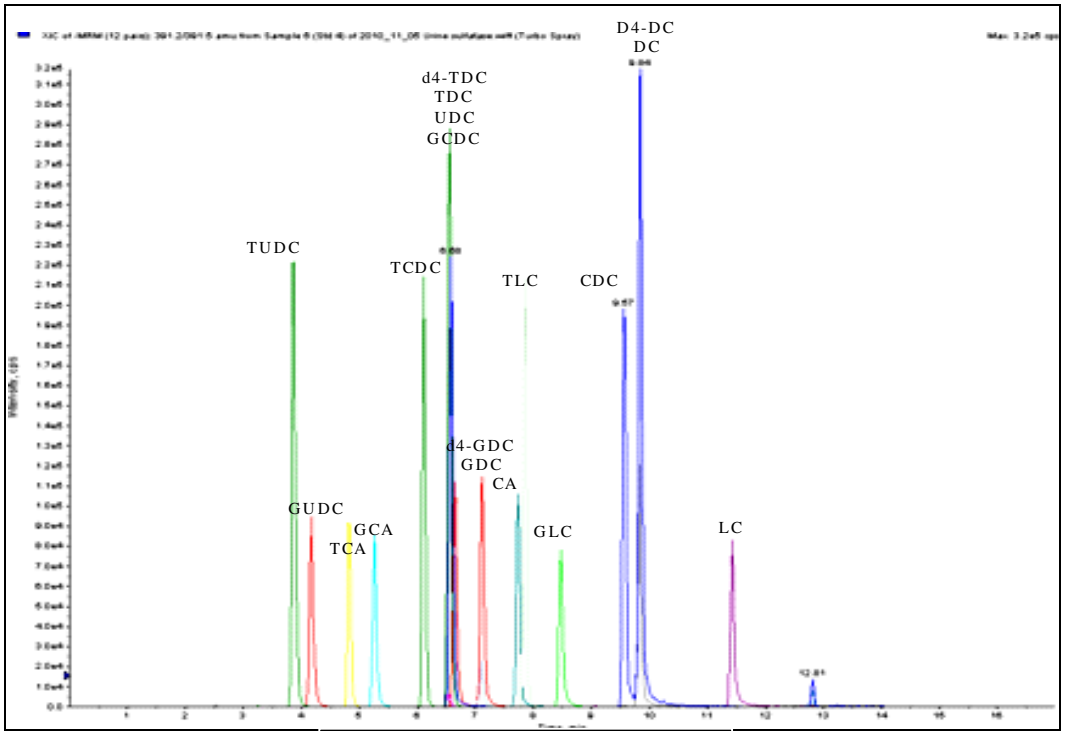


Figure 6: A chromatogram of a 1 µmol/L bile acid standard mixture showing all the fractions measured (from left to right: TUDC-Tauroursodeoxycholic acid, GUDC-Glycoursodeoxycholic acid, TCA-Taurocholic acid, GCA- Glycocholic acid, TCDC-Taurochenodeoxycholic acid, UDC-Ursodeoxycholic acid, GCDC-Taurodeoxycholic-2,2,4,4-d4 acid, D4-DC-Deoxycholic-2,2,4,4-d4 acid, GDC-Glycodeoxycholic acid, CA-Cholic acid, TLC-Taurolithocholic acid, GLC-Glycolithocholic acid, CDC-Chenodeoxycholic acid, DC- Deoxycholic acid, LC- Lithocholic acid).

Table 1: Bile acid fractions with retention times

Bile Acid Fraction	Retention Time (min)
Tauroursodeoxycholic acid (TUDC)	3.91
Glycoursodeoxycholic acid (GUDC)	4.27
Taurocholic acid (TCA)	4.85
Glycocholic acid (GCA)	5.27
Taurochenodeoxycholic acid (TCDC)	6.53
Taurodeoxycholic acid (TDC)	6.60
Ursodeoxycholic acid (UDC)	6.60
Glycochenodeoxycholic acid (GCDC)	6.60
Glycodeoxycholic acid (GDC)	7.10
Cholic acid (CA)	7.77
Taurolithocholic acid (TLC)	7.69
Glycolithocholic acid (GLC)	8.55
Chenodeoxycholic acid (CDC)	9.60
Deoxycholic acid (DC)	9.83
Lithocholic acid (LC)	11.50

2.2.4. Hindgut

2.2.4.1. Faecal fat excretion (Free fatty acids by chemical titration)

Three day Ffat excretion was measured by the method described by van de Kamer in 1949. The dry weight of the three day faecal collection was measured. The samples were then homogenised with hot water to produce a smooth suspension. All samples were analysed in duplicates and run over three consecutive days. 15mL of the homogenized samples was transferred into a 250mL weighed conical flask and weight of the faecal samples was derived. A recovery sample was processed for each day. To the 15mL sample 45mL of 5% ethanolic potassium hydroxide was added along with a few anti-bumping granules. The flasks were boiled for a minimum of 30 minutes over a steam bath and then allowed to cool to room temperature. Then 15mL of working hydrochloric acid (HCL; 330g in 1L) was added and mixed followed by 25mL of petroleum ether. The flasks were shaken vigorously for two minutes. Then they were left in the fume cupboard in order for the phases to separate (the hydrolysed fatty acids moved to the upper layer while the mineral acids remained at the bottom). Then 7mL of the upper phase was transferred into glass tubes and centrifuged as 1000 RPM for two minutes. 5mL of the aliquot was then tranfered into a 100mL stopped conical flask and 1mL ethanol was added to each sample along with three drops of phenolphthalein indicator. Using a 1mL burette calibrated to 0.2mLs, M/20 tetramethyl ammonium hydroxide (TMAH) was used to titrate until the indicator turned to a permanent pink colour. The total fatty acids were calculated to obtain 24 hour Ffat excretion. The recovery was within 90-110%.

The normal reference range for faecal fat excretion in adults is 16 - 25 mmol/day (Duncan & Hill, 2007).

2.3. Statistical analysis

The distribution of all variables was tested using Shapiro-Wilk W test. As the data was non-parametric the groups were compared using, Kruskal-Wallis analysis of variance (ANOVA). Mann-Whitney pair wise test was used to compare between groups where significance was obtained with Kruskal-Wallis ANOVA, with Bonferroni correction for the four comparisons. Correlations were assessed using Spearman's correlation test. All statistical analysis was done on Analyse-it® (version 2.21, Microsoft®, Leeds, UK) software. P value of <0.05 was considered to be statistically significant. Data is expressed as median (IQR).

Chapter Three

Results

3. Results

The demographics of the four groups are summarised in Table 2 and the recruitment and sample analysis are summarised in Figure 7. All the biochemical investigations in the four groups are summarised in Table 3.

Time since operation to recruitment were similar between the surgical groups (p=NS). However, there was a wide range in time since operation where AGB were recruited between 5 to 29 months, RYGB between 3 to 65 months and the BPD-DS between 9 to 95 months. The BPD-DS group was significantly heavier than AGB and RYGB groups (p=0.02, p=0.02 respectively). This is expected as those with BMI >60 kg/m^2 often opted for BPD-DS given the greater weight reduction seen in this procedure. This is further supported by post operative weight loss of 68 (65-98) kg compared to 18 (15-22) kg in AGB (p<0.001) and 30 (26-31) kg in RYGB (p=0.02). The control group were not matched for post operative BMI of the surgical groups and were heavier than all the three surgical groups (all p<0.05). The Roux limb length of the RYGB group was approximately 150 cm and the common channel for the BPD-DS group was approximately 100 cm.

Table 2: Demographics of the four groups.

	Control	AGB	RYGB	BPD-DS	p value
Number (n)	7	6	7	5	
Male: Female (ratio)	2:5	1:5	2:5	1:4	NS
Age (years)	45 (43-51)	46 (32-61)	48 (44-55)	40 (43-47)	NS
Preoperative weight (kg)	N/A	124 (115-131)	128 (123-138)	161 (147-187)	0.004
Preoperative BMI (kg/m^2)	N/A	44 (43-47)	47 (42-51)	60 (53-62)	0.003
Current weight (kg)	132 (129-140)	103 (99-106)	104 (99-108)	92 (89-93)	0.001 NS*
Current BMI (kg/m^2)	47 (44-50)	38 (37-39)	36 (34-38)	30 (25-38)	<0.0001 NS*
Weight loss (kg)	N/A	18 (15-22)	30 (26-31)	68 (65-98)	<0.0001
Time since operation (m)	N/A	21 (11-26)	10 (8-17)	30 (19-64)	NS

Results expressed as median (IQR) or numbers. P value was obtained by Kruskal–Wallis Analysis of Variance (ANOVA) between the four groups. * denotes P values obtained between the three surgical groups. Abbreviations: AGB, adjustable gastric banding; RYGB, Roux-en-Y gastric bypass; BPD-DS, bilio-pancreatic diversion-duodenal switch; BMI, body mass index; m, months; NS, not significant.

