

# **The role of gut hormones on food intake and carbohydrate tolerance**

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To Jane & Ben

# Abstract

The Roux-en-Y Gastric Bypass (RYGB) operation for obesity results in sustained weight loss and improvements in carbohydrate tolerance, that no current medical treatment can replicate. The mechanism that underlies the metabolic improvements observed after RYGB remains unclear, but there is an association with increased post-prandial concentrations of several gut hormones; glucagon like peptide-1 (GLP-1), oxyntomodulin (OXM), peptide YY (PYY) and glucagon. Each of these hormones have multiple and different actions that include reductions in food intake, increases in energy expenditure, satiety and improvements in glucose tolerance. Previous studies investigating the metabolic effects of these hormones have used single or dual infusions.

In this thesis, I have investigated for the first time the effect of co-infusing three gut hormones; GLP-1, OXM and PYY together on food intake. The dose used of each hormone was designed to replicate the postprandial levels observed after RYGB. This triple hormone infusion resulted in a significant reduction in food intake relative to saline and subsequently supports the role of gut hormones in the metabolic benefits observed after RYGB

In addition, I have also investigated the effect of co-infusing GLP-1 and glucagon on carbohydrate tolerance. Glucagon is elevated post RYGB but is known to increase plasma glucose by promoting glycogenolysis and gluconeogenesis. This conflicts with the improved glucose tolerance observed post RYGB. I have demonstrated for the first time that co-infusion of GLP-1 and glucagon results in improved glucose tolerance.

Finally, I have investigated a metabolic complication of RYGB in which gut hormones have been implicated, postprandial hypoglycaemia (PPH). In this thesis, I have shown an association between PPH and elevated GLP-1 and glucagon. These data provide a potential target for treating PPH as well as demonstrating a potential adverse event of elevated gut hormone concentrations.

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# Declaration of Originality

I declare that the text is of my own writing. Where appropriate I have cited other researchers' work.

The majority of the work described in this thesis was done myself. All collaboration and assistance is detailed below.

**Chapter 2** Human studies were performed in collaboration with Dr Rachel Troke, Dr Jaimini Cegla Dr Katherine McCullough and Dr Ben Field. Assistance with radioimmunoassays was performed by Dr Jaimini Cegla and Dr Ben Jones.

**Chapter 3** Human studies were performed collaboration with Dr Preeshila Behary, Dr Nicola Guess and Miss Werd Al-Najim. Assistance with assays was performed by Dr Preeshila Behary and Dr Nicolai Albrechsten.

**Chapter 4** Human studies were performed in collaboration with Dr Preeshila Behary. Assistance with assays was performed by Dr Preeshila Behary.

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# Abbreviations

$\alpha$ -MSH	$\alpha$ -Melanocytes Stimulating Hormone
ADRR	Average daily risk
AgRP	Agouti-Related Protein
ANOVA	Analysis of Variance
AP	Area postrema
ARC	Arcuate Nucleus
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body Mass Index
CART	Cocaine and amphetamine regulated transcript
CNS	Central Nervous System
CONGO	Continuous overlapping net glycaemic action
DEBQ	Dutch Eating Behaviour Questionnaire
DIO	Diet Induced Obesity
DMN	Dorsomedial Nucleus
DMV	Dorsal motornucleus of the vagus nerve
DPP-4	Dipeptidyl Peptidase IV
DVC	Dorsal vagal complex
ELISA	Enzyme-linked Immunosorbent Assay
FGF	Fibroblast Growth Factor
GI	Gastrointestinal
GIP	Glucose-dependent insulintropic peptide
GLP-1	Glucagon-like Peptide-1
GLP-1R	Glucagon-like Peptide-1 Receptor
GPCR	G-protein-coupled receptor
GRPP	Glicentin-related polypeptide
HbA1c	Glycated Haemoglobin
HBGI	High blood glucose index
IP	Intraperitoneal
IP	Intervening Peptide
IP-1	Intervening Peptide-1
IP-2	Intervening Peptide-2
IV	Intravenous
IGTT	Intravenous glucose tolerance test
LBGI	Low blood glucose index
LH	Lateral hypothalamus
MAGE	Mean amplitude glucose excursion
MC4R	melanocortin-4 receptor



ME	Median Eminence
MODD	Mean of daily differences
MPGF	Major pro-glucagon fragment
NICE	National Institute of Clinical Excellence
NEFA	Non-esterified fatty acids
NPY	Neuropeptide Y
NTS	Nucleus Tract Solitarius
OXM	Oxyntomodulin
PC	Prohormone Convertase
POMC	Proopiomelanocortin
PPH	Postprandial Hypoglycaemia
PVN	Periventricular Nucleus
PWL	Percentage weight loss
PYY	Peptide YY
RIA	Radioimmunoassay
RCT	Randomised Controlled Study
RYGB	Roux-en Y Gastric Bypass
SC	Subcutaneous
SGLT1	Sodium Glucose Transport protein 1
SEM	Standard error of the mean
SON	Supraoptic Nucleus
SOS	Swedish Obesity Subject
T1DM	Type one diabetes mellitus
T2DM	Type two diabetes mellitus
UK	United Kingdom
USA	United States of America
VAS	Visual Analogue Scale
VLED	Very low energy diet

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# **Chapter 1**

## **General Introduction**

## **1.1 Obesity – a pandemic**

The rise in obesity represents a modern day pandemic. Its prevalence has doubled in the last 30 years from 5 and 8% of men and women respectively in 1980 to 10 and 14% in 2008(1).

The rapid rise in obesity reflects a positive imbalance between energy intake and energy expenditure. However, the causes for this imbalance are multifactorial and represent a complex interplay between environmental factors, a genetic predisposition and changes in human behaviour.

### **1.1.1 Central control of energy balance**

Energy balance is regulated at a central level, specifically the hypothalamus and brain stem. Multiple different processes are co-ordinated at these centres. These include,

- The homeostatic control of energy intake;
- The processing of gustatory, visual and olfactory sensation;
- Determination of the rewarding/emotional aspects of food;
- Higher cortical functions involved in motivation and impulse control;
- Control of energy expenditure through metabolic rate and activity.

In order to tightly regulate energy balance it is essential for these centres to receive information regarding the environment. Peripheral signals are conveyed via neural and humoral pathways. The latter is facilitated by specific areas of the brain where the blood brain barrier is incomplete. These include the area postrema (AP) and the median eminence (ME). The brainstem receives signals from the periphery via the AP, and visceral vagal afferents carrying signals from the gut. The hypothalamus is influenced by higher centres, the brainstem and by peripheral humoral signals via the ME.

Particularly relevant to the regulation of energy balance is the arcuate nucleus of the hypothalamus (ARC). Within the ARC are two distinct sets of neurons with opposite functions. The lateral ARC is specific for anorexigenic neurons co-expressing pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (2). POMC is processed to produce  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) that in turn activates the melanocortin-4 receptor (MC4R). A further group of neurons in the medial ARC co-express neuropeptide Y (NPY) and agouti gene related peptide (AgRP) (3). These neurotransmitters stimulate food intake and energy seeking behaviour, i.e. they are 'orexigenic'. In particular, AgRP acts to antagonise  $\alpha$ -MSH at the MC4R. The balance of activity between these two groups of neurons regulates body weight via

the paraventricular nucleus (PVN). This in turn influences higher centres in the cerebral cortex that regulate food seeking behaviour, the hypothalamo-pituitary-thyroid axis and the sympathetic nervous system to regulate the body's basal metabolic rate and resting energy expenditure(4).

### **1.1.2 The health burden of obesity**

Obesity is associated with multiple co-morbidities. These include cancer(5), obstructive sleep apnoea(6), hypertension(7), hyperlipidaemia(8), type 2 diabetes mellitus (T2DM)(9). Whilst obesity in itself is a risk factor for cardiovascular disease(10), the addition of the latter co-morbidities increases morbidity and mortality through myocardial infarction and stroke(11).

## **1.2 Treatment of obesity**

### **1.2.1 Lifestyle modification**

Lifestyle modification (diet and exercise) remains the initial treatment for obesity and diabetes as endorsed by multiple guidelines (12-14). Dietary changes can refer to both calorie restriction and/or specific macronutrient modification. There is evidence that calorie restriction in the form of very low energy diets can result in significant weight loss with associated improvements in glycaemia. A Very Low Energy Diet (VLED) is defined

as a diet of 800kcal/day or less. Lim *et al* demonstrated that a VLED for 8 weeks in 11 patients with T2DM resulted in significant weight loss (mean percentage weight loss of  $15\pm 1\%$ ) with concomitant improvements in fasting glucose, hepatic insulin sensitivity and plasma lipids relative to non diabetic controls(15). However, this was a small, short term study and whether the observed improvements in diabetes can be maintained using this approach in the longer term remain unknown. Specifically, in terms of weight loss, a meta-analysis of VLED has shown a modest average percentage weight loss of 6.79% at 5 years(16). However, dietary modifications are noted to have high attrition rates.

### **1.2.2 Pharmacotherapy**

The history of obesity pharmacotherapy is burdened by numerous medications that have been removed due to unfavourable side effect profiles. Subsequently, increased scrutiny has resulted in a limited armory of treatments. Furthermore, there are discrepancies between the licensed anti-obesity medication in the United Kingdom (UK) and the United States of America (USA). In the UK, there remains only one licenced pharmacotherapeutic approved by the National Institute of Clinical Excellence (NICE); orlistat, whilst in the USA there are 4 additional anti-obesity drugs. The current anti-obesity drugs are

- Orlistat

- Lorcaserin
- Phentermine/toperimate
- Naltrexone/bupropion
- Liraglutide

#### **1.2.2.1 Orlistat**

This is a gastrointestinal lipase inhibitor that results in excretion of 30% of ingested fat and modest weight reduction(17). However, the use of orlistat is limited by considerable gastrointestinal side effects, such that only 10% of patients remain adherent after 1 year (18).

#### **1.2.2.2 Lorcaserin**

Lorcaserin is an agonist for 5-HT receptors on anorectic POMC neurones. Previous 5-HT agonists used as anti-obesity drugs include fenfluramine, which was subsequently withdrawn due to increased risk of valvular heart disease(19). This side effect is mediated via 5-HT<sub>2B</sub>. Lorcaserin avoids this adverse event by being specific to the 5-HT<sub>2C</sub> receptor(20). Relative to placebo, it achieves weight loss of 3.0-3.6% in phase 3 trials(21,22). In addition it has been reported to reduce glycated haemoglobin (HbA1c) by 0.5% in T2DM(23). Whilst approved by the Federal Drugs Agency (FDA) in 2012, the European Medical Agency (EMA) has asked for further safety data prior to licensing.

### **1.2.2.3 Phentermine/topiramate**

Both topiramate and phentermine are powerful anti-obesity monotherapies but their use has been limited by serious side effects. Phentermine is an amphetamine mimetic. The weight loss actions of this drug are mediated through enhanced norepinephrine and dopaminergic stimulation of POMC neurons. Similar amphetamine based drugs were popular anti-obesity treatments in the Seventies but concerns regarding addiction resulted in their use only short term. Topiramate was developed as an anti-epileptic medication but its effect on weight loss was noted during initial drug trials(24). However, dose limiting, neuropsychiatric symptoms have prevented further development as a monotherapy for obesity. The mechanism by which topiramate achieves weight loss remains unclear but there is evidence for both orexigenic glutamate signalling and increased energy expenditure(25,26).

Use of these drugs in combination is attractive for a number of reasons. Since each act via different mechanisms, their combined effect on weight loss is less likely to be diminished by compensatory mechanisms. Additionally, the side effects that occurred with the high doses needed to achieve weight loss may be attenuated by using lower doses of the individual drugs that in combination still yield significant weight loss.

The FDA has licensed two separate doses of these agents in combination.



The lower dose of 7.5mg phentermine/46mg topiramate yields placebo subtracted weight loss of 6.6%(27). Doubling this dose increases weight loss to 9.3% but is only approved to those who lose insufficient weight on the lower dose preparation(28).

#### **1.2.2.4 Naltrexone/bupropion**

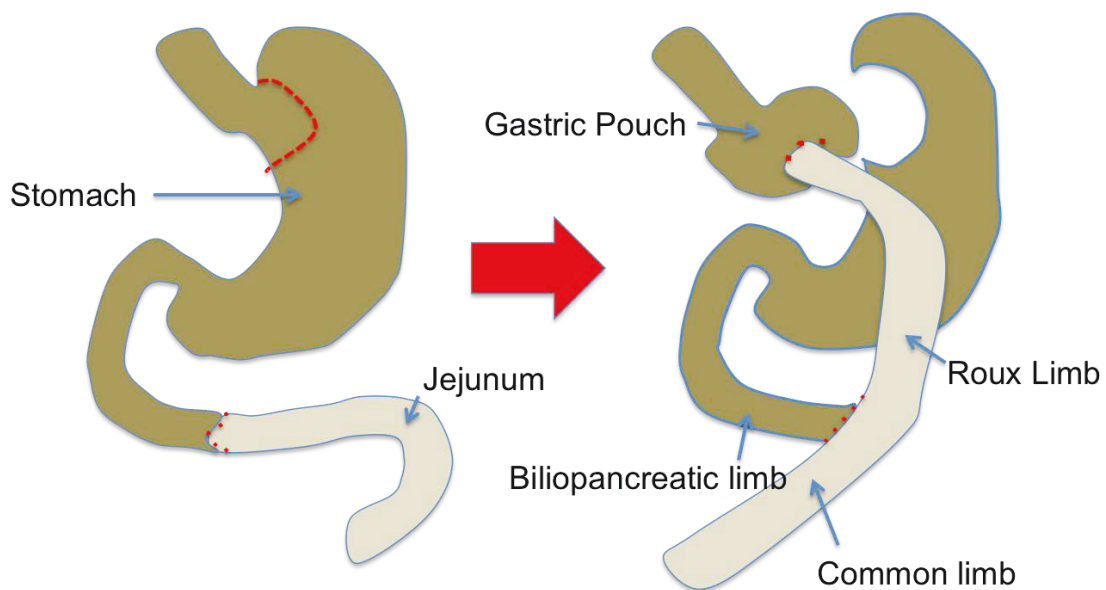
This is another combination drug, combining naltrexone (an opioid receptor antagonist) and bupropion (a mixed dopamine and norepinephrine reuptake inhibitor)(29,30). Both these drugs are used individually for the treatment of addiction; naltrexone being used for alcohol dependence and bupropion for smoking cessation.

Phase 3 studies have demonstrated a 3.2-5.2% weight loss greater than placebo with a 0.5% reduction in HbA1c in T2DM(31-34).

#### **1.2.3 Bariatric Surgery**

Bariatric surgery is an umbrella term encompassing a variety of operations that manipulate the gastrointestinal tract to achieve weight loss. There are a variety of operations that are performed around the world, but the three most common are gastric banding, laparoscopic sleeve gastrectomy and the Roux-en-y gastric bypass (RYGB)

The most common procedure is the RYGB surgery (35). This operation combines a restrictive component by the creation of a small gastric pouch and a bypass component in which nutrients are diverted straight into the jejunum (Figure 1.1).



**Figure 1.1 The anatomy of the gastrointestinal tract following RYGB surgery.** The stomach is resected to make a small gastric pouch. This is anastomosed to the jejunum. The bypassed small bowel is renamed the biliopancreatic limb. The distal small bowel that is anastomosed to the gastric pouch is renamed the Roux limb. Where the biliopancreatic and Roux limbs meet and continue is named the common limb.

### **1.2.3.1 Weight loss after RYGB**

Two recent randomised controlled trials (RCTs) comparing bariatric surgery to intensive lifestyle changes have shown that RYGB results in significantly greater weight loss and improvement in diabetes(36,37). Weight loss is durable as evidenced by the Swedish Obesity Subject (SOS) study, a prospective controlled trial which demonstrated that 10 years after RYGB, percentage weight loss (PWL) is maintained at  $25\pm 11\%$ (38).

Whilst the rate of obesity and its co-morbidities rise, there are limited non-surgical treatments. RYGB is the most effective treatment for durable weight loss, it is not without cost or risk. A meta-analysis quoted a 30-day mortality of 1 in 200(39) . Furthermore, some patients are reluctant to have surgery despite the low mortality. There remains an urgent need for a nonsurgical intervention for obesity. Understanding how RYGB is so effective may result in new more effective treatments.

The mechanisms underlying the metabolic improvements after RYGB remain unclear but multiple theories have been postulated. The most supported mechanism involves changes in the concentration of postprandial gut hormones (discussed in section 1.3). Other potential

drivers of weight change post RYGB include bile acids, Fibroblast Growth Factor (FGF) 19 and microbiota.

Bile acids and FGF 19 are metabolic regulators of glucose and energy metabolism and are elevated post RYGB(40). Bile acids have the potential to stimulate GLP-1 release through TGR5 receptors, found on L cells of the small bowel(41). Bile acids also regulate FGF 19 release in the gut, which have been shown to inhibit hepatic gluconeogenesis(42).

RYGB has been shown to change the composition of gut microbiota in rodent models of bariatric surgery. Li et al. performed either RYGB or sham operations in 30 male Wistar (non-obese) rats, and collected stool samples after surgery. Gut microbiota analysis demonstrated that post surgery there was a decrease in *Firmicutes* and *Bacteroidetes* but an increase in *Proteobacteria* (43). Another study comparing gut microbiota in 10 diet induced obese (DIO) mice that underwent RYGB to sham operated mice replicated the increased prevalence of *Proteobacteria*. The same group went on to transfer the gut microbiota from RYGB mice to germ free mice via caecal contents. This resulted in a significant decrease in PWL of  $5.0 \pm 1.8\%$  after two weeks compared to germ free mice that were inoculated with gut microbiota from sham operated mice (44).

There have been two human studies assessing the effect of RYGB on gut microbiota. Zhang et al, examined the gut microbiota from three groups: RYGB operated, obese and normal weight. There were only three subjects per group. The RYGB operated group demonstrated a relative increase in *Proteobacteria* and decrease in *Firmicutes* post surgery, compared to the obese and normal weight volunteers. However, this small study had issues in methodology. Specifically, the post RYGB patients had their stool samples collected at different time points spread between 8-15 months. Additionally the pattern of weight loss after RYGB was heterogeneous, with two patients continuing to lose weight whilst one had a plateau in their weight loss (45). The postulated mechanisms by which changes in gut microbiota contribute to weight loss post RYGB include, a reduction in systemic inflammation and changes in short chain free fatty acids production, which potentially lead to increased gut hormone production and reduced energy harvesting from substrates(46).

### **1.3 Gut hormones**

Whilst the mechanism by which RYGB is so effective remains elusive, there is an associated increase in various gut hormones post-surgery. An understanding of how these hormones regulate energy balance may help us to design effective anti-obesity treatments

### **1.3.1 Glucagon**

Glucagon was first identified in the 1922 as a “hyperglycaemic factor of the pancreas” as part of studies investigating the effects of pancreatic extracts(47). It has emerged that glucagon has a key role not only in glucose homeostasis, but also in energy balance, affecting both appetite(48) and energy expenditure(49).

#### **1.3.1.1 Processing from the pro-glucagon**

Pro-glucagon, the precursor of glucagon, was identified in 1983 by Bell *et al*(50). It consists of 160 amino acids and is expressed mainly in pancreatic  $\alpha$  cells, in the gut L cells and in the CNS(51). Later studies have established that these cells exhibit differential posttranslational processing of this precursor to produce tissue specific hormones (Figure 1.2)(52,53).

#### **1.3.1.2 Biological actions of glucagon**

##### **1.3.1.2.1 Glucose metabolism**

The etymology of glucagon reveals its most well known function ie *gluc* and *agon* meaning sweet and to lead respectively. Glucagon increases plasma glucose levels via multiple mechanisms;

- Promotes glycogenolysis
- Inhibits glycogenesis

- Promotes gluconeogenesis
- Inhibits glycolysis

The net result of these effects is a rise in plasma glucose (54).

#### **1.3.1.2.2 Lipid metabolism**

Glucagon has two major actions on lipid metabolism. Firstly, chronic (21 days) subcutaneous injection of glucagon in rats results in a reduction of plasma cholesterol and triacylglycerol of 40 and 70%(55). The mechanism for this remains unclear but there is an associated increase in cholesterol transformation into bile acids(55). Alternatively, mouse studies have demonstrated that administration of glucagon to wild type mice resulted in an increase in beta oxidation of non-esterified fatty acids (NEFA). This effect may account for the increase in plasma triglycerides observed in glucagon knock out mice (56).

Glucagon also promotes lipolysis in white adipocyte tissue(57). This is thought to relate to glucagon's action on stimulating hormone sensitive lipase, which in turn increases NEFA (58).

#### **1.3.1.2.3 Food Intake**

Glucagon at physiological levels reduces food intake in rats(59) and men(60). The evidence for this is twofold. Firstly, portal infusions of glucagon antibodies administered to rats prior to a meal result in

glucagon antagonism and increased food intake(61). Secondly, an infusion of glucagon at a physiological dose in man resulted in a reduction in food intake(62). The mechanism for the observed anorectic action of glucagon may be via vagal afferent stimulation, specifically the hepatic branch, as selective hepatic vagotomy results in attenuation of glucagon satiety effect(63).

#### **1.3.1.2.4 Energy Expenditure**

In addition to its action as an anorectic hormone, glucagon also increases energy expenditure, suggesting a potential dual action on achieving weight loss.

Glucagon role in increasing energy expenditure is supported by the observation of increased oxygen consumption in rats that were infused with pharmacological doses of glucagon(64). This increase in energy expenditure is thought to be mediated via brown adipose tissue (BAT), through uncoupling of the respiratory chain resulting in non-shivering thermogenesis. This is also supported by the observation that serum glucagon levels increase after cold exposure (a potent stimulator of BAT)(65).



Glucagon-stimulated increased oxygen consumption was blocked by the use of propranolol (a beta blocker) suggesting that glucagon exerts its effects on energy expenditure via the sympathetic nervous system(66). This is further supported by the observation that denervated BAT blunts thermogenesis induced by glucagon(67).

However a recent study by Salam *et al* failed to show an increase in metabolic activity in BAT following a glucagon infusion in man, despite evidence of an increase in energy expenditure(68). This suggests that the effect of glucagon on energy expenditure is independent of BAT. An alternative mechanism for glucagon stimulated energy expenditure is futile substrate cycling, in which cyclical metabolic pathways produce no net product but consume energy(69).

### **1.3.1.3 Glucagon in diabetes**

Glucagon was first postulated as a potential factor in the pathogenesis of T2DM in the “bihormonal hypothesis” by Unger *et al* in the 1970’s(70). This theory suggests that the cause of T2DM is not only a reduction in insulin but also dysfunctional secretion of glucagon. In support of this, patients with T2DM have higher baseline and postprandial glucagon levels compared to those with normal glucose tolerance(71). This is in contrast to the suppressed glucagon response to a carbohydrate meal in

subjects with normal glucose tolerance. Further evidence to support the role of glucagon in the pathology of diabetes is a study in which a glucagon receptor antibody administered to type 1 diabetic (T1DM) mice resulted in improvements in HbA1c.(72) In contrast to these findings, RYGB with normal glucose tolerance have elevated postprandial glucagon(73,74). In addition chronic administration of glucagon to Wistar rats resulted in reduced plasma glucose as opposed to any detrimental effect on glucose homeostasis(55).

### **1.3.2 Glucagon-like peptide-1 (GLP-1)**

#### **1.3.2.1 Structure and processing of GLP-1**

GLP-1, like glucagon and oxyntomodulin (OXM), is produced by post-translational processing of pro-glucagon. The products of this process are tissue specific and dependent on the prevalence of prohormone convertases. Whilst GLP-1 and OXM are products of tissues that contain prohormone convertases 1/3, the presence of prohormone convertase 2 in pancreatic  $\alpha$  cell yields glucagon (53,75). Total or full-length GLP-1<sub>1-37</sub> has limited biological activity. However, the removal of six amino acids from the N-terminal results in the two bioactive forms; GLP-1<sub>7-37</sub> and GLP-1<sub>7-36</sub> amide of which the latter is more predominant.

Both bioactive forms have a short half-life of 1-2 minutes due to the activity of the ubiquitous enzyme Dipeptidyl-Peptidase 4 (DPP-4) which cleaves two amino acids to produce the biologically inactive GLP-1<sub>9-36</sub> amide(76). Active GLP-1 is also a substrate for the peptidase neprilysin (neutral endopeptidase 24.11)(77). In vitro studies have identified six cleavage sites in the central and C-terminal regions of the GLP-1 peptide that neprilysin acts on(78). These are

1. Glu<sup>27</sup>-Phe<sup>28</sup>
2. Trp<sup>31</sup>-Leu<sup>32</sup>
3. Asp<sup>15</sup>-Val<sup>16</sup>
4. Ser<sup>18</sup>-Tyr<sup>19</sup>
5. Tyr<sup>19</sup>-Leu<sup>20</sup>
6. Phe<sup>28</sup>-Ile<sup>29</sup>.

### **1.3.2.2 Distribution and secretion of GLP-1**

GLP-1 is found in both the intestine and the CNS(51,79). Within the intestine, GLP-1 is secreted from the enteroendocrine L cell. L cells secrete multiple hormones including OXM and PYY(80,81). The peptide products of the L cell vary depending on their position within the gastrointestinal tract. The highest concentration of L cells secreting GLP-1 are found within the ileum(82).

GLP-1 expressing neurons are particularly prevalent in the caudal portion of the NTS(79). Ascending axons from GLP-1 producing neurons spread to the forebrain and brainstem regions associated with metabolic and autonomic control in the mouse, including the ARC, PVN, rostral ventrolateral medulla and DMN(83). These projections correlate well with the pattern of GLP-1R expression throughout the rat brain(84).

### **1.3.2.3 Regulation of GLP-1 release in the intestine**

Intestinal GLP-1 secretion is linked to meal ingestion with release lowest in the fasting phase and highest in the postprandial phase(85). Carbohydrates, fats and proteins all stimulate GLP-1 release(86,87). There are multiple mechanisms by which L cell secretion of GLP-1 is linked to nutrient sensing and involves neural, paracrine and endocrine mechanisms.

Neural and hormonal mechanisms are particularly relevant to the acute phase of GLP-1 secretion (rapid rise in the first 30-60 minutes of eating). The vagal nerve can stimulate GLP-1 secretion as demonstrated in rat studies in which stimulation of the sub diaphragmatic vagus nerve results in an increase in plasma concentrations of GLP-1(88). Glucose dependent

insulintrophic peptide (GIP) released from L cells on the proximal small bowel can also stimulate GLP-1 secretion (88).

The chronic phase of release is mediated by direct sensing of nutrients in the enteral lumen. Glucose sensing by enteroendocrine cells involves a number of mechanisms. A critical component of glucose sensing in the gut is Na<sup>+</sup>-coupled glucose uptake by sodium glucose transport protein 1 (SGLT1), which generates small currents that trigger depolarization and voltage-gated Ca<sup>2+</sup> entry(89). Glucose metabolism, involving glucokinase and the closure of ATP-sensitive (K<sub>ATP</sub>) channels(90), and basolateral/plasma glucose concentration may also play a role in glucose-stimulated gut hormone release.

#### **1.3.2.4 Receptors and signalling cascades**

The GLP-1 receptor (GLP-1R), is a seven trans-membrane domain, G-protein-coupled receptor (GPCR) in the secretin-like class B family(91). Expression of GLP-1R is distributed in pancreatic islets, lung, central and peripheral nervous system , stomach, kidney and heart (91,92).

GLP-1R binding by an agonist results in activation of two separate secondary messenger pathways. The first involves cAMP production via the G<sub>s</sub> G-protein(93). An alternative pathway for stimulated cAMP

involves recruitment of the scaffold protein  $\beta$ -arrestin and downstream activation of ERK, CREB, and increased IRS-2 expression (94).

### **1.3.2.5 Biological actions of GLP-1**

#### **1.3.3.5.1 Glucose metabolism**

GLP-1 has a number of actions that affect carbohydrate tolerance. Firstly it functions as an incretin hormone, (it is a hormone that increases circulating insulin levels in response to an oral glucose load relative to an intravenous challenge) (95). It stimulates pancreatic  $\beta$  cell proliferation and differentiation(96), insulin gene expression(97) and insulin secretion and is capable of normalising the blunted insulin responses to glucose seen in type 2 diabetic patients(98). In addition to its insulintropic effect, GLP-1 also suppresses glucagon release in a glucose-dependent fashion from  $\alpha$  cells, likely via somatostatinergic mechanisms (99,100). In addition, the effects of GLP-1 on gastric emptying(101) and food intake(102) also contribute to a net reduction in postprandial glucose levels.

#### **1.3.3.5.2 Food Intake**

GLP-1 has been shown to inhibit food intake in both rats as well as man(103-105).In rats this anorectic effect has been observed with both central and peripheral administration of GLP-1(103,104). GLP-1's

anorectic effect is likely due to a combination of peripheral and central action. Delayed gastric emptying subsequent to GLP-1 administration is likely to contribute to its anorectic effect(106), although it has been reported that this effect may be temporary due to tachyphylaxis(107). The central action of GLP-1 is demonstrated by the activation of multiple areas within the brain including the PVN, SON of the hypothalamus, NTS and AP(108-110). However, the mechanism is likely to be independent of activation of the MC4R as studies using a MC4R antagonist (AgRP(83-132) did not attenuate GLP-1's anorectic effect(111).

Evidence of a vagus mediated anorectic effect of GLP-1 is demonstrated by the presence of the GLP-1R gene expression in the vagal nodose ganglion(112) and that a truncal vagotomy yields a reduction in GLP-1's effect on food intake(110).

#### **1.3.3.5.3 Cardiovascular actions**

Beyond GLP-1 actions on glucose metabolism and weight loss it also noted to have an important impact on the cardiovascular system(113). These effects include an increase in heart rate(114), a lowering of blood pressure(115) and an improvement in vascular endothelial dysfunction which is a risk factor for atherosclerosis(116). The mechanism underlying

the rise in heart rate and the clinical significance of this remains unknown. The benefits on blood pressure may be due to increased natriuresis(117) or vasodilation(118). These effects (except the increase in heart rate) suggest a cardio-protective effect and this has been observed in clinical trials(119).

