University of Nottingham




# The Gut Hormone, Glucagon-Like Peptide 2, Contributes to the Improved Glycaemic Control Observed Post-Bariatric Surgery, as a Result of its Intestinotrophic Effects

A thesis submitted for the degree of Doctor of Philosophy at the University of Nottingham

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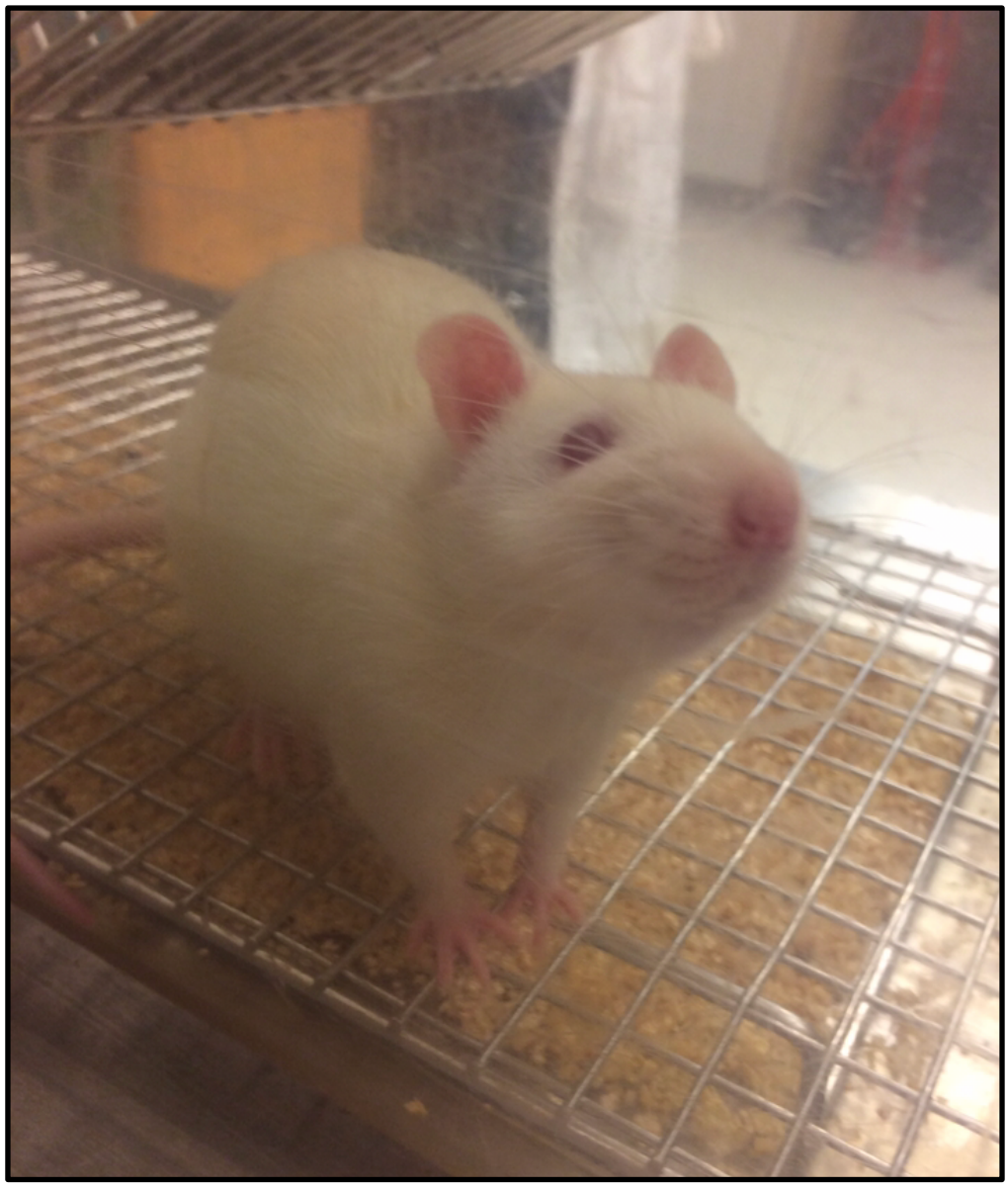
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# PUBLICATIONS AND PRESENTATIONS

## PUBLICATIONS

**Role of GLP-2 in the Metabolic Benefits of Bariatric Surgery**

*Rudge, EJM, Mielewczyk, TC, Tavakkoli A, Sheu, EG*

Abstract from Scientific Forum Podium Presentation at Clinical Congress, 2016

**Bariatric Surgery for Autoimmunity?**

*Rudge, EJM, Sheu, EG*

JAMA Surg, 2017, 1;152(4):349-350

## PRESENTATIONS

**Acute Effect of Systemic Infusion of GLP-2 on Glucose Homeostasis**

*Rudge, EJM, Tavakkoli A, Sheu, EG*

5th Annual Harvard Surgery Research Day, Boston, Massachusetts, May 2016

**The Role of GLP-2 in the Metabolic Benefits of Bariatric Surgery**

*Rudge, EJM, Tavakkoli A, Sheu, EG*

- Presented locally at the Harvard Metabolic Lab, Harvard Medical School, May 2016; regionally at New England Surgical Society Resident and Fellow Research Day, Waltham MA, May 2016, and internationally at Clinical Congress: American College of Surgeons, Washington DC, October 2016

**Acute Proximal Jejunal Exclusion Lowers Intestinal Glucose Absorption**

*Rudge, EJM, Tavakkoli A, Sheu, EG*

New England Surgical Society Resident and Fellow Research Day, Waltham MA, May 2017

# LIST OF ABBREVIATED TERMS

**AUC** – Area Under the Curve

**GLP-2** – Glucagon-Like Peptide 2

**GLP-2RA** – Glucagon- Like Peptide 2 Receptor Antagonist

**(GLY)GLP-2** – Teduglutide

**$G_P$** – Portal Glucose

**$G_{PS}$** – Porto-systemic Glucose Gradient

**$G_S$** – Systemic Glucose

**GTT** – Glucose Tolerance Test

**HFD** – High Fat Diet

**IPGTT** – Intra-peritoneal Glucose Tolerance Test

**OGTT** – Oral Glucose Tolerance Test

**PBS** – Phosphate Buffered Saline

**SC** – Standard Chow

**SD** – Sprague Dawley

**TED** – Teduglutide

**ZDF** – Zucker Diabetic Fatty

# ABSTRACT


## BACKGROUND

Bariatric surgery, specifically Roux-en-Y Gastric Bypass (RYGB), leads to durable weight loss as well as significant improvements in Type 2 Diabetes (T2D). Despite multiple randomised studies showing superiority of RYGB to maximal medical therapy for treatment of T2D, surgery remains grossly underutilised with only 1-2% of patients who qualify for the surgery proceeding with it. The limited acceptance of surgery highlights the need for less invasive interventions that can replicate the metabolic success of RYGB. A better understanding of the anti-diabetic mechanisms of surgery is therefore required in order to allow the development of less invasive T2D therapies (e.g. less invasive surgery, a medical device, or medication) which can then be offered to a wider patient population. RYGB appears to exert its anti-diabetic effects through multiple mechanisms. Work previously carried out within this lab has suggested that, post-RYGB surgery, there is a decrease in intestinal glucose absorption, with an increase in intestinal glucose utilisation. This thesis seeks to gain corroborating data in order to further decipher glucose utilisation and absorption patterns post-RYGB surgery. Additionally, it aims to determine whether these glucose modulations could be linked to the intestinal hypertrophy observed post-RYGB, and whether it could be mediated by Glucagon-Like Peptide 2 (GLP-2), a potent intestinotrophic hormone found at higher concentrations following RYGB.

## METHODS

A series of experiments were conducted to look at the effect of administering acute GLP-2 and chronic GLP-2 (in the form of Teduglutide, a degradation-resistant GLP-2 analogue), in both fasted and fed states, and within healthy and pathological rat

models, when compared to phosphate buffered saline (PBS) control. Aided by a portal, systemic and small bowel rat catheterisation model (previously developed within this laboratory) which allowed infusion of substances into the venous system and into the intestine, the effect of GLP-2 on blood glucose levels was assessed primarily. Additionally, the effects of GLP-2 on glucose transporter (GLUT-1, GLUT-1, SGLT-1), glucose sensor (SGLT-3) genes, GLP-2 receptors, intestinal proliferation genes (C-MYC and C-FOS), gut hormones (GLP-1, GIP, glucagon and insulin), and intestinal morphology, were studied. Further experiments included: GLP-2 receptor antagonism (GLP-2RA) studies, altered Biliopancreatic (BP) limb length studies, and experiments aiming to replicate RYGB physiology using a non-RYGB model, in order to assess effects on intestinal metabolism, in terms of glucose utilisation and absorption.

**RESULTS**

GLP-2 had statistically significant effects on glucose handling. Acute GLP-2 infusion resulted in significantly lower post-prandial portal vein glucose levels compared to control, within a healthy rat model. Teduglutide caused significant bowel hypertrophy in experimental animals versus control, and within both healthy and pathological models. Furthermore, increased intestinal hypertrophy was seen within the pathological group compared to the healthy group. Additionally, Teduglutide significantly raised portal and systemic glucose levels at the start of oral glucose tolerance tests (OGTT) but, subsequently, produced a profound hypoglycaemic effect compared to control. This was observed in both healthy and pathological rat models, with the response amplified within the latter of the two models. Despite significant upregulation of SGLT-1, GLUT-1, GLUT-2 and SGLT-3 within the pathological

model, no statistically significant difference in glucose utilisation or absorption was observed. GLP-2 antagonism experiments were largely unsuccessful; potential explanations include suboptimal dosing of the GLP-2 receptor antagonist (GLP-2RA), or the possibility of a deleterious effect of GLP-2RA when used within a pathological model. Altered BP limb length studies showed differences in glucose handling, with 40cm BP limb length showing significantly increased levels of glucose absorption. Significantly decreased glucose absorption was demonstrated within the proximal jejunal exclusion studies.

**CONCLUSION**

This thesis set out to explore mechanisms behind improved glucose handling, observed following RYGB surgery, and to investigate a potential role for GLP-2 in mediating this. Although it would be ambitious to claim that GLP-2 contributes to improvements in glycaemic control, it certainly appears to cause a glucose-related effect in animals treated with it, either acutely or chronically. Additionally, these effects were more dramatic within a pathological animal model. Furthermore, PCR experiments revealed significant upregulation of glucose transporter/sensor genes within the pathological model. Given that pathological animals displayed significantly increased small bowel hypertrophy compared to healthy GLP-2 treated rats, it is possible that Teduglutide may convey a beneficial effect to hyperglycaemic animals. The mechanisms underlying the potential impact of GLP-2 upon glucose metabolism are yet to be established, and further studies are required to delineate these effects and to prove definitive links between GLP-2 mediated bowel hypertrophy and improved modulation of glucose.

# CHAPTER 1

## 1.1 INTRODUCTION

The obesity epidemic has fuelled a parallel epidemic in Type 2 diabetes (T2D), a condition that now affects 29 million people in the United States and 400 million people worldwide. Trends indicate that this epidemic will continue to rise, with over 50 million pre-diabetic patients in the US and nearly 500 million such patients in China (Xu, Wang et al. 2013). In 2014, the cost of diabetes care worldwide was over $600 billion (2014). It is clear, therefore, that obesity and T2D pose a huge threat to world health. Given these figures and the fact that T2D is the leading cause of amputations, renal failure and blindness in the western world, substantial research efforts into pharmaceutical treatments have expanded the classes of diabetes medications beyond metformin and insulin. Despite this, only half of diabetics achieve the target HbA1c of < 7% using the current intensive medical treatment algorithms on offer to them.

As a result, bariatric surgery has emerged as a potential alternative to medical treatment in those patients with diabetes resistant to lifestyle modification and drug therapy. Although bariatric surgery, such as Roux-en-Y Gastric Bypass (RYGB) and Sleeve Gastrectomy (SG), was originally carried out for the specific endpoint of weight loss, a multitude of clinical data, including randomised control trials, have shown that this type of surgery to be superior to optimal medical therapy in managing T2D (Mingrone, Panunzi et al.), (Mingrone, Panunzi et al.), (Schauer, Bhatt et al.) and (Schauer, Kashyap et al.). Indeed, clinical data has provided us with evidence that bariatric surgery, as well as improving glycaemic control, can even lead to complete resolution of T2D, and sometimes within a matter of days post-operatively (Goldfine,

Shoelson et al. 2009). In fact, 80% of patients who undergo RYGB experience an early, and weight independent, remission of their T2D. However, the risks of surgery, along with permanent changes to gastrointestinal anatomy, have hampered widespread acceptance; less than 1-2% of Americans who qualify for weight loss surgery actually undergo the procedure. These considerations highlight the critical need for less invasive interventions to reproduce the metabolic successes of bariatric surgery.

There is widespread consensus that the metabolic benefits of RYGB may offer clues in creating novel therapeutic avenues. Unfortunately progress on this front has been hindered by a lack of detailed mechanistic understanding. The overall goal of this laboratory (the Laboratory for Surgical and Metabolic Research, Boston, MA) is to uncover the mechanism/s, in order to contribute to the effort to develop less invasive interventions that can be offered to millions of people afflicted with T2D, a goal with major public health value and impact.

## 1.2 LITERATURE REVIEW

### *1.2.1 Diabetes Mellitus*

Diabetes mellitus is a disorder of metabolism. In healthy individuals, the gastro-intestinal tract breaks down carbohydrates into glucose, and other monosaccharides, which are then absorbed into the bloodstream. This then stimulates the secretion of insulin, a hormone produced and secreted by the beta cells of the pancreas. Insulin enables cells in the body to take up the glucose and use it as energy. Glucose disposal is mostly accomplished by muscle and fat, which store the excess postprandial glucose as glycogen and fat, respectively. Those with diabetes, however, suffer from diminished insulin action, meaning that glucose builds up in the blood instead of being absorbed by cells. This results in hyperglycaemia and the cells of the body are starved of energy, despite high blood glucose levels (Lavin 2009).

Diabetes can occur as a result of reduced insulin secretion, a reduction in the effectiveness of the insulin, or a combination of the two. The distinction between the two types is a clinical one, based on whether the condition requires insulin to prevent ketoacidosis (a serious and dangerous condition where the liver breaks down fat and proteins in response to a perceived need for respiratory substrate). If it does, then the patient has Type 1 Diabetes (T1D). If not, then the patient has T2D. If the patient has T2D, but insulin is required to maintain acceptable glycaemic control, then the patient has insulin-treated T2D.

Those with diabetes have an increased risk of developing a number of serious health problems. Consistently high blood glucose levels can lead to serious diseases affecting the heart and blood vessels, eyes, kidneys and nerves. In addition, people with diabetes also have a higher risk of developing infections. In almost all high-income

countries, diabetes is a leading cause of cardiovascular disease, blindness, kidney failure, and lower limb amputation (2014).

### *1.2.2 Epidemiology*

Given that 1 in every 12 people on the planet suffers from diabetes, it is a condition that is extremely relevant to world health. The International Diabetes Federation (IDF) estimates that almost 400 million people currently have the condition (**Figure 1**), with T2D responsible for almost 90% of cases. More worryingly still, half of those with the disease remain undiagnosed. The IDF predicts that the number of people with the condition will increase to over 600 million by 2035. They also estimate, that in 2014, diabetes was responsible for the death of nearly 5 million people, with one person dying from the condition and its complications every 7 seconds. These figures demonstrate the huge burden that diabetes places on healthcare services throughout the world. They also demonstrate how crucial it is for research to identify a way to control diabetes, in order to reduce the morbidity and mortality associated with the condition.

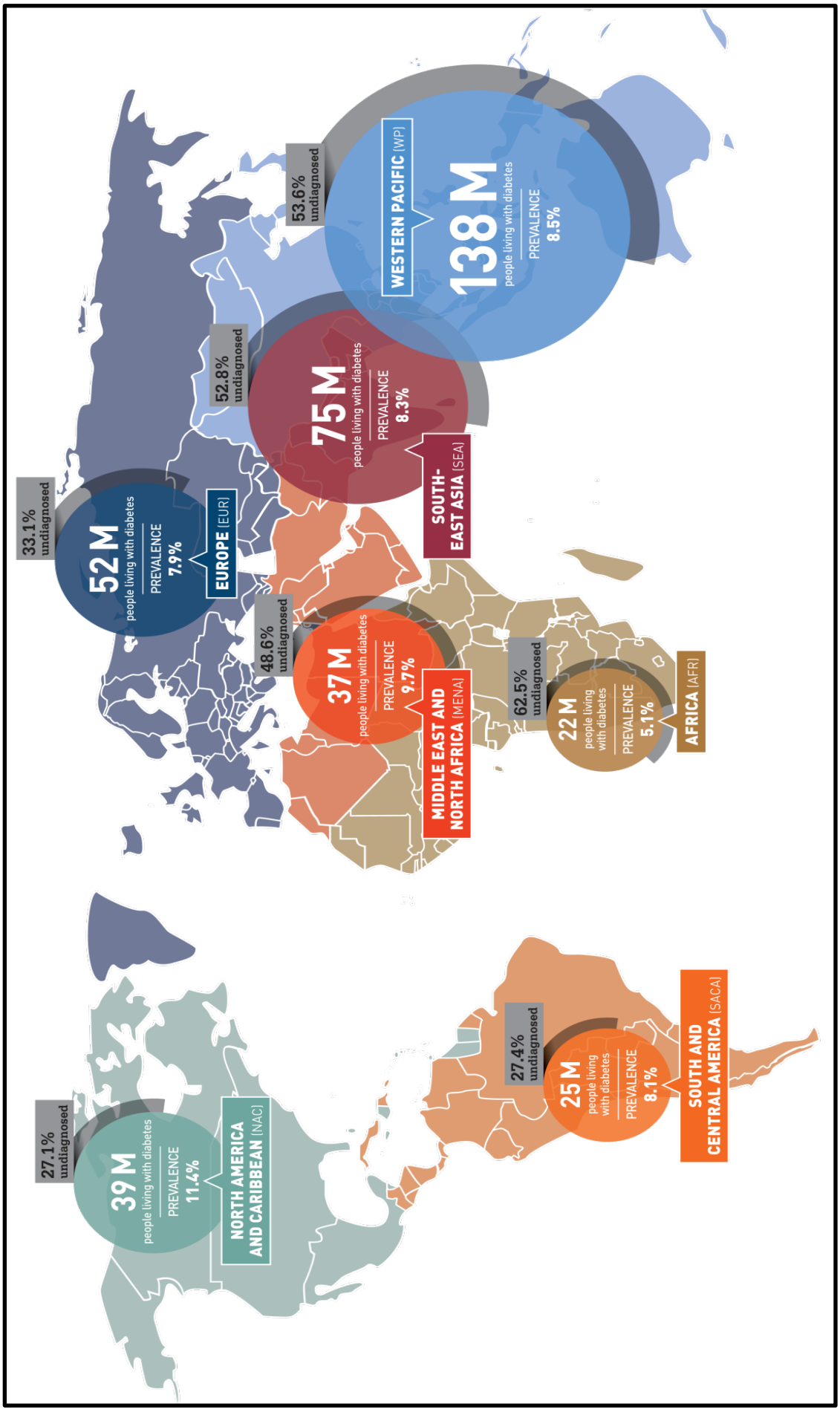



**Figure 1:** The number of people around the world living with diabetes.

### *1.2.3 Type 1 Diabetes Mellitus*

T1D results from an autoimmune destruction of insulin-producing β-cells in subjects genetically predisposed to the disease (Atkinson 2005). The reason this occurs is not fully understood. People with T1D produce very little or no insulin. The disease may affect people of any age, but usually develops in children or young adults. People with this form of diabetes need injections of insulin every day in order to control blood glucose levels.

### *1.2.4 Type 2 Diabetes Mellitus*

T2D accounts for at least 90% of all cases of diabetes. It is characterised by insulin resistance and relative insulin deficiency. Either, or both, may be present at the time of diagnosis. T2D can occur at any age and may remain undetected for many years. A diagnosis is often made when a complication appears, or a routine blood or urine glucose test is carried out. It is often, but not always, associated with being overweight or with obesity, which in itself can cause insulin resistance and lead to high blood glucose levels. People with T2D can often initially manage their condition through lifestyle modification alone, for example through increasing exercise and improving their diet. However, over time, most people will require oral drugs and/or insulin to control the disease (IDF 2014). Some patients are unable to control their blood sugar levels through lifestyle modification and medications alone, and bariatric surgery is now being offered as a treatment in those who qualify for it. This project seeks to contribute to the treatment of *Type 2* diabetes, and so it is this particular form of diabetes that will be mainly referred to within the confines of this thesis.

### *1.2.5 Aetiology*

The origin of T2D is multifactorial, including genetic and environmental factors (Lavin 2009). The concordance rate for T2D in monozygotic twins is heading towards 100% - much higher than in T1D. For siblings of patients with T2D, the lifetime risk of developing the condition is approximately 40%. Western diet, lack of exercise, obesity and hypertension have all been implicated in the aetiology of type 2 diabetes.

### *1.2.6 Pathophysiology*

T2D is a complex, progressive disease. Research suggests that T2D results from the combination of three factors; pancreatic $\beta$-cell deficiency, insulin resistance within the adipose tissues and skeletal muscles, and excessive hepatic glucose production (Lavin 2009). The earliest defect in patients with T2D is insulin resistance. With disease progression, fasting blood glucose levels increase further, despite increased pancreatic insulin secretion. Results from the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that affected individuals may have lost 50% of their $\beta$-cell function by the time of diagnosis. Further decline in insulin release leads to inadequate suppression of hepatic gluconeogenesis (the generation of glucose from non-carbohydrate carbon substrates such as pyruvate, lactate, glycerol and amino acids), resulting in further fasting and postprandial (the time period occurring after a meal) hyperglycaemia. Thus, the combination of insulin resistance, diminished insulin secretion, along with increased glucose formation by the liver, creates the setting for profound hyperglycaemia.

Despite extensive investigation, the molecular mechanisms of decreased insulin secretion and increased insulin resistance have not been fully clarified. Chronic

elevation of free fatty acids, a characteristic of T2D, as well as structural changes in the islets of Langerhans as a result of amyloid accumulation may contribute to reduced insulin secretion and apoptosis. Histopathological findings have not been elucidated in insulin resistance, but weight loss has been demonstrated to improve insulin sensitivity. Other hormones have also been demonstrated to have a role in the pathogenesis of T2D. These include incretin hormones, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). New therapies for T2D are now targeting these hormonal pathways.

### *1.2.7 Risk Factors*

The main risk factors for T2D, relevant to this project, include:

- family history of diabetes
- cardiovascular disease
- overweight or obese status
- sedentary lifestyle
- hypertension
- increased triglycerides
- low concentrations of high-density lipoprotein cholesterol
- having previously been identified as having impaired glucose tolerance or impaired fasting glucose (Lavin 2009)

### *1.2.8 Diagnosis*

Individuals at risk of developing T2D may be identified before the clinical onset of the disease. The diagnostic criteria, according to the World Health Organisation

(WHO) guidelines, require the following to be met:

Diabetes symptoms (e.g. polyuria, polydipsia and unexplained weight loss for T1D) plus:

- a random venous plasma glucose concentration ≥ 11.1 mmol/l

  *or*

- a fasting plasma glucose concentration ≥ 7.0 mmol/l (whole blood ≥ 6.1 mmol/l)

  *or*

- two-hour plasma glucose concentration ≥ 11.1 mmol/l after consuming 75g glucose in an oral glucose tolerance test (OGTT).

### *1.2.9 Management*

When managing diabetes there are two aims:

- to prevent hypo- and hyperglycaemia, and maintain a level of glycaemic control appropriate to the patient's age, duration of disease, existing complications, compliance, and lifestyle on a day to day basis
- to prevent/treat as necessary the long-term micro- and macrovascular complications of diabetes

The management of T2D may involve one or more of:

- lifestyle modifications, including a very low calorie (600-700/day) diet
- therapy with oral hypoglycaemic agents
- insulin therapy
- surgery

There are several classes of diabetic medications available. New drug approaches have included medications modulating peroxisome proliferator-activated receptors (PPAR) and those that augment the effect of glucagon-like peptide -1 (GLP-1), a hormone that both increases insulin secretion and suppresses glucagon secretion, thereby improving glycaemic control. Unfortunately, safety and cost concerns of new drugs have limited their use to second- or third-line agents that are added to control blood glucose as the disease progresses.

Despite developments and improvements in the medical treatment of T2D, $HbA_{1c}$ (a form of haemoglobin that is measured primarily to identify the average plasma glucose concentration over prolonged periods) targets of < 6.5% are still not being achieved in around half of all diabetics (Selvin, Parrinello et al. 2015). Furthermore, underlying aetiology of T2D is not reversed by the medical therapies on offer. This has therefore positioned bariatric surgery, with its associated improvement and, often, complete reversal of T2D, as an exciting treatment modality.

## 1.3 BARIATRIC SURGERY

The past decade has seen an enormous increase in the number of bariatric operations performed. This trend is likely to continue, mirroring the epidemic of obesity around the world and its rising prevalence among children. Bariatric surgery is considered by many to be the most effective treatment for obesity in terms of maintenance of long-term weight loss and improvement in obesity-related comorbid conditions, specifically T2D. Two of the most used and most effective procedures are Laparoscopic Roux-en-Y Gastric Bypass (RYGB) and Laparoscopic Sleeve Gastrectomy (SG). Laparoscopic Gastric Band has fallen out of favour in the last few years due to an increased number of patients requiring reoperations and revisions, because of failure to lose weight, weight-gain and progressive side effects, such as dysphagia.

### *1.3.1 Laparoscopic Roux-en-Y Gastric Bypass*

RYGB involves gastric size reduction and intestinal rearrangement, such that ingested food bypasses approximately 95% of the stomach, the entire duodenum, and a short portion of the jejunum (**Figure 2**). The procedure consists of the creation of a small gastric pouch (15–30ml) on the lesser curvature of the stomach, which is completely divided from the gastric remnant and then anastomosed to the jejunum, creating an alimentary or Roux limb of approximately 100–150 cm. Bowel continuity is restored by an entero-entero-anastomosis between the excluded biliopancreatic limb (BP) and the Roux limb to form a common channel/limb (Sandoval 2015).

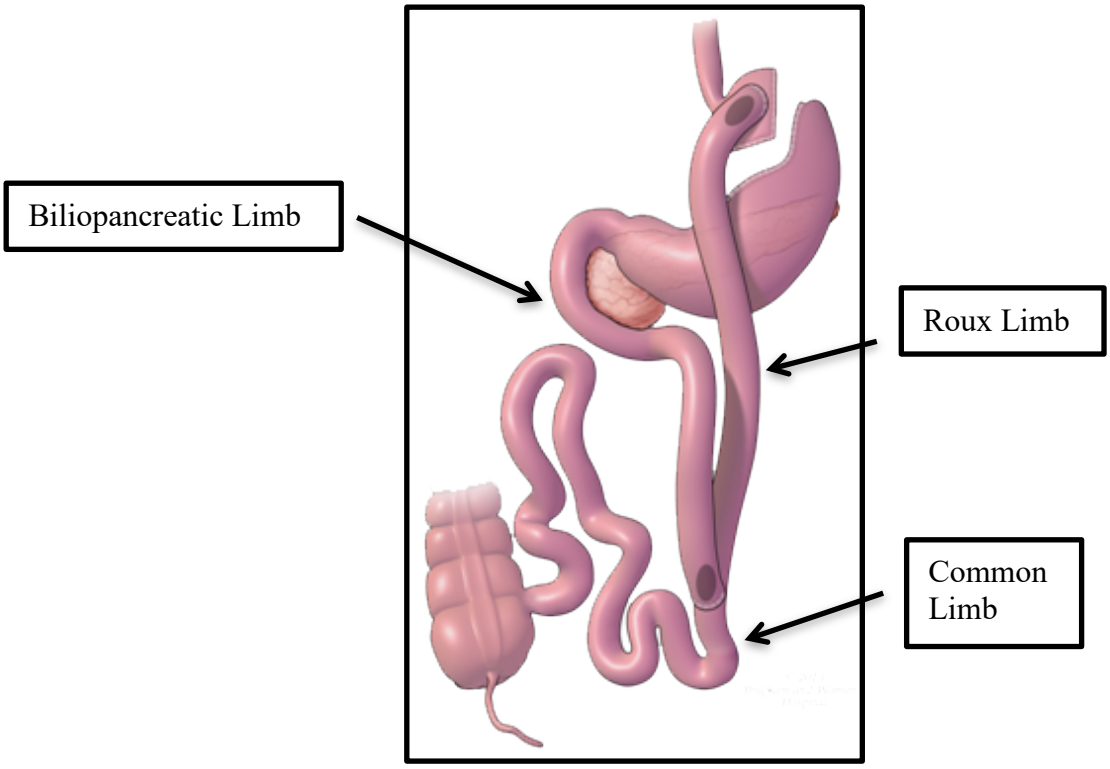



**Figure 2:** Anatomical structure of Roux-en-Y gastric bypass (Brigham and Women's Hospital).

RYGB achieves sustained total body weight loss of 25-35%, with a reduction in obesity related comorbidities, such as T2D, coronary heart disease, hypertension, cerebrovascular accident, gallbladder disease, respiratory disease, osteoarthritis, and some cancers. Although initially evolving from procedures designed to cause malabsorption, subjects undergoing RYGB report only transient gastrointestinal side effects, and the incidence of malabsorption is low. Whilst the beneficial outcomes of bariatric surgery have been repeatedly documented, the mechanisms by which these operations achieve stable weight reduction, with only moderate clinical evidence of malabsorption, have only recently been explored.

The RYGB procedure allows undigested nutrients to bypass most of the stomach, duodenum and proximal jejunum, and to enter the distal jejunum before pancreatic secretions are reunited with the alimentary canal through a second distal anastomosis. As a consequence, digested nutrients have access to a reduced absorptive surface area of the small intestine. Subjects may report early gastro-intestinal side effects, but

clinical malabsorption rarely persists. The degree to which RYGB is tolerated with only a minimal degree of malabsorption has remained perplexing and suggests that the gut may accommodate the altered anatomy and reduced surface area through an adaptive mechanism. RYGB is considered by many to be the gold standard bariatric operation, however, it is now no longer the most commonly performed bariatric operation in the United States, with SG now being the more popular option amongst surgeons (ASMBS , Surgery 2015).

### *1.3.2 Laparoscopic Sleeve Gastrectomy*

SG involves removal of part of the stomach along the greater curvature with no intestinal rearrangement (Sandoval 2015). The procedure consists of subtotal vertical gastrectomy with preservation of the pylorus, including longitudinal resection of fundus, body and antrum, to create a tubular duct along the lesser curvature (**Figure 3**). Resection comprises approximately 80% of the stomach and the gastric remnant has a capacity of approximately 200mL.

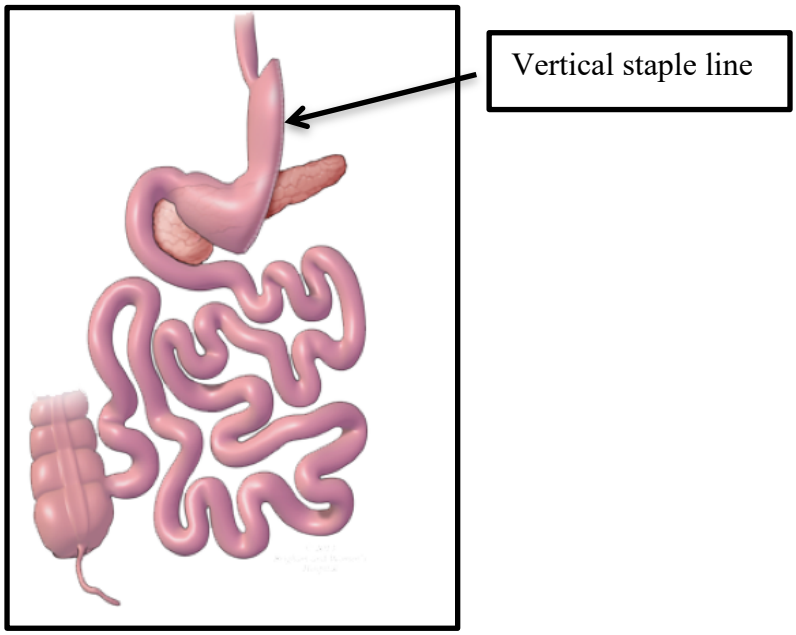



**Figure 3:** Anatomical structure of sleeve gastrectomy (Brigham and Women's Hospital).

### *1.3.3 Bariatric Surgery Outcomes*

*Weight Loss*

Long-term follow-up data is available, in some cases up to nearly two decades. Weight loss averages 65% for most patients, with over 85% of patients losing and maintaining 50% initial excess weight loss. Contemporary series have documented mortality rates of approximately 0.1%, and serious early complication rates of 5%. Long-term issues with fat malabsorption, protein-energy malnutrition and micronutrient deficiencies are relatively uncommon and can usually be managed with oral supplementation. Reoperations are infrequently needed for failures or complications. Despite the high likelihood of success both in weight loss and correction of obesity-related medical conditions, the operation requires advanced laparoscopic surgical skills with a learning curve as long as 100 cases, and a 10-15% long-term failure rate (Madura and Dibaise 2012).

In the UK, NICE Guidelines state that bariatric surgery is a treatment option for people with obesity if the following criteria are fulfilled (Guidelines 2019):

- Patients have a BMI of 40 kg/m2 or more, or between 35 kg/m2 and 40 kg/m2 and other significant disease (for example, type 2 diabetes or high blood pressure) that could be improved if they lost weight.
- All appropriate non-surgical measures have been tried but the person has not achieved or maintained adequate, clinically beneficial weight loss.
- The person has been receiving or will receive intensive management in a tier 3 service.
- The person is generally fit for anaesthesia and surgery.
- The person commits to the need for long-term follow-up.

*T2D Resolution*

Randomised trials have recently demonstrated the superiority of bariatric operations, together with medical therapy, compared with medical therapy alone (Schauer, Bhatt et al.). In a systematic review, Meijer and others report a T2D reversal rate of 83% in patients undergoing the RYGB procedure. Interestingly, more recent non-surgical weight loss studies, including the primary care-based Diabetes Remission Clinical Trial (DiRECT) have shown that 46% of people with T2D could achieve remission at 12 months, and 36% at 24 months, through low calorie diet-mediated weight loss (Taylor, Al-Mrabeh et al. 2019). Additionally, although it has been known for more than 50 years that bariatric surgery can result in remission of T2D, the time course of change had not previously been observed. In a 2006 bariatric surgery study in women, the average participant bodyweight was 152 kg, and it was calculated that they would require about 2700 calories per day to meet their basal energy requirements. But on the evening before bariatric surgery, a severe restriction of calorie intake was instigated (nil by mouth for approximately 24 h then intravenous feeding intended to provide 1800 kcal per day). This restriction is relevant because a year earlier, Petersen and colleagues had shown rapidly decreasing levels of liver fat and complete normalisation of hepatic insulin sensitivity following calorie restriction (to 1200 kcal per day) in people with type 2 diabetes. This effect could account for the mechanism behind the rapid fall in plasma glucose observed post-bariatric surgery.

Nevertheless, the positive effect of bariatric surgery has led to a number of consensus statements supporting bariatric surgery for the treatment of T2D, and has elicited calls for lowering the BMI criteria for surgical intervention in less obese diabetics (Madura and Dibaise 2012). For example, whilst T2D is often associated with obesity, in many

instances, especially in the Asian population, T2D patients are not obese (Xu, Wang et al. 2013). In addition, based on the currently available data for those patients with drug refractory T2D with BMI<30 kg/$m^2$, bariatric surgery might improve glycaemic control and weight loss in a very limited range of patients. However, and more importantly, there is an associated doubled surgical complication rate. Research suggests that it remains too premature to provide bariatric surgery for non-obese T2D patients. (Baskota, Li et al. 2015). Instead, the more appealing alternative is to develop a detailed mechanistic understanding of the metabolic benefits of RYGB, with the goal of harnessing this knowledge to develop less invasive alternatives.

### *1.3.4 Bariatric Surgery Mechanisms*

It has long been observed that, in those with T2D undergoing RYGB and SG, hyperglycaemia often resolves in the early postoperative period, and independently of weight loss. Indeed, observational studies have shown impressive improvements in glycaemic control after bariatric surgery, with rates of improvement in diabetes ranging from 55 to 95% (Schauer, Bhatt et al.), with the greater effect observed after RYGB. The weight-loss independent changes imply an, as of yet, incompletely defined role of the entero-insular axis in this process, with the result of enhanced beta-cell sensitivity to glucose and normalised peripheral insulin sensitivity.

The fact that RYGB and SG have different anatomical rearrangements, and yet demonstrate similar outcomes, is a clear challenge to historical hypotheses that bariatric surgery efficacy is driven by mechanical factors, that is, restriction of meals and/or malabsorption of nutrients, rather than changes in physiology. Bariatric surgery induces numerous physiological changes that have nothing to do with meal restriction or nutrient malabsorption, and which are also comparable between RYGB and SG. These changes include, but are not limited to, a 10-fold increase in nutrient-induced glucagon-like peptide (GLP)-1, elevation of plasma bile acids, alteration of the microbiome, early improvement in hepatic insulin sensitivity, increased insulin response to a meal, and increased gastric emptying rate (Sandoval 2015). With specific regards to RYGB, which is the bariatric procedure to be examined in more detail within the context of this thesis, current research has revealed several post-operative changes that may explain its anti-diabetic effects (**Figure 4**). Considering the complexity of the surgery, the observation of a number of changes is not surprising, and identification of a critical pathway has been elusive.

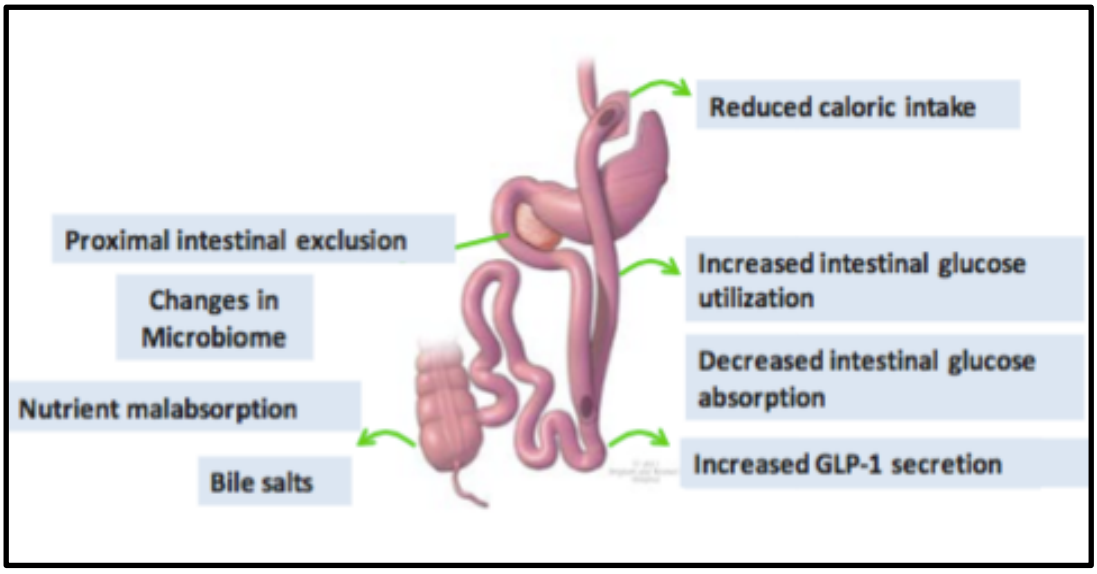



**Figure 4:** Post-operative changes helping to explain anti-diabetic effects of RYGB (Brigham and Women's Hospital).

An interesting difference between the RYGB and SG, however, focuses on their effect on intestinal morphology. The impact of bariatric surgery on intestinal cell growth and its potential direct link to improved glucose homeostasis was explored in a recent manuscript by Cavin et al (Cavin, Couvelard et al. 2015). Data from this study adds to an ever-expanding body of literature that RYGB alters intestinal morphology. RYGB has been found to increase bowel width, villus height, crypt depth, and cell proliferation in the Roux and common intestinal limbs, but only bowel width in the biliopancreatic limb (Taqi, Wallace et al. 2010). Interestingly, similar changes in intestinal morphology have not been observed in SG. However, partial small bowel resection, along with other types of bariatric surgery (such as duodenal jejunal bypass, placement of a duodenal endoluminal sleeve, and ileal interposition) all result in a massive proliferation of the intestinal mucosa (Shaw, Gohil et al. 2012). The question is, however, whether this alteration in intestinal morphology is a crucial requirement when it comes to the metabolic impact of bariatric surgery.

Along with changes in morphology, Cavin et al observed increases in gene and protein expression of several glucose transporters, and a functional increase in glucose transport into enterocytes from the basolateral, rather than the luminal, side of the intestinal wall in the roux limb of RYGB rats (Cavin, Couvelard et al. 2015). This team suggest that the increased intracellular glucose is used by the intestine as a fuel for the observed intestinal hypertrophy and hyperplasia.

It is the laboratory's belief that isolating the proximal bowel from nutrients is an important component of the metabolic benefits of RYGB (Rubino, Forgione et al. 2006), (Stearns, Balakrishnan et al. 2009). Indeed, a non-surgical bariatric technique, the duodenal- jejunal bypass liner (DJBL, GI Dynamics, Lexington, MA), was developed to treat obesity. The DJBL is a 60-cm long impermeable liner which is delivered and retrieved endoscopically. Once in place, it mimics the intestinal bypass component of the RYGB. DJBL treatment has been shown to result in weight loss and improvement of T2D (de Jonge, Rensen et al. 2016). However, unfortunately, the effect of weight reduction of initial DJBL treatment seems to be diminished after 4 years of follow-up (van Rijn, Roebroek et al. 2019).

Studies carried out here have since shed light on an underlying mechanism, which involves isolation of glucose nutrient sensors in the proximal bowel. In addition, increasing amounts of human and animal data carried out by other groups have shown that the early post-operative benefits of surgery on glucose homeostasis stem from an early drop in hepatic insulin resistance (Bojsen-Moller, Dirksen et al.). These considerations have led the lab to believe that changes in portal vein nutrient and hormone levels are critical to the metabolic benefit of RYGB. Lab colleagues have generated data that supports the role of the portal vein as a site of nutrient sensing,

especially after RYGB. Additionally, a potentially critical role for the intestinotrophic hormone, Glucagon Like Peptide 2 (GLP-2) has been identified, in mediating the anti-diabetic effects of RYGB.

### *1.3.5 Intestinal Glucose Metabolism Post-RYGB*

Improvements in glucose homeostasis occur early after RYGB, and before any appreciable weight loss. Indeed, patients are often able to discontinue their anti-diabetic medications before hospital discharge, with approximately 40% of obese patients with T2D going into remission within days or weeks after RYGB (Abdeen and le Roux 2015). The precise mechanisms underlying the resolution of diabetes after RYGB have not been determined. Several studies (including those within this laboratory) have described morphological changes, characterised by hypertrophy in the Roux limb, after RYGB. However, the importance of the morphological adaption of the Roux limb with regards to the metabolic effects of RYGB remains largely unknown. The Roux limb of RYGB-treated rats exhibits reprogramming of intestinal glucose metabolism to meet the increased anabolic demands of intestinal tissue growth and maintenance, and Saeidi et al used PET/CT to confirm that RYGB induces enhanced intestinal glucose absorption and utilisation (Saeidi, Meoli et al.).

They found that the intestine became a major tissue for glucose disposal after RYGB; specifically, glucose disposal per gram of tissue doubled in the intestine of RYGB-treated rats, with the glucose absorption by the Roux limb and common limb being higher in comparison to glucose absorption in the jejunum, in SHAM-operated rats. Significantly, there was a positive correlation between the improvement in glycaemic control after RYGB and the intestinal glucose uptake. Their study concluded that

changes in the metabolism of the Roux limb itself may play a direct role in the improvement in glucose homeostasis after RYGB.

### 1.4 THE PORTAL VEIN HYPOTHESIS

Data from this lab (Laboratory of Surgical and Metabolic Research, Boston, MA) has highlighted significant changes that occur within the portal milieu after RYGB. This is an under-investigated area, with these experiments representing some of the most detailed analyses performed so far. Studies of the anti-diabetic effects of RYGB have been traditionally focused on GLP-1, a known incretin (a hormone that stimulates a decrease in blood glucose levels) with insulinotropic effects. However, with increasing data questioning the importance of GLP-1 (Vetter, Wadden et al. 2015) (Ye, Hao et al. 2014), GLP-2 studies represent a novel focus that could define a potentially critical role for this hormone in metabolic benefits of surgery and have significant impact in this field.

During studies conducted here, using portal vein catheterisation to assess intestinal glucose utilisation absorption, it was noted that the baseline portosystemic glucose gradient ($G_{PS}$ - difference between glucose in the portal vein and the systemic glucose level) (**Figures 5 and 6**) was negative.

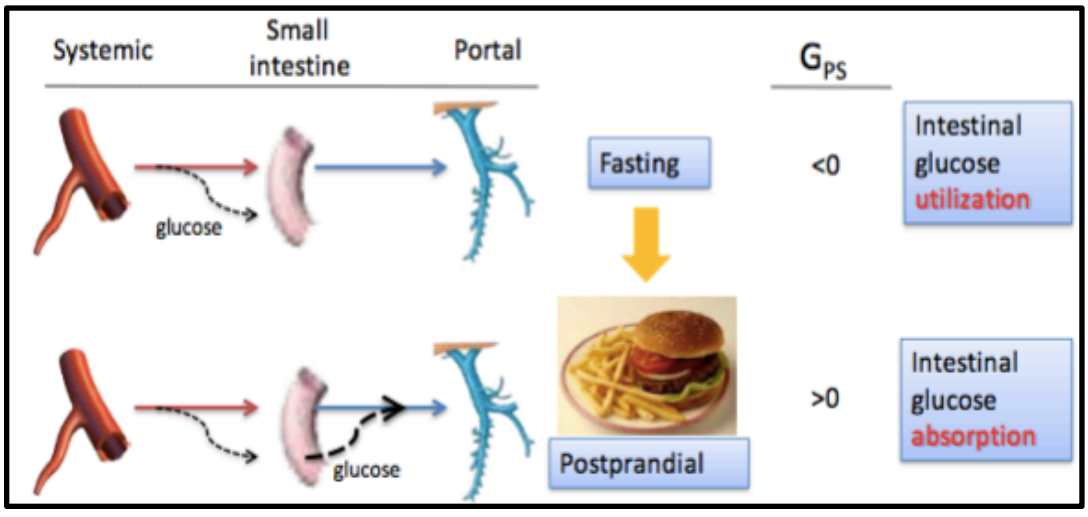



**Figure 5:** Explanation of the Portosystemic Gradient Concept (Tavakkoli 2014).

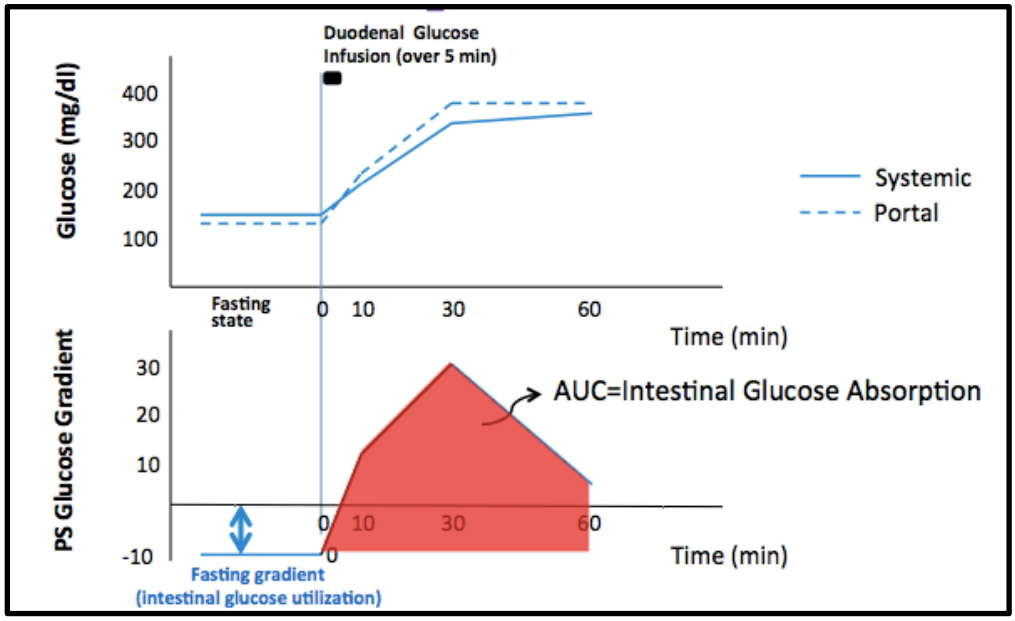



**Figure 6:** Portosystemic Gradient changes observed upon duodenal glucose infusion (Tavakkoli 2014).

This $G_{PS}$ negativity corroborates the fact that the intestine utilises glucose as an energy source. Since lab colleagues had observed intestinal mucosal hypertrophy following RYGB (Stearns, Balakrishnan et al. 2009), with an increase in mucosal thickness, it was hypothesised that intestinal glucose utilisation may have increased after RYGB and this, therefore, initiated experiments to measure any changes in $G_{PS}$ following RYGB. Coincidentally, around this time, Saeidi et al. published a paper documenting an increase in intestinal glucose utilisation following RYGB using PET imaging (Saeidi, Meoli et al.). This observation was then confirmed using the laboratory's catheterisation technique, validating the hypothesis that increased intestinal glucose utilisation contributes to diabetes resolution after RYGB.

To understand how intestinal glucose utilisation increases after RYGB, this laboratory demonstrated an increase in intestinal hexokinase activity and a decrease in intestinal glucose-6-phosphatase after RYGB, consistent with an increase in intestinal glucose utilisation and decrease in intestinal gluconeogenesis. It is believed these biochemical

changes are in large part driven by the hypermetabolic intestine as it undergoes mucosal hypertrophy. To better understand the mechanism behind intestinal hypertrophy, systemic and portal levels of GLP-2, a potent intestinotrophic hormone, were measured. This laboratory's studies revealed a marked increase in systemic and portal GLP-2 levels (**Figure 7**). GLP-2, a gastrointestinal hormone, is known to stimulate intestinal mucosal hypertrophy (le Roux, Borg et al. 2010).

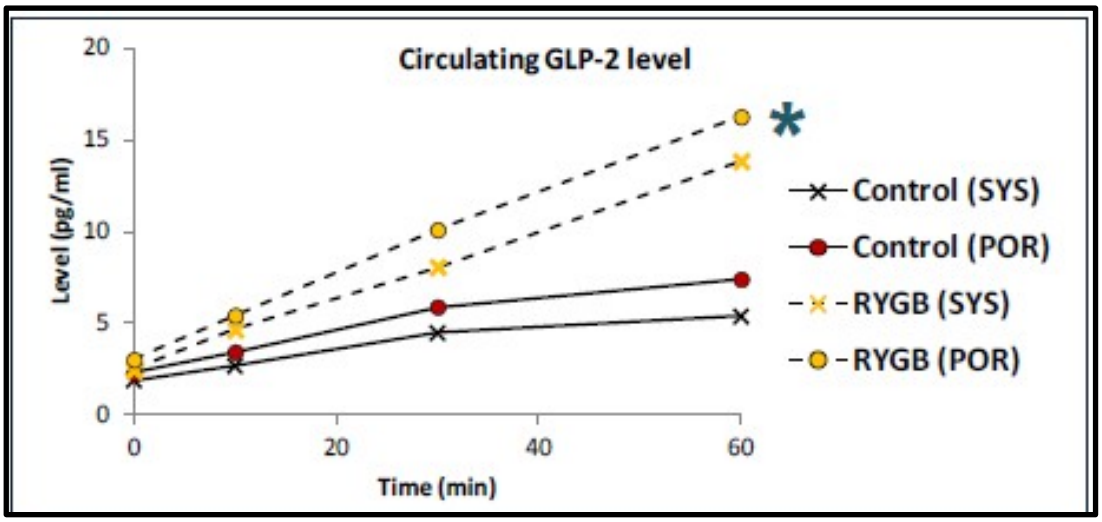



**Figure 7:** Following RYGB, there was a significant increase in both portal and systemic circulating levels of GLP-2. *p<0.05 for RYGB animals compared to corresponding values from controls (Tavakkoli 2014).

## 1.5 GASTROINTESTINAL HORMONES

Being a key organ for digestion, absorption, and assimilation, the gastrointestinal tract plays a crucial role in controlling energy intake (Shi, Zhou et al. 2013). It is no surprise therefore that multiple peptide hormones produced within the gastrointestinal tract aid in the regulation of energy homeostasis and metabolism (Estall and Drucker 2006). In the fight to stem both obesity and T2D, attention has turned to exploring the role of these gastrointestinal hormones. Gut hormones convey nutrient-regulated signals to the enteric nervous system and to distal organs, acting as circulating hormones secreted in the fasting and postprandial state (Drucker 2015). Interactions

between food, the gut and the brain form a key feedback loop, around which food and energy intake is regulated. Within this regulatory system, bioactive peptides play a major role in both the periphery and the brain, and in the signalling between the two, contributing to interconnected metabolic, endocrine, neural and neuroendocrine systems (Mercer 2015).

Of particular interest are the hormones glucagon-like peptide 1 (GLP-1) and GLP-2, both made and secreted by entero-endocrine cells, known as L-cells, and by neurons in the CNS. Whereas GLP-1 has been found to act as an incretin, that is, a hormone which both promotes secretion of insulin and inhibits secretion of glucagon, and as a result stimulates a decrease in blood glucose levels, GLP-2 is better known for its ability to enhance intestinal function. Less is known about GLP-2's effect on blood glucose levels.

### *1.5.1 GLP-2*

GLP-2 is a 33 amino acid peptide (**Figure 8**) and is related in sequence to GLP-1, glucagon, glucose-dependent inhibitory peptide (GIP) and other members of the glucagon peptide super-family (**Figure 9**).

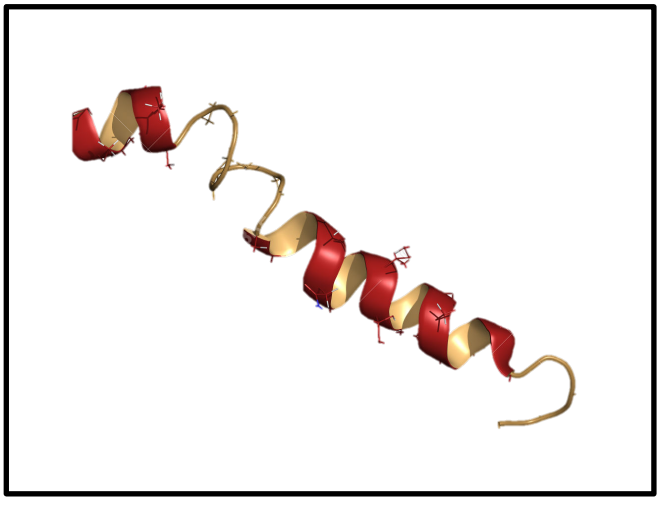


**Figure 8:** GLP-2 Structure (http://www.123rf.com/photo_24079276_glucagon-like-peptide-2-glp-2-peptide-molecule-its-recombinant-analog-teduglutide-is-used-to-treat-s.html).

GLP-2 is produced and secreted by the intestinal L-cell, in response to nutrient ingestion (Rowland and Brubaker 2011). The L-cell is a specialised entero-endocrine cell most commonly found in the ileum and colon, however, it can also be found more proximally within the small intestine, including the jejunum and even the ileum. GLP-2 is also synthesised in the brain, predominantly in the brain stem, from where it may be transported to distal regions of the central nervous system to exert more-localised actions.

GLP-2 was first discovered as an intestinotrophic (stimulating or regulating growth of intestinal tissue) factor in 1996 by Drucker et al (Drucker, Erlich et al. 1996). GLP-2 is now recognised as a hormone that influences multiple functions, specifically in the gastro-intestinal tract. For example, it increases mesenteric blood flow and activates pro-absorptive pathways in the gut, facilitating nutrient absorption. GLP-2 also enhances gut barrier function and induces proliferative and cytoprotective pathways in the small bowel (Drucker 2015).

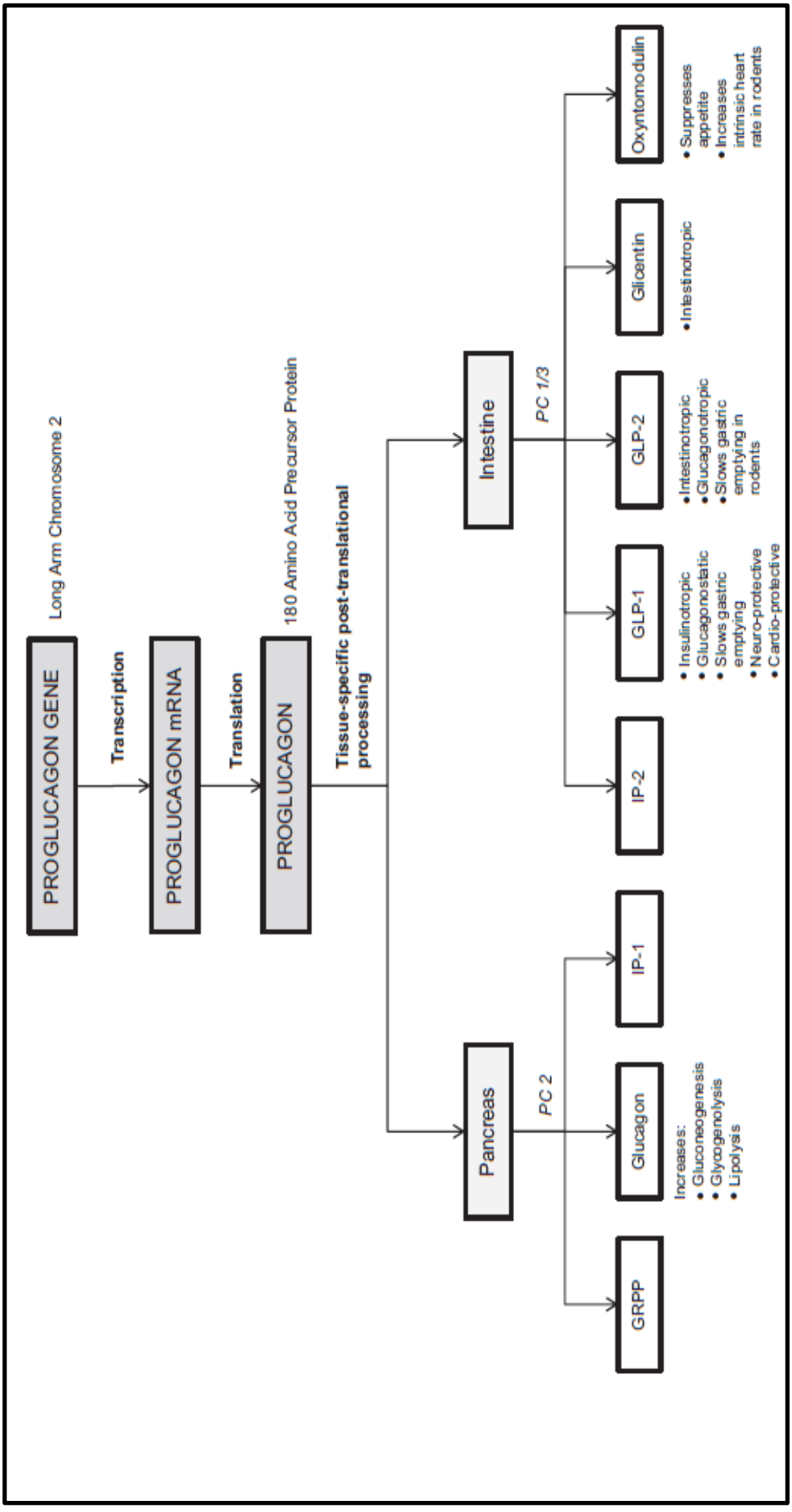



**Figure 9:** Diagram showing how the peptides, glucagon, GLP-1 and GLP-2, share a common precursor gene, proglucagon (Marathe, Rayner et al. 2013).

The discovery of GLP-2's potent growth-promoting and cytoprotective effects in the GI tract stimulated interest in its use as a therapeutic agent for the treatment of GI disease involving malabsorption, inflammation and/or mucosal damage (Estall and Drucker 2006). Within the gut, GLP-2 increases nutrient absorption, crypt cell proliferation, and mesenteric blood flow and decreases gut permeability (Cani, Possemiers et al. 2009) and motility, epithelial cell apoptosis, and inflammation. Outside the gut, GLP-2 reduces bone resorption, can suppress appetite, and is cytoprotective in the lung (Connor, Evock-Clover et al. 2015)

The biological half-life of circulating GLP-2 is relatively short (approximately 7 minutes in humans), due to extensive renal clearance and rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP-4) (Hartmann, Harr et al. 2000). The importance of DPP-4 in the regulation of GLP-2 bioactivity was originally shown in rats, as administration of exogenous native GLP-2 had only a modest effect on bowel growth due to high levels of endogenous DPP-4 activity. Administration of a GLP-2 analogue lacking the N-terminal DPP-4 cleavage site significantly increased bowel weight in these rats relative to administration of native GLP-2. The metabolite produced as a result of DPP-4 cleavage, GLP-2 (3-33), was demonstrated to act as a competitive antagonist of the GLP-2 receptor (GLP-2RA), and inhibited GLP-2-induced mucosal growth in rodents (Shin, Estall et al. 2005), (Thulesen, Knudsen et al. 2002). Interestingly, GLP-2 (3-33) may also act as a weak GLP-2R agonist at

pharmacological concentrations. Thus, the biological actions of GLP-2 may be limited both by its rapid enzymatic degradation and renal clearance, and through competition with the products of its own degradation. Accordingly, the degradation-resistant

analogue of GLP-2 (Teduglutide, TED) is now used in the treatment of human gastrointestinal disease, such as short-bowel syndrome (Jeppesen 2012).

### *1.5.2 GLP-2 Receptor*

In order to understand GLP-2 analogues, a better understanding of the GLP-2 receptor (GLP-2R) is required. The GLP-2R is a protein that in humans is encoded by the GLP-2R gene located on chromosome 17. Responses to GLP-2 are mediated via the GLP-2R. It is a member of the glucagon/secretin G protein-coupled receptor (GPCR) super-family. Precise tissue distribution of GLP-2R expression remains poorly understood in both humans and rodents. It has been reported that GLP-2R expression is mainly confined to the GI tract, particularly to the stomach, duodenum and proximal small bowel. Some studies have revealed weak expression of GLP-2R in the colon. Extra-intestinal expression of GLP-2R has been reported in the CNS, lung and pancreas in humans and rodents. At a cellular level, GLP-2R is expressed in the enteroendocrine L cells, located within intestinal subepithelial myofibroblasts. They can also be found in the enteric neurons of the submucosal and myenteric plexus, pancreatic alpha cells and astrocytes in the brain (El-Jamal, Erdual et al. 2014).