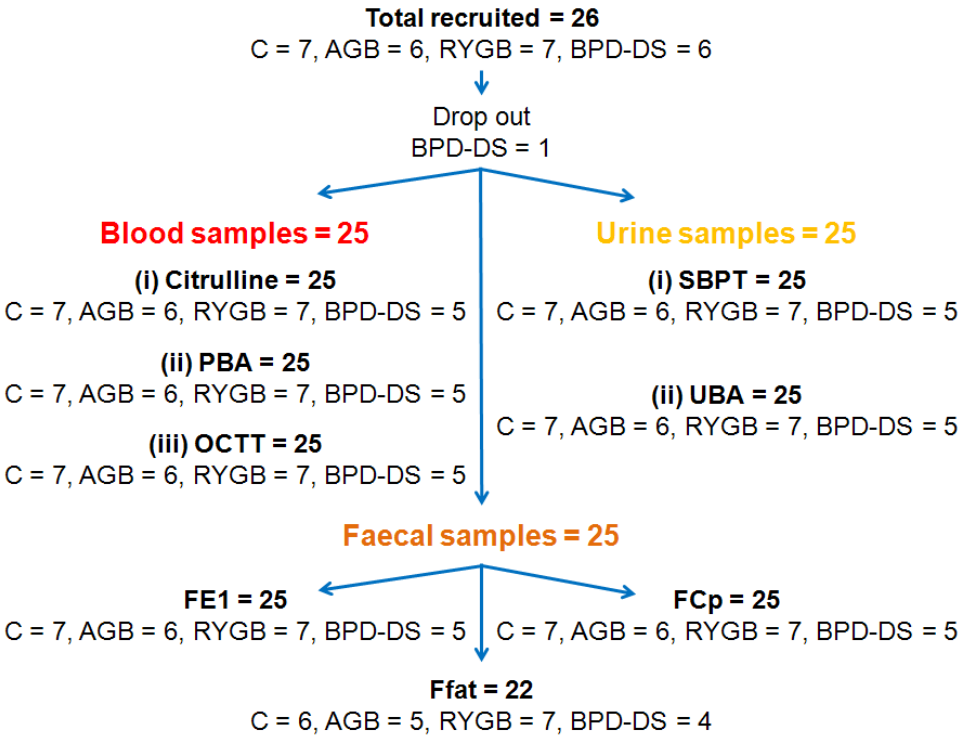


Figure 7: Flowchart demonstrating participant recruitment and sample analysis. Abbreviations: C, control; AGB, adjustable gastric banding; RYGB, Roux-en-Y gastric bypass; BPD-DS, biliopancreatic diversion-duodenal switch; FE1, faecal elastase-1; FCp, faecal calprotectin; SBPT, small bowel permeability test; OCTT, oro-caecal transit time; Ffat, faecal fat; PBA, plasma bile acid; UBA, urine bile acid.

Table 3: All the biochemical investigations done on urine, blood and faecal samples in the four groups.

Investigation	Control	AGB	RYGB	BPD-DS	K-W ANOVA
OCTT (min)	180 (165-240)	210 (158-240)	180 (180-203)	180 (150-210)	0.935
Citrulline (µmol/L)	21 (20-22)	22 (21-24)	21 (19-26)	20 (19-22)	0.819
FE1 (µg/g faeces)	500 (500-500)	500 (289-500)	366 (292-382)	376 (245-377)	0.012
SBPT Lactulose (%)	0.205 (0.124-0.246)	0.155 (0.089-0.124)	0.261 (0.197-0.390)	0.662 (0.660-0.889)	0.002
D-Xylose (%)	28.1 (20.1-38.2)	20.1 (10.1-23.5)	28.2 (19.4-31.9)	22.0 (21.0-24.9)	0.406
L-Rhamnose (%)	9.9 (7.2-12.1)	6.2 (3.6-7.7)	9.8 (7.2-12.6)	7.6 (7.0-7.7)	0.133
L:R ratio	0.014 (0.009-0.018)	0.003 (0.001-0.006)	0.010 (0.005-0.017)	0.117 (0.089-0.123)	0.002
FCp (µg/g faeces)	15.6 (15.6-17.7)	32.7 (15.6-56.0)	48.1 (25.4-81.3)	18.5 (17.3-20.0)	0.038
PBA (µmol/L)	2.588 (2.280-3.387)	2.819 (2.336-3.961)	3.507 (2.110-4.120)	12.127 (11.203-12.996)	0.014
UBA (nmol/mmol)	195.0 (131.0-214.0)	250.1 (160.0-445.4)	398.7 (328.2-481.4)	1227.3 (645.7-1562.0)	0.006
Ffat (mmol/d)	18.5 (14.7-26.9)	23.9 (11.7-35.1)	61.7 (49.3-72.9)	292.3 (212.4-377.0)	0.002
FWt (g/72 hr)	233 (171-422)	306 (266-390)	310 (241-409)	1583 (1092-1909)	0.056

Abbreviations: SBPT, small bowel permeability test; L: R, lactulose: L-Rhamnose; AGB, adjustable gastric banding; RYGB, Roux-en-Y gastric bypass; BPD-DS, bilio-pancreatic diversion-duodenal switch; K-W ANOVA, Kruskal–Wallis Analysis of

Variance; OCTT, oro-caecal transit time; FE1, faecal elastase-1; SBPT, small bowel permeability test, FCp, faecal calprotectin; PBA, total plasma bile acid; UBA, total urine bile acid; Ffat, faecal fat; FWt, faecal weight. FE1 >500 µg/g faeces was taken as 500 for analysis. P value was obtained by K-W ANOVA between the four groups and $p<0.05$ was considered significant. Results are expressed as median (IQR).

3.1. Entire gut

Oro-caecal transit time: Sulphapyridine was detected at 180 (165-240) min in the control group, 210 (158-240) min in the ABG group, 180 min (180-203) in the RYGB group and 180 min (150-210) in the BPD-DS group. There was no difference in OCTT between the four groups (p=0.935) [Figure 8].

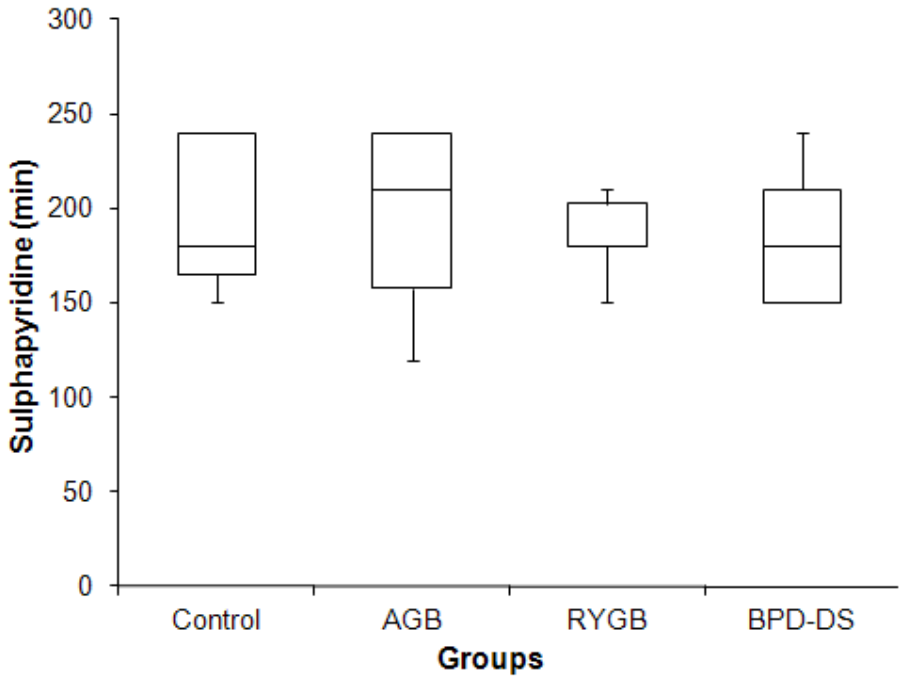


Figure 8: Box and Whisker plots of the sulphapyridine concentration between the four groups. The measurement of oro-caecal transit time (OCTT) using orally administered sulphasalazine relies on the principle that colonic microorganisms produce azo-reductase which converts sulphasalazine to sulphapyridine. Hence, the detection of sulphapyridine in timed plasma samples is a measure of OCTT. There was no difference in the OCTT between the four groups.

Functional enterocyte mass: Plasma citrulline concentration a surrogate marker of functional enterocyte mass was 21 (20-22) µmol/L in the control group, 22 (21-24) µmol/L in the AGB group, 21 (19-26) µmol/L in the RYGB group and 20 (19-22) µmol/L in the BPD-DS group. There was no difference in functional enterocyte mass between the four groups (p=0.819) [Figure 9].

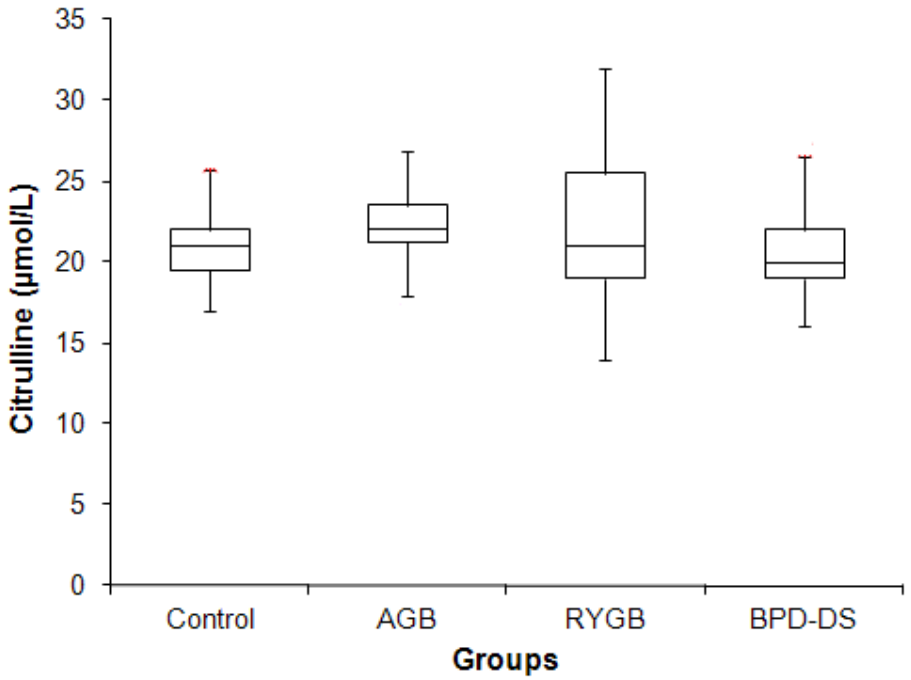


Figure 9: Box and Whisker plots of citrulline concentration between the four groups. Citrulline is synthesised exclusively in small intestinal enterocytes, hence, it is a surrogate marker of the functional enterocyte mass. There was no difference in the functional enterocyte mass between the four groups.