#### **1.3.2.6 GLP-1 physiology in T2DM**

The potential of GLP-1 as a treatment for T2DM is based on the following observations on GLP-1 and the pathology of T2DM. Firstly, patients with T2DM have a reduction in postprandial GLP-1 which may contribute to the attenuation in stimulated insulin secretion(120). Secondly, patients with T2DM continue to be sensitive to the incretin effects of exogenous GLP-1, unlike GIP, where the incretin effect is significantly blunted in these patients. Lastly, infusion of GLP-1 rapidly normalises hyperglycaemia in patients with T2DM(99,117)

This has led to the rapid development of a number of GLP-1 analogues that have been designed to be resistant to DPP-4 and so prolong their half-life relative to the native peptide. The first of these is exenatide (exendin-4, Bristol-Meyers Squibb). Exenatide was originally isolated from the saliva of the Gila monster lizard. It shares a 53% homology with human



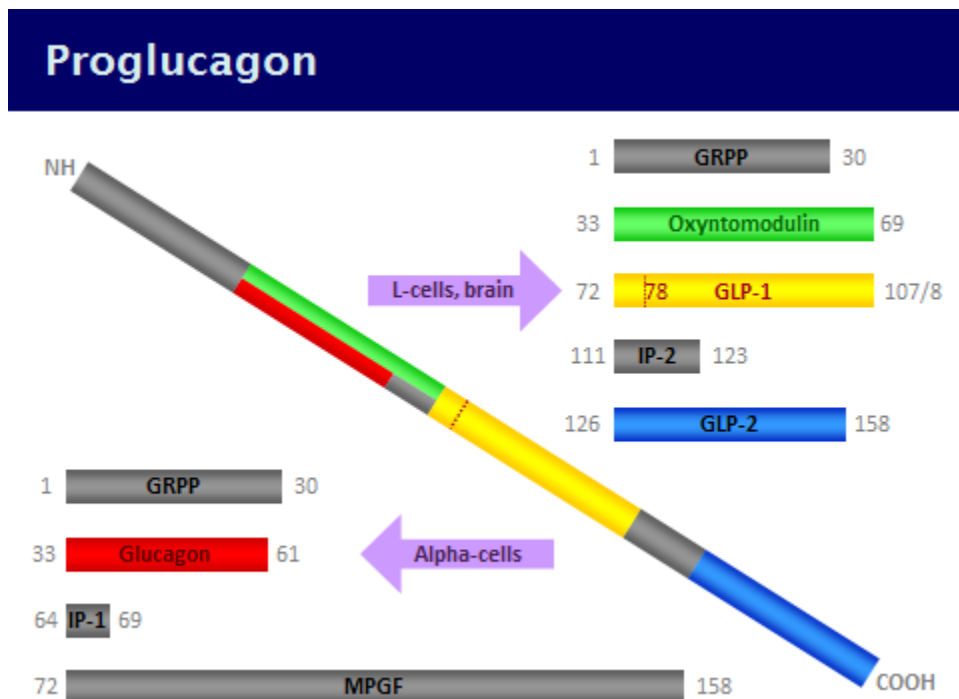
GLP-1 and is given as a subcutaneous (SC) injection twice a day. An improvement in HbA1c by 0.8-1.11% and reduction in weight between 1.6 to 2.8 kg has been reported with twice daily exenatide (121). The most common side effect with exenatide is that of nausea and vomiting, both of which attenuate with time and are reduced by a slow titration up in dose.

However, dissatisfaction with a therapy that requires self-injection twice a day has resulted in the pharmaceutical development of GLP-1 treatments moving forward in two separate pathways.

As an alternative to injectable GLP-1 analogues is the development of oral DPP4 inhibitors that act to increase endogenous GLP-1 levels. Examples of these include sitagliptin, vildagliptin and saxagliptin. Sitagliptin is taken once a day and causes a reduction in HbA1c of 0.7%(122). However, one drawback of the gliptins is that they are weight neutral.

The alternative approach has been to attempt to improve patient satisfaction by prolonging the half-life of GLP-1 agonists. The first of these was liraglutide (Novo Nordisk). This analogue is 97% identical to native GLP-1, but contains an amino acid substitution (K34R) to allow a

palmitate fatty acid group to be linked via a  $\gamma$ - glutamic acid spacer. The fatty acid group binds to albumin, increasing the half-life of liraglutide to 13 hours. In contrast to the DPP4 inhibitors, liraglutide can generate weight loss and is now licensed for the management of obesity by the FDA. Phase 3 trials of liraglutide given SC daily have shown a weight loss of 5.6% subtracted from placebo(123).



**Figure 1.2 Proglucagon cleavage.** Proglucagon expression is centred in the pancreas, GI tract and brain. Glucagon is the primary secretory product in the pancreas, whereas GLP-1 and OXM production takes place

mainly in the GI tract and brain. Abbreviations: GRPP, glicentin-related polypeptide; IP, intervening peptide; GI, gastrointestinal; GLP, glucagon-like peptide; MPGF, major pro-glucagon fragment. Location in the pro-glucagon peptide sequence is indicated by numbers. Adapted from Field *et al* (124).

### **1.3.3 OXM**

#### **1.3.3.1 OXM- discovery and structure**

OXM derives its name for its ability to modulate gastric acid secretion from gastric oxyntic glands(125). It was initially identified in 1968 from gastric extracts that cross-reacted with glucagon in radioimmunoassays(126). However, it was in 1981 when the structure of OXM was elucidated. It is a 37 amino acid peptide that contains the entire 29 amino acid sequence of glucagon combined with 8 amino acid carboxy-terminal extension. It is a product of post-translational of the preproglucagon precursor. Production of OXM is tissue specific and dependent on the presence of prohormone convertase 1/3(53,75). OXM has a similar distribution to GLP-1; ileum, jejunum, colon as well as neurons in the NTS and hindbrain.

#### **1.3.3.2 Secretion of OXM**

OXM is released from the intestinal L cells 5–10 minutes after meal ingestion, in amounts proportional to the calorie intake (127,128). Circulating levels of OXM peak at around 30 min after ingestion(129).

OXM demonstrates a diurnal variation, independent of food intake, with levels highest in the evening and lowest in the early morning(128).

### **1.3.3.3 OXM signalling**

At present, no separate OXM receptor has been identified. However, OXM binds to and is an equipotent agonist of both the GLP-1 and glucagon receptors, although its affinity to those receptors is reduced relative to their cognates (130).

#### **1.3.3.3.1 OXM and GLP-1 receptor.**

Evidence that anorectic actions of OXM are mediated via the GLP-1 receptor is based on the following two observations. Firstly, the weight loss seen in wild type mice injected with OXM is abolished in GLP-1R<sup>-/-</sup> mice(131). Secondly, the administration of exendin<sub>9-39</sub> (a GLP-1 antagonist) into the cerebral ventricles of mice inhibits the anorectic actions of both GLP-1 and OXM(131).

There is emerging data that OXM binding to the GLP-1R may have a differing biological action than GLP-1. This is based on contrasting secondary messenger recruitment. Specifically OXM is a functional antagonist in  $\beta$ -arrestin 2 activation. This distinction in signalling may

also account for the observed difference in hypothalamic pathway activation between OXM and GLP-1(132).

### **1.3.3.3.2 OXM and the glucagon receptor**

Whereas the anorectic actions of OXM were abolished in GLP-1R knockout mice, there were other effects that persisted such as increased heart rate, energy expenditure and glycogenolysis(131). It has been postulated that these actions are mediated via the glucagon receptor. Evidence to support this comes from experiments that have utilized an OXM analogue that has been modified via a mutation of gln to glu at position 3. The resultant analogue known as OXMQ3E, has diminished binding of the glucagon receptor(133). Studies comparing OXM to OXMQ3E have demonstrated the former induces greater glycogenolysis, weight loss, and reduction in lipid levels(134). This suggests that activation of the glucagon receptor is relevant to the weight loss observed with OXM.

### **1.3.3.4 Biological actions of OXM**

#### **1.3.3.4.1 Weight loss**

OXM causes weight loss in humans and rodents(131,135-138). Overweight and obese subjects receiving SC administration three times

daily of OXM (400 nmol pre-prandially) over a 4-week period resulted in an average weight loss of 2.3 kg(139).

In animal studies, OXM was shown to lower body weight as well as increase core temperature compared to a pair-fed group. This implies that some of the weight loss produced by OXM is mediated via an increase in energy expenditure(136,138). This effect was later confirmed by indirect calorimetry(131).

In humans, there is an acute anorectic effect of exogenous OXM which is sustained with chronic administration(135,139). Furthermore, in overweight and obese volunteers, SC self-administered OXM was effective at reducing weight due to a combination of reduced food intake and increased energy expenditure(137).

#### **1.3.3.4.2 Glucose metabolism**

OXM improves glucose metabolism in both mice and men(140,141). Similar to GLP-1, the mechanism for this could be due to an incretin effect and a reduction in gastric emptying(142,143).

### **1.3.4 Peptide YY (PYY)**

PYY is a peptide hormone that was first isolated from porcine small intestine in 1980(144). Its name reflects a high proportion of tyrosine residues, specifically one at both the N- and C-terminals.

#### **1.3.4.1 Structure of PYY**

PYY is a 36 amino acid peptide hormone. It is a member of the PP fold family that includes PP and NPY which all share a common hairpin-like U-shaped fold tertiary structure.

#### **1.3.4.2 Processing of PYY**

The precursor of PYY is pre-proPYY which has an additional signal peptide, and a C-terminal extension peptide that are joined to PYY (145). Processing of pre-proPYY involves cleavage of the signal peptide, amidation of the C-terminal tyrosine, which is necessary for biological activity, and proteolytic cleavage by a prohormone convertase to give PYY1-36, the 36 amino acid form of the peptide, which is secreted. The secreted PYY1-36 is a target for DPP-4 which cleaves the amino terminal tyrosine proline dipeptide to generate PYY3-36, which is both the biological active and predominant circulating form of PYY(146,147).

#### **1.3.4.3 Distribution and secretion of PYY**

PYY is secreted from the entero-endocrine L cells of the gastrointestinal tract, Whilst L cells are distributed throughout the small and large bowel,

PYY expression progressively increases from the small bowel to its highest concentration in the rectum (148). PYY is also produced in the gigantocellular reticular nucleus of the hindbrain and the pancreas. (149,150)

Plasma levels of PYY peak at 1-2 hours following a meal and remain elevated for up to 6 hours (148). Calorie and macronutrient content are independent factors in determining the amount of PYY secreted. The peak postprandial levels of PYY are proportionate to the calorific content of food ingested (151). It remains clear which macronutrient is the most potent stimulator of PYY secretion. Studies investigating different isocaloric meals of different macronutrient content have demonstrated that protein is the most potent macronutrient stimulus for PYY release, followed by fat and then carbohydrate (152). However, when an intraduodenal route was used, fat was more potent than protein in promoting PYY release (153).

Neural or hormonal regulation may be more relevant to PYY release, as studies in humans have shown that circulating PYY starts to rise 15 minutes postprandially, before direct stimulation of the L cell is likely to occur. (154).



#### **1.3.4.4 PYY Receptors**

PYY binds to the Y family of G protein coupled receptors. In mammals, five receptor subtypes have been cloned, namely Y1, Y2, Y4, Y5 and Y6(155). In humans, PYY1-36 binds to all receptors in the Y family (Y1, Y2, Y4 and Y5). PYY3-36, the predominant circulating form of PYY, is more selective for the Y2 and Y5 receptors, and has a lesser affinity for Y1 and Y4 receptors(156)

#### **1.3.4.5 Biological Actions of PYY**

##### **1.3.4.5.1 Inhibition of Appetite and Food Intake**

PYY 3-36 has an inhibitory effect on food intake in rats when injected peripherally or centrally into the ARC(157). This anorexic effect is lost when PYY3-36 is given centrally to Y2 receptor null mice suggesting that the satiating action of PYY3-36 is mediated via Y2(157). The effects of PYY3-36 on food intake are reduced in both Y1 receptor and Y5 receptor null mice, implying that Y1 and Y5 receptors also play a part in central control of appetite (158). Nevertheless, the predominant receptor mediating PYY3-36 anorectic action is likely to be the Y2 receptor(159). This is supported by the fact that a selective Y2 receptor antagonist, when injected into the ARC, blocks the anorexigenic effect of peripherally administered PYY3-36(160).

Further evidence demonstrating the physiological role of PYY in appetite regulation is demonstrated by observations regarding *PYY*- null mice. They are known to be hyperphagic(152) and MRI imaging demonstrates that they have greater levels of fat than controls(152). Treatment with PYY3-36 reverses their obesity phenotype(152).

The anorexic effect of PYY3-36 is also applicable to humans as observed when an acute intravenous infusion (dosed to replicate postprandial levels) resulted in a reduction in food intake in both non-obese and obese individuals. These healthy subjects also had a significant reduction in the subjective feeling of hunger as assessed by visual analogue scores (VAS)(157).

The mechanism of PYY's anorectic action is multifactorial. There is a contribution of central inhibition of appetite, specifically an inhibition of the orexigenic NPY/AgRP neurons within the hypothalamus. PYY3-36 when administered directly into the ARC, acts via pre synaptic Y2 receptors to inhibit NPY/AgRP(157). Exposure to PYY3-36 results in suppression of *Npy* mRNA(157). Furthermore, electrophysiology studies showed that PYY3-36 activates anorexigenic POMC/CART neurons(157). The proposed mechanism for this was a reduction in the GABA-mediated tonic inhibition of the anorexigenic POMC/CART

neurons by the NPY/AgRP neurons. However, in contrast to this observation, PYY3-36 administration produces weight loss in the POMC knock-out mouse(161). Furthermore, there is now evidence that PYY 3-36 inhibits POMC neurons via a post synaptic Y2 receptor(162).

The presence of Y2 receptors on the vagal nerve implicates its involvement in the anorectic action of PYY3-36(163). Vagal afferents Furthermore vagotomy abolished the anorectic effects of IV PYY3-36(163).

However, peripherally administered PYY3-36 is still able to activate POMC/CART neurons in the ARC even with vagotomy(163).Therefore, PYY3-36 can suppress food intake via two sites of action, the hypothalamus and the vagus nerve. A further indirect mode of inhibition of appetite is that in humans, administration of PYY3-36 resulted in a decrease in the orexigenic hormone ghrelin(164).

#### **1.3.4.5.2 Effects on Glucose Metabolism**

Two separate observations support a role for PYY in glucose homeostasis. Firstly, both *pyy* and Y1 receptor knock out mice exhibit hyperinsulinaemia(165,166). Secondly, pancreatic islets cells express

PYY, specifically  $\alpha$  and  $\delta$  but not  $\beta$  cells(167). This suggests a potential paracrine role.

PYY1-36 and PYY 3-36 have contrasting effects on glucose homeostasis. Whilst the administration of PYY1-36 to isolated islet cells results in a decrease in glucose dependent insulin secretion(168), injections of PYY3-36 or Y2 receptor agonist to rodents improves glucose homeostasis(169-171).

The postulated paracrine effect of PYY is likely to be specific to PYY1-36 as mRNA encoding Y1 and Y4 receptors has been detected in both human and mouse islets(166,172). Y2 receptor mRNA in islets cell has not been detected suggesting any effect of PYY3-36 on insulin secretion is indirect(173).

Alternative mechanisms by which PYY3-36 may affect glucose homeostasis include an increase in either GLP-1 secretion or insulin sensitivity(169,174). Evidence supporting a role for PYY3-36 stimulation of GLP-1 comes from mouse studies by Chandarana *et al* that demonstrated an increase in the concentration of GLP-1 within the hepatic portal vein following injection of PYY3-36, with no increase observed in the systemic circulation. Chandarana *et al* have also

demonstrated that the improvement in glucose homeostasis observed after PYY3-36 injection is diminished in the presence of the GLP-1 antagonist, Exendin9-39(174). An improvement in insulin sensitivity has been demonstrated by van den Hoek *et al* using euglycaemic clamp studies involving PYY3-36 infusions in mice. However, as this study did not measure GLP-1 concentrations during the PYY3-36 infusion, it remains unclear whether effect may relate to GLP-1(169).

Whilst the above *in vitro* and rodent studies support the role of PYY in glucose regulation, there are conflicting data obtained from human studies. Sloth *et al*, using a 0.2 pmol/kg/min infusion of PYY3-36 in man demonstrated an increase in insulin secretion(175). However, an infusion of 0.15 pmol/kg/min of PYY3-36 by Tan *et al*, in humans during an Intravenous Glucose Tolerance Test (IVGTT) demonstrated neither an increased insulin sensitivity nor insulin secretion (176). However, the contradictory data may reflect the multiple differences in methodologies such as different doses of PYY and alternative deliveries of glucose (via an oral route by Sloth *et al* relative to an intravenous route by Tan *et al*).

### **1.3.5 Evidence that gut hormones are important in weight loss after RYGB**

Whilst the mechanism behind the rapid improvement in diabetes and sustained weight loss after RYGB remains unclear, changes in levels of gut hormones have been postulated as being responsible. Post RYGB subjects when given a meal or glucose challenge have postprandial levels of gut hormones that are significantly higher than weight matched controls given the same stimulus (177-179). These increased postprandial levels of GLP-1 and PYY are seen early after the operation and this change appears durable (180). Interestingly, the meal stimulated rise in PYY increments persists over the course of a year but this does not occur with GLP-1 (181,182). There is currently no data on how long circulating OXM remains elevated following surgery.

Elevated postprandial levels of glucagon after RYGB are increasingly being recognised(73,74,183). There is discordance in the literature over how long this increase persists, with some studies reporting elevated levels for two weeks to three months before returning to pre-surgery levels at one year(73,74) whilst other demonstrating a persistently raised postprandial glucagon level at one year (184).

Elevated glucagon following bariatric surgery is an interesting observation. Whilst its role in increasing energy expenditure and reducing appetite would correlate with the weight loss observed after RYGB, the expected hyperglycaemia induced by glucagon is inconsistent with amelioration in diabetes that occurs post-operatively. It is possible that any glucagon-induced hyperglycaemia is counterbalanced by the elevated post-prandial incretins.

Although these data suggest that the increased secretion of gut hormones are important in the observed weight loss after RYGB, there is a paucity of evidence that directly links the two phenomena. There is some evidence from rodent models. Chandarana *et al.* created a mouse model of RYGB that led to greater weight loss than sham operated mice (185). This difference in weight loss was not present in *Pyy* knockout mice, suggesting that PYY secretion was a key factor in mediating the weight loss, at least in this particular surgical model of RYGB.

## **1.4 Postprandial Hypoglycaemia- a metabolic complication of RYGB surgery**

The RYGB operation is considered a relatively safe surgical procedure. It has a 30-day mortality of 1 in 200 giving it a safety profile comparable to

other elective procedures such as a laparoscopic cholecystectomy(39). However, as the rate of RYGB operations increases in response to the rapid rise in obesity, previously rare metabolic complications are becoming more common. An example of this is postprandial hypoglycaemia (PPH). PPH in the context of RYGB was first reported in a case series of 6 patients in 2005. Since this first recognition of hyperinsulinemic hypoglycaemia after RYGB, there has been a dramatic rise in cases reports as well as studies aimed at exploring the pathology and treatment of this condition.

#### **1.4.1 Incidence of PPH**

The reported incidence of PPH post RYGB is reported as 0.1%(186). However, this study has several limitations. It was a retrospective review of a Swedish database of surgical patients. As such it was dependent on self-reporting of a condition that remains under recognised by both patients and physicians.

#### **1.4.2 Pathogenesis of PPH**

Data regarding this is conflicting. In the first case series of PPH, Service *et al*, went on to perform partial pancreatectomies on his cohort of six patients(187). A comparison analysis between the histopathology of pancreata between the PPH patient and a control group revealed islet cell hyperplasia. It was subsequently postulated that RYGB may result in a



form of nesidioblastosis. However, when another group reanalysed the pancreata from this cohort and compared the histology to a BMI matched control set, there was no significant difference in the size of the  $\beta$  cell, implying that the features observed in the pancreata were secondary to obesity as opposed to RYGB(188). It should also be noted that of the six patients in the original cohort who were operated on, 50% had recurrence of symptoms(187).

Since then, there has been speculation within the literature as to the whether the altered hormonal milieu that occurs after RYGB may account for the hyperinsulineamic hypoglycaemia. Specifically, it has been postulated that the elevated levels of GLP-1, an insulinotropic hormone, could be the causative factor. Studies investigating this hypothesis have produced conflicting data. Goldfine *et al* have demonstrated elevated fasting and post prandial levels of GLP-1 in a group of 12 patients with PPH in comparison to 12 asymptomatic RYGB patients(189). Furthermore, Salehi *et al* infused exendin<sub>9-39</sub> (a GLP-1 antagonist) in patients with PPH and demonstrated amelioration in biochemical hypoglycaemia. However, in contrast to these results, patients with PPH who undergo reversal of their operation have a reduction in their GLP-1 levels but do not see an improvement in their symptoms(190). Also conflicting with this is a case series of patients with PPH that have successfully been treated with liraglutide, a GLP-1 analogue(191).

## **1.5 Aim of Studies**

This thesis will describe investigation of post RYGB physiology, focusing on glucagon, GLP-1, OXM and PYY. Specifically, these studies have examined:

- The effects of co-infusion of glucagon and GLP-1 on carbohydrate tolerance after a standard mixed meal test.
- The effects of chronic co-infusion of GLP-1, OXM and PYY on food intake
- The hormonal milieu in postprandial hypoglycaemia

## **1.6 Hypotheses**

The presented studies have addressed the following specific hypotheses.

1. Co-infusion with GLP-1 and glucagon will attenuate the hyperglycaemia observed when glucagon is administered alone
2. Co-infusion of GLP-1, OXM and PYY can reduce food intake
3. GLP-1 is elevated patients with postprandial hypoglycaemia.

## **Chapter 2:**

# **The acute effects of co-infusion of glucagon and GLP-1 on carbohydrate tolerance in humans**

## 2.1 Introduction

Currently, the most effective treatment for obesity is RYGB surgery, resulting in a mean 27% sustained reduction in pre-surgical weight(192). In addition, there is a rapid improvement in glycaemic control that occurs shortly after surgery suggesting mechanisms independent of weight loss(193,194). The mechanism by which RYGB achieves weight loss and diabetes remission remains unclear. The associated postprandial increase in a number of gut hormones suggests a potential causative mechanism(177). One such gut hormone is GLP-1; a 36 amino acid peptide secreted postprandially from enteric L cells in response to macronutrients. GLP-1 has multiple actions that could contribute to the metabolic benefits observed after RYGB, including a satiating effect(102), a delay in gastric emptying(101), an insulinotropic action (the so-called incretin effect)(95), and suppression of glucagon secretion (195).

These observations have led to the development of GLP-1 receptor agonists that are currently used in the treatment of obesity and diabetes(196). However, the therapeutic potential of these medications has been effected by dose-limiting nausea. As a result, the weight loss induced by GLP-1 agonists is modest relative to RYGB surgery(123).

Combining GLP-1 with other gut hormones that are elevated after RYGB may result in greater weight loss. One potential example of this is the combination of GLP-1 with glucagon.

Glucagon is a 29 amino acid peptide secreted from the  $\alpha$  cells of the pancreas. It stimulates gluconeogenesis and glycogenolysis, which in turn increases plasma glucose. Despite promoting hyperglycaemia, glucagon has many other actions that make it an attractive anti-obesity therapeutic agent, including an increase in satiety, a reduction in food intake, and an increase in resting energy expenditure(49,62,197).

The rationale for the development of dual GLP-1/glucagon treatments reflects previous studies of another gut hormone, the 37 amino acid peptide OXM. Following RYGB, plasma OXM concentrations increase(179). Administration of OXM to healthy overweight and obese volunteers, causes a reduction in appetite, weight and an increase in energy expenditure(135,139). Interestingly, OXM has no known specific receptor but is a weak agonist of both the GLP-1 and glucagon receptors(130). Furthermore, OXM has a longer half life than either GLP-1 or glucagon. Hence, it has been postulated that the benefits of OXM are mediated via dual agonism of GLP-1 and glucagon receptors.

This hypothesis has been supported by a series of studies. Two separate groups have tested dual GLP-1/glucagon agonists and demonstrated greater weight loss in rodents than achievable with GLP-1 agonists alone(198,199). An earlier study from this department has co-infused sub-anorectic doses of glucagon and GLP-1 in rodents, demonstrating greater appetite suppression than with administration of either individual peptide(200), and this observation was replicated more recently in human volunteers(201). Furthermore, it has been shown that the addition of glucagon to GLP-1 produces a significant increase in energy expenditure when compared to saline or GLP-1 alone(49). These data not only support the notion that dual agonism of GLP-1 and glucagon receptors mediates the effects of OXM, but also highlights its potential as an anti-obesity treatment.

Historically, glucagon's deleterious effects on glucose metabolism have prevented its development as a treatment for obesity. The addition of GLP-1 to glucagon may ameliorate glucagon's hyperglycaemic effect. The observation of attenuation in postprandial glucose levels with GLP-1 and glucagon, relative to glucagon alone is consistent with this, (201). However, conclusions from this study should be interpreted with caution as an *ad libitum* meal, rather than a standardised meal, was used. Hence, the significant reduction in food intake achieved following co-

infusion of glucagon and GLP-1 relative to glucagon alone might explain the reduction in postprandial glucose concentration.

Further support for the attenuation of postprandial hyperglycaemia by combining GLP-1 with glucagon comes from observations in patients after RYGB. In addition to the established rise in GLP-1, there is a parallel exaggerated elevation in plasma glucagon after ingestion of a mixed meal, with a doubling in peak postprandial levels relative to fasting levels(73,74,183). As RYGB patients have improved carbohydrate tolerance, this suggests that either glucagon in this setting does not cause an elevation in postprandial glycaemia, or that the observed raised GLP-1 levels attenuate any glucagon-induced hyperglycaemia.

At present, no study has evaluated the effects of a co-infusion of GLP-1 and glucagon infusion on carbohydrate tolerance in man. To address this, I have studied the effects of infusions of GLP-1 and glucagon, either individually or in combination, on glucose tolerance.



## **2.2 Hypothesis and aims**

### **2.2.1 Hypothesis**

The hyperglycaemic effects of intravenous glucagon can be attenuated by the co-administration of intravenous GLP-1 in man.

### **2.2.2 Aim**

To investigate the acute effects of co-infusion of glucagon and GLP-1 on glucose tolerance in healthy, overweight volunteers.

## **2.3 Materials and Methods**

### **2.3.1 Peptides**

Glucagon and GLP-1<sub>7-36</sub> amide were purchased from Novo Nordisk (Crawley, UK) and Bachem Ltd (Switzerland) respectively. Glucagon was dissolved in the solvent provided by Novo Nordisk (1 ml water for injection, lactose monohydrate, hydrochloric acid and sodium hydroxide for pH adjustment) whilst GLP-1<sub>7-36</sub> amide was dissolved in 1 ml sterile 0.9% saline (Bayer, Haywards Heath, UK). Gelofusine was supplied by B. Braun Medical Ltd (Sheffield, UK).

### **2.3.2 Subjects**

Ten healthy overweight volunteers, 6 men and 4 women, of mean age  $31.9 \pm 10.2$  years (range 20 – 49 years), mean weight  $81.1 \pm 8.0$ kg (range 67.4-93.0kg), mean height  $1.72 \pm 0.07$ m (range 1.63-1.86m), mean BMI  $27.4 \pm 2.04$ kg/m<sup>2</sup> (range 25.1-30.4kg/m<sup>2</sup>) were recruited. Inclusion criteria were: age 18 years and over, BMI of 25–35 kg/m<sup>2</sup>, with stable weight for at least three months. Exclusion criteria were: history of any medical, psychological or other condition, or use of any medications, including over-the-counter products, which, in the opinion of the investigators, would either interfere with the study or potentially cause harm to the

volunteer, without access at home to a telephone, or any other factor likely to interfere with the ability to participate reliably in the study, history of hypersensitivity to any of the components of the infusions, treatment with an investigational drug within the preceding 2 months or who had donated blood during the preceding 3 months or intention to do so before the end of the study. Women who were currently pregnant, breastfeeding or unable to maintain adequate contraception for the duration of the study and for one month afterwards were also excluded.

All subjects were screened and determined to be in normal health (or have no significant disease process), by medical history, physical examination, 12 lead electrocardiogram and routine biochemistry and haematology. Women of child bearing age were advised to avoid pregnancy during the study and underwent urine tests to exclude pregnancy prior to each study. Any abnormal eating behavior was assessed using the Dutch Eating Behaviour Questionnaire (DEBQ) and the SCOFF questionnaire(202,203). The study was approved by the Hammersmith & Queen Charlotte's Ethics Committee (reference no. 10/H0707/80). All subjects gave written informed consent, and the study was planned and performed in accordance with the Declaration of Helsinki and Good Clinical Practice.

### 2.3.3 Protocol

Each subject attended for five study visits. The first visit was to acclimatise the subject to the clinical environment and to experimental procedures. This acclimatisation visit was run in identical fashion to subsequent, randomised double-blinded visits, except that the infusion always consisted only of vehicle. Data from the acclimatisation visit were not included in the analysis. The subsequent four visits followed a randomised, double-blind, placebo-controlled crossover design comparing four different infusions: (1) Vehicle alone (Gelofusine® – B. Braun Medical Ltd, Sheffield, UK); (2) glucagon alone (1.4 pmol/kg/min); (3) GLP-1<sub>7-36</sub> amide alone (0.4 pmol/kg/min); (4) combination (glucagon + GLP-1<sub>7-36</sub> amide together (1.4 pmol/kg/min and 0.4 pmol/kg/min respectively)). A sub-anorectic dose of GLP-1 previously reported in the literature was used(102,201,204). This dose has been previously shown to produce plasma active GLP-1 concentrations of 15-20 pmol/l, similar to concentrations observed in post-prandial RYGB patients(205,206).

With regards to glucagon, in a previous study, nausea was experienced by three of the 13 volunteers during infusion with 2.8 pmol/kg/min of glucagon(201). Given that the nausea uniformly occurred in the postprandial state, the current study protocol incorporated a lower dose of

glucagon. Previous studies have used infusions of glucagon at 0.86 pmol/kg/min to reproduce the non-suppressed plasma glucagon levels observed in diabetes patients(207). We administered a dose of 1.4 pmol/kg/min to simulate the elevated postprandial glucagon levels seen following RYGB(208). The randomisation was carried out by an independent clinician not otherwise involved in the study.