Other groups have suggested that given only modest evidence for GLP-2R expression in enterocytes (intestinal lining cell), it is more likely that the proliferative and cytoprotective effects of GLP-2 observed are indirect. Indeed, findings imply an indirect model for GLP-2 action whereby GLP-2 released from enteroendocrine L-cells or rodent neurons stimulates the release of downstream mediators of GLP-2 action, with these mediators responsible for the proliferative, anti-apoptotic, and pro-absorptive effects (Mayo, Miller et al. 2003) (**Figure 10**).

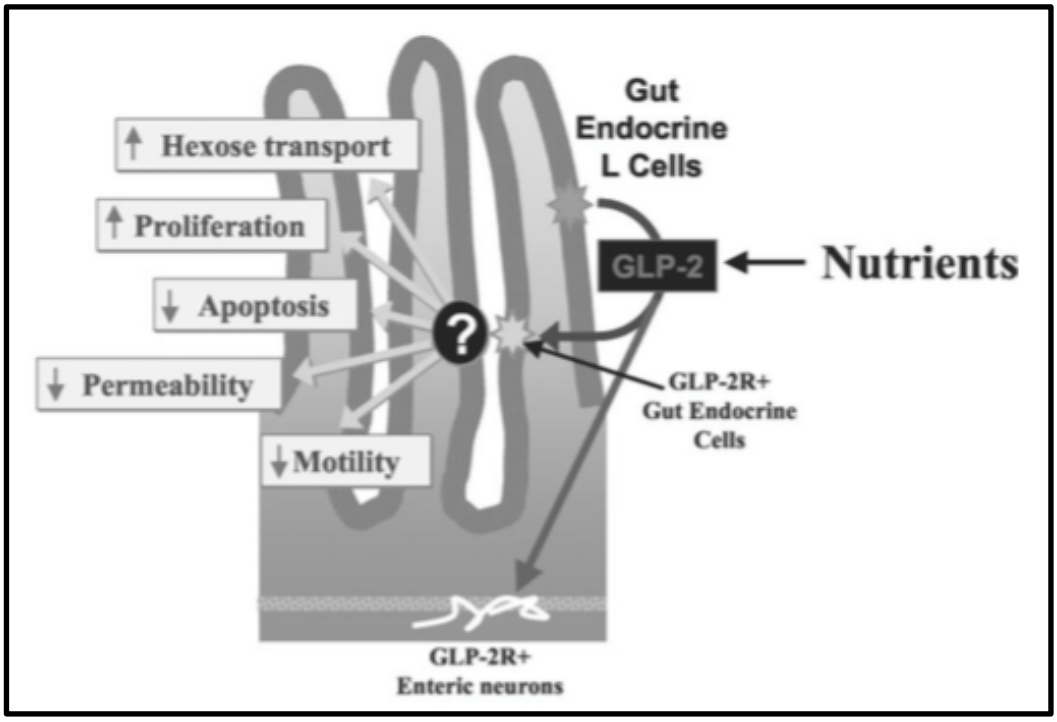



**Figure 10:** Schematic representation of GLP-2 action in the gastrointestinal epithelium via GLP-2 receptors expressed in the enteric nervous system (mouse) or enteroendocrine cells (human) (Mayo, Miller et al. 2003).

*1.5.3 GLP-2 Studies Within This Laboratory*

As part of the on-going challenge to understand how RYGB contributes to the amelioration of hyperglycaemia, this laboratory has found that changes in GLP-2 levels post-RYGB mediate intestinal hypertrophy and contribute to improved glucose balance. With regards to intestinal hypertrophy, whole bowel weight (and specifically Roux limb weight) in post-RYGB rats was significantly higher at 4 weeks compared to bowel in control rats (which had been subjected to SHAM-laparotomy) (**Figure 11**).

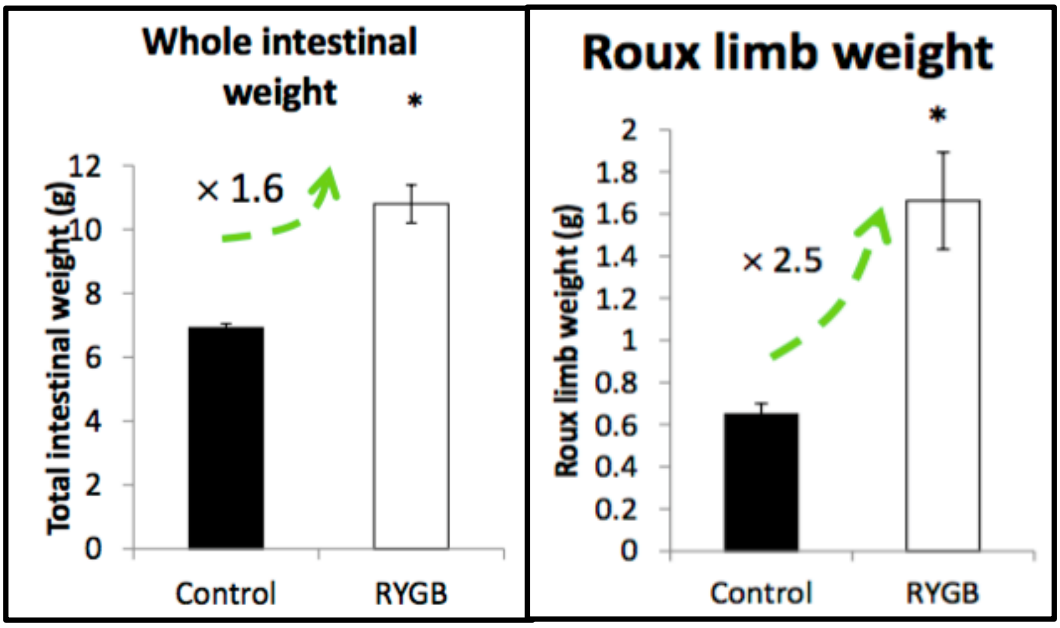



**Figure 11:** Increase in whole intestinal weight, and Roux limb weight, in control RYGB rats (*p<0.05) (Pal 2014).

Intestinal glucose balance was calculated by measuring hexokinase activity (the enzyme involved in converting glucose in to glucose-6-phosphate). A rise in hexokinase activity signified an increase in intestinal glucose utilisation. Glucose-6-phophatase (the enzyme required to convert glucose-6-phosphate into glucose) and phosphoenolpyruvate carboxykinase (PEPCK), another enzyme used in the metabolic pathway of gluconeogenesis, were also measured. Both were found to have decreased in RYGB rats after a period of 4 weeks from day of surgery, thus signifying a decrease in intestinal gluconeogenesis. The results showed an overall net increase in intestinal glucose utilisation and are summarised in **Figure 12**.

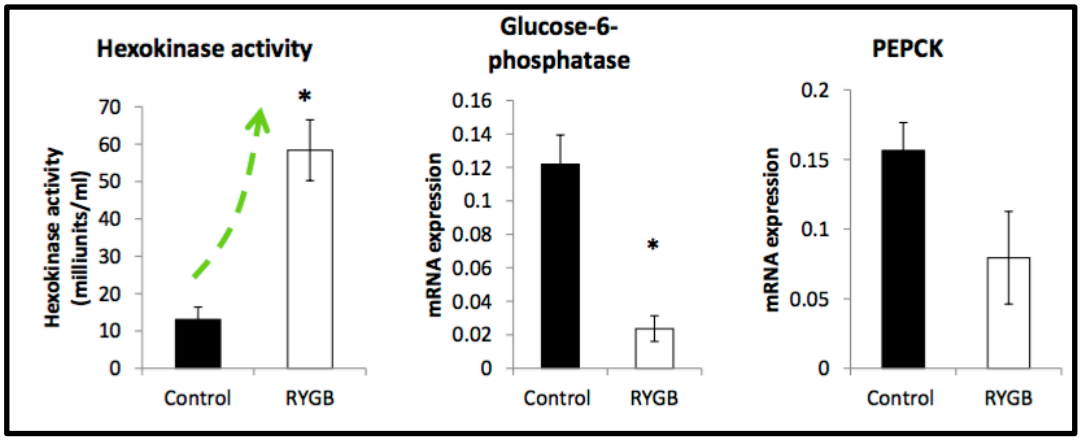



**Figure 12:** Hexokinase activity, and mRNA expression of glucose-6-phosphatase and PEPCK in control and RYGB rats (Pal 2014).

This is perhaps more clearly summarised in **Figure 13**.

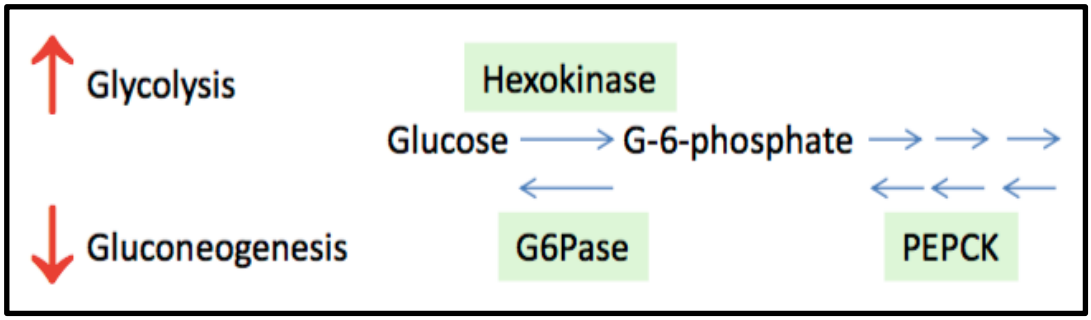



**Figure 13:** Simplified version of pathways in glycolysis and gluconeogenesis (Pal 2014).

Levels of GLP-2 were also found to be significantly higher in RYGB rats at 60 minutes post duodenal glucose infusion compared to controls (**Figure 14**).

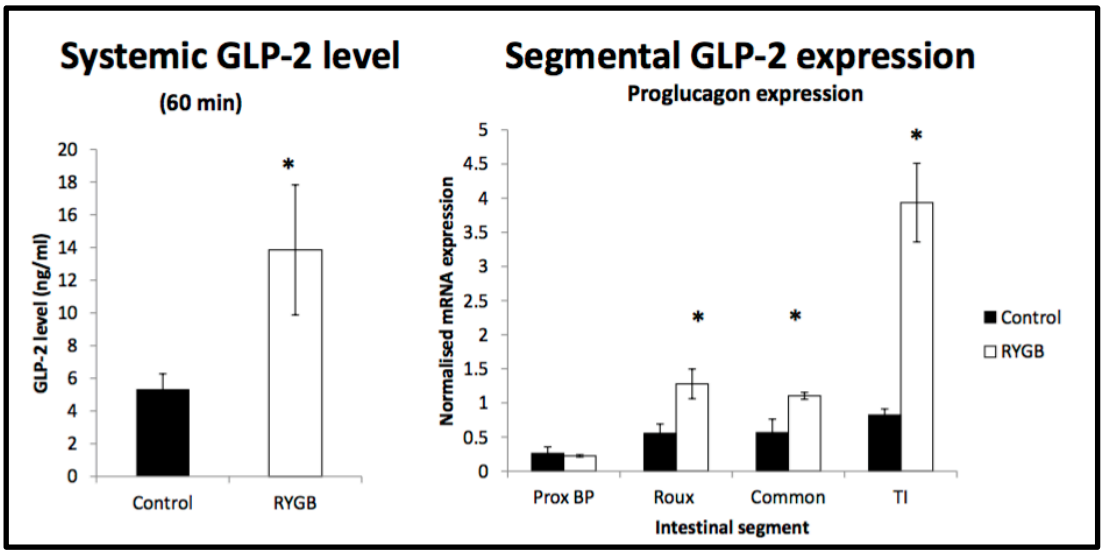



**Figure 14:** Systemic blood GLP-2 levels, and mRNA expressions of GLP-2 within the intestine, in control and RYGB rats (Pal 2014).

GLP-2 is well known for its intestinotrophic role. Recently, research, including that carried out in our lab, has revealed that, like GLP-1, it may also have a role in the maintenance of glucose homeostasis (Shi, Zhou et al. 2013), (Bahrami, Longuet et al. 2010). It is apparent that GLP-2 plays a complex role within the intestine - the seemingly overwhelming interest attracted by GLP-2 as a trophic factor may have somewhat clouded the importance of the peptide in other gastrointestinal processes (Baldassano and Amato 2014). As this lab has already suggested, RYGB causes a large increase in net glucose to be taken up by the gut from the systemic circulation and this increased glucose uptake correlates with intestinal hypertrophy and increased expression and secretion of GLP-2 (Pal 2014). More research into GLP-2's metabolic action, in particular, its contribution to glucose homeostasis, is required as this hormone may reveal novel targets for the treatment of one of the most dangerous conditions threatening world health: T2D.

### *1.5.4 Other GLP-2 Studies*

Most GLP-2 administration studies that have been conducted have been done so in the context of looking at the intestinotrophic effects of GLP-2 on enteropathies. As part of these studies, some teams have looked at the effect of GLP-2 on glucose and hormone levels. These are discussed below.

#### *Human Studies*

One group acknowledged that the acute effects of GLP-2 on glucose and lipid homeostasis in humans are less well characterised than in animal studies. They infused healthy male volunteers with GLP-2 (2 picomol/kg/min), or placebo, over 120 and 390 minutes in the fasting and postprandial states, respectively (Meier, Nauck et

al. 2006). Specifically, they found that GLP-2 administration markedly increases glucagon concentrations during both fasting and post-prandial states. They suggested that this was a novel result as only one previous study had measured plasma glucagon concentrations during GLP-2 administration. In these experiments, intravenous administration of a 5-fold lower GLP-2 dose led to miniscule elevations in glucagon plasma concentrations. They also found that, with regards to glucose levels in the fasting state, plasma glucose concentrations did not change during the administration of either placebo or GLP-2. Meal ingestion led to an increase in plasma glucose concentrations, but no differences in glucose levels occurred between the experiments with GLP-2 or placebo administration.

Another study examined the acute effects of GLP-1 and GLP-2, alone and in combination, on intestinal absorption. The effect of these infusions on glucose were also studied. A dose of 1 picomol/kg/min of both GLP-1 and GLP-2 was used and infused for 24-hour time periods. At 15 minutes after start of infusions, GLP-1 and GLP-1 + 2 lowered the glucose levels significantly compared to placebo. The same was seen at 60 minutes. At 120 minutes, glucose levels were elevated in relation to GLP-2 treatment compared to placebo, GLP-1 and GLP-1 + 2 treatments. At 180 minutes, glucose concentrations reverted to baseline levels and no differences were seen between treatments (Madsen, Askov-Hansen et al. 2013).

*Rodent Studies*

Given that this lab has approval for the use of rats within its RO1 Grant, the following studies involving GLP-2 infusion in rats were useful in terms of dosage guidance. Kaji et al studied the effects of variations in dose and method of administration on GLP-2 activity in the rat, with the primary outcome being the biological response of the

intestinal mucosa (Kaji, Tanaka et al. 2008). They did not assess the effect of GLP-2 on blood glucose levels. Sprague-Dawley rats (**Figure 15**) were infused with GLP-2 (along with Trans-Parenteral Nutrition - TPN) through a central venous catheter (via the right external jugular vein). Results showed that high dose, continuous infusion of GLP-2 (240 μg/kg/day) resulted in the most significant results in terms of small bowel adaptation. The researchers were surprised to find that the high dose group showed a significant increase in total body weight compared with the other groups, despite receiving identical nutritional support.

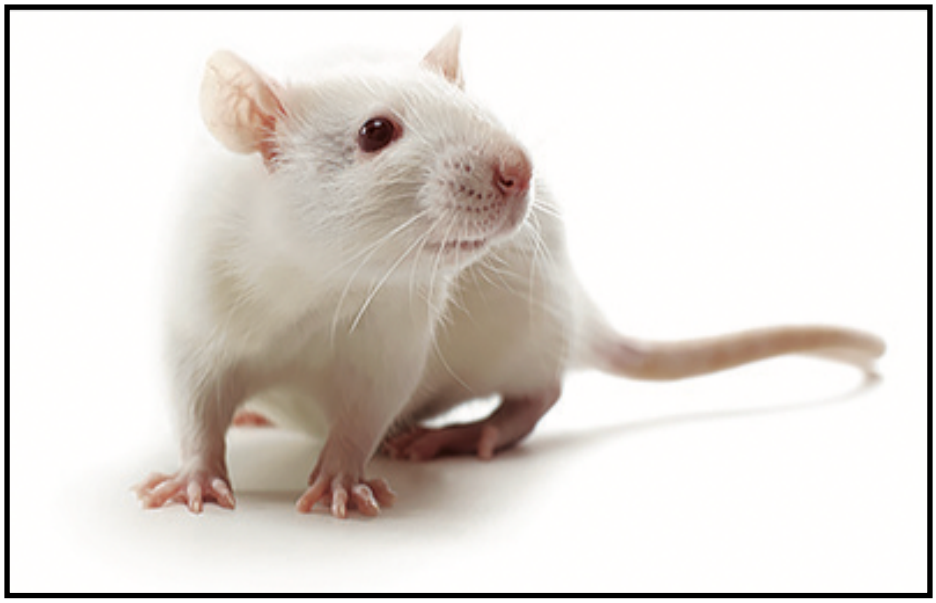


**Figure 15:** Sprague-Dawley rat (https://www.criver.com/sites/default/files/resource-files/spague-dawley-rats-us-pricing.pdf)

A literature review has not revealed any rat GLP-2 infusion studies that measure blood glucose as a primary or secondary endpoint. However, one team did look at the effects of GLP-2 infusion on glucose transporter activity in rat jejunum (Cheeseman 1997). The transport capacity for hexoses in the small intestine of many species can be changed to meet the dietary load over a period of a few days. This allows the animal to take advantage of the altered energy content of the food and at the same time ensure that hexose transporter protein expression is sufficient to allow for complete absorption of hexoses from the lumen to the bloodstream.

Two major transport proteins are known to be involved in this process; sodium-glucose linked transporter 1 (SGTL-1) and glucose transporter 2 (GLUT-2). Expression of these transport proteins can be altered to match the dietary hexose content such that SGLT-1 and GLUT-2 will increase in response to an elevated glucose intake. Details of the signals and mechanisms of this control mechanism are

not known, but control appears to be exerted at both the transcriptional and translational level. GLP-2 was infused into the external jugular vein, and at a concentration of 800 picomol/hour for 2 hours. Cheeseman et al found that acute GLP-2 infusion led to an increase in abundance of SGLT-1 within the rat jejunal brush border membrane (BBM). Additionally, Au et al demonstrated that GLP-2 appears to promote the insertion of GLUT-2 into the rat jejunal BBM, providing a low-affinity/high-capacity route of entry for dietary hexoses (Au, Gupta et al. 2002). Additionally, another member of the sodium/glucose cotransporter gene family, SGLT-3, has been identified. It is thought that this protein has a role in glucose *sensing*, rather than glucose *transport* (Ashley Blackshaw and Young 2011).

### *1.5.5 Other Gastrointestinal Hormones of Note*

During recent years, it has become clear that the gastrointestinal tract constitutes the largest and most varied endocrine organ of the body. More than 40 hormones originate from the gastrointestinal tract, and several of these exert considerable impact on glucose metabolism and appetite regulation. The different gastrointestinal hormones originate from specialised enteroendocrine cells scattered throughout the gastrointestinal tract, with certain enteroendocrine cells located in specific regions and others more widespread and some with different density along the gastrointestinal tract. The most important tasks of this complicated system of diverse enteroendocrine cells integrated amid muscle, nerve, and exocrine tissues are as follows: *1*) to sense the amount and type of nutritional content in the lumen of the gut and *2*) to signal to tissues and organs involved in metabolism and deposition of nutrients, making them react in unison to provide optimal short- and long-term conditions for handling of

nutrients (Knop and Taylor 2013).

Some other gastrointestinal hormones which have been explored for their function within bariatric surgery mechanisms include the following: peptide YY (PYY), GLP-1, ghrelin, glucose dependent insulinotropic polypeptide (GIP) and leptin. PYY inhibits gastrointestinal mobility, along with gastric, pancreatic and intestinal secretion. It induces satiety and reduces nutrient intake in both obese and non-obese. Interestingly, obese individuals appear to have a PYY deficiency that could affect satiety signals and could thus reinforce obesity. Ghrelin, on the other hand, stimulates appetite, increases food intake and promotes the storage of fat. In a comparative study of laparoscopic sleeve gastrectomy and gastric bypass using a randomised, double-blind design, both fasting and postprandial PYY levels, were increased similarly postoperatively (Karamanakos, Vagenas et al. 2008). The markedly reduced ghrelin levels in addition to increased PYY levels after sleeve gastrectomy are associated with greater appetite suppression and excess weight loss compared with gastric bypass. The authors hypothesised that the reduced ghrelin after sleeve gastrectomy has an additive effect upon the PYY response on appetite control. An animal study supports this hypothesis by demonstrating that ghrelin attenuates the inhibitory effect of PYY and GLP-1 on food intake and gastric emptying in a dose-dependent manner (Chelikani, Haver et al. 2006). Both RYGB and Sleeve Gastrectomy (SG) have been shown to increase GLP-1. Data has shown that the postprandial rise in GLP-1 might affect feeding behaviour after RYGB and to a lesser extent SG, where the increase in GLP-1 is less marked.

Finally, secretion of leptin decreases food intake and increases energy expenditure through its effects on the hypothalamus. Leptin circulates in pro-portion to whole-

body adipose tissue mass. For example, increased body fat results in increased leptin, which ultimately stimulates reduced food intake, and the converse is also true with decreased body fat. However, increased leptin levels do not prevent obesity, therefore, it has been suggested that the progression of obesity is not a result of leptin deficiency but instead leptin resistance. The leptin data are not as consistent as those reported for other gastrointestinal hormones. Some studies have demonstrated significantly lower fasting leptin concentrations in the post-RYGB and SG patient population compared to pre-RYGB/SG subjects, whereas others did not find a statistically significant difference between study populations.

Given that a potential role for GLP-2, in terms of its contribution to bariatric surgery T2D outcomes, has only recently come to light, and given the work already carried out within this lab, it was decided that experimental studies to help delineate GLP-2's role further would be conducted. The overriding aim was to discover whether GLP-2 could provide the answer, or at least part of an answer, to the question that scientists and surgeons have been asking since the link between bariatric surgery and the amelioration of T2D was first made over 15 years ago.

# CHAPTER 2

## 2.1 OVERALL HYPOTHESIS

"The gut hormone, glucagon-like peptide 2, contributes to the improved glycaemic control observed post-RYGB, as a result of its intestinotrophic effects."

In order to investigate this hypothesis, five aims were developed:

## 2.2 AIMS

- **Aim 1:** Investigate the effects of acute GLP-2 administration (via venous infusion), in healthy Sprague-Dawley (SD) rats, fed standard chow (SC).

- **Aim 2:** Investigate the effects of chronic GLP-2 administration (via subcutaneous injection), in SD rats fed:

    - standard chow and
    - high fat diet (HFD) (pathological model)

- **Aim 3:** Investigate the effects of RYGB on glucose homeostasis.

- **Aim 4:** Investigate the effects of GLP-2 Receptor Antagonism post-RYGB (with altered biliopancreatic limb length of 40cm v 20cm) in HFD fed rats (pathological model).

- **Aim 5:** Investigate the effects of mimicking RYGB acutely by comparing proximal and mid-jejunal glucose infusion.

## 2.3 AIM 1: ACUTE GLP-2 SYSTEMIC VENOUS INFUSION

- Investigate the effects of acute GLP-2 administration (via infusion), in healthy SD rats, fed standard chow (SC).

### *2.3.1 Overview*

*Assess the acute effects of systemic infusion of GLP-2 on systemic and portal blood glucose levels in normal, healthy Sprague-Dawley (SD) rats, fed standard chow:*

- Non-survival surgery.
- Portal and systemic catheterisation technique.
- Portal and systemic blood sampling to assess fasting glycaemia in portal circulation (hepatic-portal vein) and systemic circulation (external jugular vein).
- Systemic GLP-2 infusion.
- Postprandial glycaemia in portal and systemic circulations assessed (after duodenal glucose infusion – catheter placed in duodenum).
- Calculate portosystemic gradient ($G_{PS}$) to assess intestinal glucose utilisation in fasting state and glucose absorption in postprandial states.
- Portal and systemic blood taken for GLP-2 levels, to be analysed by ELISA (Luminex unable to offer GLP-2 analysis).
- Portal and systemic blood taken for analysis of other hormones (insulin, glucagon, GLP-1, GIP); to be analysed using Luminex.
- Jejunal mucosa (at 26cm from pylorus and equivalent to Roux limb) harvested and PCR conducted to look for evidence of glucose transporter gene upregulation (GLUT-1 and GLUT-2), and an increase in cell proliferation genes (C-MYC, C-FOS).

*2.3.2 Hypothesis*

*It is hypothesized that infusing GLP-2 into the systemic circulation, in the fasted state, will lead to:*

- A lowering of the level of portal blood glucose (compared to control) due to increased glucose utilisation by the intestine, and reduction of glucose entering the portal drainage system as a result of this.
- A lowering of the level of systemic blood glucose due to increased glucose uptake from the systemic circulation by the intestine.
- A greater decrease in portal blood glucose levels compared to the decrease in systemic blood glucose levels, hence resulting in a more negative $G_{PS}$.
- Evidence of increased intestinal glucose utilisation in order to exert adaptation effects i.e. increase in glucose transporter genes/proteins and an increase in cell proliferation genes/proteins.

*It is hypothesized that infusing GLP-2 into the systemic circulation, in the post-prandial state, will lead to:*

- An even more pronounced GLP-2 driven hypoglycaemic effect given the increased availability of glucose secondary to luminal glucose absorption in the presence of GLP-2, meaning a predicted quicker return to baseline blood glucose levels.
- Upregulation of glucose transporter and intestinal proliferation genes and, therefore, proteins.

## 2.4 EXPERIMENTS

### *2.4.1 Materials and Methods*

*Glucagon-like-peptide 2 (GLP-2)*

500µg of lyophilised GLP-2 was purchased from Abcam (ab142306, Cambridge, MA) (**Figure 16**). The product had been internally validated by Abcam. The chemical structure was given as:

"His-Ala-Asp-Gly-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Thr-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp"

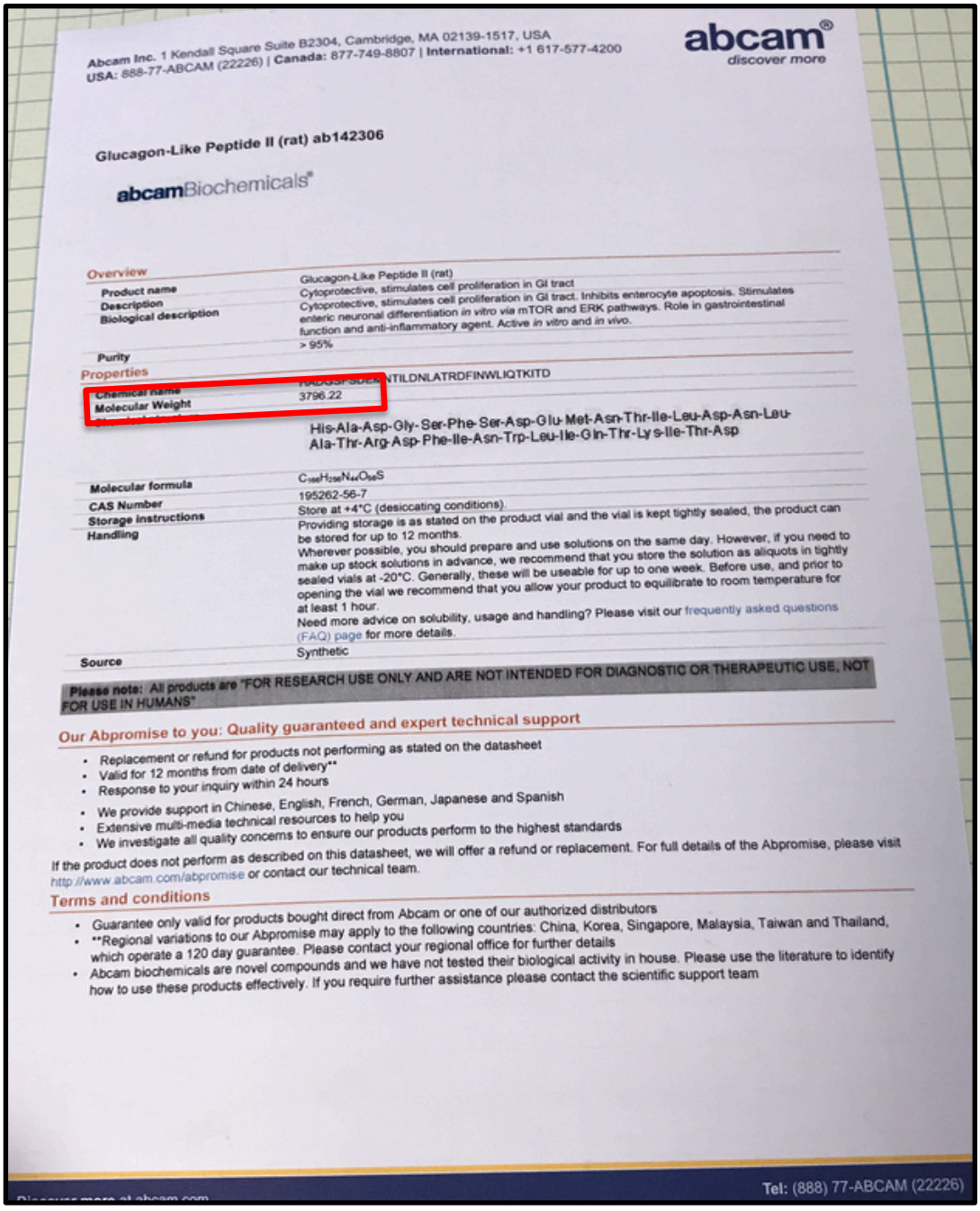
With a chemical formula of:

$C_{166}H_{256}N_{44}O_{56}S$

With a molecular weight of:

3796.22g/mol

(Theoretical weight: 3796.2g/mol).



Abcam Inc. 1 Kendall Square Suite B2304, Cambridge, MA 02139-1517, USA
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abcam discover more

Glucagon-Like Peptide II (rat) ab142306

abcamBiochemicals

Overview

| | |
|---|---|
| Product name | Glucagon-Like Peptide II (rat) |
| Description | Cytoprotective, stimulates cell proliferation in GI tract |
| Biological description | Cytoprotective, stimulates cell proliferation in GI tract. Inhibits enterocyte apoptosis. Stimulates enteric neuronal differentiation *in vitro* via mTOR and ERK pathways. Role in gastrointestinal function and anti-inflammatory agent. Active *in vitro* and *in vivo*. |
| Purity | > 95% |

Properties

| | |
|---|---|
| Chemical name | [illegible]NTILDNLATRDFINWLIQTKITD |
| Molecular Weight | 3796.22 |

His-Ala-Asp-Gly-Ser-Phe-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Thr-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp

| | |
|---|---|
| Molecular formula | $C_{158}H_{256}N_{44}O_{56}S$ |
| CAS Number | 195262-56-7 |
| Storage instructions | Store at +4°C (desiccating conditions) |
| Handling | Providing storage is as stated on the product vial and the vial is kept tightly sealed, the product can be stored for up to 12 months.<br>Wherever possible, you should prepare and use solutions on the same day. However, if you need to make up stock solutions in advance, we recommend that you store the solution as aliquots in tightly sealed vials at -20°C. Generally, these will be useable for up to one week. Before use, and prior to opening the vial we recommend that you allow your product to equilibrate to room temperature for at least 1 hour.<br>Need more advice on solubility, usage and handling? Please visit our frequently asked questions (FAQ) page for more details. |
| Source | Synthetic |

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**Figure 16:** Abcam GLP-2 data sheet.

*GLP-2 Dose Calculations for Experiments 1-5*

**EXPERIMENT 1**

As guided by studies carried out by Kaji et al, who demonstrated that continuous infusion of GLP-2 (240 µg/kg/day) resulted in the most significant results in terms of small bowel adaptation (Kaji, Tanaka et al. 2008), it was decided that a dose of 240µg/kg would be used per rat.

**To limit freeze/thaw cycle to once only for each GLP-2 infusion, 6 aliquots were prepared:**

6 aliquots of 80µg (Abcam sheet suggests GLP-2 purity >95%)

6 x 80 = 480µg

NB: The measurement of 80µg was chosen as this would be the amount required for a 330g rat. Most rats were expected to weigh below 300g, however, as rats weighing 220-240g were purchased.

**As protein keeps better in a more concentrated solution:**

500µg GLP-2 was dissolved in 1ml phosphate buffered saline (PBS, a buffer with a neutral pH of 7.5)

0.5µg GLP-2 in 1µl PBS

1µg GLP-2 in 2µl PBS

So, 80µg GLP-2 in 160µl PBS

Each of the 6 aliquots required to contain 160µl of GLP-2/PBS

**According to the amount of GLP-2 required i.e. 240µg/kg:**

For a 250g rat, 60µg GLP-2 is required

**To dilute up to 2ml (for 3ml syringe used in infusion pump):**

Add 1.84ml PBS (2ml – 0.160ml (160µl) = 1.84ml)

Each aliquot contains 80µg of GLP-2 in 2ml, so the concentration of GLP-2 is 40µg/ml.

For a 250g rat, according to calculations, 1.5ml of infusate is required.

Taking in to consideration the volume required to prime the 15cm catheter:

**Total infusate = 1.531ml.**

A 2ml eppendorf was used to store the 2ml GLP-2/PBS.

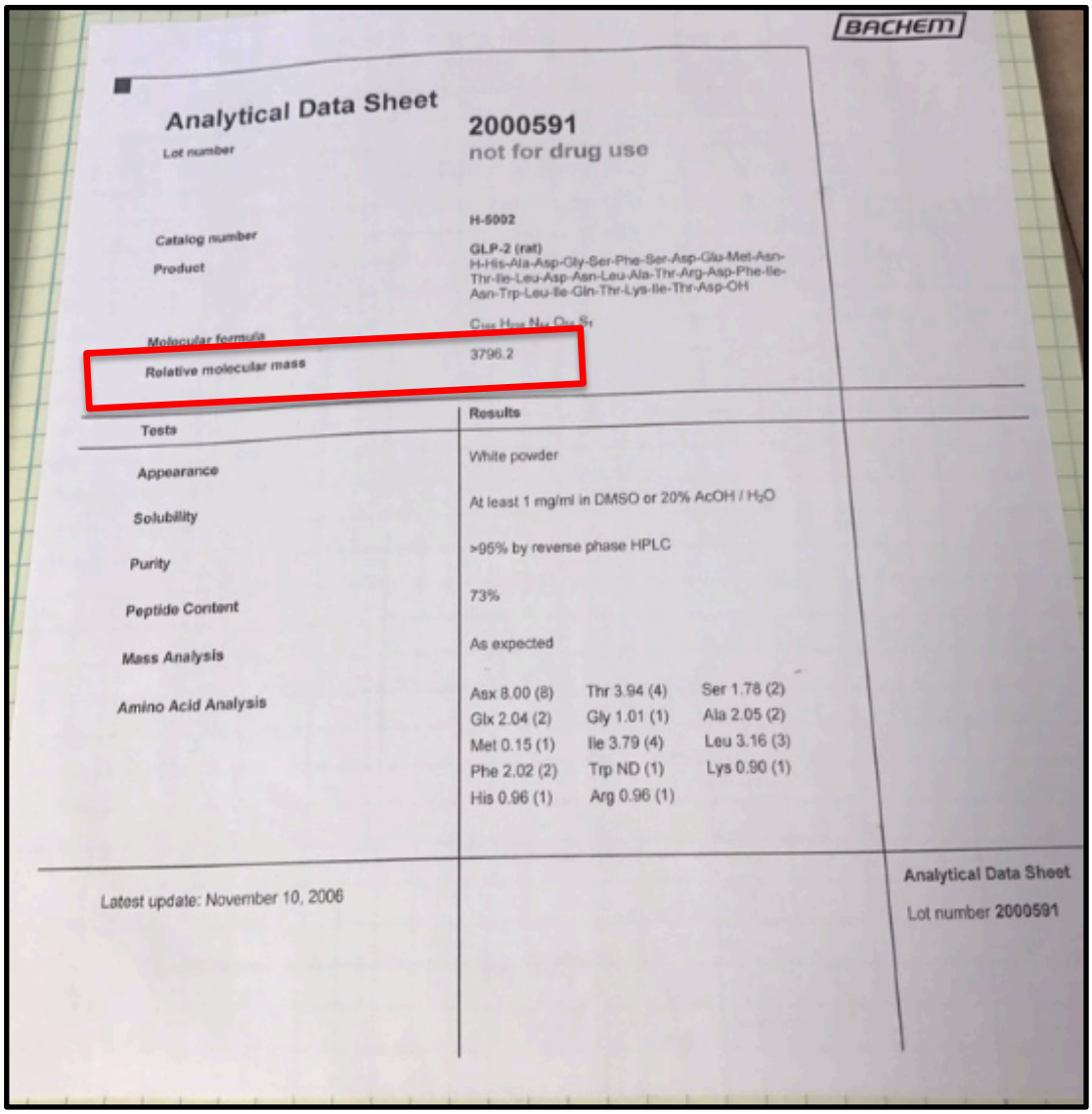
As a control, 6 eppendorfs containing 2ml of PBS only were also prepared.

The 12 eppendorfs were stored at -20˚C.

A needle was used to aspirate the 2ml of GLP-2/PBS, or PBS only, into a 3ml syringe, which was then placed in the infusion pump.

**EXPERIMENTS 2-4**

Lyophilised GLP-2 (2000591, Bachem, Torrance, CA) was purchased. It was necessary to switch from Abcam, as they were unable to provide any further GLP-2 for at least 6 weeks, which was felt to be an unacceptable delay. It was ensured that the peptide purchased had the same chemical structure and formula as that used in Experiment 1, and that it had been internally validated by Bachem. The molecular weight was confirmed as 3796.2g/mol (theoretical weight: 3796.2g/mol), (**Figure 17)**.



BACHEM

**Analytical Data Sheet**

Lot number **2000591**
not for drug use

| | |
|---|---|
| Catalog number | H-5002 |
| Product | GLP-2 (rat)<br>H-His-Ala-Asp-Gly-Ser-Phe-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Thr-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp-OH |
| Molecular formula | $C_{155}H_{240}N_{44}O_{49}S_1$ |
| Relative molecular mass | 3796.2 |

| Tests | Results |
|---|---|
| Appearance | White powder |
| Solubility | At least 1 mg/ml in DMSO or 20% AcOH / $H_2O$ |
| Purity | >95% by reverse phase HPLC |
| Peptide Content | 73% |
| Mass Analysis | As expected |
| Amino Acid Analysis | Asx 8.00 (8) Thr 3.94 (4) Ser 1.78 (2)<br>Glx 2.04 (2) Gly 1.01 (1) Ala 2.05 (2)<br>Met 0.15 (1) Ile 3.79 (4) Leu 3.16 (3)<br>Phe 2.02 (2) Trp ND (1) Lys 0.90 (1)<br>His 0.96 (1) Arg 0.96 (1) |

Latest update: November 10, 2006

Analytical Data Sheet
Lot number **2000591**

**Figure 17:** Bachem GLP-2 data sheet.

The dose was increased to 1000µg/kg (quadruple the dose used in Experiment 1), as guided by the results gained from Experiment 1 (section 2.6.1).

**To limit the freeze/thaw cycle to once only for each GLP-2 infusion, 6 aliquots were prepared:**

6 aliquots of 330µg (Bachem sheet suggests GLP-2 purity >95%)

NB: As with Experiment 1, 330µg was chosen as this would be the amount needed for a 330g rat – most rats should weigh below 300g, however (rats purchased at 220-240g weight).

**As protein keeps better in more concentrated solution:**

1000µg GLP-2 dissolved in 1ml PBS (buffer with pH of 7.5)

1µg in 1µl

So, 330µg in 330µl

Each of the 6 aliquots required to contain 330µl of GLP-2/PBS.

**According to the amount of GLP-2 required i.e. 1000µg/kg: (4 x 240µg, rounded to 1mg/kg)**

For 250g rat, 250µg GLP-2 is required

**To dilute up to 2ml (for 3ml syringe used in infusion pump), add:**

1.67ml PBS (2ml – 0.330ml (330µl) = 1.67ml)

Aliquot contains 330µg GLP-2 in 2ml, so concentration of GLP-2 is 157µg/ml.

For a 250g rat, according to calculations, 1.5ml of infusate required.

Taking in to consideration volume required to prime the 15cm catheter:

**Total infusate = 1.531ml.**

A 2ml eppendorf was used to store the 2ml of GLP-2/PBS.

As a control, 6 eppendorfs containing 2ml of PBS only were prepared.

The 12 eppendorfs were stored at -20˚C.

A needle was used to aspirate the 2ml of GLP-2/PBS into a 3ml syringe, which was then placed in the infusion pump.

**EXPERIMENT 5**

12 aliquots of 330µg GLP-2/PBS were prepared and stored using the same method as for Experiments 2-4. On the day of surgery, each experiment required 2 aliquots worth of GLP-2/PBS and were aspirated using a needle into a 5ml syringe, ready for infusion. A duodenal glucose infusion was given over a 5-minute period, from minute 45 to minute 50.

*Animals*

Animal studies were performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. Male Sprague-Dawley rats (SD; 220–240g, Harlan, IN) were used. The SD rat is a healthy rodent model. Rats were acclimatised for 7 days under a 12:12 light: dark cycle (lights-on at 07.00) with *ad libitum* access to standard rat chow (Purina 5053). After an overnight fast, they were anesthetised using isoflurane (1-3% in oxygen). Two experiments were carried out each day, starting at 09.00. The order of experiment and control surgeries was alternated from Experiment 4 onwards to avoid the confounding factor of diurnal variation.

*Catheter Placement*

Portal and systemic venous blood was sampled, before and during a systemic GLP-2 infusion, in order to measure glucose, from which portosystemic gradients were calculated, and hormone levels in order to measure hormone secretion. For experiments 1-5, GLP-2 was infused into the systemic vein.

For systemic sampling, a silastic catheter (Dow Corning, ID 0.02in) was advanced through the right jugular vein into the right atrium, allowing sampling of mixed systemic venous blood, and then secured with a silk suture (**Figures 18 and 19**). After each blood sample was taken, the catheter was flushed with heparinised saline (20µl of 20 units/ml) to maintain patency throughout the experiment.

For portal blood sampling, a silastic catheter (Dow Corning, ID 0.012in) was inserted into the superior mesenteric vein (SMV). A short length of SMV was controlled using curved vascular clamps (Fine Science Tools) (**Figures 20 and 21**), the SMV punctured (using a 27-gauge needle), and the catheter inserted. The catheter was advanced 4-5cm so as to have its tip positioned within the hepatic portal vein. The portal vein diameter is approximately 2.2mm and the portal catheter outer diameter is 0.64mm so the catheter occupies less that 10% of the portal vein area. For all experiments, portal and systemic blood glucose levels were measured at each time-point using a glucometer (LifeScan OneTouch). Total blood volume removed from each rat did not exceed 2.8ml (10-15% total blood volume can be removed if fluid replacement given i.e. for a 300g rat, circulating volume is 64ml/kg, which is equal to 64 x 0.3). Thus, 10-15% of 19.2ml allowed a range of 1.92 – 2.8ml blood volume whilst limiting physiological stress on the rats.

For experiments 2-5, blood taken for hormone analysis was placed in K2-EDTA tubes containing DPP-IV inhibitor (1mcl per 0.1ml blood, to prevent breakdown of GIP, GLP-1 and GLP-2), and Aproptonin (5mcl per 0.1 ml blood, to prevent breakdown of glucagon) and kept on ice. Tubes were centrifuged at 4000G for 15 minutes at 4°C. The blood plasma was aspirated and stored at -80°C.

For Experiment 2, a commercially available GLP-2 ELISA kit (catalogue number EZGLP2-37K, Millipore, Darmstadt, Germany) was used to measure portal and systemic GLP-2 levels at 0- and 60-minute time points, in duplicate.

For Experiment 3, a commercially available Luminex rat metabolic hormone panel (catalogue number RMHMAG-84K, Millipore, Darmstadt, Germany) was used to measure portal and systemic plasma GIP, GLP-1, insulin, glucagon and C-peptide levels at 0-, 20- and 60-minute time points in duplicate.

Experiment 5 involved an additional catheter, placed directly into the duodenum and secured with a silk suture (**Figure 22**). A glucose bolus of 2mg/g of glucose, (glucose tolerance test, GTT), an amount equivalent to that given to rats in an oral glucose tolerance test (25%; dissolved in water), was then administered directly to the small bowel lumen over a 5-minute period.

For Experiments 4-5, a 10cm section of jejunum (26cm distal to the pylorus, equivalent to the Roux limb) was harvested at the end of the surgery, but prior to euthanasia. Mucosa was separated from the muscle layers and stored at -80°C.



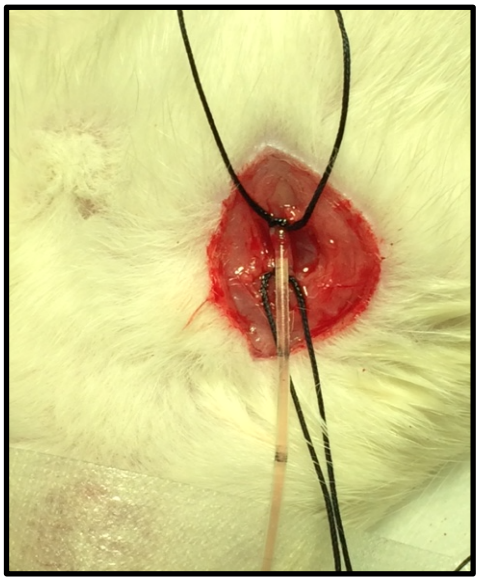
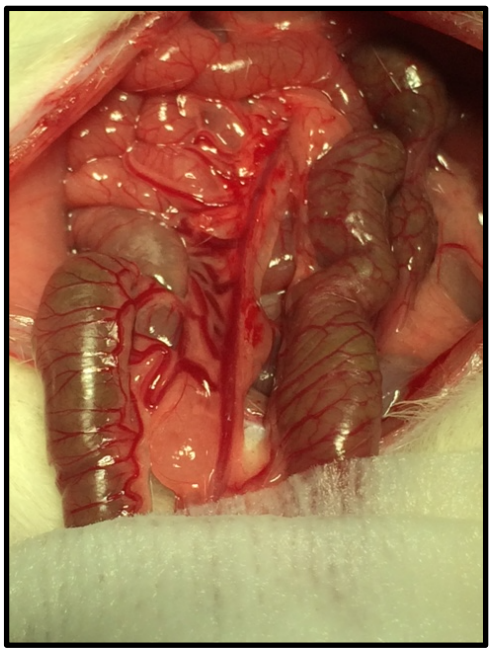


**Figure 18:** Right External Jugular Vein showing puncture ready for catheter, and **Figure 19:** Subsequent placement of catheter.



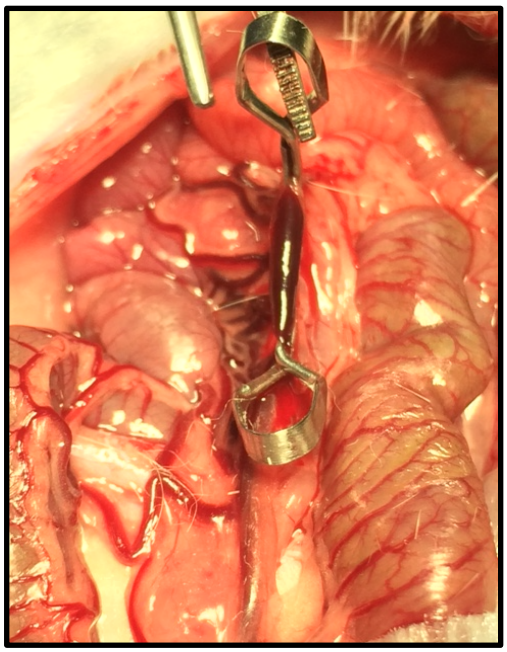


**Figure 20:** Superior Mesenteric Vein (SMV) exposed and **Figure 21:** SMV clamped proximally and distally prior to catheterisation.

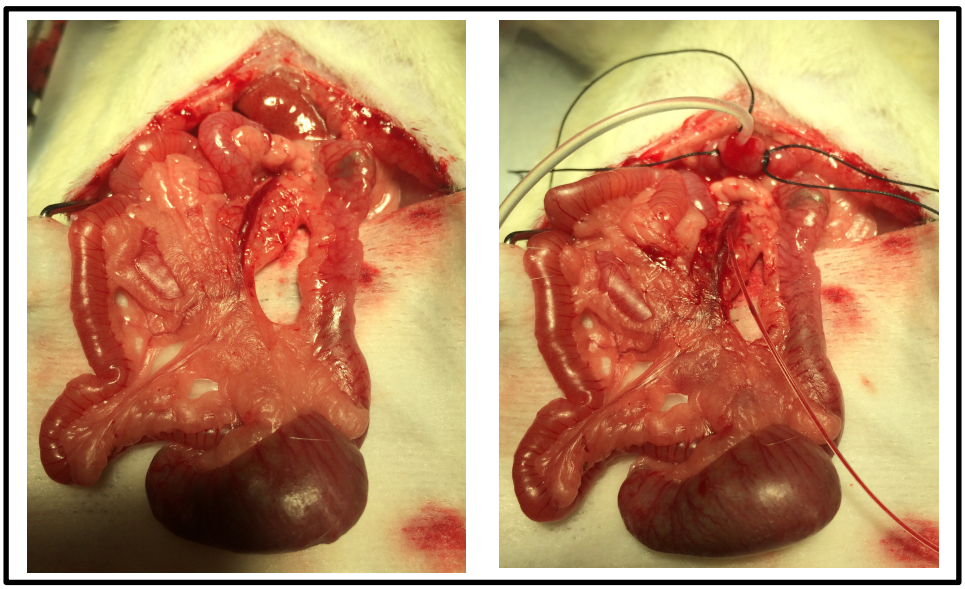


**Figure 22:** Before and after SMV and duodenal catheterisation.

*Statistical Analysis*

Data analysis was performed using Microsoft Excel and GraphPad Prism 8.0 statistical software (GraphPad, San Diego, CA). The two-tailed unpaired Student's t-test was used for planned comparison of two groups and, unless specified otherwise, this was the test used for all data analysed. Data is presented as mean +/- standard error of the mean.

## 2.5 EXPERIMENT PROTOCOL AND TIMELINES

### *2.5.1 Experiment 1*

- 12 healthy, male, fasted SD rats
- GLP-2 dose: 240µg/kg i.e. for 220g rat = 52.8µg GLP-2 for 120 minutes
- All rats received similar levels of Buprenorphine at induction
- All rats received similar levels of Isoflurane throughout the surgery
- Control (PBS) rat operated on first, followed by Experiment (GLP-2) rat each day
- Mean rat weights: GLP-2 = 302.4g, PBS = 295g. GLP-2 + PBS Mean = 298.7g

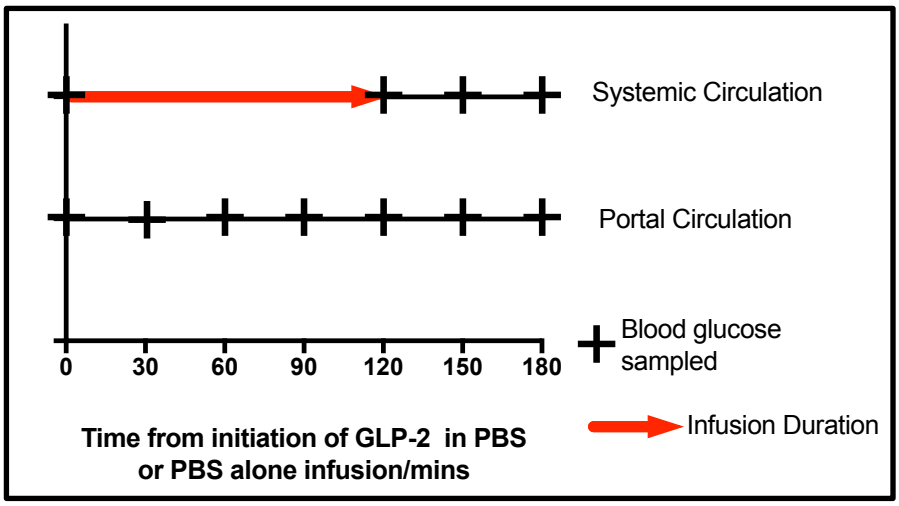



**Figure 23:** Experiment 1 – Timeline.

### *2.5.2 Experiment 2*

Findings of Experiment 1 (section 2.6.1) led to:

- 12 healthy, male, fasted SD rats
- Quadrupling the GLP-2 dose: 1000µg/kg
- Infusion time reduced from 120 min to 60 min

- Decision made to pause the infusion at 30 minutes so as to be able to take a systemic blood glucose sample and have a full set of results to compare to the portal blood glucose results (see red arrow, **Figure 24**)
- All rats received similar levels of Buprenorphine at induction.
- All rats received similar levels of Isoflurane throughout the surgery
- PBS rat operated first, followed by GLP-2 rat each day
- Blood collected for Enzyme-linked Immunosorbent Assay (ELISA) hormone analysis at 0 and 60 minutes (see **Appendix 1**).
- Mean rat weights: GLP-2 = 284g, PBS = 271.2g. GLP-2 + PBS Mean = 277.6g

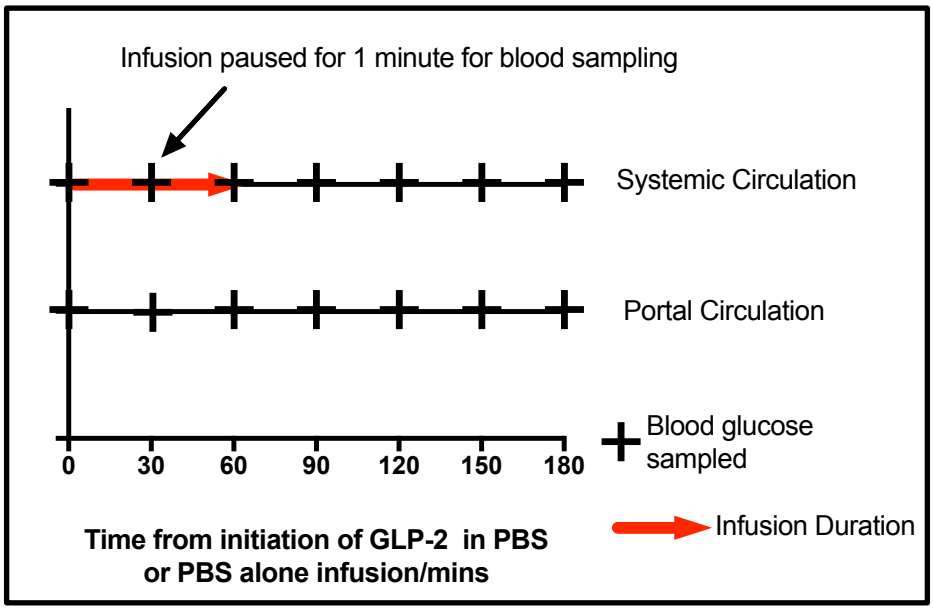



**Figure 24:** Experiment 2 – Timeline.

*2.5.3 Experiment 3*

Findings of Experiment 2 (see section 2.6.2) led to:

- 12 healthy, male, fasted SD rats
- GLP-2 dose kept the same at 1000µg/kg

- GLP-2 infusion rate reduced from 60 min 20 min, to see if results were reproducible with a shorter anaesthetic time
- All rats received similar levels of Buprenorphine at induction
- All rats received similar levels of Isoflurane throughout the surgery
- PBS rat operated on first, followed by GLP-2 rat each day
- Blood collected for Luminex hormone analysis (see **Appendix 2**). Hormones of interest were insulin, glucagon, GIP, GLP-1 and C-peptide
- Mean rat weights: GLP-2 = 261.3 g, PBS = 247.3g. GLP-2 + PBS Mean = 254.3g

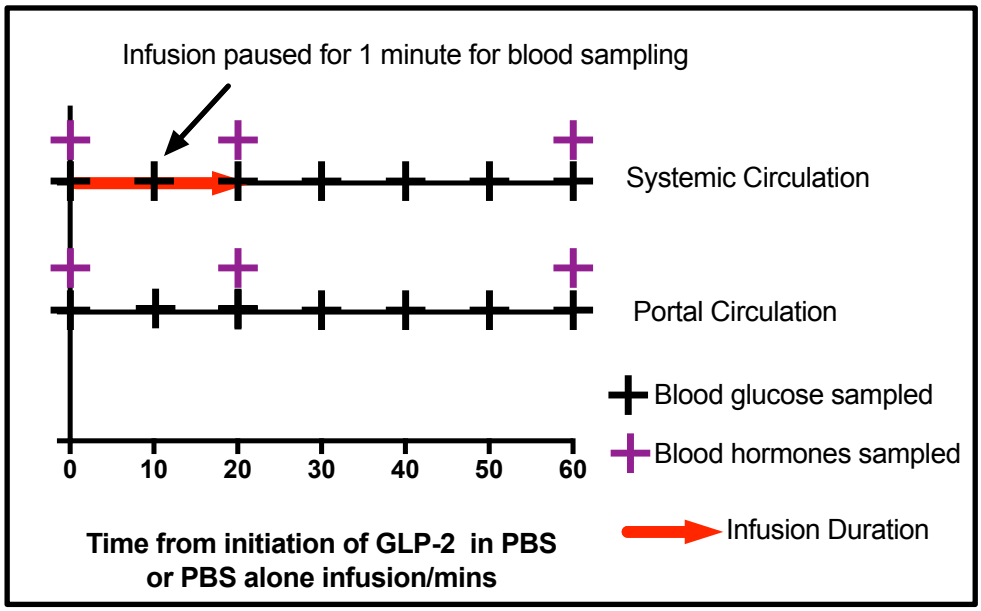



**Figure 25:** Experiment 3 – Timeline.

*2.5.4 Experiment 4*

Findings of Experiment 3 (see section 2.6.3) led to:

- 12 healthy, male, fasted SD rats
- GLP-2 dose kept the same at 1000µg/kg for 20 minutes

- Experiment duration lengthened to 120 minutes as rats in Experiment 3 struggled with such frequent blood sampling
- All rats received similar levels of Buprenorphine at induction
- All rats received similar levels of Isoflurane throughout the surgery
- Jejunal tissue (taken 26cm from pylorus and, therefore, equivalent to Roux limb) harvested for Polymerase Chain Reaction (PCR) (see **Appendix 3**). Genes of interest were GLUT-1, GLUT-2, C-MYC and C-FOS. Cyclophylin A was used as the housekeeping gene (**Figure 26)**
- PBS and GLP-2 rat operating order alternated each day to try and control for diurnal rhythm, and to achieve similar baseline glucose levels in both groups
- Mean rat weights: GLP-2 = 257.6g, PBS = 271.6g. GLP-2 + PBS Mean 264.6g

| | **Forward primer** | **Reverse primer** |
|---|---|---|
| **GLUT-1** | 5'-GTGCTCGGA TCCCTGCAGTTCG-3' | 5'-GGGATGGACTCTCCATAGCGGTG-3' |
| **GLUT-2** | 5'-CTCGGGCCTTACGTGTTCTTCCTT-3' | 5'-TGGTTCCCTTCTGGTCTGTTCCTG-3' |
| **C-MYC** | 5'-GGAAGGACTATCCAGCTGCC-3' | 5'-TGGAGCATTTGCGGTTGTTG-3' |
| **C-FOS** | 5'-TACTACCATTCCCCAGCCGA-3' | 5'-TGGCACTAGAGACGGACAGA-3' |
| **Cyclophylin A** | 5'-GTGGTCTTTGGGAAGGTGAA-3' | 5'-GTCGGAGATGGTGATCTTCTTG-3' |

**Figure 26:** PCR Primer Sequences.

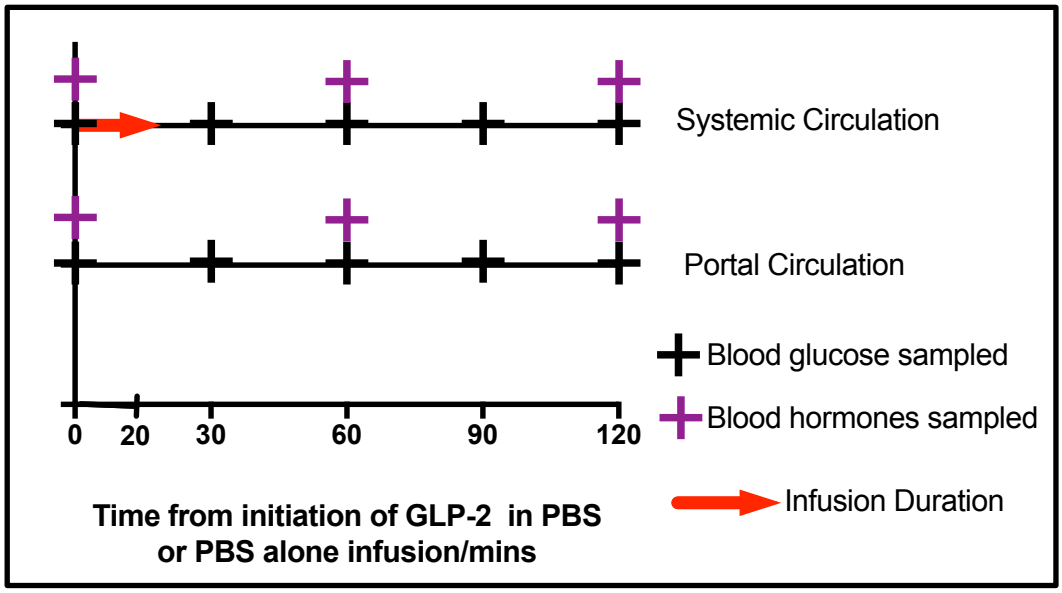



**Figure 27:** Experiment 4 – Timeline.

*2.5.5 Experiment 5*

Findings of Experiment 4 (see section 2.6.4) led to:

- Doubling the dose of GLP-2 (compared to experiments 2-4) to 2000µg/kg, and increasing the infusion time to 40 minutes (compared to 20 minutes in Experiment 4)
- Followed by a 5-minute infusion of glucose, and blood sampled until 120 minutes
- 12 healthy, male, fasted SD rats
- All rats received similar levels of Buprenorphine at induction.
- All rats received similar levels of Isoflurane throughout the surgery
- PBS and GLP-2 rat operating order alternated each day to control for diurnal variation

- Jejunal tissue (equivalent to Roux limb) harvested for PCR – genes of interest were GLUT-1, GLUT-2, C-MYC and C-FOS (see **Appendix 3**)
- Mean rat weights: GLP-2 = 247.6g, PBS = 255.5g. GLP-2 + PBS Mean = 251.5g

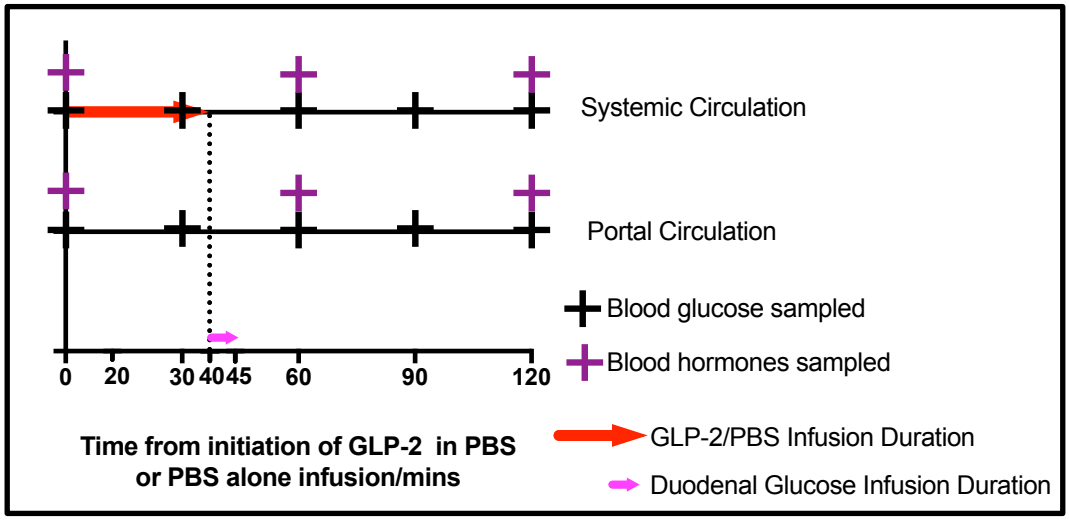



**Figure 28:** Experiment 5 – Timeline.

## 2.6 RESULTS

Statistical analysis was carried out as described in section 2.4.1.