3.2. Foregut

Faecal elastase-1: FE1 concentrations were 500 (500-500) µg/g faeces in the control group, 500 (289-500) µg/g in the AGB group, 366 (292-382) µg/g in the RYGB group and 376 (245-377) µg/g in the BPD-DS group. FE1 was lower in the RYGB group compared to the control ($p=0.002$), with no difference between any other group comparisons (all $p>0.05$) [Figure 10].

The total three day (72 hours) faecal weight was 233 (171-422) g in the control group, 306 (266-390) g in the AGB group, 310 (241-409) g in the RYGB group and 1583 (1092-1909) g in the BPD-DS group (Table 3). When FE1 concentration was corrected for the total three day faecal collection, there was no difference between the groups; 116500 (85250-210750) µg/72 hours in control, 133000 (71000-195000) µg/72 hours in the AGB, 118730 (78187-147484) µg/72 hours in the RYGB and 425296 (293436-565924) µg/72 hours in the BPD-DS group ($p=0.231$).

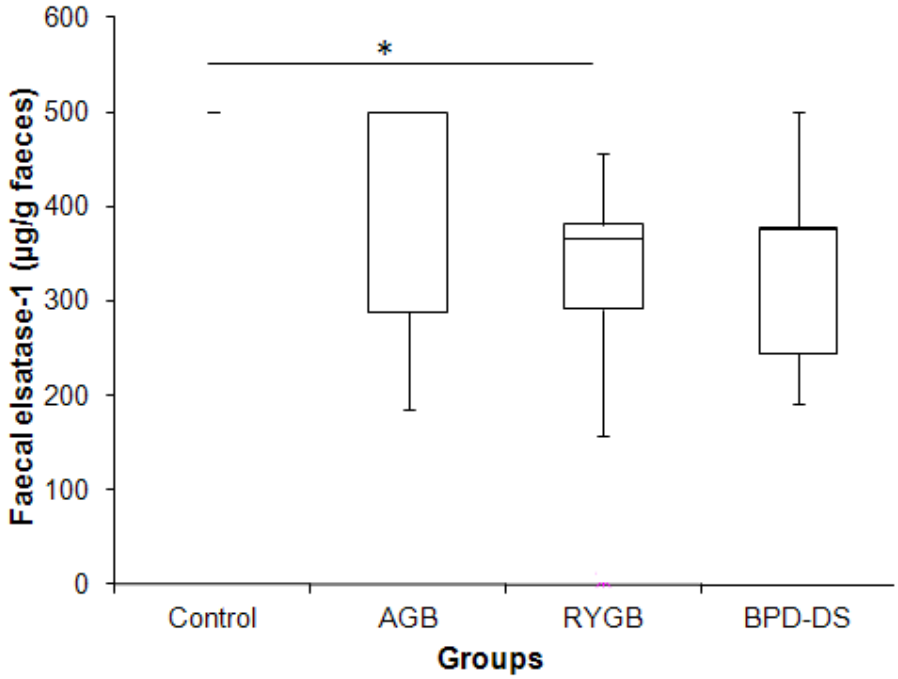


Figure 10: Box and Whisker plots of faecal elastase-1 (FE1) between the four groups. FE1 is a sensitive and specific marker of exocrine pancreatic function. FE1 ≤200 µg/g indicates exocrine pancreatic insufficiency.* denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. FE1 >500 µg/g faeces was reported as 500 for analysis. FE1 was lower in the RYGB group compared to the controls ($p=0.002$), with no difference between any other group comparisons (all $p>0.05$).

3.3. Mid-gut

Small bowel permeability test (SBPT): The recovery of D-Xylose in the urine was 28.1 (20.1-38.2) % in the control group, 20.1 (10.1-23.5) % in the AGB group, 28.2 (19.4-31.9) % in the RYGB group and 22.0 (21.0-24.9) % in the BPD-DS group. The recovery of L-Rhamnose in the urine was 9.9 (7.2-12.1) % in the control, 6.2 (3.6-7.7) % in the AGB, 9.8 (7.2-12.6) % in the RYGB and 7.6 (7.0-7.7) % in the BPD-DS. There was no differences in recovery of D-Xylose ($p=0.406$) or L-Rhamnose ($p=0.133$), suggesting similar absorptive capacity for the two mono-saccharides in all four groups. The recovery of Lactulose in the urine was 0.205 (0.124-0.246) % in the control group, 0.155 (0.089-0.124) % in the AGB group, 0.261 (0.197-0.390) % in the RYGB group and 0.662 (0.660-0.889) % in the BPD-DS group. The recovery of Lactulose was higher in the BPD-DS compared to the control ($p=0.020$) and the AGB ($p=0.016$), with no difference between other group comparisons (all $p>0.05$).

The L:R ratio was 0.014 (0.009-0.018) in the control group, 0.003 (0.001-0.006) in the AGB group, 0.010 (0.005-0.017) in the RYGB group and 0.117 (0.089-0.123) in the BPD-DS group. The L:R ratio in BPD-DS was higher compared to the control, ($p=0.012$), the AGB ($p=0.016$) and the RYGB ($p=0.012$) groups. There was no difference in the L:R ratio between the other three groups (all $p>0.05$) [Figure 11].

Figure 11: Box and Whisker plots of lactulose:rhamnose (L:R) ratio between the four groups. The L:R ratio is part of the small bowel permeability test and a L:R ratio >0.05 indicates increased small bowel permeability. * denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. L:R ratio in biliopancreatic diversion-duodenal switch (BPD-DS) was higher compared to the control, ($p=0.012$), the adjustable gastric banding (AGB) ($p=0.016$) and the Roux-en-Y gastric bypass (RYGB) ($p=0.012$) groups. There was no difference in the L:R ratio between the other three groups (all $p>0.05$).

Faecal calprotectin: FCp concentration was 15.6 (15.6-17.7) µg/g faeces in the control group, 32.7 (15.6-56.0) µg/g in the AGB group, 48.1 (25.4-81.3) µg/g in the RYGB group and 18.5 (17.3-20.0) µg/g in the BPD-DS group. FCp was higher in the RYGB, compared to control group (p=0.016). In the RYGB group three out of the seven patients had concentrations >60µg/g faeces. There was no difference between the control, AGB and BPD-DS, all p>0.05 [Figure 12]. One patient in the AGB group and one patient in the BPD-DS group had elevated FCp of 218 and 109 µg/g faeces respectively.

When FCp concentration was corrected for the total three day faecal collection, there was no difference between the groups; 5522.4 (2659.8-8251.8) µg/72 hours in control, 11949.6 (4149.6-15238.8) µg/72 hours in the AGB, 26141.4 (6183.8-34515.4) µg/72 hours in the RYGB and 28436.1 (177420.0-77431.7) µg/72 hours in the BPD-DS group (p=0.076).

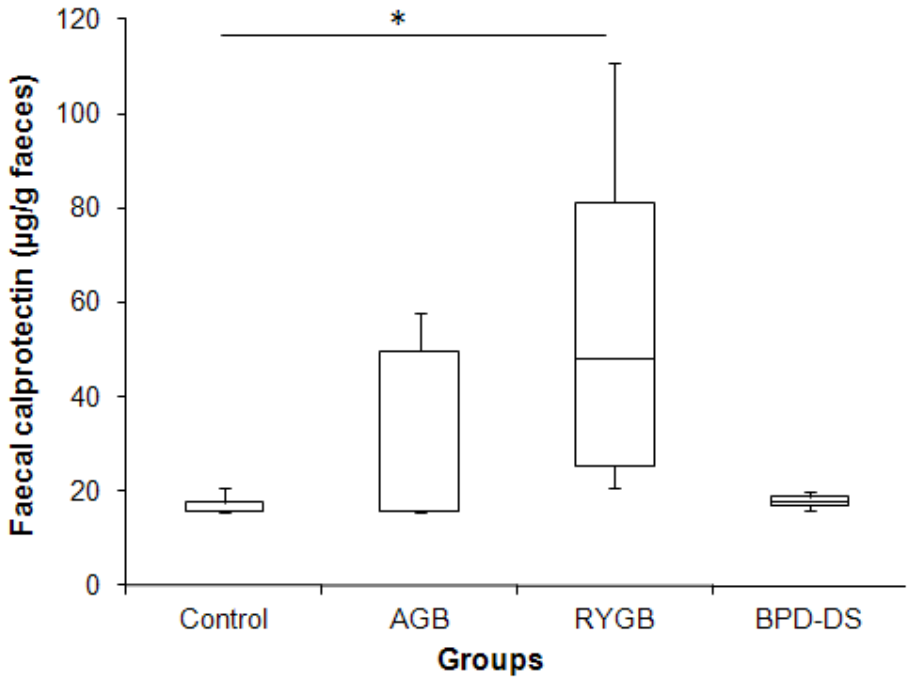


Figure 12: Box and Whisker plots of faecal calprotectin (FCp) concentration between the four groups. Calprotectin accounts to nearly 50% of the neutrophil protein in both blood and faeces. FCp >60 µg/g faeces indicates gut inflammation. * denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. FCp was higher in the RYGB, compared to control group ($p=0.016$) with no difference between the other three groups (all $p>0.05$).