In order to limit adsorption of peptide to the infusion apparatus Gelofusine® was used as the vehicle for all peptide infusions, to dissolve the contents of the randomised vials of peptide and to prime all syringes and infusion lines(209). Each peptide was drawn up under sterile conditions in a separate 50 ml syringe and, to allow the use of two different infusion rates, delivered by a separate syringe driver (Graseby 3100, SIMS Graseby Ltd, Watford, UK, or Asena GH Mk III, Alaris Medical Systems Ltd, Basingstoke, UK). Thus, on a visit when the volunteer received only one peptide, the second syringe delivered vehicle only, set at the delivery rate calculated for the other hormone.

During the 24-hour period prior to each study visit, participants refrained from strenuous exercise and alcohol consumption. They fasted from 10 p.m. the night before the study, drinking only water. On the morning of each study visit, participants attended a dedicated Clinical Investigation

Unit at the Hammersmith Hospital. Female participants had a urine  $\beta$ -hCG test to exclude pregnancy before the peptide infusion was started. Two cannulae were inserted into the participant's peripheral veins. One cannula was used for sampling, and the other one was used to administer peptide infusion. The infusion containing the peptide hormone(s) was started at 0 minutes. In order to rapidly achieve a steady state, ramping of the gut hormone infusions was performed. Ramping was carried out at four times the nominal infusion rate for five minutes, then twice the nominal infusion rate for a further five minutes, and was then reduced to the nominal rate for the remainder of the 220 minute infusion. At 30 minutes, a meal was served, consisting of liquid (Ensure Plus, 13.8 g of protein, 10.8 g of fat, 44.4 g of carbohydrates, 330 kcal, 220 ml, Abbott Laboratories, Abbott Park, IL) and solid components (Nutri-Grain bar, 1.5 g of protein, 3 g of fat, 26 g of carbohydrates, 133 kcal, Kellogg Company, UK). Participants were allowed 10 minutes to consume the meal. The hormone infusion continued for 220 minutes in total. Participants remained in the study room for 60 minutes following termination of the infusion, at which point the cannulae were removed. The participant was then discharged home.

Blood samples were collected at 0, 30, 40, 50, 60, 70, 80, 90, 100, 115, 130, 145, 160, 190, and 200 minutes, into lithium heparin-coated BD

Vacutainer tubes (International Scientific Supplies Ltd, Bradford, UK) containing 1000 kallikrein inhibitor units (0.1ml) (Trasyolol, Bayer Scherin Pharma, Berlin, Germany) for gut hormone analysis. In addition, blood samples were collected into plain serum Vacutainer tubes containing clot activators and fluoride oxalate tubes for insulin and glucose assay respectively. The insulin samples were allowed to clot for ten minutes at room temperature, after which they were centrifuged and separated and stored at -20°C until analysis. All other samples underwent immediate centrifugation at 4°C, after which plasma was promptly separated and stored at -20°C until analysis.

The pulse and blood pressure of each subject was measured at t=0, 15, 30, 60, 100, 130, 160, and 220 minutes. Volunteers asked to complete a VAS sheet (appendix 1) at the same time points as pulse and blood pressure.

## **2.3.4 Plasma Hormone and Other Assays**

### **2.3.4.1 Glucose and insulin**

Plasma glucose and serum insulin samples were analysed in the Department of Chemical Pathology, Imperial College Healthcare NHS Trust. Plasma glucose was assayed using an Abbott Architect automated analyser (Maidenhead, UK), utilizing a hexokinase- glucose-6-phosphate

dehydrogenase method. The analytical range was 0.278–44.4 mmol/L, with an intra-assay coefficient of variation of 0.65–1.98% and an inter-assay coefficient of variation of 0.84–0.93%. Serum insulin was measured on an Abbott Architect platform using Abbott reagents which has a intra-assay coefficient of variation of 10%.

#### **2.3.4.2 Glucagon**

Glucagon was measured using established in-house radioimmunoassays (RIAs)(127). All samples were assayed in duplicate. Glucagon was purchased from Bachem Ltd (Switzerland). All other reagents and materials were supplied by Sigma (Poole, Dorset, UK). The glucagon labels were prepared by Professor M. Ghatei (Professor of Regulatory Peptides, Metabolic Medicine, Faculty of Medicine, Imperial College) who iodinated the peptide using the iodogen method and was purified by reverse-phase HPLC.

Assays were performed in veronal buffer (11 distilled water containing 10.3g sodium barbitone, 0.3g sodium azide), at pH 8 with 0.3% BSA (and 0.02% Tween for the GLP-1 assay) (VWR, UK). Standard curves were prepared in assay buffer at 0.5 pmol/ml for glucagon, added in duplicate at volumes of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100  $\mu$ l. The glucagon antibody (RCS5) was raised in rabbits against the C-terminal of glucagon



and is therefore specific for pancreatic glucagon. It does not cross react with either glicentin or oxyntomodulin. The antibody was used at a dilution of 1:50000. Experimental samples of 50 µl, 100 µl glucagon antibody solution and 100 µl of glucagon label solution were used and all tubes were buffered to a total volume of 700 µl with assay buffer. The assays were incubated for 96 hours at 4°C. Free peptide was separated from bound using charcoal adsorption. To each tube, 4 mg of charcoal, suspended in 0.06 M phosphate buffer with gelatine was added immediately prior to centrifugation. The samples were then centrifuged at 1500 rpm, 4°C, for 20 minutes. Bound and free label were separated and both the pellet and supernatant counted for 180 seconds in a  $\gamma$ -counter (model NE1600, Thermo Electron Corporation). Plasma glucagon concentrations in the samples were calculated using a non-linear plot (RIA Software, Thermo Electron Corporation) and results calculated in terms of the standard.

#### **2.3.4.3 Active GLP-1**

Active GLP-1 immunoreactivity was measured using a commercially available ELISA kit (Millipore Corporation, MA, USA), with the following specificities: GLP-17-36amide 100%, GLP-17-37 72%, GLP-11-36 OR GLP-11-37 <2%.

#### **2.3.4.4 Measure of $\beta$ cell sensitivity and function**

The Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) which is a measure of insulin resistance in the fasting state (210) was measured using the HOMA-IR2 calculator by the Oxford Diabetes Trial Unit(211).

The insulinogenic index is a measure of  $\beta$  cell function and is calculated as the ratio of change in insulin from fasting to 30 minutes post meal to the change in glucose from fasting to 30 minutes post meal(212).

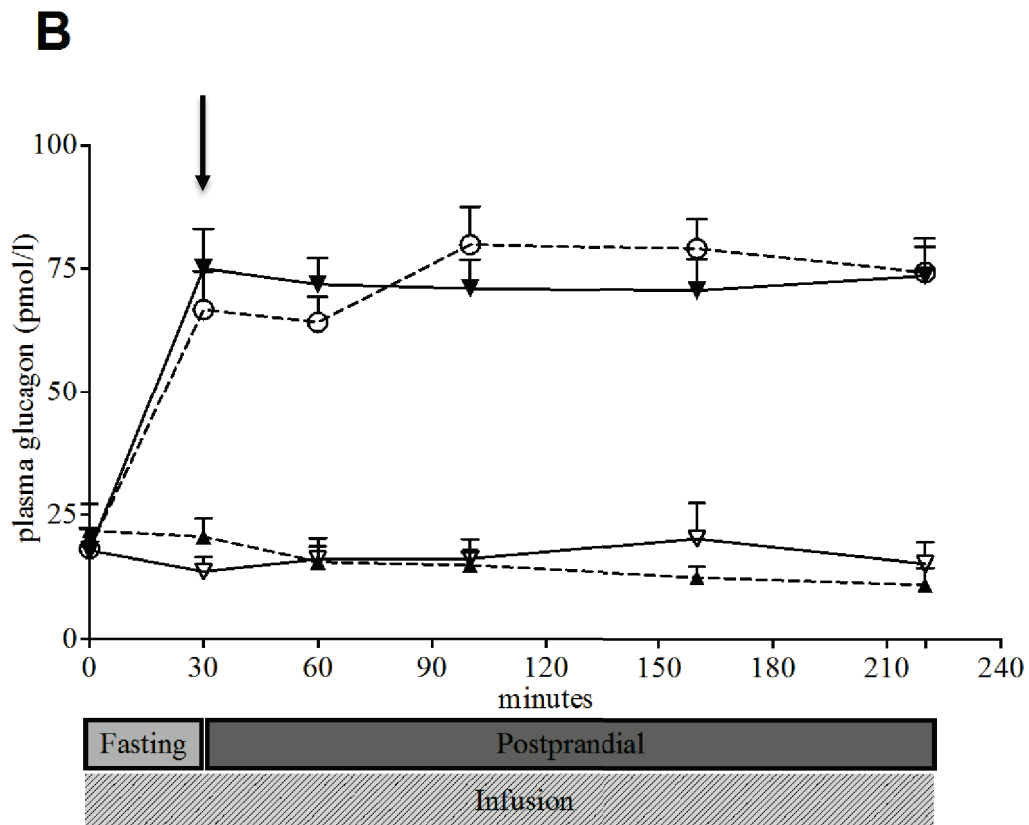
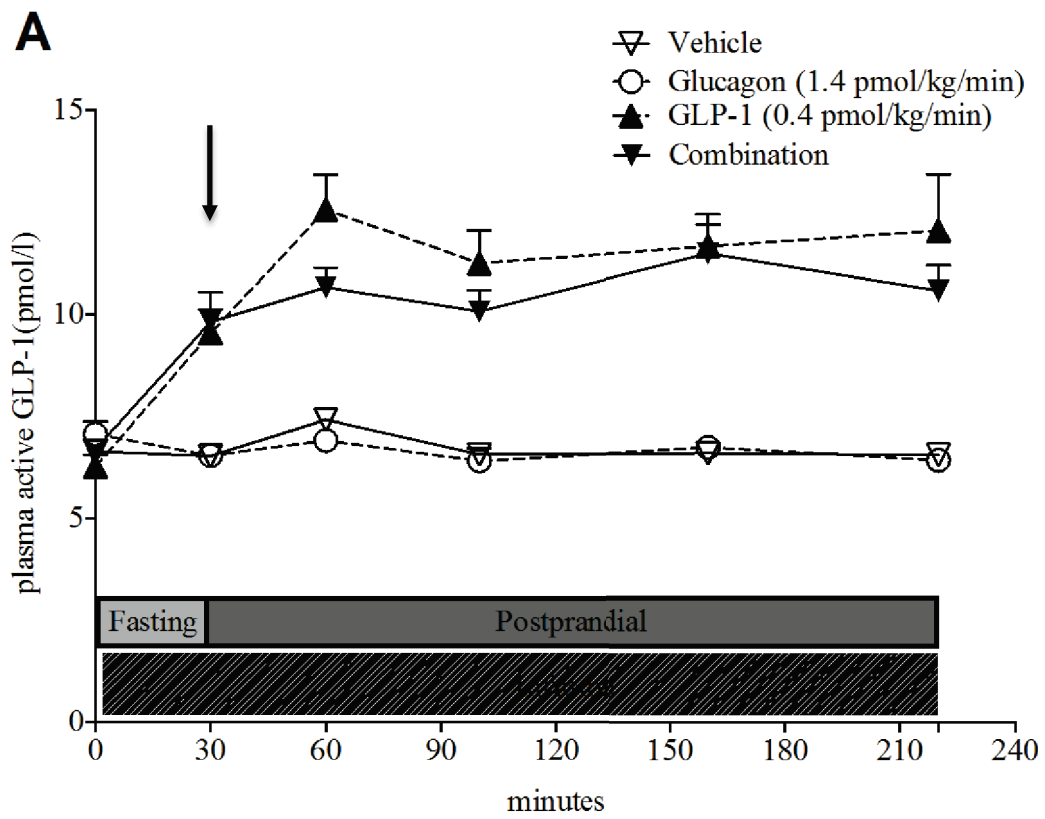
#### **2.3.5 Statistical Analysis**

All data are expressed as mean  $\pm$  SEM unless specified. Two-way repeated measures ANOVA with Bonferroni *post hoc* test was used to compare differences in glucose, insulin, pulse, blood pressure and VAS scores at specific time points. One-way ANOVA with Tukey *post hoc* test was used to compare differences between AUC insulin and glucose. Statistical significance was defined as  $P < 0.05$ .

## 2.4 Results

### 2.4.1 Gut hormone assays

Mean plasma concentration of active GLP-1 30 minutes before the infusion was  $6.63 \pm 0.13$  pmol/l. In the experimental groups receiving GLP-1 alone or GLP-1 combined with glucagon, the mean concentration rose to  $11.57 \pm 0.51$  pmol/l. There was no significant difference in the concentration of active GLP-1 between those that received GLP-1 alone or in combination with glucagon (Figure 2.1A). Mean plasma concentration of glucagon 30 minutes prior to infusion was  $18.90 \pm 2.22$  pmol/l, rising to  $70.97 \pm 5.51$  pmol/l at 30 minutes in those receiving glucagon, as a single infusion or in combination with GLP-1 (Figure 2.1B).



**Figure 2.1 Plasma active GLP-1 (A) and glucagon (B) concentrations after hormone infusion.** Data are given as mean  $\pm$  SEM. Duration of infusion denoted by labelled grey hashed bar. A mixed meal was given at T=30 minutes (shown by arrow). N = 10 for each infusion.

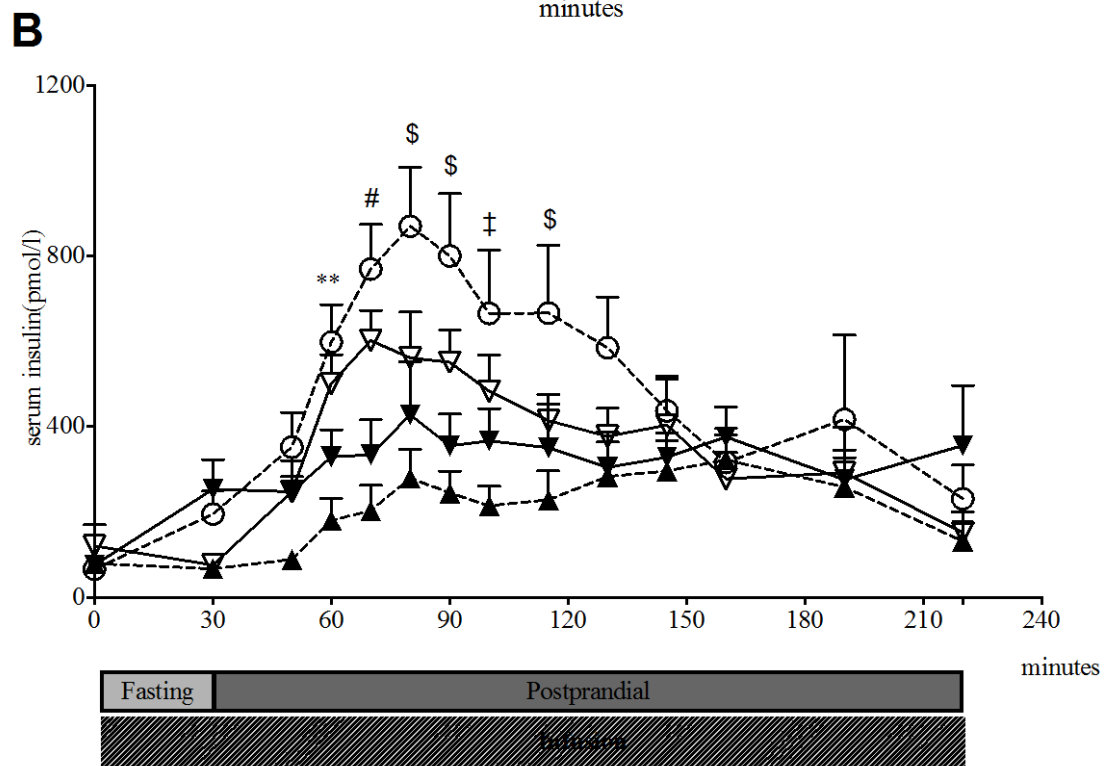
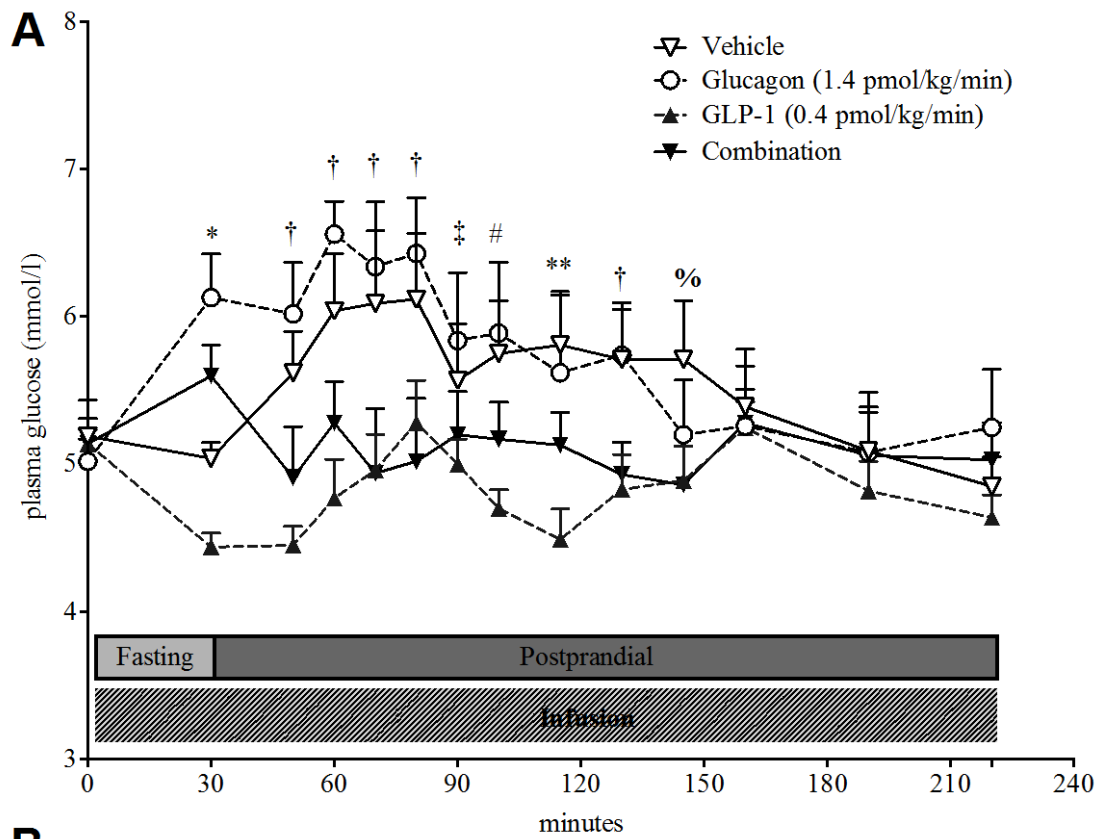
## 2.4.2 Glucose and Insulin results

The effects of vehicle, glucagon, GLP-1 or GLP-1/glucagon in combination on plasma glucose and serum insulin are shown in Figure 2.2. Prior to infusion, mean plasma glucose was  $5.12 \pm 0.08$  mmol/l with no significant difference between groups. In the vehicle group, mean plasma glucose was constant for the first 30 minutes, but then rose in response to consuming the mixed meal to  $6.12 \pm 0.45$  mmol/l at 80 minutes. Mean plasma glucose then returned to a baseline level at 160 minutes. In all infusion groups, plasma glucose concentration returned to baseline at this time point.

In those receiving a glucagon infusion, mean pre-prandial plasma glucose rose to  $6.13 \pm 0.30$  mmol/l after 30 minutes, which was significantly different to the vehicle group (mean plasma glucose:  $5.04 \pm 0.11$  [vehicle] vs.  $6.13 \pm 0.30$  mmol/l [glucagon],  $P < 0.01$ ). Plasma glucose peaked at  $6.56 \pm 0.22$  mmol/l at 30 minutes after meal commencement (T=60 minutes), before dropping to baseline at T=160 minutes. AUC glucose<sub>T=30-160</sub> was unchanged relative to vehicle (Figure 2.3A). Administration of GLP-1 alone significantly decreased mean plasma glucose compared to vehicle (mean plasma glucose<sub>T=30</sub>:  $5.14 \pm 0.10$  [GLP-1] vs  $4.44 \pm 0.10$  mmol/l [vehicle  $P < 0.001$ ]). Glucose concentrations fluctuated during the GLP-1 infusion but never exceeded pre-infusion fasting values. Mean

AUC glucose  $T=30-160$  in the GLP-1 group was significantly reduced compared to glucagon (Mean AUC glucose  $T=30-160$   $615.3 \pm 42.3$  [GLP-1] vs  $764.8 \pm 17.3$  mmol·min/l [glucagon]  $P < 0.05$ ). The combination of GLP-1 and glucagon significantly attenuated the increase in plasma glucose observed by glucagon alone (Mean AUC glucose  $T=30-160$   $643.9 \pm 32.6$  [combination] vs  $764.8 \pm 17.3$  mmol·min/l [glucagon]  $P < 0.05$ ).

Following consumption of the mixed meal, serum insulin rose in all infusion groups peaking 50 minutes later. Relative to vehicle, the AUC insulin  $T=30-160$  was significantly lower in those receiving GLP-1, but there was no difference when compared to glucagon alone or co-infusion of glucagon and GLP-1. Infusion of glucagon alone produced the highest AUC insulin  $T=30-160$ . However, the AUC insulin  $T=30-160$  was significantly lowered by the addition of GLP-1 to glucagon compared to administration of glucagon alone (AUC insulin  $T=30-160$   $73606 \pm 12484$  [glucagon] vs  $42841 \pm 7457$  pmol·min/L [combination]  $P < 0.01$ )(Figure 2.3B).





**Figure 2.2 Effects of glucagon, GLP-1 and combination on glucose (A) and insulin (B) concentrations.** Data are given as mean  $\pm$  SEM. Duration of infusion denoted by labelled gray and hashed bar. 10 volunteers received each infusion. Significant differences are:

\*Glucagon vs Vehicle  $P < 0.05$ , GLP-1 vs Glucagon  $P < 0.05$ , Combination vs GLP-1  $P < 0.05$ .

†GLP-1 vs Vehicle  $P < 0.05$ , combination vs vehicle  $P < 0.05$ , GLP-1 vs Glucagon  $P < 0.05$ , Combination vs Glucagon  $P < 0.05$ .

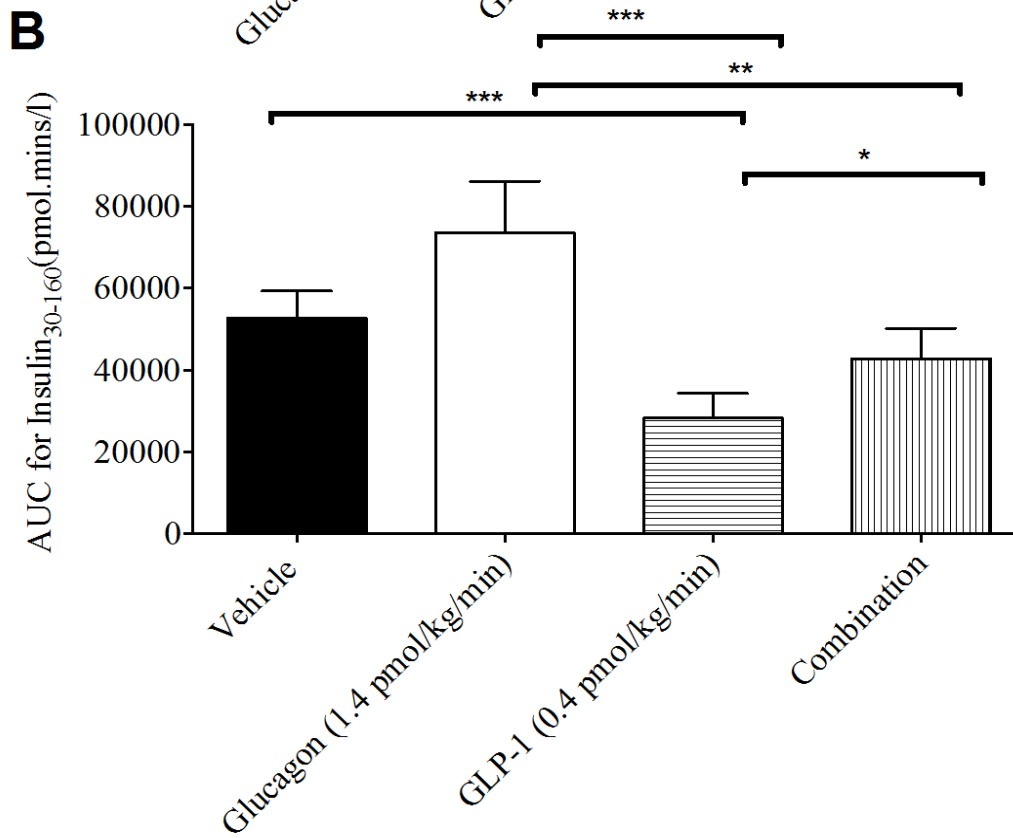
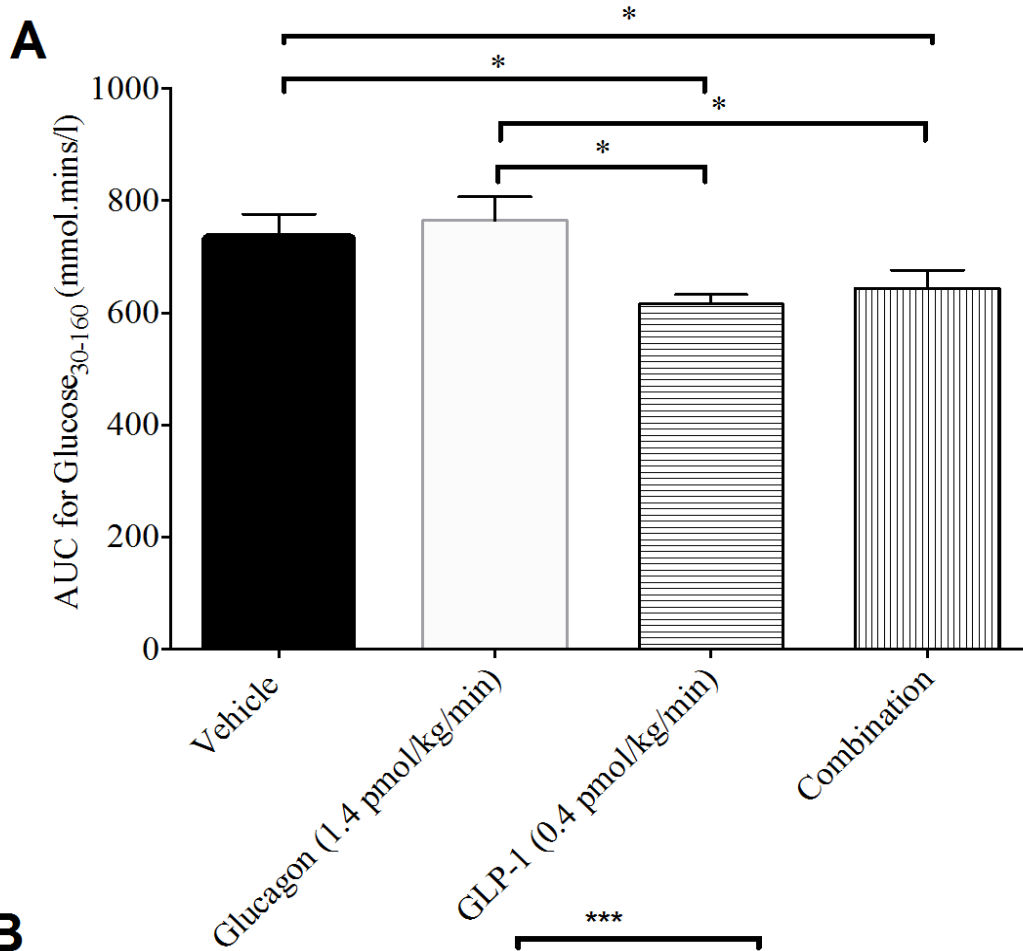
‡GLP-1 vs Glucagon  $P < 0.05$ .

#GLP-1 vs Vehicle  $P < 0.05$ , GLP-1 vs Glucagon  $P < 0.05$ , Combination vs Glucagon  $P < 0.05$ .

\*\*GLP-1 vs Vehicle  $P < 0.05$ , GLP-1 vs Glucagon  $P < 0.05$ .

%GLP-1 vs Vehicle  $P < 0.05$ , Combination vs Vehicle  $P < 0.05$

\$ GLP-1 vs Glucagon  $P < 0.05$ , Combination vs Glucagon  $P < 0.05$



**Figure 2.3 Effects of glucagon, GLP-1 and combined combination on mean AUC glucose (A) and mean AUC insulin (B) during infusion.** Data are mean AUC  $\pm$  SEM shown. 10 volunteers received each infusion. Significantly different mean AUC values are indicated as follows \* P<0.05, \*\*P<0.01 \*\*\*P<0.001.

### **2.4.3 Measures of insulin sensitivity and insulin secretion**

The effects of each hormone infusion on insulin resistance and secretion are shown in Table 21. As expected, baseline hepatic insulin resistance (as measured by HOMA-IR) prior to infusion was similar between the groups. There was no significant increase in the insulinogenic index comparing all four infusion groups.