### *2.6.1 Experiment 1*

This was the first acute GLP-2 experiment conducted and so it was interesting to see whether there would be any observed effect from the dosing of GLP-2 that had been chosen (240mcg/kg). Out of the total of 12 rats, 9 survived the experiment (3 GLP-2 rats and 4 PBS rats). The other 3 died at 0, 90 and 120 minutes and were therefore not included in the analysis.

With regards to portal blood glucose levels, GLP-2 (n=5) and PBS (n=4) rats demonstrated a similar trend in that blood glucose increased in both groups (**Figure 29**). With GLP-2 rats, this increase peaked at 60 minutes, with the trend being a decrease for the remainder of the experiment. With PBS rats, blood glucose peaked at 30 minutes, before decreasing, but with a second increase from 60 to 120 minutes.

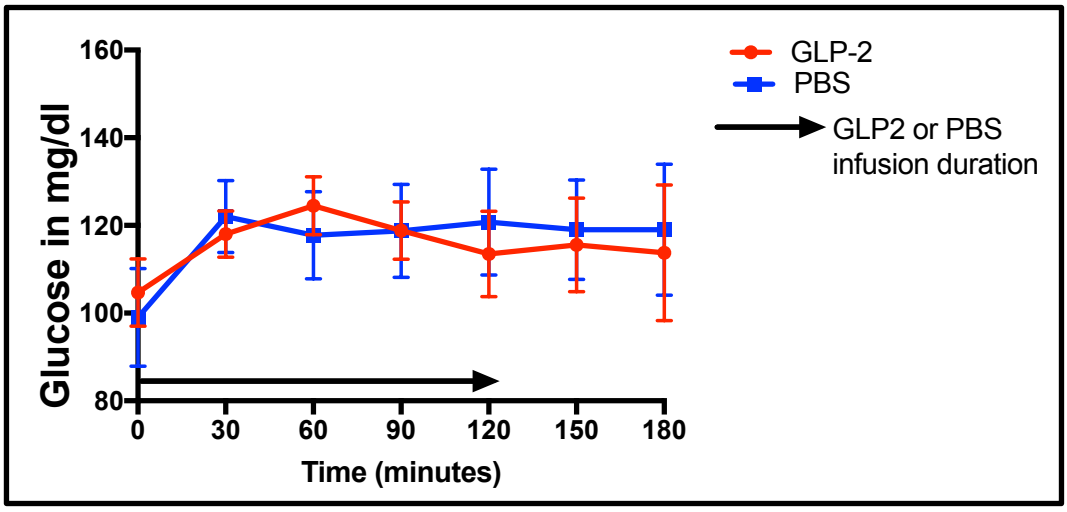



**Figure 29:** Experiment 1 – Mean portal glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 240mcg/kg; GLP-2 infusion duration, 120 minutes; experiment duration, 180 minutes).

With regards to systemic blood glucose levels there were, again, similar trends for both GLP-2 and PBS groups. This time, there were fewer results available as blood was only taken at four time points due to the GLP-2 infusion being administered via this catheter over the first 120 minutes of the experiment. Interestingly, and in opposition to portal glucose levels, both GLP-2 and PBS systemic blood glucose levels tended to decrease from 0 to 120 minutes. Following the termination of GLP-2 infusion, both groups then saw a rise in blood from 120 to 150 minutes, with the GLP-2 group glucose levels continuing to rise at 180 minutes. PBS systemic glucose levels decreased again from 150 to180 minutes (**Figure 30**).

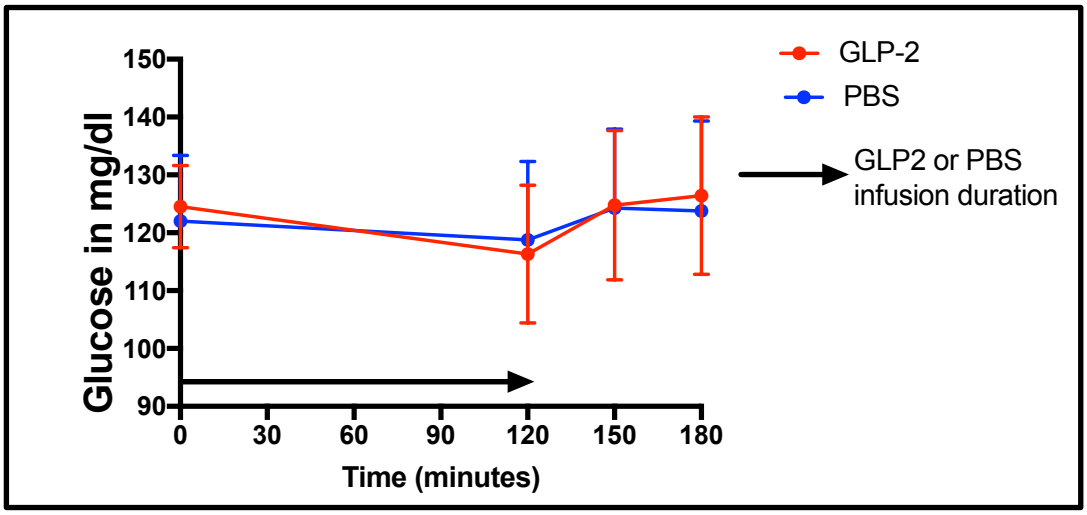



**Figure 30:** Experiment 1 – Mean systemic glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 240mcg/kg; GLP-2 infusion duration, 120 minutes; experiment duration, 180 minutes).

Once again, there was a similar trend in each group regarding the portosystemic glucose gradients, with a negative $G_{PS}$ in the GLP-2 group (-20) and in the PBS group (-23) at 0 mins. Both became less negative until 120 minutes, with the PBS group becoming positive. Both groups then became more negative again until 150 minutes, with the GLP-2 group continuing this trend, whilst the PBS group plateaued until 180 minutes. There were no statistically significant differences between the two groups

regarding portal and systemic blood glucose, or regarding portosystemic glucose gradients (**Figure 31**).

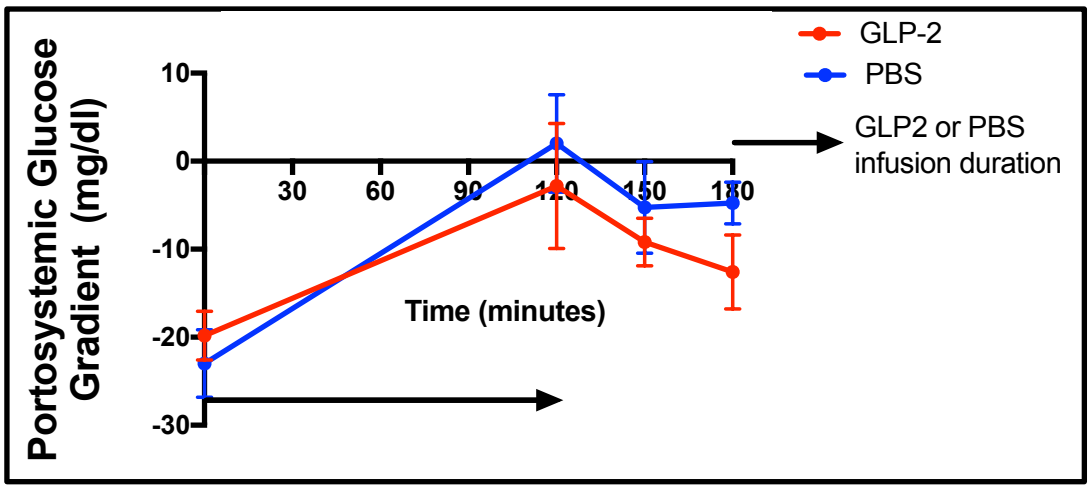



**Figure 31:** Experiment 1 – Mean portosystemic gradient following GLP-2 or PBS infusion (GLP-2 dose, 240mcg/kg; GLP-2 infusion duration, 120 minutes; experiment duration, 180 minutes).

*2.6.2 Experiment 2*

In the second acute GLP-2 infusion experiment, 10 out of 12 rats survived – 1 PBS rat died at 0 minutes and 1 GLP-2 rat suffered irreversible bowel ischaemia secondary to prolonged SMV clamp time and, thus, were not included in the analysis.

Unlike in Experiment 1, GLP-2 (n=5) and PBS (n=5) groups did not share a similar trend with regards to portal glucose levels. In the PBS group, the blood glucose rose slowly over the 180 minutes, with an overall increase of 12 mg/dl. In the GLP-2 group, there was a marked increase in blood glucose, starting at 30 minutes and reaching its peak at 90 minutes. The blood glucose then decreased from 90-120 minutes, before rising slightly and plateauing at 150 minutes. The 60- and 90-minute peaks in the GLP-2 group were found to be statistically significant compared to the PBS group p=0.012 and p=0.049, respectively (**Figure 32**).

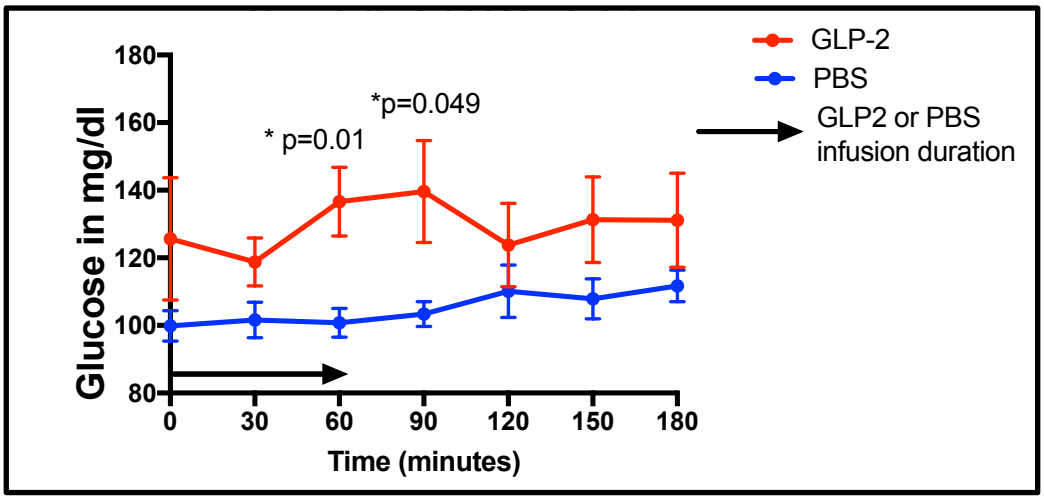



**Figure 32:** Experiment 2 – Mean portal glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 60 minutes; experiment duration, 180 minutes).

With regards to systemic blood glucose levels, an almost identical trend was observed. 60-minute and 90-minute peaks were seen in the GLP-2 group, both of which were statistically significant compared to the PBS group (p=0.03 at both time points). The PBS control group systemic glucose trend was also seen to be very similar to that of the GLP-2 experiment group portal glucose trend, which was reassuring. Additionally, a full set of blood results were taken systemically to match portal blood samples by pausing the infusion at the 30-minute time point (**Figure 33**).

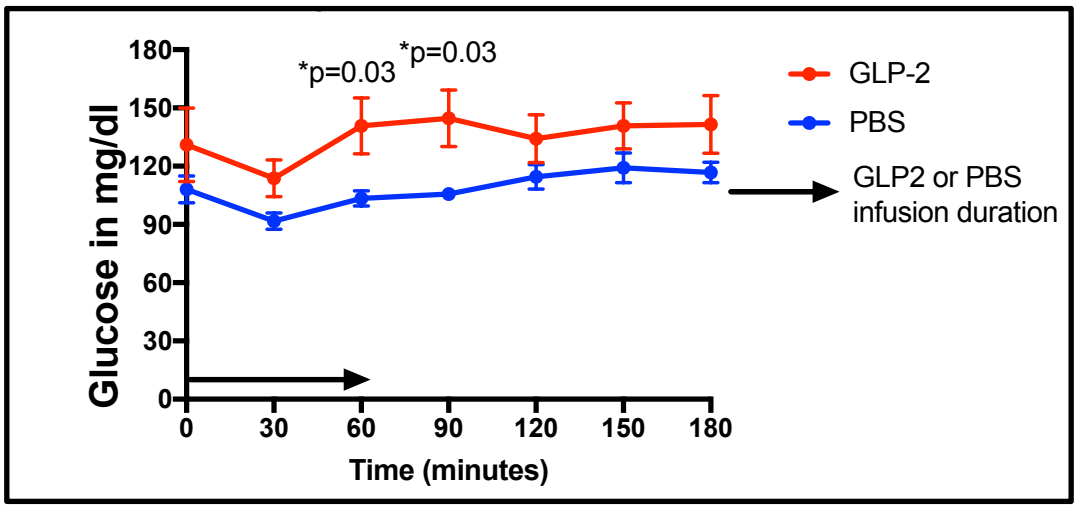



**Figure 33:** Experiment 2 – Mean systemic glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 60 minutes; experiment duration, 180 minutes).

The portosystemic gradient was more negative within the GLP-2 group than within the PBS group, but not significantly so (**Figure 34**).

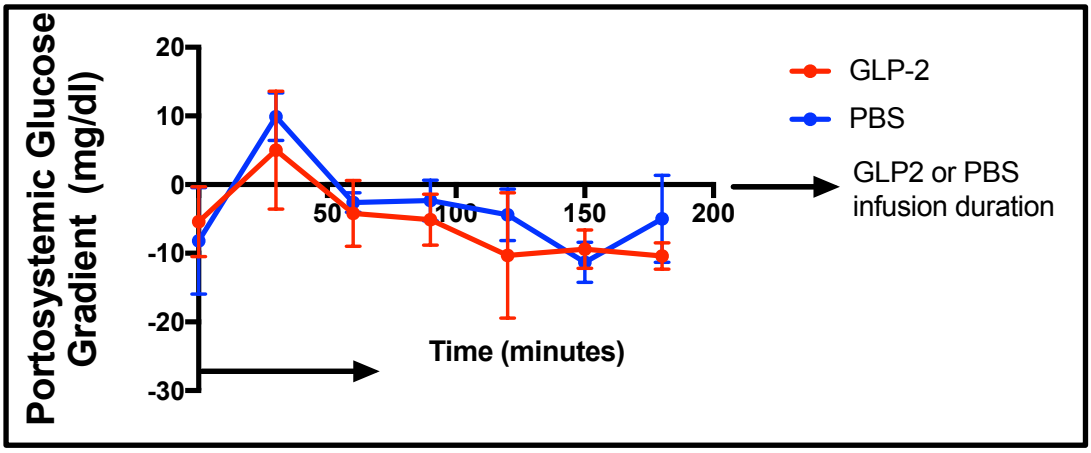



**Figure 34:** Experiment 2 – Mean portosystemic gradient following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 60 minutes; experiment duration, 180 minutes).

ELISA results showed statistically significant higher levels of GLP-2 in the GLP-2 group (n=5) at 60 minutes compared to the PBS group (n=5), and in both portal and systemic blood, confirming that the dose of GLP-2 administered was effectual (**Figure 35**).

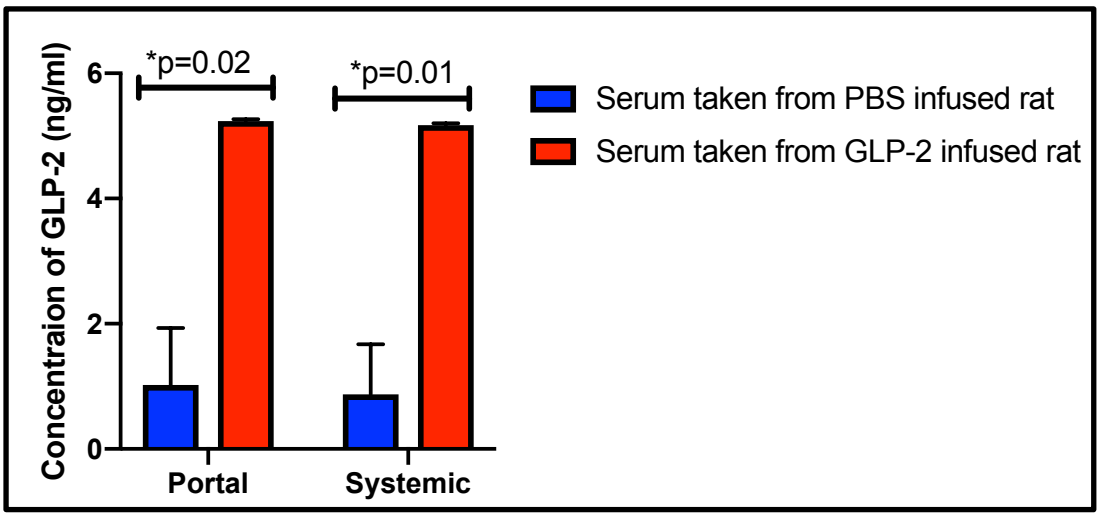



**Figure 35:** Experiment 2 – Enzyme-linked assay showing levels of GLP-2 levels at 60 minutes, in portal and systemic blood.

*2.6.3 Experiment 3*

Experiment 3 saw nine rats surviving until the end of the experiment – 6 GLP-2 rats and 3 PBS rats. One PBS rat died at 52 minutes, and 2 other PBS rats suffered excessive bleeding at SMV cannulation. Therefore, these 3 rats were not included in the analysis.

Portal blood glucose was similar within both groups in that there was an observed upward trend. However, interestingly, the GLP-2 rats (n=6) had a higher (although, not significantly so) baseline blood glucose than the PBS rats (n=3). An explanation for this was thought to be due to the fact that, as part of the protocol for these acute GLP-2 infusion experiments, 2 rats were operated on per day, always starting with a PBS control rat. There was concern that the differing baseline glucose levels were due to diurnal variation. Nevertheless, by the 10-minute time point, GLP-2 and PBS group mean blood glucose levels are almost matching. This is important because the GLP-2 group then go on to show an increase in blood glucose levels, so much so that at the 50- and 60-minute time points, glucose levels were significantly higher (p=0.02 and p=0.01, respectively) compared to the glucose levels in the PBS group (**Figure 36**).

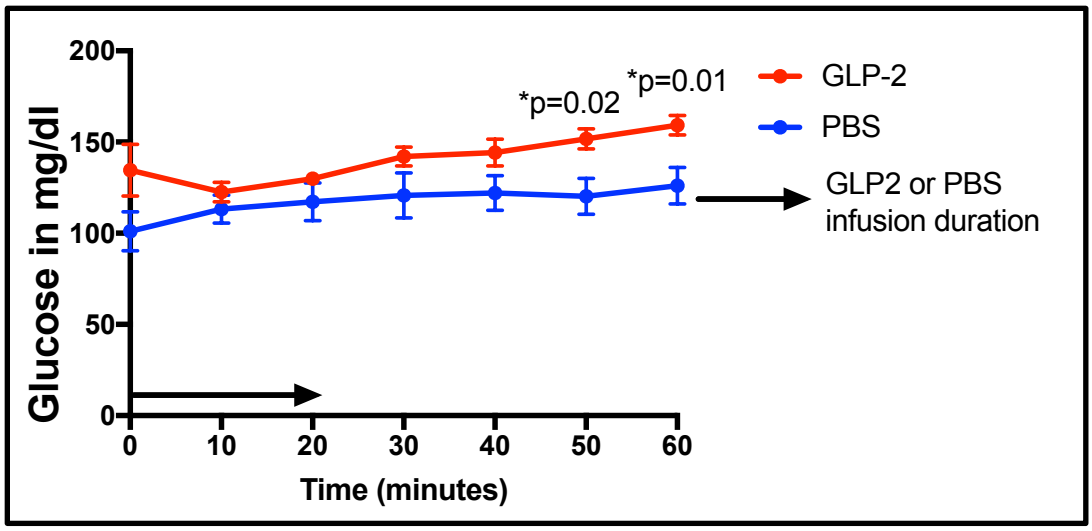



**Figure 36:** Experiment 3 – Mean portal glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 60 minutes).

Systemic blood glucose trends were similar to portal in that there was a differing baseline glucose level between the two groups, and that at the 10-minute time point, glucose levels were almost on a par. As with the portal glucose levels, systemic glucose in the GLP-2 group continued to increase significantly more than in the PBS group, and statistical significance was reached at the 40-, 50- and 60- minute time points (p=0.04, p=0.02 and p=0.02, respectively) (**Figure 37**).

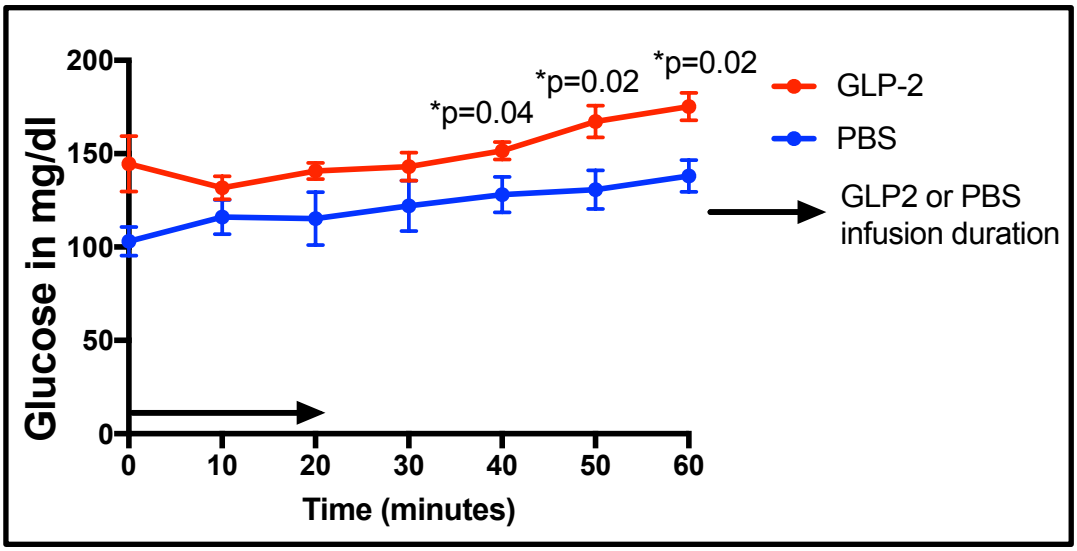



**Figure 37:** Experiment 3 – Mean systemic glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 60 minutes).

The portosystemic gradient in the GLP-2 group was more negative than in the PBS group, but not significantly so (**Figure 38**).

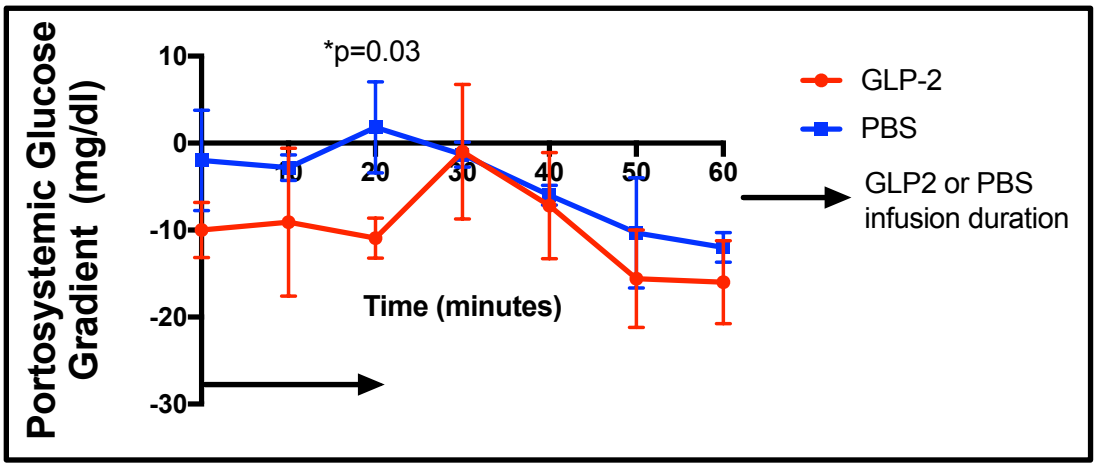



**Figure 38:** Experiment 3 – Mean portosystemic gradient following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 60 minutes).

Although only an n=1 in each group, Luminex hormone analysis, looking at GLP-1, GIP, glucagon and insulin, revealed some interesting hormonal trends. The stand out result is the presumed effect of GLP-2 on insulin levels, both in portal (**Figure 40**) and systemic blood (**Figure 42**), particularly at the 20-minute time point, compared to PBS. Pointedly, this is the time at which GLP-2 infusion had just finished. In terms of the other hormones, there were only minimal differences to be seen between the GLP-2 and PBS groups, in either portal or systemic blood (**Figures 39, 40, 41 and 42).**

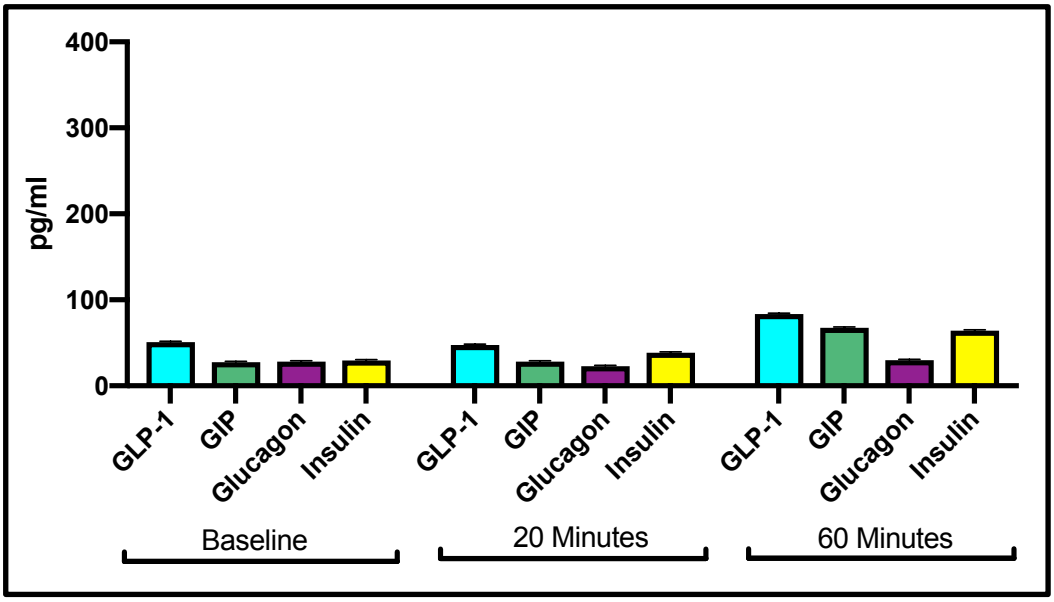



**Figure 39:** Experiment 3 – Luminex hormone analysis of portal blood following PBS infusion, at baseline (0 minutes), 20 and 60 minutes.

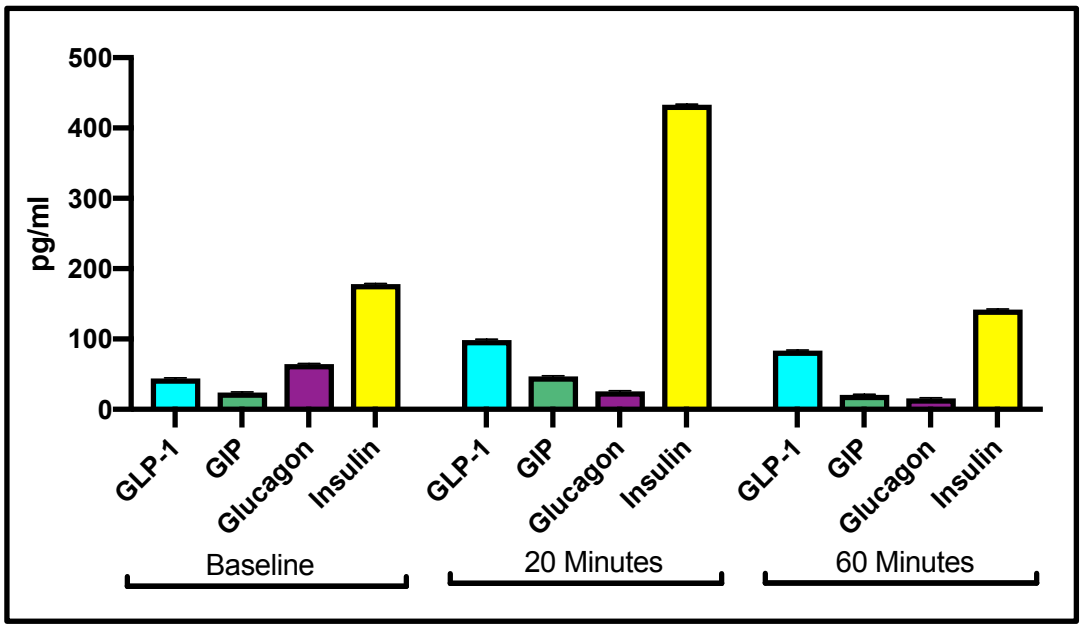



**Figure 40:** Experiment 3 – Luminex hormone analysis of portal blood following GLP-2 infusion, at baseline (0 minutes), 20 and 60 minutes.

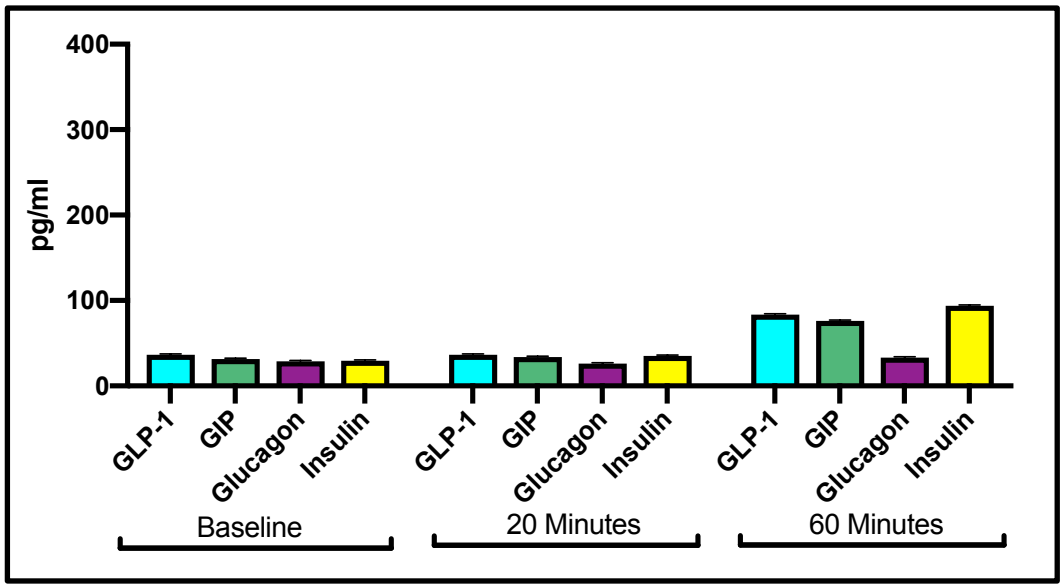



**Figure 41:** Experiment 3 – Luminex hormone analysis of systemic blood following PBS infusion, at baseline (0 minutes), 20 and 60 minutes.

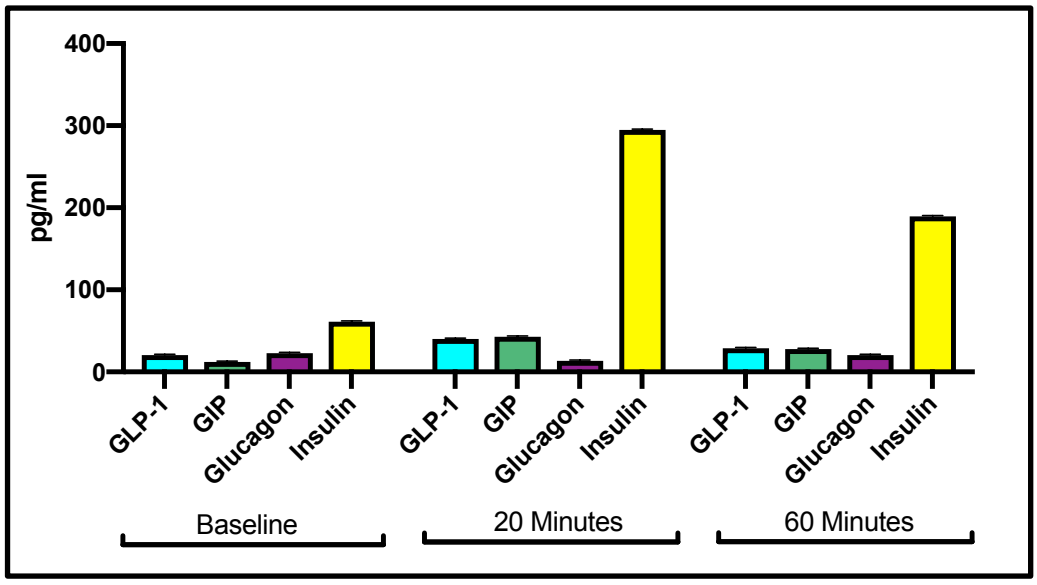



**Figure 42:** Experiment 3 – Luminex hormone analysis of systemic blood following GLP-2 infusion, at baseline (0 minutes), 20 and 60 minutes.

*2.6.4 Experiment 4*

Experiment 4 of the acute GLP-2 infusion experiments saw 10 rats in total survive until the end of the surgery. 1 PBS rat died at 90 minutes, and 1 GLP-2 rat died at 0 minutes due to excessive bleeding at SMV cannulation. Data from these rats was not included.

With regards to portal blood glucose levels, there was an upward trend in both GLP-2 (n=5) and PBS (n=5) groups. The GLP-2 group exhibited a quicker rise in glucose but, at 50 minutes, this then switched so that the GLP-2 group revealed a lower blood glucose than the PBS group by the end of the surgery. Although there were no statistically significant results within this experiment, it was reassuring to see that, by alternating GLP-2 and PBS rats in terms of which type of rat was operated on first each day, a similar average baseline blood glucose value was obtained for the two groups (**Figure 43**).

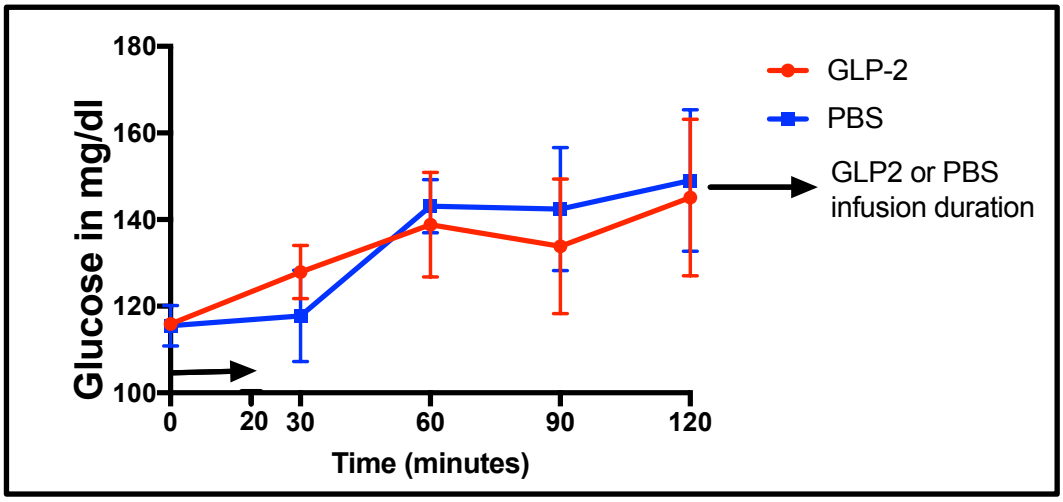



**Figure 43:** Experiment 4 – Mean portal glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 120 minutes).

Systemic blood glucose results revealed a slight difference compared to portal results in that there was no switch between GLP-2 and PBS groups – glucose levels in the GLP-2 group were higher from the outset, although not significantly so. As with the portal results, it was reassuring to see that baseline values were similar between the two groups (**Figure 44**).

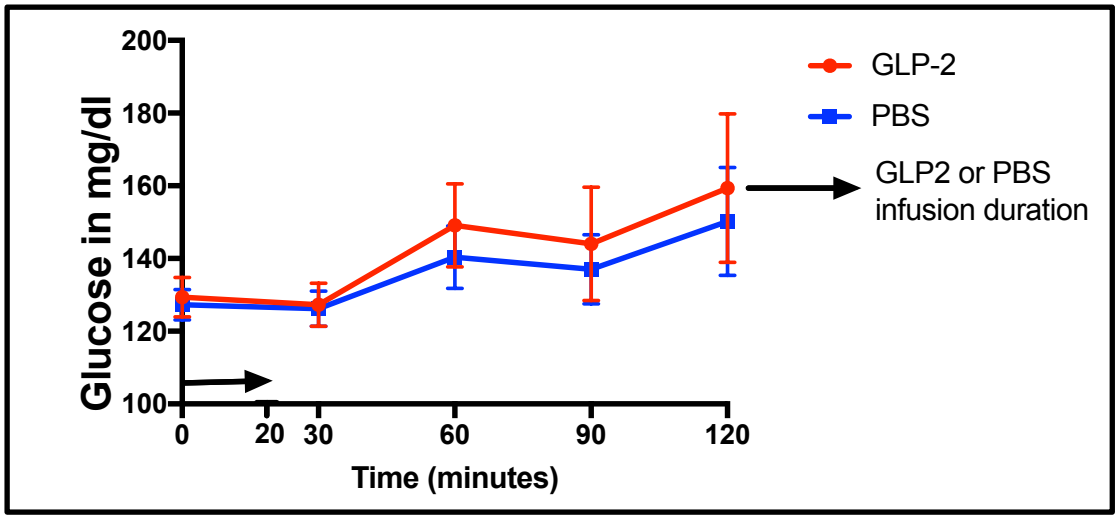



**Figure 44:** Experiment 4 – Mean systemic glucose levels following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 120 minutes).

With regards to the portosystemic gradient, there did appear to be a difference between GLP-2 and PBS groups, with the GLP-2 group $G_{PS}$ becoming positive by 30 minutes, but then becoming negative for the rest of the experiment. The PBS group $G_{PS}$ took longer to become positive, only doing so at around 50 minutes. It became noticeably more positive than the GLP-2 group, although not significantly so, with statistical significance just missing out at the 60-minute time point (p=0.07) (**Figure 45**).

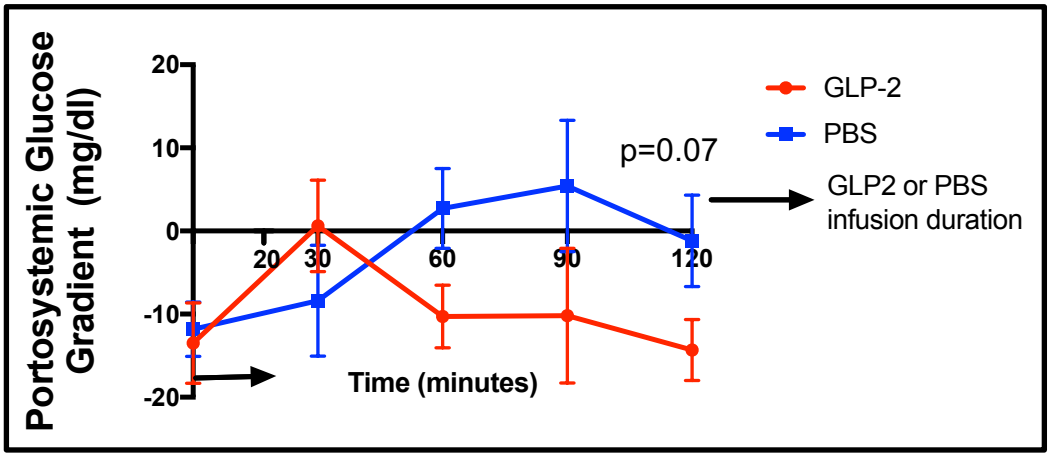



**Figure 45:** Experiment 4 – Mean portosystemic gradient following GLP-2 or PBS infusion (GLP-2 dose, 1000mcg/kg; GLP-2 infusion duration, 20 minutes; experiment duration, 120 minutes).

PCR showed no difference in GLUT-1 expression in the GLP-2 group (n=5) compared to the PBS group (n=5) (**Figure 46**).

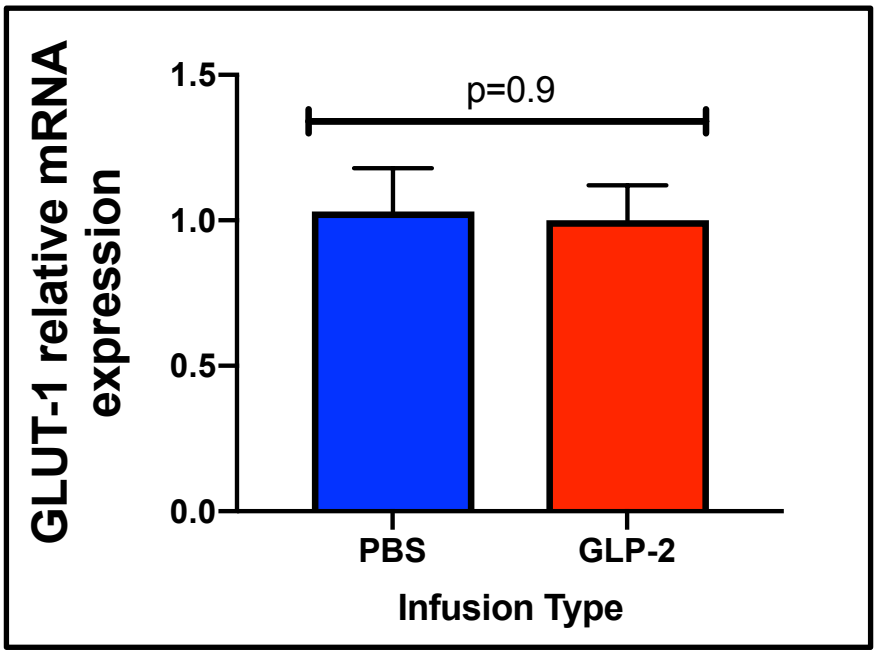



**Figure 46:** Experiment 4 – GLUT-1 expression in fasted state.

The same result was seen regarding GLUT-2 (**Figure 47**), and the proliferation genes, C-MYC and C-FOS (**Figures 48 and 49**). This is in opposition to results observed in the post-prandial state (see section 2.6.5).

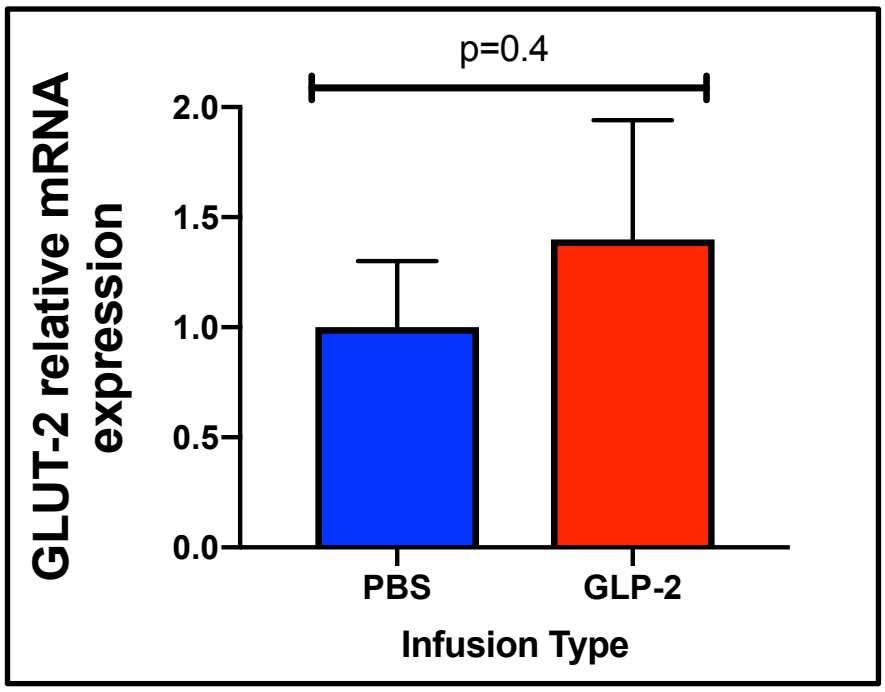



**Figure 47:** Experiment 4 – GLUT-2 expression in fasted state.

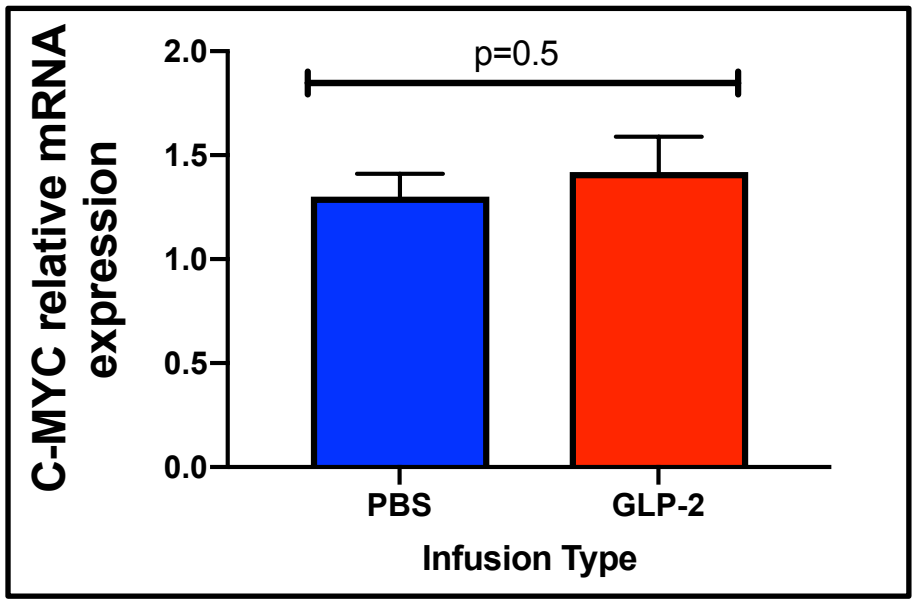



**Figure 48:** Experiment 4 – C-MYC expression in fasted state.

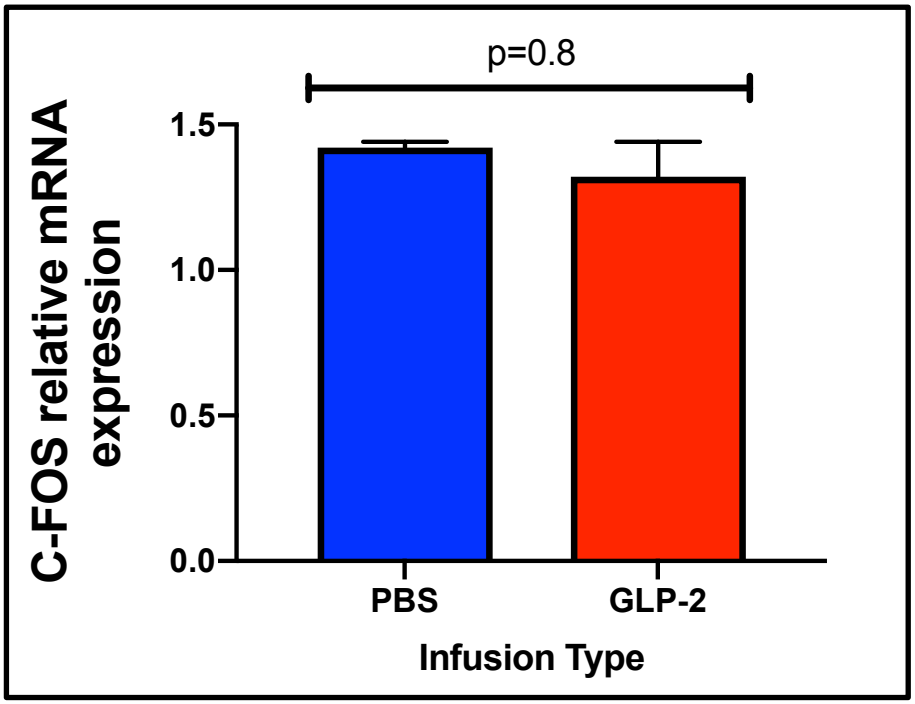



**Figure 49:** Experiment 4 – C-FOS expression in fasted state.

*2.6.5 Experiment 5*

Experiment 5, the final acute GLP-2 infusion study, resulted in the survival of 10 rats by the end of the procedure. 1 PBS rat died at 90 minutes (secondary to a suspected air embolism caused by the systemic catheter). Another PBS rat died at 30 minutes – this rat had a difficult SMV cannulation and it is probable that the rat suffered from irreversible bowel ischaemia. Data from these 2 rats was not included.

Portal blood glucose levels were seen to be similar between the GLP-2 group (n=5) and the PBS group (n=5) at baseline. Glucose levels went on to rise in both groups following the glucose infusion at the 40-minute time point. At 120 minutes, the blood glucose level in the GLP-2 group was significantly lower than that of the PBS group (p=0.01) (**Figure 50**).

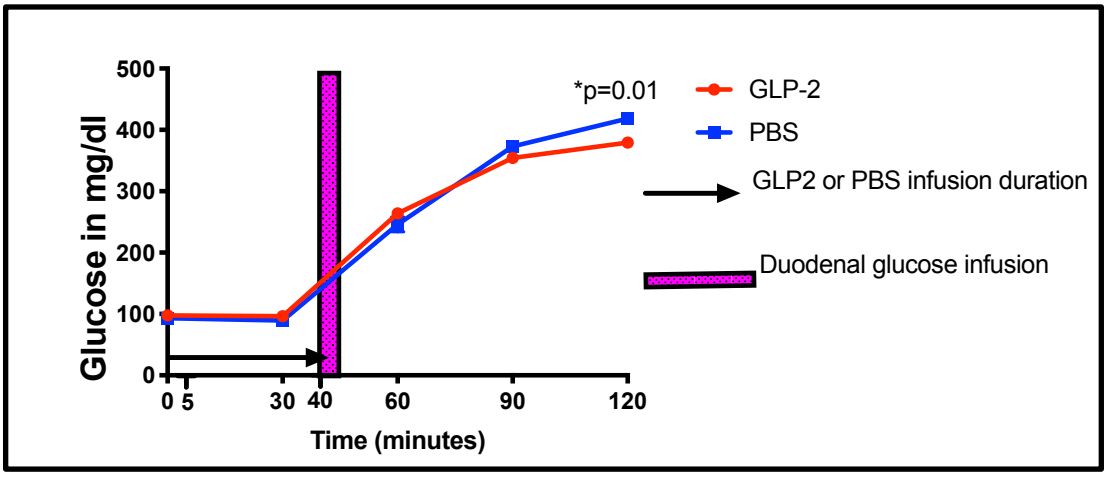



**Figure 50:** Experiment 5– Mean portal glucose levels following GLP-2 or PBS infusion, with concurrent duodenal glucose infusion (GLP-2 dose, 2000mcg/kg; GLP-2 infusion duration, 40 minutes; experiment duration, 120 minutes + 5-minute duodenal glucose infusion).

The systemic blood glucose levels behaved in a similar way to the portal results, although did not reach significance (**Figure 51**).

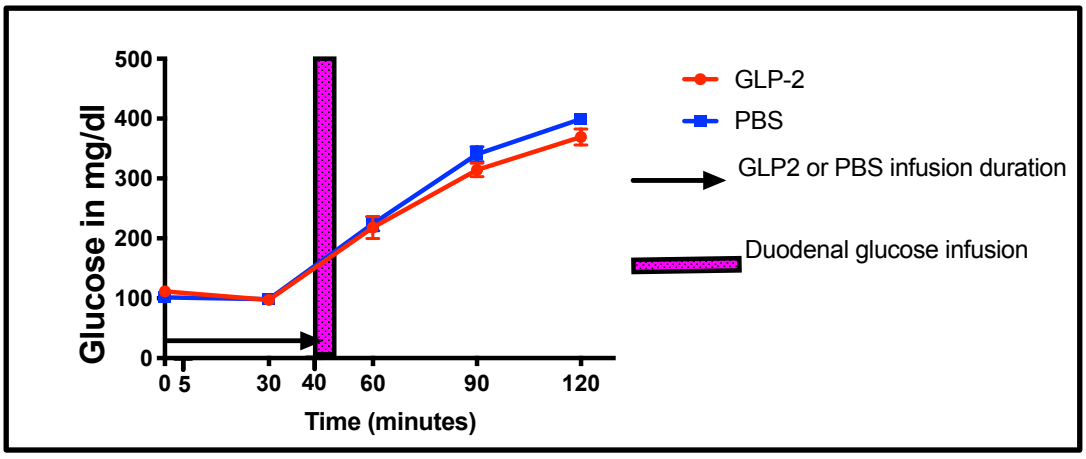



**Figure 51:** Experiment 5 – Mean systemic glucose levels following GLP-2 or PBS infusion, with concurrent duodenal glucose infusion (GLP-2 dose, 2000mcg/kg; GLP-2 infusion duration, 40 minutes; experiment duration, 120

Interestingly, the GLP-2 group $G_{PS}$ became more positive at the 60-minute time point compared to the PBS group. This is in contrast to non-glucose infused rats (in previous experiments), where the $G_{PS}$ gradient becomes more negative than the PBS rats. However, by 120 minutes, the GLP-2 $G_{PS}$ had become the more negative of the two groups once again. Statistical significance was not reached (**Figure 52**).

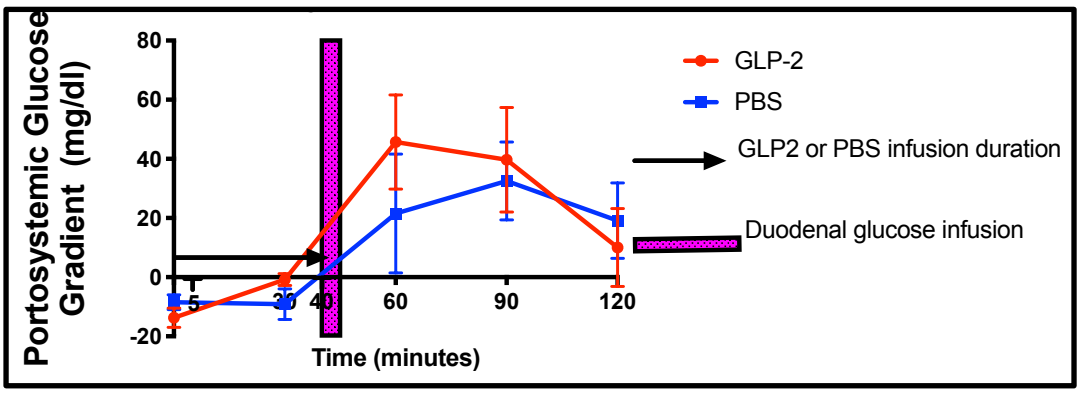



**Figure 52:** Experiment 5 – Mean porto-systemic gradient following GLP-2 or PBS infusion, with concurrent duodenal glucose infusion (GLP-2 dose, 2000mcg/kg; GLP-2 infusion duration, 40 minutes; experiment duration, 120

In comparison to Experiment 4 (in which the rats remained fasted), PCR revealed some interesting results. GLP-2 appeared to upregulate production of GLUT-1 compared to PBS control (**Figure 53**). This missed out on statistical significance, however.

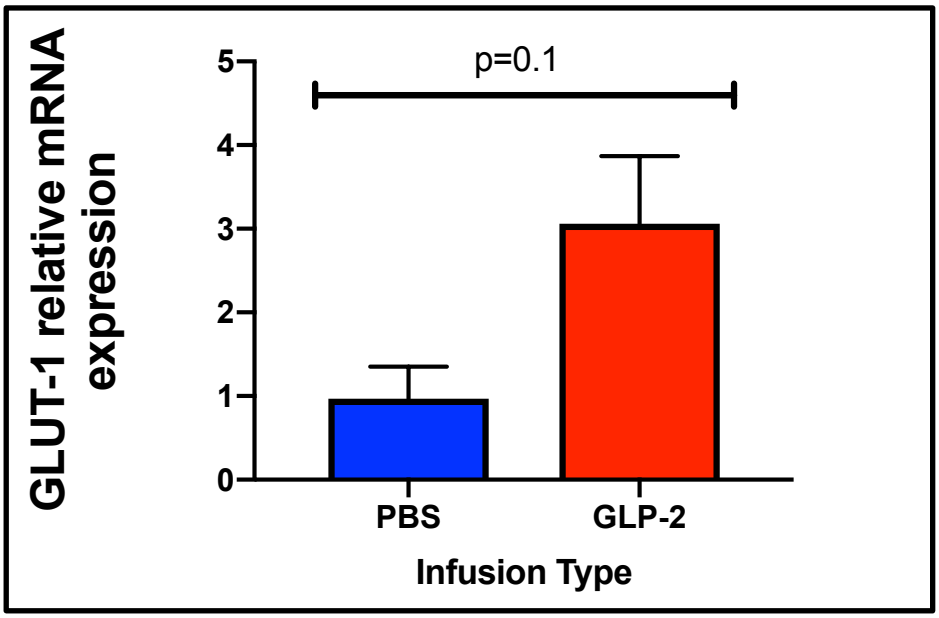



**Figure 53:** Experiment 4 – GLUT-1 expression in post-prandial state.

GLUT-2, on the other hand, was shown to be significantly upregulated by GLP-2 compared to PBS (p=0.04) (**Figure 54**).

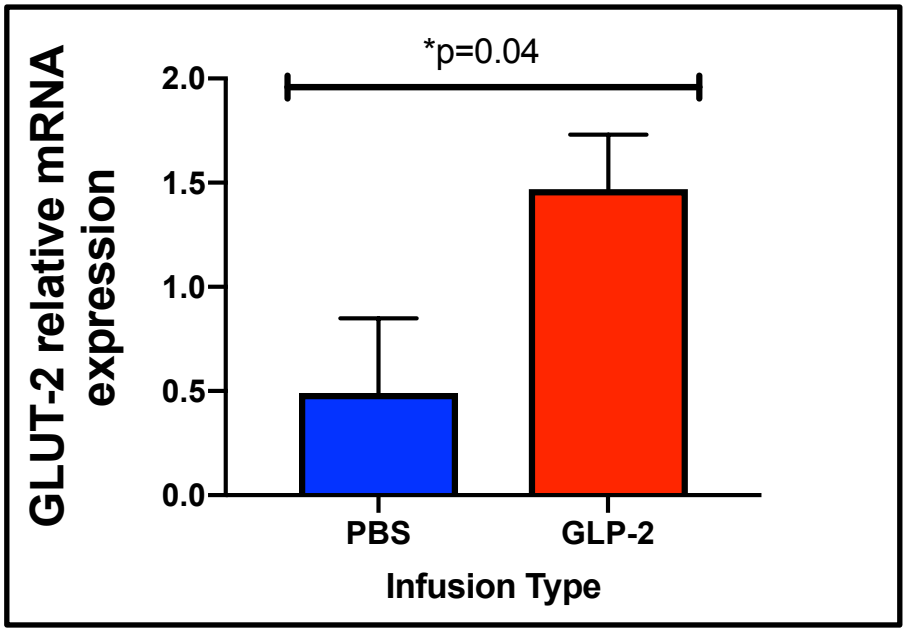



**Figure 54:** Experiment 4 – GLUT-2 expression in post-prandial state.

C-MYC and C-FOS production also appeared to increase following GLP-2 infusion, but significance was not achieved (p=0.5 and p=0.09, respectively) (**Figures 55 and 56**).