Bile acids: The total BAs in the fasting plasma sample were 2.588 (2.280-3.387) µmol/L in the control group, 2.819 (2.336-3.961) µmol/L in the AGB group, 3.507 (2.110-4.120) µmol/L in the RYGB and 12.127 (11.203-12.996) µmol/L in the BPD-DS group. The total plasma BA was elevated in the BPD-DS group compared to the control, the ABG and the RYGB groups (all $p<0.05$) with no difference between other group comparisons [Figure 13a]. The primary BAs (CA & CDC) and secondary BAs (DC, LC & UDC) did not show any difference between the groups (all $p>0.05$). However, the total conjugated BAs were higher in the BPD-DS group 5.129 (4.154-6.204) µmol/L compared to the control group 1.365 (1.127-1.529) µmol/L, the AGB group 1.947 (1.684-2.419) µmol/L and the RYGB group 1.449 (1.276-1.775) µmol/L (all $p<0.05$).

The total BAs in the fasting urine sample were 195.0 (131.0-214.0) nmol/mmol in the control group, 250.1 (160.0-445.4) nmol/mmol in the AGB group, 398.7 (328.2-481.4) nmol/mmol in the RYGB and 1227.3 (645.7-1562.0) nmol/mmol in the BPD-DS group. The total urine BA was elevated in the BPD-DS group compared to the control, the ABG and the RYGB groups (all $p<0.05$) with no difference between other group comparisons [Figure 13b]. The primary BAs and the secondary BAs were higher in the BPD-DS group compared to the control, AGB and RYGB groups (all $p<0.05$) with no difference between the other group comparisons (data not shown). However, the total conjugated BAs were not different between the groups; control 91.8 (80.4-141.1) nmol/mmol, AGB 166.0 (101.3-280.8) nmol/mmol, RYGB 252.3 (175.8-374.4) nmol/mmol and BPD-DS 541.2 (306.4-856.9) nmol/mmol (all $p>0.05$).

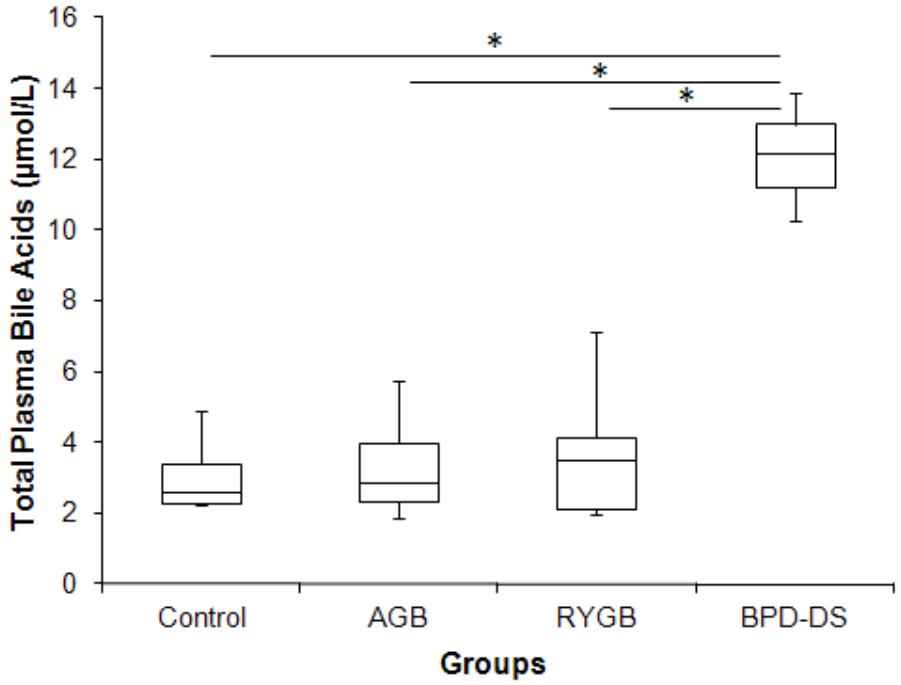


Figure 13a: Box and Whisker plots of the total plasma bile acid concentration between the four groups. * denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. The total plasma BA was elevated in the BPD-DS group compared to the control, the ABG and the RYGB groups (all $p<0.05$).

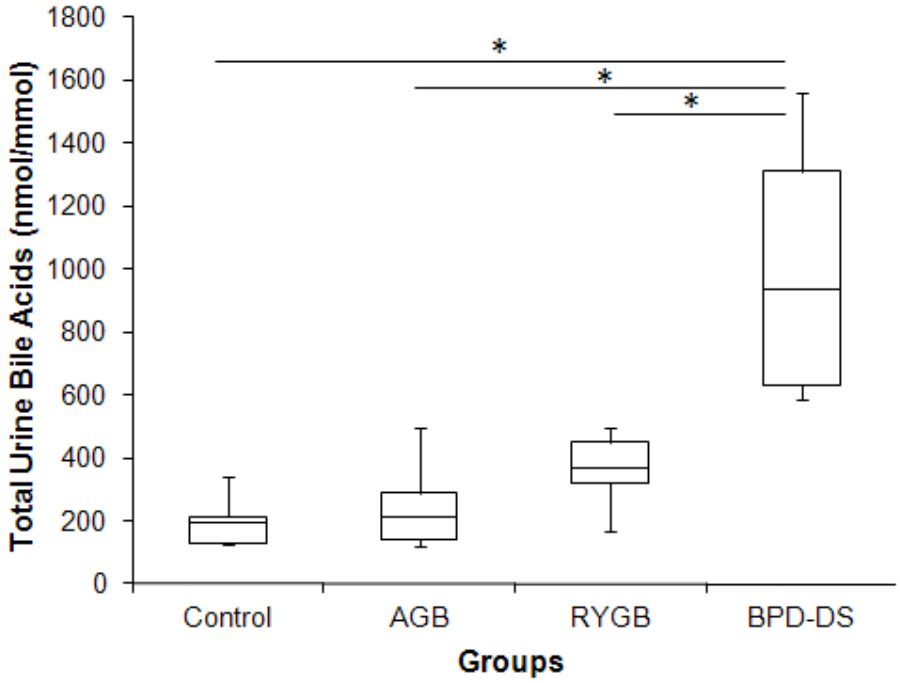


Figure 13b: Box and Whisker plots of the total urine bile acids concentration between the four groups. * denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. The total urine BA was elevated in the BPD-DS group compared to the control, the ABG and the RYGB groups (all $p<0.05$) with no difference between other group comparisons.

3.4. Hindgut

Faecal fat excretion: The Ffat excretion was 18.5 (14.7-26.9) mmol/d in the control group, 23.9 (11.7-35.1) mmol/d in the AGB group, 61.7 (49.3-72.9) mmol/d in the RYGB group and 292.3 (212.4-377.0) in the BPD-DS group. The BPD-DS group had higher Ffat content compared to the control ($p=0.038$), the AGB ($p=0.046$) and the RYGB ($p=0.024$) groups. The RYGB group had higher Ffat content compared to the control group ($p=0.033$). There was no difference between the RYGB and AGB groups ($p=0.808$) [Figure 14].

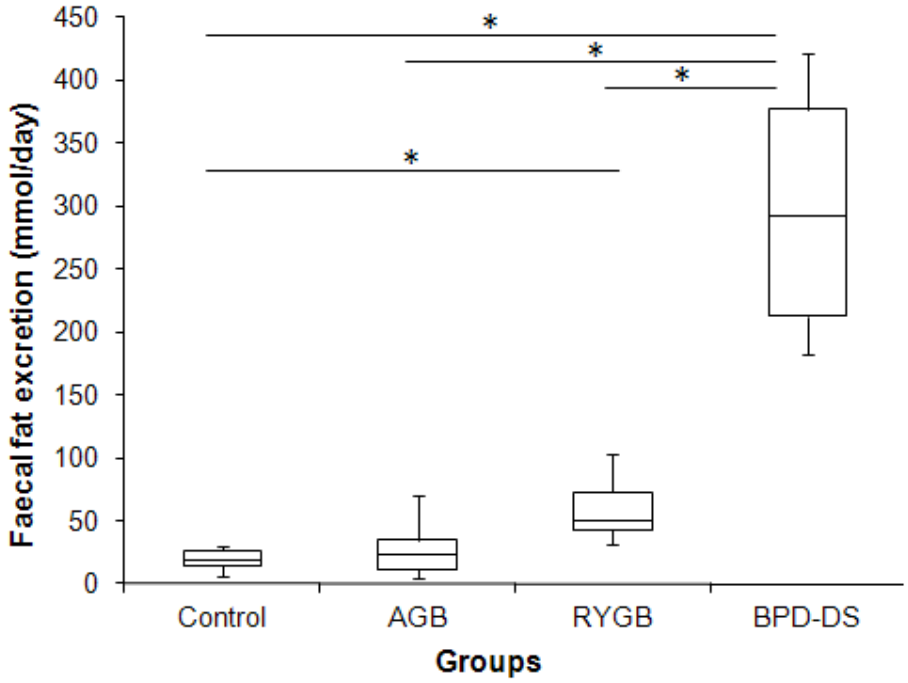


Figure 14: Box and Whisker plots of faecal fat excretion between the four groups. * denotes significance ($p<0.05$). P value was obtained by Mann-Whitney pair wise test, with Bonferroni correction for the four comparisons. The BPD-DS group had higher Ffat content compared to the control ($p=0.038$), the AGB ($p=0.046$) and the RYGB ($p=0.024$) groups. The RYGB group had higher Ffat content compared to the control group ($p=0.033$). There was no difference between the RYGB and AGB groups ($p=0.808$).

Spearman correlation between FCp and FE1 is shown in Figures 15. Figures 16a and 16b show Spearman's correlation between weight loss as a percentage (%) and rate of weight loss as kg/month and Ffat excretion.