**Table 2.1 Measures of glucose homeostasis during a mixed meal test following infusion of GLP-1, glucagon or a combination of both**

	<b>Vehicle</b>	<b>Glucagon</b>	<b>GLP-1</b>	<b>Combination</b>	<b>Significant differences between groups</b>
<b>Fasting glucose (mmol/L)</b>	5.19±0.25	5.02±0.15	5.14±0.10	5.14±0.17	
<b>Fasting glucose after 30 minutes infusion (mmol/L)</b>	5.04±0.11	6.13±0.30	4.44±0.09	5.60±0.21	Glucagon>Vehicle P<0.001 Glucagon>GLP-1 P<0.0001 Glucagon + GLP-1>GLP-1 P<0.0001
<b>Peak insulin (pmol/l)</b>	749.9±103.3	936.2±132.7	426.2±75.1	666.1±140.9	Glucagon>GLP-1 P<0.05
<b>Mean time to peak insulin (mins)</b>	82.5±7.7	82.5±7.4	125.0±11.6	105.5±17.6	
<b>HOMA-IR (t=0) (mmol·uIU/L<sup>2</sup>)</b>	1.95±0.66	1.22±0.24	1.47±0.30	1.41±0.34	
<b>AUCglucose<sub>30-160</sub> (mmol·min/l)</b>	733.1±43.0	764.8±17.3	615.3±42.3	643.9±32.6	Glucagon>GLP-1 P<0.05 GLP-1<Vehicle P<0.05 Glucagon + GLP-1<Vehicle P<0.05
<b>AUC Insulin<sub>30-160</sub> (pmol·min/l)</b>	52646±6742	73606±12484	28277±6118	42841±7457	Glucagon>GLP-1 P<0.001 GLP-1<Vehicle P<0.0001 Glucagon + GLP-1>GLP-1 P<0.05 Glucagon + GLP-1<Glucagon P<0.01
<b>AUC insulin<sub>30-160</sub>/glucose<sub>30-160</sub> (pmol/mmol)</b>	43.3±4.8	57.5±8.0	26.1±5.4	36.6±5.5	Glucagon>GLP-1 P<0.01
<b>Insulinogenic index (pmol/mmol)</b>	618.2±273.0	907.1±435.9	235.2±67.0	160.7±123.4	

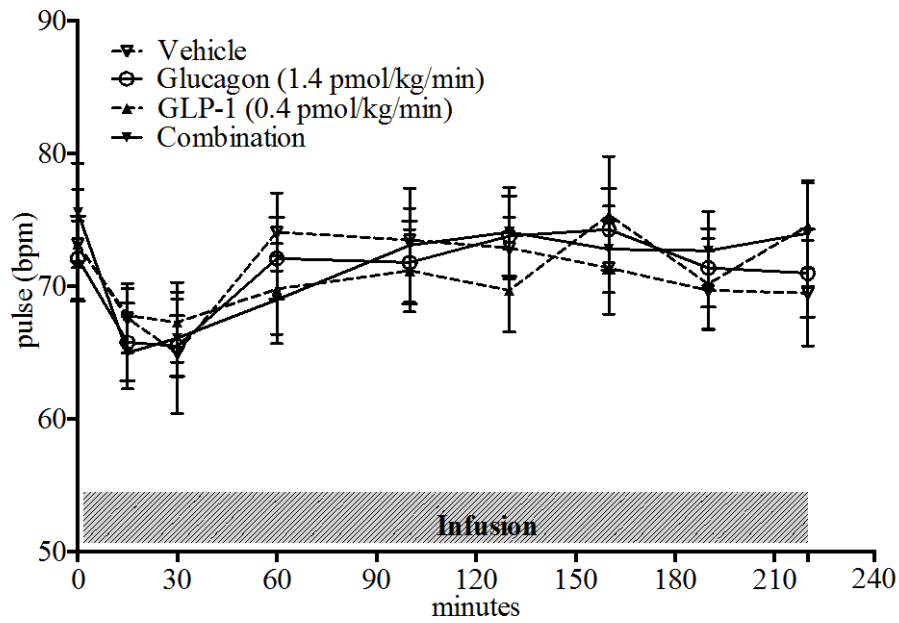
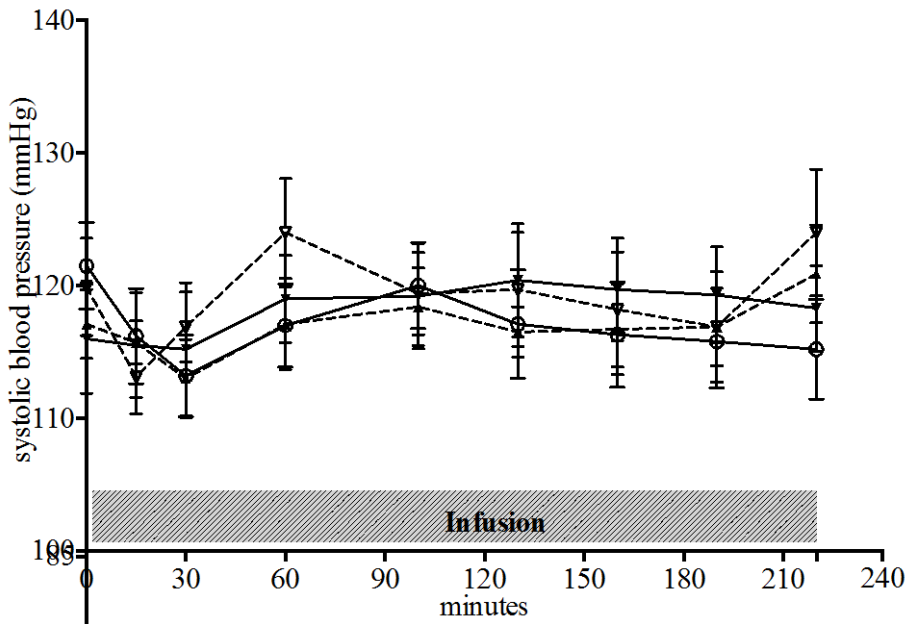
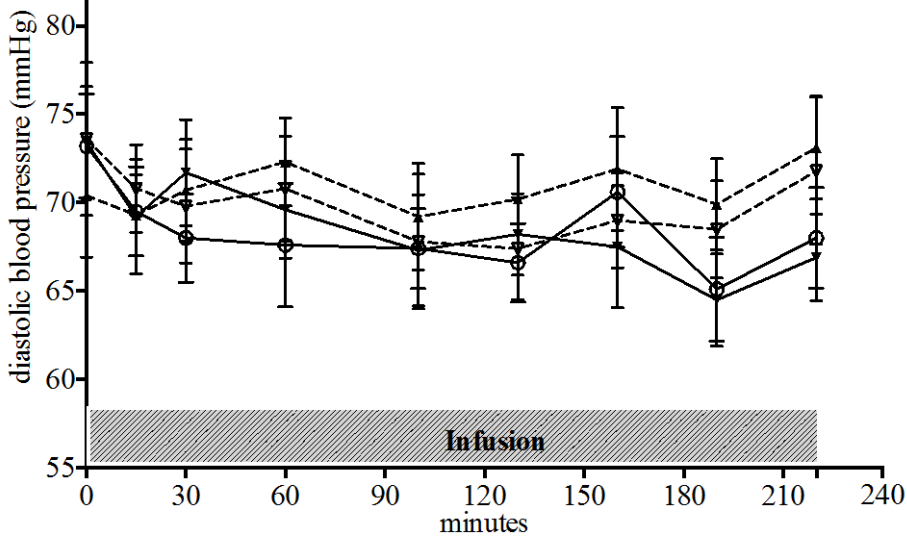
**Table 1. Measures of glucose homeostasis during a mixed meal test following infusion of GLP-1, glucagon or a combination of both**

Insulinogenic index is calculated as change in insulin from T=30 to T=60, divided by the change in glucose from T=30 to T=60. 10 volunteers received each infusion.

## **2.4.4 Safety data**

### **2.4.4.1 Blood Pressure and Pulse**

There were no significant changes in pulse rate or blood pressure throughout the study (Figure 2.4).

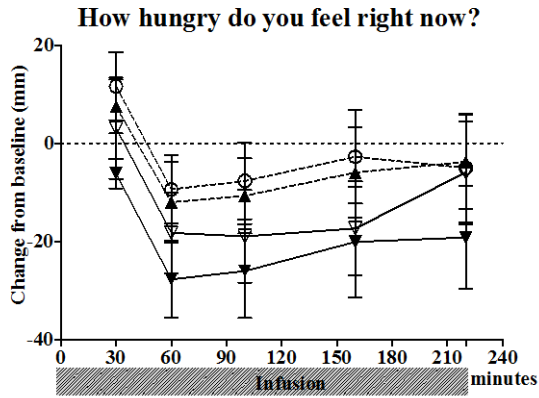
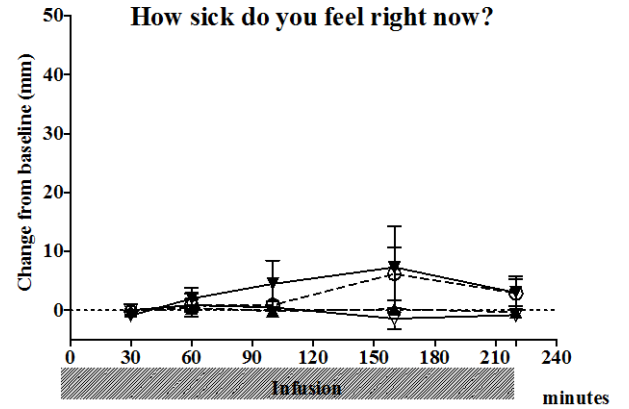
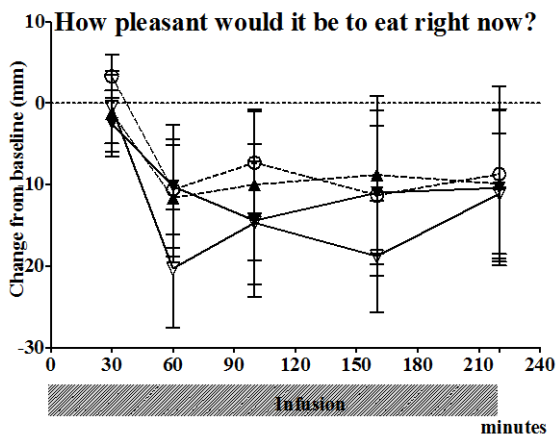
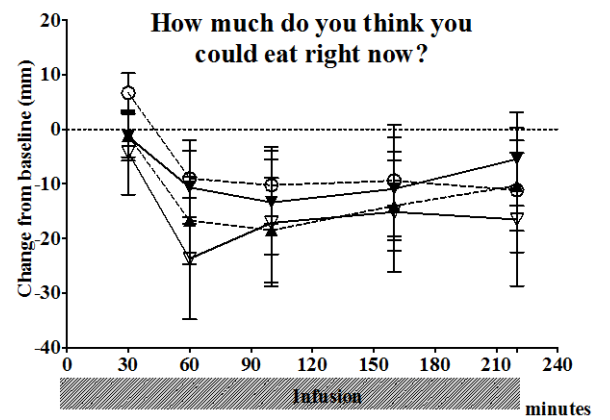
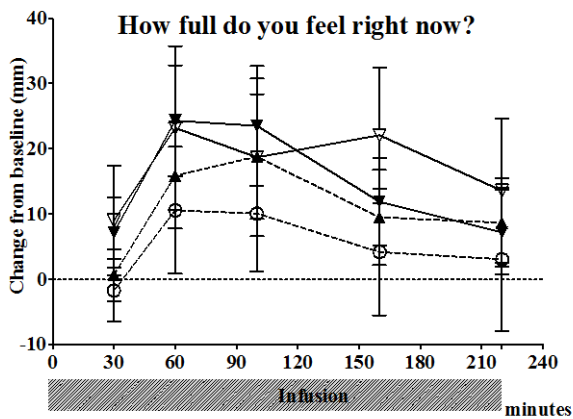
**A****B****C**



**Figure 2.4 Cardiovascular parameters during infusion of GLP-1, glucagon and combination of both.** Heart rate (A), Systolic (B), and Diastolic (C) blood pressure changes in response to infusions. Two-way, repeated measures ANOVA showed no significant differences in mean between treatment groups. Data are mean  $\pm$  SEM. 10 volunteers received each infusion. Duration of infusion reflected by labelled grey and hashed bar.

**2.4.4.2 VAS results performed during acute infusion of GLP-1, glucagon or combination of both.**

There was no significant change in levels of nausea as measured by VAS (Figure 2.5).

**A****B****C****D****E**

- ▽ Vehicle
- ⊖ Glucagon (1.4 pmol/kg/min)
- ▲ GLP-1 (0.4 pmol/kg/min)
- ▼ Combination

**Figure 2.5 VAS results performed during acute infusion of GLP-1, glucagon or combination of both.**

Visual analogue scores (A) How hungry do you feel right now? (B) How sick do you feel right now? (C) How pleasant would it be to eat right now? (D) How much do you think you could eat right now? (E) How full do you feel right now? Two-way repeated measures ANOVA showed no significant differences in mean VAS score between treatment groups. Data are mean  $\pm$  SEM. Duration of infusion represented by labelled grey and hashed bar.

## 2.5 Discussion

This clinical study, has demonstrated that the combination of GLP-1 and glucagon significantly attenuates the rise in pre-prandial glycaemia induced by glucagon alone. This is similar to previous observations by this department (49,201). Notably, this is the first time that infusion of GLP-1 and glucagon in combination has been shown to significantly reduce postprandial glycaemia (relative to glucagon alone) in response to a fixed meal stimulus. There was a further rise in glucose postprandially during the glucagon infusion, but this was similar to the post-prandial glucose levels during the vehicle arm. There was a significant reduction in AUC glucose with combination GLP-1 and glucagon, relative to vehicle.

A previous study has shown that administration of intravenous glucagon at 3 ng/kg/min (0.9 pmol/kg/min) in obese subjects for 6 hours did not significantly increase glucose levels following an OGTT, relative to saline(207). This finding has also been replicated in overweight subjects with a mixed meal stimulus(213). The absence of postprandial hyperglycaemia in response to glucagon infusion compared to vehicle may be due to a compensatory rise in insulin, either a direct insulinotropic effect of glucagon or indirectly via a rise in portal concentrations of glucose. This is consistent with an earlier study of patients with insulin-dependent diabetes, receiving an infusion of glucagon (3

ng/kg/min), which resulted in a significantly higher postprandial glucose concentrations relative to saline, implying that postprandial insulin secretion may be responsible for this effect(214). However, inconsistent with this in the current study, is the observation that there was no significant difference in AUC insulin comparing the glucagon alone and vehicle arms. In addition, Frank *et al.* showed that glucagon infusion did not increase C-peptide levels compared to a control infusion, making a glucagon-mediated increase in insulin secretion an unlikely explanation(213). An alternative explanation is that there is enhanced hepatic glucose uptake during the glucagon infusion(215). Metabolic labelling studies will be necessary to study the metabolic fluxes in response to glucagon infusion and ingestion of a meal in order to delineate this phenomenon.

The infusion of GLP-1 alone resulted in a reduction in postprandial glucose and insulin relative to the vehicle infusion. The ratio of AUC insulin to AUC glucose and insulinogenic indices suggest that the GLP-1 infusion was the least insulinotropic infusion. Whilst GLP-1 is thought of as an insulinotropic hormone, this relative reduction in post-prandial insulin is well established and likely relates to slowed gastric emptying and an attenuation in postprandial glucose rise(106).

The combination of GLP-1 and glucagon resulted in a significant reduction in both plasma glucose and serum insulin (as measured by AUC) relative to

glucagon alone. However, relative to GLP-1 alone, the dual infusion of GLP-1 and glucagon resulted in a significantly higher AUC of glucose and insulin. Both GLP-1 and glucagon are known to be insulinotropic, but in our study, both the glucagon alone, and the combination infusion, stimulated a higher insulin response relative to GLP-1 alone. These observations offer a valuable insight into the action of these hormones in the postprandial phase. Following RYGB, it has been observed that carbohydrate tolerance is improved with a significant rise in postprandial insulin secretion(183). As the combination infusion was a more potent stimulus for insulin secretion than GLP-1, the observed postprandial rise in glucagon after RYGB may contribute to the elevated insulin levels seen after this surgery.

A recent study by Bagger *et al* also examined the physiological effects of glucagon plus GLP-1 combination(216). There were significant differences in experimental design compared to the current study. Firstly, the main experimental outcome in the current study was carbohydrate tolerance, in contrast to energy expenditure and food intake. In the current study, the mixed meal challenge was initiated once the hormone infusions had achieved elevations of glucagon and GLP-1 at a steady state, whereas in the study by Bagger *et al*, the combined infusion and mixed meal were given simultaneously. Secondly, the current study was performed in an overweight population (mean BMI 27.2 kg/m<sup>2</sup>) whereas the cohort studied by Bagger *et al*. had a normal

BMI. The target treatment group for any dual agonist treatment would be likely to be obese or overweight. Lastly, the ratio of glucagon to GLP-1 dose utilised in our study was 3.5:1 as opposed to 0.8:1, closer to the ratio of binding affinities of OXM to the receptors of glucagon and GLP-1 at 2.5:1.

There are several limitations to the interpretation of the current study should be noted. These data relate to the acute effects of GLP-1 and glucagon infusions and as yet the chronic effects of these hormones remain unknown. Assuming weight loss occurs in response to the anorexigenic effects and increase in energy expenditure, one would anticipate that insulin sensitivity might improve in response to more prolonged treatment, leading to further reductions in glycaemia. Furthermore, chronic glucagon administration would not be expected to yield a persistently elevated fasting glucose, as any increase in glycogenolysis should only produce a transient rise in plasma glucose and the glucagon-induced enhancement of gluconeogenesis is sensitive to insulin inhibition. Due to limitations within the study design, only fixed doses of GLP-1 and glucagon were investigated. Future work should aim to study different doses hormones administered either individually or in combination in order to identify the optimal doses and dose ratio. Whilst the current study design attempted to replicate the two-fold increase in plasma glucagon concentration observed after RYGB, the concentration achieved was three-fold higher relative to fasting levels. This may suggest that glucagon concentrations observed post



RYGB are less likely to cause hyperglycaemia. It will be important to investigate whether the observed beneficial effects of a combined GLP-1-glucagon infusion as shown in the current study translate to an obese diabetic population, particularly where there are limitations to stimulated insulin secretion.

In conclusion, this study has demonstrated that the combination of GLP-1 and glucagon results in improved carbohydrate tolerance relative to vehicle and relative to glucagon alone in the context of a fixed mixed meal stimulus. These data provide further support for the safety of dual GLP-1 and glucagon agonists in the treatment of obesity and diabetes.

## **Chapter 3:**

**The acute effects of co-infusion of  
GLP-1, Oxyntomodulin and PYY on  
food intake and nausea**

### **3.1 Introduction**

The dual observations that after RYGB surgery there is an elevation in postprandial concentrations of GLP-1, OXM and PYY, and that these hormones are powerful anorectic agents has led to speculation regarding a causative role in the metabolic benefits of RYGB surgery(102,135,157,177,180). Furthermore, such observations support the premise that these gut hormones could be developed as anti-obesity therapeutics. However, this potential has yet to be realised. At present the only anti-obesity medication based on a gut hormone, is liraglutide; a long acting GLP-1 receptor agonist(123). Further development of gut hormone based therapeutics has two barriers that must be overcome, a dose-limiting nausea and a patient acceptable drug delivery process.

The weight loss observed with liraglutide is relatively modest compared to RYGB (10% vs 30% of pre-intervention weight)(123,217). Whilst the anorectic effect of GLP-1 is dose-proportional, it also has a dose-limiting side-effect; nausea(102). Nausea has also been observed with PYY and OXM(218). One potential solution to overcome the nausea is to use lower doses of single gut hormones in combination to achieve an additive anorectic effect. This concept has been tested in studies that have co-infused PYY with GLP-1 and PYY with OXM, and compared their effect on food intake relative to individual hormone infusions(204,218). Both studies demonstrated that the co-infusion of

two hormones had an additive effect on food reduction but without any significant nausea. The success of dual infusions could be further improved by the co-infusion of multiple hormones.

Another potential barrier to the use of gut hormones as therapeutic agents is their mode of delivery. Due to their short half life, studies investigating the effects of GLP-1, OXM and PYY, have used an intravenous route for infusion(49,102,204,218,219). An IV administration has limitations to chronic use. Alternative modes of delivery include SC injections. OXM has been given as a SC injection but due to its short half life required dosing four times per day(139). Long acting analogues can reduce the rate of injection; Bydureon®(Astra Zenica, United Kingdom) (a GLP-1 long acting agonist) is administered once per week(220). However, at present there are no long acting analogues of PYY or OXM.

Another alternative route of delivery is a continuous SC infusion. This route of drug delivery is currently used in patients with T1DM and Parkinson's disease(221,222). GLP-1 has been infused by this route and resulted in weight loss(223). However, no study has investigated SC infusion of PYY or OXM.

To address these issues, the current study was designed to investigate the effects of combined SC co-infusion of GLP-1, OXM and PYY, on food intake.

## **3.2 Hypothesis and aims**

### **3.2.1 Hypothesis**

I hypothesised that GLP-1, OXM and PYY infused in combination will reduce food intake without nausea.

### **3.2.1 Aim**

To determine doses of GLP-1, OXM and PYY that could be infused together at a safe and tolerable dose. Subsequently, to investigate the effects of co-infusing GLP-1, OXM and PYY on food intake and nausea.

## **3.3 Methods**

### **3.3.1 Peptides**

GLP-17-37 amide, OXM and PYY were purchased from Insight Biotechnology limited (Wembley, UK). Representative vials were randomly selected and sent for microbial examination and for detection of pyrogen via a Lumulus Amoebocyte Lysate (LAL) test. Microbial analysis (conducted by the Department of Microbiology, Hammersmith Hospital, London) determined that the vials were sterile on bacterial and extended fungal culture. LAL testing (performed by Associates of Cape Cod, Liverpool, UK) determined that endotoxin levels were within the safe range for human infusion.

To calculate actual peptide content, for the purpose of calculating accurate peptide doses, further representative vials was sent for amino acid analysis (Bachem, St Helens, UK).

Further vial samples were used for toxicity studies. Forty mice were injected, via an IP injection at a dose 10 times greater than the maximum intended dose for humans (in pmol/kg body weight). Prior to sacrifice, the mice were observed for any abnormal behaviour. Short and long term toxicity was assessed by sacrificing 20 mice at 48 hours after injection and the other 20 mice 14 days after injection. An independent histopathologist received the following organs for assessment of toxicity; liver, pancreas, kidneys, heart, brain, lungs and

reproductive organs.

### **3.3.2 Subjects**

Subjects were recruited via advertisement on the internet and newspapers.

Inclusion criteria for the study were healthy males or females aged 18 -70 years, with a BMI < 30 kg/m<sup>2</sup> and stable weight over the last three months. Exclusion criteria were: smokers, recreational drug users, significant past or current history of physical or psychiatric illness, pregnancy or breastfeeding. Prior to inclusion to the study all subjects were screened and determined to be healthy by medical history, physical examination, haematological and biochemical testing and 12 lead electrocardiogram.

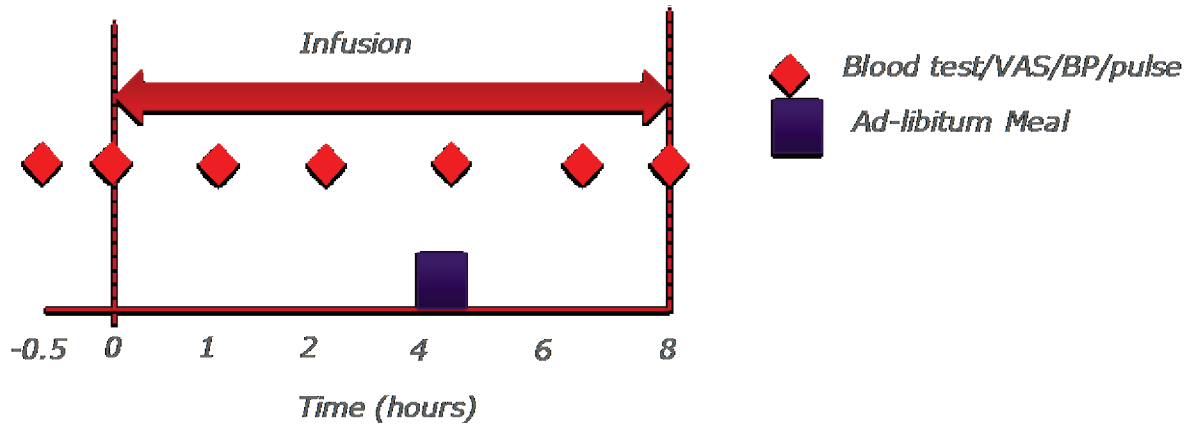
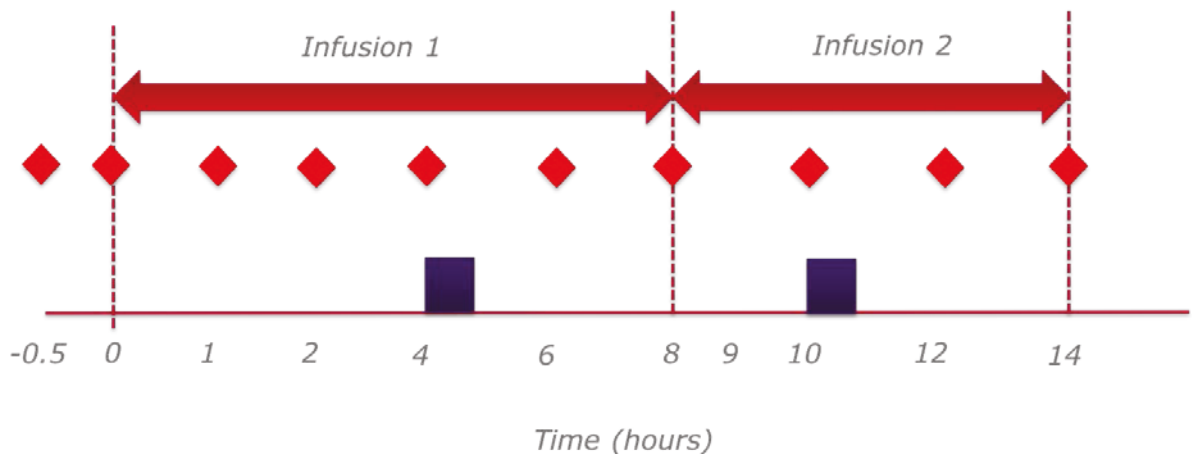
The study was approved by the West London Research Ethics Committee (ref no 13/LO/1510). Written informed consent was obtained for all volunteers and the study was carried out according to the principles of the Declaration of Helsinki.

### **3.3.3 Protocol**

The study was designed as a randomised, single-blinded, placebo-controlled study comparing the effects of a SC co-infusion of GLP-1/OXM/PYY infusion to saline.

Whilst GLP-1 has been given as a SC infusion, there are no published studies investigating the effects of SC infusions of either OXM or PYY either as alone or in combination. During the dose finding study, doses of GLP-1, OXM and PYY were administered either alone or in combination. The protocol for the dose finding study is shown in Figure 3.1A. A peptide infusion was commenced at T=0 during which a range of doses of GLP-1 (2-20 pmol/kg/min), OXM (5-30 pmol/kg/min) and PYY (0.5-1.5 pmol/kg/min) were infused either alone or in combination. An *ad-libitum* meal was served at four hours. Regular blood samples, VAS scores and vital sign measurements were taken at the times indicated.



**A****B**

**Figure 3.1 Protocol for dose finding study (A) and main study (B).** In both studies volunteers were cannulated for venous blood sampling. Blood samples, VAS scores and vital signs were checked at the times indicated. In the dose finding study only one *ad-libitum* meal was given at four hours after the infusion was started. In the main study, two *ad-libitum* meals were given at four and eight hours after the infusion was started.

Following the dose finding study the protocol was amended to include a second *ad-libitum* meal and extended infusion duration as shown in Figure 3.1B.

Subjects arrived to the clinical research facility at the Hammersmith Hospital having fasted overnight. Upon arrival, female volunteers were asked to pass a sample of urine that was tested to confirm that they were not pregnant. A peripheral cannula was inserted to allow blood sampling. At T=-30 minutes, a subcutaneous cannula (Quick-set infusion set, Meditronic, Watford, UK) was placed in the subject's abdomen. At T=0 the SC infusion was started. Subjects received either a co-infusion of GLP-1 (4 pmol/kg/min), OXM (4 pmol/kg/min) and PYY (0.4 pmol/kg/min) or saline. Prior to making up the infusion, both syringes and infusion tubing used to deliver the hormones or saline was soaked in gelafusine to reduce adsorption of the peptides.

The rate of infusion was delivered by a Chrono® pump (Cane SPA, Rivoli, Italy). The infusion was continued for eight hours after a new infusion was started. The need for two infusions was due to the stability of GLP-1 within the syringe. At T= 14 the subject had their cannulae removed and was discharged home.

Energy intake was assessed via two *ad-libitum* meals that were given at T=4h and T=10h respectively. The *ad-libitum* meal consisted of one of a choice

of 6 standardised ready meals (Sainsbury's®) of known macronutrient and calorific content; tomato and mozzarella pasta bake (127kcal/100g), macaroni cheese (189 kcal/100g), chicken tikka masala with rice (178 kcal/100g), chicken korma with rice (154 kcal/100g), chicken jalfrezi with rice (156 kcal/100g) and beef in black bean sauce with rice (130 kcal/100g). Subjects were given 20 minutes to eat the ad-libitum meal and were instructed to eat until comfortably full. For both the dose finding and main study, each volunteer was given the same *ad-libitum* meal. The weight of the food was measured pre- and post-meal.

### **3.3.4 Blood assay analysis**

Insulin and glucose were measured as described in Chapter 2. Plasma active GLP-1 (GLP-17–36) and total PYY (PYY3–36 + PYY1–36), were prepared using the Milliplex MAP magnetic bead-based multi-analyte, metabolic panel, 4-plex immunoassay (HMHMAG-34K; Millipore, St. Charles, MO, USA) and measured using Luminex Magpix with xPONENT software (Luminex Corporation, Austin, TX, USA). The kit is a capture sandwich immunoassay which uses a capture antibody attached to a microsphere and a detection antibody that incorporates a fluorescent label.

OXM was measured by the Panum Institute, Kobenhavn University, Denmark, using an in-house radio-immunoassay.

### **3.3.5 Statistical analysis**

Two-way repeated ANOVA with Bonferroni *post hoc* test were used to compare differences in glucose, insulin, and changes in blood pressure and pulse. A paired t- test was used to analyse changes in food intake. Results are presented as mean  $\pm$  SEM and statistical significance was defined as  $P < 0.05$ .