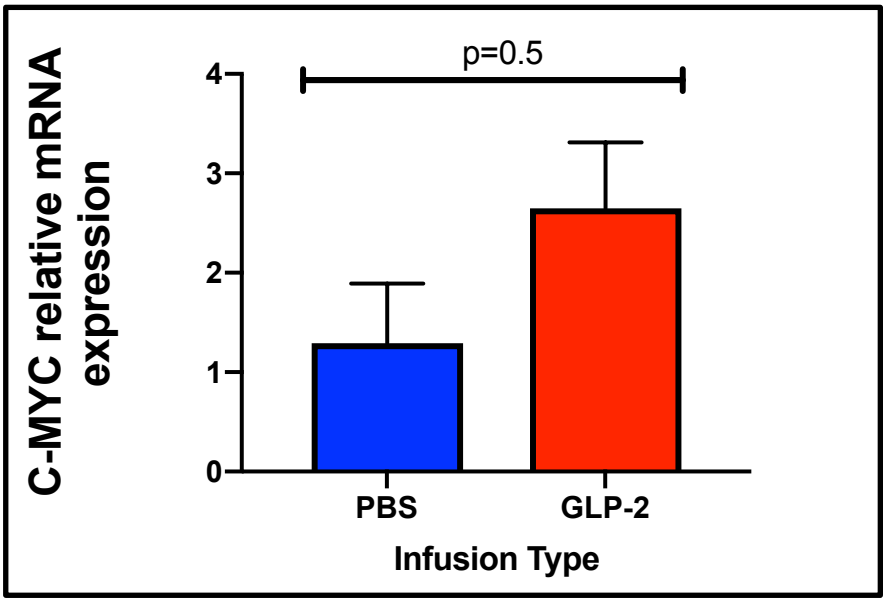



**Figure 55:** Experiment 4 – C-MYC expression in post-prandial state.

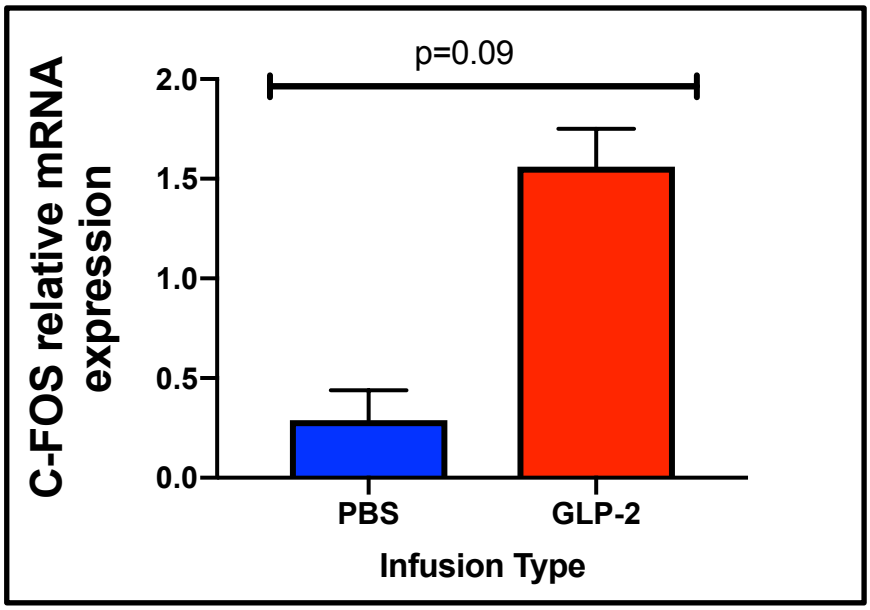



**Figure 56:** Experiment 4 – C-FOS expression in post-prandial state.

## 2.7 DISCUSSION

As per the literature review, groups who have investigated the effect of acute GLP-2 infusions had not used glucose levels as either the primary or secondary outcome, within a rat model or, indeed, any animal model. Thus, it was interesting to see how GLP-2 might affect glucose homeostasis in an acute setting, and both in fasted and fed states.

Although the first 3 fasted experiments initially showed some promising results, with GLP-2 appearing to cause a rise in glycaemia compared to PBS, once baseline glucose levels were matched between experiment and control rats (from experiment 4 onwards), it was hard to replicate the results obtained from experiments 2 and 3. However, when the experiment was switched from a fasted to a post-prandial setup, some interesting results were observed. In this case, it was intriguing to see that portal glucose levels were actually significantly lower (by approximately 10%) in the GLP-2 group at 60 minutes compared to the PBS group, with a trend to lower in the GLP-2 group within the systemic circulation. This was an important result as it appeared to agree with the original hypothesis; that GLP-2 infusion would lead to a lowering of portal and systemic blood glucose levels compared to control. Indeed, portosystemic gradient was also shown to be lower at 120 minutes, although not significantly so.

Experiment 4 showed that, at baseline, both groups had a negative $G_{PS,}$ presumably representing the net use of glucose by the intestine in the fasted state. As with experiments 1-3, both GLP-2 and PBS group $G_{PS}$ were seen to become more positive, despite the experiments being carried out in the fasted state. It is likely that this trend was seen because of the combined stress of anaesthesia and surgery. Interestingly, and perhaps mediated by the GLP-2 infusion, there appears to be a turning point at 30

minutes in the GLP-2 group - the $G_{PS}$ was seen to become positive, but then trended negative for the rest of the time period, in contrast to the positive $G_{PS}$ seen within the PBS control group. At 60 minutes, p=0.07, just missing out on significance. It is possible that this $G_{PS}$ negativity within the GLP-2 group could represent increased glucose utilisation by the intestine in response to the GLP-2. In turn, this could support the hypothesis that GLP-2 improves the body's glucose management by causing increased intestinal uptake in order for it to exert its more chronically seen adaptive effects i.e. mucosal growth of small intestine.

Within Experiment 5, and following a 5-minute glucose infusion, the GLP-2 group $G_{PS}$ becomes more positive than the PBS group $G_{PS}$, but this was not shown to be significant. This was in opposition to the hypothesis that $G_{PS}$ would become more negative in the GLP-2 group. It was interesting that glucose levels did not begin to decrease in either groups overall – this may be because the experiment duration was not long enough to see the expected harvest GTT decrease in blood glucose.

It was reassuring to see a statistically significant increase in plasma GLP-2 levels within the GLP-2 infusion group at 60 minutes via ELISA. This seemed to confirm that the peptide was not only correct, but that it was at least at a high enough concentration to be detected and, thus, likely effectual. In terms of the Luminex hormone analysis carried out in Experiment 3, it was not possible to say whether the insulin level discrepancy between the two groups is statistically significant as the blood sampling was from 1 rat per group. However, it was felt that this particular bench technique would be helpful as part of future aims to delineate GLP-2's effects on both incretin and other hormones secreted by the GI tract.

When jejunal mucosa, harvested at the end of Experiment 4 (without duodenal glucose infusion), was analysed there was no difference in GLUT-1 expression between the two groups. However, the jejunal mucosa harvested at the end of Experiment 5 (with duodenal glucose infusion) showed an increased trend in GLUT-1 expression. This could be an important finding because GLUT-1 is known to be present in fetal intestine but is thought to progressively disappear in adulthood. However, Saeidi et al also found an increase in GLUT-1, following a glucose meal in an RYGB model (Saeidi, Meoli et al.), and thus Experiment 5's results were in parallel with Saeidi's results, thus, upregulation of GLUT-1 could be an important mechanism for increasing glucose utilisation, mediated by GLP-2.

Results regarding GLUT-2 expression were also intriguing. It had been decided upon to look at GLUT-2 because a potential role for GLP-2 to influence transient GLUT-2 upregulation from the basolateral aspect of enterocytes to the jejunal brush-border had been previously suggested (Cheeseman et al, 2002). In the fasted state, there was a trend to increased GLUT-2 expression, however, in the post-prandial state, a significant increase in GLUT-2 expression was seen, thus supporting the findings by Cheeseman et al. Once again, this could reveal an important role for GLUT-2 in GLP-2 mediated glucose utilisation.

Intestinal proliferator expression, represented by C-MYC and C-FOS, was also studied. In the fasted state, there was almost no difference between GLP-2 and PBS groups regarding either mRNA expression. However, both proliferators were seen to increase in the postprandial state, with C-MYC mRNA expression levels in the GLP-2 group just missing out on reaching significance. It's difficult to know why significant increases in proliferation mRNA following GLP-2 infusion was not observed, given

that GLP-2 is known to be a potent proliferator. It is possible that the experiment was not powered adequately, or that these particular proliferation genes weren't sensitive enough to GLP-2, or to the dose of GLP-2 used, in order to see pronounced effects. Nevertheless, the fact that a trend in proliferator gene increase is seen does go some way to matching the original hypothesis in terms of providing evidence for suggesting that GLP-2 increased glucose utilisation within the intestine in order to mediate hypertrophy.

In summary, it was found that acute GLP-2 administration significantly lowers portal glucose in the postprandial state in healthy, SD rats. It also significantly increases GLUT-2 intestinal mucosal mRNA expression, whilst trending towards significantly increasing GLUT-1, C-MYC and C-FOS expression. As a result of these five experiments, it was felt that further studies were required to determine fully whether GLP-2 has a role to play in glucose homeostasis. The decision was made to move away from *acute* infusion studies and, instead, design an experiment that involved administering *chronic* GLP-2, in both healthy and pathological rat models. Additionally, it was felt that any potential GLP-2 effects on glycaemia could have been compromised by high levels of the GLP-2 degradation enzyme, DPPIV (Dipeptidyl peptidase-4) within in blood, leading to circulating GLP-2 having a biological half-life of 7 minutes (Hartmann, Harr et al. 2000). Thus, Teduglutide, a long acting GLP-2 agonist was selected in order to extend the half-life and improve efficacy of the peptide.

# CHAPTER 3

## 3.1 AIM 2: CHRONIC GLP-2 SUBCUTANEOUS ADMINISTRATION

- Investigate the effects of chronic GLP-2 administration (via subcutaneous injection), in Sprague Dawley rats, fed:
    - standard chow (SC) and
    - high fat diet (HFD) (pathological model)

### 3.1.1 *Overview*

*Assess the effect of chronic subcutaneous GLP-2 injection on intestinal glucose utilisation and absorption in standard chow (SC) and high fat diet (HFD) rats:*

- SD rats fed either SC, or HFD for 7 weeks in order to induce glucose intolerance.
- Daily subcutaneous injection of Teduglutide, ($Gly^2$)GLP-2, a DPP IV-resistant and, therefore, longer-acting analogue of GLP-2, or phosphate buffered saline (PBS: control) for 3 weeks.
- Assess OGTT over 3-week period.
- Assess portal system (hepatic-portal vein) and systemic system (external jugular vein) glycaemia at end of 3-week period – pre and post duodenal glucose infusion.
- Calculate portosystemic gradient ($G_{PS}$) to calculate intestinal glucose utilisation and absorption in fasting state and post-prandial state at end of 6-week period.
- Assess weight of rats over 3-week period.

- Weigh food twice a week to measure consumption between control and GLP-2 groups.
- Weigh small intestine at end of 3-week period.
- Luminex analysis of GLP-1 and GIP, glucagon and insulin.
- PCR to analyse GLP-2 effect on glucose-transporters/sensors, GLP-2R (glucagon-like peptide 2 receptor), IGF-1 (insulin-like growth factor 1), T1R2 (sweet taste receptor).

### *3.1.2 Hypothesis*

*It is hypothesised that chronic subcutaneous administration of GLP-2 will:*

- Reduce fasting and postprandial blood glucose levels.
- Increase intestinal weight secondary to its hypertrophy effects.
- Alter rat total body weight, although difficult to predict in which direction.
- Make the portosystemic gradient less positive (compared to control) indicating increased intestinal glucose utilisation.
- Upregulate glucose transporter/sensor and IGF-1* and T1R2** gene expression.
- *Further reading had revealed a possible mechanism suggesting that luminal nutrients stimulate intestinal growth by increased expression of both GLP-2 and IGF-1 (Nelson, Murali et al. 2008).
- **Additionally, RYGB has been shown to lead to decreased luminal absorption via exclusion of sweet-taste pathways in the proximal bowel (Bhutta, Deelman et al. 2014).

## 3.2 VALIDATION PROTOCOL 1

*Materials and Methods*

*Teduglutide*

Teduglutide, (Gly$^2$)GLP-2, (referred to as TED in this thesis) was synthesised by and purchased from the Peptide/Protein Core Laboratory at Massachusetts General Hospital (MGH). The structure of the peptide was confirmed by this laboratory as follows:

"His-gly-asp-gly-ser-phe-ser-asp-glu-met-asn-thr-ile-leu-ala-ala-arg-phe-ile-asn-trp-leu-ile-gln-thr-lys-ile-thr-asp".

Unlike the validated GLP-2 purchased from Abcam and Bachem in Aim 1, the Teduglutide was synthesised especially for these studies and had not been internally validated by MGH. The laboratory suggested that their peptide was 98% pure by high performance liquid chromatography (HPLC). However, its activity still needed to be confirmed. Hence, a validation experiment was devised.

*Animals*

Animal studies were performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. Six male SD rats (200-220g, Charles River, MA) were acclimatised for one day under a 12:12 light: dark cycle (lights-on at 07.00) with *ad libitum* access to standard rat chow (Purina 5053). The SD rat is a healthy rodent model. Three were selected to receive 0.1mg/kg/day (0.1mcg/g) subcutaneous TED, dissolved in 200µl PBS. In order to reduce the number of freeze-thaw cycles, aliquots of the TED solution were made up

in advance. The following calculation was used to ensure rats received 100mcg/kg/day, given that their weight naturally increased over the 6-day validation injection period:

- To make stock solution:
    - Daily dose to be given as based on literature review (Kaji, Tanaka et al. 2008) = 0.1mg/kg (0.1µg/g).
    - Therefore, a 220g rat requires 22µg.
    - Stock solution to have a concentration of 0.11µg/µl in order to achieve total volume of at least 200µl per rat.
    - Take 1mg of peptide and dissolve in 9.09ml of PBS to give a concentration of 0.11mg/ml (equivalent to 0.11µg/µl).

- Example calculation for a rat weighing 210g:
    - Rat requires 21µg of TED.
    - Therefore, amount of TED solution = 21/0.11
      = 190.9µl.
      = 0.19ml to be administered using a 1.0ml syringe.

Three rats were selected to act as control animals and received 200µl PBS only. Each day at 10:00, all 6 rats were placed in a DecapiCone (a tapered plastic film tube, providing quick and easy restraint of rats) and received a weight-adjusted subcutaneous injection (of either TED or PBS) into the loose skin over the neck. Rats were given a total of 6 injections in total, with a total study duration of 7 days. On day 7, all six rats were harvested, with small intestine weighed. Haematoxylin and Eosin staining was used to measure and compare villous and crypt depth between

experiment and control animals. The first 5cm of proximal jejunum intestine, just distal to the Ligament of Treitz (which was used as an anatomical landmark for reproducibility of method purposes) was taken for histology, as it was felt that it was the section of bowel most likely to show most obvious morphological changes (Thymann, Stoll et al. 2014).

*Haemtoxylin and Eosin Staining Method*

**Frozen Section Method - Using OCT Solution**

Intestinal tissue was opened up length-wise with scissors. The tissue was rinsed with PBS. It was then fixed in freshly made 4% paraformaldehyde solution (PFA) for at least 1 hour, after which it was placed in a refrigerator. Next day, the tissue was rinsed in PBS (3 rinses for 5 minutes each). Tissue was then placed in 30% sucrose until it sank. It was then dragged through optimum cutting temperature (OCT) solution three times in order to prevent the formation of ice crystals. A receptacle containing 2-methylbutane was placed in liquid nitrogen. A cryomold containing the tissue (placed in top-right corner of mold) was submerged in the 2-methylbutane. Frozen cryomolds were wrapped in foil and stored in a -80°C freezer until sent for cryosection at Beth Israel Deaconess Medical Centre (BIDMC) Histology Core.

*Harvest Protocol*

Performed as in section 2.4.1.

*Results and Discussion*

Statistical analysis was carried out as described in section 2.4.1. Although a statistically significant increase in total rat body weight was observed in the TED group (n=3) versus the PBS group (n=3) (**Figure 57**), as well as a significant increase

in combined weight of jejunum and ileum within the TED group (**Figure 58**), unfortunately, no difference in villus length or crypt depth was seen (**Figure 59**).

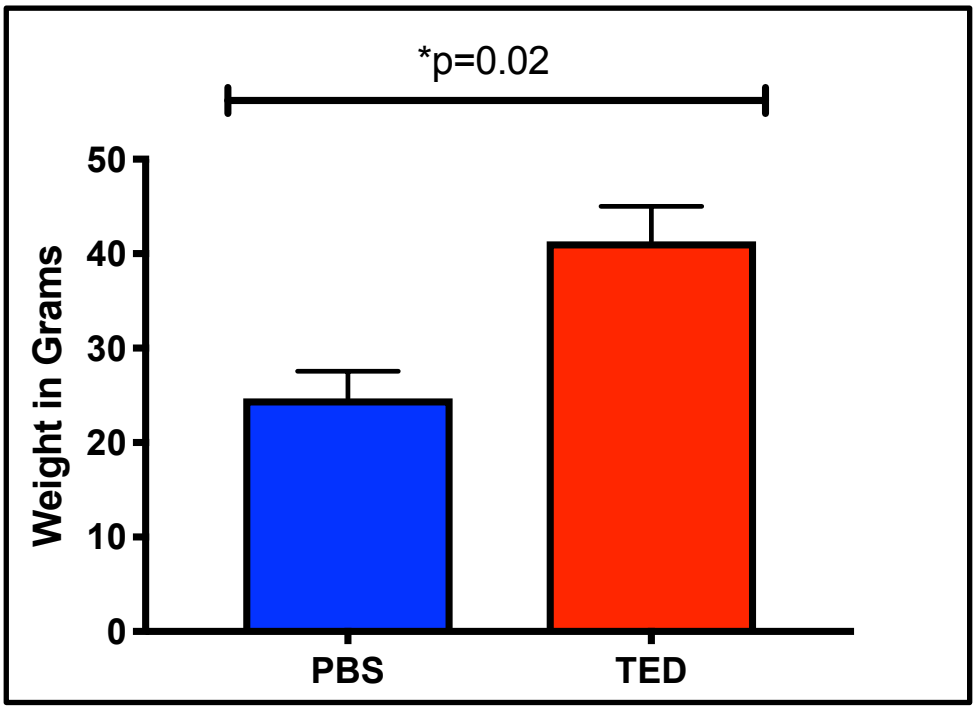



**Figure 57:** Validation Protocol 1 – Mean change in total body weight.

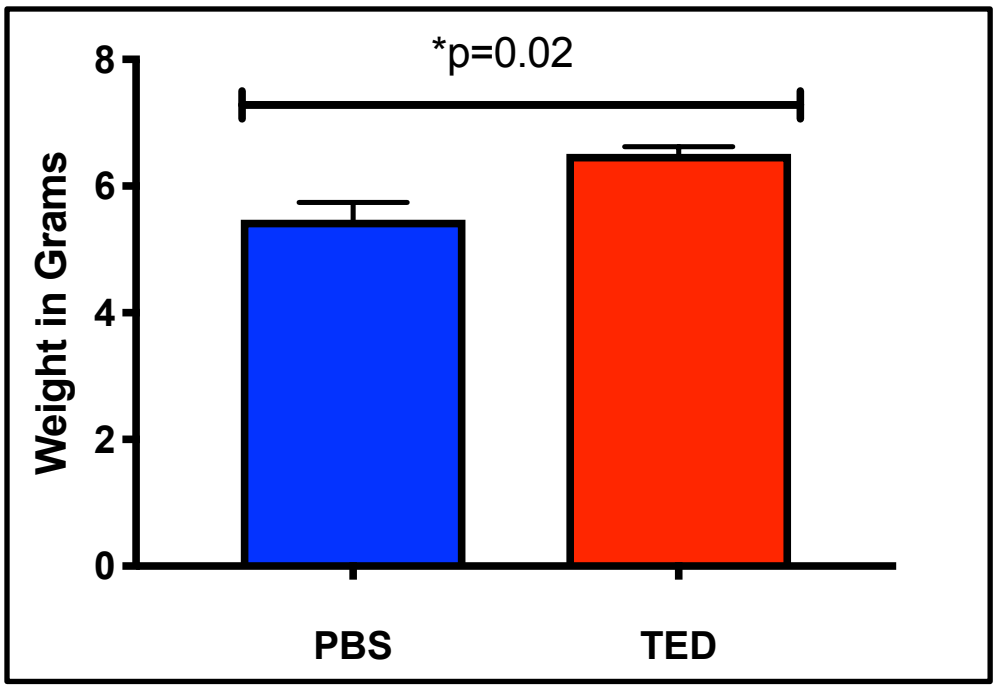



**Figure 58:** Validation Protocol 1 – Mean combined jejunum and ileum weight.

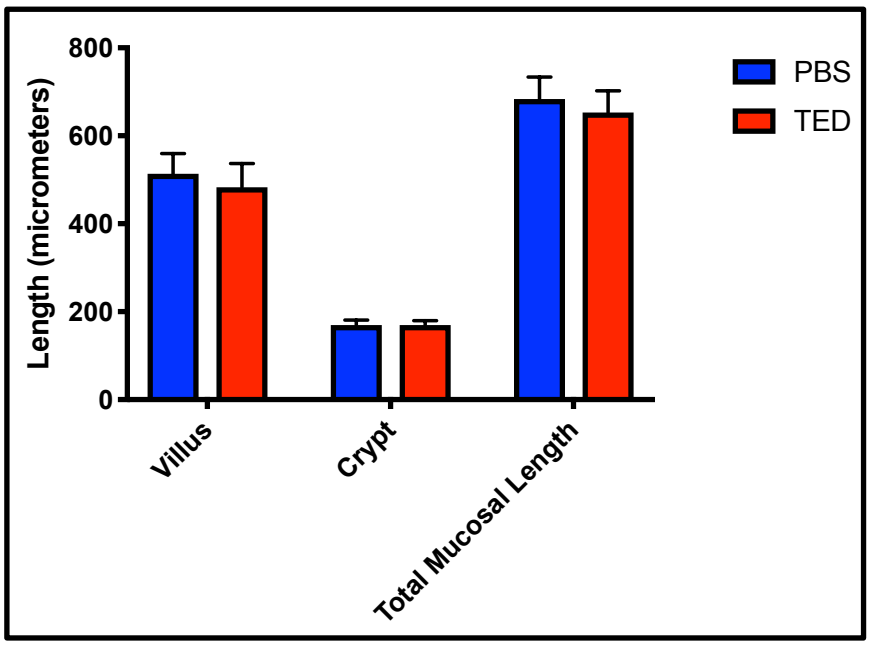



**Figure 59:** Validation Protocol 1 – Histological measurements (OCT cut 1).

The histology core was asked to cut new slides to see if there might be a problem with the original slides. However, once again, there was no difference in villus and crypt measurements between the two groups (**Figure 60**).

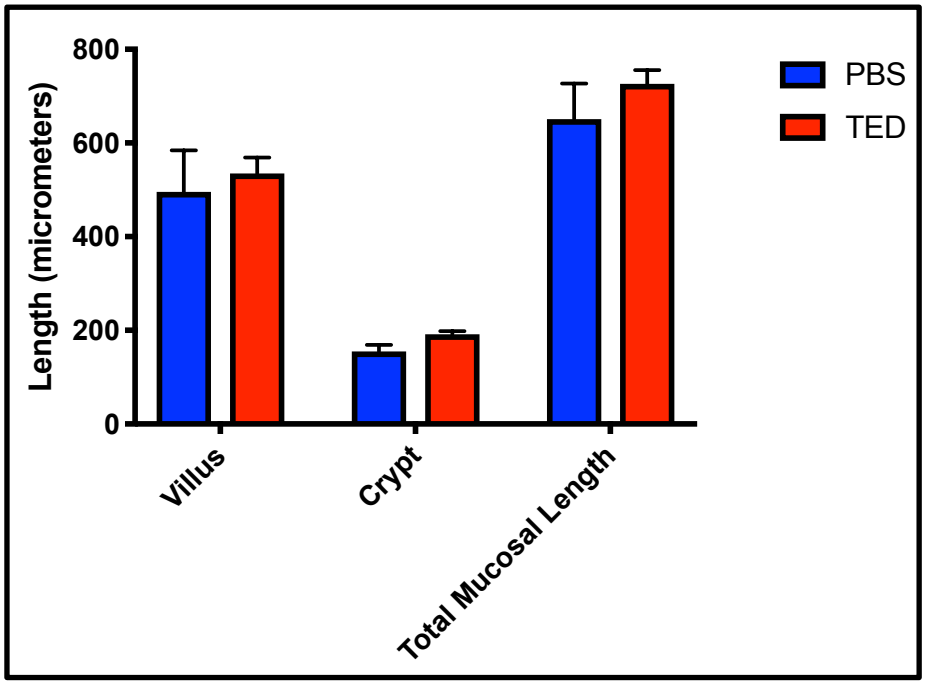



**Figure 60:** Validation Protocol 1 – Histological measurements (cut 2).

Given that the hypothesis for this thesis centres around GLP-2's known effects on bowel hypertrophy there were concerns regarding the peptide's functionality. The protocol was altered so that both dosage and duration of TED administered was increased. The dosing adjustments were based on work done by another group who assessed the effects of GLP-2 of diurnal gene expression (Ramsanahie, Berger et al. 2004). They also used Teduglutide and at a dose of 1000µg/kg/BD, i.e. 2000µg/kg/day for 10 days. Validation Protocol 1 used a dose of 100µg (0.1mg)/kg/day for 6 days. Thus, it was determined that it would not be unreasonable to try a higher dose of TED, and for a longer duration.

There were also concerns regarding the quality of imaging that the frozen OCT method was offering, and whether it might have been affecting the accuracy of the morphological analysis (**Figure 61**). Therefore, as guided by the BIDMC histological core, a Paraffin staining method was adopted (as described in section 3.3).

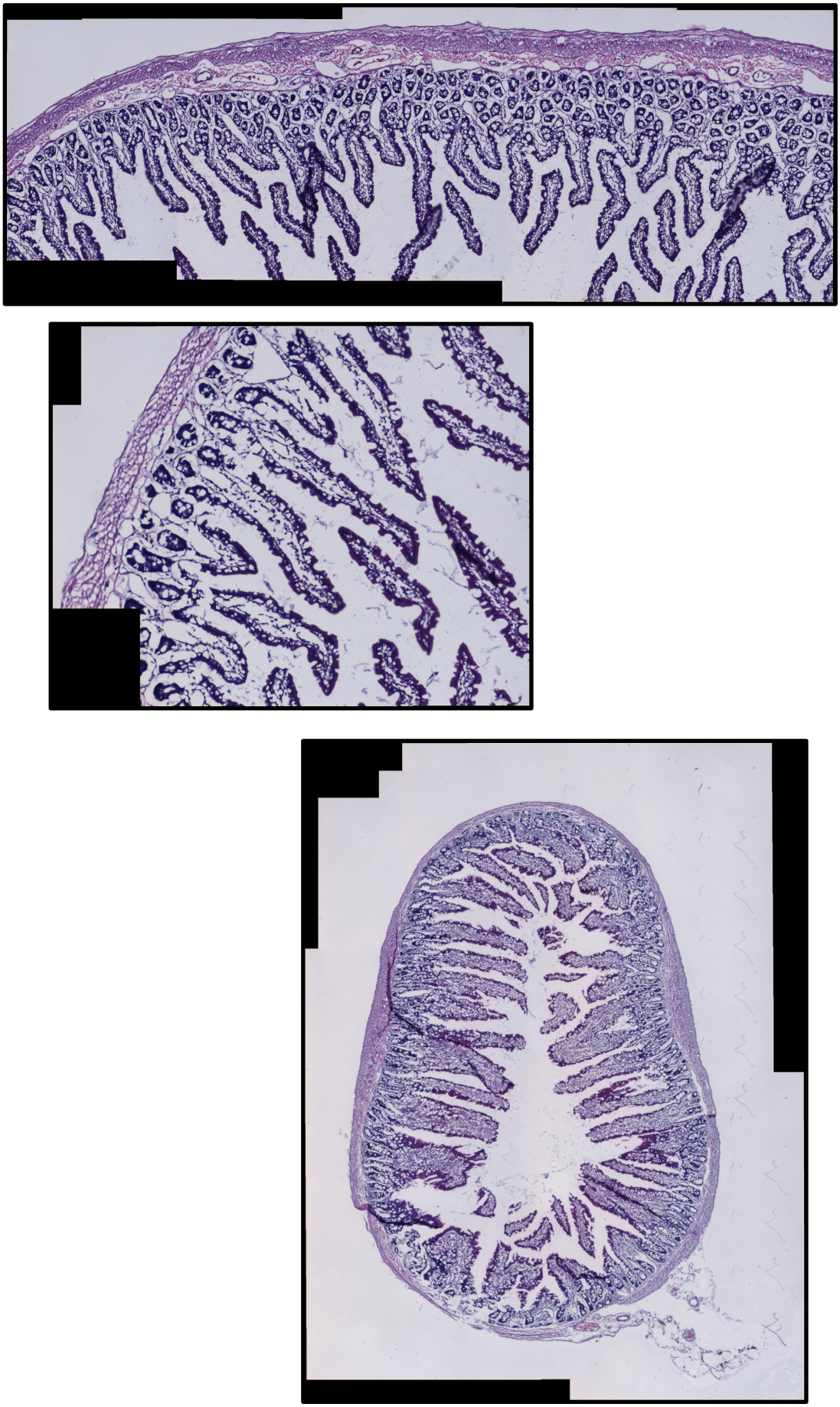


**Figure 61:** Examples of histological imaging gained from the frozen OCT fixing method.

## 3.3 VALIDATION PROTOCOL 2

*Materials and Methods*

*Teduglutide*

As with Validation Protocol 1, TED was purchased from the Protein/Peptide Core at Massachusetts General Hospital.

*Animals*

A further six male SD rats (Charles River, MA) were acclimatised in the same way as in Validation Protocol 1. However, this time 0.4mg (400µg)/kg in 200µl PBS (increased from 0.1mg/kg) was injected in 3 rats, and 200µl PBS alone in another 3 rats, for a total of 8 days (instead of 6 days). A stock solution of 0.44µg/µl of TED dissolved in PBS was made up.

*Haemtoxylin and Eosin Staining Method*

**Paraffin Method**

Concerns with the quality of sections that were gained from the OCT frozen method resulted in the paraffin method being used. As with the frozen method above, intestinal tissue was opened up length wise and rinsed with PBS. It was then directly placed in freshly made 4% PFA and stored in a refrigerator overnight. Next day, the samples were taken to BIDMC Histology Core, where they were embedded in paraffin wax and cut in to 5µm sections.

*Harvest Protocol*

Performed as in section 2.4.1.

*Results and Discussion*

Statistical analysis was carried out as described in section 2.4.1. This time there was no difference in the change in total body weight between the two groups (**Figure 62**). There was, however, a statistically significant increase in combined jejunum and ileum weight in the TED group compared to the PBS group (p=0.02) (**Figure 63**).

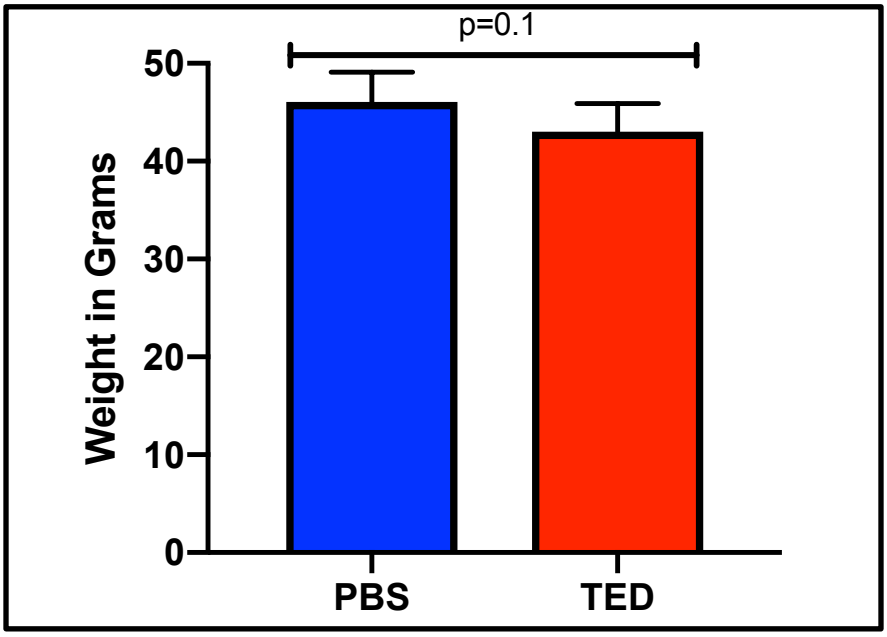



**Figure 62:** Validation Protocol 2 – Mean change in total body weight.

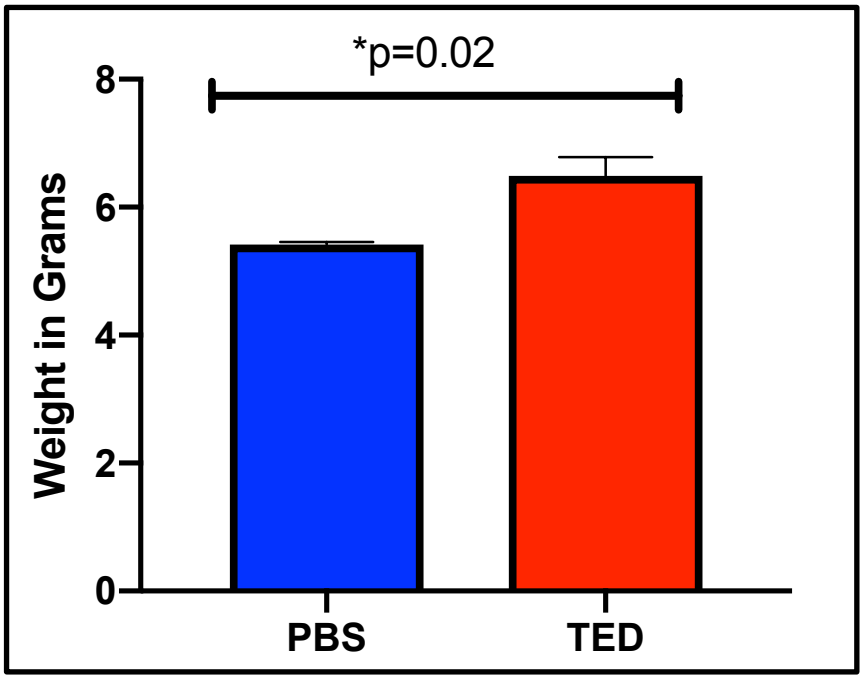



**Figure 63:** Validation Protocol 2 – Mean combined jejunum and ileum weight.

Although it was felt that the overall quality of images gained from the paraffin histology method was an improvement (**Figure 64**), compared to the frozen OCT method (**Figure 61**), the first paraffin block cut that we received gave non-significant results regarding villus and crypt measurements (**Figure 62**).

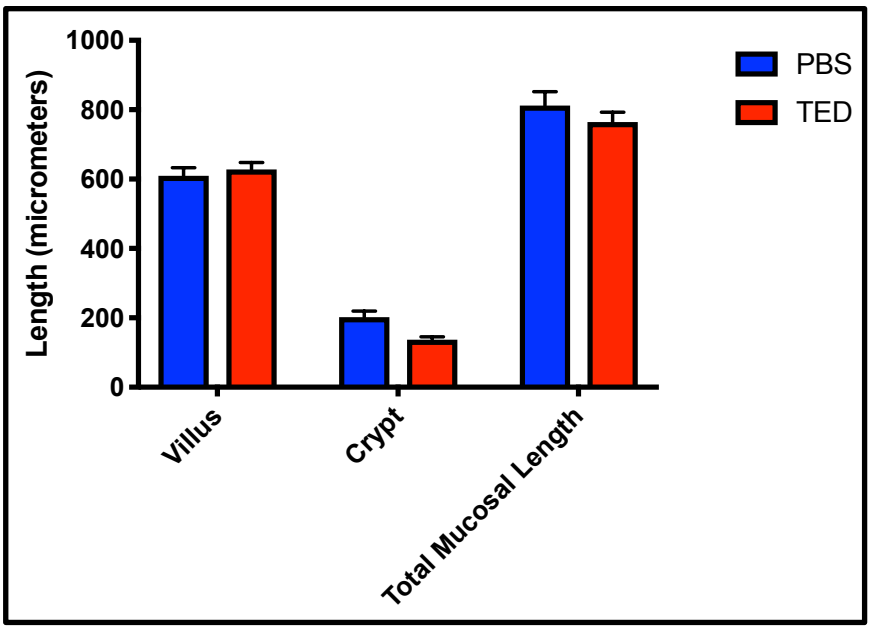



**Figure 62:** Validation Protocol 2 – Histological measurements (PARA cut 1).

A repeat cut from the paraffin block was requested and, with measurements more promising this time (**Figure 63**).

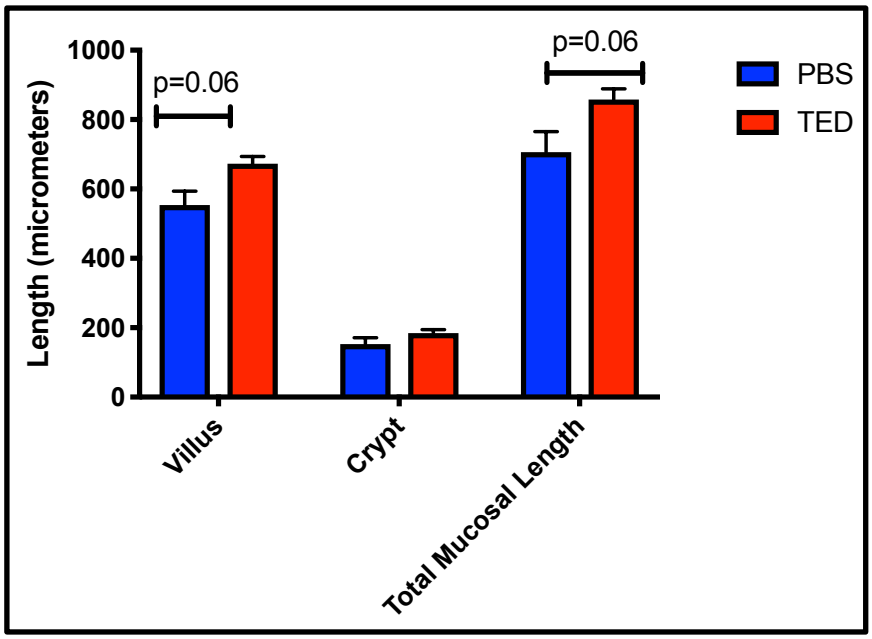
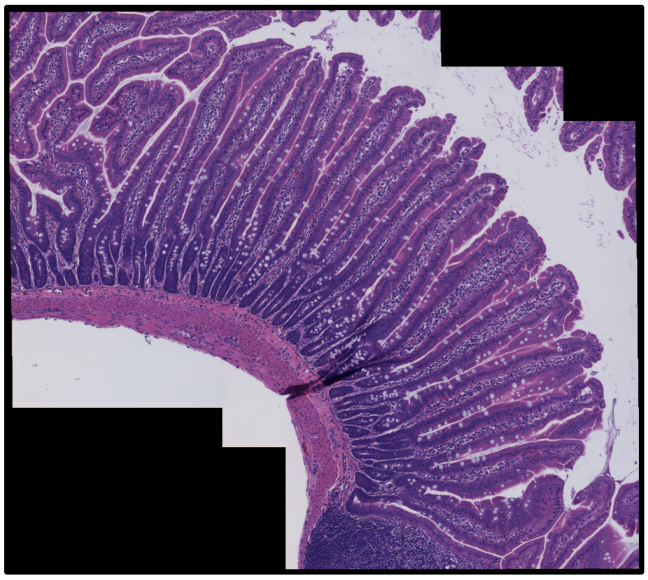



**Figure 63:** Validation Protocol 2 – Histological measurements (PARA cut 2).





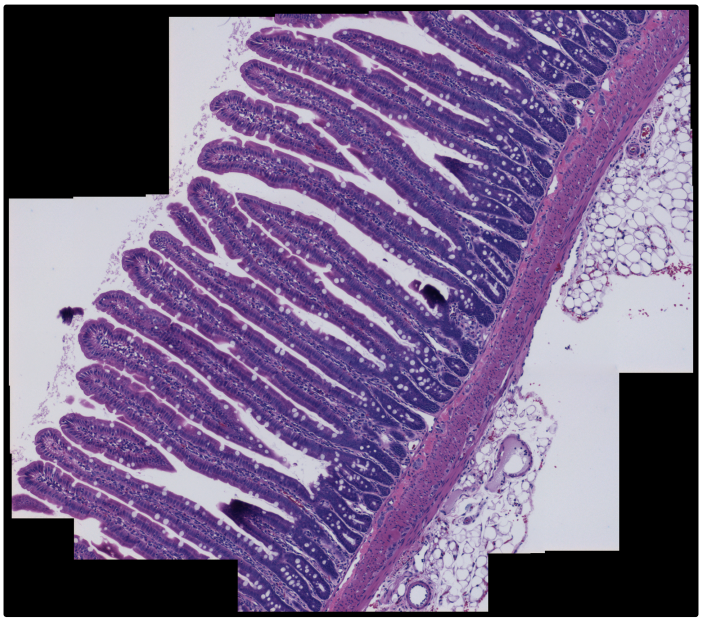


**Figure 64:** Examples of histological imaging gained from the paraffin fixing method.

At this point, it was also decided to check that measurement analysis was being conducted as accurately as possible. It was discovered that the measuring technique being employed often didn't measure a villus right from the tip to the start of the crypt. It was felt that this could account for why it had been difficult to prove the validity of the Teduglutide peptide. After agreeing on a more accurate measuring method, the second paraffin cut from Validation Protocol 2 was reanalysed. This time, a trend towards significant increase in villous length (p=0.06) was observed in the TED group, along with a statistically significant increase in crypt depth (p=0.01) in the TED group (**Figure 65**).

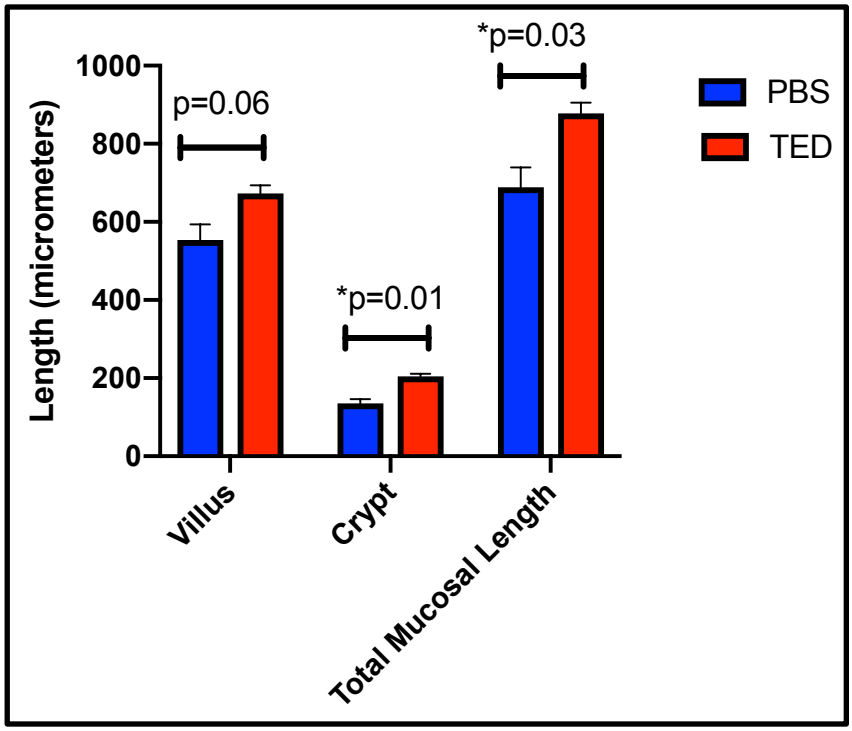



**Figure 65:** Validation Protocol 2 – Histological measurements (PARA cut 2, re-measurement).

Given these results, it was felt that there was no real reason to distrust the peptide, thus it was decided upon to enter the main experiment.

## 3.4 EXPERIMENT 1

### *3.4.1 Materials and Methods*

*Teduglutide*

As with Validation Protocols -1 and 2, TED was purchased from the Protein/Peptide Core at Massachusetts General Hospital.

*Animals*

A further six male SD rats (200-220g, Charles River, MA) were acclimatised for 7 days under a 12:12 light: dark cycle (lights-on at 07.00) with *ad libitum* access to standard rat chow (Purina 5053). The dose of 0.4mg (400µg)/kg in 200µl PBS, used in Validation Protocol 2, was injected subcutaneously into 3 rats, and 200µl PBS alone into another 3 rats, for a total of 24 days, with a total study duration of 33 days. Oral glucose tolerance tests (OGTT) and intraperitoneal glucose tolerance tests (IPGTT) were carried out as per the study timeline (**Figure 66**).

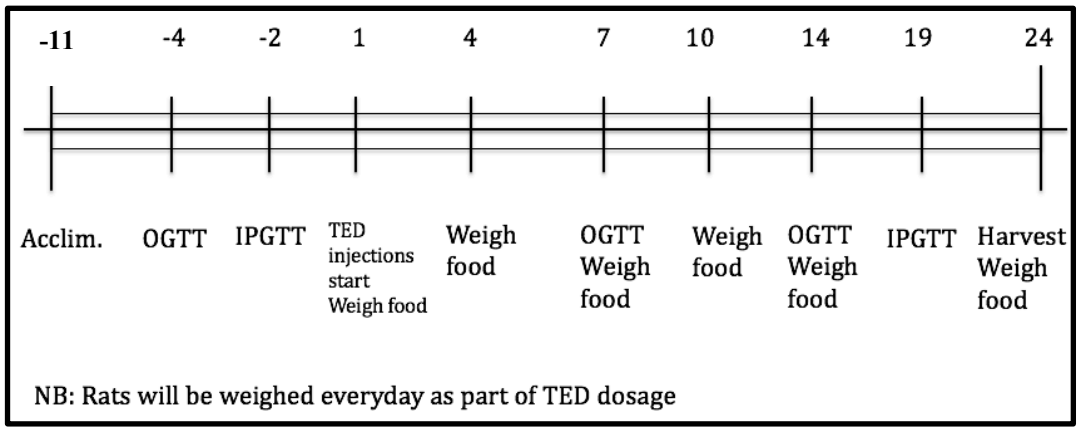



**Figure 66:** Experiment timeline.

*OGTT Protocol*

All animals underwent a 15 hour fast on a wire tray, with water ad libitum. Each OGTT started at 10:00 in the morning. Rats were weighed using the same set of electronic scales each time. A 25% glucose solution (2g/kg of D-glucose dissolved in $dH_2O$) was gavaged using a gavage needle. A drop of tail vein blood was taken using a 27G needle at baseline (before glucose administered) and at 15-, 30-, 60-, 90- and 120-minute time points, and glucose measured using a handheld glucometer (OneTouch Ultra Mini). After collection of blood, direct pressure was applied to the puncture site until haemostasis was achieved. In agreement with local protocol, if there was any sign of respiratory distress post-injection the animal would be euthanised immediately.

*IPGTT Protocol*

All animals underwent a 15 hour fast on a wire tray, with water ad libitum. Each OGTT started at 10:00 in the morning. All 12 rats were weighed and given a general anaesthetic (1-3% isoflurane in oxygen). As with OGTT, a 25% glucose solution (2g/kg of D-glucose dissolved in $dH_2O$) was prepared. Each rat was held at a 45-degree angle with the abdomen exposed and the head positioned upwards. A short 25G needle was inserted cranially into the right lower abdomen at a 30- to 45-degree angle caudal to the umbilicus and lateral to the midline to avoid the liver, bladder and caecum. Blood samples were collected from the tail vein at 0, 15, 30, 60, 90 and 120 minutes post-intraperitoneal glucose injection.

*Harvest Protocol*

*Catheter Placement*

Portal and systemic venous blood was sampled in the same way as described above, (section 2.4.1), before and after duodenal glucose infusion, and over a 60-minute time-frame. Duodenal infusion involved giving a 25% glucose solution infused over 5 minutes into the duodenum located 1cm distal to the pylorus.

*Blood Analysis*

As with prior experiments, portal and systemic blood glucose was measured at each time-point using a glucometer (LifeScan OneTouch). Additionally, portal and systemic blood was taken for hormone analysis using ELISA and Luminex techniques (see **Appendix 1 and 2**, respectively), at specific time points. DPP-IV Inhibitor and Aproptonin were once again added to eppendorphs before blood collection. Blood was centrifuged at 4°C at 4000*G* for 15 min, the plasma aspirated, and stored at -80°C. Portosystemic glucose ($G_{PS}$) gradient was calculated ($G_{PS} = G_P - G_S$, where $G_P$ is portal glucose level and $G_S$ is systemic glucose level at a given time point), and this was used to demonstrate changes in intestinal glucose utilisation and absorption.

*Morphological Preparation*

At the 60-minute time point, after the last blood sample had been taken, the jejunum (the start of which was taken to be at the duodenal-jejunal junction) and ileum (down to where it enters the caecum at the ileo-caecal valve) was removed from the abdomen. The first 5cm of jejunum was removed and prepared using the paraformaldehyde technique for subsequent paraffin cutting and morphological

analysis, because of the improved images that paraffin offered over OCT (see section 3.3).

*Weighing of Tissue*

The rest of the jejunum and ileum, still intact, was then rinsed with distilled water and weighed.

*Termination of Experiment*

Each experiment was ended by puncturing the diaphragm with scissors and cutting the heart in two, allowing exsanguination under anaesthesia.

*3.4.2 Results*

Statistical analysis was carried out as described in section 2.4.1. The main experiment revealed diverse results. OGTT seemed to show that chronic Teduglutide administration was having an effect on glucose tolerance. At baseline (5 days pre-injections), the two groups (n=3, both groups) were matched in terms of blood glucose levels, with no significant difference between them at any time point following an oral glucose challenge (**Figure 67**).

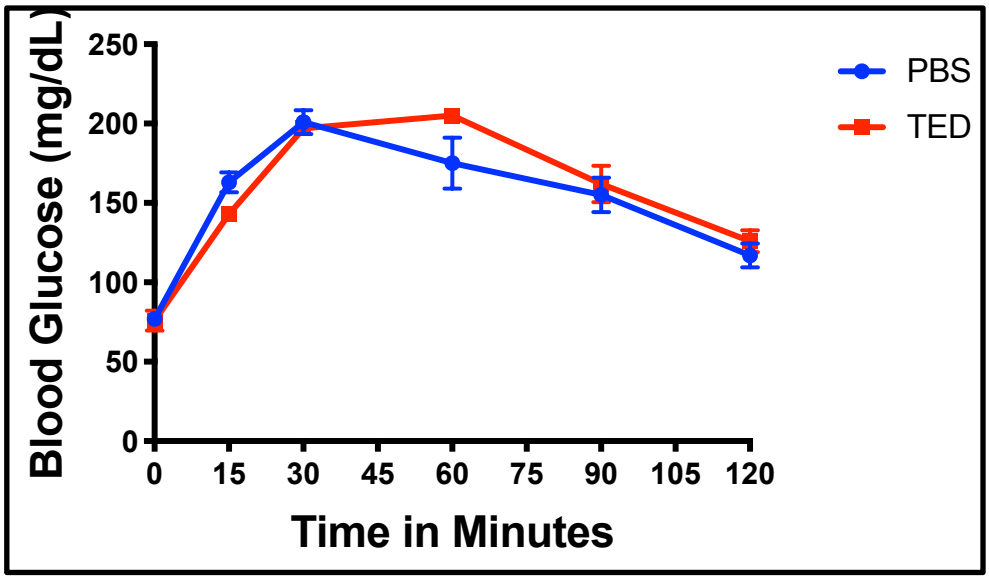



**Figure 67:** Oral glucose tolerance test 1 (baseline; 5 days pre-initiation of injections).

However, at week 1 (**Figure 68**) and week 2 (**Figure 70**) post-initiation of injections, blood glucose levels in the TED group became significantly lower at 60- and 90-minute time points (p=0.03, p=0.0004 and p=0.01, p=0.03). Area under the curve (AUC) was also calculated for OGTT as an additional way of visualising and interpreting the data (**Figures 69 and 71**). Despite the promising results gained from the glucose tolerance tests, there was no significant difference to be found between the two groups according to AUC.

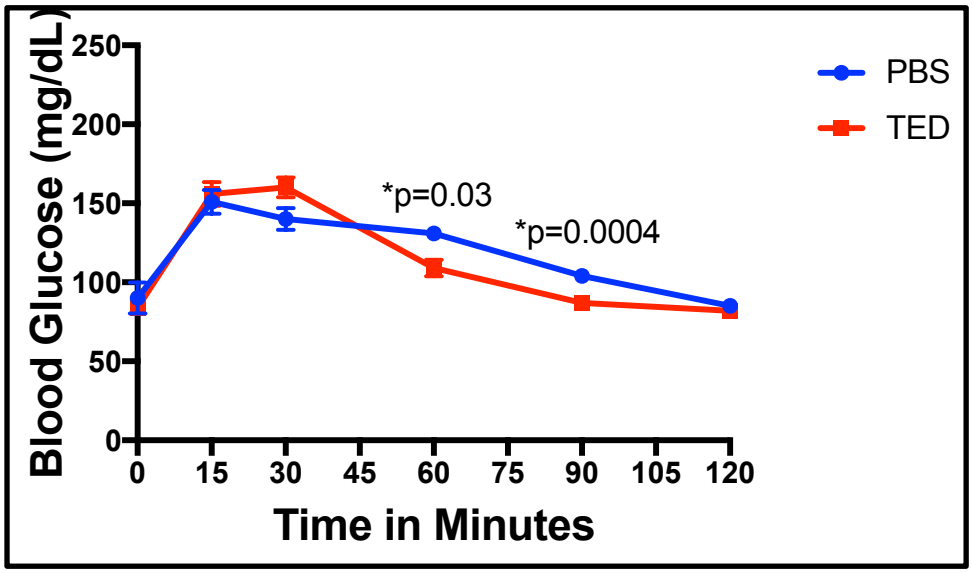



**Figure 68:** Oral glucose tolerance test 2 (day 7 post-initiation of injections).

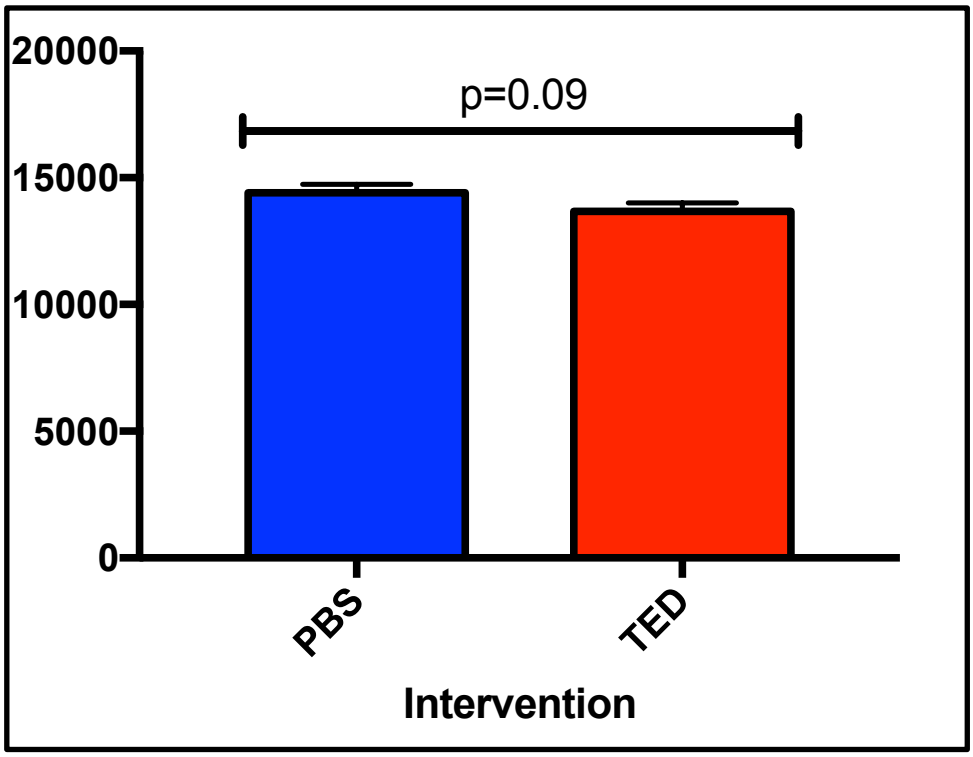



**Figure 69:** Area under the curve for oral glucose tolerance test 2.

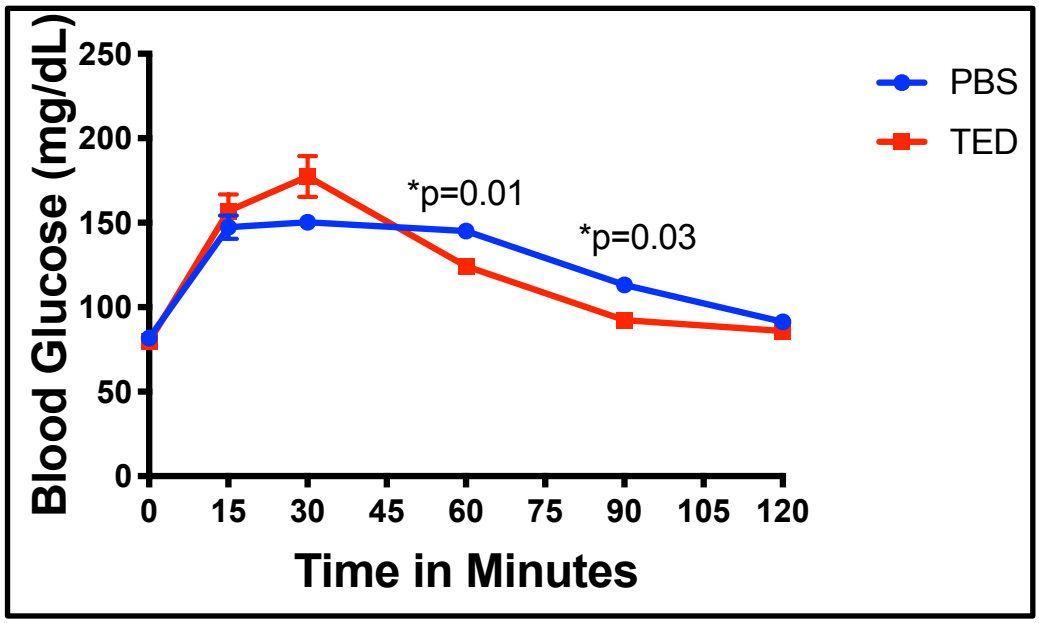



**Figure 70:** Oral glucose tolerance test 3 (day 14 -post-initiation of injections).

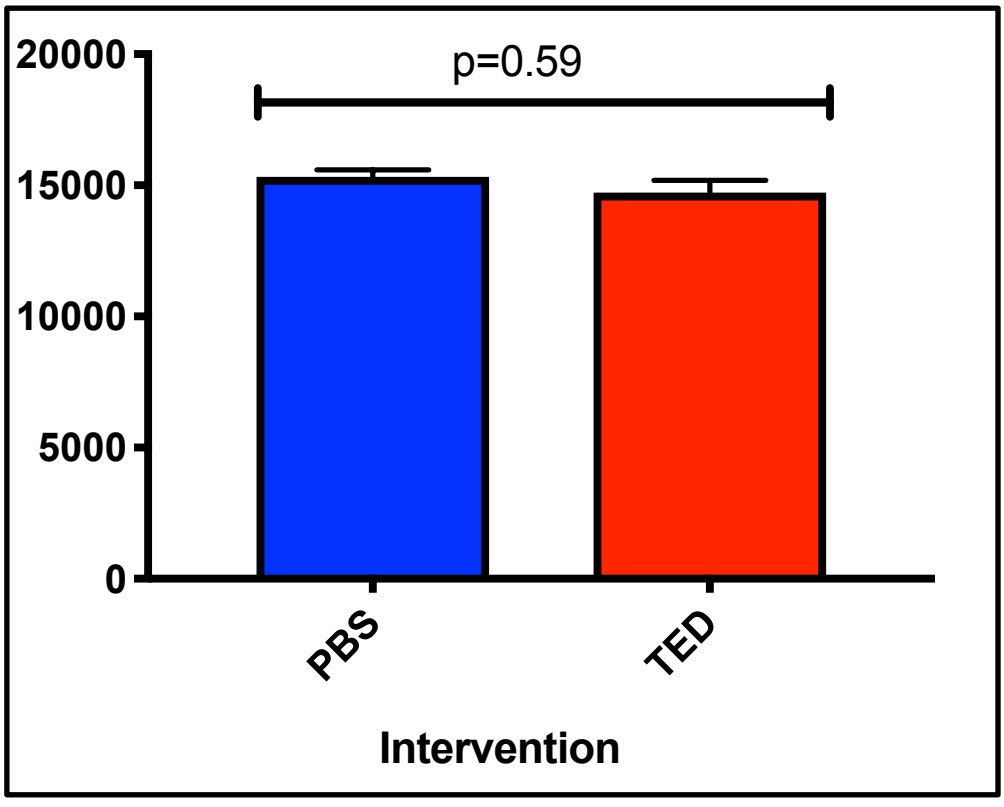



**Figure 71:** Area under the curve for oral glucose tolerance test 3.

This pattern was also reflected in the harvest glucose tolerance test (GTT) at day 24, with the TED group glucose levels, in both portal and systemic blood, dropping at 60 minutes compared to glucose levels in the PBS group (although, not reaching statistical significance) (**Figures 72 and 73**).