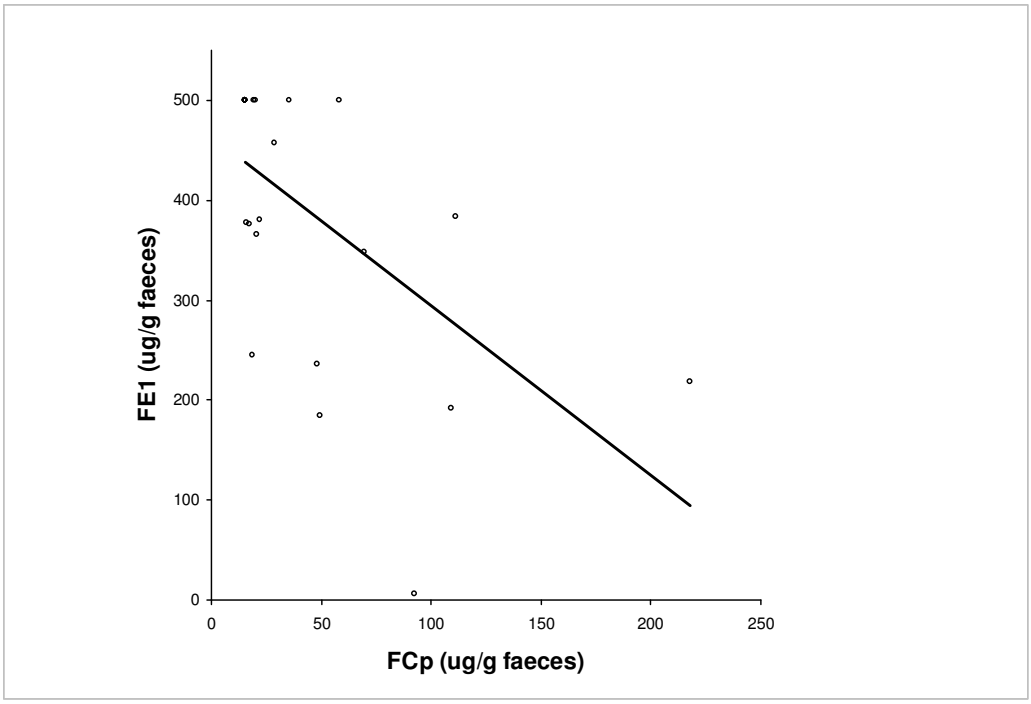


Figure 15: Spearman correlation between FCp (faecal calprotectin) and FE1 (faecal elastase-1) in all the groups. There was a negative correlation between the FCp and FE1 concentrations (R^2 -0.69, p=0.001).

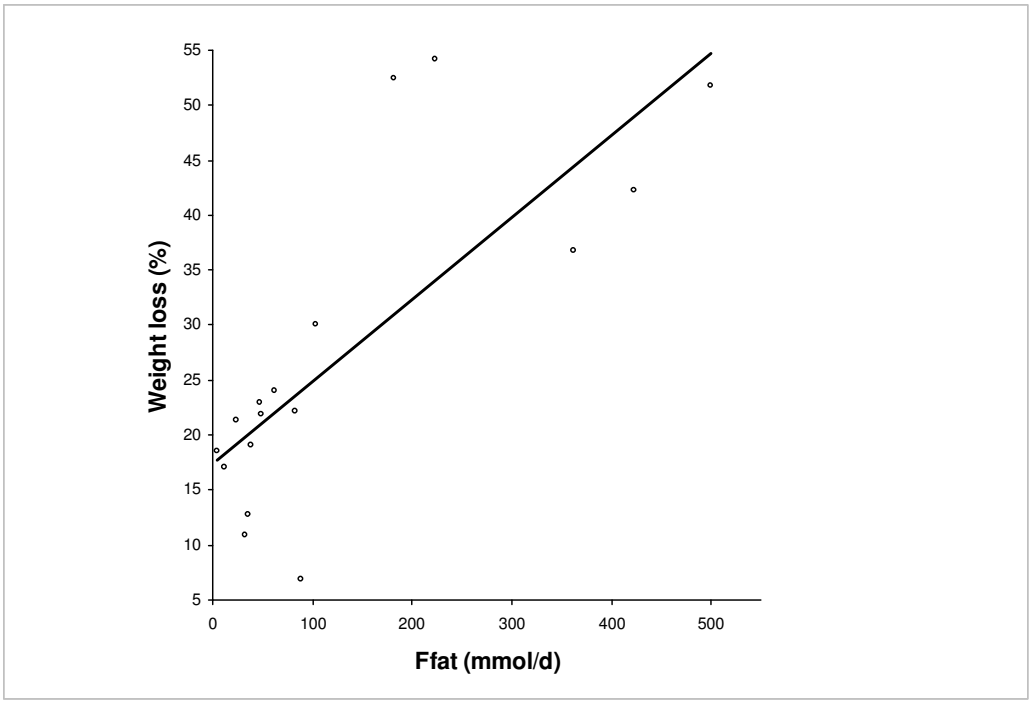


Figure 16a: Spearman's correlation between weight loss in % and faecal fat (Ffat) excretion in all the groups. There was a positive corrletaion between weight loss in % and Ffat excretion (R^2 0.77, p=0.003).

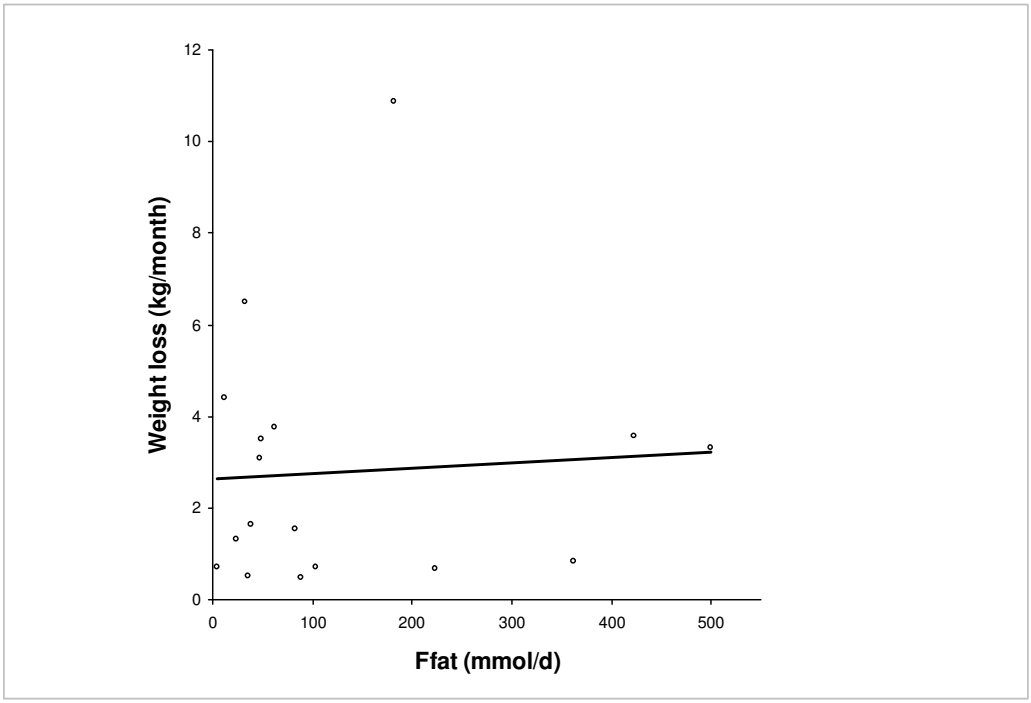


Figure 16b: Spearman's correlation between rate of weight loss (kg/month) and faecal fat (Ffat) excretion in all the groups. There was no correaltion between rate of weight loss (kg/month) and the Ffat excretion (R^2 -0.01, p=0.971).

Chapter Four

Discussion

4. Discussion

Despite the increasing popularity of bariatric surgery, the mechanisms contributing to weight loss following these surgical procedures remain elusive. This study investigated patients who had undergone different bariatric procedures for evidence of malabsorption. We investigated this comprehensively via intestinal function tests including small bowel permeability test and direct assay of faeces for inflammatory marker calprotectin; we further evaluated the exocrine pancreatic function by FE1 and functional length of the bowel with an established marker of enterocyte mass; citrulline. Any alterations to gut transit was assessed by the measurement of sulphapyridine appearance in the plasma after oral administration of sulphasalazine and finally three day collection of faecal samples were quantitated for free fatty acids excretion by a well established chemical tritation method. Changes in BA metabolism were also assessed by measuring urine and plasma BAs in fasting samples.

This study has demonstrated that; AGB does not affect oro-caecal transit time, functional enterocyte mass, exocrine pancreatic function, intestinal permeability, absorption of sugars, gut inflammation, BA metabolism or absorption of fats. RYGB does not affect oro-caecal transit time, functional enterocyte mass, intestinal permeability, absorption of sugars or BA metabolism. However, there is some reduction in exocrine pancreatic function and fat absorption and increase in gut inflammation. BPD-DS does not affect oro-caecal transit time, functional enterocyte mass, exocrine pancreatic

function, absorption of sugars or gut inflammation. However, it increases intestinal permeability, BA turnover and excretion and also reduces fat absorption compared to AGB and RYGB procedures.