## **3.4 Results**

### **3.4.1 Dose finding study investigating the acute effects of GLP-1, OXM and PYY on energy intake and nausea in humans.**

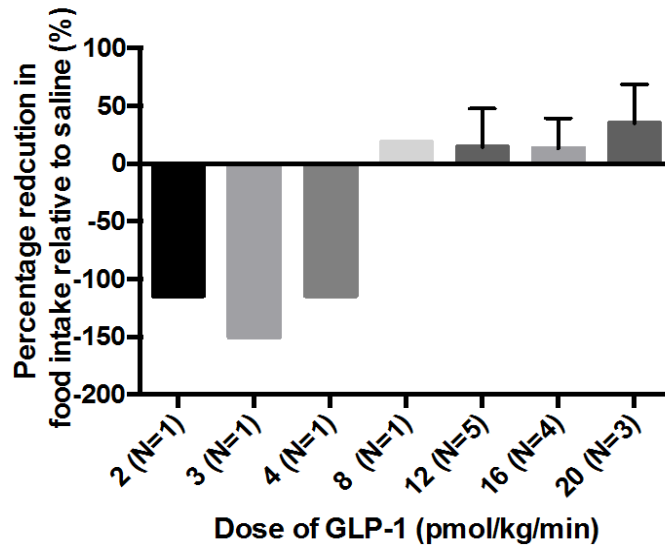
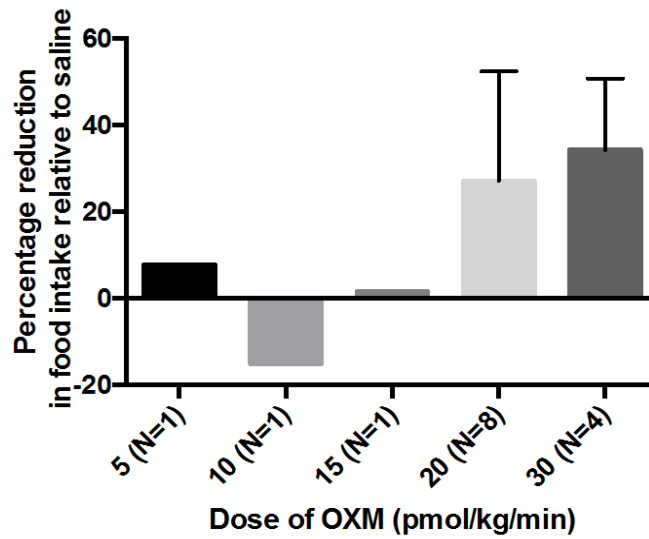
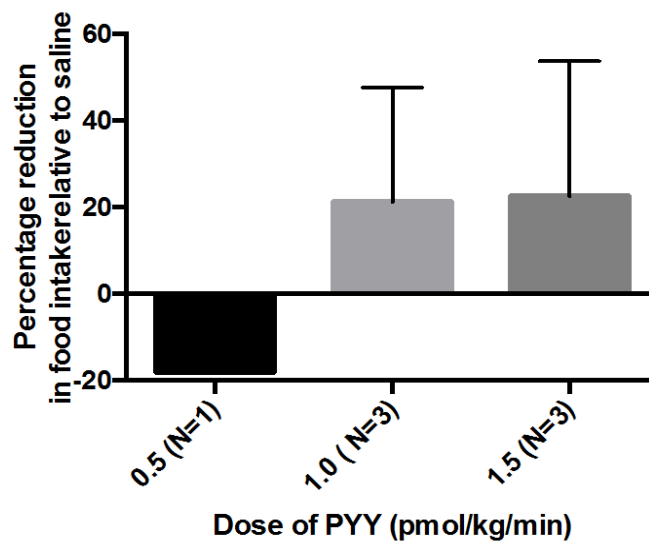
A dose finding study was undertaken to determine the appropriate subcutaneous dose of GLP-1, OXM and PYY that could reduce food intake without nausea compared to saline. The dose finding study took part in three phases. The first phase was a parallel arm study in which single infusions of GLP-1, OXM and PYY were given. The second phase involved the use of combined infusion of GLP-1 and OXM. The final phase was co-administration of all three hormones.

#### **3.4.1.1 Investigation of infusions of subcutaneous GLP-1, OXM or PYY alone on food intake and nausea**

The effects of GLP-1, OXM and PYY infusions, administered alone, were tested to determine the effects on food intake (Figure 3.2) and nausea (Table 3.1).

The lowest effective dose of GLP-1 that was tolerated in terms of nausea was 16 pmol/kg/min. Both 20 and 30 pmol/kg/min doses of OXM were effective at reducing food intake relative to saline but less nausea was observed in those receiving 20 pmol/kg/min. Tolerable doses of PYY were lower than both GLP-1 and OXM. Both 1.0 and 1.5 pmol/kg/min doses of PYY were anorectic but

more nausea was observed with 1.5 pmol/kg/min.

**A****B****C**

**Figure 3.2 Percentage reduction in food intake following subcutaneous administration of GLP-1 (A), OXM (B) and PYY (C) relative to saline.** Volunteers received an infusion of either saline (control) or one of the following hormones; GLP-1, OXM or PYY. The infusion duration was eight hours. An *ad-libitum* meal was given four hours after the infusion was started. The number of volunteers who received each infusion is shown in brackets.

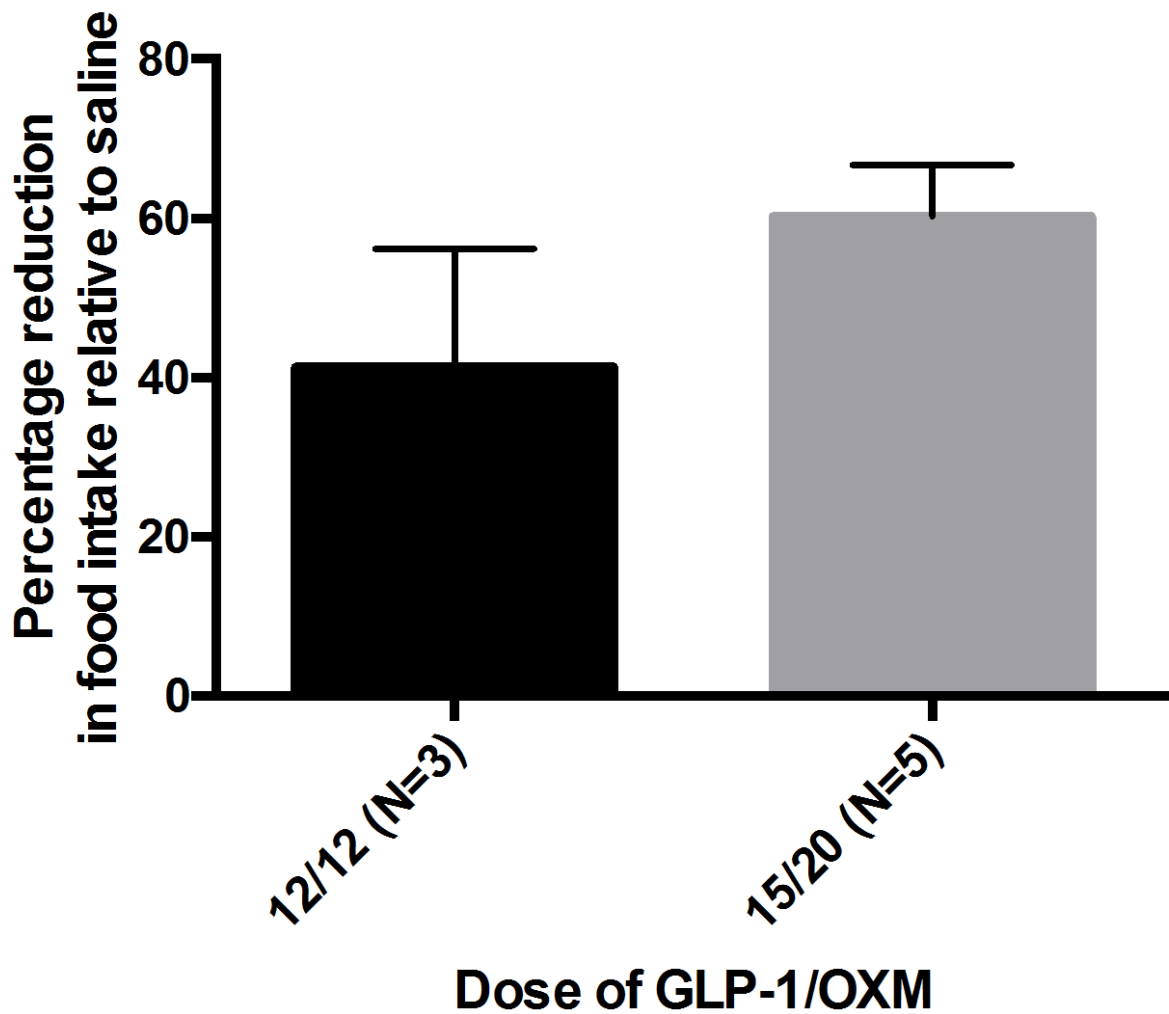


Peptide	Dose (pmol/kg/min)	Number of subjects	Number of subjects with nausea
GLP-1	2-8	1	0
	12	5	1
	16	4	1
	20	3	3
OXM	5-15	1	0
	20	8	0
	30	4	2
PYY	0.5	1	0
	1.0	3	1
	1.5	3	2

**Table 3.1 Investigation of the effects of varying doses of subcutaneous infusions of GLP-1, OXM or PYY alone on nausea.** Volunteers received infusions of GLP-1, OXM and PYY at different doses for a duration of eight hours. Nausea was either recorded on VAS score sheets at specific time points or reported to the investigator if occurring between time points.

### **3.4.1.2 Investigation of the acute effects of subcutaneous co-administration of GLP- 1 and OXM on food intake and nausea.**

Further dose finding was performed to determine the effect of co-infusion of GLP-1 and OXM. Doses of 16 and 20 pmol/kg/min of GLP-1 and OXM were selected based on the results of the previous dose finding study. However, as both peptides are infused via a single pump that uses one infusion rate for both peptides, it was more practical to infuse 15 and 20 pmol/kg/min of GLP-1 and OXM. Whilst this dose was effective at reducing food intake (Figure 3.3) there was a high rate of nausea (Table 3.2) and so both peptide doses were reduced to 12 pmol/kg/min. This combination at a lower dose produced less nausea.



**Figure 3.3 Percentage reduction in food intake achieved by subcutaneous co-infusion of GLP-1 and OXM at varying doses relative to saline.** Volunteers received an infusion of either saline or co-infusion of GLP-1 and OXM. The infusion duration was eight hours. An *ad-libitum* meal was given four hours after the infusion was started. The number of volunteers who received each infusion is shown in brackets.

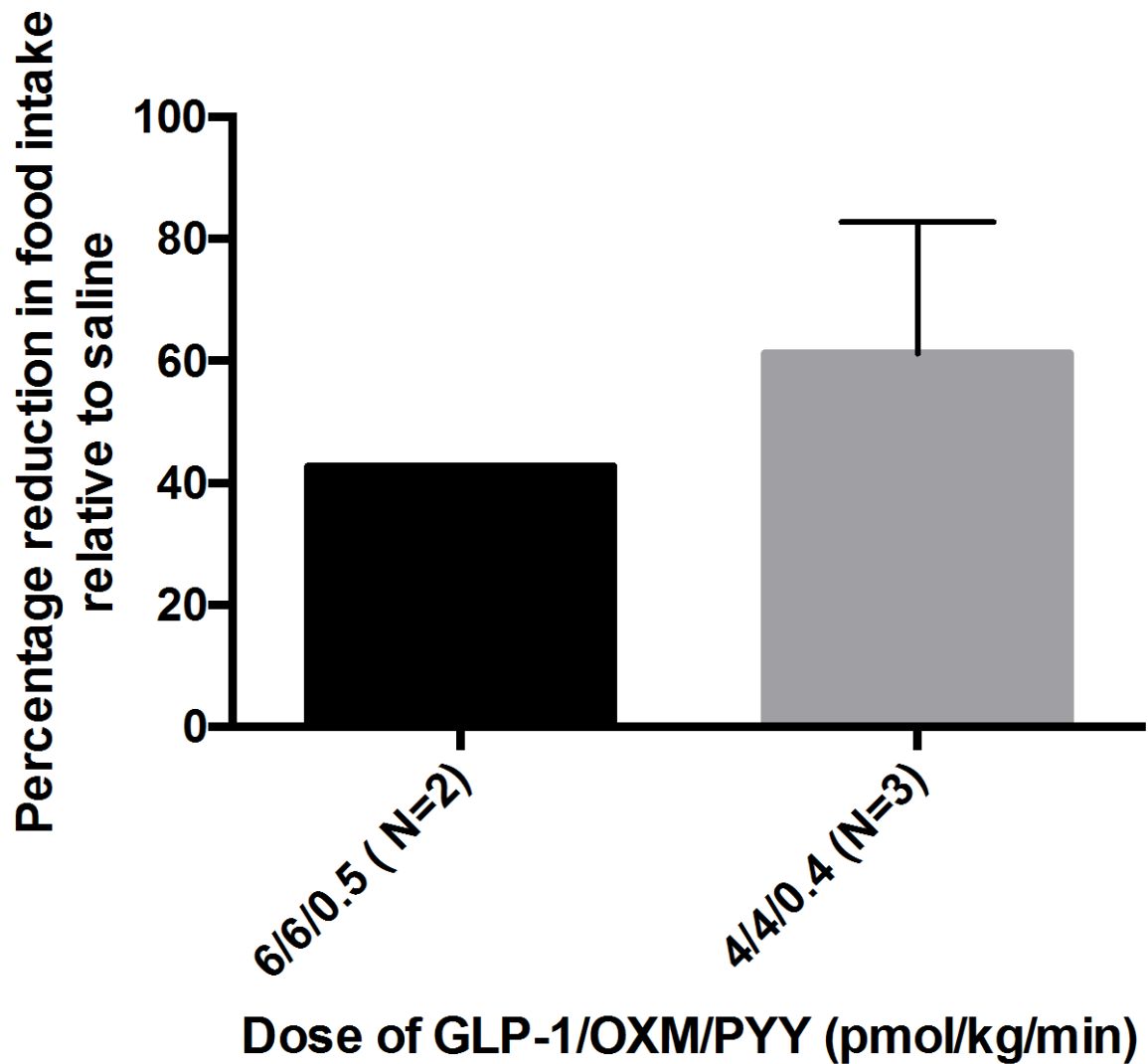
<b>Dose of GLP-1/OXM (pmol/kg/min)</b>	<b>Number of subjects</b>	<b>Number of subjects with nausea</b>
12/12	3	0
15/20	5	4

**Table 3.2 Effect of different doses of combined subcutaneous infusions of GLP-1 and OXM on nausea.** Volunteers received co-infusions of GLP-1 and OXM for a duration of eight hours. Nausea was either recorded on VAS score sheets at specific time points reported to the investigator if occurring between time points.

### **3.4.1.3 Investigation of the acute effects of subcutaneous co-administration of GLP-1, OXM and PYY on food intake and nausea.**

The final dose finding phase aimed to identify a tolerable dose of each peptide when co-infused together. Whilst co-infusion of GLP-1 and OXM (each at 12 pmol/kg/min) was well tolerated, both doses were reduced to 6 pmol/kg/min before addition of PYY. The rationale for this was that the experience from moving to single to combination infusions had demonstrated that nausea was induced at lower doses when multiple hormones were co-infused.

The first trial dose for triple peptide infusion was 6, 6 and 0.5 pmol/kg/min of GLP-1, OXM and PYY respectively. The effects of this combination on food intake and nausea are shown in Figure 3.4 and Table 3.3. Since administration of this combination at these doses produced nausea, subsequent peptide doses were reduced to 4, 4 and 0.4 pmol/kg/min respectively. This combination of doses reduced food intake but produced less nausea.



**Figure 3.4 Effect of subcutaneous co-administration of GLP-1, OXM and PYY at varying doses on food intake.** Volunteers received a combined infusions of GLP-1, OXM and PYY infusion or saline. The infusion duration was eight hours. An *ad-libitum* meal was given four hours after the infusion was started. The number of volunteers who received each infusion is shown in brackets.

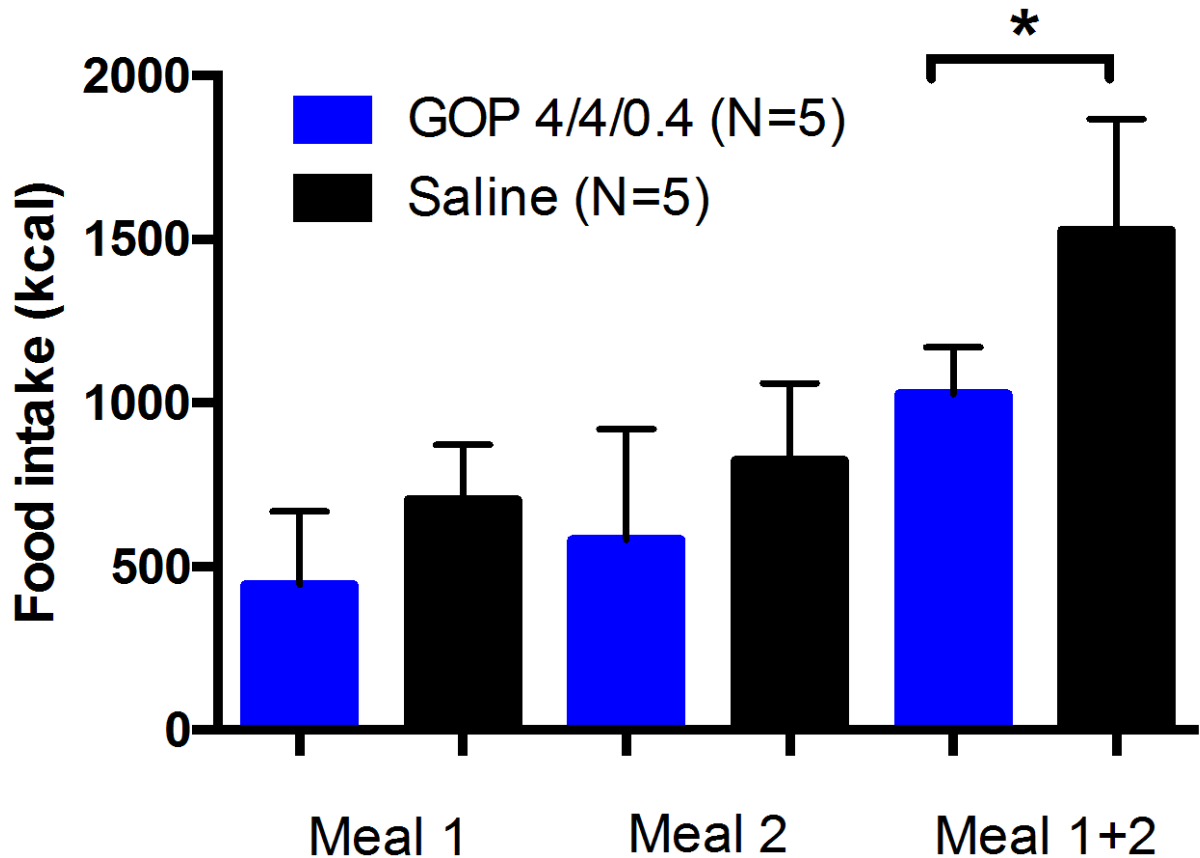
<b>Dose of GLP-1/OXM/PYY (pmol/kg/min)</b>	<b>Number of subjects</b>	<b>Number of subjects with nausea</b>
6/6/0.5	2	2
4/4/0.4	3	0

**Table 3.3 Effect of subcutaneous co-infusions of GLP-1, OXM and PYY of varying doses on nausea.** Volunteers received co-infusions of GLP-, OXM and PYY at varying doses for a duration of eight hours. Nausea was either recorded on VAS score sheets at specific time points or reported to the investigator if occurring between time points.

### **3.4.2 Investigation of the acute effects of subcutaneous co-administration of GLP-1, OXM and PYY on food intake and nausea.**

Co-administration of GLP-1, OXM and PYY resulted in a significant reduction in mean total food intake (meal 1 and meal 2) relative to saline (mean total food intake  $1027 \pm 145$  [GLP-1/OXM/PYY] vs  $1528 \pm 152$  kcal [saline]  $P < 0.05$ , Figure 3.5).



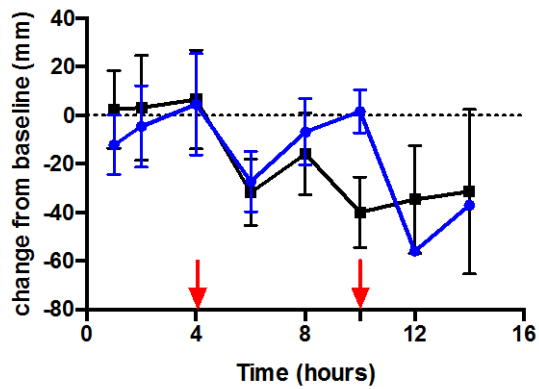


**Figure 3.5 Energy intake following ad-libitum meal in human subjects.** Participants were given a meal of known quantity and calorie content during an infusion of saline or GLP-1, OXM and PYY (GOP) (4/4/0.4 pmol/kg/min). Data were analysed using a paired t test. \*  $P < 0.05$ .  $N = 5$ . Meals 1 and 2 were *ad-libitum* meals, was given four and eight hours after the infusion was started.

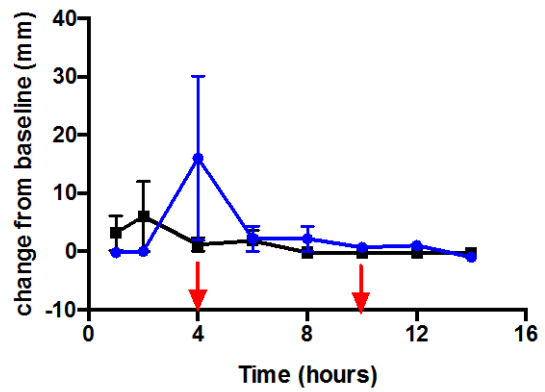
### **3.4.2.2 Investigation of the acute effects of subcutaneous co-administration of GLP-1, OXM and PYY on nausea and satiety.**

During the GLP-1, OXM and PYY infusions, one volunteer developed nausea four hours after the infusion was started. No other volunteer receiving the co-infusion of peptides had nausea. There was no significant difference in VAS scores between those receiving co-infusion of GLP-1, OXM and PYY relative to saline (Figure 3.6).

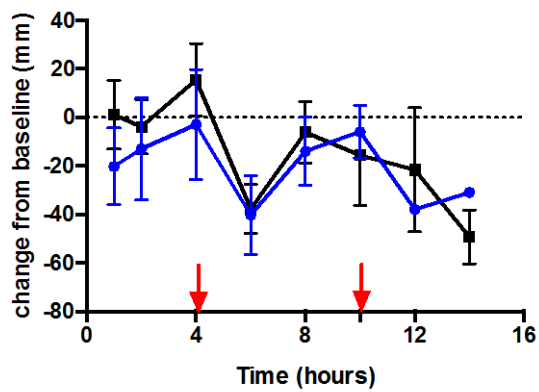
**A** How hungry do you feel right now?



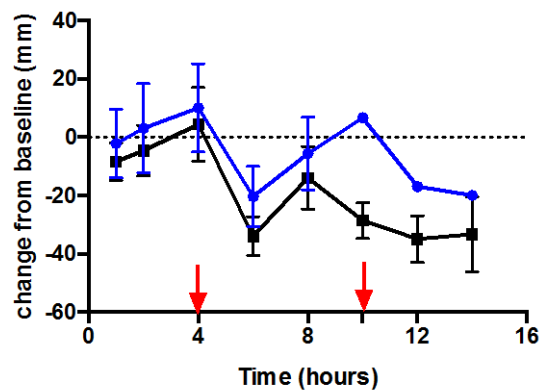
**B** How sick do you feel right now?



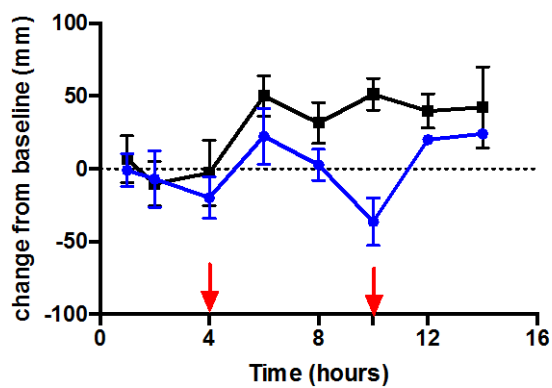
**C** How pleasant would it be to eat right now?



**D** How much could you eat right now?



**E** How full do you feel right now?



● GOP 4/4/0.4 (N=5)  
■ Saline (N=5)  
↓ Ad-libitum meal

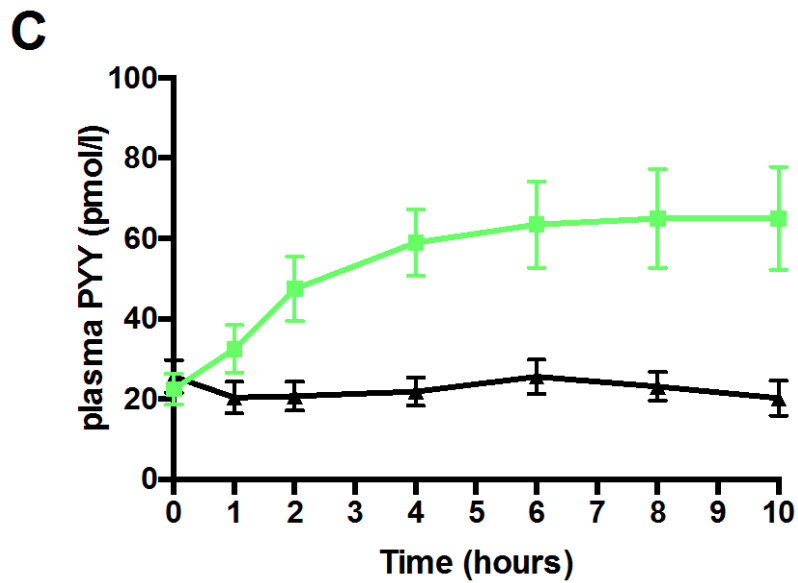
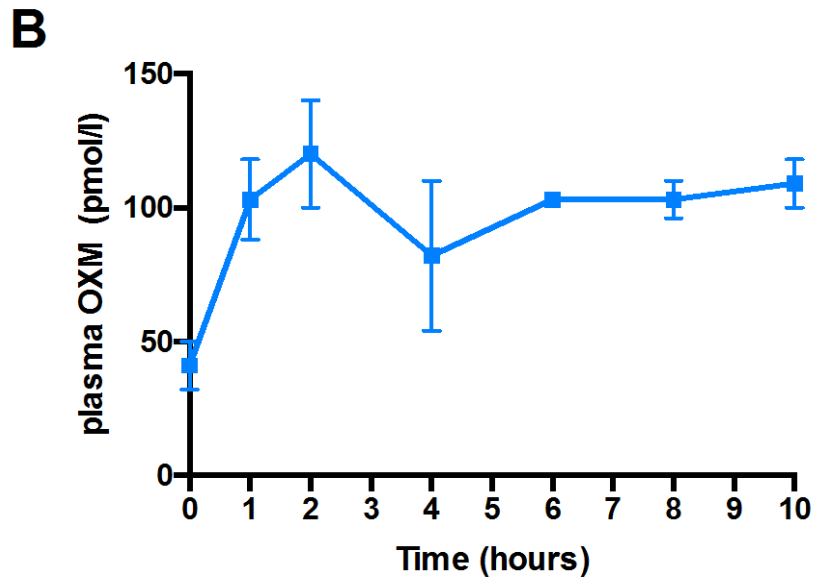
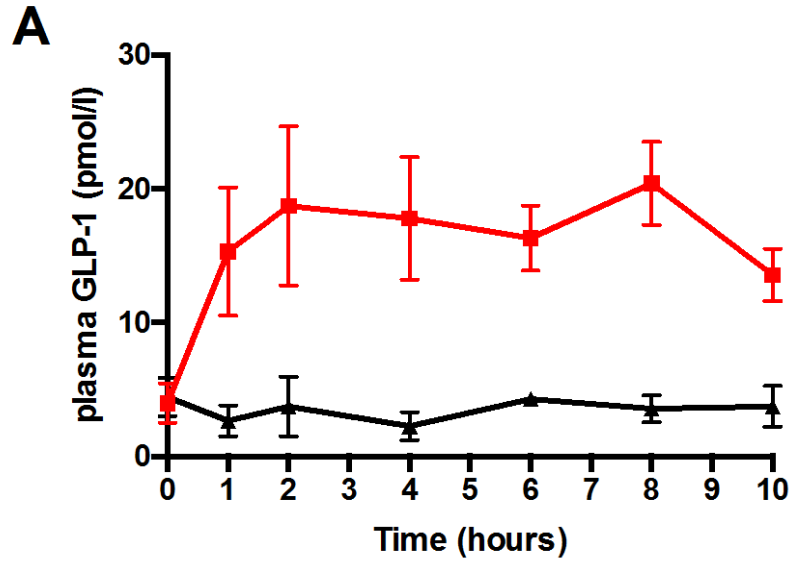
**Figure 3.6 Change from baseline in visual analogue scale (VAS) scores in subjects.** VAS scores were measured at each time point in response to the following questions: (A) ‘How hungry do you feel right now?’, (B) ‘How sick do you feel right now?’, (C) ‘How pleasant would it be to eat right now?’, (D) ‘How much do you think you could eat right now?’, (E) ‘How full do you feel right now?’ and (F) ‘How tasty was the meal?’. Volunteers received either an infusion of GLP-1, OXM and PYY ( 4/4/0.4 pmol/kg/min) or saline for 14 hours minutes. Data were analysed with two-way repeated measures ANOVA with Bonferroni post test (A-E). Abbreviations: GLP-1, OXM and PYY (GOP).

### **3.4.2.3 Investigation of the gut hormone levels following an acute subcutaneous co-administration of GLP-1, OXM and PYY.**

Prior to infusion, mean plasma concentration of GLP-1 was  $4.0 \pm 1.5$  pmol/l (Figure 7). Two hours after the infusion was started, a peak plasma GLP-1 concentration of  $18.7 \pm 6.0$  pmol/l was achieved. There was no significant rise in the concentration of GLP-1 in the volunteers when infused with saline.

Mean fasting concentration of OXM was  $41.0 \pm 9.0$  pmol/l. This rose to a mean peak concentration of  $120.0 \pm 20.0$  pmol/l, two hours after the combined infusion was initiated. Samples taken from subjects receiving saline were not measured due to the limited amount of samples I was able to send to my collaborators.