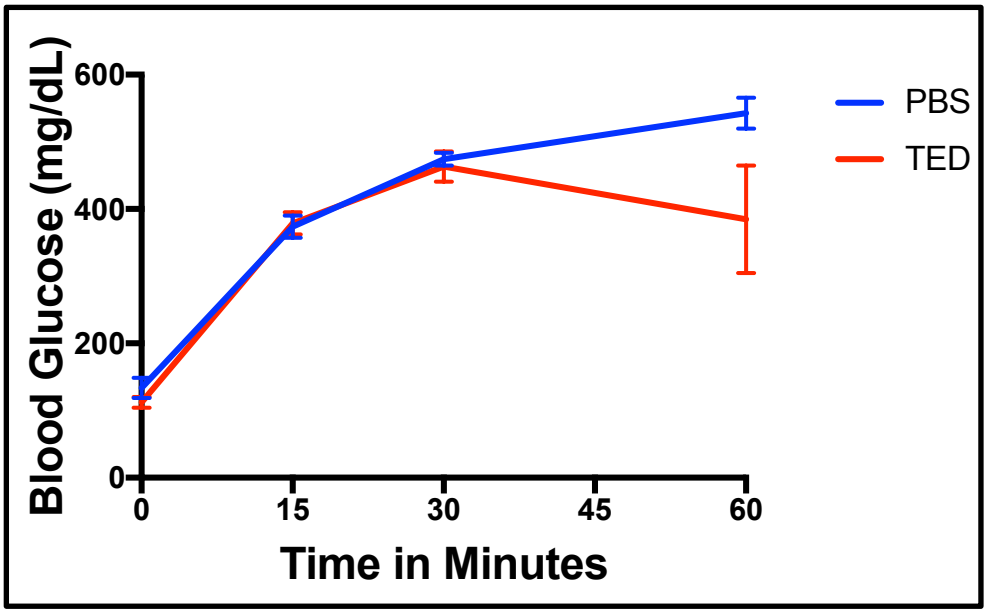



**Figure 72:** Mean portal glucose levels at harvest GTT (day 24).

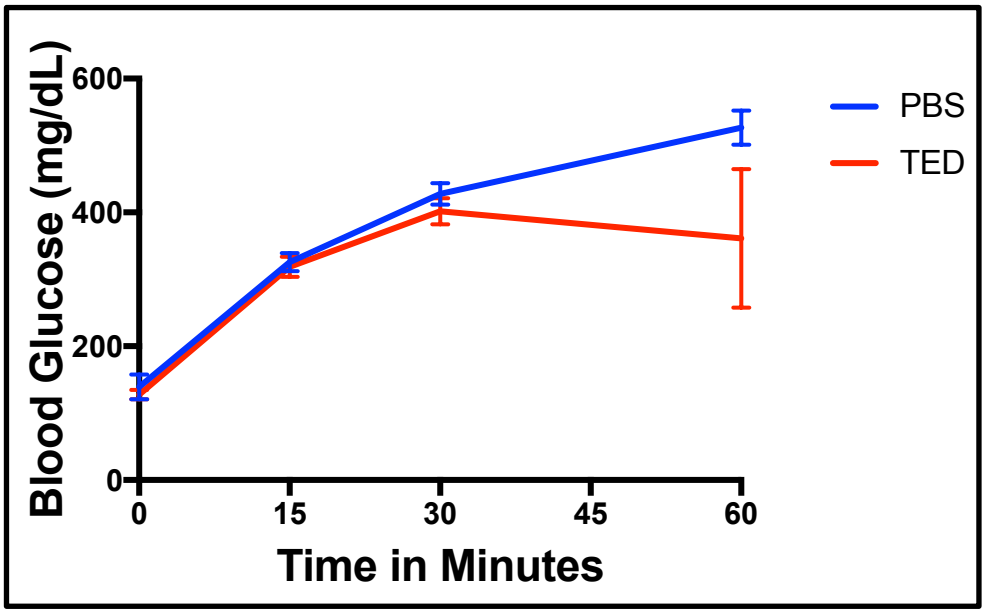



**Figure 73:** Mean systemic glucose levels at harvest GTT (day 24).

Portosystemic gradient in the TED group became more positive than in the PBS group by 15 minutes and stayed more positive until the end of the experiment, although this was not found to be significant, either (**Figure 74**).

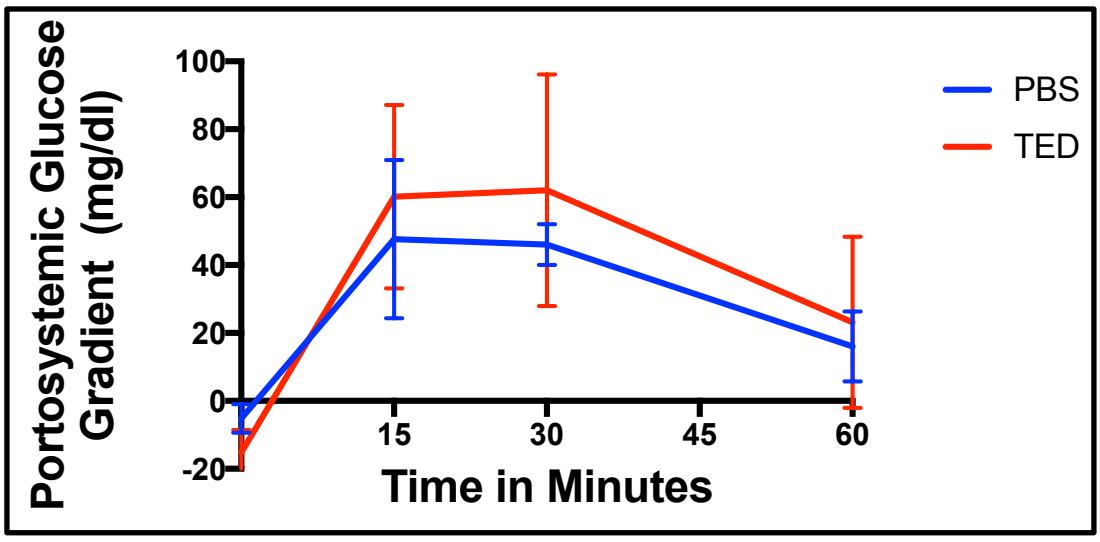



**Figure 74:** Mean porto-systemic glucose gradient at harvest GTT (day 24).

IPGTT seemed to conflict with the OGTT results. The first IPGTT, carried out 2 days before initiation of injections, showed that the two groups were well matched, with no significant difference between them (**Figure 75**). However, by day 19 of the study, when IPGTT was carried out again, blood glucose levels in the TED group were seen to be higher than the PBS group. At 15 minutes there was a clear TED group glucose peak (not significant), but instead of dipping under the PBS group glucose levels (as with the OGTTs), the TED group glucose levels remained high, and significantly so at 60 minutes (p=0.01) (**Figure 76**). Once again, AUC was calculated for IPGTT (**Figure 77**).

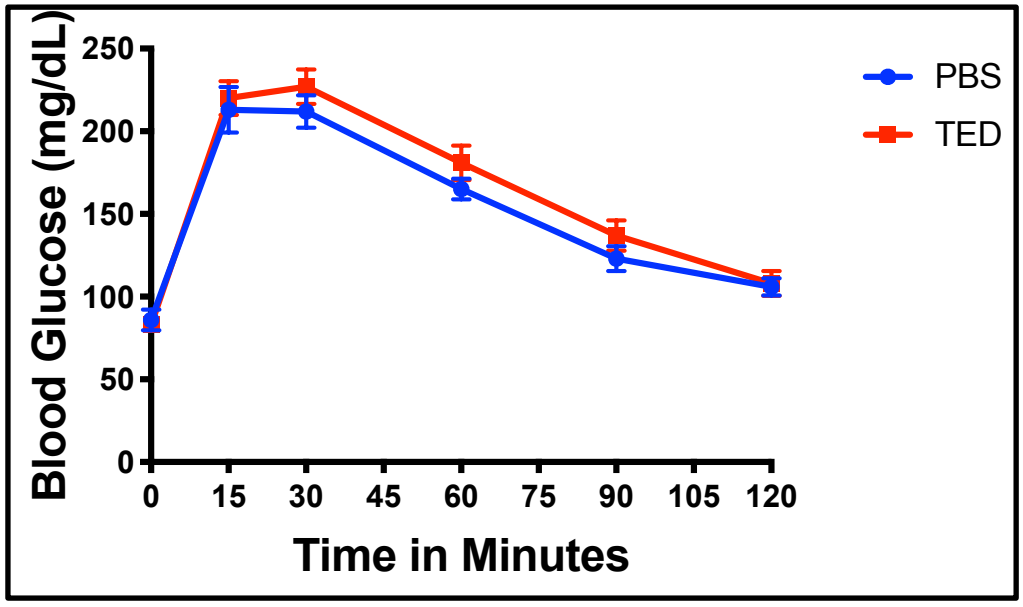



**Figure 75:** Intra-peritoneal glucose tolerance test 1 (baseline; 2 days pre-initiation of injections).

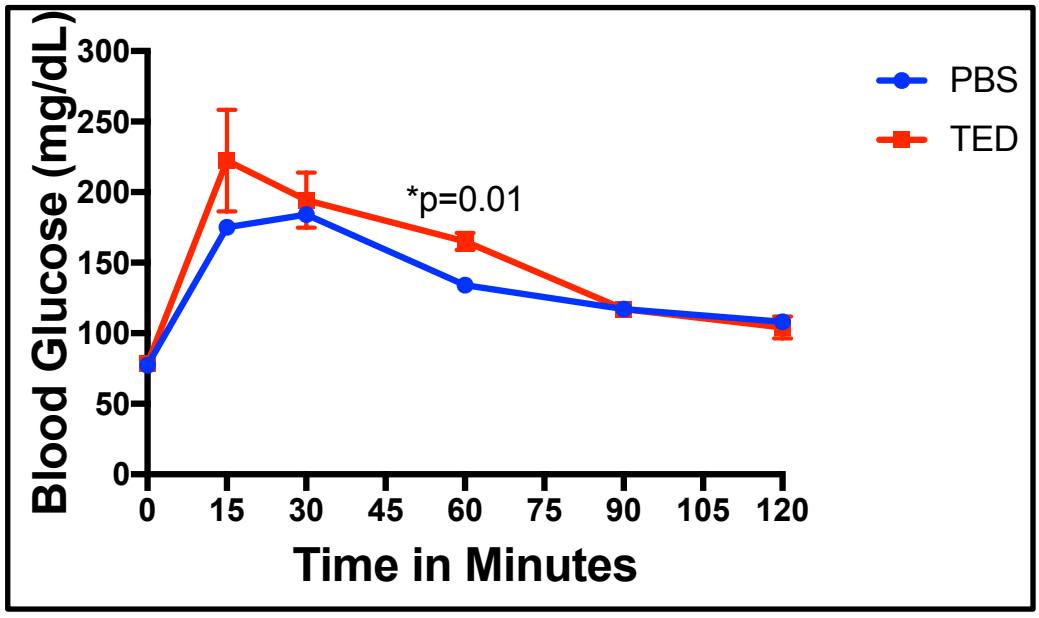



**Figure 76:** Intra-peritoneal glucose tolerance test 2 (16 days pre-initiation of injections).

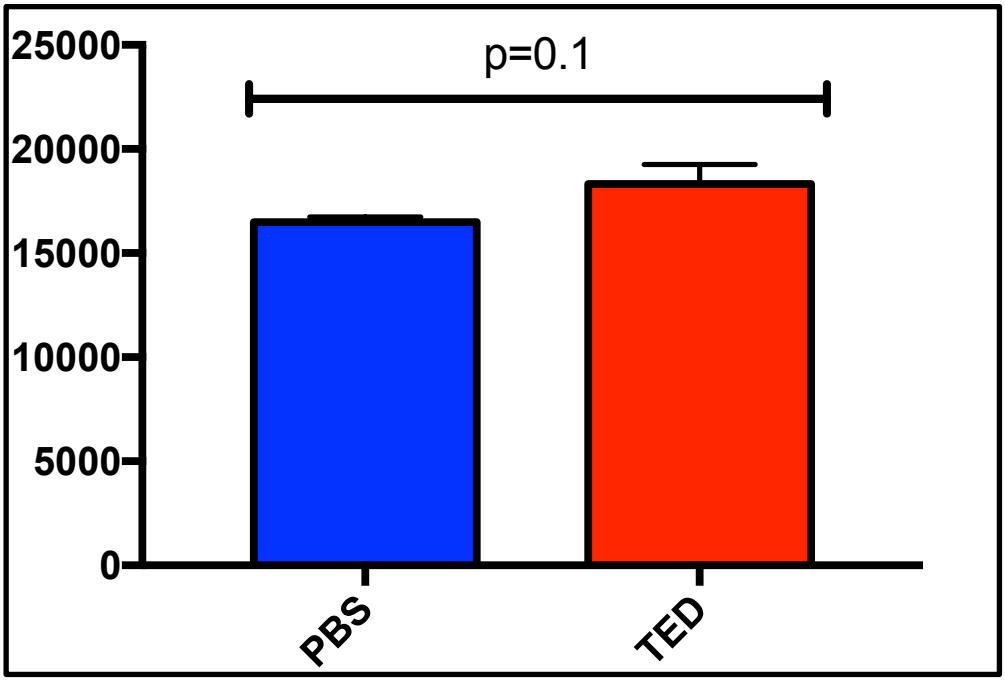



**Figure 77:** Area under the curve for intra-peritoneal glucose tolerance test 2.

As with the OGTT results, there was no statistical significance to be found between the two groups according to AUC. There was no significant change in mean total body weight between the two groups. (**Figure 78**).

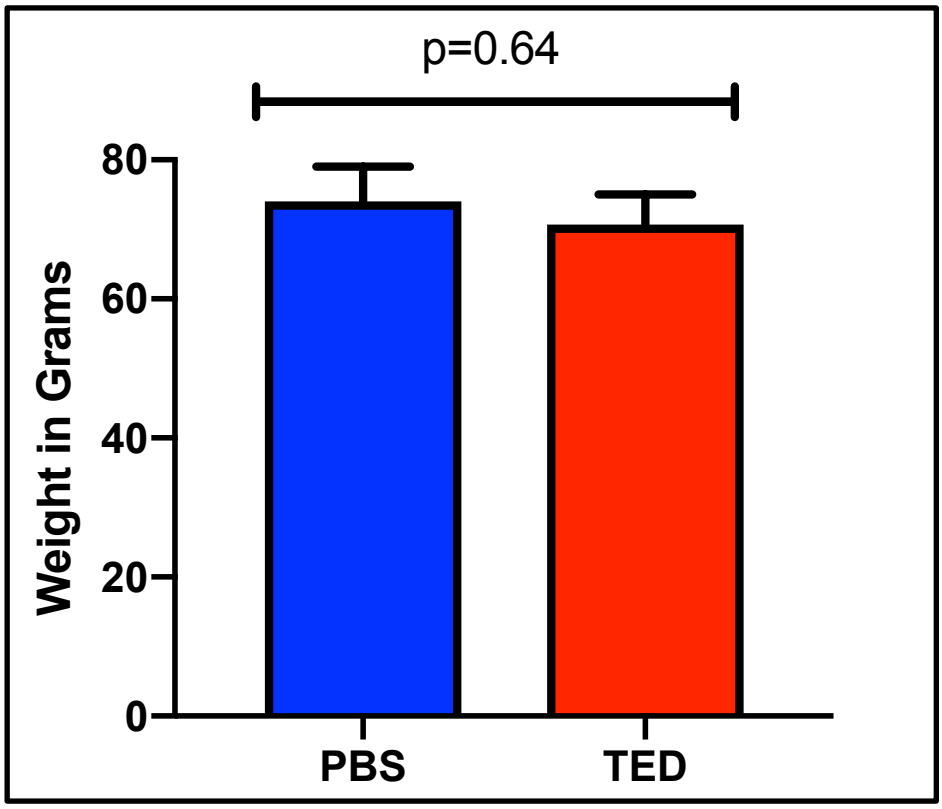



**Figure 78:** Mean change in total body weight.

Mean total food consumption appeared to be higher in the TED group, although this was not found to be significant (**Figure 79**).

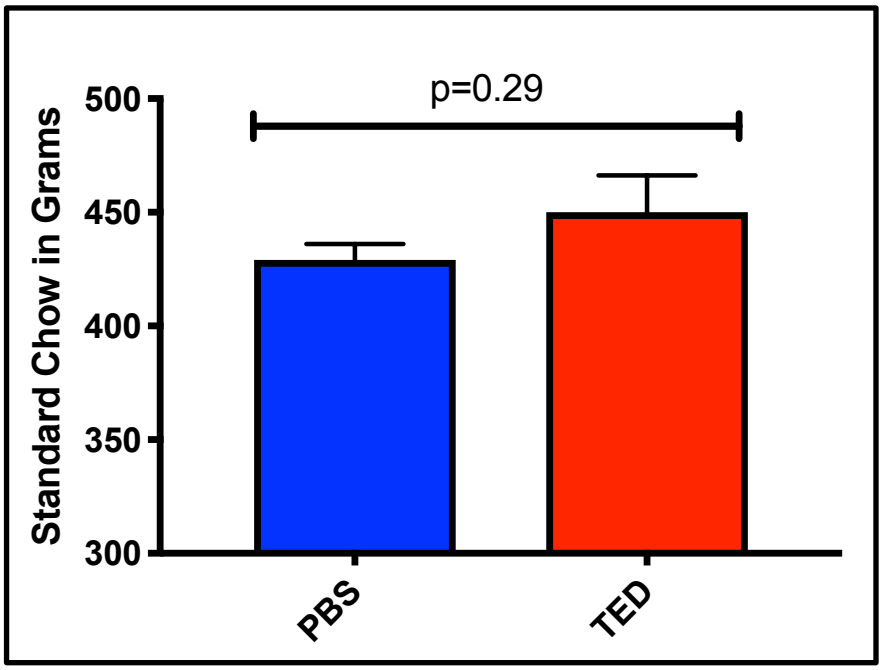



**Figure 79:** Mean total food consumption.

Interestingly, and as with the previous two validation protocols carried out, there was a significantly higher combined jejunal and ileal weight in the TED group (7.58g) versus the PBS group (6.24g) (p=0.01) (**Figure 80**).

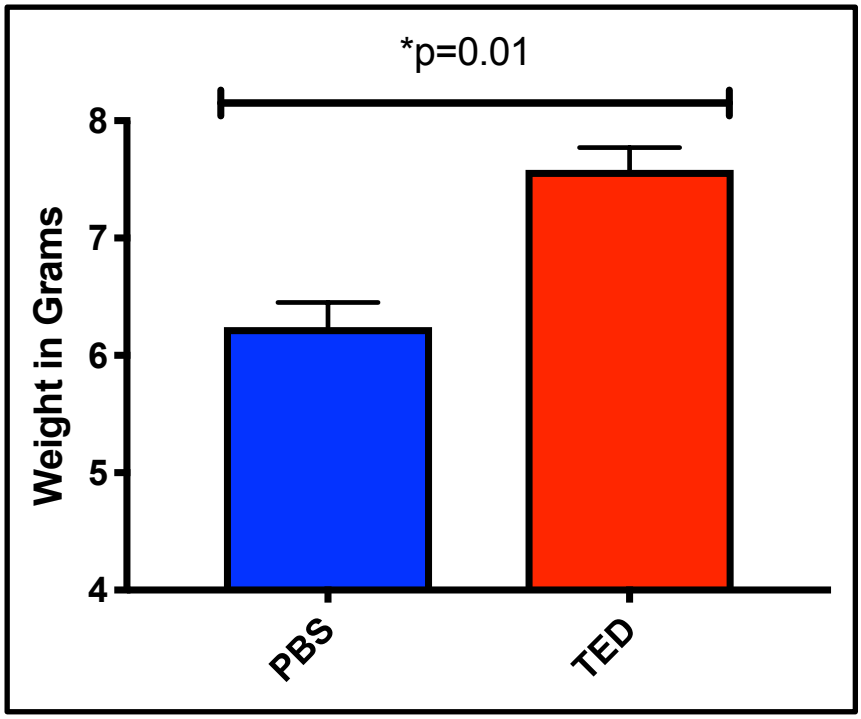



**Figure 80:** Mean combined jejunum and ileal weight.

Disappointingly, this wasn't supported by histological analysis of the proximal jejunum, with neither of the two paraffin block cuts showing any significant difference in villus length or crypt depth between the two groups (**Figures 81 and 82**).

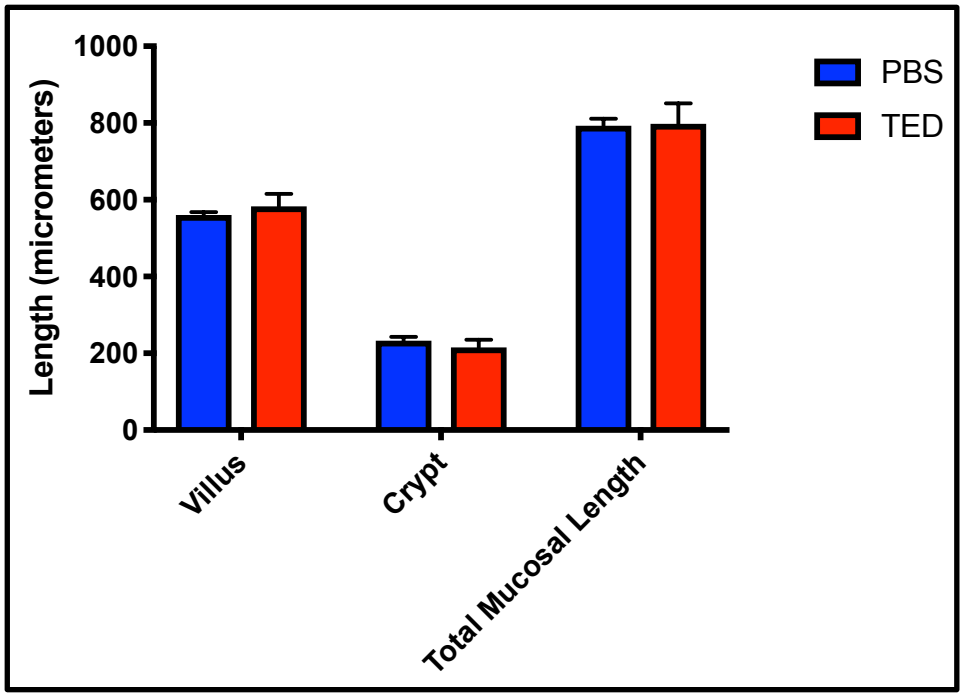



**Figure 81:** Mean villus length and crypt depth (paraffin technique, cut 1).

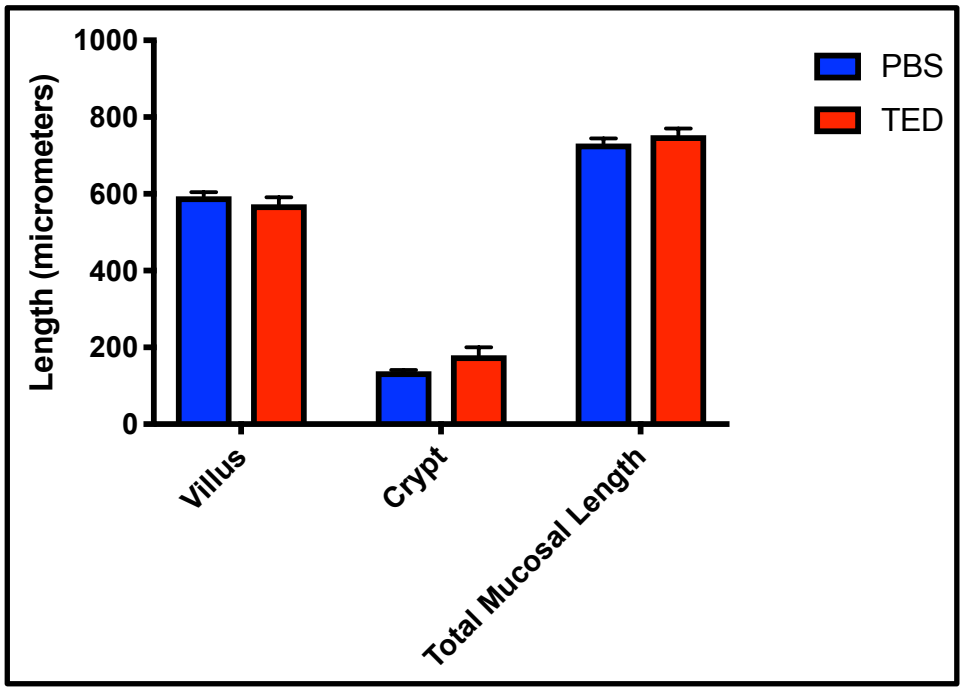



**Figure 82:** Mean villus length and crypt depth (paraffin technique, cut 2).

*3.4.3 Discussion*

Within this chronic Teduglutide administration study, two validation protocols were conducted in order to look for evidence of intestinal hypertrophy in order to prove the functionality of the purchased peptide. Peptide dose was altered, as was the chosen morphological preparation and the analysis method. A promising signal was eventually seen – a small, but significant, difference in the level of intestinal mucosal adaptation between experimental and control intestine. Thus, the main experiment was entered, and TED administration given for 21 days (instead of 8 days in the second validation protocol). Disappointingly, evidence of increased intestinal adaptation was not observed within the main experiment. It was concluded, therefore, that either the peptide was not functioning in the desired or optimum way, or the structure of the peptide was not correct. After closer inspection of the certificate of analysis it noticed that the mass given (via mass spectroscopy) by the lab supplying the peptide at MGH, was 3746.7g/mol (**Figure 83**). The theoretical weight of Teduglutide is known to be 3752g/mol.

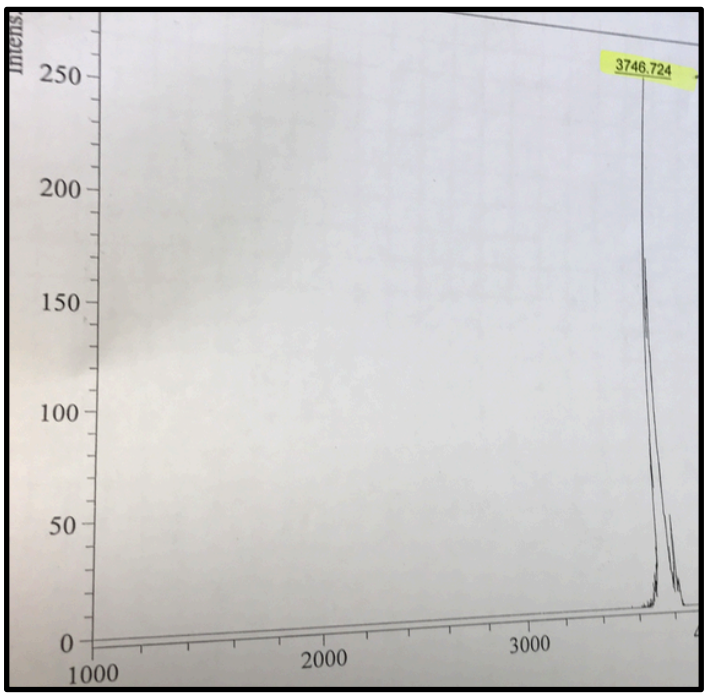



**Figure 83:** MGH lab certificate of Teduglutide analysis, showing a mass spectroscopy peak at 3746.7g/mol.

The MGH lab was contacted and they gave the explanation that the mass spectrometer that they used to validate the weight of the peptide was a low end MALDI-MS that they said would give results within +/- 5 Daltons i.e. it was accurate to within 5g/mol. Accordingly, there were concerns over this potential inaccuracy. As a result, the decision was made to abandon studies involving this peptide and to start looking for other peptide manufacturers who could provide a more accurately quantified peptide.

CASLO, a company based in Denmark, was identified as one such manufacturer. They were able to offer more accurate mass spectroscopy results (**Figure 84**, section 3.5). Therefore, the decision was made to repeat the study in section 3.4 with CASLO's Teduglutide peptide. As before, a validation protocol was carried out as the peptide had not been internally validated by CASLO.

## 3.5 VALIDATION PROTOCOL 3

*Methods and Materials*

*Teduglutide*

Given the suspected failure of the peptide supplied by the Peptide Core Laboratory at Massachusetts General Hospital, the decision was made to source the peptide from a different company. CASLO is a Danish company, affiliated with the Technical University of Denmark. It was founded in 2005 and the company focuses on a range of services within peptide chemistry. The structure of the peptide was confirmed by this laboratory (via mass spectrometry) as 3753.07g/mol (**Figure 84**), within 1g/mol of the expected theoretical weight of Teduglutide (3752.16 g/mol). Nevertheless, a validation experiment needed to be carried out in order to confirm the functionality of the peptide.

*Animals*

Six male SD rats were purchased (200-220g, ENVIGO, New Jersey). Unfortunately, Charles River (the usual lab supplier) were unable to supply SD rats within an acceptable time frame and, thus, a different animal supplier was chosen. From here on in, all rats were purchased from ENVIGO. These six rats were acclimatised in the same way as in section 3.4.1. The dose used in Validation Protocol 2 was again selected (0.4mg/kg in 200µl PBS), and was injected into 3 rats, and 200µl PBS alone into another 3 rats, for a total of 21days. Oral glucose tolerance tests were carried out in the same was as above, and as per the experimental timeline (**Figure 85**). It was decided that IPGTT would not be carried out as it was felt that too many separate experiments along the study time-line could potentially have a negative impact on the harvest day results.

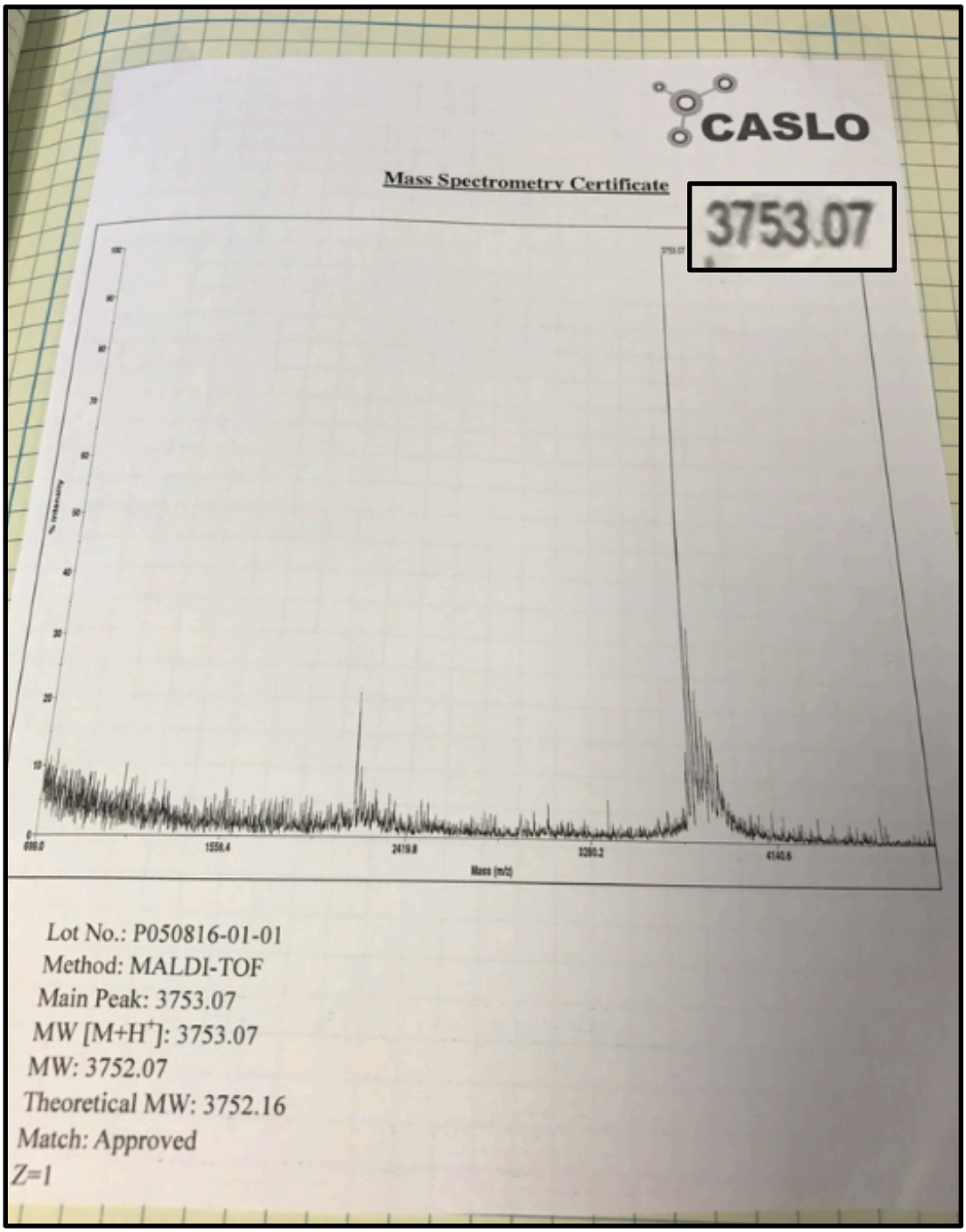



**Figure 84:** CASLO laboratory certificate of Teduglutide analysis, showing a mass spectroscopy peak at 3753.07g/mol.

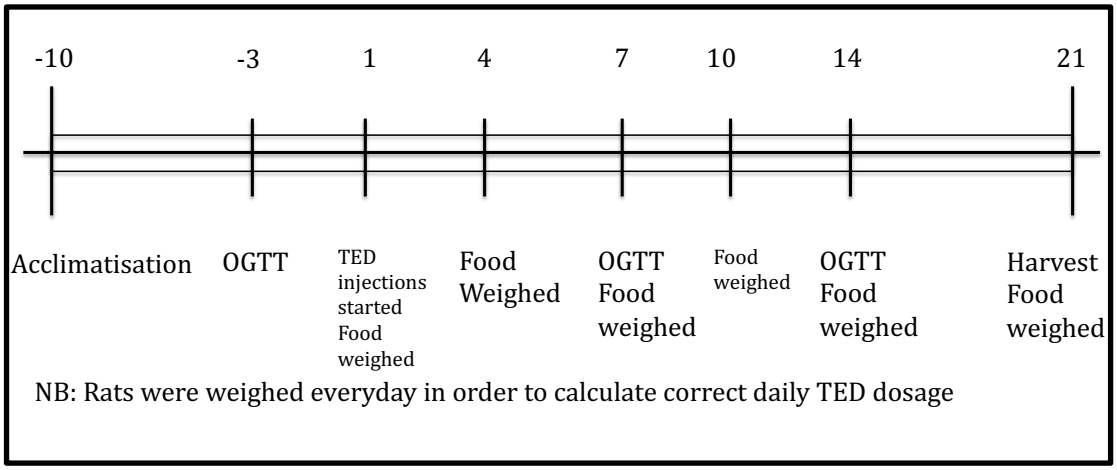



**Figure 85:** Validation Protocol 3 experiment timeline.

*Harvest Protocol, With Explanation of Intestinal Glucose Utilisation and Absorption Calculations*

Catheter placement, blood analysis and morphological preparation were performed as in section 3.4.1. New to this particular experiment, 10cm of jejunum was removed and divided into proximal and distal 5cm lengths. As in section 3.3.1, the proximal 5cm length was weighed and then added to paraffin to be sent for morphological assessment. The second, distal, 5cm length was used for PCR experiments. Additionally, intestinal glucose fluxes were calculated using the portosystemic glucose gradient:

$$G_{PS} = G_P - G_S$$

where $G_P$ is portal glucose level and $G_S$ is systemic glucose level at a given time point.

At baseline (0 min), before the intestinal glucose infusion was started, $G_{PS}$ was negative (i.e. $G_P < G_S$) reflecting the net use of glucose by the intestine in the fasting state. Glucose utilisation at ($G_{util}$; mg/h) was calculated:

$$G_{util} = G_{PSx} \times 60 \times PBF \times 10^{-2}$$

where $G_{PSx}$ is the portosystemic gradient at a given time-point (x) and PBF is portal blood flow (ml/min).

After starting the intestinal glucose infusion, $G_{PS}$ became positive, indicating glucose absorption by the intestine into the portal bloodstream. The area-under-curve of $G_{PS}$ was used to estimate intestinal glucose absorption ($G_{absorp}$; mg/h) as follows:

$$G_{absorp} = G_{PS}(AUC) \times PBF \times 10^{-2}$$

where $G_{PS}(AUC)$ is area-under-curve, or 'portosystemic area', of $G_{PS}$ from 0 to 60 min (mg/dl min).

Portal flow was calculated as previously described by the laboratory (Pal, Rhoads et al. 2018):

"A transabdominal ultrasound Doppler (Vevo 2100, VisualSonics, Toronto, Canada) was used to measure portal blood flow. The portal vein, which runs from the splenic vein-SMV confluence to its bifurcation into left and right branches, was identified. The mean diameter was calculated from two measurements (at the confluence and the bifurcation). The mean flow velocity was measured in the sagittal section view. Portal blood flow was calculated as the product of vein area and mean velocity. Portal vein diameter was 2.2 ±0.09 mm and portal flow was 9.6 ±0.7 ml/min in SD rats".

*PCR Preparation and Weighing of Tissue*

A 5cm length of jejunum was opened up, rinsed and the mucosa removed by scraping. Mucosa was then placed into an eppendorph, which was then placed directly into

liquid nitrogen. Eppendorphs were transferred to a -80°C freezer to be stored for subsequent PCR experiments (see **Appendix 3**). The rest of the jejunum and ileum was then weighed.

*Termination of Experiment*

As in section 3.4.1, each experiment was terminated by puncturing the diaphragm with scissors and cutting the heart in two, allowing exsanguination under anaesthesia.

*Assays*

ELISA, Luminex and PCR were all carried out as per **Appendices 1, 2 and 3**. For this study, glucose transporter proteins (SGLT-1, GLUT-1 and GLUT-2), a glucose sensor protein (SGLT-3), GLP-2 receptor, insulin-like growth factor (IGF-1), and sweet taste receptor (T1R2) were studied. Primers were designed using PrimerQuest® Tool (Intergrated DNA Technologies) (**Figure 86**). All primers were purchased from ThermoFisher Scientific).

| | **Forward primer** | **Reverse primer** |
|---|---|---|
| **SGLT1** | 5′-CCAAGCCCATCCCAGACGTACACC-3’ | 5′-CTTCCTTAGTCATCTTCGGTCCTT-3’ |
| **SGLT3** | 5′-GTCGGTCATGTTGGCTTCT-3′ | 5′-CTCTCTGATGCCTTCTTCCTTATC-3’ |
| **GLUT 1** | 5’-GTGCTCGGA TCCCTGCAGTTCG-3’ | 5’-GGGATGGACTCTCCATAGCGGTG-3’ |
| **GLUT 2** | 5’-CTCGGGCCTTACGTGTTCTTCCTT-3’ | 5’-TGGTTCCCTTCTGGTCTGTTCCTG-3’ |
| **GLP-2R** | 5’-ATCCGTCTCCTGTCGCTCCG-3' | 5’-CGTTGATTTCGCCAATCTGTATTT-3' |
| **IGF-1** | 5’-GTAGGTCTTGTTTCCTGCAC-3' | 5’-CACATCTCTTCTACCTGGC-3' |
| **T1R2** | 5’-TTCTTTCACGAGGTGCTCCG-3’ | 5’-AGGACTCAGAGGCGATCCACAC-3’ |
| **Cyclophylin A** | 5’-GTGGTCTTTGGGAAGGTGAA-3’ | 5’-GTCGGAGATGGTGATCTTCTTG-3’ |

**Figure 86:** PCR Primer Sequences.

*Results and Discussion*

Statistical analysis was carried out as described in section 2.4.1. All six rats survived to the end of the study, thus n=3 in both groups for all results. The validation protocol revealed some interesting, along with some statistically significant, results.

The first OGTT, carried out 3 days prior to the initiation of injections, reassuringly showed that the two groups were well matched (**Figure 87**).

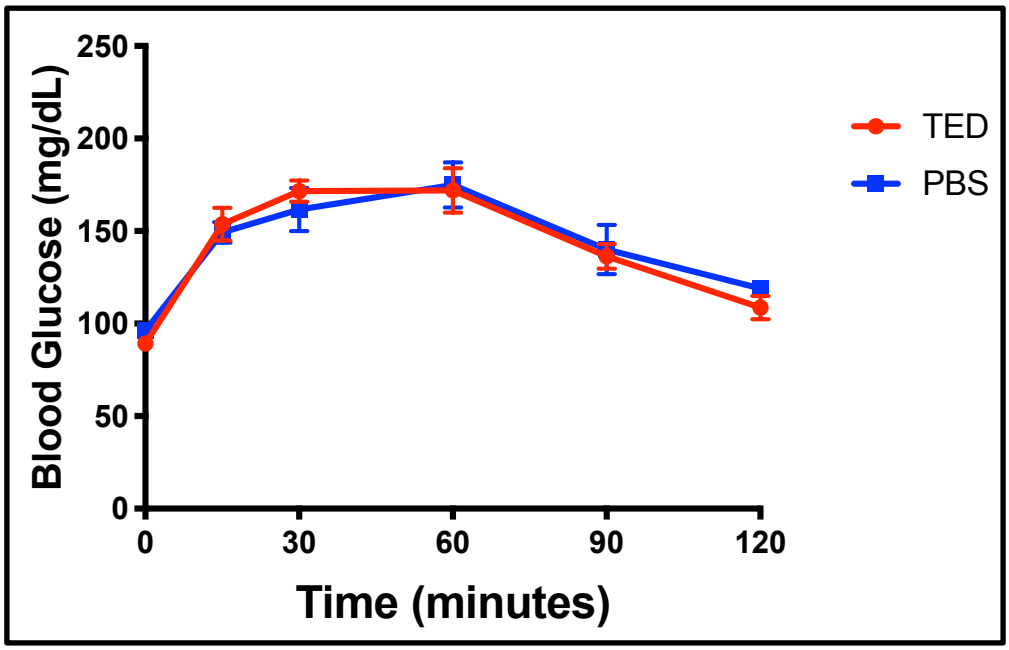



**Figure 87:** Validation Protocol 3 - Oral glucose tolerance test 1 (baseline; 3 days pre-initiation of injections).

The OGTT result at day 7 (**Figure 88**) was interesting as it was very similar to the day 7 OGTT in the main experiment using the peptide from the Massachusetts General Hospital (MGH) lab (**Figure 68**). Again, a 30-minute glucose peak in the TED group was seen, followed by a hypoglycaemic effect compared to the PBS group from 60 to 90 minutes, with the latter time point showing a significant difference (p=0.03), before both groups return almost back to baseline at 120 minutes.

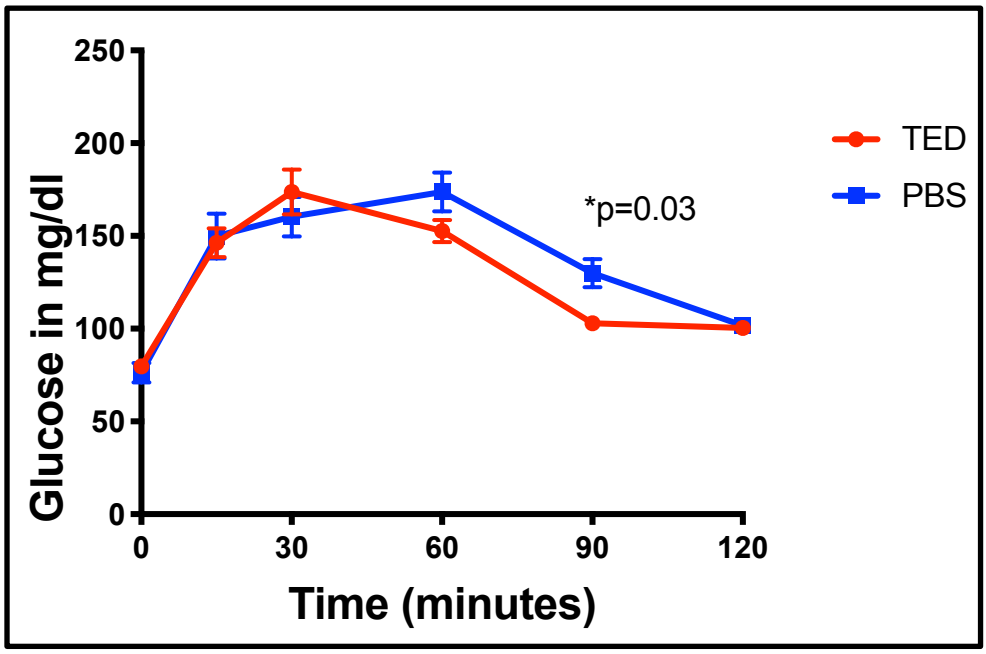



**Figure 88:** Validation Protocol 3 - Oral glucose tolerance test 2 (7 days post-initiation of injections).

The day 14 OGTT once again showed a clear and, this time, significant (p=0.03) peak glucose at 30 minutes in the TED group. There was a subsequent glucose level dip in this group compared to PBS, however, this was not found to be significant (**Figure 89**). Pointedly, all three OGTT PBS curves were very similar in shape, thus suggesting that the changes being observed in the TED group were real, compared to control.

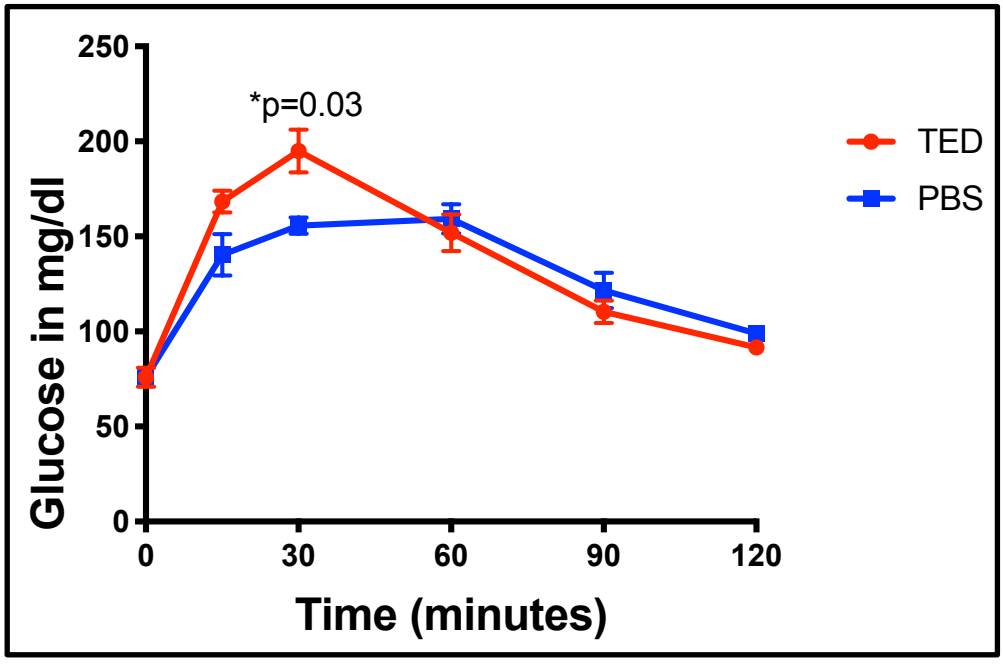



**Figure 89:** Validation Protocol 3 - Oral glucose tolerance test 3 (14 days post-initiation of injections).

At harvest, there was a steep rise in blood glucose over the first 30 minutes within the TED group, both within portal (**Figure 90**) and systemic (**Figure 91**) blood, and with the systemic glucose level reaching significance at both the 15- and 30-minute time points (p=0.005 and p=0.001, respectively). Systemic blood glucose AUC was the only AUC to show significance (**Figure 92**).

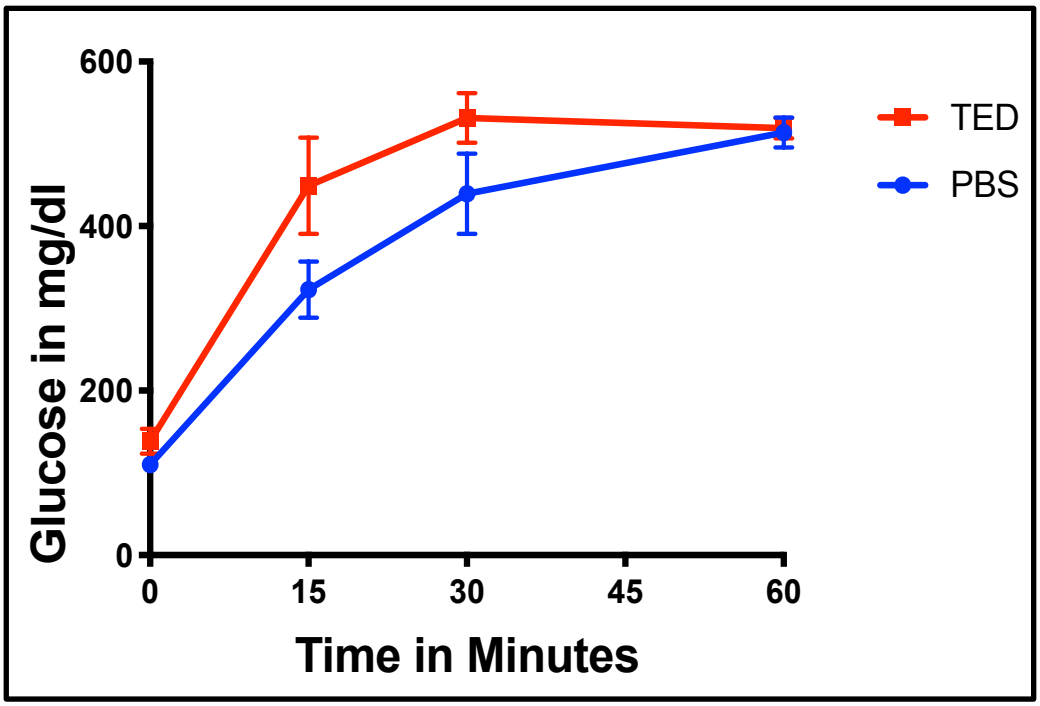



**Figure 90:** Validation Protocol 3 - Mean portal glucose levels at harvest GTT (day 21).

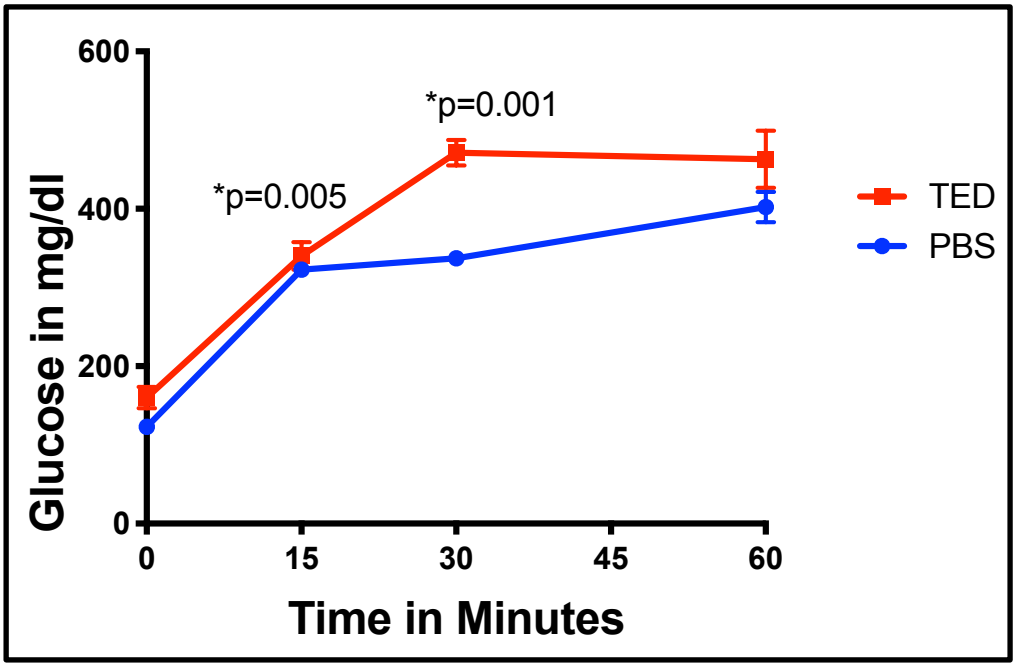



**Figure 91:** Validation Protocol 3 - Mean systemic glucose levels at harvest GTT (day 21).

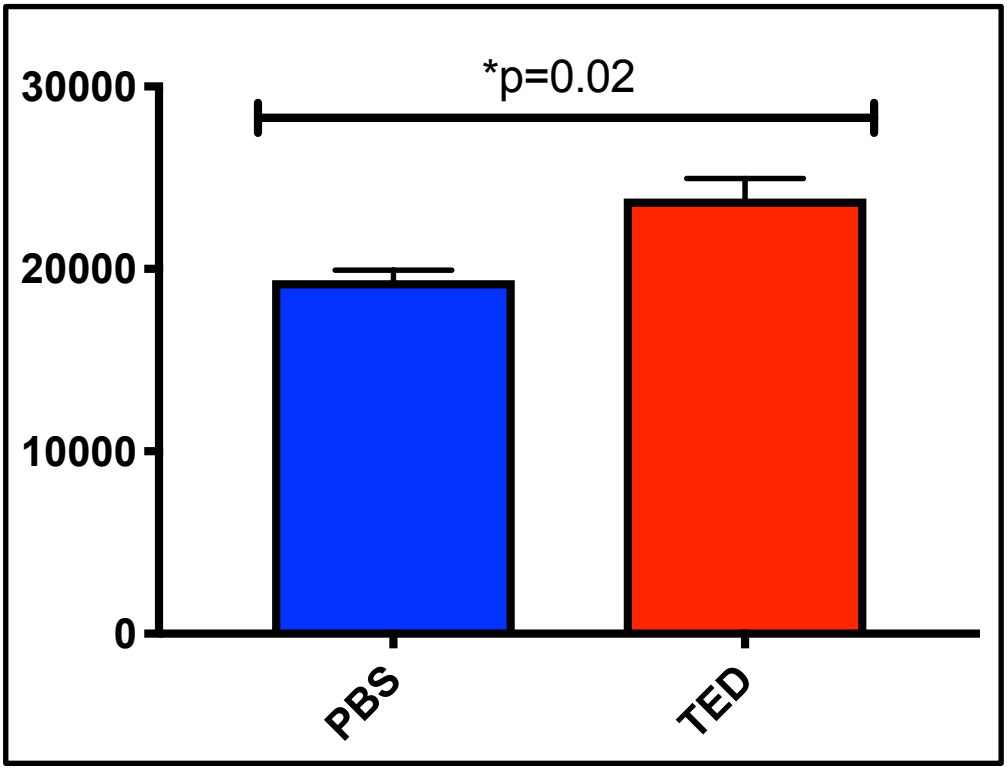



**Figure 92:** Validation Protocol 3 - Area under the curve for systemic glucose levels at harvest GTT (day 21).

The portosystemic gradient in the TED group became more positive than the PBS group, but from around 20 minutes, was more negative until the end of the 60-minute procedure (**Figure 93**).

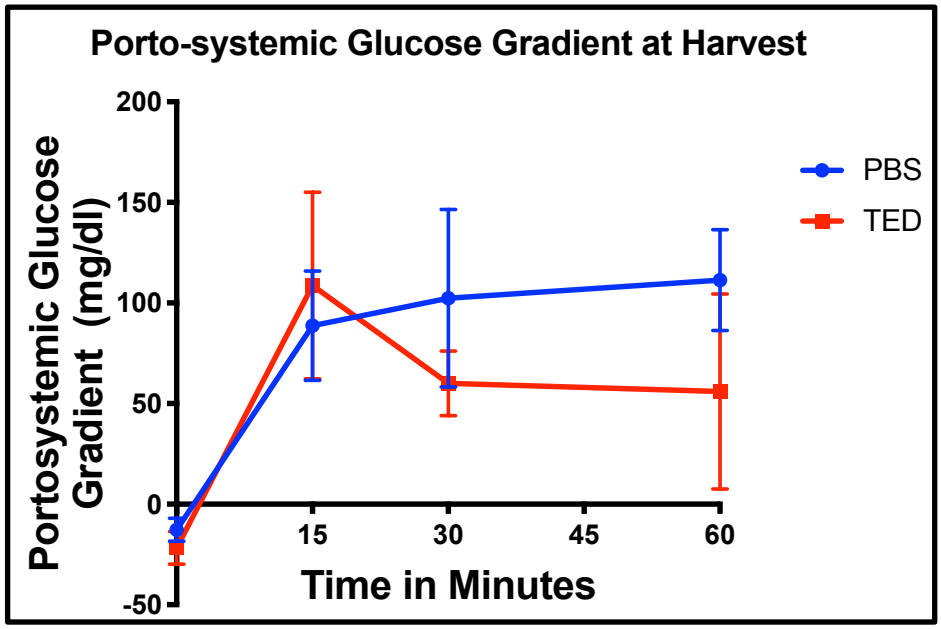



**Figure 93:** Validation Protocol 3 - Mean porto-systemic glucose gradient at harvest GTT (day 21).

Total bodyweight remained matched between the two groups throughout the study (**Figure 94**), as did the weight of chow consumed (**Figure 95).**

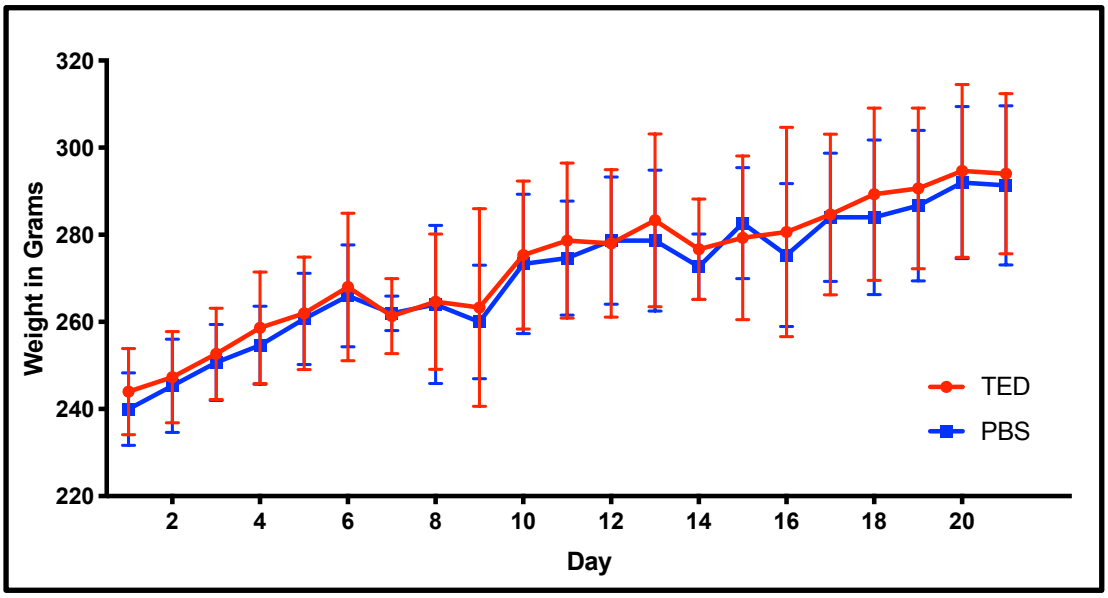



**Figure 94:** Validation Protocol 3 - Mean total bodyweights throughout study.

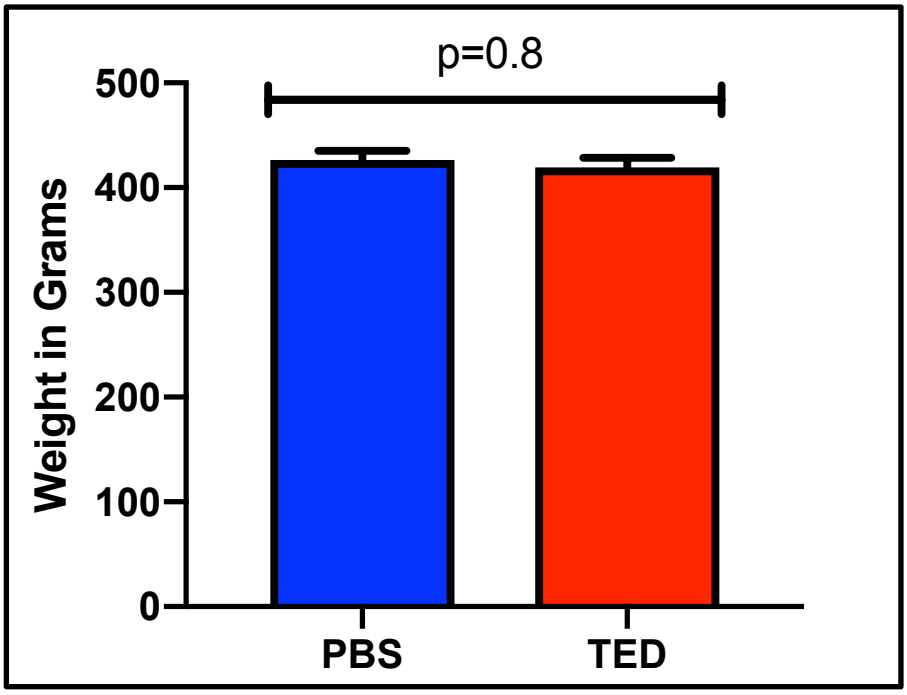



**Figure 95:** Validation Protocol 3 - Mean total weight of chow consumed.

The combined weight of jejunum and ileum was significantly higher (p=0.01) in the TED group (5.89g) compared to the PBS group (4.51g) (**Figure 96**).

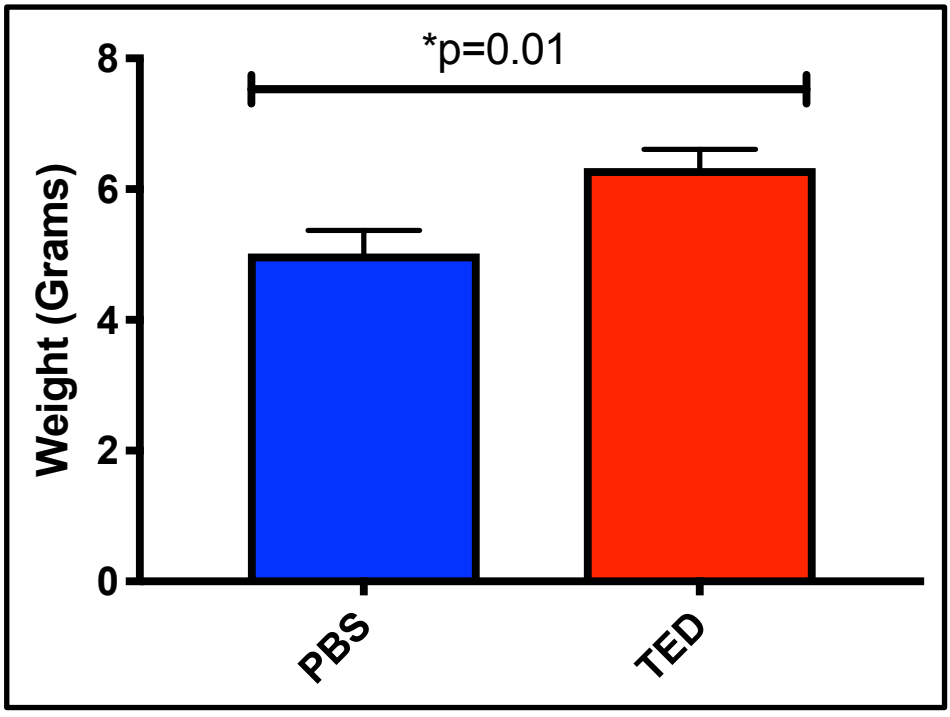



**Figure 96:** Validation Protocol 3 - Mean combined jejunum and ileal weight.

When weight-adjusted, calculated by dividing jejunal and ileal weight by the total weight of the rat at start of harvest procedure, the TED group still had a significantly increased combined jejunal and ileal weight (p=0.03) (**Figure 97**).