A phenomenon that is poorly understood in the context of malabsorption after bariatric surgery is intestinal adaptation. The absorptive capacity of the small bowel far exceeds normal physiological needs. Most nutrients (carbohydrate, protein and water soluble vitamins) are absorbed in the proximal 100-200cm of jejunum with fat absorption distributed over a broader length of the small bowel dependent on the amount of fat consumed (Borgstrom *et al*, 1957). In spite of the greater structural and functional absorptive role of jejunum significant resection of ileum is poorly tolerated and has a greater chance of resulting in SBS compared to equivalent loss of jejunum. A study that assessed intestinal adaptation after ileal interposition surgery (this operation moves upwards a distal part of the ileum which is repositioned in the proximal jejunum) in rats demonstrated, at six weeks post surgery there was a significant increase in length and number of villi in the interposed ileal segment and that the calculated surface area of the interposed ileal segment was comparable to that of normal jejunum (Kohli *et al*, 2010). These features demonstrate that the interposed ileal segment underwent adaptation to a more jejuna-like histological and gene expression phenotype. Gut adaptation is a gradual process and involves both structural (hypertrophy and hyperplasia) as well as functional changes (reduction in permeability, transit time and bile salt absorption) (DiBasie *et al*, 2004). Hence, although initially evolving from procedures that were meant to cause malabsorption the

modified techniques used in RYGB and BPD-DS result only in transient gastrointestinal side effects and the incidence of malabsoption is low and more importantly clinical malabsorption rarely persist (Skroubis *et al*, 2006).

4.1. Entire gut

Oro-caecal transit time:

Gut transit time has been studied using different methods in normal population, and different cohorts with known gastrointestinal pathologies or surgeries. Gut transit (i.e. OCTT) consist of two parts; gastric emptying and jejunal to caecal transit time. The rates of gastric emptying and small bowel transit seem to be largely independent of one another (Read, 1984). Furthermore, the size of the meal and its fat content has been shown to delay gastric emptying on a weight-for-weight basis without affecting the small bowl transit (Read *et al*, 1982). On the other hand, un-absorbable disaccharide lactulose accelerated small bowel transit, but does not significantly influence gastric emptying (Read *et al*, 1982). The rate of gastric emptying has been shown to influence the transit of food down the first 70 cm of small intestine in normal subjects, however; it has little or no influence on the rate of transit down the entire small intestine (Read *et al*, 1982). Even though we did not investigate for SIBO in our cohort, SIBO does not affect transit time measured by sulphapyridine unlike the lactulose hydrogen breath method. Furthermore, different doses of sulphasalazine also do not alter transit times (Sharpstone *et al*, 1999).

Other studies on gut transit times after bariatric surgery showed increased gastric emptying after RYGB and BPD-DS (Morinigo *et al*, 2006; Hedburg *et al*, 2011). One study showed that after RYGB there was no change or even slower gastric emptying after a solid meal (Naslund & Beckman, 1987). Pellegrini *et al* (1986) demonstrated delayed small intestine transit post-total gastrectomy and oesophago-jejunostomy. These differences have been explained by changes in intra-luminal content viscosity such as higher-fibre reduces bolus propulsion (Gonlachanvit *et al*, 2004) and potential augmentation of gut transit due to increased postprandial gut hormone (GLP-1 and PYY) secretion following RYGB and BPD-DS (Flint *et al*, 2001).

Our findings are consistent with Naslund *et al* (1987), as we found no difference between any of our groups. In our study participants took sulphasalazine capsule along with the sugar drink (including lactulose) for the SBPT. However, all followed the same protocol. None of our patients were on medications known to affect gastric emptying or small bowel transit such as opiates, anti-diarrhoeals, anti-emetics, alcohol and antibiotics. Some authors have described a method to assess the gastric emptying and small bowel transit separately using two different markers based on their site of absorption (3-O-methyl-D-glucose in jejunum and sulphapyridine in caecum) (Sharpstone *et al*, 1999). This is a limitation of our study as even though there was no difference in oro-caecal transit time (Table 3) we could not account for possible difference in gastric emptying.

Dumping syndrome is well documented after RYGB (Dapri *et al*, 2011) and rapid gastric emptying following the procedure could contribute to this symptom. By the time food reaches the ileum it is considerably reduced in bulk and may not stimulate propulsive activity hence, passage through the ileum could normalise the OCTT after surgery. Timings for sample collection was determined based on our previous findings (Sharpstone, 1999), but two patients in the control, two in AGB and one in BPD-DS group had no sulphapyridine detected within the four hour plasma sampling time. Future studies using the same protocol should collect samples for more than four hours in order to accommodate for prolonged OCTT.

In our study, none of the bariatric procedures altered oro-caecal transit time. Thus, weight loss following these procedures cannot be attributed to rapid intestinal transit leading to malabsorption. The main factor in the anatomical alteration between the three different procedures that might influence oro-caecal transit is the pylorus. The pylorus is bypassed in RYGB but not in AGB or BPD-DS. Therefore, RYGB may lead to rapid gastric emptying [which is in keeping with the higher incidence of postprandial hypoglycaemia following RYGB (Valderas *et al*, 2012)], which was not assessed in this study. Clinically, it has been shown, that the amount of weight loss after RYGB correlates poorly with the postprandial hypoglycaemia, implying that it is unlikely to play a major role (Cummings *et al*, 2004).

Functional enterocyte mass:

Hypertrophy of small bowel has been reported following BPD-DS (Stock-Damge *et al*, 1986) and more recently after RYGB in rats (le Roux *et al*, 2010). le Roux *et al*, (2010) have shown an elevation in glucagon-like peptide 2 (GLP-2) following RYGB in both rats and humans. GLP-2 is the only known hormone that has direct effect on bowel mucosa and contributes to increase in absorptive surface area by stimulating enterocyte proliferation and inhibiting apoptosis (Drucker, 2003). These findings are in keeping with publications which demonstrated increase in mucosal and muscular layers using radiology following intestinal bypass procedures (Nadreau *et al*, 2006 & Murray *et al*, 1993). The incidence of calorie malabsorption remains low and clinical malabsorption tends to be transient following RYGB and even BPD-DS due to intestinal adaptation (Skroubis, 2006). This is a gradual process involving both structural (hypertrophy and hyperplasia) and functional changes such as reduction in permeability, transit time and bile salt absorption (DiBasie, 2004). The above findings are further supported by our study employing a biochemical marker of functional enterocyte mass; citrulline (Table 3). Postoperative subjects having similar citrulline concentration i.e. functional length of bowel compared to controls, indicating that the absorptive capacity is not compromised. This suggests that after RYGB and BPD-DS the gut had already undergone hypertrophy to allow similar absorptive capacity to the patients after AGB or the obese control group. Even though all groups had a similar citrulline concentration they were all below the normal reference range of 30-50 µmol/L reported in normal weight individuals (Rabier & Kamoun, 1995). However, our results are in keeping with other publications on citrulline

measurement in obese subjects where fasting plasma concentrations were on average 19 and 28 µmol/L (Sapir *et al*, 1974; Swendseid e*t al*, 1969). As nitric oxide (NO) is metabolised to arginine and citrulline, the relatively lower concentration of citrulline in the obese can be attributed to the reduced NO bioavailability in the obese (Gruber *et al*, 2008).

In our study, none of the bariatric procedures altered citrulline concentrations (functional enterocyte mass). Unlike in AGB, in RYGB the first 75 cm of small bowel and in BPD-DS the entire duodenum and jejunum are bypassed and do not come in contact with consumed food. Hence, the citrulline concentrations which are similar to the control and the AGB groups following RYGB and BPD-DS are suggestive of gut hypertrophy as an adaptive mechanism in order to minimise malabsorption.

4.2. Foregut

Exocrine pancreatic function:

The role of biliopancreatic secretions following bariatric procedures is poorly understood. The anatomical alterations in RYGB and BPD-DS facilitate digestion to take place in a short common channel compared to normal individuals. Some authors have demonstrated decrease in lipase and protease activity with relative preservation of amylase activity during enzyme transit through the small bowel related to activity, inhibition and auto-digestion of the enzymes themselves (Layer *et al*, 1986). Interestingly gut hormones such as PYY and GLP-1 which are elevated after RYGB and BPD-DS have a negative feedback on exocrine pancreatic secretion (Gracia-Fuentes *et al*,

2008). Furthermore, the most potent stimulus for exocrine pancreatic secretion; the presence of nutrients in the duodenum, is compromised in RYGB and BPD-DS. Exocrine pancreatic insufficiency, defined as inadequate pancreatic enzyme activity for digestion, caused by insufficient enzyme production, insufficient enzyme activation or disturbed enzyme deactivation is a common presenting feature following gastric resections (Domínguez-Muñoz, 2007). It may reflect a reduced enzyme-release response to endogenous stimulation as discussed above or to reduced enzyme activation caused by SIBO (Kahl & Malfertheiner, 2004).

Postprandial pancreatic secretion is first neurally stimulated by fundus relaxation, which triggers a vagal reflex. The release of CCK in response to the nutrient-stimulated duodenal secretion of CCK-releasing peptide represents the major hormone-mediated postprandial stimulation of exocrine pancreatic secretion (Domínguez-Muñoz, 2009). Following bariatric surgery number of mechanisms can disrupt this neural and hormonal stimulus resulting in pancreatic insufficiency; (i) disturbance of fundus relaxation caused by the disappearance of antro-fundic and duodeno-fundic reflexes, (ii) absence of neurally stimulated pancreatic secretion caused by the lack of fundus relaxation, (iii) reduction in CCK mediated stimulation of pancreatic secretion secondary to duodenal bypass, (iv) large and hard-to-digest nutrient particles reaching the jejunal lumen because of resection of the distal stomach, (v) asynchrony between the gastric emptying of nutrients and bilio-pancreatic secretion as a result of anatomical reconstruction (Domínguez-Muñoz, 2009).

Nonetheless, for clinical symptoms of severe exocrine pancreatic insufficiency more than 90% of maximally stimulated pancreatic exocrine function needs to be lost (DiMango *et al*, 1973). The pancreatic gland normally secretes more than 2L of juice per day which is composed of water, bicarbonates and enzymes (Pezzilli, 2009), and normally produces more enzymes than is necessary for food digestion (Pezzilli, 2009). Recently, it has been demonstrated that gastric lipase can compensate pancreatic lipase even if it is not capable of complete lipolytic activity (Pezzilli, 2009). Hence, it is not surprising that in our study RYGB and BPD-DS subjects had suppression of exocrine pancreatic function compared to normal and AGB subjects (Table 3), but at the same time they did not present with clinical symptoms of malabsorption.