Before the combined infusion was started, the mean basal concentration of PYY was  $22.4 \pm 3.8$  pmol/l. Mean peak concentration was achieved six hours after the start of the infusion at  $56.2 \pm 1.3$  pmol/l. There was no significant rise in the concentration of PYY in the volunteers when infused with saline.



**Figure 3.7**

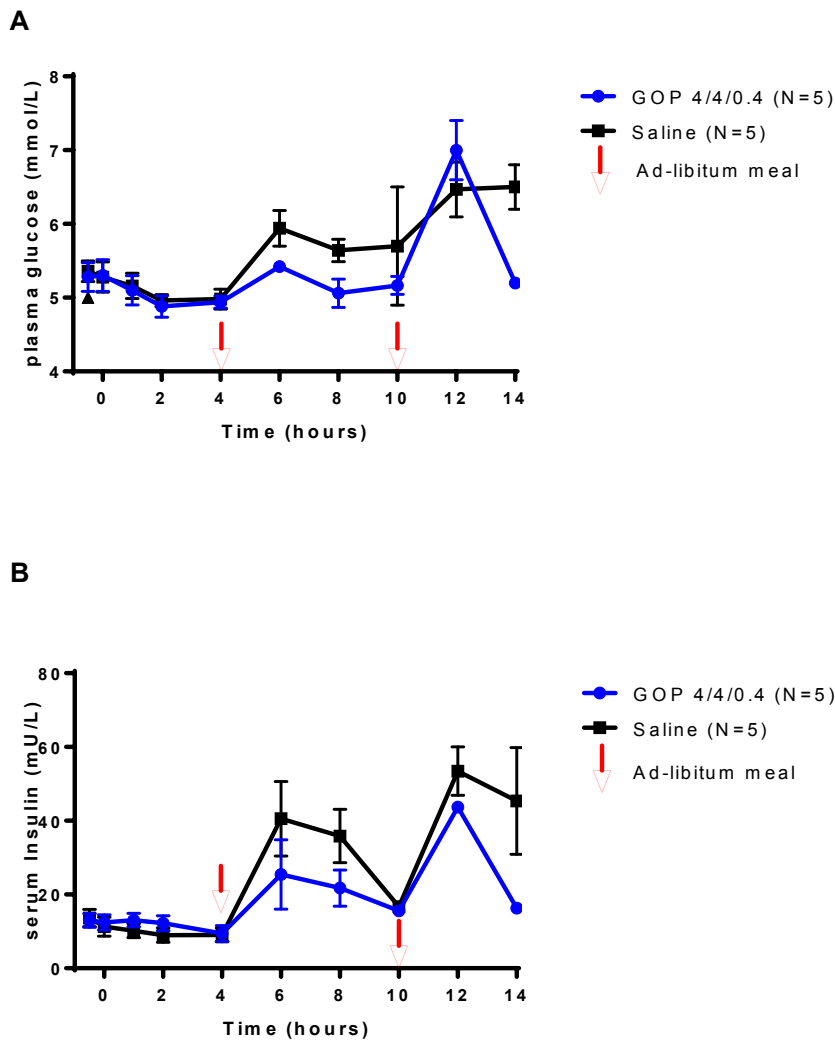
**Mean plasma concentration of GLP-1 (A), OXM (B) and PYY (CC) during infusion of GLP-1, OXM and PYY.** Subjects were infused with GLP-1, OXM and PYY for 14 hours. Samples for hormone measurement were taken at the times indicated. N = 5

### **3.4.2.3 Investigation of the acute effects of subcutaneous co-administration of GLP-1, OXM and PYY on glucose and insulin.**

The mean baseline plasma glucose in all subjects was  $5.3 \pm 0.1$  mmol/l prior to infusion (Figure 3.8). Mean plasma glucose concentration rose after both *ad-libitum* meals. However, there was no significant difference in plasma glucose concentration in subjects receiving co-infusion of GLP-1, OXM and PYY (4/4/0.4 pmol/kg/min) relative to saline throughout the duration of 14 hour infusion.

Mean baseline serum insulin concentration for all subjects was  $11.9 \pm 1.4$  mIU/L in all subjects prior to infusion. Insulin concentration increased in response to the elevation in post-prandial plasma glucose but there was no significant difference between the two infusion groups

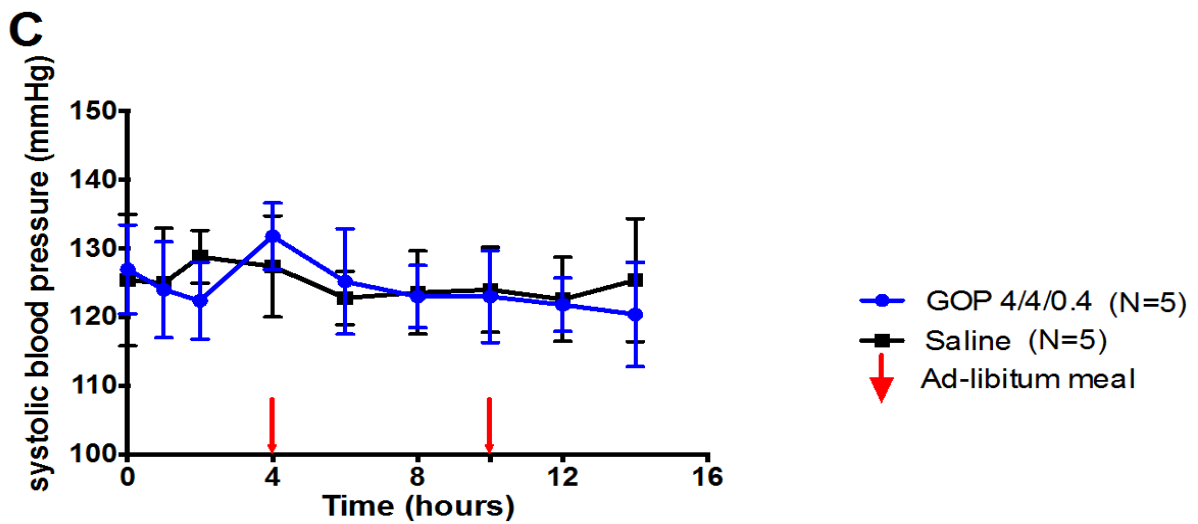
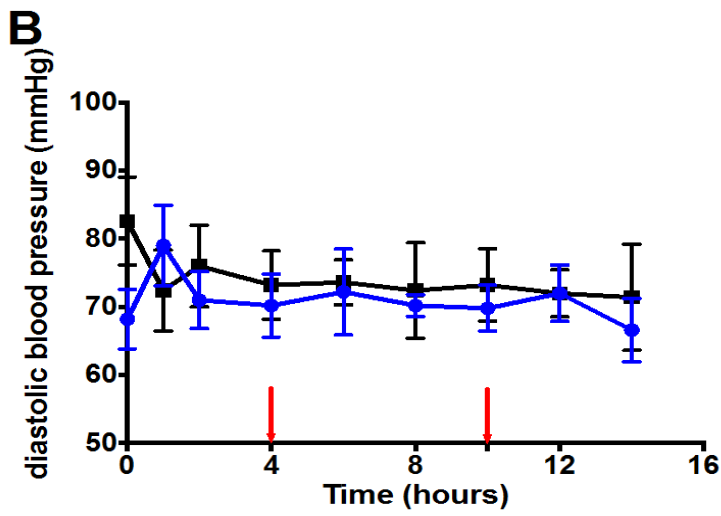
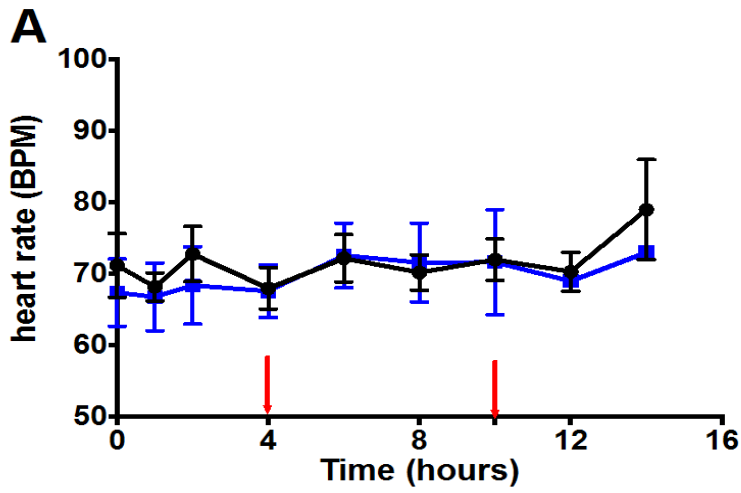




**Figure 3.8 Comparison of changes in glucose (A) and insulin (B) following co-infusion of GLP-1, OXM and PYY.** Volunteers received a combined infusion of either GLP-1, OXM and PYY (GOP) (4/4/0.4 pmol/kg/min) or saline. Glucose and insulin samples were collected at the time points indicated and two *ad-libitum* meals were consumed at the four and eight hours after infusion was started. Data was analysed using two-way ANOVA with Bonferroni *post hoc* test. Number of volunteers in each group shown in brackets.

#### **3.4.2.4 Investigation of the acute effects of subcutaneous co-administration of GLP-1, OXM and PYY on cardiovascular parameters.**

There was no significant difference in pulse rate or systolic and diastolic blood pressure in subjects receiving co-infusion of GLP-1, OXM and PYY relative to saline (Figure 3.9).



**Figure 3.9 Changes in pulse rate (A), systolic (B) and diastolic (C) blood pressure following co-infusion of GLP-1, OXM and PYY relative to saline.** Subjects were infused over 14 hours a co-infusion of GLP-1, OXM and PYY (4/4/0.4pmol/kg/min) or saline or Data were analysed using two-way ANOVA with Bonferroni multiple *post hoc* test. Number of subjects in each group shown in brackets.

### 3.5 Discussion

This study demonstrates that co-infusion of GLP-1, OXM and PYY can significantly reduce food intake. This data is novel for two reasons. Firstly, it is the first study to investigate the co-infusion of these three gut hormones. Secondly, whilst there are data on the use of a continuous SC pump to deliver GLP-1, this route of administration has not studied previously with PYY or OXM(223). The anorectic effect demonstrated supports the further development of delivering these gut hormones in combination as a treatment for obesity in humans.

Co-infusion of GLP-1, OXM and PYY resulted in a mean reduction in total energy intake of  $501 \pm 128$  kcal. This is equivalent to a 32.8% reduction in daily food intake. This reduction in food intake is comparable to 34% reduction in calorie intake observed in RYGB patients 2 years post surgery(224). If future work demonstrates that the reduction in food intake with co-infusion of GLP-1, OXM and PYY is sustained, this could provide an alternative treatment for obesity to surgery.

This is the first study in man to infuse three gut hormones together to study the effects on food intake. Others have previously co-infused two gut hormones. Field *et al* used a combination of OXM and PYY whilst Neary *et al* co-infused

GLP-1 and PYY(204,218). These studies demonstrated reductions in energy intake of 43 and 27% respectively but neither is directly comparable to the current investigation due to differences in both the route of administration as well as the doses used. Both previous studies demonstrated that the co-infusion was additive in its anorectic effect, by comparing the co-infusion to single hormone infusion. The data from this study cannot provide further insight into the additive anorectic as single infusions were not employed beyond the dose finding stage. However, during the dose finding period, it was necessary to reduce the doses of gut hormones administered each time a new gut hormone was added to the co-infusion due to nausea. This suggests that the effects of the triple hormone co-infusion may be additive but further controlled studies will need to be performed to confirm this.

Only one of the five subjects experienced nausea during the triple hormone infusion. However, if the cohort size was larger, there may have been more nausea observed. A future larger study would be able to address this further.

Whilst the primary outcome of this study was been food intake, an alternative mechanism to achieve weight loss is through increasing energy expenditure. It has been shown that specific gut hormones such as glucagon and OXM can induce an increase in energy expenditure(49,137). An increase in energy expenditure is also observed after RYGB surgery which may be related to the

increased concentration of OXM and glucagon post surgery(225). Determining whether a co-infusion containing OXM can also increase energy expenditure is an important step in developing it as an anti-obesity therapeutic.

There are specific limitations to this study that should be noted. Only a small cohort of five patients was used and the study was only single-blinded. Furthermore whilst the duration of the infusion was longer than in most studies investigating the effects of gut hormones on food intake (mean duration was 60 minutes), it would be useful to assess whether the anorectic effect is maintained beyond 24 hours(102). A final limitation was the absence of oxyntomodulin concentrations in those receiving saline.

In conclusion, this study has shown that a subcutaneous combined infusion of GLP-1, OXM and PYY can reduce food intake in healthy obese human volunteers. This is an important step in the development of combination gut hormones as anti obesity treatments. Future work should evaluate the effects of a triple hormone infusion on energy expenditure, and determine whether the observed energy intake reduction persists when administered over the longer term.

## **Chapter 4**

# **The role of gut hormones in the pathophysiology of postprandial hypoglycaemia**



## 4.1 Introduction

In Chapters 2 and 3 I have demonstrated that administration of gut hormones can result in improvements in carbohydrate tolerance and a reduction in food intake. This data supports the role that the elevated postprandial concentration of gut hormones post RYGB may contribute to the improvement in weight and diabetes that occur after the operation. Furthermore, it supports the use of gut hormones as a potential treatment for obesity and diabetes. However, if gut hormones are responsible for the metabolic benefits after RYGB they may also account for some of the metabolic complications that occur too.

Laparoscopic RYGB is considered a relatively safe surgical procedure with a morbidity and mortality of 0.16% which is similar to low risk surgery such as laparoscopic cholecystectomy(226). However, as the operation is becoming more frequently performed to match the rise in obesity prevalence, unusual metabolic complications are becoming more recognised. These include nutritional deficiencies such as pernicious anaemia which is likely related to the reduction in gastric size and other conditions such as a loss of bone mass that at present has no clear pathogenic mechanism(227,228). PPH, in which patients develop low glucose concentrations after eating is another metabolic complication of RYGB that has implicated gut hormones as a potential cause(184).

PPH is considered a relatively new phenomenon having first been described by Service *et al* in 2005 in 6 patients(187). However, there have been case reports and series of PPH occurring after partial gastrectomies (for gastric ulcer disease) that have been reported since the 1930s(229). Whilst the bariatric and dyspeptic operations differ in technique, the alteration in postprandial glucose profile is common in both and a similar pathophysiology is likely.

PPH may reflect an extreme instance of the changes in glucose homeostasis that occurs after RYGB. After surgery there is a dramatic alteration in glucose tolerance(230). Beyond a reduction in fasting and postprandial plasma glucose levels, there is also a change in the glucose tolerance curve. Instead of a steady rise in plasma glucose that plateaus before returning to baseline, there is a sharp rise with a peak glucose at 30 minutes with a subsequent rapid drop(230). In patients with PPH the rapid decrease in glucose concentrations can result in hypoglycaemia. It remains unclear why certain patients post RYGB develop PPH whilst others do not.

The rapid oscillations in postprandial glucose demonstrate the significant increase in glucose variability that occurs post RYGB. This increase in glycaemic variability after RYGB has been documented with the use of continuous glucose monitoring, relative to obese subjects who have not had RYGB surgery(231). Increased glycaemic variability is not exclusive to patients

who have diabetes prior to surgery but also occurs in patients with no prior history of diabetes. It is unclear if RYGB patients with PPH have increased glycaemic variability relative to RYGB patients who do not.

Whilst the cause behind PPH remains unclear, it has been established that the hypoglycaemia is hyperinsulinemic in nature(232). Post RYGB there is a rapid postprandial rise in insulin but the mechanism underlying the increased insulin secretion remains unknown(233). Previous work has focused on incretins (insulinotropic hormones that are secreted in response to an oral glucose load) and in particular GLP-1. Some studies have shown elevated postprandial GLP-1 levels in patients with PPH relative to asymptomatic patients whilst others have failed to confirm this(184,234). Furthermore, both GLP-1 antagonists and agonists have been shown to improve PPH(191,235). As such, the role of gut hormones including GLP-1 in the pathophysiology of PPH remains unclear and requires further investigation.

## **4.2 Hypothesis and Aims**

### **4.2.1 Hypothesis**

I hypothesise that patients with PPH after RYGB have increased glycaemic variability and that this is associated with changes in levels of gut hormones.

### **4.2.2 Aims**

My aims were to investigate a cohort of patients with PPH:

1. To measure their glycaemic variability using continuous glucose monitoring.
2. To measure the secretion of postprandial gut hormones.
3. To examine the relationship of postprandial gut hormone secretion to the type of meal stimulus.

## **4.3 Methods**

### **4.3.1 Subjects**

Subjects were recruited from the Imperial Weight Centre who were referred with symptoms suggestive of PPH. For inclusion to the study patients must have either autonomic (this includes symptoms such as sweaty, palpitations tremor) or neuroglycopenic symptoms (such as drowsiness, pre-syncope, or loss of consciousness). These symptoms had to occur within five hours after eating and resolved with carbohydrate ingestion. Exclusion criteria were any current medication known to affect glucose metabolism or a history of symptoms that predated the operation.

The study was done as part of routine clinical care for patients suspected to have PPH and as such no ethical approval was required.

Previous studies that have investigated PPH have compared bariatric surgery patients who are known to have symptoms of PPH with a group of asymptomatic patients(236,237). However, in many of these studies, it is apparent that during the provocation test used in the studies not all the symptomatic group had a hypoglycaemia and at the same time, some of the asymptomatic group appear to have a biochemical hypoglycaemia without symptoms. This illustrates two important observations. First, in the RYGB

cohort, there is a high prevalence of asymptomatic hypoglycaemia. Secondly, in those patients who have previously shown to have PPH, the reproduction of this condition appears variable.

If a patient who has previously been demonstrated to have PPH undergoes a provocation test but does not experience biochemical hypoglycaemia, it is possible that the hormonal changes that may cause PPH are not present during that study. As the aim of the current study was to determine if gut hormones are associated with biochemical hypoglycaemia, subjects were subdivided into those that had biochemical hypoglycaemia during a given provocation test and their gut hormone response to a provocation test was compared to those that did not experience hypoglycaemia .

### **4.3.2 Continuous Glucose Monitoring**

Two types of continuous glucose monitor (CGM) were used.

1. Medtronic iPro®2
2. Abbott Navigator® II

The Medtronic iPro®2 CGM was the initial choice for continuous glucose monitoring but because of a high sensor failure rate, subsequently the Navigator II®2 CGM was used.

Subjects were asked to wear a CGM for 5 days. Both the Medtronic iPro®2 and Abbott Navigator® II comprise a light-weight memory storage unit which is attached to a sensor. Using a spring-loaded device, the sensor is placed in the subcutaneous layer of tissue of the abdomen where it measures glucose concentrations in interstitial fluid. Both sensors measure interstitial glucose using an amperometric enzyme electrode based on the glucose oxidase reduction-oxidation reaction. The Medtronic iPro®2 measures glucose at 5 minute intervals whilst the Abbott Navigator® II has a 10 minute interval.

For both CGMs, subjects were asked to measure their capillary blood glucose (CBG) using the finger prick method, in order to calibrate their machine. They were provided with Abbott Freestyle Lite® blood strips for this purpose. For the Medtronic iPro®2, subjects were asked to perform a minimum of 3 CBG measurements per day. For the Abbott Navigator® II, subjects were asked to perform CBG at 1, 2, 10, 24 and 72 hours after the sensor was inserted.

After five days of wearing the sensor, the subjects returned the CGM storage unit. The data was uploaded onto internet-based platforms (Carelink® and Freestyle Copilot® for iPro®2 and Navigator®II respectively), which use proprietary algorithms to relate capillary glucose measurements to subcutaneous interstitial fluid glucose measurements.

The recorded data were analysed for mean glucose and glycaemic variability measures including Mean Amplitude Glycaemic Excursions (MAGE), Continuous Overlapping Net Glycaemic Action (CONGA), Mean of Daily Differences (MODD) and risk indices such as Low Blood Glucose Index (LBGI), High Blood Glucose Index (HBGI) and Average Daily Risk Ratio (ADRR). These values were calculated using the easyGV 9.0 software(238). The equations used to calculate these measures of glycaemic variability are shown in Table 4.1.



Variability measure	Formula	Discriminating feature
MAGE	$\sum \frac{\lambda}{x} \text{ if } \lambda > v$	Used most extensively
CONGA	$\sqrt{\frac{\sum_{t=t_1}^{t_k} (D_t - \bar{D})^2}{k-1}}$ $\bar{D} = \frac{\sum_{t=t_1}^{t_k} D_t}{k} \quad D_t = G_t - G_{t-m}$	Specifically developed for CGM
MODD	$\frac{\sum_{t=t_1}^{t_k}  GR_t - GR_{t-1440} }{k^*}$	Assess intra-day variability
LBGI	$\frac{1}{N} \sum_{i=1}^N rl(x_i)$	Gives a risk score for hypoglycaemia
HBGI	$\frac{1}{N} \sum_{j=1}^N rh(x_j)$	Gives a risk for hyperglycaemia
ADDR	$\frac{1}{N} \sum_{t=1}^N [LR + HR]$	Gives a measure of glycaemic control

**Table 4.1 Measures of glycaemic variability.** MAGE=mean amplitude glucose excursion; CONGA=continuous overlapping net glycaemic action; MODD=mean of daily differences, HBGI= high blood glucose index; LBGI=low blood glucose index, ADDR =average daily risk.  $\lambda$  = blood glucose changes from peak to nadir  $\chi$  = number of observation  $v$  = 1 standard deviation of mean glucose for a 24 hour period  $\kappa$  = total number of observations  $rl$  = risk value associated with a low glucose (if  $\chi < 0$ ),  $rh$ = risk value associated with high glucose (if  $\chi > 0$ ),  $\chi_I$  = nonlinear transformation of glucose measured, LR = risk value attributed to low glucose HR = risk value attributed to high glucose. Adapted from Siegelaar *et al.* (239)

The normal reference values for a non diabetic population are shown in Table 4.2.

	Lower limit	Upper limit
<b>MAGE</b>	0.0	2.8
<b>CONGA</b>	3.6	5.5
<b>MODD</b>	0.0	3.5
<b>LBGI</b>	0.0	6.9
<b>HBGI</b>	0.0	7.7
<b>ADRR</b>	0.0	8.7

**Table 4.2 Normal reference range for measures of glycemic variability.** MAGE=mean amplitude glucose excursion; CONGA=continuous overlapping net glycaemic action; MODD=mean of daily differences, HBGI= high blood glucose index; LBGI=low blood glucose index, ADRR =average daily risk ratio. Adapted from Nathan Hill *et al*(240)

### 4.3.3 Mixed Meal Test (MMT) Study

Patients arrived at the planned investigation unit at 0830h having fasted from 2200h the night before. Upon arrival, subjects had a peripheral venous cannulae inserted for the purpose of blood sampling. At 0900h subjects were given an Ensure Plus® (13.8 g of protein, 10.8 g of fat, 44.4 g of carbohydrates, 330 kcal, 220 ml, Abbott, Maidenhead, UK) to consume. They were advised to consume the Ensure Plus® within 10 minutes and were observed by the investigator to confirm complete consumption. Blood sampling for glucose, insulin, C-peptide and gut hormones were taken at the following time points, 0, 30, 60, 90, 120, 150, 180, 210 and 240 minutes. Vacutainer tubes (International Scientific Supplies Ltd, Bradford, UK) containing clot activators and fluoride oxalate tubes were used to collect insulin and glucose samples respectively. For the purpose of collecting gut hormones, lithium heparin-coated BD Vacutainer

tubes were prepared with the addition of 1000 kallikrein inhibitor units (0.1ml) (Trasylol, Bayer Schering Pharma, Berlin, Germany). Samples were stored on ice until centrifugation (4°C, 4000 rpm, 10 minutes) after which, plasma was separated immediately and stored at -20°C until analysis. In addition, a CBG sample was taken if subjects complained of symptoms of hypoglycemia. Blood pressure and pulse were also measured at those time points. The study was stopped if subjects developed neurological symptoms of hypoglycemia and had a capillary blood glucose less than 2.0 mmol/l. Biochemical hypoglycemia was defined as a venous plasma glucose <3.0 mmol/l in keeping with the consensus guidelines on hypoglycemia (241).

#### **4.3.4 Prolonged Oral Glucose Tolerance Test (POGTT)**

The protocol for the prolonged oral glucose tolerance test was identical to the mixed meal test aside from the use of an oral 75 g glucose load in substitution for the Ensure Plus®. The oral glucose challenge was prepared by dissolving 75 g of anhydrous glucose in 500 ml of water.

#### **4.3.5 Glucose, Insulin and C-peptide assays**

Glucose, insulin and C-peptide samples were measured by the Department of Chemical Pathology, Imperial College Healthcare National Health Service Trust. Plasma glucose was assayed using an Abbott Architect automated

analyzer (Maidenhead, UK), utilizing a hexokinase-glucose-6-phosphate dehydrogenase method. The analytical range was 0.278–44.4 mmol/L, with an intra-assay coefficient of variation of 0.65–1.98% and an inter-assay coefficient of variation of 0.84–0.93%. Serum insulin levels were measured on an Abbott Architect platform using Abbott reagents with an inter-assay coefficient of variation of 10%.

#### **4.3.6 Gut Hormone assays**

Plasma active GLP-1 (GLP-17–36), total PYY (PYY3–36 + PYY1–36), GIP and Glucagon were measured using the Milliplex MAP magnetic bead-based multi-analyte, metabolic panel, 4-plex immunoassay (HMHMAG-34K; Millipore, St. Charles, MO, USA) and Luminex Magpix with xPONENT software (Luminex Corporation, Austin, TX, USA). The manufacture protocol was used (Appendix B). The cross reactivity between the antibody for glucagon and oxyntomodulin is 0.7%. The kit is a capture sandwich immunoassay in which a capture antibody is attached to a microsphere and a detection antibody that incorporates a fluorescent label.

#### **4.3.7 Statistical Analysis**

Two-way repeated measures ANOVA with Bonferroni *post hoc* test was used to compare differences in glucose, insulin, active GLP-1, GIP, PYY and glucagon

at different time points. One-way ANOVA with Tukey *post hoc* test was used to compare differences in AUCs when more than two groups were being compared. Unpaired Student t-tests were used to compare differences between groups when only two groups were being compared. When data was not normally distributed (assessed by D'Agostino-Pearson omnibus normality test) due to small number, a Mann-Whitney test was used. Results are presented as mean  $\pm$  SEM and statistical significance defined as  $P < 0.05$ .

## **4.4 Results**

### **4.4.1 Demographics of subjects**

The demographics of the subjects recruited for the study are shown below in Table 4.3. Sixteen subjects who had symptoms suggestive of PPH after RYGB were classified as symptomatic RYGB. Two control groups were also analysed; a group of obese patients who had not had RYGB surgery (obese no-RYGB) and a group consisting of RYGB patients who had not had symptoms of PPH (asymptomatic RYGB). Further details on the volunteers with PPH are shown in Table 4.4.

	<b>Symptomatic RYGB</b>	<b>Obese-No RYGB</b>	<b>Asymptomatic RYGB</b>
<b>Number of subjects</b>	16	9	5
<b>Gender</b>	9F 7M	7F 2M	4F 1M
<b>Age (years)</b>	48.2±2.5	43.6±3.9	41.8±3.6
<b>HbA1c (mmol/l)</b>	38.4±1.2	39.8±1.7	37±2.2
<b>Years since surgery (years)</b>	2.5±1.4		2.6±0.7
<b>Presurgical weight (kg)</b>	127.3±5.7		134.7±4.8
<b>Percentage weight loss at 1 year(%)</b>	30.8±7.9		29.8±6.9

**Table 4.3 Demographics of subjects in study. F=Female, M=Male.**

<b>Patient No.</b>	<b>Year of Surgery</b>	<b>Type of Surgery</b>	<b>Age (years)</b>	<b>Diabetes pre surgery</b>	<b>Presurgical weight (Kg)</b>	<b>Percentage weight loss at 1 year (%)</b>	<b>Onset of symptoms from surgery (years)</b>
1	2010	RYGB	50	Yes	117	32	2
2	2010	RYGB	52	No	130	37	1
3	2007	RYGB	44	Yes	126	41	3
4	2007	RYGB	26	No	151	27	3
5	2010	RYGB	44	Yes	131	33	2
6	2010	RYGB	60	No	118	28	1
7	2006	Oesophagectomy and Roux-en-Y anastomosis	46	No	90	20	4
8	2011	RYGB	52	Yes	127	35	2
9	2010	RYGB	51	yes	98	38	1
10	2005	Gastrectomy for peptic ulcer disease	49	No	101	26	7
11	2012	RYBB	46	yes	118	29	1
12	2012	RYGB	49	No	167	31	1
13	2010	RYGB	71	Yes	121	36	3
14	2011	RYGB	43	Yes	153	43	2
15	2013	RYGB	33	No	168	25	1
16	2012	RYGB (conversion from sleeve)	55	No	121	12	2

**Table 4.4 Demographics of PPH subjects. 16 subjects who were suspected of having PPH were investigated in this study**

#### **4.4.1 Investigation into glycaemic variability in patients with PPH**

Seven subjects (patient numbers 2, 3, 5, 7, 9, 11 and 13 from table 4.4) who had symptoms of PPH post RYGB wore CGMs for five days. Their data was compared to three RYGB patients who were asymptomatic for PPH (Table 4.5) There were no significant differences in the presurgical weight or percentage weight loss between the two groups. There was no significant difference between the two groups CGM data, although the MAGE score approached significance.



<b>GV Measure</b>	<b>Asymptomatic (N=3)</b>	<b>Symptomatic (N=7)</b>	<b>P value</b>
<b>Mean (mmol/l)</b>	5.49±0.42	6.12±0.24	0.20
<b>SD</b>	1.78±0.57	2.04±0.23	0.61
<b>MAGE</b>	2.39±0.69	4.93±0.75	0.07
<b>CONGA</b>	4.76±0.50	5.05±0.19	0.52
<b>LBGI</b>	4.84±1.46	3.49±0.70	0.37
<b>HBGI</b>	4.61±3.29	4.36±2.72	0.93
<b>% time in hypoglycaemia &lt;3.0</b>	2.1±1.6	1.0±0.6	0.48
<b>% time in range, 3.0-7.0</b>	85.1±4.8	74.2±3.4	0.11
<b>% time in hyperglycemia &gt;7.0</b>	12.8±3.6	21.9±5.2	0.29

**Table 4.5 Comparison of measures of glycaemic variability between patients with PPH and those without.**

Differences in measures of glycaemic variability (GV). There were no significant differences in the methods used. SD=standard deviation; MAGE=mean amplitude glucose excursion; CONGA=continuous overlapping net glycaemic action; HBGI= high blood glucose index; LBGI=low blood glucose index.