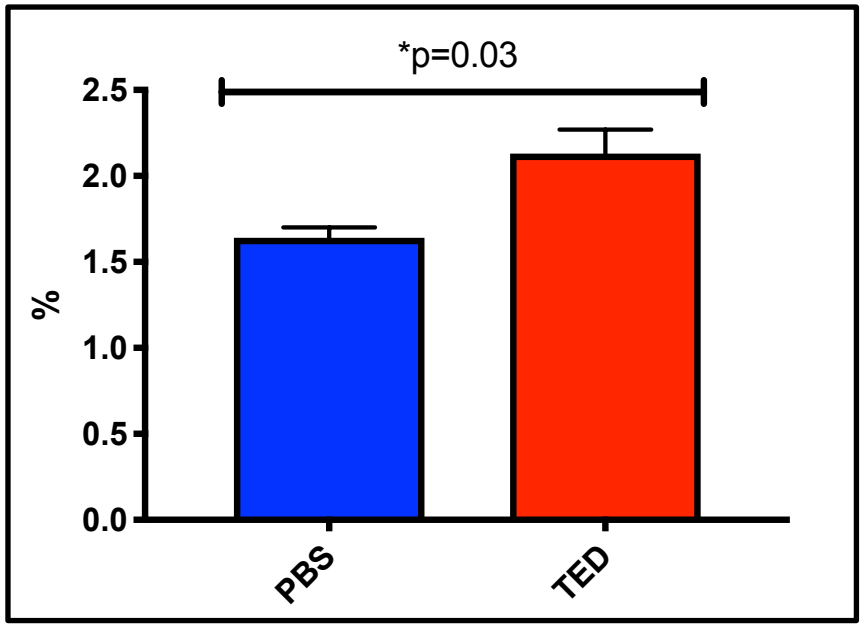



**Figure 97:** Validation Protocol 3 - Mean combined jejunum and ileal weight expressed as % bodyweight.

The first 5cm of jejunum was weighed in each rat. The TED group were shown to have a significantly heavier first 5cm of jejunum compared to the PBS group (0.38g v 0.27g, p=0.002) (**Figure 98**).

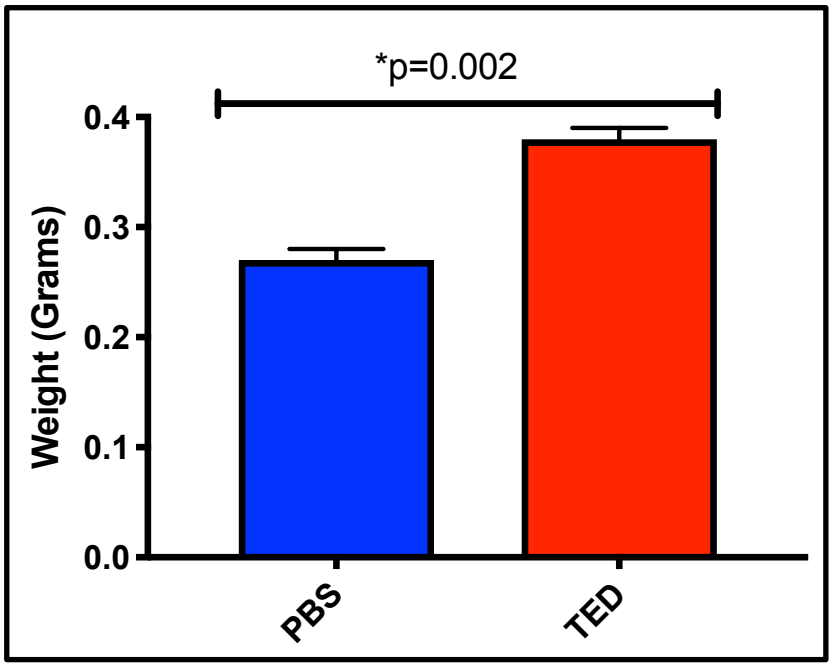



**Figure 98:** Validation Protocol 3 - Mean weight of 5cm of jejunum.

Again, when weight-adjusted, the TED group still had a significantly heavier first 5cm of jejunum (p=0.0001) (**Figure 99**).

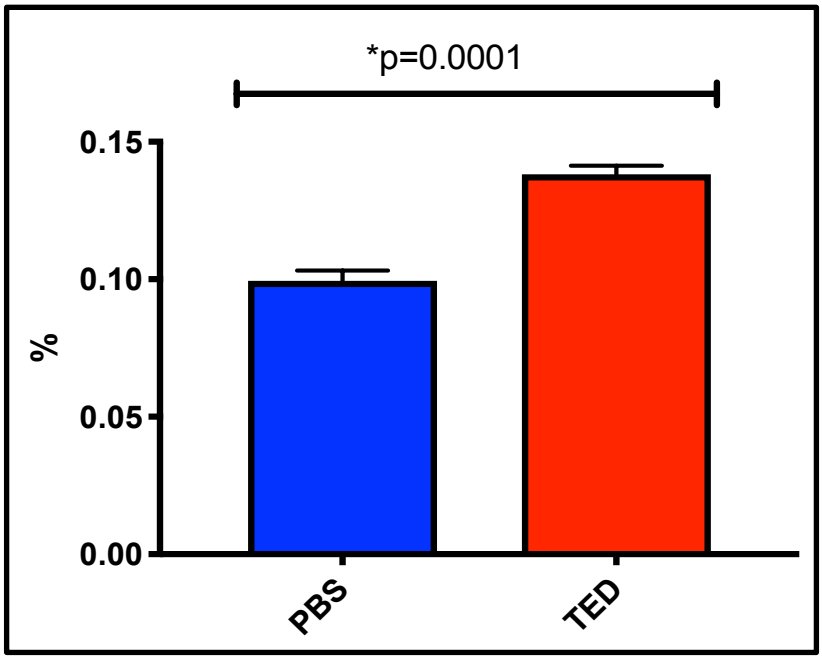



**Figure 99:** Validation Protocol 3 - Mean weight of 5cm of jejunum expressed as % bodyweight.

In terms of histological analysis, there was a reassuring trend in villus length in the TED versus the PBS group (497.2µm v 407.8µm, p=0.09) (**Figure 100**).

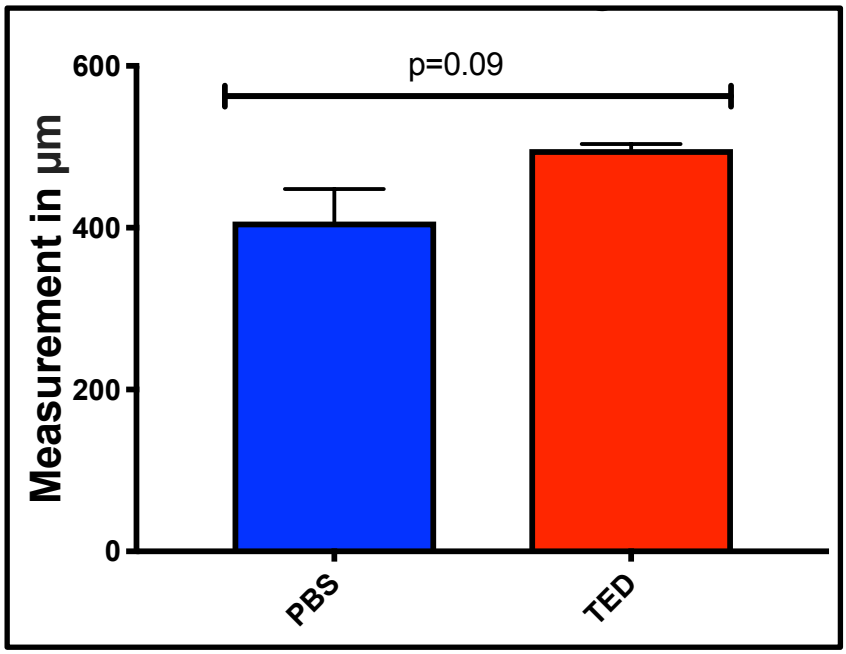



**Figure 100:** Validation Protocol 3 - Mean villus length.

TED crypts were deeper (169.9µm v 153.2µm, p=0.14), although this was not found to be significant (**Figure 101**).

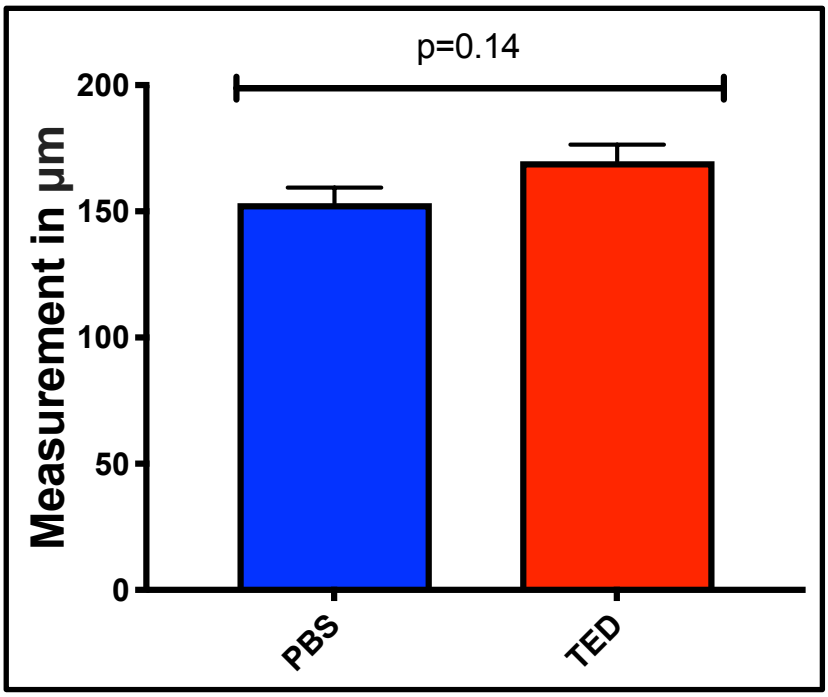



**Figure 101:** Validation Protocol 3 - Mean crypt depth.

Nevertheless, if villus length and crypt depth were to be taken as a whole i.e. 'total mucosal length', then a significant comparison is seen with a TED total mucosal length of 667.1µm and a PBS total mucosal length of 561.0µm (p=0.049) (**Figure 102**).

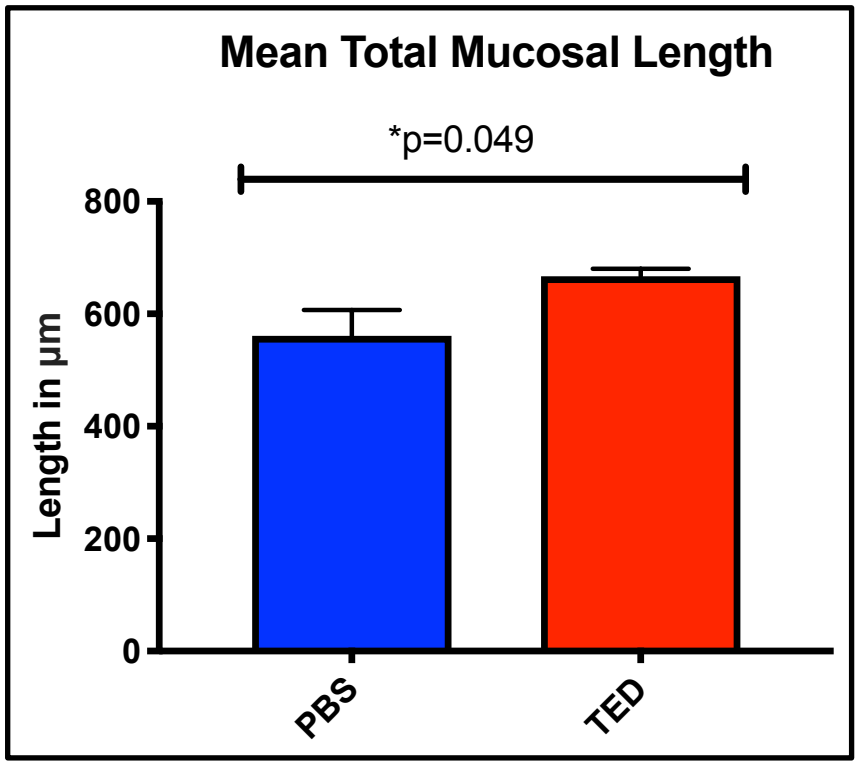



**Figure 102:** Validation Protocol 3 - Mean total mucosal length.

## 3.6 EXPERIMENT 2

### *3.6.1 Materials and Methods*

Taking Validation Protocol 3 (section 3.5) results in to consideration, it was felt that that there was enough evidence to suggest that the CASLO Teduglutide peptide was working in the desired way. The decision was made to increase the number of rats to 6 in each group. Thus, the experiment was repeated using the method described within section 3.5.

### *3.6.2 Results*

**CASLO PEPTIDE, STANDARD CHOW, n=6**

The baseline OGTT, performed 3 days prior to initiation of injections, and with the addition of 6 rats (3 to each group, so that n=6 in both groups), remained well matched (**Figure 103**).

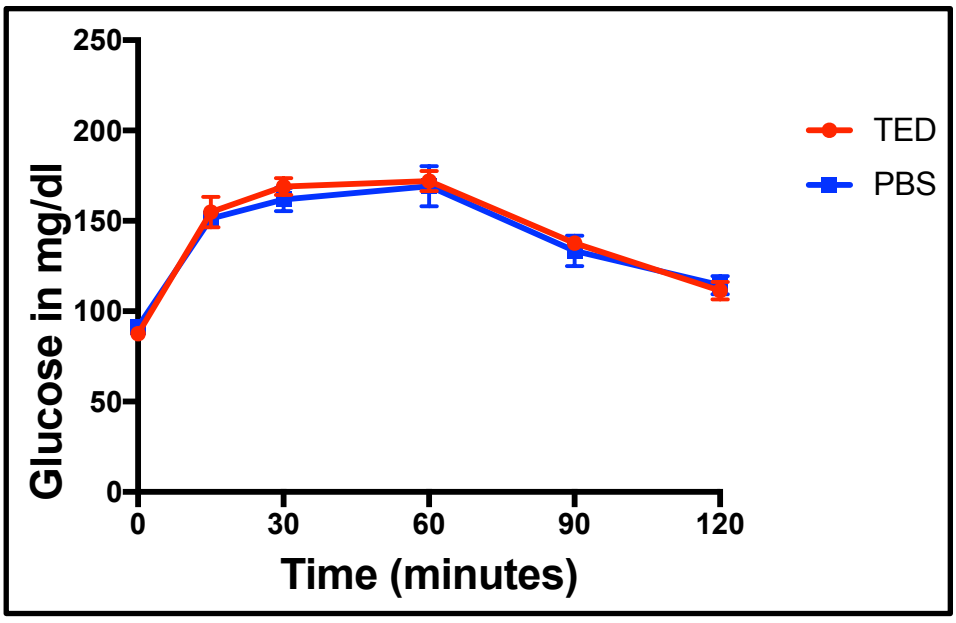



**Figure 103:** Oral glucose tolerance test 1 (baseline; 3 days pre-initiation of injections).

After 7 days of injections, the usual trend began to appear; that is, a TED group blood glucose peak at 30 minutes, this time just missing out on significance (p=0.06), with a subsequent dip in glucose levels from 60 to 90 minutes, again, just missing out on significance at the 90-minute time point (p=0.09) (**Figure 104**).

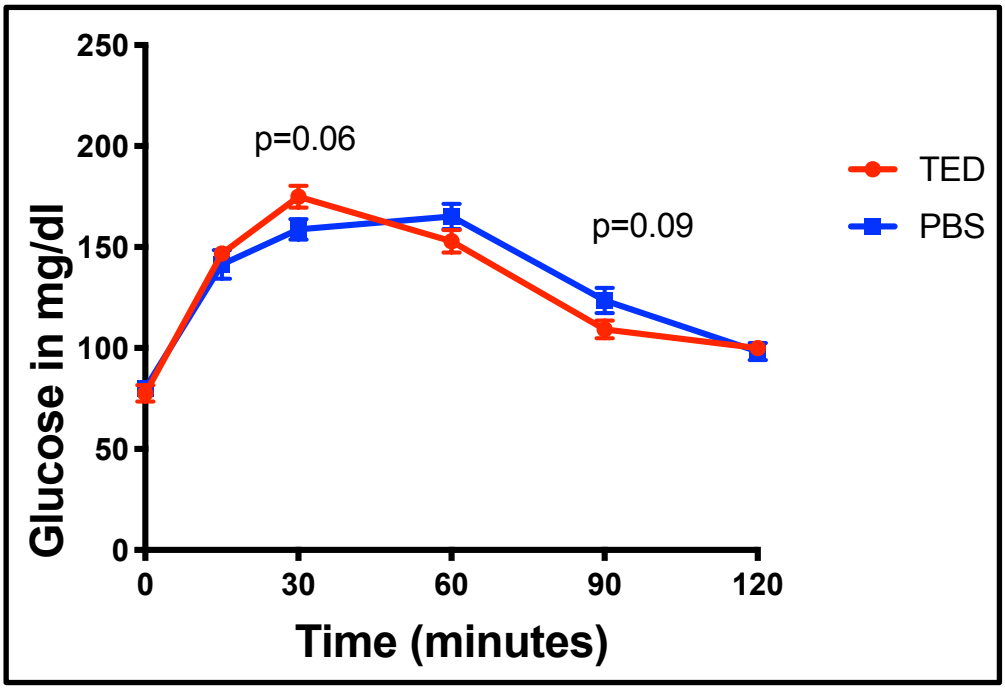



**Figure 104:** Oral glucose tolerance test 2 (7 days post-initiation of injections).

However, the 14-day OGTT showed a more dramatic result. This time there was a statistically significant peak in the TED group at 30 minutes, with a peak value of 195.0mg/dl v 155.6mg/dl in the PBS group (p=0.005). Additionally, there was a significant increase in the TED group at the 15-minute time point (p=0.01). The expected dip in blood glucose at 60 minutes was once again seen, however, results weren't significant (**Figure 105**).

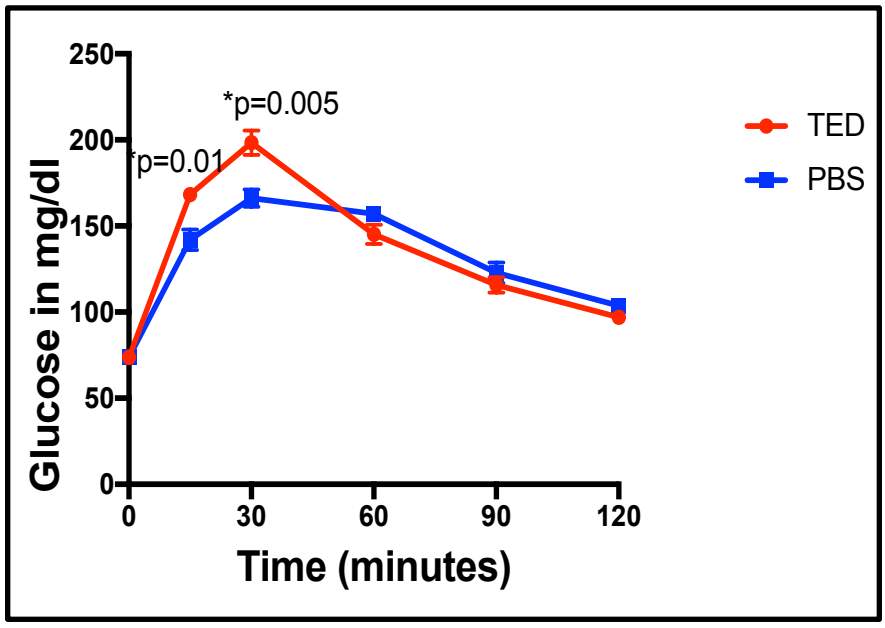



**Figure 105:** Oral glucose tolerance test 3 (14 days post-initiation of injections).

Harvest blood glucose levels, following the usual interoperative duodenal glucose infusion, also showed some convincing trends. Both portal (**Figures 106 and 107**) and systemic (**Figures 108 and 109**) blood glucose levels followed the same trend; a steep increase up to a peak at 60 minutes, followed by a plateau up until the 60-minute time point. Portal and systemic results at 15 minutes and 30 minutes were all found to be significantly increased compared to the PBS group blood glucose levels (P15 p=0.01, P30 p=0.0006 and S15 p=0.049, S30 p=0.001). The 60-minute time points in the TED group portal and systemic blood just missed out on reaching significance (both p=0.06).

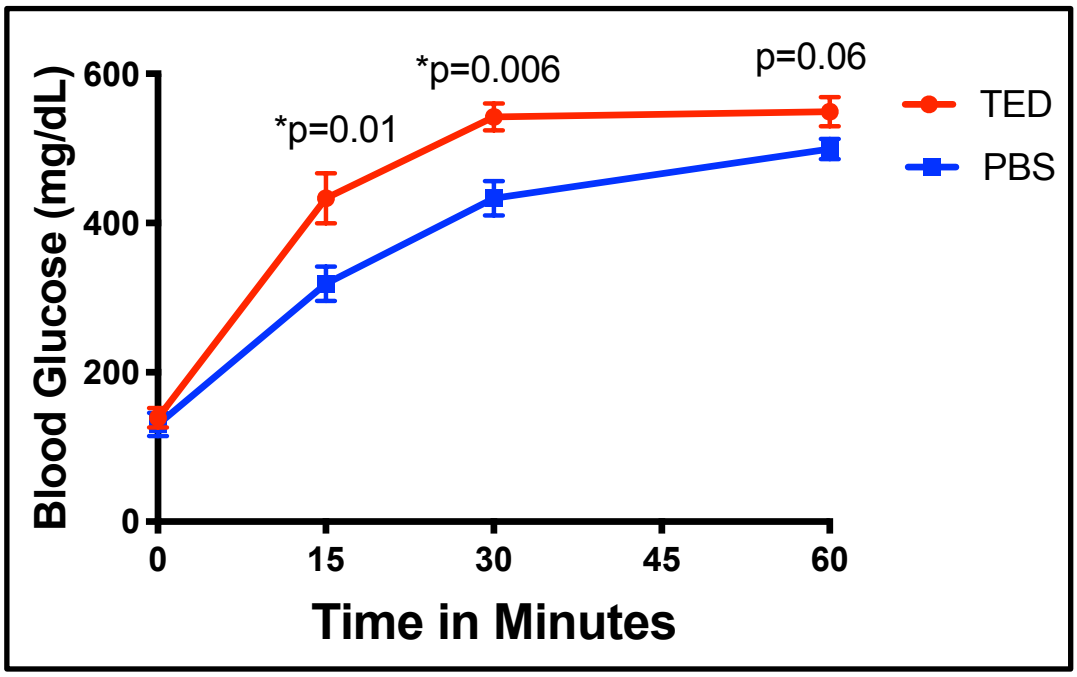



**Figure 106:** Mean portal glucose levels at harvest GTT (day 21).

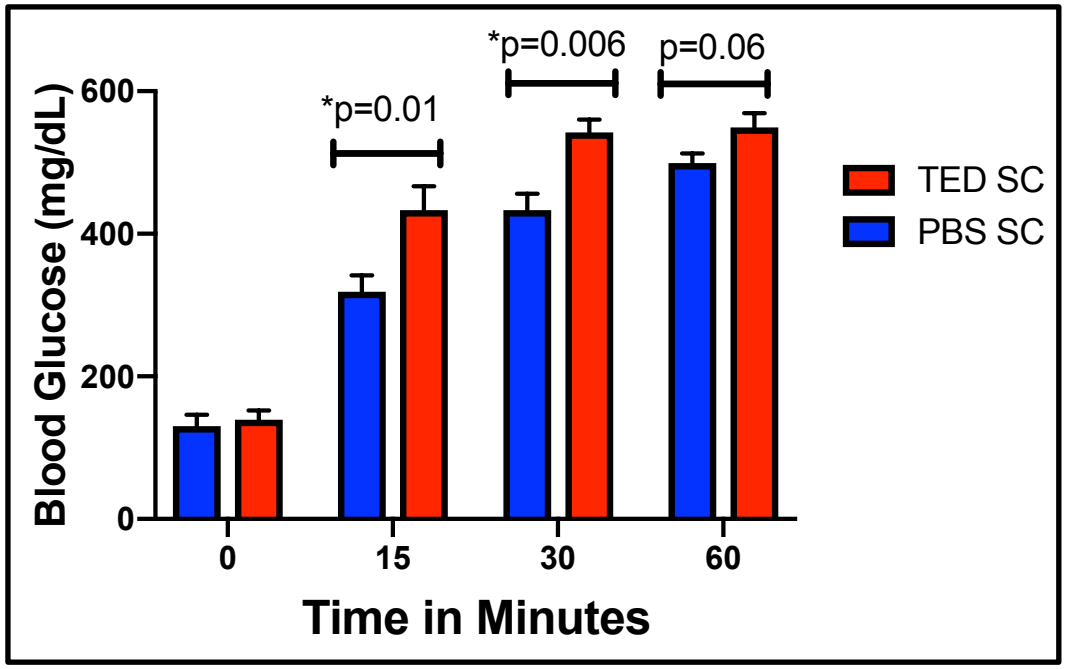



**Figure 107:** Mean portal glucose levels at harvest GTT (day 21). (Displayed in bar chart format).

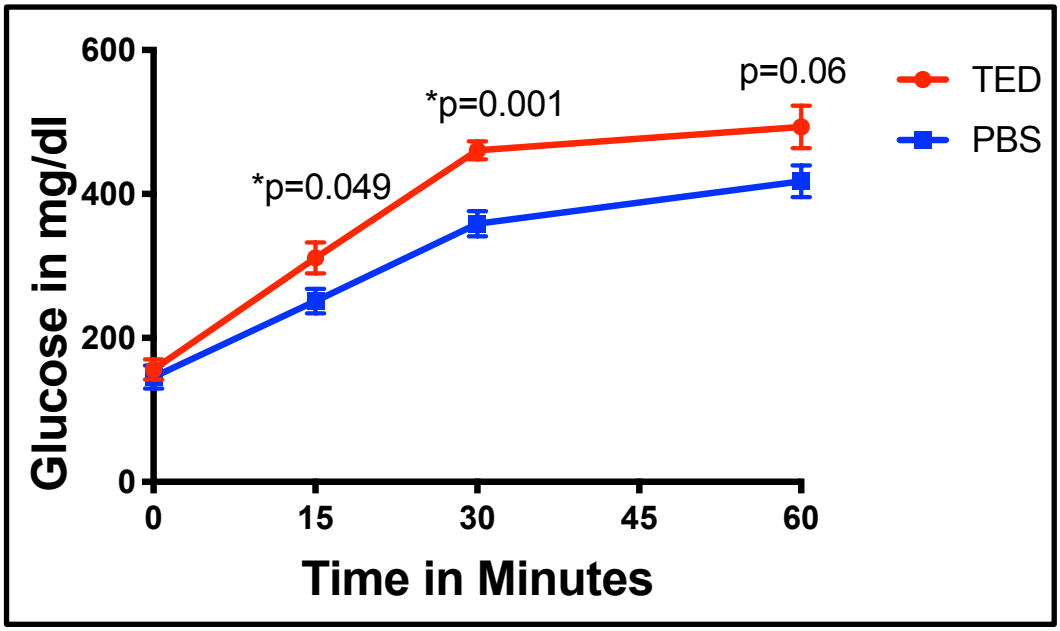



**Figure 108:** Mean systemic glucose levels at harvest GTT (day 21).

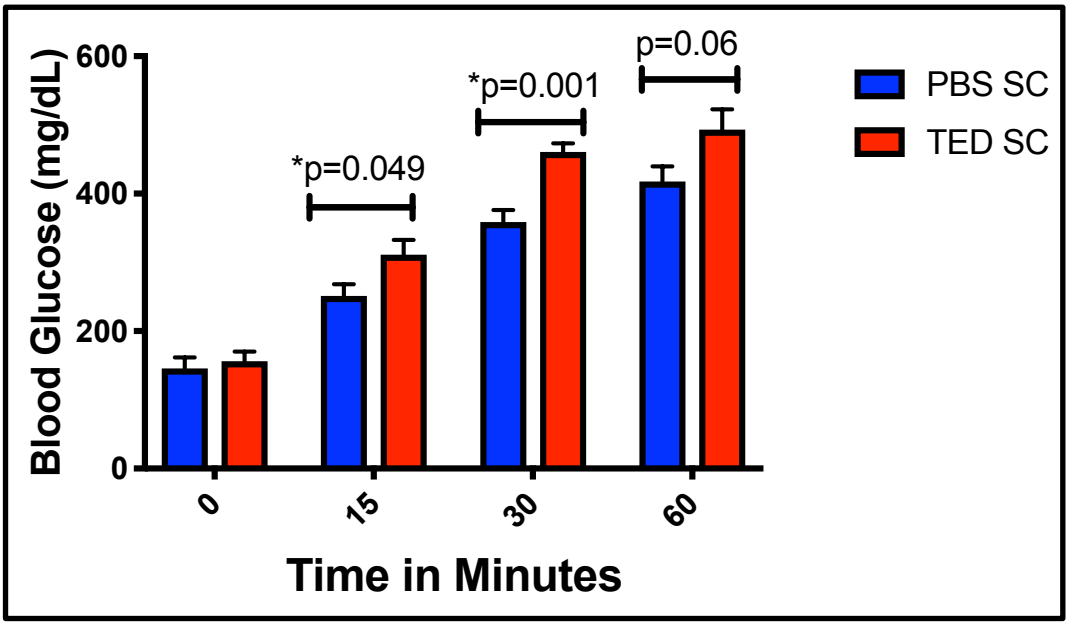



**Figure 109:** Mean systemic glucose levels at harvest GTT (day 21). (Displayed in bar chart format).

Portosystemic gradient in the TED group was shown to be more positive at 15 minutes (not significantly), and this then switched so that the TED group $G_{PS}$ became less positive than the PBS group at 60 minutes (again, not reaching significance) (**Figure 110**).

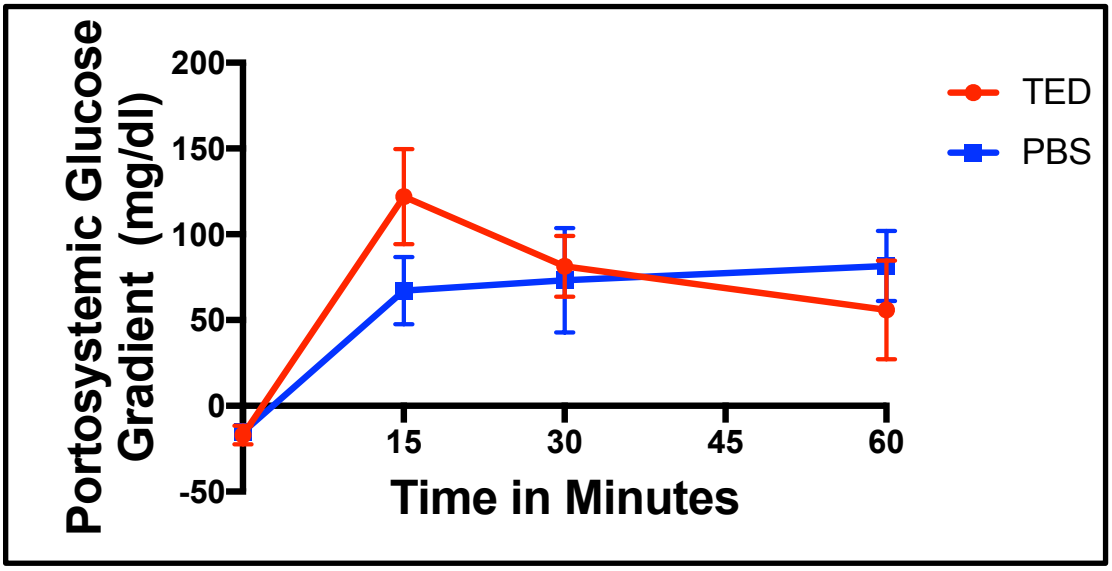



**Figure 110:** Mean porto-systemic glucose gradient at harvest GTT (day 21).

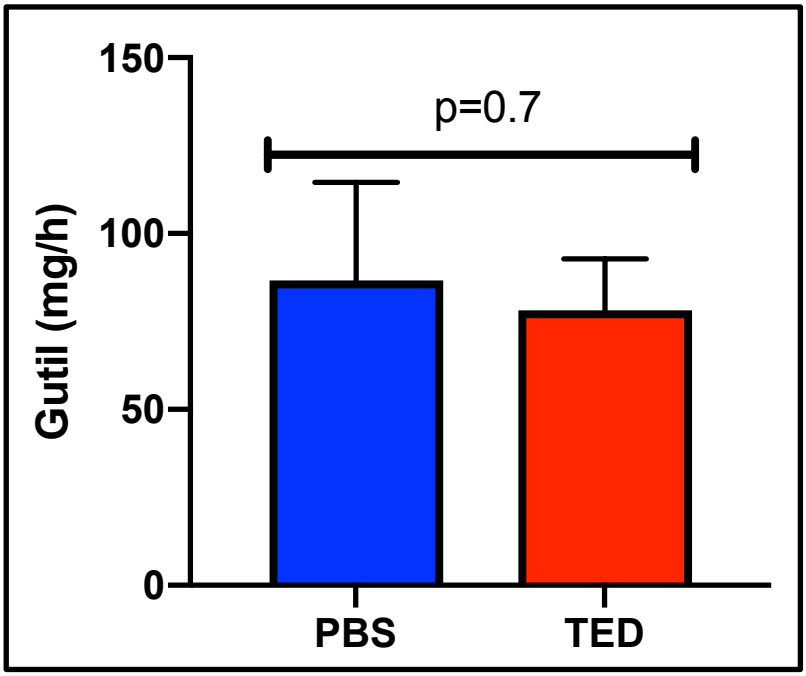



**Figure 111:** Intestinal glucose utilisation.

There was no significant difference in glucose utilisation, at baseline, or glucose absorption over the duration of the experiment, between the two groups (**Figures 111 and 112**).

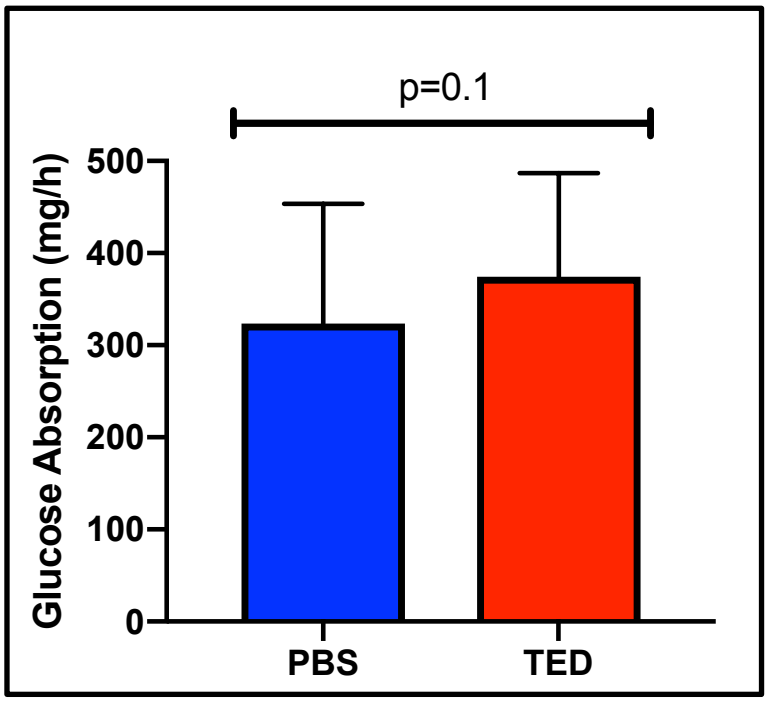



**Figure 112:** Intestinal glucose absorption.

Total body weight remained matched between the two groups throughout the study (**Figure 113**), as did the weight of the standard chow consumed (**Figure 114**).

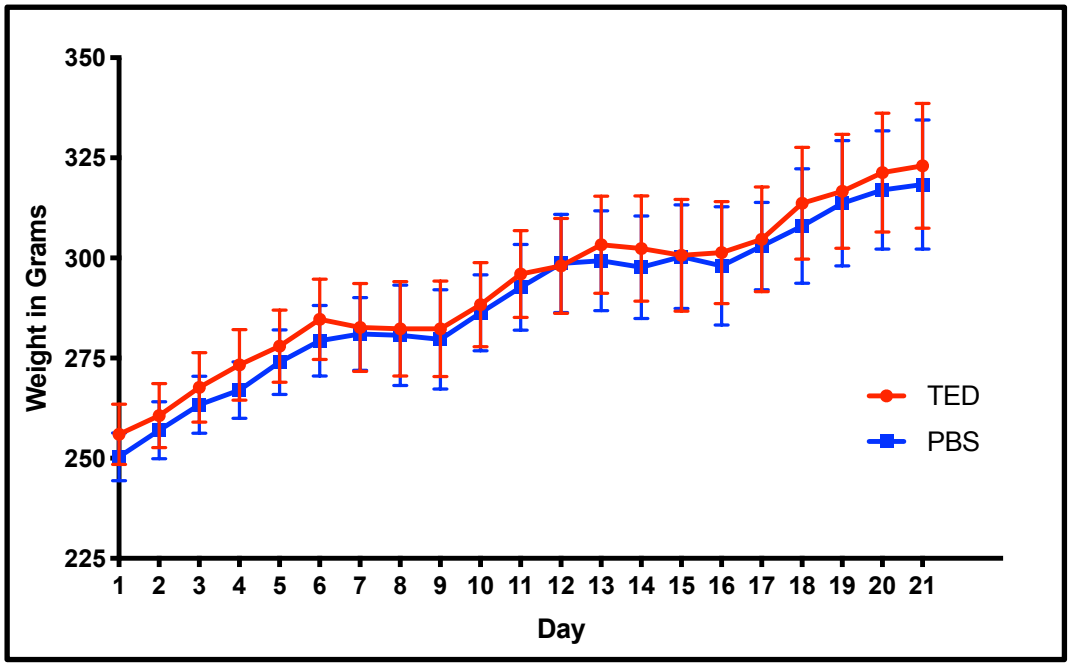



**Figure 113:** Mean total bodyweights throughout study.

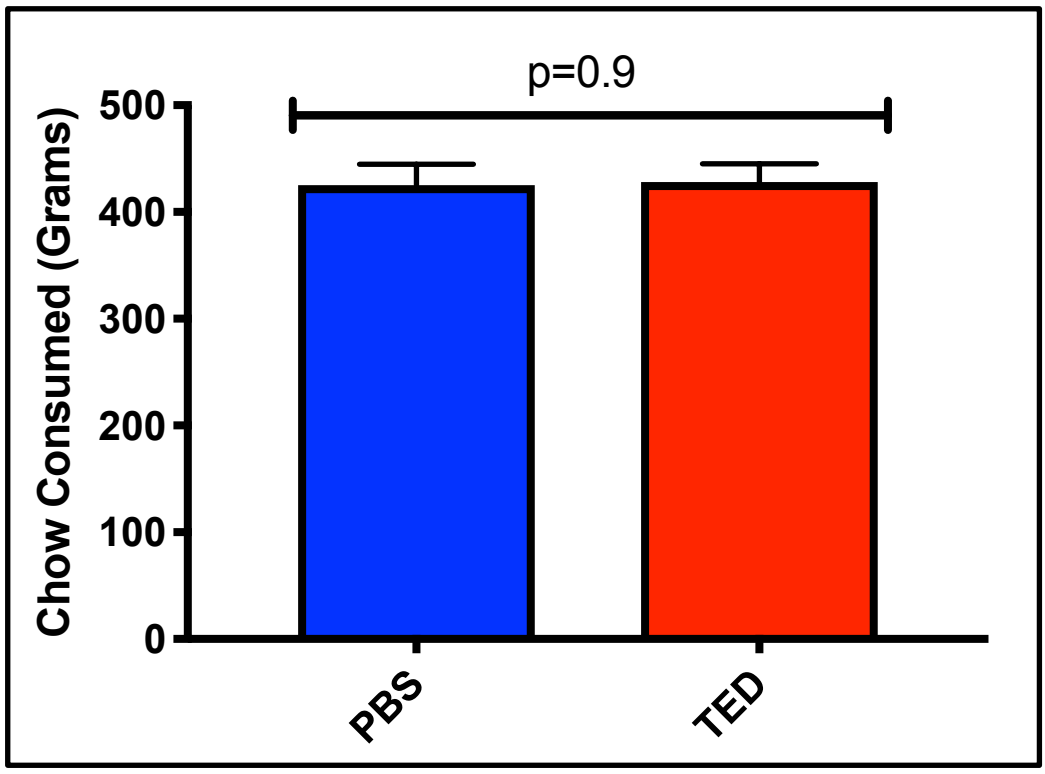



**Figure 114:** Mean total weight of chow consumed.

The combined weight of jejunum and ileum was significantly higher in the TED group compared to the PBS group (6.32g v 5.02g, p=0.01) (**Figure 115**).

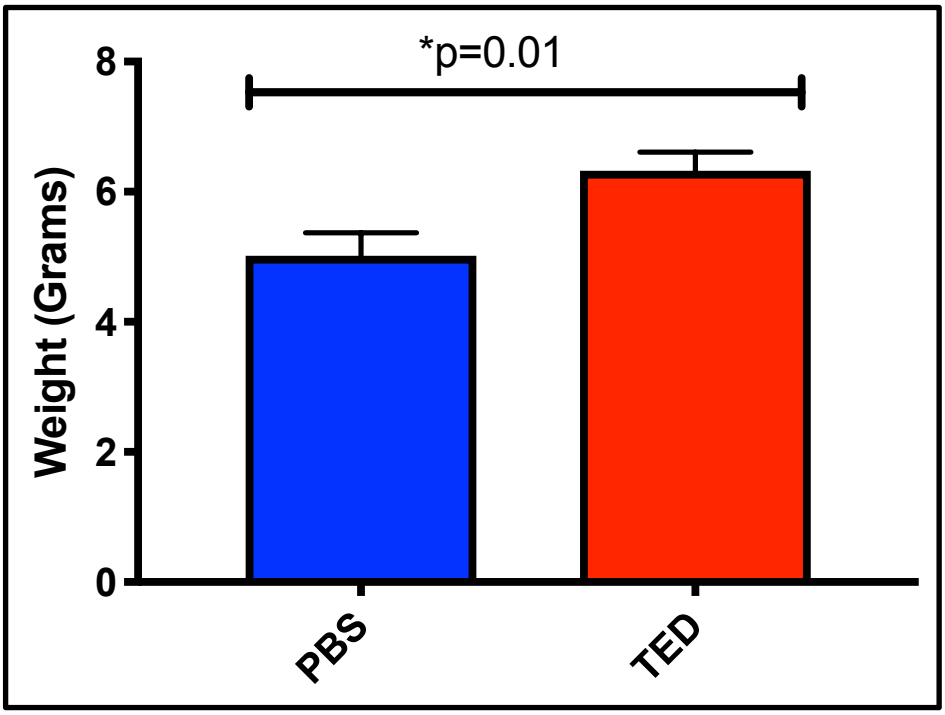



**Figure 115:** Mean combined jejunum and ileal weight.

When weight-adjusted the TED group still had a significantly increased combined jejunal and ileal weight (p=0.003) (**Figure 116**).

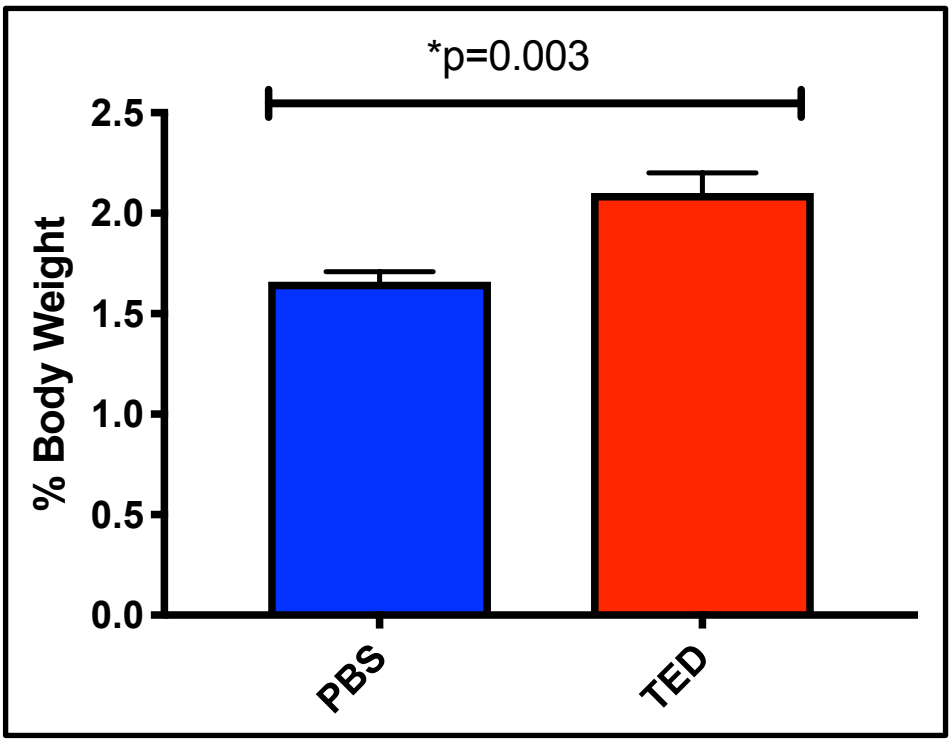



**Figure 116:** Mean combined jejunum and ileal weight expressed as % body weight.

Again, when the first 5cm of each jejunum was weighed the TED group were shown to have a significantly heavier first 5cm of jejunum compared to the PBS group (0.39g v 0.28g, p=0.0005) (**Figure 117**).

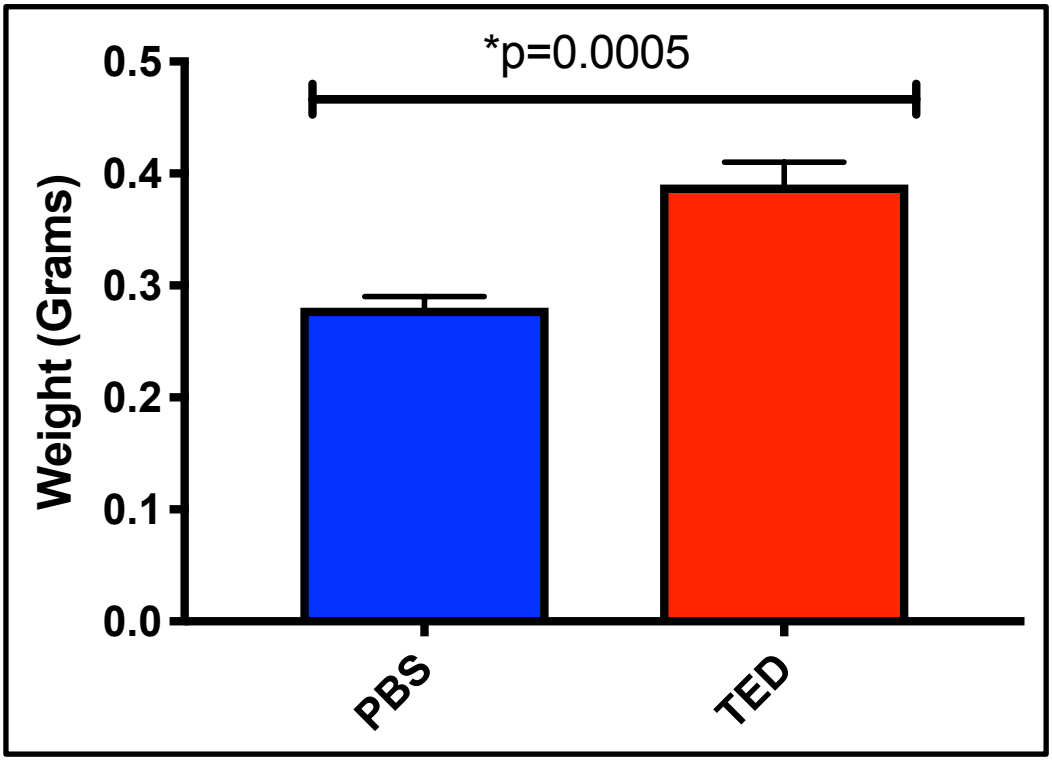



**Figure 117:** Mean weight of 5cm of jejunum.

When weight-adjusted, the TED group still had a significantly heavier first 5cm of jejunum (p=0.001) (**Figure 118**).

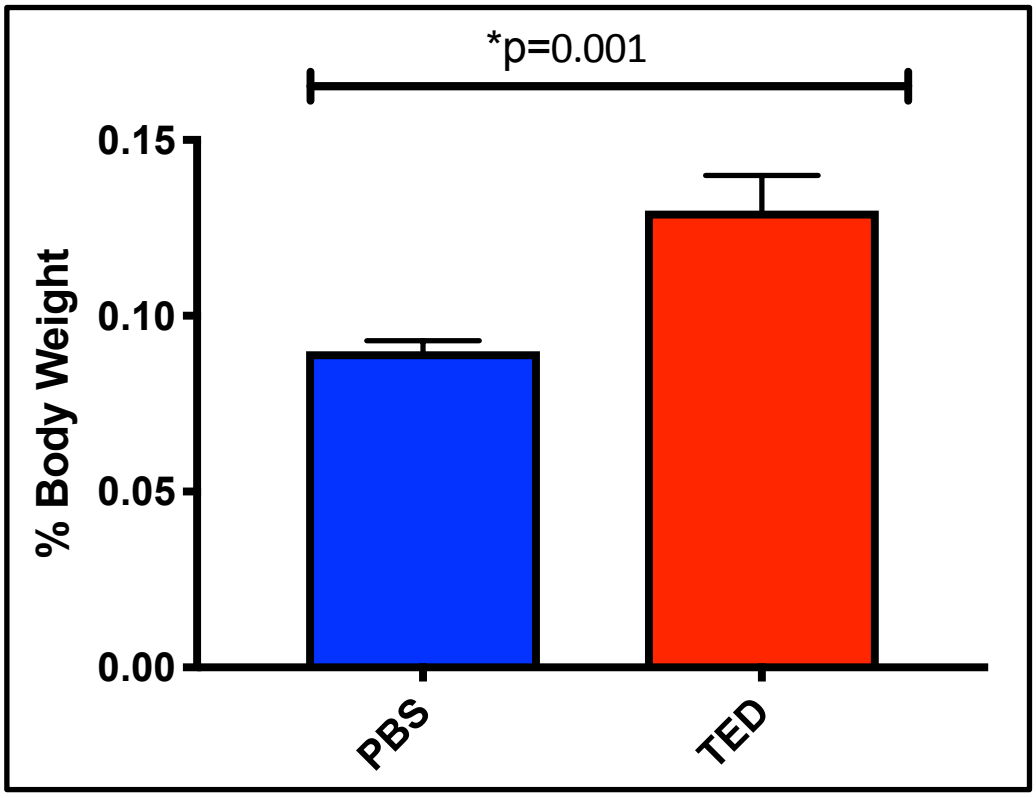



**Figure 118:** Mean weight of 5cm of jejunum expressed as % bodyweight.

In terms of histological analysis, there was a significant increase in villus length in the TED versus the PBS group (494.8µm v 403.9µm, p=0.005) (**Figure 119**).

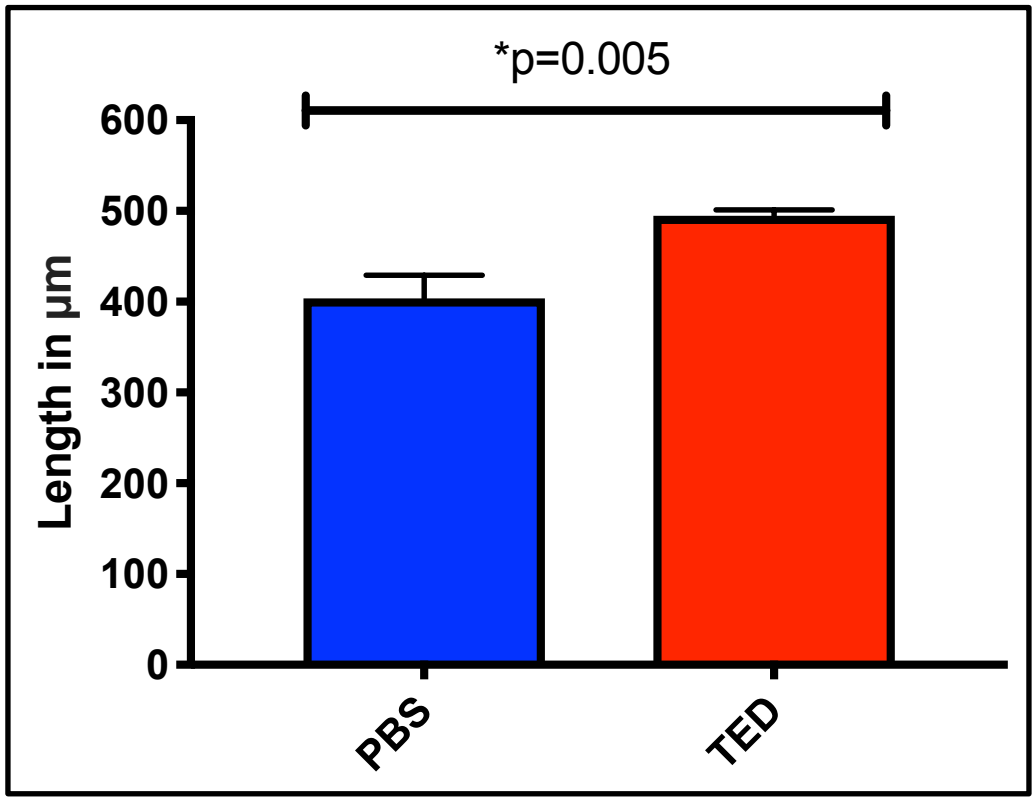



**Figure 119:** Mean villus length.

With regards to crypt depth, TED crypts were significantly deeper (167.0µm v 148.7µm, p=0.04) (**Figure 120**).

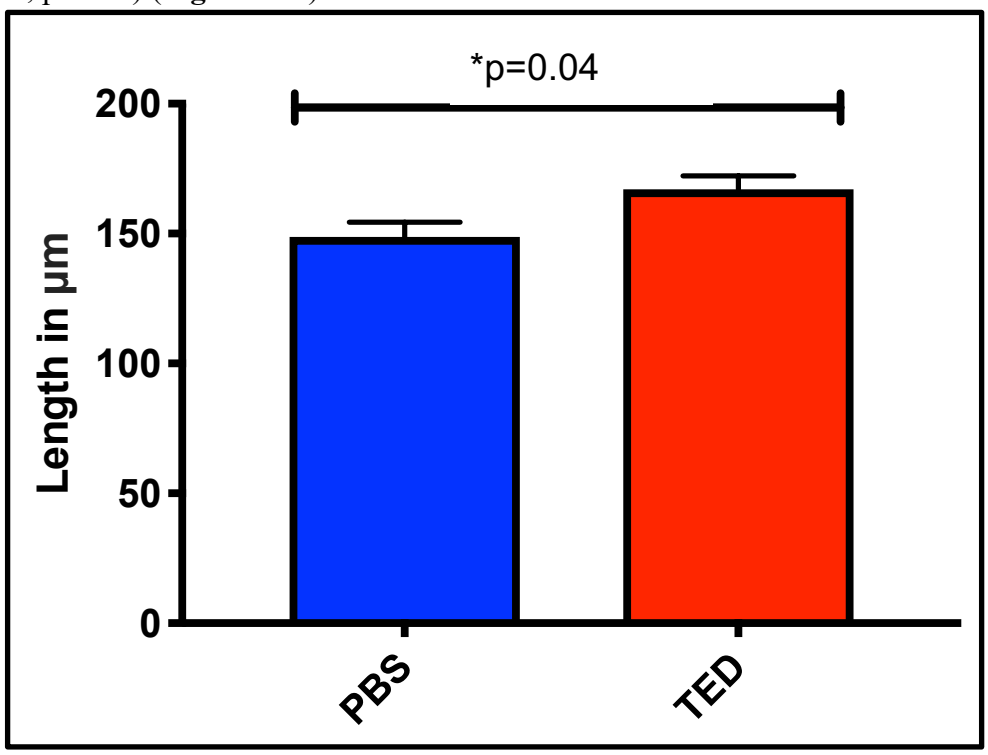



**Figure 120:** Mean crypt depth.

The overall total mucosal length was thus also significantly increased in the TED group (661.8µm v 552.7µm, p=0.001) (**Figure 121**).

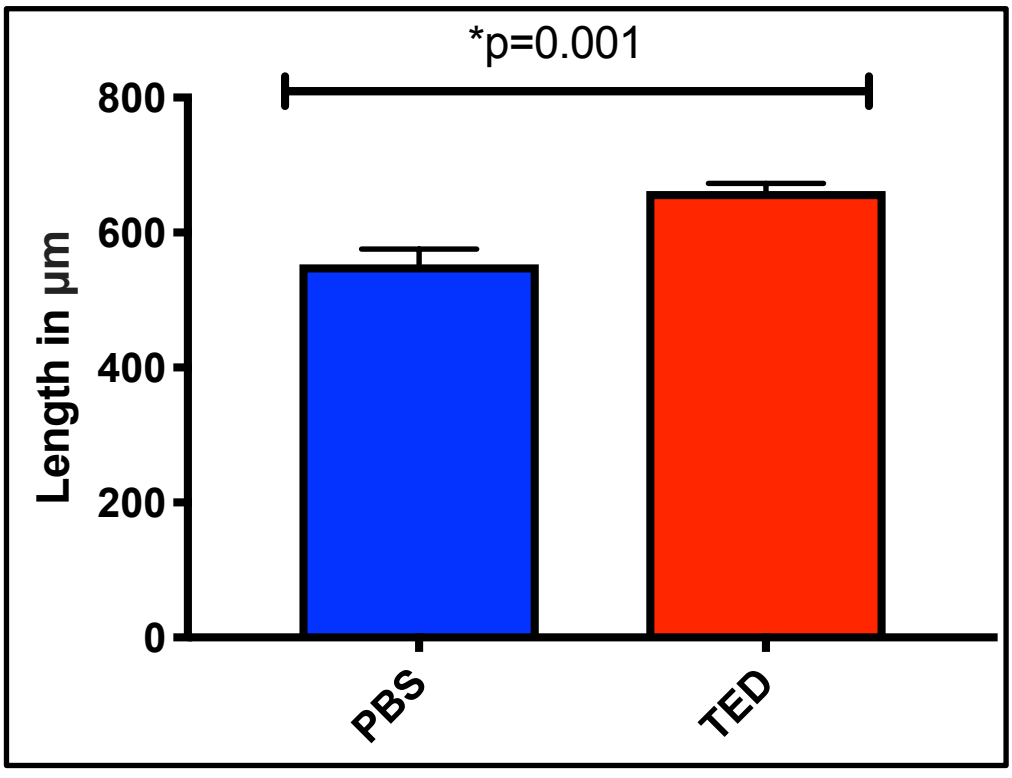



**Figure 121:** Total mucosal length.

A GLP-2 ELISA was conducted to see what effect exogenous GLP-2 might be having on endogenous GLP-2 levels. Blood had been taken at the harvest procedure, from both the portal and systemic catheters, in both the TED (n=6) and PBS (n=6) groups, and at 0-, 30- and 60-minute time points. At baseline, both groups were matched, portally and systemically (**Figure 122**).

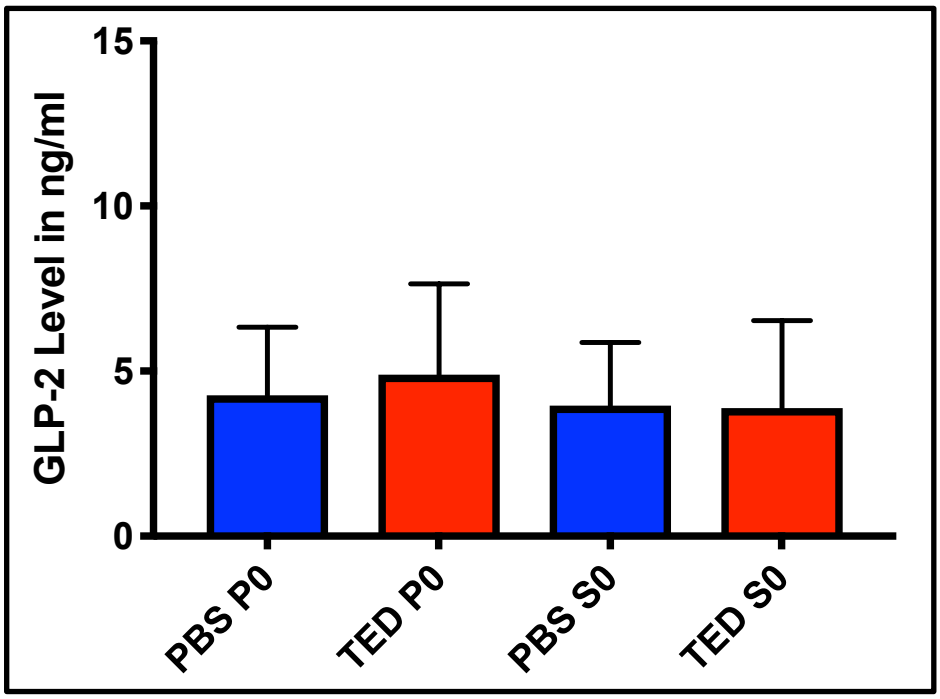



**Figure 122:** GLP-2 levels at baseline (0 minutes) in portal (P) and systolic (S) blood.

At the 30-minute time point, an increase in GLP-2 in the TED group was seen, portally and systemically, although neither reached significance (**Figure 123**).