In our study, AGB did not alter the exocrine pancreatic function whereas as RYGB and BPD-DS suppressed it. The complex enterohoromonal feedback system that allows gut content to regulate exocrine pancreatic function is disrupted following RYGB and BPD-DS in different ways. After RYGB, gastric acid secretion will be reduced in both the alimentary and bilio-pancreatic limb, which may in turn minimise secretin and CCK secretion and diminish exocrine pancreatic function (Mason *et al*, 1976; Ponsky *et al*, 2005). Our findings are consistent with this hypothesis, in that FE1 was reduced in the RYGB. After BPD-DS, gastrin and gastric acid secretion are also expected to be attenuated as less acidic chyme enters the first part of the duodenum, with in turn reduces the stimulation of CCK and secretin.

4.3. Mid-gut

Small bowel permeability:

SIBO is another way in which the postoperative gut flora can impact absorption via changes to intestinal permeability and other changes such as bile salt de-conjugation and reduced bile slat solubilisation (Lakhani *et al*, 2008). SIBO is defined as an overgrowth of bacteria in the proximal small intestine (in excess of $>10^5$ colony forming units (CFU)/mL) and typically diagnosed by hydrogen breath test (Khoshini *et al*, 2008). Modern Bariatric procedures reduce the incidence of SIBO compared to JIB by avoiding blind loop nonetheless, mechanisms that prevent SIBO can be disrupted such as gastric acid secretion, pancreatic enzyme activity and an intact migrating motor complex (Prachand & Alverdy, 2009). Hence, not surprisingly studies have demonstrated SIBO following RYGB (Ishida *et al*, 2007), BPD-DS (Michielson *et al*, 1996) and vagotomy (Reddymasu & McCallum, 2010). Some degree of vagal nerve damage is associated with AGB and other bypass procedures. Furthermore, high prevalence of SIBO has been shown even in morbidly obese which is associated with hepatic steatosis (Sabaté *et al*, 2008). Evidence suggests that the increase in small bowel permeability can be attributed to SIBO in the jejunum potentially inducing mitotic changes, as normal jejunum is devoid of micro organisms (Lakhani *et al*, 2008). These findings are in keeping with the results of our study as all groups had to an extent a degree of increased excretion to at least two saccharides (Table 3). However, the overall L:R ratio was significantly elevated in all the BPD-DS patients, a procedure which would most likely contribute to SIBO compared to other bariatric surgeries. Possible mechanisms for an increase in permeability

following bypass procedures could be increased crypt cell proliferation (gut hypertrophy) due to elevated GLP-2 which is evident as early as 23 days postoperatively (le Roux *et al*, 2010) as well as an adoptive process of the gut following surgery (Welters *et al*, 2001).

In our study the absorption of sugars was similar following all three procedures. The small bowel permeability on the other hand was not altered following AGB and RYGB but increased following BPD-DS. The gut hypertrophy following RYGB and BPD-DS seem to allow for similar absorptive capacity for the monosaccharide probes used in this study. The elevated L:R ratio after BPD-DS can be explained by two possible mechanisms; (i) the common channel in BPD-DS is about 100 cm in length whereas it is over 100 cm in RYGB. Hence, the greater hypertrophy for length of the small bowel may contribute to increased number of tight junctions between cells in BPD-DS compared to RYGB. This in turn can result in an increase in passive diffusion of lactulose, (ii) SIBO which has been shown to be more prevalent following BPD-DS may have contributed to the increased permeability.

Gut inflammation:

Calprotectin appear to play a regulatory role in the inflammatory process (Brun *et al*, 1994). It is realised from the cells during cell activation or cell death and accounts for up to 50% of the neutrophilic cytosolic protein. Neutrophil activation is a fundamental process in innate immune response and morbid obesity is associated with activation of the innate immune response (Nijhuis *et al*, 2009). Furthermore, FCp has been shown to be

elevated in obese subjects compared to normal weight individuals (Nijhuis *et al*, 2009). In our cohort FCp was within the normal reference range (<60µg/g faeces) in the obese controls. However, all surgical groups had a least one elevated FCp (AGB =1, RYGB=3 and BPD-DS=1). It may be argued that the elevations were due to inflammatory process following surgery as they were not known to have inflammatory bowel disease. Recruitment into the study since surgery for the five elevated FCp was 3, 7, 14, 20 and 64 months. Studies have shown that SIBO is also not associated with elevated FCp (Montalto *et al*, 2008). Another possible explanation could be the alteration in bile flow following the procedure, as UDC has been shown to exert anti-inflammatory action (Martinez-Augustin & Sanchez de Medina, 2008) whereas DC is predicted to aggravate inflammation (Martinez-Augustin & Sanchez de Medina, 2008). However, in our study the five with elevated FCp did not have an elevated DC (p=0.69) or UDC (p=0.20) compared to the others. On the other hand our study showed a significant negative correlation (R^2 -0.69, p=0.001) between FCp and FE1 (Figure 15), which is in keeping with what others have reported (Pezzilli *et al*, 2007). Post surgical exocrine pancreatic insufficiency may cause the observed intestinal inflammation, due to modification of the intestinal ecology and subsequent release of pro-inflammatory cytokines (Pezzilli *et al*, 2007).

In our study there was no evidence of gut inflammation following AGB and BPD-DS but following RYGB there was elevated FCp concentrations. As the AGB does not involve any anatomical modification of the small bowel this is expected. However, if the RYGB group showed an elevated FCp the same

would be expected in the BPD-DS group. The possible explanations for the elevated FCp in the RYGB group include; (i) subtle post-operative alteration of pancreatic exocrine function (see above) and causative or resulting alteration in gut pH may increase intestinal inflammation and FCp through modification of gut microbiota and pro-inflammatory cytokine release, (ii) increased enterocyte turnover following RYGB. The BDP-DS group may have not shown an elevated FCp due to the alteration in bile flow which may attenuate FCp as BAs have anti-inflammatory actions (see below).

Bile acid metabolism:

We assessed for alteration in BA metabolism following the different bariatric procedures by measured plasma and urine BA from sample collected after an overnight fast. Limitations in our protocol in BA measurements are as follows; (i) we did not have an established method for measuring faecal BA hence, data on faecal loss of BA was not available, (ii) BA secretion is stimulated following food intake hence, the postprandial BA response data was not available. However, this is the first study to demonstrate significant elevation in BA after BPD-DS compared to the AGB and RYGB procedures. This BA elevation may be a result of undiluted BA reaching the terminal ileum due to the anatomical alterations. This in part may explain the remission in T2DM after BPD-DS, as BA can stimulate GLP-1 (incretin hormone) release via its action on the L-cell TGR5 receptor (Prawitt & Staels, 2010). We failed to observe elevated BA after RYGB, a procedure which also results in remission of T2DM prior to any associated weight loss (Nakatani *et al*, 2009; Patti *et al*, 2009) however, this might have been due to type II error.

A study which looked at BA metabolism in high-fat diet-fed rats that underwent ileal interposition surgery (this operation moves upwards a distal part of the ileum which is repositioned in the proximal jejunum) found that plasma BA concentrations increased in operated rats, and that faecal BA excretion decreased (Kohli *et al*, 2010). Furthermore, characteristic features of the ileum adaptation included increase in the active bile salt re-absorption protein, apical sodium bile acids transporter (ASBT) (Kohli *et al,* 2010).

Increased BA in the terminal ileum spilling over into the colon can result in watery diarrhoea (Johnston *et al*, 2011). It has been known for over four decades that BAs can cause diarrhoea and much work has been done to understand BA absorption, malabsorption and to unravel the underlying causes (Johnston *et al*, 2011). BA malabsorption can be divided into three types (Johnston *et al*, 2011). Type 1 / secondary: occurs in patients with terminal ileal resection or with impaired terminal ileal function secondary to disease. Hence, BAs are malabsorbed and spill over into the colon where they cause chloride and water secretion (Fromm & Malavolti, 1986). Type 2 / idiopathic: occurs in patients with a histological normal terminal Ileum and is also known as primary BA diarrhoea (Williams *et al*, 1991). Type 3: occurs in patients following gastric surgery, post cholecystectomy or in celiac disease (Fromm & Malavolti, 1986). Although BA malabsorption is not life threatening, it can lead to copious watery diarrhoea, the passage of mucus, urgency, faecal incontinence that can significantly impair quality of life (Johnston *et al*, 2011). However, in our study none of the patients in the RYGB or BPD-DS groups reported symptoms suggestive of BA diarrhoea.

The total urine BA concentrations were higher in the BPD-DS group compared to the other three groups however; the total conjugated BAs in the urine were similar in all four groups which is suggestive of similar degree of conjugation. The primary and secondary BAs in the urine were also higher in the BPD-DS group compared to the other three groups which reflected the increased re-absorption of BAs in BPD-DS and possible saturation of the conjugation pathways in the liver.

In our study there was no change in BA metabolism after AGB and RYGB whereas following BPD-DS there was a rise in total and conjugated BAs in plasma and total BAs in the urine. In the RYGB the bilio-pancreatic limb is around 75 cm in length whereas in BPD-DS it consists of the entire duodenum and jejunum. Therefore, in BPD-DS BAs flow undiluted through a large part of the small intestine prior to reaching the terminal ileum which has the highest expression of the BA transporter ASBT and is the usual site for the greatest proportion of active BA re-absorption (Shneider, 2001). This will result in re-absorption of a higher concentration of BAs and subsequent conjugation following BPD-DS compared to AGB and RYGB.