#### **4.4.2. Comparison of changes in glucose and insulin following a Mixed Meal Test in patients suspected of having PPH**

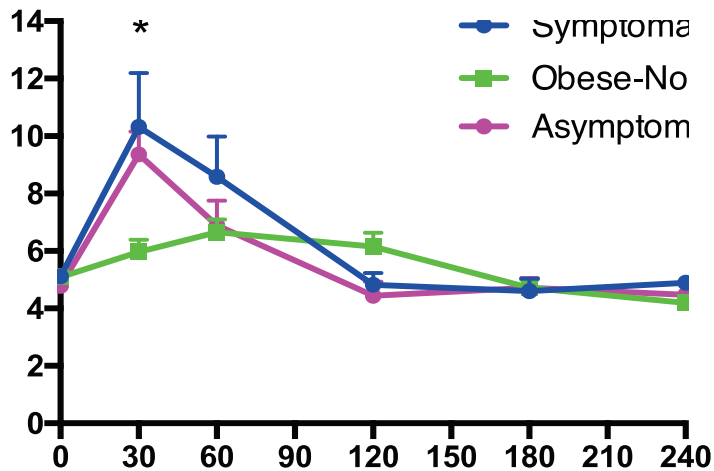
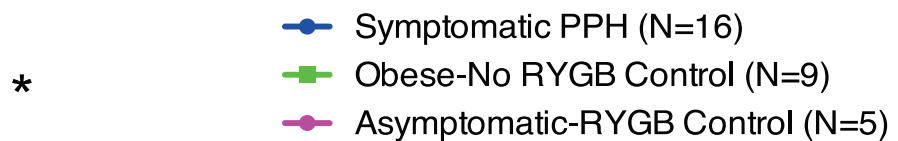
Thirty subjects underwent a MMT. The subjects were separated into three groups;

- 16 subjects with symptoms of PPH (Symptomatic RYGB)
- 5 subjects who had had RYGB surgery but had no symptoms of PPH (Asymptomatic RYGB)
- 9 subjects who were obese but had not had RYGB (Obese-No RYGB).

There was no significant difference in the fasting glucose concentration in all three groups (Figure 4.1A and Table 4.6). Thirty minutes following the consumption of the mixed meal, there was a significant difference in plasma glucose concentration between both surgical cohorts relative to Obese-No RYGB group (Glucose<sub>30</sub>: 9.4±0.8 [Symptomatic RYGB] vs 6.1±0.4 mmol/l [Obese-No RYGB], P<0.05; 10.3±1.9 [Asymptomatic RYGB] vs 6.1±0.4 mmol/l [Obese-No RYGB], P<0.05).

There was no significant difference in the concentration of fasting insulin between all three groups (Figure 4.1B and Table 4.6). Thirty minutes following the consumption of the mixed meal, there was a significant difference in serum insulin concentration between both surgical cohorts relative to Obese-No RYGB group (Insulin<sub>30</sub>: 130.2±24.7 [Symptomatic RYGB] vs 39.9±7.7 mU/l

[Obese-No RYGB],  $P < 0.05$ ;  $152.5 \pm 44.3$  [Asymptomatic RYGB] vs  $39.9 \pm 7.7$  mU/l [Obese-No RYGB],  $P < 0.05$ ). Both RYGB groups concentration of serum insulin returned to baseline at 120 minutes whilst the Obese-No RYGB group's serum insulin was elevated till 180 minutes.

**A****B**

**Figure 4.1 Changes in plasma glucose (A) and serum insulin (B) following a mixed meal in patients suspected to have PPH, a non- surgical obese group and an asymptomatic RYGB group.** Mixed meal was given at T=0. Numbers of subjects in each group shown in brackets. Significant differences are \*Suspected PPH vs Obese-No RYGB P<0.001, Asymptomatic-RYGB vs Obese-No RYGB P<0.001. \*\* Suspected PPH vs Obese-No RYGB P<0.001.

Outcome measures	Symptomatic RYGB (N=16)	Obese-No RYGB (N=9)	Asymptomatic RYGB (N=5)
Mean fasting glucose (mmol.l <sup>-1</sup> )	4.8±0.2	5.0±0.3	5.1±0.2
Mean peak glucose (mmol.l <sup>-1</sup> )	9.8 ± 0.7	7.1 ± 0.3	10.8 ± 1.5*
Mean nadir glucose (mmol.l <sup>-1</sup> )	3.4 ± 0.2	4.1 ± 0.1	4.3 ± 0.5
Mean glucose <sub>30</sub> (mmol.l <sup>-1</sup> )	9.4 ± 0.8*	6.0 ± 0.4	10.3 ± 1.9*
Mean time to peak glucose (min)	35.6 ± 3.0*	80.0 ± 13.2	42.0± 12.0*
Mean time to nadir glucose (min)	146.3 ± 20	220 ± 14.1	120.0±28.5
Mean AUC glucose (mmol.l <sup>-1</sup> .min)	1346.7±82.9	1333.3±57.4	1485±81.4
Fasting insulin (mU.l <sup>-1</sup> )	7.0±0.1	9.6±1.0	10.4±2.1
Mean peak insulin (mU.l <sup>-1</sup> )	139.0± 23.1	61.1 ± 9.1	178.4 ± 51.0*
Mean insulin <sub>30</sub> (mU.l <sup>-1</sup> )	130.2 ± 24.7*	39.9 ± 7.7	152.5 ± 44.3*
Mean time to peak insulin (min)	39.4 ± 4.5*	73.3±12.4	42.0 ± 7.3
Mean AUC insulin (mU.l <sup>-1</sup> .min)	8233.3±1004.4	6729.2±988.9	13004.6±4159.0

**Table 4.6 Comparison of measures of glucose homeostasis following a mixed meal in symptomatic RYGB group, a non- surgical obese group and an asymptomatic RYGB group.** Significant differences are \* vs Obese No RYGB, P < 0.05.

### **4.4.3 Investigation into changes in glucose and insulin in PPH patients: Hypo vs Non-Hypo groups**

Six of the 16 patients referred with symptoms suggestive of PPH had plasma glucose concentrations below 3.0 mmol/l following a MMT (Table 4.7). In order to further investigate what causes PPH in a post RYGB patient, the subjects data was categorised into those that had biochemical hypoglycaemia (MMT Hypo) and those that did not (MMT Non-hypo).

Glucose and insulin responses to the MMT are shown in Figure 4.2 and Table 4.8. There was no significant difference in baseline plasma glucose concentrations between those that did and did not have biochemical hypoglycaemia. As expected, the MMT Hypo group had a significantly lower nadir glucose relative to the MMT Non-hypo group (Nadir glucose  $2.39 \pm 0.19$  [MMT-Hypo group] vs  $3.40 \pm 0.16$  mmol/l [MMT-Non-Hypo group]). With reference to the glycaemic profile there was no other significant difference between the two groups.

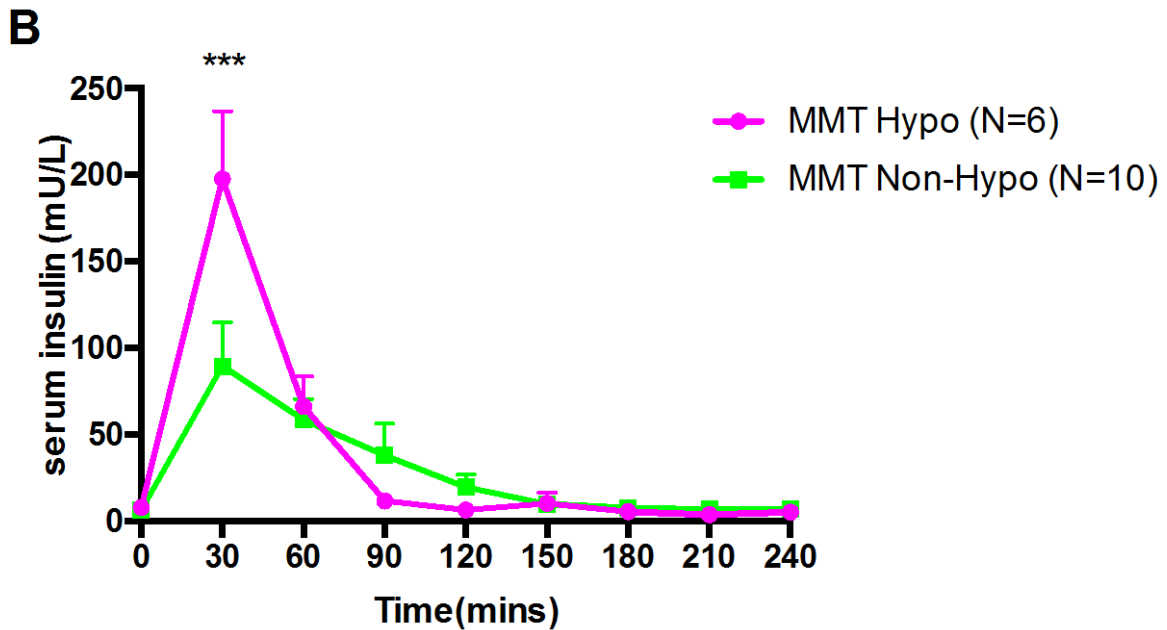
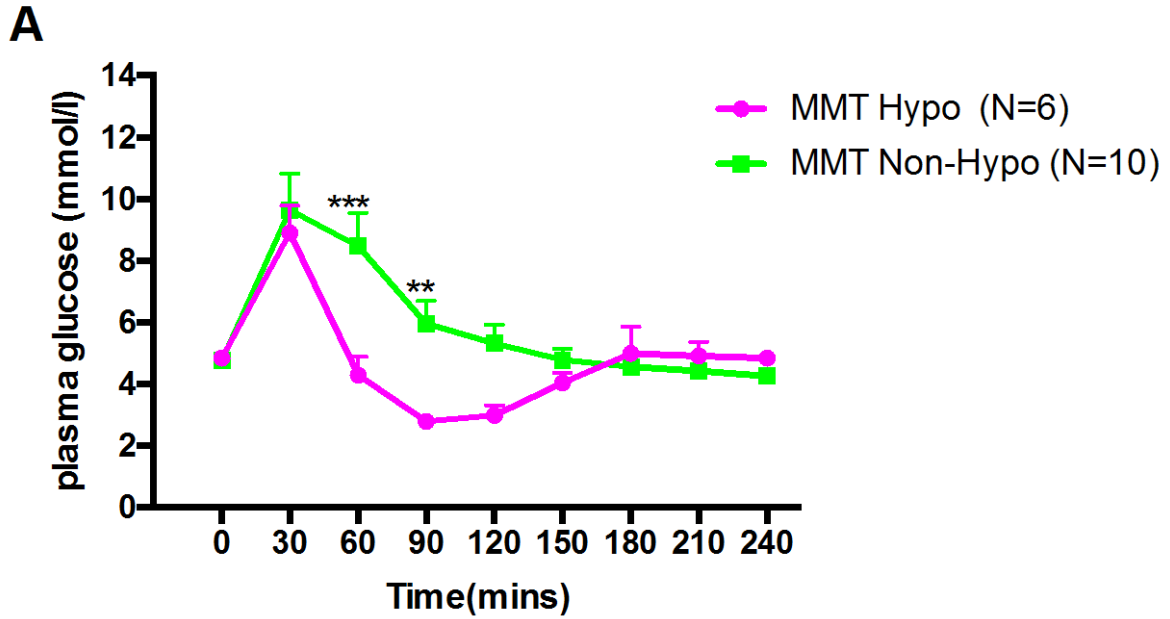
There was no significant difference in the baseline insulin concentration between the two groups. However, after consuming the mixed meal, the MMT Hypo group had a significantly higher mean peak serum insulin concentration

(Peak insulin  $197.8 \pm 39.2$  [MMT Hypo group] vs  $103.8 \pm 23.3$  [MMT Non-Hypo group]  $P < 0.05$ ).

Patient No.	MMT		POGTT	
	Hypo or Non-Hypo	Nadir glucose (mmol/l)	Hypo or Non-Hypo	Nadir glucose (mmol/l)
1	Non-Hypo	3.7	N/A	N/A
2	Hypo	2.0	Hypo	1.7
3	Hypo	1.7	N/A	N/A
4	Non-Hypo	3.5	Hypo	2.2
5	Non-Hypo	3.9	Non-hypo	3.0
6	Non-Hypo	4.3	Non-hypo	3.4
7	Hypo	2.5	Hypo	1.7
8	Hypo	2.8	N/A	N/A
9	Non-Hypo	4.6	Non-hypo	3.4
10	Non-Hypo	3.8	Hypo	2.3
11	Non-Hypo	3.9	Hypo	2.3
12	Non-Hypo	4.7	Hypo	2.9
13	Hypo	2.9	Non-hypo	3.8
14	Non-Hypo	3.4	Hypo	2.8
15	Hypo	2.5	Hypo	2.2
16	Non-Hypo	3.6	Hypo	2.8

**Table 4.7 Prevalence of hypoglycaemia and nadir glucose during either a MMT or POGTT.** 16 subjects had a MMT whilst only 13 had a POGTT. Abbreviations, N/A- not applicable (subject did not have a POGTT)





**Figure 4.2 Changes in glucose (A) and insulin (B) following a mixed meal test in patients with suspected PPH.** Patients classified as either hypo (n=6) or non hypo (n=10), depending if their nadir glucose was less than 3 mmol/l. Significant differences are \*\*P<0.01, \*\*\*P<0.0001.

<b>Outcome measures</b>	<b>MMT Hypo (N=6)</b>	<b>MMT Non Hypo (N=10)</b>
<b>Mean peak glucose (mmol.l<sup>-1</sup>)</b>	8.90±0.90	10.31±1.04
<b>Mean nadir glucose (mmol.l<sup>-1</sup>)</b>	2.40±0.19	3.94±0.14*
<b>Mean time to peak glucose (min)</b>	30.00±0.00	39.00±4.58
<b>Mean time to nadir glucose (min)</b>	95.00±9.22	177.00±17.58*
<b>Glucose<sub>30</sub> (mmol.l<sup>-1</sup>)</b>	8.90±0.90	9.64±1.18
<b>Mean peak insulin (mU.l<sup>-1</sup>)</b>	197.80±39.17	103.78±23.22
<b>Mean time to Peak insulin (mins)</b>	30.00±0.00	45.00±6.71
<b>Insulin<sub>30</sub> (mU.l<sup>-1</sup>)</b>	162.1±45.94	103.1±19.16*
<b>Mean AUC glucose (mmol.l<sup>-1</sup>.min)</b>	1131.33±39.10	1429.50±111.22
<b>Mean AUC insulin (mU.l<sup>-1</sup>.min)</b>	9325.17±1529.82	7113.20±1300.73
<b>Mean AUCinsulin:glucose (mU.mmol<sup>-1</sup>)</b>	8.15±1.38	5.14±0.98
<b>Mean Insulinogenic index (mU.mmol<sup>-1</sup>)</b>	53.85±17.19	21.70±6.24

**Table 4.8 Comparison of measures of glucose homeostasis following a mixed meal in PPH patients.** The symptomatic RYGB patients were further categorised into two groups, those who had biochemical hypoglycaemia (MMT Hypo) and those that did not (MMT Non-Hypo) Significant differences are \* P < 0.05.

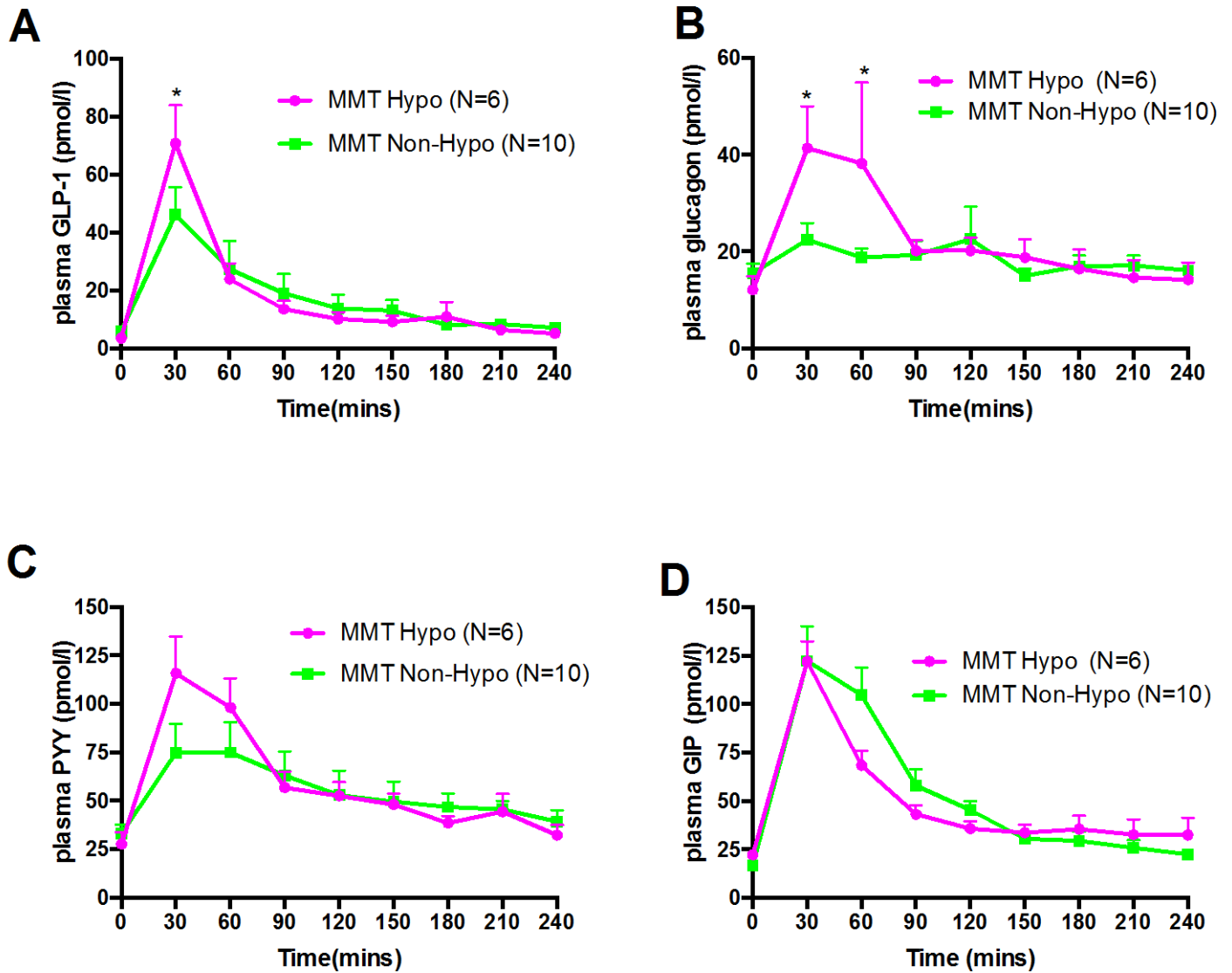
### **4.4.3 Investigation into changes in gut hormones in PPH patients: Hypo vs Non-Hypo groups**

There was no significant difference in the baseline plasma GLP-1 levels prior to the MMT in both group (Figure 4.3). GLP-1 concentration following a mixed meal rose in both groups and after 30 minutes there was a significant difference (GLP-1<sub>30</sub> 70.9±13.2 [MMT Hypo group] vs 40.3±9.4 pmol/l [MMT Non-Hypo group] P<0.05). By 60 minutes and for the remainder of the MMT there was no difference in the concentration of GLP-1 between the groups.

Baseline fasting glucagon levels were not significantly different between the PPH Hypo group and the PPH Non-Hypo group. Following the MMT glucagon concentration increased in both groups but was significantly higher in the PPH Hypo group at 30 and 60 minutes following consumption of the MMT (glucagon<sub>30</sub> 41.4±8.6 [MMT Hypo group] vs 22.5±3.4pmol/l [MMT Non-Hypo group] P<0.05, glucagon<sub>60</sub> 38.2±10.7 [MMT Hypo group] vs 18.8±1.9 [MMT Non-Hypo group] P<0.05).

PYY and GIP concentrations rose after consumption of the mixed meal. The post prandial concentration of PPY and GIP in both groups peaked at

30 minutes. There was no significant difference in concentrations of PYY and GIP at any time point during the mixed meal between both groups.



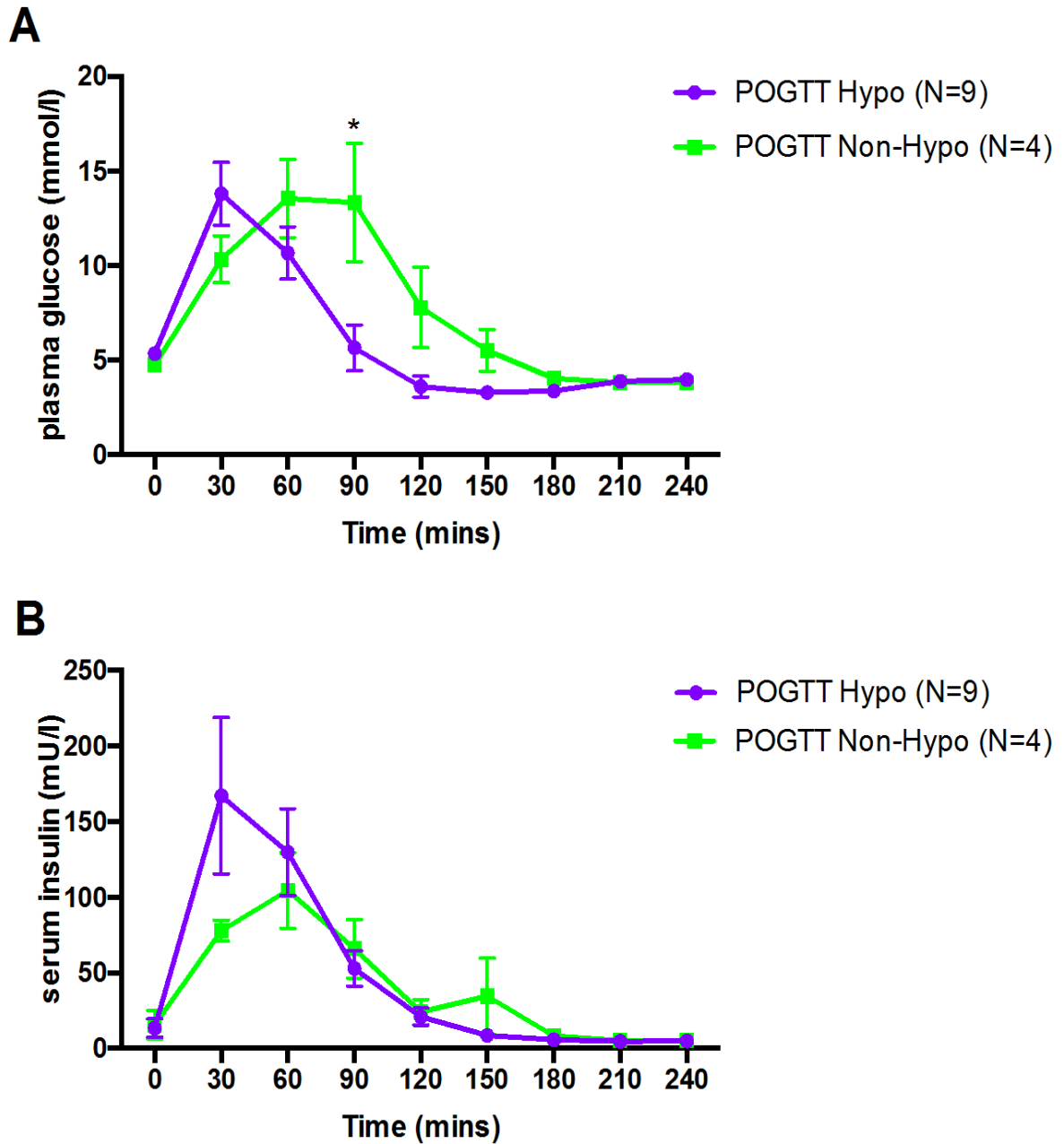
**Figure 4.3** Changes in serum GLP-1 (A), glucagon (B), PYY (C) and GIP (D) following a mixed meal test. Patients classified as either MMT Hypo (N=6) or MMT Non-Hypo (N=10), depending if their nadir glucose was less than 3 mmol/l. Significant differences are \*  $P < 0.05$ .

#### **4.4.4 Investigation into changes in glucose and insulin following a POGTT in patients with PPH.**

Only 13 of the 16 patients suspected to have PPH completed the POGTT. Of those 13 subjects, 9 had evidence of biochemical hypoglycaemia (Table 4.7). Similarly, to the previous experiment using a MMT, the subjects were divided into two groups based on the presence of biochemical hypoglycaemia; POGTT Hypo and POGTT Non-Hypo.

The glucose and insulin profiles of these subjects are shown in Figure 4.4 and Table 4.9. Both groups had a similar baseline glucose prior to drinking the oral glucose challenge. After consumption, the plasma glucose rose in both groups. The PPH Non-Hypo group had a peak glucose that appeared to be later than the PPH Hypo group but this was not statistically significant.

As expected, the nadir glucose was significantly lower in the PPH Hypo group (nadir glucose:  $2.39 \pm 0.19$  [POGTT Hypo group] vs  $3.40 \pm 0.16$  mmol/l [POGTT Non-Hypo group]  $P < 0.01$ ).



**Figure 4.4 Changes in glucose (A) and insulin (B) during a POGTT.** Patients were classified as either hypo (N=9) or non hypo (N=6). Significant differences are \*  $P < 0.05$ .

Outcome measures	POGTT Hypo (N=9)	POGTT Non-Hypo (N=4)
Mean peak glucose (mmol.l <sup>-1</sup> )	14.51±1.50	15.90±1.95
Mean nadir glucose (mmol.l <sup>-1</sup> )	2.34±0.17	3.45±0.17*
Mean time to peak glucose (min)	41.25±5.49	67.50±14.36
Mean time to nadir glucose (min)	146.25±14.39	195.00±19.37
Glucose <sub>30</sub> (mmol.l <sup>-1</sup> )	13.79±1.67	10.33±1.24
Mean Peak Insulin (mU.l <sup>-1</sup> )	202.70±44.70	114.27±17.14*
Mean time to Peak insulin (mins)	48.75±7.89	52.50±7.50
Insulin <sub>30</sub> (mU.l <sup>-1</sup> )	162.1±45.94	103.1±19.16*
Mean AUC glucose (mmol.l <sup>-1</sup> .min)	1131.33±39.10	1429.50±111.22
Mean AUC insulin (mU.l <sup>-1</sup> .min)	167.00±51.79	77.83±6.80
Mean AUCinsulin:glucose (mU.mmol <sup>-1</sup> )	7.80±1.50	5.25±0.81
Mean Insulinogenic index (mU.mmol <sup>-1</sup> )	45.62±32.42	17.52±2.31

**Table 4.9 Comparison of measures of glucose homeostasis following a POGGT in PPH patients.** The symptomatic RYGB patients were further categorised into two groups, those who had biochemical hypoglycaemia (POGTT Hypo) and those that did not (POGTT Non-Hypo) Significant differences are \* P < 0.05

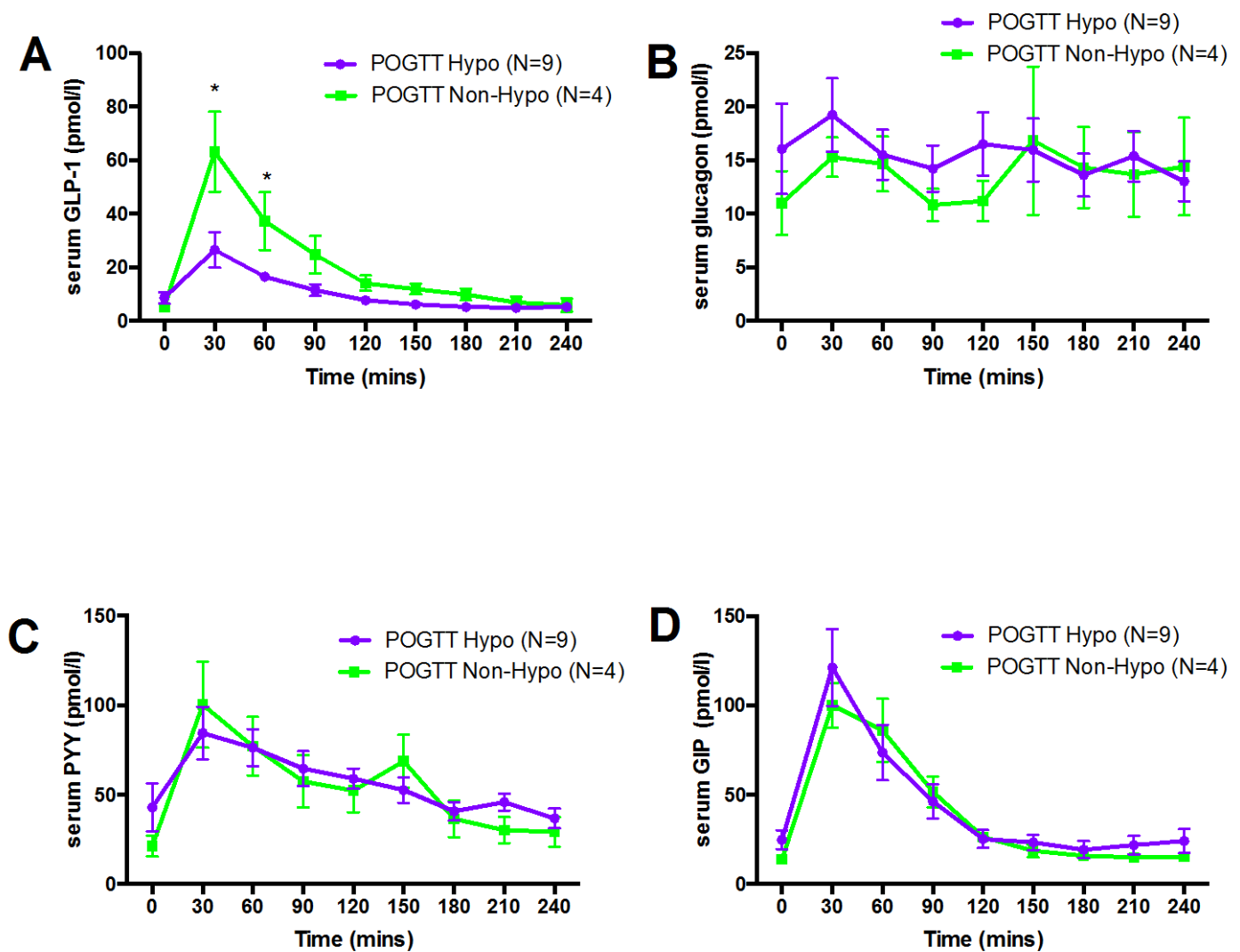


#### **4.4.5 Investigation into changes in gut hormones following a POGTT in patients with Hypo and without Hypo during the test**

There was no significant difference in baseline GLP-1 between the groups (Figure 4.5). Following the oral glucose challenge GLP-1 rose in both groups. The peak concentration of GLP-1 was significantly higher in the POGTT Non-Hypo group (Peak GLP-1:  $63.13 \pm 14.91$  [POGTT Non-Hypo group] vs  $25.31 \pm 6.78$  pmol/l [POGTT Hypo group]  $P < 0.0001$ ). GLP-1 concentrations returned to baseline at 180 minutes post consumption of the oral glucose challenge.