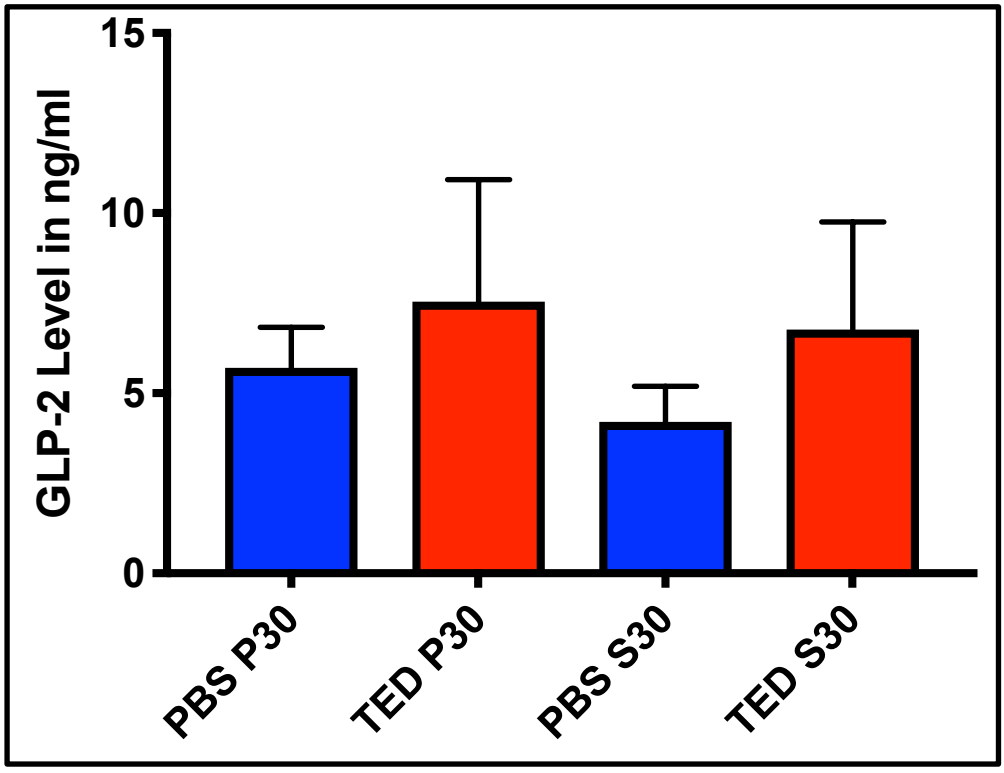



**Figure 123:** GLP-2 levels at 30 minutes, in portal (P) and systolic (S) blood.

By 60 minutes, the GLP-2 levels in the TED group were noticeably (although, not significantly) lower than in the PBS group (**Figure 124**).

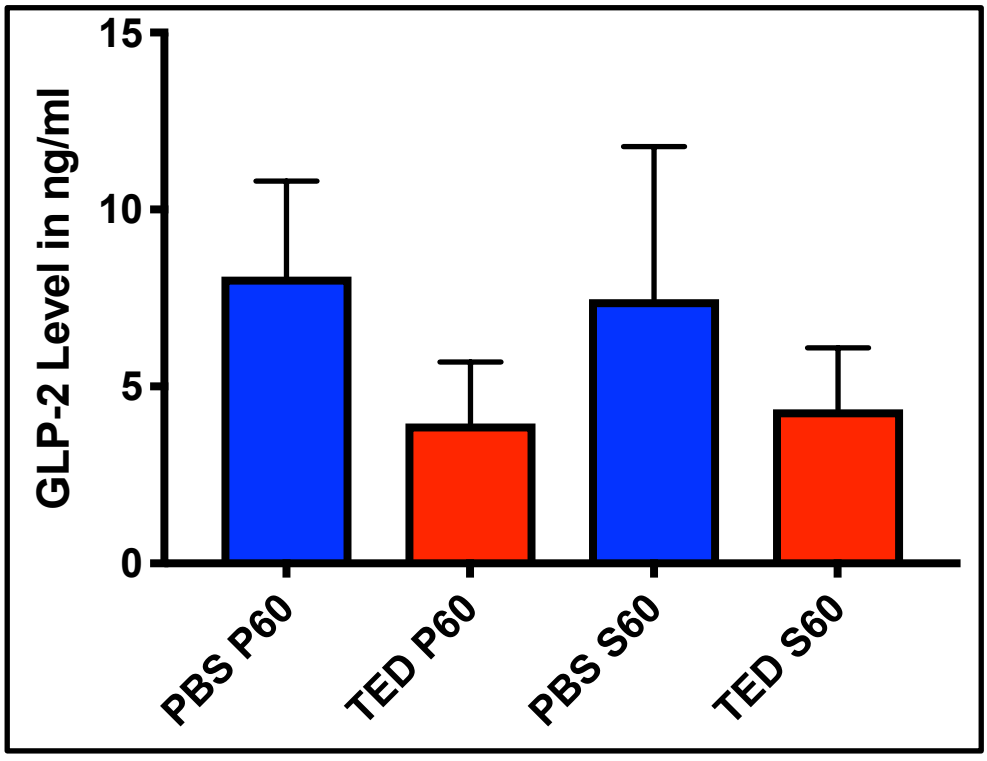



**Figure 124:** GLP-2 levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic GIP hormone analysis via Luminex. At baseline, GIP levels were noticeably lower in the TED group compared to the PBS group, showing significance within the systemic blood (18.07pg/ml v 72.48pg/ml, p=0.001) (**Figure 125**).

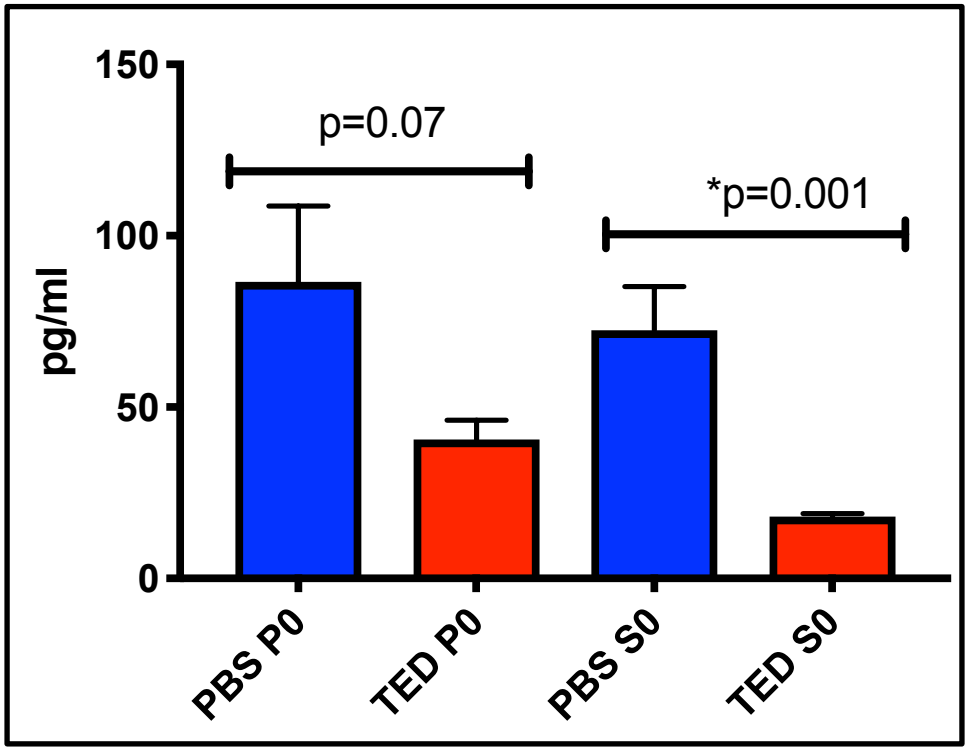



**Figure 125:** GIP levels at baseline (0 minutes), in portal (P) and systemic (S) blood.

This shifted after the duodenal glucose infusion, so that at 30 minutes, portal glucose levels in the TED group became significantly higher than in the PBS group (225.9pg/ml v 115.0pg/ml, p=0.03) (**Figure 126**).

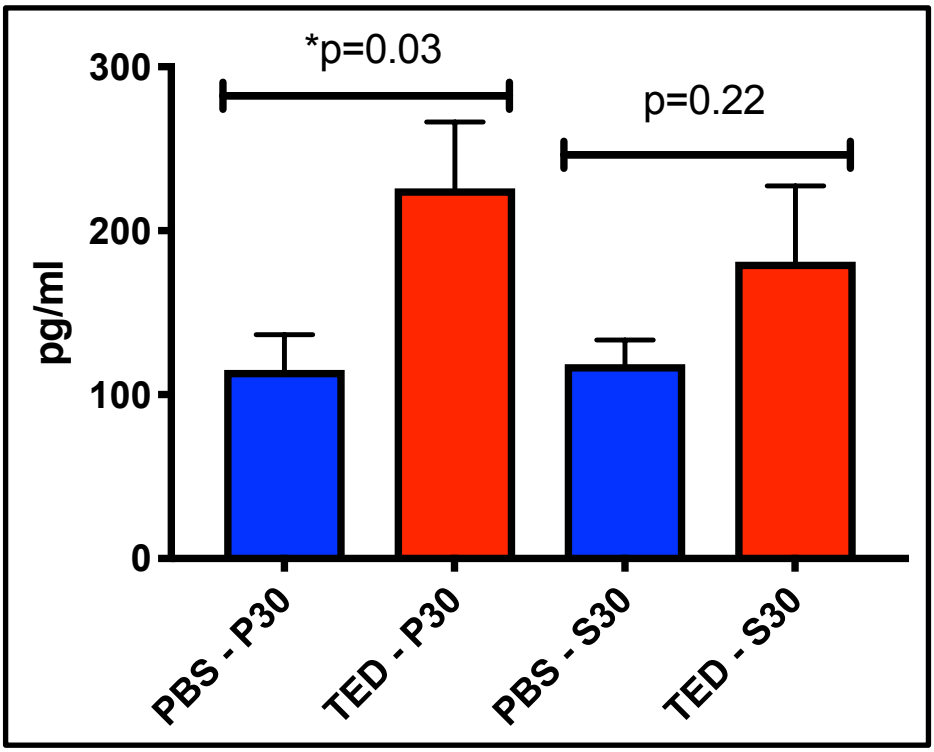



**Figure 126:** GIP levels at 30 minutes, in portal (P) and systolic (S) blood.

The same trend was seen in the systolic blood, although this did not reach significance. However, at the 60-minute time point, both portal and systemic blood glucose levels in the TED group were significantly higher than in the PBS group (p=0.00004 and p=0.03, respectively) (**Figure 127**).

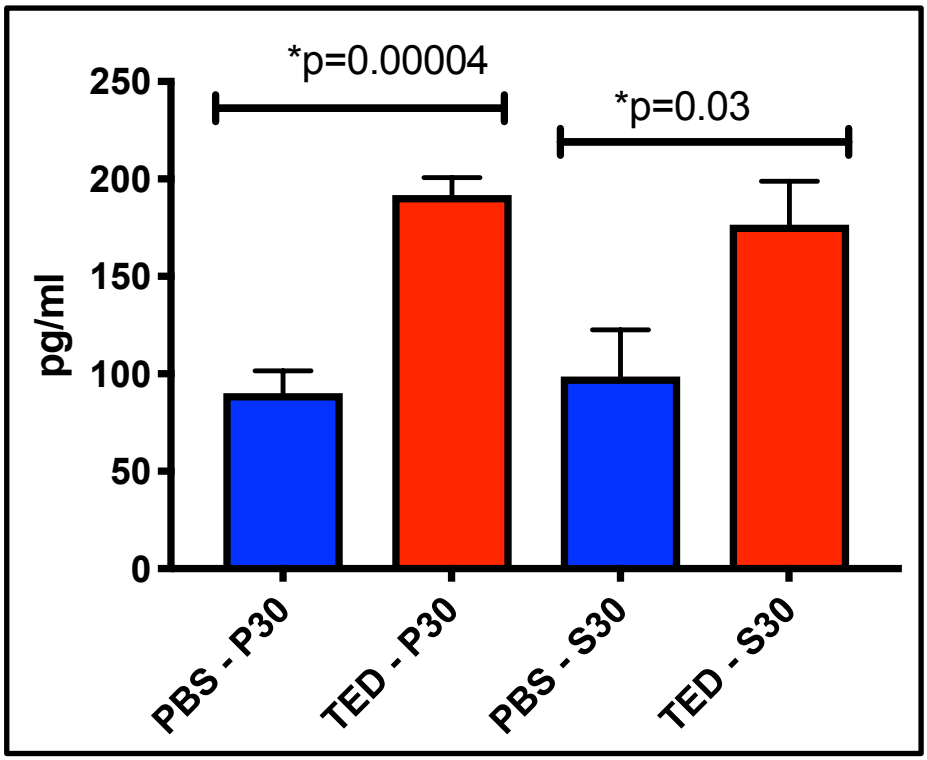



**Figure 127:** GIP levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic GLP-1 hormone analysis, via Luminex. There was only one statistically significant result regarding GLP-1, which was seen at the 60-minute time point where, in the TED group, glucose levels in the portal blood were significantly lower (342.7pg/ml v 1214.3pg/ml, p=0.03) (**Figure 130**). The lower systemic blood glucose levels in the TED group at 60 minutes just missed reaching significance (p=0.07).

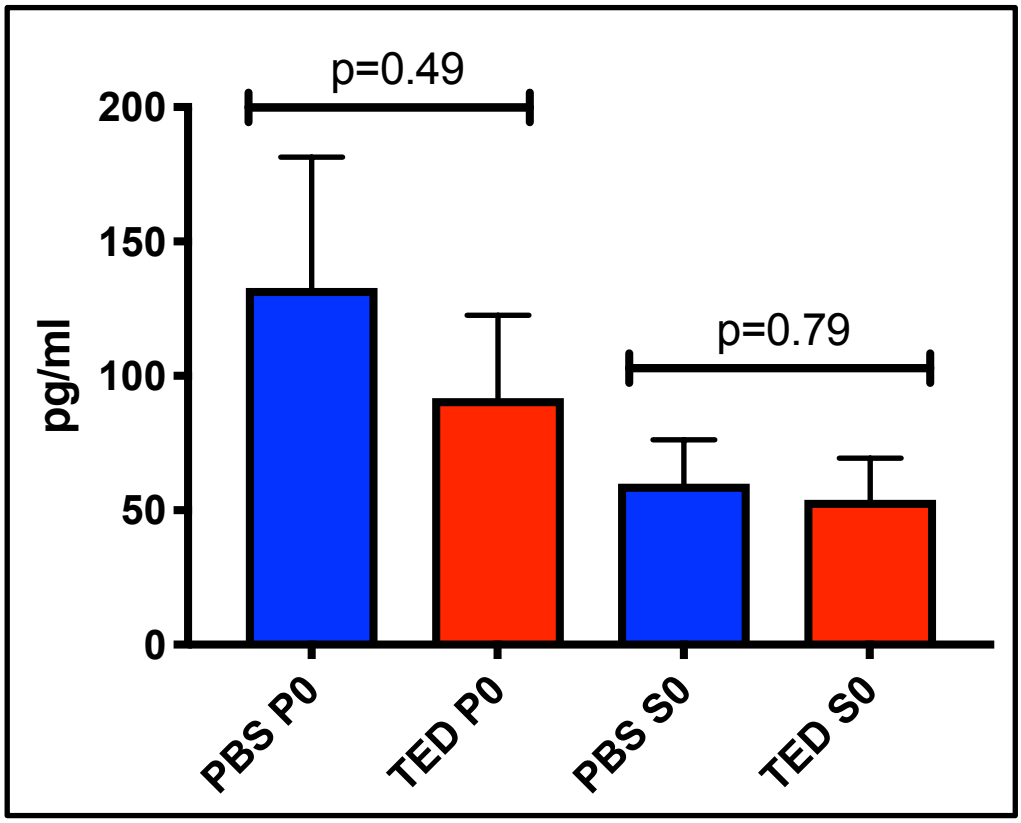



**Figure 128:** GLP-1 levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

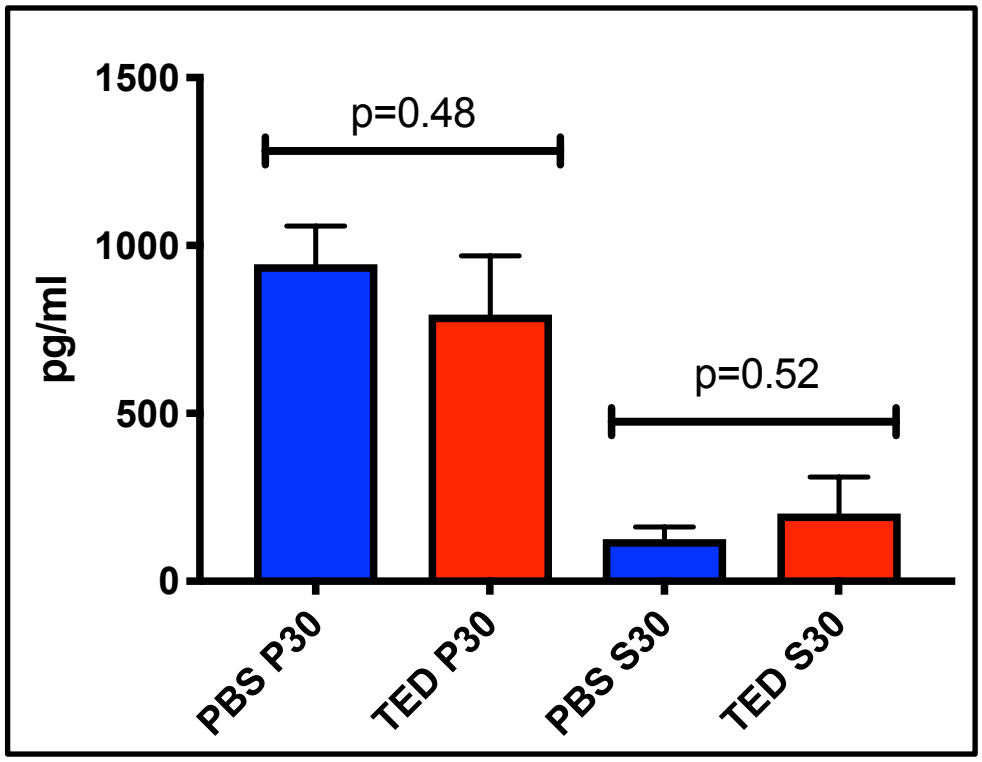



**Figure 129:** GLP-1 levels at 30 minutes, in portal (P) and systolic (S) blood.

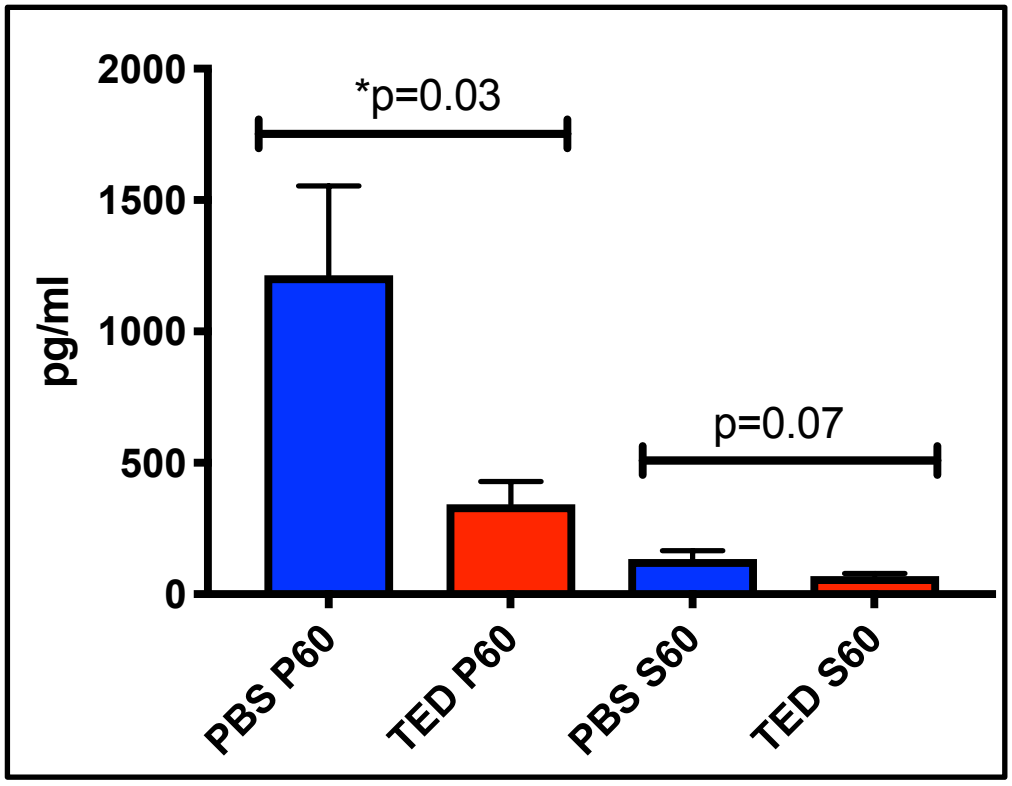



**Figure 130:** GLP-1 levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic glucagon hormone analysis, via Luminex. Surprisingly, there were no statistically significant results, although the trend was for a higher glucagon level within the TED group at all time points, and in both types of blood (apart from systolic blood at 60 minutes) (**Figures 131, 132 and 133**).

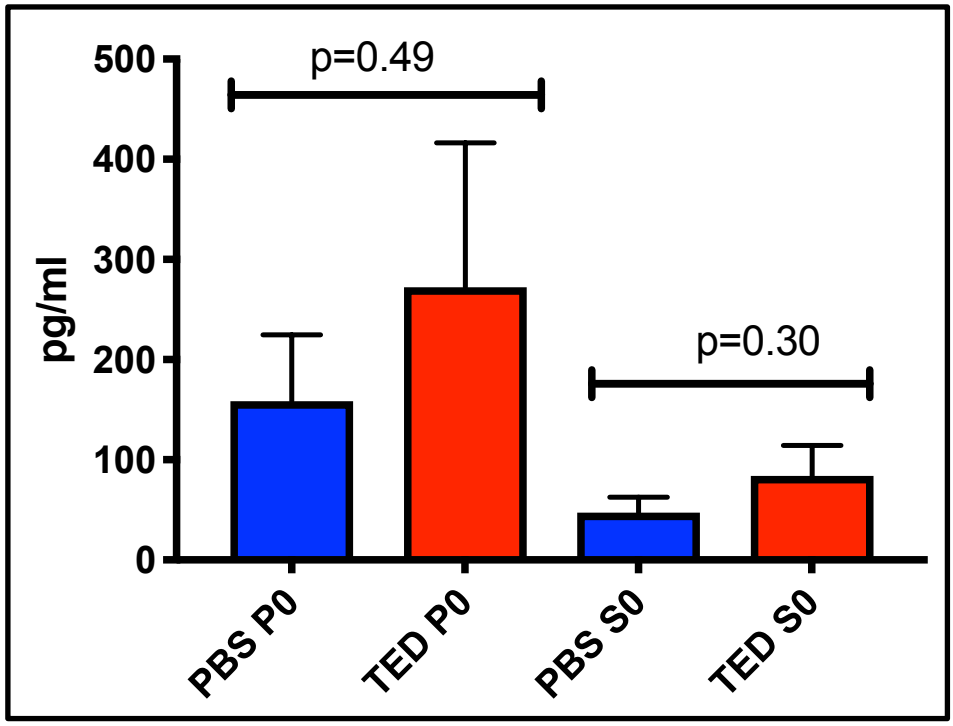



**Figure 131:** Glucagon levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

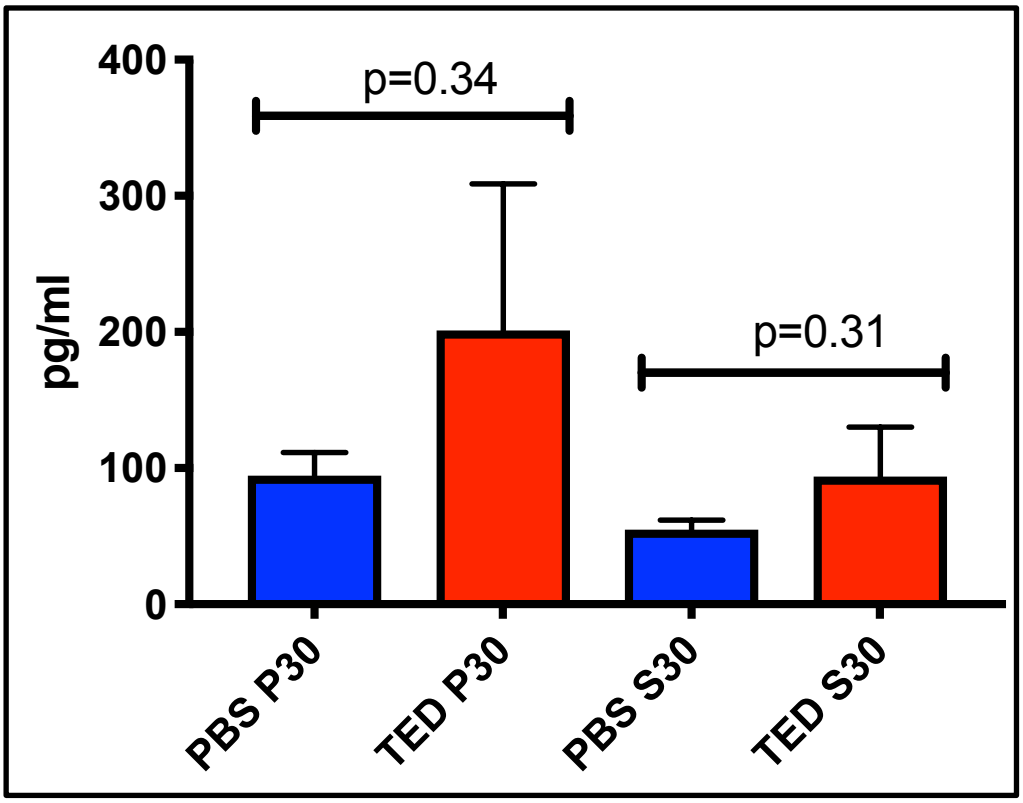



**Figure 132:** Glucagon levels at 30 minutes, in portal (P) and systolic (S) blood.

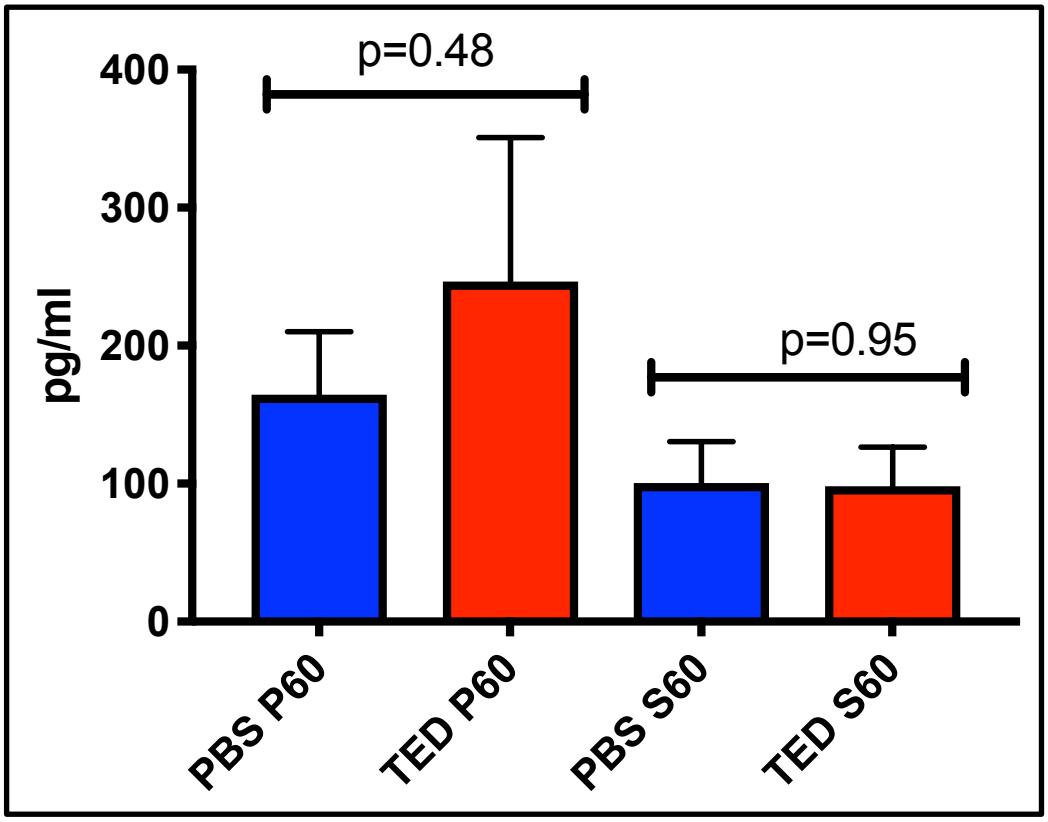



**Figure 133:** Glucagon levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic insulin hormone analysis, via Luminex. As with glucagon, there were no statistically significant results (**Figures 134, 135 and 136**).

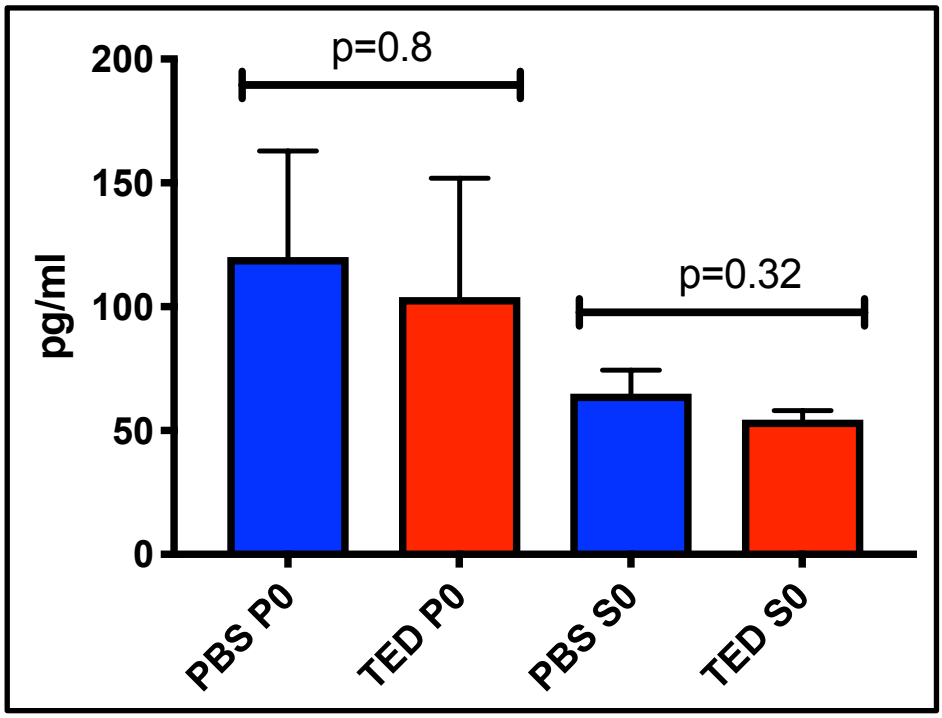



**Figure 134:** Insulin levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

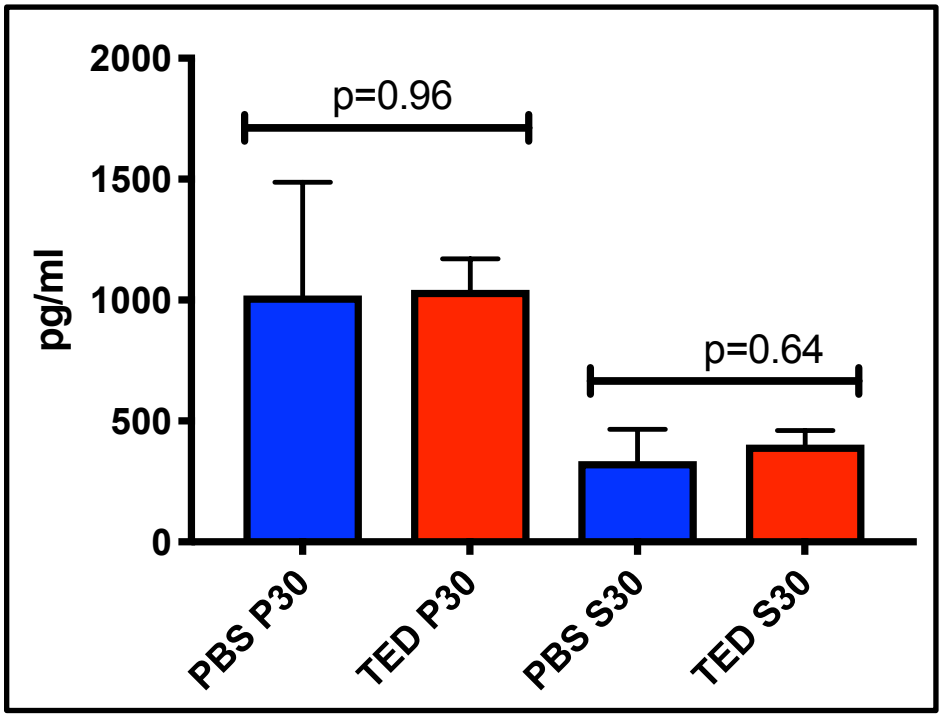



**Figure 135:** Insulin levels at 30 minutes, in portal (P) and systolic (S) blood.

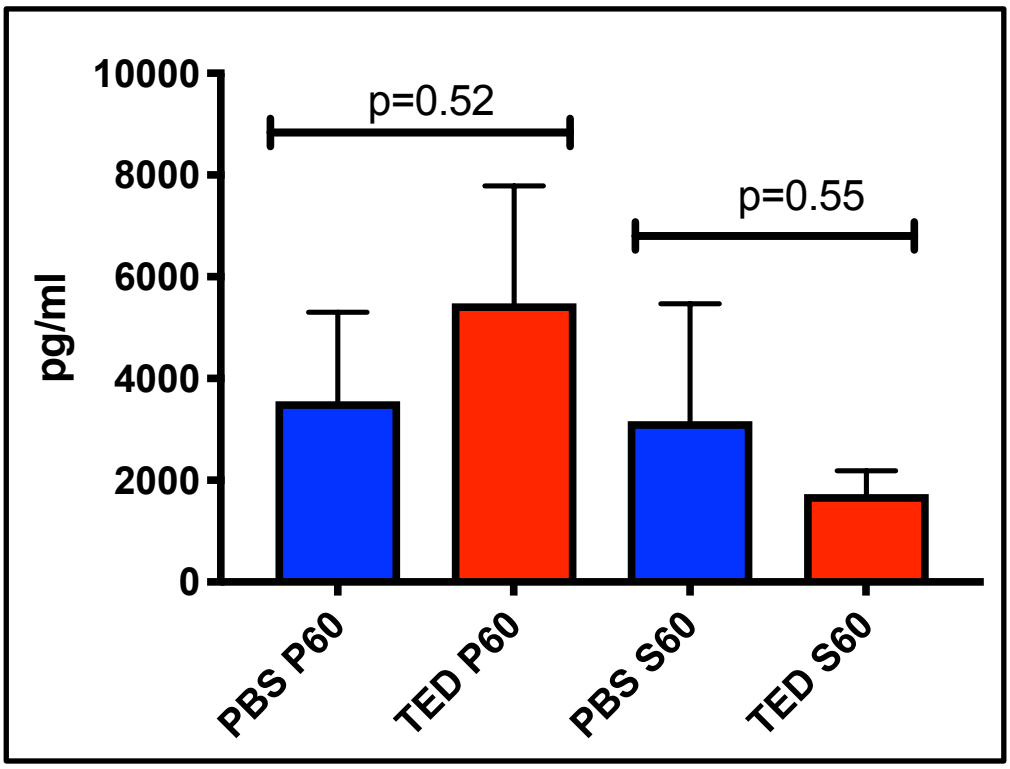



**Figure 136:** Insulin levels at 60 minutes, in portal (P) and systolic (S) blood.

Real-Time PCR was carried out on jejunal mucosa to quantify the following the following genes: SGLT-1, SGLT-3, GLUT-1, GLUT-2, GLP-2R, IGF-1 and T1R2. N=6 in both groups. Each of the following figures has an accompanying primer melt curve – the more matching the curves the more likely that the primer is generating a specific PCR product and not producing non-specific amplification.

Jejunal SGLT-1 expression was found to be significantly higher in the TED group, with a 3.8-fold change (p=0.03) (**Figure 137**).

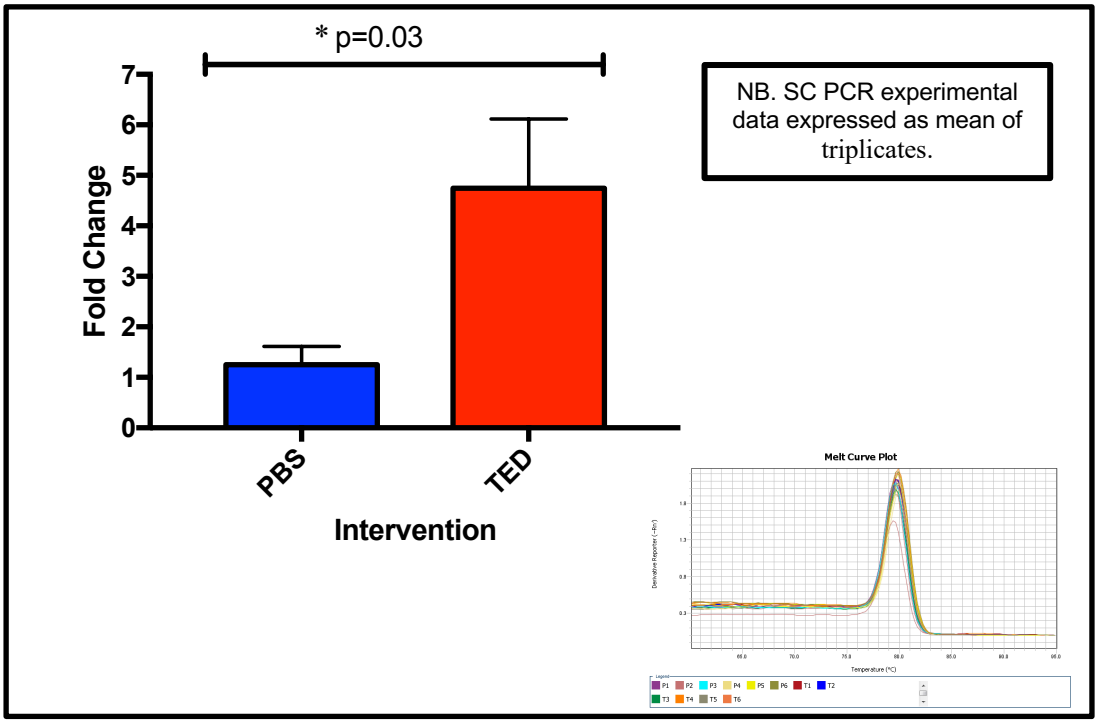



**Figure 137:** Jejunal SGLT-1 expression.

Jejunal SGLT-3 expression was also found to be significantly higher in the TED group compared to the PBS group, with a 2.6-fold change (p=0.02) (**Figure 138**).

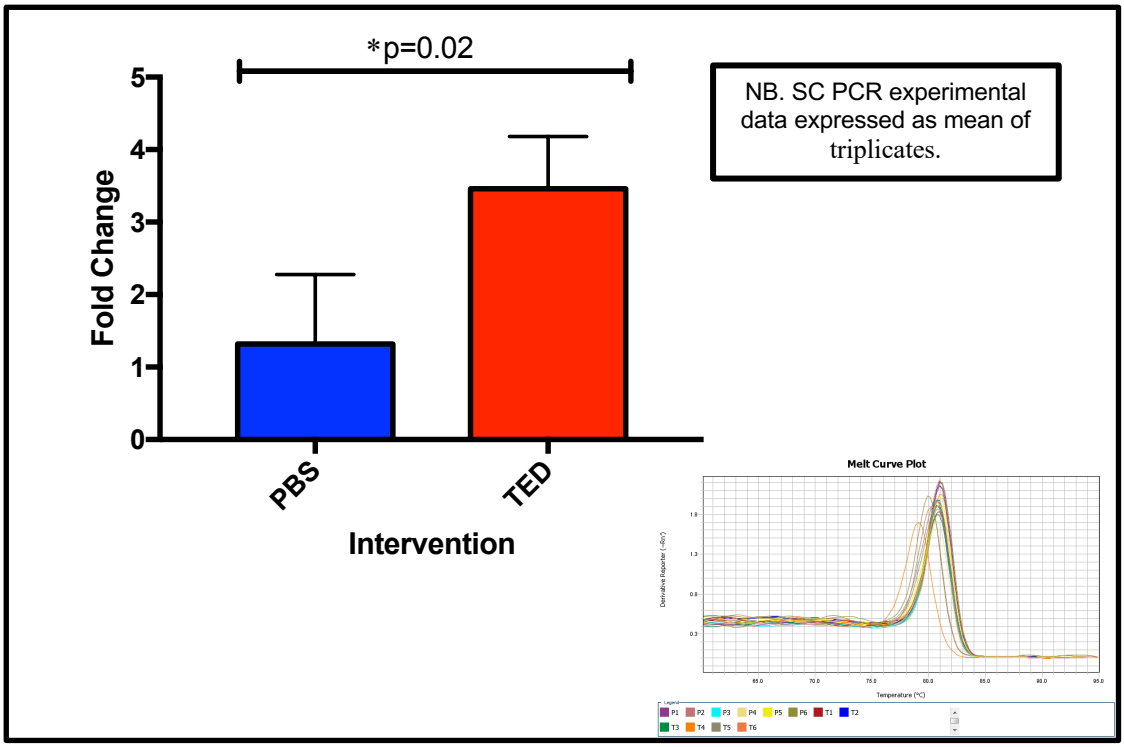



**Figure 138:** Jejunal SGLT-3 expression.

There was no significant difference between groups concerning GLUT-1, GLUT-2, GLP-2R, IGF-1 or T1R2 (**Figures 139, 140, 141, 142 and 143**), although an apparent decrease in GLP-2R expression within the TED group was close to reaching significance (p=0.059) (**Figure 141**).

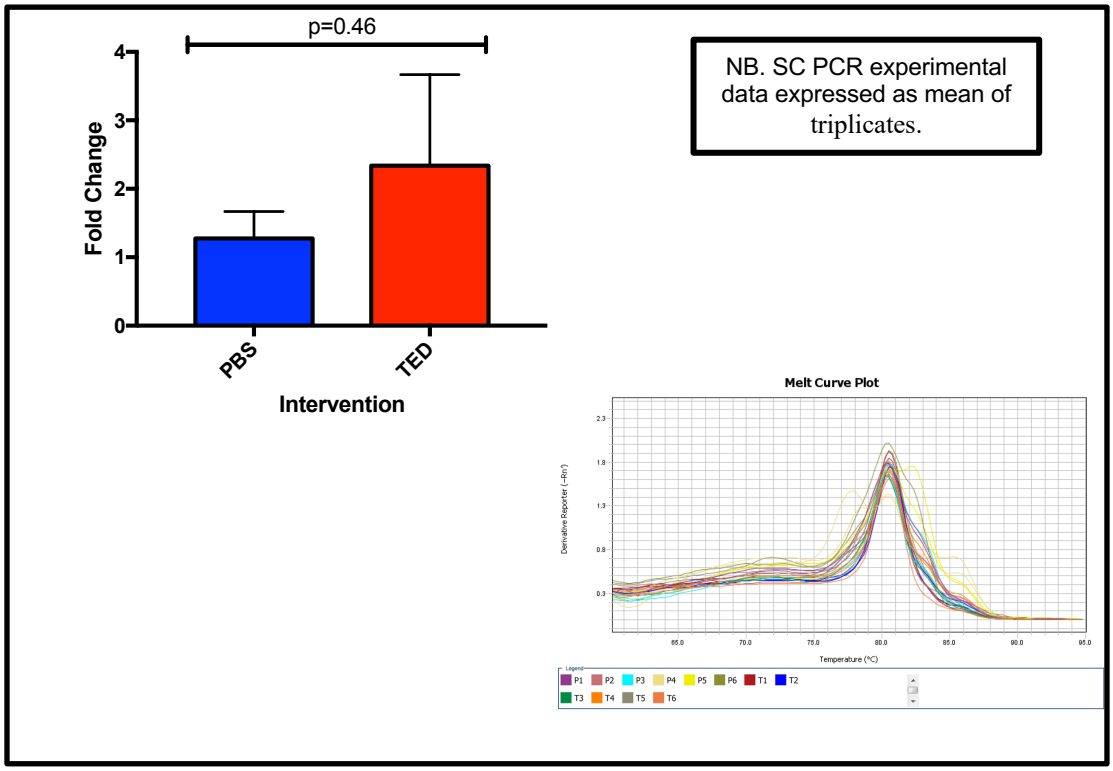



**Figure 139:** Jejunal GLUT-1 expression.

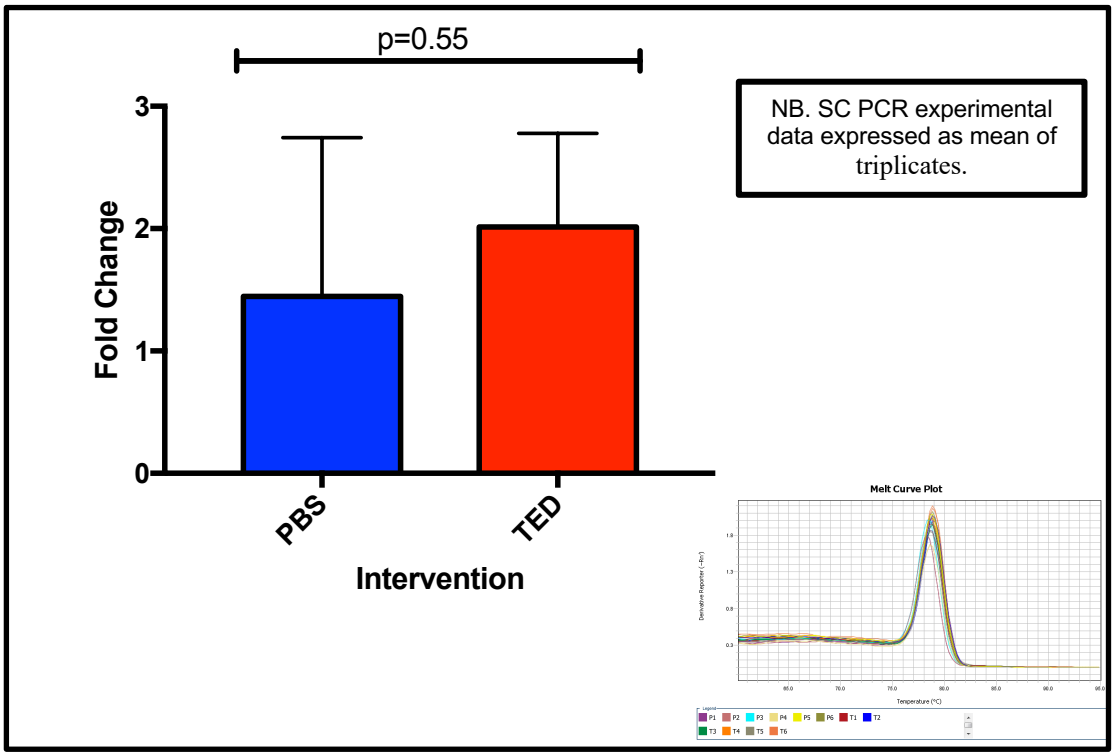



**Figure 140:** Jejunal GLUT-2 expression.

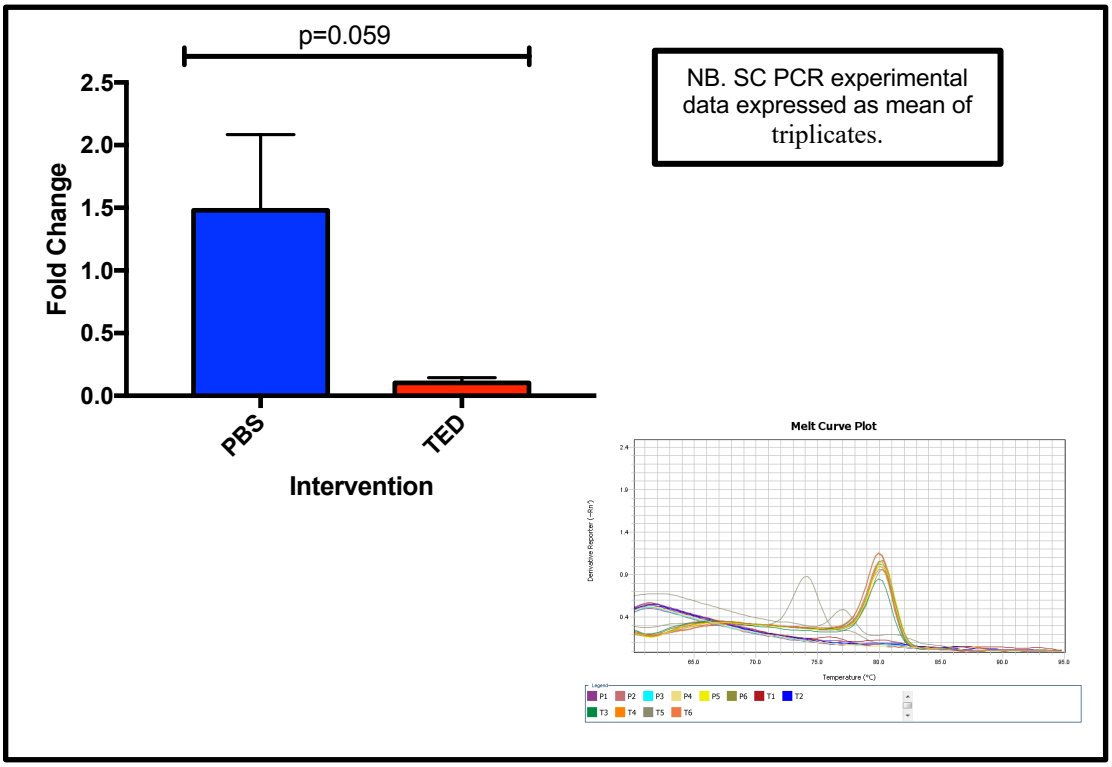



**Figure 141:** Jejunal GLP-2R expression.

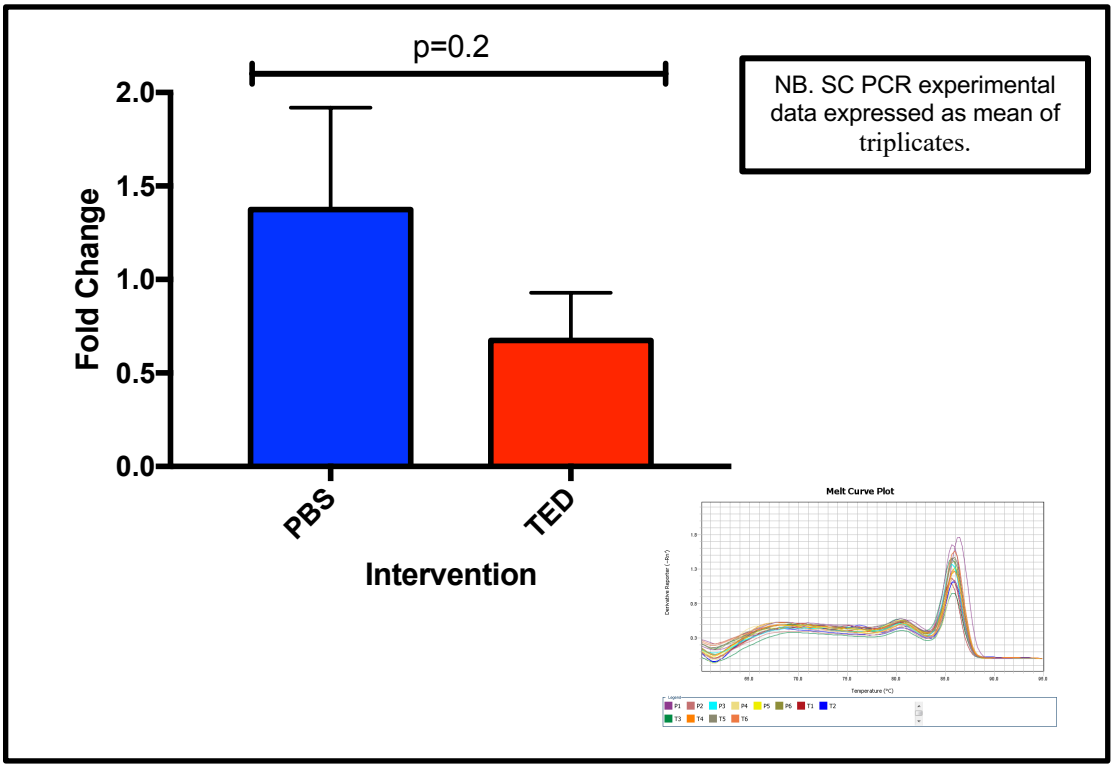



**Figure 142:** Jejunal IGF-1 expression.

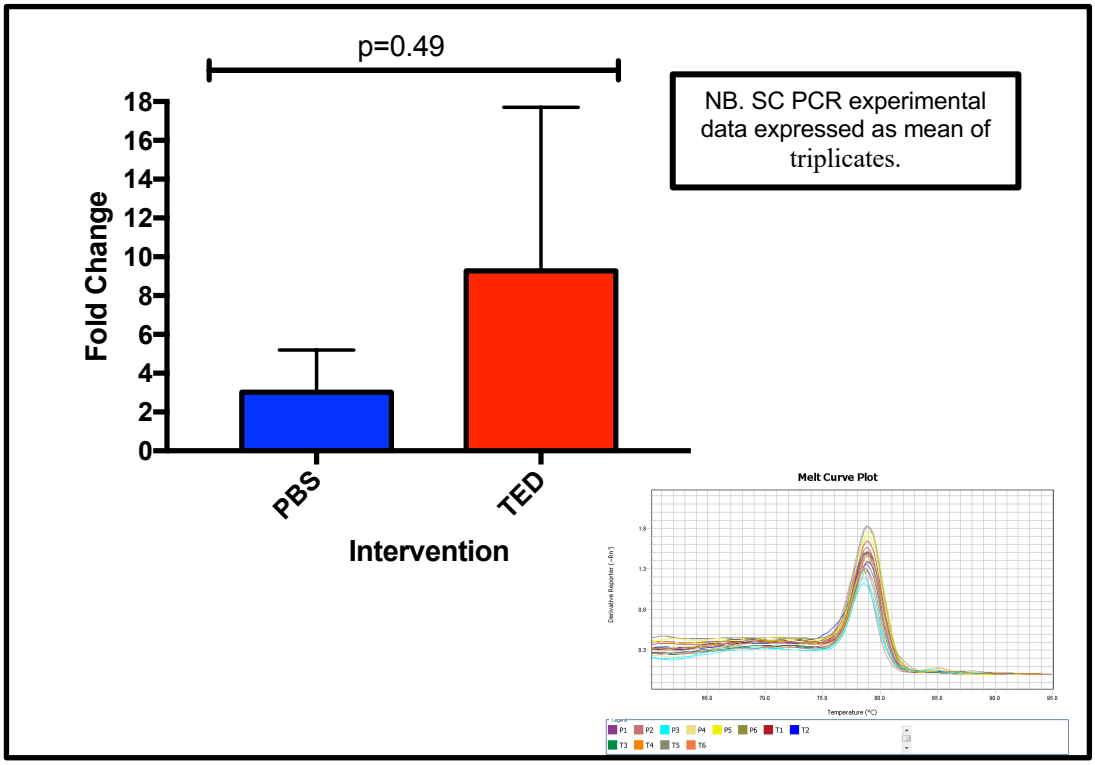



**Figure 143:** Jejunal T1R2 expression.

*3.6.3 Discussion*

**CASLO PEPTIDE, STANDARD CHOW, n=6**

Within this chronic Teduglutide administration study, using the CASLO provided Teduglutide peptide, a Validation Protocol was conducted which appeared to confirm the validity of the peptide. The main study was, therefore, entered.

Firstly, morphological assessment, in terms of crude weight of the intestine and histological measurement, suggested that the TED peptide was causing significant increase in intestinal adaptation. Secondly, there appeared to be a significant effect on glucose handling, with OGTTs showing a higher peak glucose within the TED group, but then a significant subsequent hypoglycaemic effect. This correlated with the hypothesis that Teduglutide would reduce postprandial blood glucose. One possible explanation is that TED significantly increases luminal absorption of glucose, which is then reflected in the higher glucose levels seen within the portal vein early on in the OGTTs, and within the harvest GTTs. This is in opposition to studies performed by Meier et al, who found that plasma glucose concentrations did not change during the administration of GLP-2, within fasted or fed state (Meier, Nauck et al. 2006). However, Experiment 1's results are supported by work carried out by Madsen et al, who demonstrated elevated glucose levels at 120 minutes post GLP-2 treatment compared to control in humans (Madsen, Askov-Hansen et al. 2013), and Jeppeson et al, who showed that GLP-2 increases nutrient absorption in humans (Jeppesen, Hartmann et al. 2001). The reversal of this hyperglycaemia over the next 30 minutes could be explained by increased intestinal glucose utilisation in that enterocytes could be directly using glucose in proliferation pathways to mediate hypertrophy. This concept is supported by FDG/PET studies performed, looking directly at glucose disposal within the bowel (Saeidi, Meoli et al. 2013). It was disappointing, therefore,

that this experiment did not show any significant changes in glucose absorption and utilisation overall. It is possible that by increasing the number of animals within the study that results could have shown a pattern more in line with other studies.

Nevertheless, PCR data appears to support the work carried out by Saeidi et al, demonstrating significant increased expression in the glucose transporters, SGLT-1 and glucose sensor, SGLT-3, and increased expression (although, not significantly) in GLUT-1 and GLUT-2. GLP-2R expression was found to be reduced in the TED group suggesting that the intestinotrophic effects of exogenous GLP-2 may be mediated through extra-intestinal mechanisms. Interestingly, another group found a similar reduction in GLP-2 mRNA expression following GLP-2 infusion, but within rats who had small intestine resected (Lai, de Heuvel et al. 2017) and also drew this same conclusion.

This study provided no evidence for a link between chronic GLP-2 administration and IGF-1 expression. This is a somewhat disappointing result. In vivo administration of IGF-1 has been found to have a potent hypoglycemic effect and has been proven to effectively lower blood glucose concentrations in subjects with T1D or T2D. Decreased levels of IGF-1 have also been documented in patients with T2D (Rubino and Gagner 2002). Leading on from this, studies have demonstrated that GLP-2 enhances intestinal IGF-1 expression and secretion, and that IGF-1 is required for small and large intestinal growth in response to GLP-2. These findings identify IGF-1 as an essential mediator of the intestinotrophic actions of GLP-2 (Dubé, Forse et al. 2006). It had been hypothesised, therefore, that IGF-1 could be a potential link between GLP-2's intestinotrophic effect and a role in glucose homeostasis following RYGB. T1R2 upregulation was not demonstrated, either.

Total chow consumed was identical between the two groups. This was, perhaps, unexpected given that GLP-2 has been shown to reduce gastric motility and suppress appetite (Connor, Evock-Clover et al. 2015). Furthermore, total body weight of rats was almost equal in TED versus PBS groups. This was surprising as other studies have shown a significant increase in total body weight following chronic GLP-2 administration compared to control, despite control groups receiving identical nutritional support (Kaji, Tanaka et al. 2008).

Luminex offered some interesting results. GIP hormone level was significantly higher in the TED group at 30 and 60 minutes. As an incretin, GIP lowers glucose levels. Given that GIP levels were found to be significantly higher at 30 and 60 minutes within the TED group this fits with the relative hyperglycaemia (compared to PBS group) seen in the TED group from 30 to 60 minutes. In slight conflict with this result, there was a significantly lower portal GLP-1 level at 60 minutes in the TED group, perhaps as a direct response to glucose levels starting to fall in the OGTTs and plateau in the harvest GTTs. Furthermore, there was no increase in glucagon secretion.

This first CASLO peptide study provided interesting, yet challenging, results within a healthy rat model. However, it was decided that a pathological rat model should be used to see if results were reproduced, as this would have relevant translational implications for this piece of research.

## 3.7 EXPERIMENT 3

### *3.7.1 Pathological Model*

In terms of choosing a pathological rat-based model, there were two main options available – using rats with a genetic predisposition for obesity and diabetes mellitus, of which the most common model would be the Zucker Diabetic Fatty (ZDF) rat (**Figure 144**), or, purchasing non-pathological rats (i.e SD rats) and feeding them a high-fat diet (HFD) in order to cause obesity and glucose intolerance.

*Genetic Manipulation*

*ZDF Rats*

A mutation predisposing to obesity and T2DM occurred in a colony of outbred Zucker rats in the laboratory of Dr. Walter Shaw at Eli Lilly Research Laboratories in Indianapolis, IN in 1974. Part of this colony containing the mutation was moved to another laboratory in 1977. Several groups of animals with diabetic lineage were identified and rederived in 1981, with inbreeding of selected pairs from this rederivation then carried out so that an inbred line of ZDF rat was established in 1985.



**Figure 144:** Zucker Diabetic Fatty (ZDF) rat (https://www.criver.com/products-services/find-model/zdf-rat?region=3671).

Male ZDF rats develop obesity and insulin resistance at a young age, and then with aging, progressively develop hyperglycaemia. This hyperglycaemia is associated with impaired pancreatic β-cell function, loss of pancreatic β-cell mass, and decreased responsiveness of liver and extrahepatic tissues to the actions of insulin and glucose. Of particular interest are the insights provided by studies of these animals into the mechanism behind the progressive impairment of carbohydrate metabolism. This feature among others, including the development of obesity- and hyperglycaemia-related complications, is common between male ZDF rats and humans with T2D associated with obesity.