4.4. Hindgut

Faecal fat excretion:

The assumption that the fat normally found in faeces is merely unabsorbed dietary fat is incorrect, and it has been suggested that as much as half of Ffat is endogenous in origin (Bromster *et al*, 1966). Ffat differs in composition from dietary fat, and appreciable amounts of fat persist in the faeces on fat-free diets, suggesting that the endogenous fat may be largely derived from desquamated mucosal cells (Webb *et al*, 1963).

In 45 patients following various operations (truncal vagotomy and pyloroplasty or vagotomy and gastrojejunostomy, Billroth I or "no loop" partial gastrectomy and total gastrectomy) the only patients to show lessened fat absorption compared with normal were those patients who showed it before operation or underwent total gastrectomy (Keynes & Penfold, 1971). Intestinal hurry may follow gastric operations (perhaps with rapid gastric emptying) and may be the cause of the diarrhoea. This might be a cause of diminished fat absorption, but as already highlighted steatorrhoea is not related to diarrhoea after gastric operations (Keynes & Penfold, 1971).

In a five-year follow-up study of 30 patients who had undergone BPD-DS, significant weight loss was achieved, without nutritional deficiencies (Sileo *et al*, 1995). Gastric stapling combined with BPD seems to lead to a degree of malabsorption, less intense than with the ileal bypass (therefore, without causing nutritional deficiencies), but high enough to induce a significant weight loss (Benedetti *et al*, 2000). Furthermore, the weight achieved was

maintained over a 30 month follow-up period, in spite of a considerably higher caloric intake, with respect to healthy individuals this probably reflects the effect of fat malabsorption following BPD-DS (Benedetti *et al*, 2000). On the other hand, a degree of fat malabsorption associated with BPD-DS tends to cause malabsorption of fat soluble vitamins. By the fourth year after BPD-DS, the incidence of vitamin A, D and K deficiencies were 69%, 63% and 68% respectively, with no deficiencies in either vitamin E or zinc (Slater *et al*, 2004). Based on these data it has been proposed that all patients with BPD-DS should receive supplemental vitamin A (25,000 units) and D (50,000 units) and have concentrations monitored and maintained in the normal range but vitamin K should be supplemented only if the international normalized ratio is above 1.4 (Marceau *et al*, 2001). The reported vitamin and mineral deficiencies after RYGB is 30-40% (Brolin *et al*, 2002). Initiating supplements are a common practice following both RYGB and BPD-DS (Parkes, 2006) and administering pancreatic enzymes to aid in absorption often resolves the deficiency but may result in some weight gain (Fujioka , 2005).

Sugerman *et al* (1997), were the first to report a series of patients who failed conventional RYGB and were revised by adding malabsorption (the Roux limb lengthened from 40 cm to creating a 75-100 cm common channel below the enteroenterostomy results in a further weight loss in about 50% of patients who initially failed to show sufficient weight loss). This was attributed to increased fat malabsorption (Brolin & Cody, 2007). Super obese patients who had a 75 cm common channel (distal-RYGB) achieved better weight loss than patients who had 300-500 cm common channel with a Roux limbs of 150 cm

suggesting that that some degree of malabsorption may be helpful in super-obese patients to achieve satisfactory long-term weight loss (Brolin *et al*, 2002). The 150 cm Roux limb produced significantly better weight loss in comparison to short limb procedures, with similar incidence of late metabolic sequelae (i.e. protein-calorie malnutrition was not observed in the 150 cm group). Although the distal-RYGB produced greater weight loss and better weight loss maintenance than the two shorter limb length procedures, the higher incidence of metabolic complications in distal-RYGB patients coupled with the unpredictability in postoperative follow-up mitigate against its routine use for all super-obese patients (Brolin *et al*, 2002). Hence, Brolin *et al* (2002) showed that gastric restriction combined with a modest degree of malabsorption resulted in significantly greater postoperative weight loss in super-obese patients than did conventional RYGB (i.e. jejunojejunostomy performed at 75 cm distal to the gastrojejunostomy). Because only 30 cm of jejunum is excluded from the functional digestive tract, protein-calorie malnutrition and persistent diarrhoea were not observed after the long-limb (jejunojejunostomy performed at 150 cm distal to the gastrojejunostomy) modification of RYGB. It appears that this long-limb modification of RYGB poses no greater risk of complications than the conventional technique and importantly there were no cases of protein deficiency, hepatic dysfunction or diarrhoea after both procedures (Brolin *et al*, 1992). All those in the RYGB group in our study had Roux limp length of 105-150 cm.

More recently Kumar *et al* (2011) and Odstrcil *et al* (2010) have demonstrated significant fat malabsorption following RYGB. Kumar *et al* (2011), showed

increased fat malabsorption at six and 12 months after RYGB (n=9) compared to baseline where fat excretion was expressed as g/72 hrs. They also showed an even higher degree of fat excretion in BPD-DS (n=2) subjects. Odstrcil *et al* (2010), assessed malabsorption five and 14 months after RYGB (n=9) with baseline and using the method of Jeejeebhoy (see chapter 1.9.4.1.) to estimate fat excretion. Odstrcil *et al* (2010), measured coefficients of fat absorption, whilst fat malabsorption accounted only for 6% at five months and 14% at 14 months, whereas reduced intake contributed to the majority of the weight loss noted after RYGB. On the other hand none of the patients developed protein or carbohydrate malabsorption. This is keeping with the results in our cohort, where greater weight loss correlated with higher Ffat excretion (Figure 16a; R^2 = 0.77, p=0.003), but there was no correlation between rate of weight loss and Ffat excretion (Figure 16b; R^2 = -0.01, p=0.971). A limitation in our study was not assessing the nutrition intake of the participants.

The length of the common channel in BPD-DS regulates the degree of fat absorption and therefore determines the long-term control of caloric absorption and weight maintenance. A 100 cm common channel has been shown to be better tolerated than a 50 cm channel, is associated with less diarrhoea and steatorrhoea and improves protein metabolism (Marceau *et al*, 2001). BPD-DS decrease intestinal fat absorption by delaying the mixing of gastric and pancreatic enzymes with bile until the final 50 to 100 cm of the ileum. BPD-DS originally devised by Scopinaro *et al* (1998), found that only 28% of ingested fat is absorbed after the surgery. Dietary fat however, is

normally almost completely absorbed in the upper 100 cm of the small intestine (Lundh, 1958). Obese individuals tend to oxidize more lipids than carbohydrates, compared with lean individuals (Golay *et al*, 1984). Weight loss is primarily due to a selective lipid malabsorption, while weight maintenance probably reflects the increased energy expenditure in physically active post-obese subjects. Reduced concentrations of free fatty acids following BPD-DS is probably the explanation of higher non-protein respiratory quotient observed in post-obese patients, after weight loss (Benedetti *et al*, 2000).

In our study the AGB did not alter Ffat excretion. Whereas, following RYGB there was a degree of fat malabsorption with a greater degree of fat malabsorption following BPD-DS compared to AGB and RYGB. Our finding of increased faecal fat after BPD-DS was with a common channel length of approximately 100 cm. The length of the common channel is crucial, a 100cm length being associated with less steatorrhoea and malabsorption than 50 cm (Marceau, 1998). We found a negative correlation between FE1 and Ffat, suggesting that exocrine pancreatic function directly impacts upon dietary fat absorption and therefore Ffat excretion, which is supported by the RYGB group in this study. Despite this, the BPD-DS group had higher Ffat excretion with no difference in FE1 compared all other groups, which suggests that the increased length of small bowel through which the food passes (either the alimentary limb and/or the common channel) far outweighs the effect of the attenuated gastric acid and bilio-pancreatic secretion with respect to dietary fat absorption. Further research in this field is warranted.

Limitations and future research: The major limitations in this the study were the small cohort which contributed to a number of type II errors, the cross sectional study design, the lack of control for dietary intake, not assessing for SIBO, assessment of malabsorption at different time intervals postoperatively and the BMI of the control group was not matched with the three surgical groups. A prospective design with a larger cohort which is matched for BMI and postoperative follow-up will increase the power of the findings as well as minimise variables. However, the collection of the three day faecal samples does always prove difficult; asking patients to collect faeces on two occasions may increase the number of participants that do not complete the study.

Chapter Five

Conclusion

5. Conclusion

This study assessed for evidence of malabsorption and adaptation following bariatric surgery using multiple biochemical investigations. AGB does not affect oro-caecal transit time, functional enterocyte mass, exocrine pancreatic function, intestinal permeability, absorption of sugars, gut inflammation, BA metabolism or absorption of fats. RYGB does not affect oro-caecal transit time, functional enterocyte mass, intestinal permeability, absorption of sugars or BA metabolism. Following RYGB, there is some reduction in exocrine pancreatic function and fat absorption and increase in gut inflammation. BPD-DS does not affect oro-caecal transit time, functional enterocyte mass, exocrine pancreatic function, absorption of sugars or gut inflammation. After BPD-DS there is an increase in intestinal permeability, BA turnover and excretion as well as a reduction in fat absorption compared to AGB and RYGB procedures. However, the fat excretion does not fully account for the significant and sustained weight loss after RYGB and BPD-DS procedures, suggesting that factors other than the oro-caecal transit time, functional enterocyte mass, exocrine pancreatic insufficiency, small bowel permeability, bowel inflammation and bile acid metabolism contribute for the weight loss. The pleiotropic entero-endocrine effects after bariatric surgery (alterations in appetite, satiety and energy balance mediated by gut hormones and BAs) play a substantial if not a more significant role in weight loss. Thus, mechanisms which contribute to weight loss after bariatric surgery need further exploration to help produce better medical and surgical management.

Impact on nutrient absorption can guide bariatric surgical procedure selection and validation of biochemical tests for malabsorption following bariatric surgery may aid investigation of weight loss failure and tailor choice of revision surgery.

Chapter Six

Bibliography

6. Bibliography

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