Fasting glucagon levels were not significantly different between either group. Following the oral glucose challenge test, glucagon concentrations fluctuated but did not change significantly from the baseline levels.

GIP and PYY baseline concentrations rose after consumption of the oral glucose challenge, before returning back to baseline by 180 minutes. There was no significant difference in concentration between the two groups at any time point.



**Figure 4.5** Changes in GLP-1 (A), glucagon (B), GIP (C) and PYY (D) following an oral glucose challenge. Patients were classified as either POGTT Hypo (N=9) or POGTT Non-Hypo (N=4). Significant differences are \*  $P < 0.005$ , \*\*  $P < 0.0001$ .

## 4.5 Discussion

In this investigation I have demonstrated the following

1. Subjects with PPH have increased glycaemic variability on continuous glucose monitoring relative to the normal ranges observed in a non-diabetic population.
2. After RYGB, subjects have altered glycaemic tolerance (measured by a MMT) relative to an obese population.
3. Subjects who have PPH and have biochemical hypoglycaemia during a MMT have an associated increase in both plasma glucagon and GLP-1 (Table 6).
4. Subjects who have PPH and have biochemical hypoglycaemia during a POGTT have an associated decrease in plasma GLP-1 (Table 4.10).

	<b>GLP-1</b>	<b>Glucagon</b>	<b>GIP</b>	<b>PYY</b>
<b>MMT</b>	Increased	Increased	No change	No change
<b>POGTT</b>	Decreased	No change	No change	No change

**Table 4.10 Summary of gut hormone changes in those that have biochemical hypoglycaemia following either a MMT or a POGTT.** Sixteen subjects had a MMT of which 6 had biochemical hypoglycaemia. Twelve subjects had a POGTT of which 8 had biochemical hypoglycaemia. Significant differences in peak gut hormone levels between those that have or did not have biochemical hypoglycaemia are shown.

### 4.5.1 Increased Glycaemic Variability

In this study, subjects with PPH were demonstrated to have increased glycaemic variability as measured by a CGM worn for five days. The specific modality of glycaemic variability that was increased was MAGE at  $4.93 \pm 0.75$ . The normal limits of glycaemic variability (including MAGE) were based on data collected from 70 glycaemic individuals by Hill *et al*(240).

There are at present no normal limits for glycaemic variability established for the RYGB population. Subsequently it is not possible to determine whether the changes established in this study reflect the changes caused by the RYGB as opposed to being specific to the PPH population. In the current study there was an increase in MAGE relative to asymptomatic subjects who had undergone RYGB but this was not statistically significant, which is likely due to the small cohort size.

Haniare *et al* also demonstrated an increase in MAGE in 10 RYGB patients with PPH of  $4.78 \pm 3.28$  (242). They also reported an increase in SD relative to a euglycaemic group of 10 subjects (SD  $1.72 \pm 1.0$  [RYGB-PPH] vs  $1.94 \pm 0.61$  [euglycaemic group] mmol/l,  $P=0.003$ ). The SD determined in the current study was  $2.04 \pm 0.23$  mmol/l. However, whilst the Hainaire *et al* study reported the SD in their RYGB-PPH group was

abnormal, it is within the normal reference range reported by Hill *et al*(240).

The increased glycaemic variability reported in this study and Haniare *et al* conflicts with a study by Marfella *et al* that demonstrated a reduction in MAGE after bariatric surgery (242,243). However, this conflicting data may be accounted by differences in methodology. Marfella *et al* studied a cohort of patients who had pancreaticobiliary diversion (an alternative operation to the RYGB). Furthermore, the Marfella study examined glycaemic variability in the in-hospital setting in contrast to the free-living environment that the current study examined.

The clinical relevance of increased glycaemic variability remains unclear in the context of RYGB patients. In patients with either type 1 or 2 diabetes there is an association between glycaemic variability and an increased incidence of hypoglycaemia(244). Other studies have shown an association between increased glycaemic variability with diabetic microvascular complications, cardiovascular disease and poor quality of life(245-247). Furthermore, short term studies investigating glycaemic variability in non-diabetic patients have shown an association with cardiovascular mortality and increased glycaemic variability(248). Whilst these findings have not been investigated in the RYGB population

it is noteworthy that the complications associated with increased glycaemic variability are at odds with the improved cardiovascular outcomes reported after bariatric surgery(38).

There was no significant difference in the percentage time spent hypoglycaemic between the symptomatic and asymptomatic subjects. This may reflect the small numbers of subjects analysed but would suggest that the key difference between the groups is the perception of hypoglycaemia. This observation is supported by a study by Laurenius *et al* that compared the frequency of hypoglycaemic symptoms between symptomatic and asymptomatic subjects who underwent a mixed meal test(237). Laurenius *et al* found that whilst there was no significant difference in the rate of hypoglycaemia, but that the symptomatic group had more symptoms of hypoglycaemia.

#### **4.5.2 Altered glucose tolerance in patients post RYGB measured by a MMT**

This study has demonstrated that patients post RYGB have an altered glucose tolerance relative to a control group of obese euglycemic individuals, regardless of whether they are symptomatic or asymptomatic from PPH. The altered glucose tolerance is characterised by significantly

earlier and higher glucose peak concentration following consumption of a mixed meal. This data is consistent with previous reports(230).

The higher peak concentration of glucose in the RYGB patients is in keeping with their increased glycaemic variability. Whilst all RYGB patients had normal glycated haemoglobin, the elevated postprandial glucose levels invites speculation as to whether they are at increased risk of diabetic complications. Whilst the SOS study demonstrated an improvement in macrovascular complications, smaller studies have demonstrated conflicting data on the improvement in microvascular complications(249-251). The SOS study which was a non-randomised controlled study comparing bariatric surgery to lifestyle management demonstrated a significant reduction in the cumulative incidence of microvascular events from 41.8 (control group) to 20.6 per 1000 patient-years (bariatric surgery group(249)). Miras *et al* in a prospective case study demonstrated an improvement in nephropathy at one year post surgery but this was not shown for retinopathy or neuropathy(250). Furthermore, a recent meta-analysis investigating the effects of bariatric surgery on retinopathy revealed that whilst  $19.2 \pm 12.9$  % of patients had improvement in their disease,  $23.5 \pm 18.7$  had a deterioration in retinopathy(251). The relevance of this data relates to how outcome measures after RYGB are interpreted. Many RCTs have inferred an

improvement in diabetic status based on a reduction in HbA1c(36,252). This is based on previous studies assessing intensive medical therapies demonstrating that reductions in HbA1c are a reliable surrogate marker for improvements in diabetic complications(253,254). However, whilst RYGB does improve HbA1c there is also an increase in glycaemic variability which has been shown to be an independent risk factor for an increase in microvascular complications (245). Randomised controlled studies with long term data is needed to evaluate whether the altered glucose tolerance post RYGB results in better or worse microvascular outcomes, compared to a medically treated diabetic cohort (with a similar HbA1c).

#### **4.5.3 Differences in glucose, insulin and gut hormones, between RYGB patients who have biochemical hypoglycaemia and those that do not, during a MMT**

This study demonstrated no significant difference in the peak glucose concentrations between RYGB patients that had hypoglycaemia and those that did not. However, the patients that subsequently had hypoglycaemia had a significantly higher insulin peak. The increased insulin response to glucose is associated with a higher GLP-1 and



glucagon concentration. An elevated concentration of both GLP-1 and glucagon post RYGB after consumption of a mixed meal is consistent with previous reports(73,74,183). However, whilst an increased GLP-1 secretion has been implicated in the pathology of PPH, increased glucagon secretion has not. Both hormones are insulinotropic and so could be responsible for the increased insulin peak observed in PPH patients.

Elevated postprandial plasma glucagon is considered paradoxical and is associated with diabetes(255). The source of glucagon has been previously been ascribed to the pancreas but a recent study by Holst et al have demonstrated extra-pancreatic glucagon secretion in patients who have had pancreatectomies(256). Holst et al speculate that the elevated glucagon may originate from L cells with aberrant processing of proglucagon. As there is hyperplasia of L cells after RYGB, as well as hyper-secretion of other hormones such as GLP-1 from these enteroendocrine cells it is possible that they account for the elevated postprandial glucagon levels(177,179,257). One method to asses this would be to perform pancreatectomies in rodent models of RYGB to investigate whether the observed elevated postprandial glucagon persists.

The relative contribution of GLP-1 and glucagon to the elevated insulin and subsequent hypoglycaemia remains unclear. There is conflicting data on the role of GLP-1 in PPH. Goldfine *et al* have demonstrated that patients with PPH have higher concentrations of GLP-1 than obese and symptomatic RYGB controls(184). Furthermore Salehi *et al* have previously shown that an infusion of a GLP-1 receptor antagonist (Exendin 9-39) during a mixed meal test in patients with PPH ameliorated the hypoglycaemia(235). However, in contrast to the above findings, surgical reversal of RYGB reduces the elevated GLP-1 concentration in PPH patients to presurgical levels but does not cure them of their hypoglycaemia(258). In contrast to the findings of Salehi *et al*, another group have demonstrated that GLP-1 receptor agonists can successfully treat PPH suggesting that GLP-1 action may be of benefit in this disease(191).

The mechanism by which GLP-1 receptor agonists ameliorate PPH has not been confirmed. GLP-1's known actions of reducing gastric emptying and increasing glucose-dependent insulin secretion are unlikely to contribute to its beneficial effect in patients with PPH(259). Post RYGB, patients have a small gastric pouch which rapidly empties and so is unlikely to be affected by GLP-1. Any insulinotropic action of GLP-1 would likely make hypoglycaemia worse and hence, it is counterintuitive

that a GLP-1 agonist could improve PPH. However, its glucagonostatic action may be relevant based on the current study's finding of elevated glucagon concentration in the MMT Hypo patients. GLP-1 is known to potently inhibit glucagon secretion independently of its action on insulin secretion(260). Pancreatic  $\beta$  cells have glucagon receptors and cell studies have demonstrated that glucagon stimulates glucose dependent insulin secretion(261). The presence of a glucagon antagonist resulted in a  $51\pm 6\%$  reduction in insulin secretion(261). This data supports the potential role of glucagon in the elevated postprandial insulin levels observed in PPH. Further evaluation is needed to determine whether GLP-1 receptor agonist treatment results in reduced glucagon concentration in PPH patients.

#### **4.5.4 Comparison of glucose, insulin and gut hormone responses between a MMT and POGTT in patients with PPH.**

More of the PPH patients demonstrated biochemical hypoglycaemia following consumption of an oral glucose challenge (9/13 or 69%) compared to a mixed meal stimulus (6/16 or 37.5%). Interestingly, in contrast with the MMT study, in those PPH patients who had biochemical

hypoglycaemia during the POGTT, there was a significant reduction in GLP-1 concentration. Furthermore, there was no significant increase in glucagon concentration following consumption of the oral glucose challenge in either the hypoglycaemic or non-hypoglycaemic group, in contrast to the situation with the MMT where we found an increased post-prandial secretion of glucagon in the hypoglycaemic group. The relatively high prevalence of hypoglycaemia following a oral glucose challenge relative to a mixed meal supports the notion that a diet that uses carbohydrates with low glycemic index may benefit patients with PPH(262).

The differences noted above may relate to differences in the stimulus used in either provocation test. The mixed meal provocation test uses a mixture of fat (20%), protein (16%) and carbohydrate (64%) whilst the POGTT uses 75 g of anhydrous glucose. In addition to the disparity in macronutrients, there are also differences in total calorie content (290 calories in POGTT and 330 calories in the MMT) and volume (500 ml in the POGTT and 220 ml in the MMT).

The stimulus used in the POGTT results in faster absorption of glucose by the intestine into the blood stream. This is reflected in the higher peak

glucose observed in the POGGT relative to the MMT (Peak glucose  $15.02 \pm 1.16$  [POGTT] vs  $9.78 \pm 0.73$  mmol/l [MMT]  $P < 0.001$ ).

The absence in any rise in glucagon concentration following the POGTT is likely due to the absence of protein as amino acids are a potent stimulus for glucagon secretion. Fat also causes an increase in glucagon concentration but less so than protein(263,264).

The mechanisms that underlie the significant increase in GLP-1 concentration in POGTT Non-Hypo group compared to the POGTT Hypo group remain unclear. This finding may be due to a type I statistical error due to the low number of subjects in the POGTT Non-Hypo group. Few studies have used an oral glucose stimulus, opting instead for a mixed meal test. However, those that have used a POGTT have demonstrated an increase in GLP-1 albeit with an even smaller number of subjects studied than the current study (265).

Also, the subdivision of subjects into those that have hypoglycaemia and those that do not is based the premise that in the absence of hypoglycaemia, there is no associated abnormal gut hormone response beyond what is observed after RYGB. However, if the above premise is

not true, the POGTT Non-Hypo group would not be an appropriate control group.

A further study with larger numbers of subjects and a separate control group of RYGB subjects who have no symptoms of PPH may help to explain the findings from the POGTT study. A larger cohort size would increase the power of the study.

The observations in this study have been made based on a single provocation test and should be interpreted with caution regarding the reproducibility of the provocation tests. Specifically, the oral glucose tolerance test has been shown to have variable results on repeat testing(266-268). Hence, a subject who is known to have PPH may have biochemical hypoglycaemia following a single provocation test but this may not occur on repeat testing and vice versa. To make the study tractable, I classified the subject as having PPH if they had biochemical hypoglycaemia following provocation regardless of their previous history. An alternative method would be to repeat MMTs or POGTT on each individual subject and correlate gut hormone levels to their glucose status. However, the symptoms of PPH after provocation are difficult to tolerate and subsequently it may be difficult to recruit for multiple provocation tests.

In conclusion, these studies have demonstrated a number of significant findings in the hormonal fluxes that occur in PPH. GLP-1, which is thought to underlie many of the improvements in glucose tolerance post RYGB, may also play a role in the pathogenesis of PPH. Furthermore, changes in glucagon secretion may also be important. A greater understanding of the roles of these hormones could help treat PPH patients as well as to guide future medical treatment for obesity based on gut hormones.

# **Chapter 5**

## **General Discussion**



The prevalence of obesity has rapidly risen such that 25% of the UK adult population is now obese(269). The cost of obesity can be measured both to the individual in terms of associated medical conditions and to society with respect to the costs of treating obesity.

Obesity is associated with a variety of medical conditions including T2DM, ischaemic heart disease, OSA, hypertension and cancer(270). The cumulative health burden associated with obesity is reflected in the 50% higher mortality risk in young adults with obesity relative to those who are overweight(271). For society the economic burden to the NHS, from treating overweight and obese individuals is £5.1 billion(272). Worryingly, the prevalence of childhood obesity continues to rise, which forebodes an ever increasing health burden unless suitable, effective treatments are achieved(273).

Currently, the most effective treatment for obesity is bariatric surgery resulting in sustained weight loss and improvements in obesity related co-morbidities up to 20 years later(38). The weight loss observed after RYGB surgery is greater than either of the currently available anti-obesity therapeutics; orlistat (GlaxoSmithKline, London UK) or liraglutide (NovoNordisk, Denmark)(17,38,123).

Orlistat is an intestinal lipase inhibitor(274). Weight loss with orlistat is significant but modest; at one year a systematic review showed 3.5% greater weight loss than placebo(17). The XENDOS study (which had a longer follow up of four years) revealed weight loss of 2.4% greater than placebo(275). This study also illustrated that the significant gastrointestinal side effect profile of orlistat which resulted in a 48% attrition rate(275).

Liraglutide is more effective, achieving 5.4 % greater weight loss than placebo at one year(123). This anti-obesity medication is based on a gut hormone, GLP-1, that has multiple beneficial metabolic actions including an insulinotropic effect, a delay in gastric emptying, a reduction in food intake and an increase in satiety. It is also one of a series of hormones (including glucagon, OXM and PYY) whose postprandial concentration is increased after RYGB surgery(180). Whilst the weight loss observed with liraglutide is lower than that observed after RYGB, the use of additional gut hormones combined with a GLP-1 agonist may result in greater weight loss.

A series of studies have explored the benefits of dual infusion of gut hormones on food intake. Human infusion studies have demonstrated that combining GLP-1 with PYY, OXM or glucagon has an additive anorectic

effect(201,218,276). However, there are no data on the effects of combined infusions on glucose tolerance, nor the effects on food take by the co-infusion of three hormones. This thesis builds on these previous studies, further investigating the co-infusion of GLP-1 and glucagon, but also focusing on their effects on glucose tolerance. It also investigates the effects of a triple hormone infusion on food intake in man. The studies were designed to use hormone doses that would replicate the levels observed following RYGB to assess the contribution that these hormones have on food intake and glucose tolerance as well as to explore their use as an anti-obesity treatment. Furthermore, the role of gut hormones in the pathology of a PPH (a complication of RYGB) has been investigated to address potential safety issues of raised gut hormones after RYGB.

### **Co-infusion of GLP-1, OXM and PYY on food intake in man.**

In chapter 3, I have demonstrated for the first time that co-infusion of GLP-1, oxyntomodulin and PYY resulted in a reduction in food intake relative to saline. The novelty of this study is two-fold. Firstly, it represents the first in man infusion of three gut hormones. Secondly, whilst GLP-1 has been previously delivered via a SC pump, oxyntomodulin and PYY have not.

The latter is particularly relevant to the feasibility of this treatment being used for obese patients. Previous co-infusions of gut hormones have been characterised by being intravenous as well as acute (lasting from 90 to 240 minutes and only using one *ad-libitum* meal) whilst the current study used a 14 hour infusion and assessed food intake over two meals. Given that gut hormones have a short half life ranging from 4-12 minutes, an appropriate drug delivery system has to be used(142,277,278). Evidence that a continuous subcutaneous pump would be acceptable for patients is their current use in the treatment of T1DM and Parkinson's disease(221,222). An alternative method of administering gut hormones is the use of long acting analogues. However, whilst there are numerous types of GLP-1 based analogues, there are no commercially available long acting agonists at the PYY or OXM receptors, although these are in phase I development(279-281).

The triple hormone infusion of OXM, GLP-1 and glucagon did not alter plasma glucose or serum insulin concentrations relative to saline. As subjects were given *ad-libitum* meals, it is not possible to comment on post-prandial glucose concentrations due to the confounding effects of different energy intake. However, there was no difference in the pre-prandial glucose concentrations between the triple hormone and saline infusion arms. Nuack *et al* using an intravenous infusion of GLP-1 (1.2

pmol/kg/min) demonstrated a significant reduction in fasting glucose concentration in subjects with insulin-treated T2DM(282). Furthermore, in chapter 2, an intravenous infusion of 0.4 pmol/kg/min resulted in a significant reduction in pre-prandial glucose concentrations. However, Toft-Nielsen *et al* using a dose of subcutaneous GLP-1 (2.4 pmol/kg/min) in subjects with non-insulin dependent T2DM found no significant effect on fasting glycaemia(283). The disparity between the studies may be accounted by the glycaemic status of the subjects. Alternatively, in the study by Nauck *et al* the intravenous infusion of GLP-1 resulted in a significant reduction in plasma glucagon concentrations whilst the study by Toft-Nielsen *et al* did not. An elevated plasma glucagon concentration is observed in T2DM and may partly account for the hyperglycaemia seen in this disease(70). This is supported by the finding in chapter 2, in which the infusion of glucagon resulted in higher pre-prandial glucose concentrations.

## **5.1 Co-infusion of GLP-1 and glucagon on carbohydrate tolerance in man.**

In chapter 2, I demonstrated that the addition of GLP-1 to glucagon infusion in humans attenuated the post-prandial insulinotropic effect of glucagon alone. Furthermore, the infusion of GLP-1 alone results in a

lower postprandial insulin response compared to vehicle. This conflicts with data demonstrating that both glucagon and GLP-1 stimulate insulin release via their respective receptors on pancreatic  $\beta$ -cells(284-286). The attenuated postprandial insulin concentration observed with GLP-1 alone is potentially due to its effect on delaying gastric emptying(101).

In contrast with the findings above, the elevated post-prandial insulin observed in subjects with PPH was associated with raised GLP-1 concentrations. This disparity may be due to the changes to the mechanical properties of the stomach after RYGB. After RYGB, patients have a smaller gastric pouch with no pylorus resulting in rapid gastric pouch emptying. Hence, any effect of GLP-1 on attenuation serum insulin concentrations due to gastric emptying in the post RYGB patients, may be outweighed by its insulinotropic effects.

In chapter 4, RYGB patients with hypoglycemia had higher peak postprandial insulin concentrations and subsequent lower plasma glucose concentrations. Hypoglycemia was also associated with higher levels of both GLP-1 and glucagon. Co-infusion of GLP-1 and glucagon in chapter 2, did result in a lower AUC glucose relative to placebo, but there was no evidence of hypoglycemia (blood glucose less than 4.0 mmol/L). The absence of hypoglycemia observed in the infusion studies may reflect the

small cohort size, the relatively lower concentrations of GLP-1 and glucagon or the difference between infusions delivered in the short-term and the chronic elevation of hormones observed after RYGB. This latter factor may be relevant as studies with follow up of patients receiving long-term administration of GLP-1 have shown an increase in the incidence of hypoglycemia relative to placebo(123,287). Furthermore, tumours that secrete GLP-1 have been associated with reactive hypoglycemia(288,289).

There are at present no human studies investigating the effects of chronic administration of glucagon. Tumours that secrete glucagon are generally associated with hyperglycemia and diabetes(290).However, there is a case report of a patient with a glucagonoma presenting with hypoglycemia suggesting that glucagon could be implicated in PPH(291).There has also been one case report of a patient with a tumour that co-secreted both GLP-1 and glucagon that presented with both hypo- and hyperglycemia(289). It is possible that in the former case report by Bolt *et al*, the tumour was co-secreting GLP-1 with glucagon as no GLP-1 assay was performed.

The association of elevated glucagon with hypoglycemia is counter-intuitive as whilst glucagon is known to be insulinotropic, it is also

considered a counter-regulatory hormone to insulin and is used in the treatment of hypoglycemia. Glucagon increases plasma glucose levels by promoting gluconeogenesis and glycogenolysis(292). One possible mechanism by which elevated glucagon concentration post RYGB may result in hypoglycemia is through tachyphylaxis. Tachyphylaxis is the process by which repeated administration of a ligand results in a reduction in effect and has been observed with glucagon in the context of hepatic glycogenolysis(293). It is possible that the significantly elevated glucagon concentrations observed in patients with PPH results in tachyphylaxis such that the effect of glucagon on glycogenolysis is attenuated, resulting in hypoglycemia in the context of elevated insulin. This hypothesis could be investigated using animal studies in which insulin is administered to mice that have been receiving a continuous infusion of glucagon or saline, at various durations, to assess the presence of hypoglycemia. The duration of glucagon infusion in which hypoglycemia occurs would provide information as to the timing at which tachyphylaxis occurs. Furthermore, if hypoglycemia does occur, the study could be repeated after a period in which the glucagon infusion has been stopped to determine if the tachyphylaxis is reversible.



## **5.2 Combining gut hormones as treatment for obesity**

The work presented in this thesis demonstrates that co-administration of a combination of gut hormones can reduce food intake (GLP-1, OXM and PYY) and improve glycemic control (GLP-1 and glucagon). However, these data result from short-term studies and prior to further development as anti-obesity treatments, longer duration studies are warranted.

Specific hurdles to the use of gut hormones as a treatment for obesity include the increased incidence of nausea, potential risks of pancreatitis and PPH(187,294,295). In chapter 3, the dose finding part of the study demonstrated that GLP-1, OXM and PYY can all cause a dose-dependent nausea and that combining those hormones reduces the individual peptide dose at which nausea occurs. Clinical trials investigating GLP-1 analogues have demonstrated a dose-dependent relationship with nausea that is transient(294). Hence, there may be attenuation of nausea following chronic triple hormone administration.

Concerns regarding the risk of pancreatitis with the use of GLP-1 analogues in man were first raised following an analysis of the FDA's database of reported adverse events(295) Subsequent analysis of alternative databases and meta-analysis have failed to demonstrate further

evidence supporting this safety concern (296,297). However, any future clinical research study investigating the chronic effects of administration of GLP-1 with OXM and PYY will likely need to monitor amylase and lipase (surrogates of pancreatitis) concentrations.

Gut hormones, and specifically GLP-1 have been implicated in the pathology of PPH since its first description(232) This thesis provides further evidence supporting the association of GLP-1 with PPH. In addition it also shows an association with elevated post prandial glucagon levels. However, whether the relationship between PPH and elevated GLP-1 and glucagon is causal remains unclear. Furthermore, the use of GLP-1 agonists have proven helpful in the treatment of PPH(191). Nonetheless, further clinical trials using a triple hormone combination will require glucose monitoring.

In conclusion, co-administration of GLP-1, OXM and PYY has been shown to reduce energy intake whilst GLP-1 and glucagon resulted in improved carbohydrate tolerance. These findings add support to the proposal that gut hormones have a role in the metabolic benefits observed after RYGB. However, further long-term studies are required to determine whether the acute changes observed translate to longer-term

improvements in metabolic status as well as to assess the incidence of side-effects such as nausea, pancreatitis and PPH.

## **Future work**

**A study comparing the effects of RYGB surgery with those of a chronic infusion (28 days) of a combination of GLP-1, oxyntomodulin and PYY.**

Following on from the acute study demonstrating a reduction in food intake with the triple hormone infusion, this investigation will study the effects of a chronic infusion on specific outcomes that are known to improve after RYGB. These include, food intake, body weight, energy expenditure and carbohydrate tolerance. A control group consisting of matched subjects undergoing RYGB will be used to compare outcomes. The results of this study will provide information on the contribution of elevated gut hormones on the beneficial metabolic effects of RYGB. In addition, it will provide answers regarding the feasibility of delivering gut hormones via a subcutaneous pump and potentially represent a step closer to combination gut hormone therapy becoming a treatment for obesity.

**A randomised controlled study investigating the use of liraglutide in the treatment of PPH.**

The work in this thesis has shown an association between both GLP-1 and glucagon with PPH. However, a causal link has not been established with both GLP-1 receptor antagonists and agonists demonstrating an attenuation in hypoglycaemia. The evidence regarding GLP-1 agonists is

limited to a case series. Hence, a randomised controlled, blinded study is needed to determine whether this effect is real. GLP-1 agonists suppress glucagon and so this study will be useful in determining the relative contributions of both GLP-1 and glucagon in the pathology of PPH.

# **Chapter 6**

## **Appendix**

## **6.1 Appendix 1- VAS**

## VISUAL ANALOGUE SCALE

**T=0**

**How hungry do you feel right now?**

NOT AT ALL  EXTREMELY

**How sick do you feel right now?**

NOT AT ALL  EXTREMELY

**How pleasant would it be to eat right now?**

NOT AT ALL  EXTREMELY

**How much do you think you could eat right now?**

NOTHING  A LARGE  
AMOUNT

**How full do you feel right now?**

NOT AT ALL  EXTREMELY



## 6.2 Appendix 2- Magpix Protocol

### IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), Standards 1-7, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200  $\mu\text{L}$  of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25  $\mu\text{L}$  of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25  $\mu\text{L}$  of Assay Buffer to the sample wells.
5. Add 25  $\mu\text{L}$  of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25  $\mu\text{L}$  of Sample (neat) into the appropriate wells.
7. Vortex Mixing Bottle and add 25  $\mu\text{L}$  of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker for overnight incubation (16-18 hours) at 4°C.

Add 200  $\mu\text{L}$  Assay Buffer per well

Shake 10 min, RT

Decant

- Add 25  $\mu\text{L}$  Standard or Control to appropriate wells
- Add 25  $\mu\text{L}$  Assay Buffer to background and sample wells
- Add 25  $\mu\text{L}$  appropriate matrix solution to background, standards, and control wells
- Add 25  $\mu\text{L}$  neat Samples to sample wells
- Add 25  $\mu\text{L}$  Beads to each well

Incubate overnight at 4°C

9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
10. Add 50  $\mu$ L of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 50  $\mu$ L Streptavidin-Phycoerythrin to each well containing the 50  $\mu$ L of Detection Antibodies.
13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
14. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
15. Add 100  $\mu$ L of Sheath Fluid (or Drive Fluid if using MAGPIX<sup>®</sup>) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
16. Run plate on Luminex 200™, HTS, FLEXMAP 3D<sup>®</sup> or MAGPIX<sup>®</sup> with xPONENT<sup>®</sup> software.
17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

↓  
Remove well contents and wash 3X with 200  $\mu$ L Wash Buffer

Add 50  $\mu$ L Detection Antibodies per well

↓  
Incubate 1 hour at RT

↓  
Do Not Aspirate

Add 50  $\mu$ L Streptavidin-Phycoerythrin per well

↓  
Incubate for 30 minutes at RT

↓  
Remove well contents and wash 3X with 200  $\mu$ L Wash Buffer

Add 100  $\mu$ L Sheath Fluid or Drive Fluid per well

Read on Luminex (50  $\mu$ L, 50 beads per bead set)

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