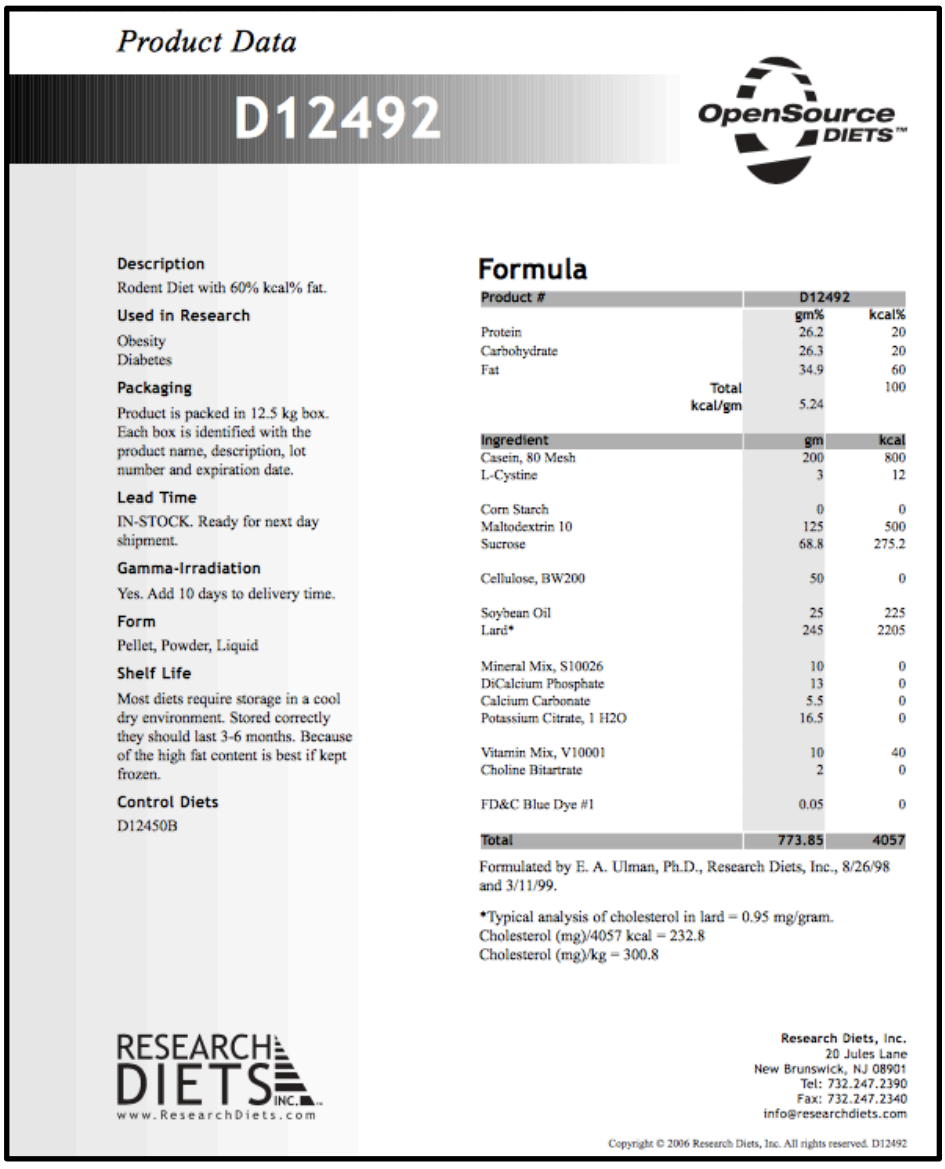
Although this sounds like the perfect model for studies into diabetes, there are some drawbacks. For example, from work done within ZDF rats in this lab, it has been apparent that they are a challenging surgical model. They have a higher fat mass, and thus, the catheterisation technique developed in this lab is far harder to master in a ZDF rat, predominantly because of fattier mesentery. They are also more susceptible to post-operative complications. Additionally, the rat model, and the type of chow required to exacerbate the development of obesity/diabetes, are both expensive. It was decided that the risks of using this model outweighed the benefits. Thus, diet-manipulation of healthy rats was chosen instead.

*Dietary Manipulation*

There were essentially two options available regarding dietary manipulation. The first was to pay an animal facility to feed healthy SD rats a high fat diet, and then provide this lab with rats exhibiting the highest weights and random/fasting blood glucose levels. The second was to purchase healthy SD rats, along with high fat diet, and feed

the rats within this lab. Although the work load would be higher, from a financial point of view, it made more sense to choose option two, thus this was the chosen option.

Research diet D12492i (i = irradiated, a requirement of this lab's animal facility) was the high fat diet chosen. D12492i consists of 60kcal % fat and is a gold standard for (DIO) Diet-Induced Obesity research (**Figure 145**).



*Product Data*

**D12492**

OpenSource DIETS™

**Description**
Rodent Diet with 60% kcal% fat.

**Used in Research**
Obesity
Diabetes

**Packaging**
Product is packed in 12.5 kg box. Each box is identified with the product name, description, lot number and expiration date.

**Lead Time**
IN-STOCK. Ready for next day shipment.

**Gamma-Irradiation**
Yes. Add 10 days to delivery time.

**Form**
Pellet, Powder, Liquid

**Shelf Life**
Most diets require storage in a cool dry environment. Stored correctly they should last 3-6 months. Because of the high fat content is best if kept frozen.

**Control Diets**
D12450B

**Formula**

| Product # | | D12492 | |
|---|---|---|---|
| | | gm% | kcal% |
| Protein | | 26.2 | 20 |
| Carbohydrate | | 26.3 | 20 |
| Fat | | 34.9 | 60 |
| | Total | | 100 |
| | kcal/gm | 5.24 | |
| **Ingredient** | | **gm** | **kcal** |
| Casein, 80 Mesh | | 200 | 800 |
| L-Cystine | | 3 | 12 |
| Corn Starch | | 0 | 0 |
| Maltodextrin 10 | | 125 | 500 |
| Sucrose | | 68.8 | 275.2 |
| Cellulose, BW200 | | 50 | 0 |
| Soybean Oil | | 25 | 225 |
| Lard* | | 245 | 2205 |
| Mineral Mix, S10026 | | 10 | 0 |
| DiCalcium Phosphate | | 13 | 0 |
| Calcium Carbonate | | 5.5 | 0 |
| Potassium Citrate, 1 H2O | | 16.5 | 0 |
| Vitamin Mix, V10001 | | 10 | 40 |
| Choline Bitartrate | | 2 | 0 |
| FD&C Blue Dye #1 | | 0.05 | 0 |
| **Total** | | **773.85** | **4057** |

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.95 mg/gram.
Cholesterol (mg)/4057 kcal = 232.8
Cholesterol (mg)/kg = 300.8

RESEARCH DIETS INC.
www.ResearchDiets.com

Research Diets, Inc.
20 Jules Lane
New Brunswick, NJ 08901
Tel: 732.247.2390
Fax: 732.247.2340
info@researchdiets.com

Copyright © 2006 Research Diets, Inc. All rights reserved. D12492

**Figure 145:** Research diet D12492 product data sheet (https://info.taconic.com/hs-fs/hub/355513/file-2452871853-pdf/Technical_Library/D12492.pdf).

The decision was made to feed SD rats on a HFD for a total of 7 weeks. Several papers guided this choice of time frame. For example, one team looked at the effect of HFD (D12492i) on bodyweight in SD rats. They noticed from as early as week 3 up until 12 weeks, that body weight was notably increased in the HFD group compared with that in the normal (standard chow) group (Guo, Ren et al. 2013) (**Figure 146**).

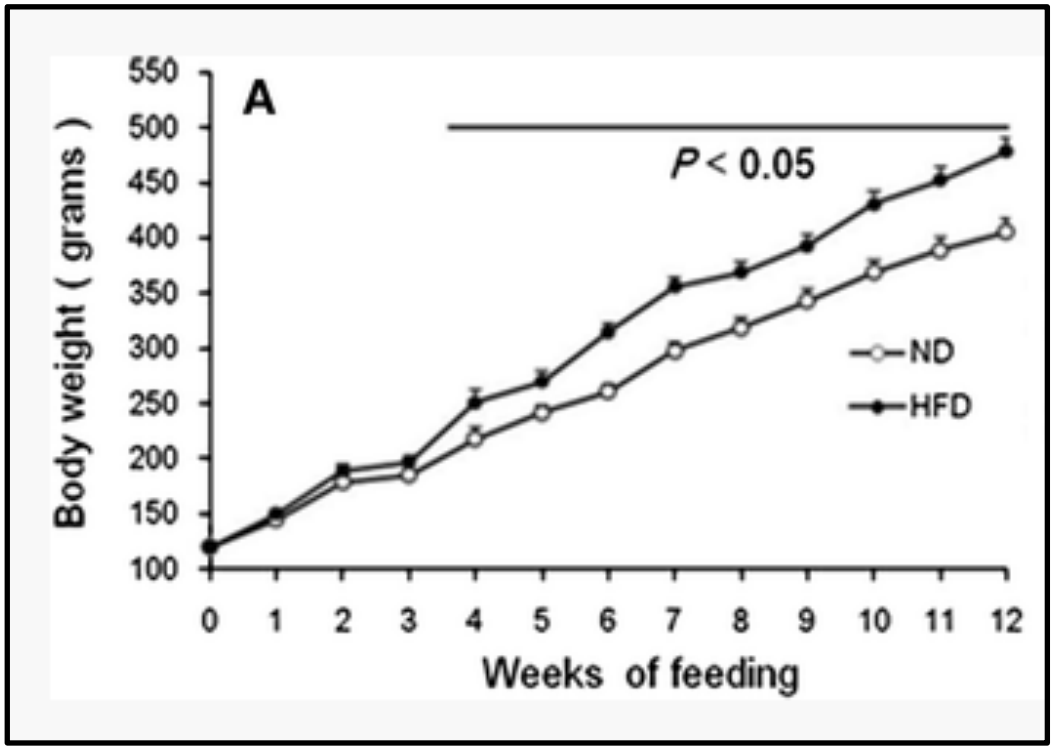



**Figure 146:** Effect of HFD (D12492i) on bodyweight in SD rats.

Another group studying high fat diets and how they might be used to replicate human metabolic disorders in rodents observed that 'obesity induction is most effective when the diet is started at a young age and continues for several weeks…Although an increase in body weight can be appreciated after as little as 2 weeks, the diet-induced phenotype becomes most apparent after several, i.e., more than 4 weeks of HFD feeding'. With regards to glucose handling they noted that 'HFD effects on blood glucose levels are described discrepantly. Normoglycemia, slight hyperglycaemia, and the development of T2D have been reported with different diet regimes. From the data

published so far, one can conclude that prolonged, i.e., several weeks, feeding with both animal and plant fat-enriched diets will eventually lead to moderate hyperglycaemia in most rat and mouse strains (most widely used are Wistar/Sprague-Dawley for rats and C57BL/6(j/n) for mice). With the diet types mentioned above, the elevation of fasting glucose levels is usually accompanied by a moderate to distinct increase in fasting plasma insulin levels'.(Buettner, Schölmerich et al. 2007).

Thus, the following methodology was used to carry out Aim 2, Experiment 3 using the CASLO Teduglutide peptide, and with rats fed a high fat diet (HFD).

### *3.7.2 Methods and Materials*

#### *Teduglutide*

Given the success of the peptide used in section 3.5, Teduglutide was once again purchased from CASLO. The mass of the peptide was confirmed by their laboratory (via mass spectrometry) as 3753.12g/mol (**Figure 147**) – again, this is almost the exact expected weight of Teduglutide. Given the statistically significant results gained from Validation Protocol 3, which now exists to serve as the control arm of the study and, given that the structure and weight of the new batch of Teduglutide received was almost identical to the previous batch, it was felt that a further validation experiment was not needed. There were no concerns regarding the functionality of the peptide provided to us by CASLO.

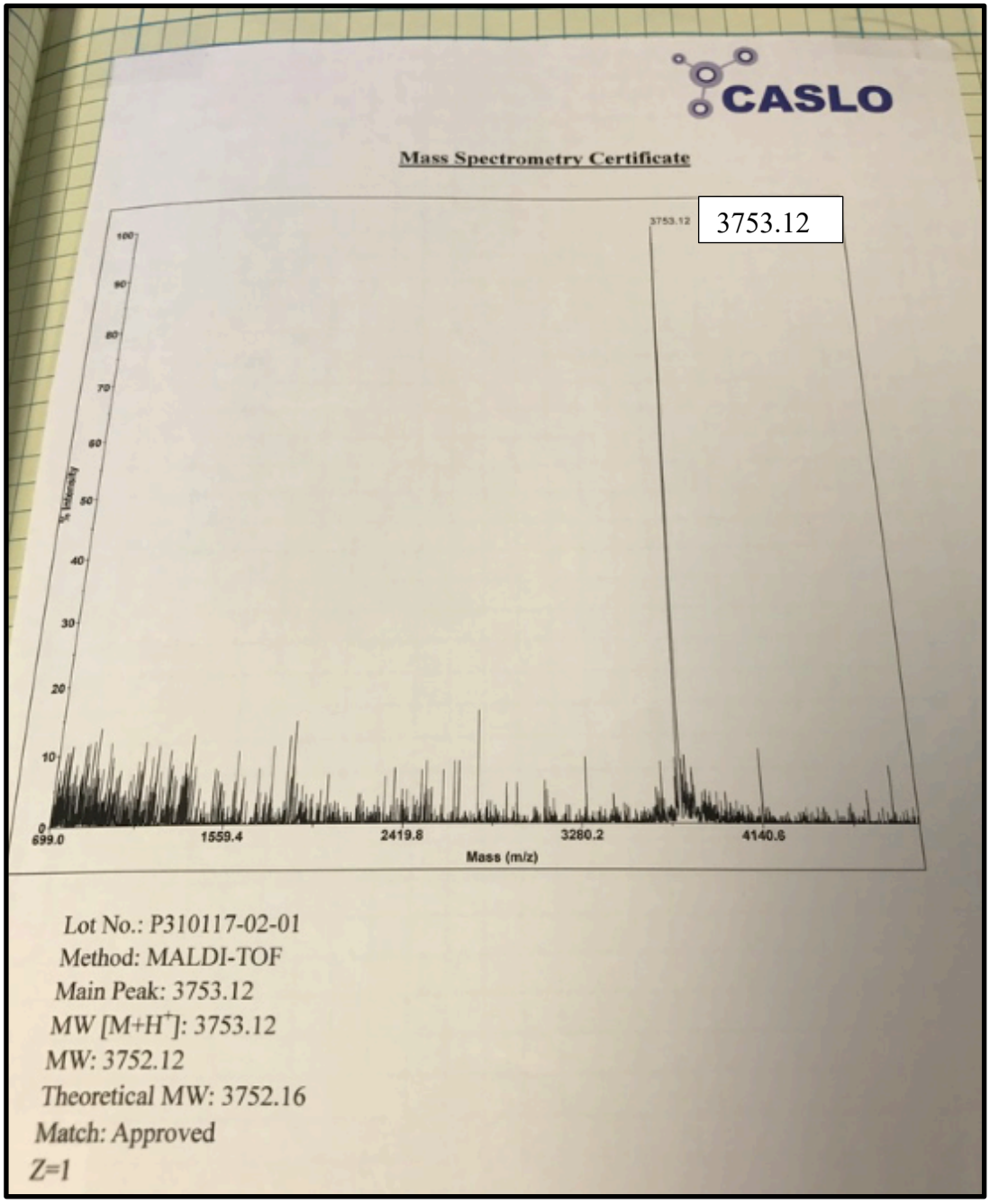



**Figure 147:** CASLO laboratory certificate of Teduglutide analysis, showing a mass spectroscopy peak at 3753.12g/mol.

*Animals*

A further twelve male SD rats (200-220g, ENVIGO, New Jersey) were acclimatised for 1 week. They were then weighed and placed in either the TED group or the PBS group, so that each group totalled a similar overall weight. All 12 rats were started on Research Diet D12492i and received 7 weeks of this chow. From day 28, 6 rats received daily injections of 0.4mg/kg Teduglutide (in 200µl PBS), whilst the other 6 rats received 200µl PBS alone. All 12 rats received TED+PBS or PBS alone, for a total of 3 weeks. Oral glucose tolerance tests were carried out as above (section 3.4.1), and as per the study timeline (**Figure 148**).

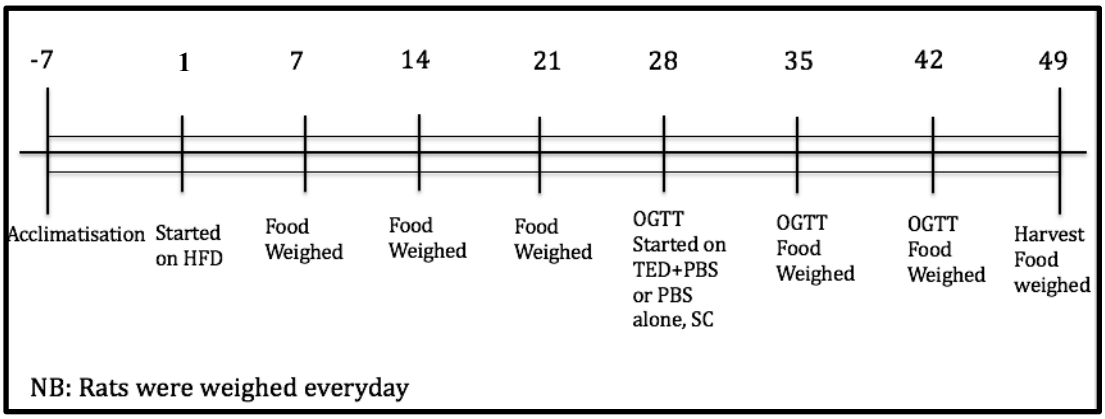



**Figure 148:** Experiment timeline.

*Harvest Protocol*

Harvest protocol, including catheter placement, blood analysis, PCR preparation and weighing of tissue, and termination of experiment were carried out as described in section 2.4.1. ELISA, Luminex and PCR were performed as described in **Appendices 1, 2 and 3**. The same primers were used as in Validation Protocol 3 (section 3.5).

*3.7.3 Results*

**CASLO PEPTIDE, HIGH FAT DIET, n=6**

Statistical analysis was carried out as described in section 2.4.1. Baseline OGTT, performed 3 days prior to initiation of injections and on all 12 HFD rats, was well matched, with no significant difference between the two groups (n=6 in both groups) at any time points (**Figure 149**).

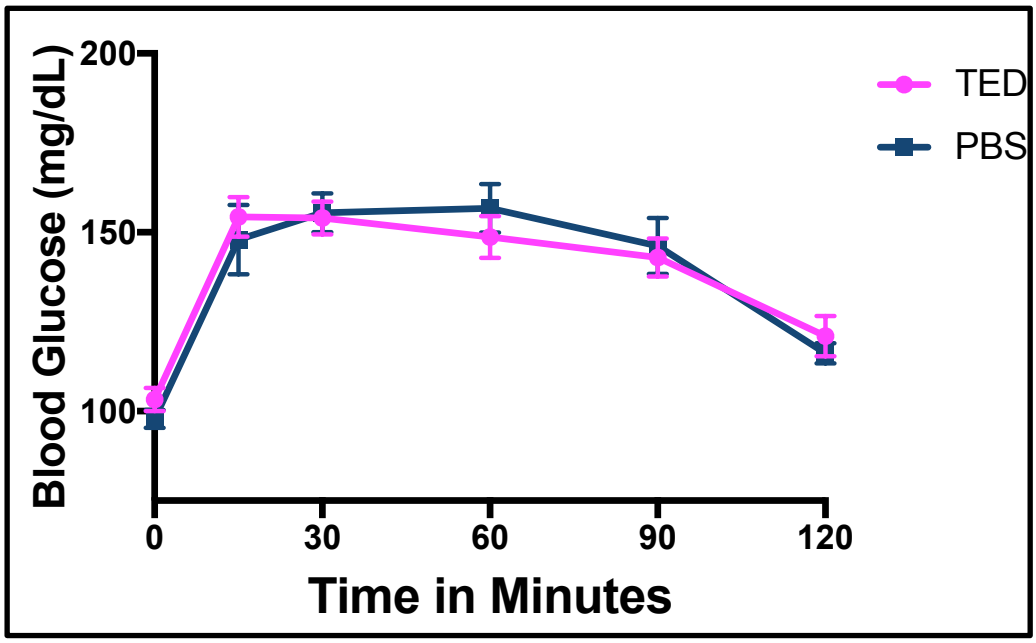



**Figure 149:** Oral glucose tolerance test 1 (baseline; 3 days pre-initiation of injections).

Baseline fasting OGTT in the HFD rats (n=12) was significantly higher than in the SC rats (n=12) (100.5mg/dl v 89.5mg/dl, p=0.001) (**Figure 150**).

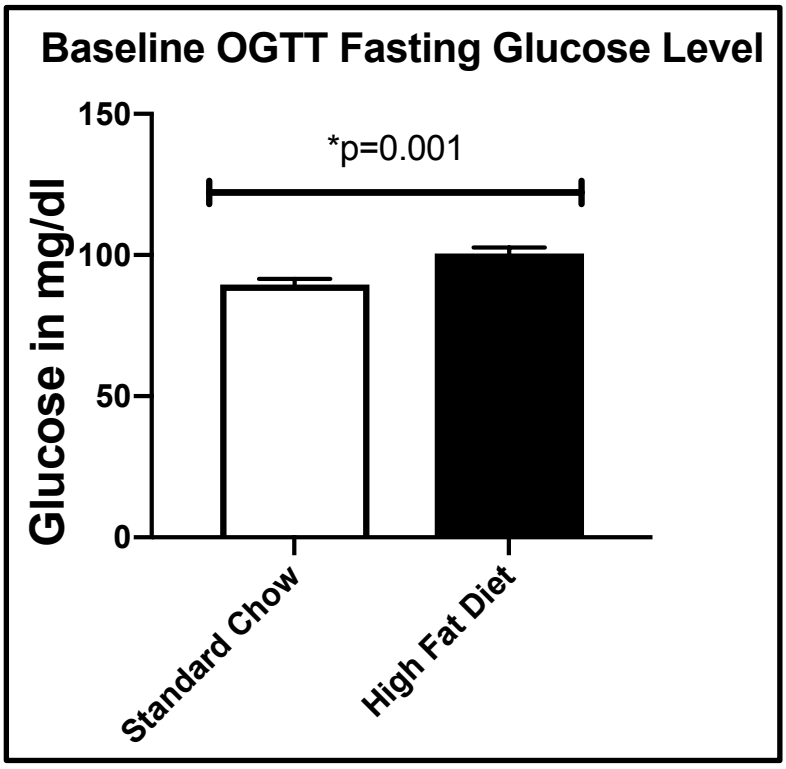



**Figure 150:** Baseline OGTT fasting glucose levels in standard versus high fat diet rats.

After 7 days of injections, a similar shaped curve was seen in the TED group to the one that was observed in the standard chow CASLO study. This time, however, the peak in blood glucose within the TED group was at the 15-minute time point, and this was significantly higher than the glucose value in the PBS group (161.6mg/dl v 133.1mg/dl, p=0.004). The 30-minute time point revealed a lower glucose level, although it was still significantly higher than the PBS group (155.2mg/dl v 144.50mg/dl, p=0.03). The familiar switch at 60 minutes, where blood glucose levels in the TED group were lower than in the PBS group (although not yet significantly), was also seen. However, both the 90- and 120-minute time points did hit significance (125mg/dl v 143.1mg/dl, p=0.002 at 60 minutes, and 106.4mg/dl v 119.33mg/dl, p=0.0002) (**Figure 151**).

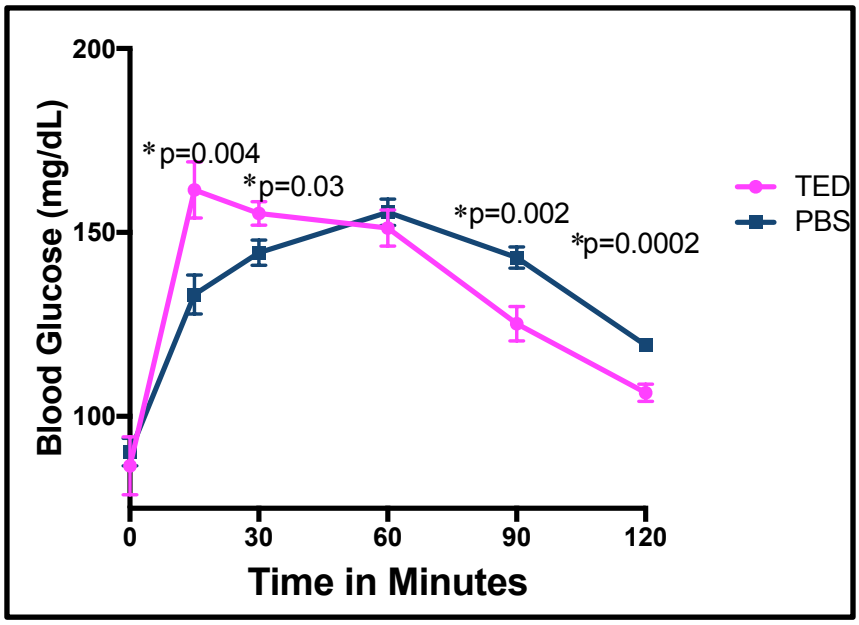



**Figure 151:** Oral glucose tolerance test 2 (7 days post-initiation of injections).

As with the baseline OGTT, HFD rats had a significantly higher mean blood glucose level at 0 minutes compared to the SC rats (88.6mg/dl v 78.3mg/dl, p=0.02) (**Figure 152**).

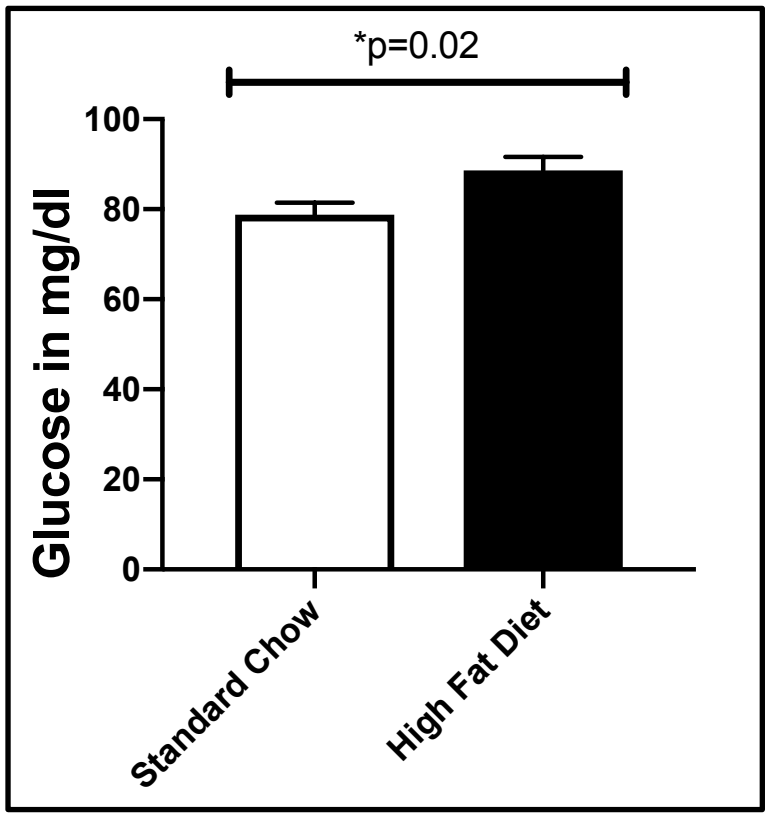



**Figure 152:** OGTT 2 fasting glucose levels in standard versus high fat diet rats.

The 14-day OGTT showed a similarly convincing result, with the 15-minute glucose level in the TED group significantly higher than the PBS group (155.5mg/dl v 130.8mg/dl, p=0.0003) and significantly higher at the 30-minute time point as well (158.5mg/dl v 140.3mg/dl, p=0.002). At the 60-minute time point glucose levels in the TED and PBS groups were fairly well matched, however, by 90 minutes, and through to 120 minutes, the TED group glucose levels were significantly lower, echoing what had been seen in the standard chow study (117.25 v 150.3, p=0.001 at 90 minutes, and 101.7mg/dl v 116.7mg/dl, p=0.002 at 120 minutes) (**Figure 153**).

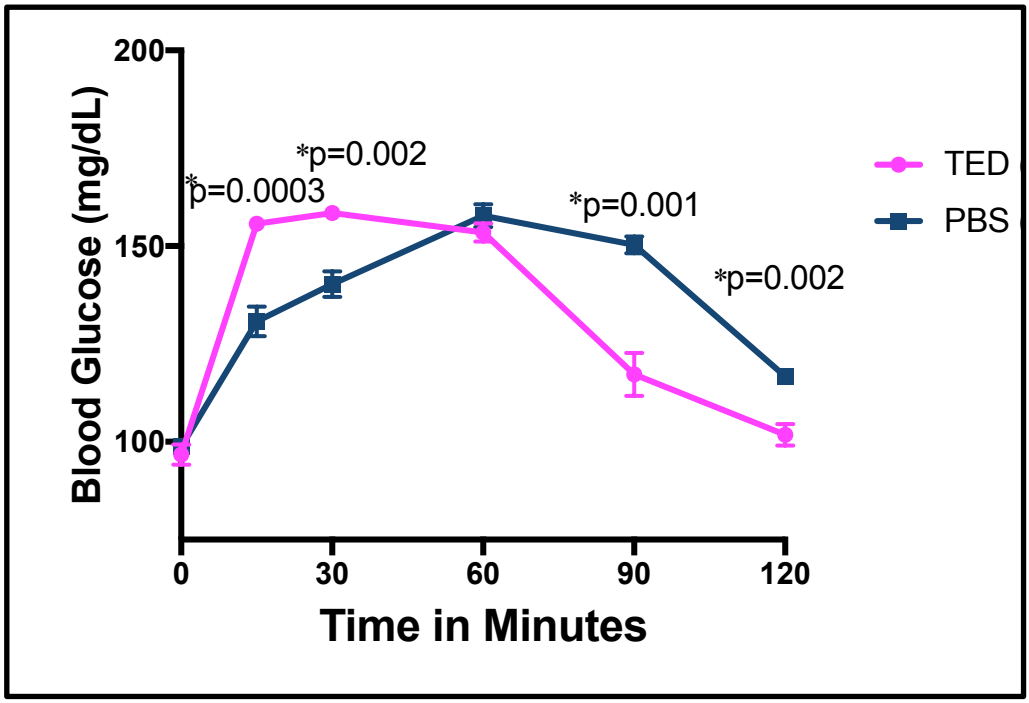



**Figure 153:** Oral glucose tolerance test 3 (14 days post-initiation of injections).

As with the baseline and 1-week OGTTs, HFD rats had a significantly higher mean blood glucose level at 0 minutes compared to the SC rats (97.8mg/dl v 73.7mg/dl, p=0.0000000001) (**Figure 154**). In line with the standard chow study, control group curves remained very similar in shape across baseline, 1- week and 2-week OGTTs.

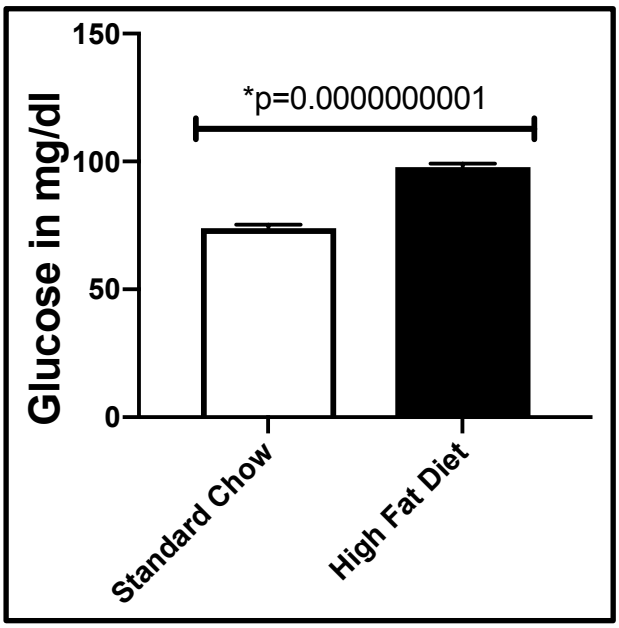



**Figure 154:** OGTT 3 fasting glucose levels in standard versus high fat diet rats.

Harvest blood glucose levels, following the usual intra-operative duodenal glucose infusion over the first 5 minutes, also showed some convincing trends. Both portal and systemic blood glucose levels followed the same trend; an increase in blood glucose up to a peak at 30 minutes, followed by a decrease down to the 60-minute time point. Portal blood glucose was found to be significantly higher in the TED group at 30 minutes (560.0mg/dl v 498.33mg/dl, p=0.02), and significantly lower than the PBS group at 60 minutes (482.0mg/dl v 540.8mg/dl, p=0.049) (**Figures 155 and 156**).

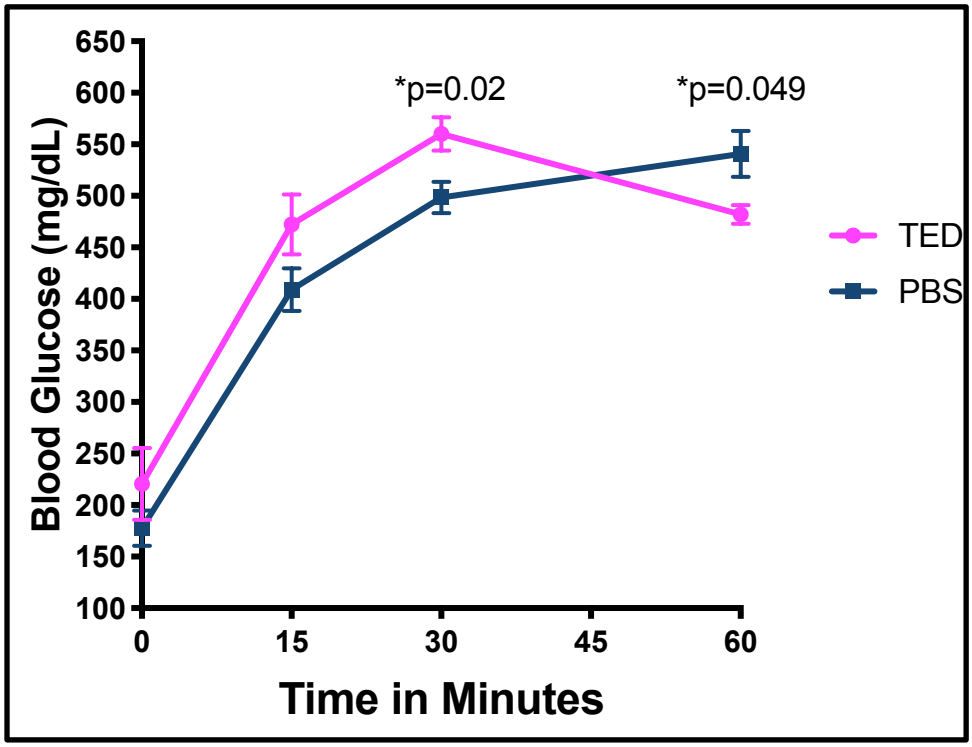



**Figure 155:** Mean portal glucose levels at harvest GTT (day 49).

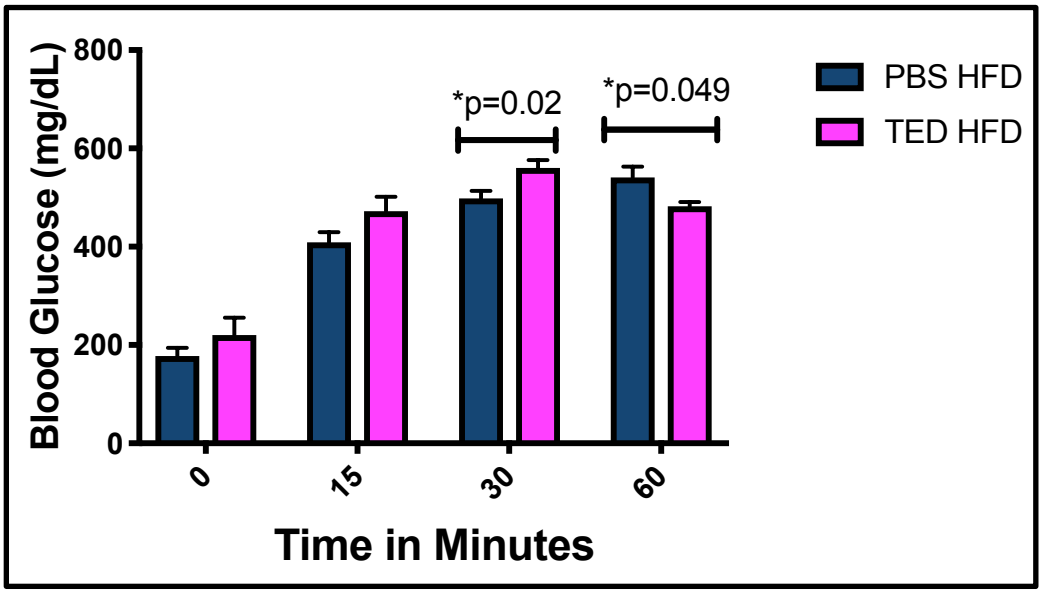



**Figure 156:** Mean portal glucose levels at harvest GTT (day 49), displayed in bar-chart format.

As with the baseline, 1- and 2-week OGTTs, HFD rats had a significantly higher mean blood glucose level at 0 minutes compared to the SC rats in both portal blood (197.0mg/dl v 134.3mg/dl, p=0.007) (**Figure 157**) and systemic blood (214.2mg/dl v 150.4mg/dl, p=0.01) (**Figure 158**).

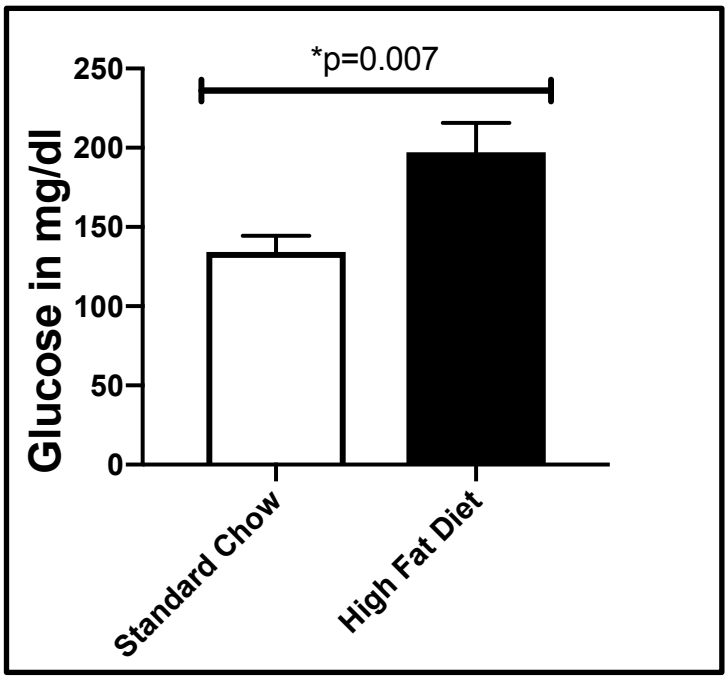



**Figure 157:** Harvest GTT fasting portal glucose levels in SC versus HFD rats.

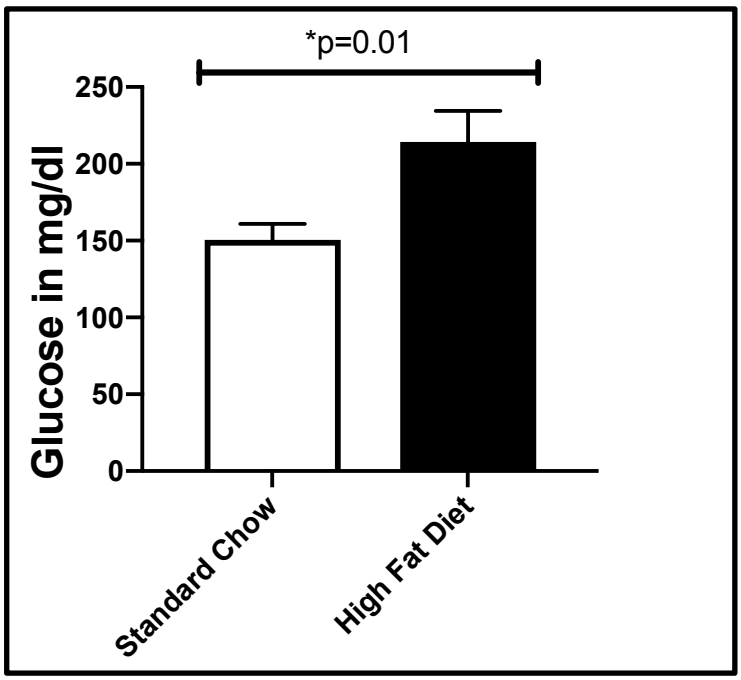



**Figure 158:** Harvest GTT fasting systemic glucose levels in SC versus HFD rats.

When 0-minute portal blood glucose levels in the TED HFD group are compared to those in the TED SC group, they can be seen to be higher, with this trending towards significance. However, by the end of the experiment (at 60 minutes), there was a noticeable switch, with TED HFD portal glucose levels becoming significantly lower than in the TED SC group (482.0mg/dl v 549.4mg/dl, p=0.01) (**Figure 159**).

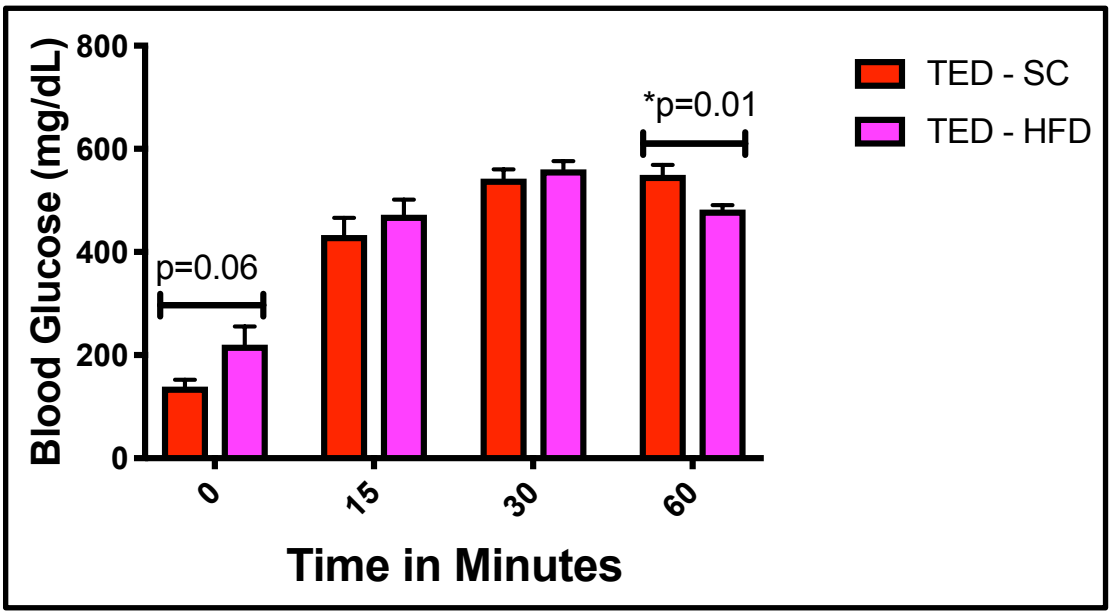



**Figure 159:** Harvest portal blood glucose levels in Teduglutide injected rats; standard chow (SC) versus high fat diet (HFD).

Systemic blood glucose in the TED group followed a similar trend to that of the PBS group up until 30 minutes. However, by the 60-minute time point, blood glucose levels in the TED group were significantly lower (346.0mg/dl v 469.67mg/dl, p=0.02) (**Figures 160 and 161**).

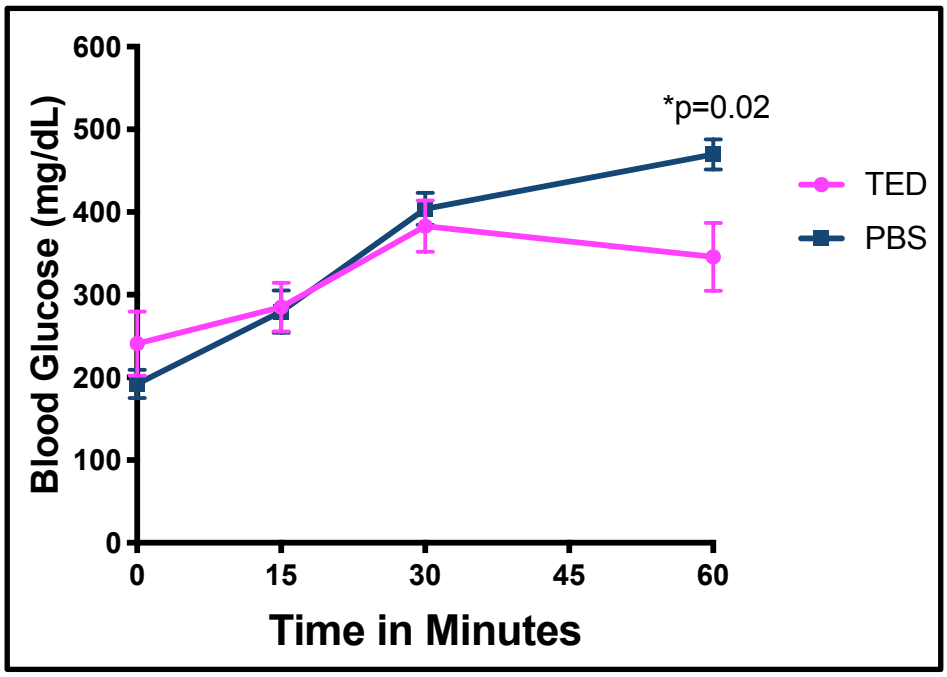



**Figure 160:** Mean systemic glucose levels at harvest GTT (day 49).

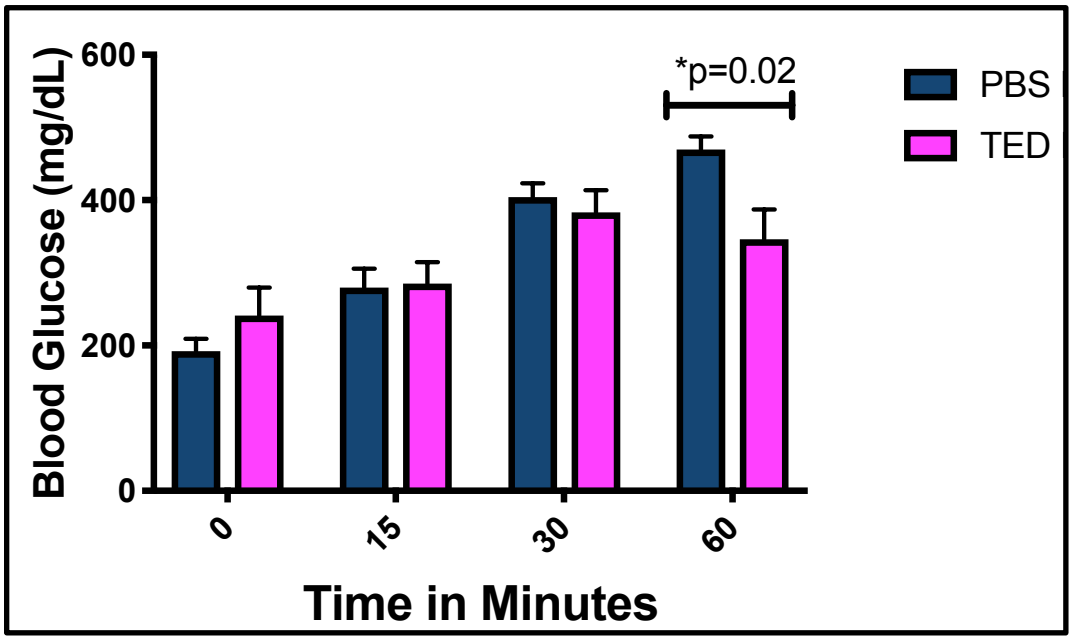



**Figure 161:** Mean systemic glucose levels at harvest GTT (day 49), displayed in bar-chart format.

**Figure 162** shows how systemic glucose levels differed between SC and HFD rats. At 0 minutes, glucose levels were trending towards being significantly higher in the TED HFD group (156.2mg/dl v 241.0mg/dl, p=0.07). However, by 30 minutes, systemic glucose levels were significantly lower in the TED HFD group compared to the TED SC group (383.0mg/dl v 460.8mg/dl, p=0.04). By 60 minutes, this switch was even more pronounced (346.0mg/dl v 492.4mg/dl, p=0.02).

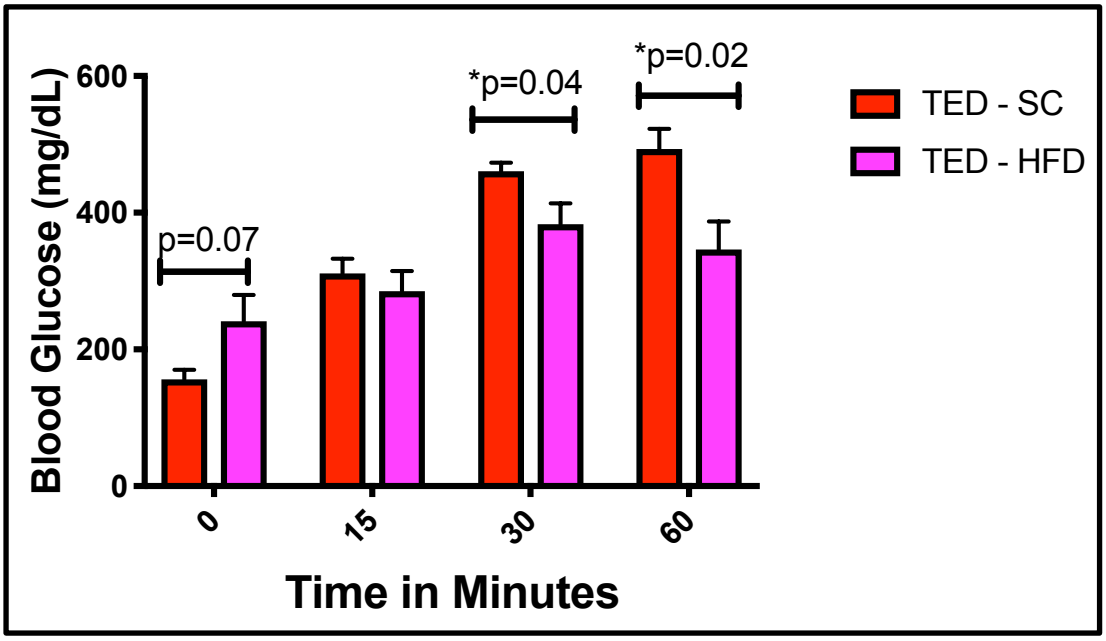



**Figure 162:** Harvest systemic blood glucose levels in Teduglutide injected rats; standard chow (SC) versus high fat diet (HFD).

Portosystemic gradient in the TED group became was shown to be more positive by the 15-minute time point (not significantly) but was found to be significantly more positive at the 30-minute time point (p=0.01). This trend continued until the 60-minute endpoint, however, the TED group $G_{PS}$ was no longer significantly more positive than the PBS group $G_{PS}$ at this point (p=0.1) (**Figures 163 and 164**).

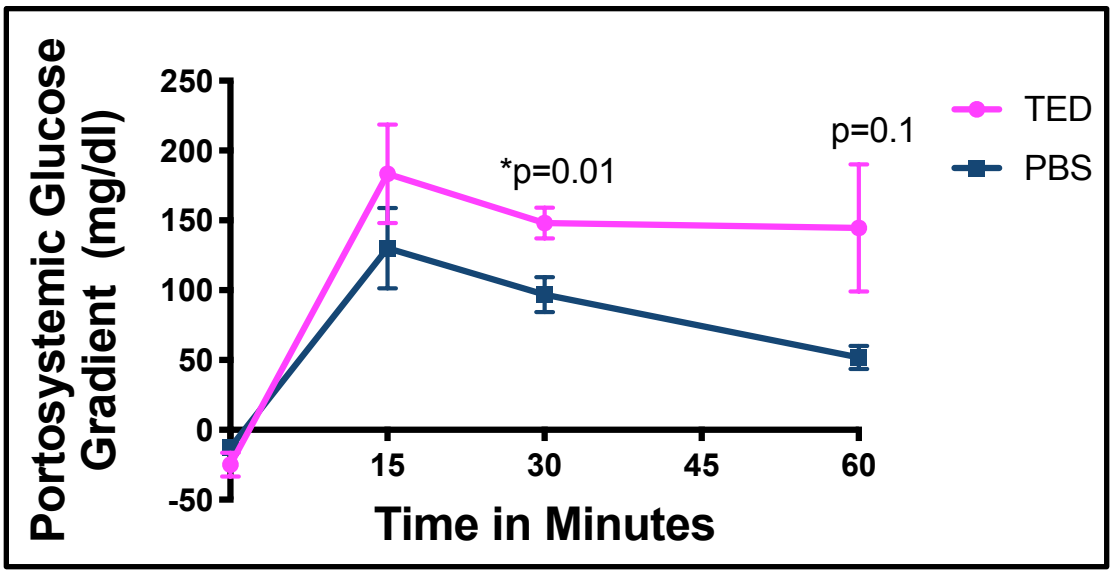



**Figure 163:** Mean porto-systemic glucose gradient at harvest GTT (day 49).

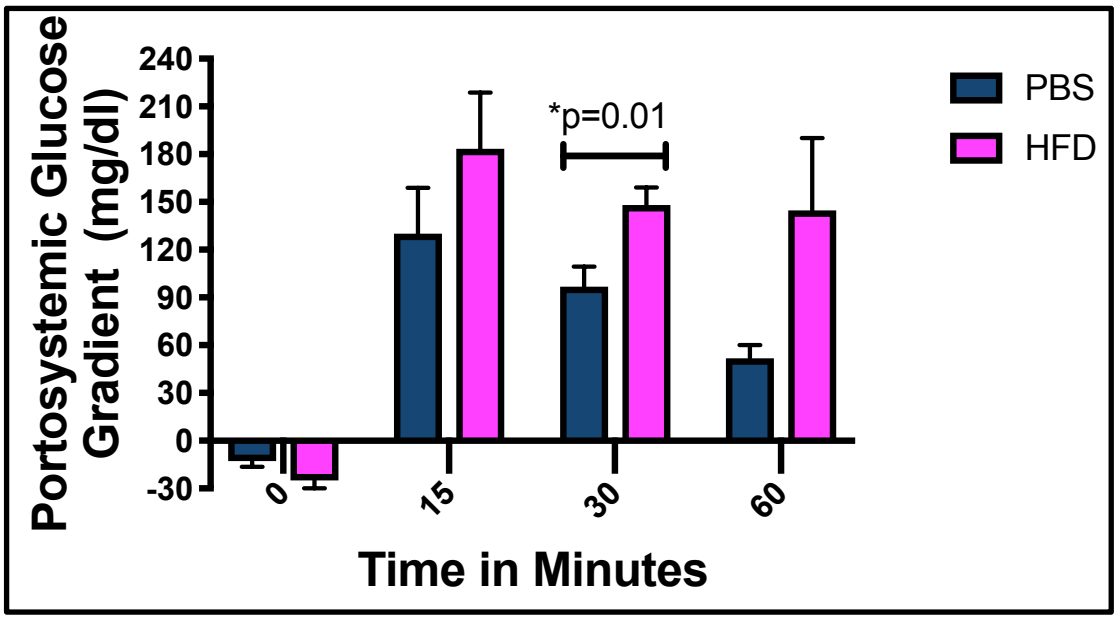



**Figure 164:** Mean porto-systemic glucose gradient at harvest GTT (day 49), displayed in bar-chart format.

When compared to the TED SC group, TED HFD $G_{PS}$ started as more negative than the TED SC, but by 30 minutes had become significantly more positive than the TED SC group (148.0mg/dl v 81.4mg/dl, p=0.02) (**Figure 165**).

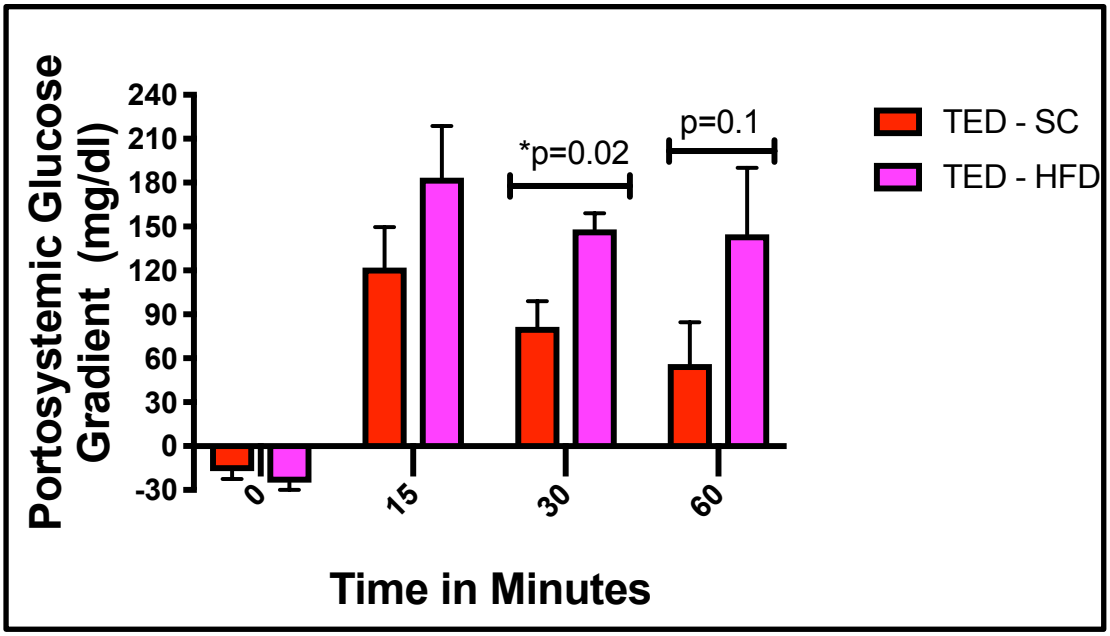



**Figure 165:** Harvest porto-systemic glucose gradient in Teduglutide injected rats; standard chow (SC) versus high fat diet (HFD).

As with the standard chow studies, there was no significant difference in glucose utilisation, at baseline, or glucose absorption over the duration of the experiment, between the two HFD groups (**Figures 166 and 167**).

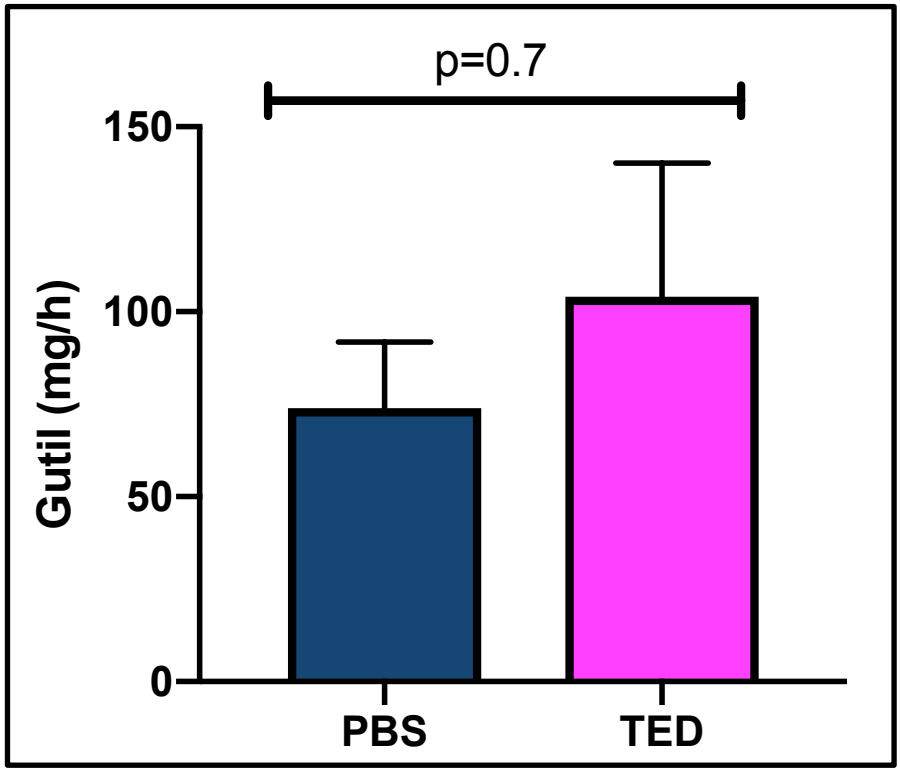



**Figure 166:** Intestinal glucose utilisation.

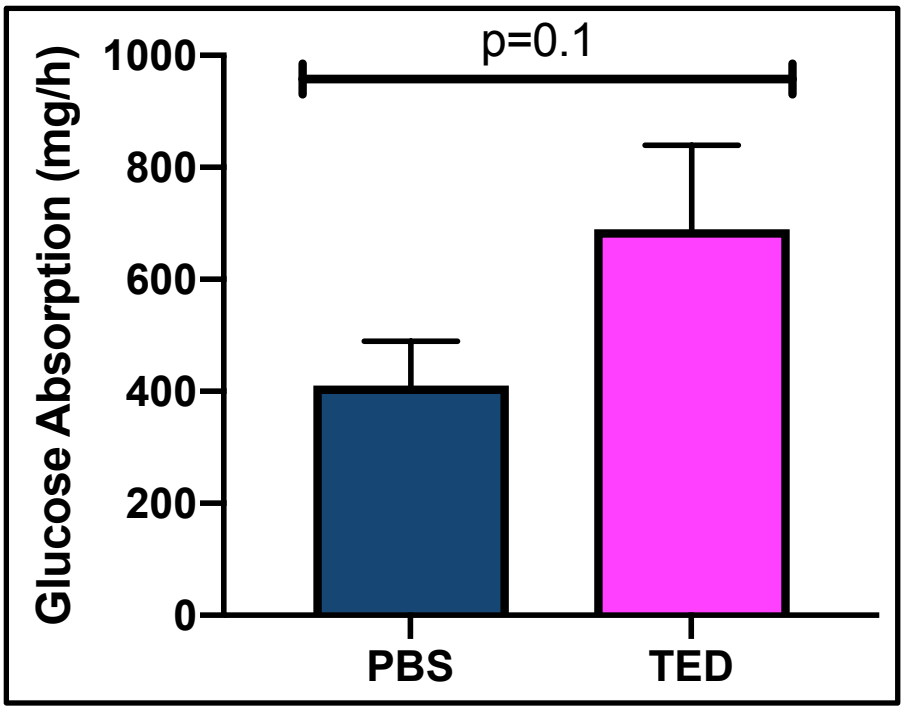



**Figure 167:** Intestinal glucose absorption.

Total body weight remained matched between the two groups throughout the study (**Figure 168**).

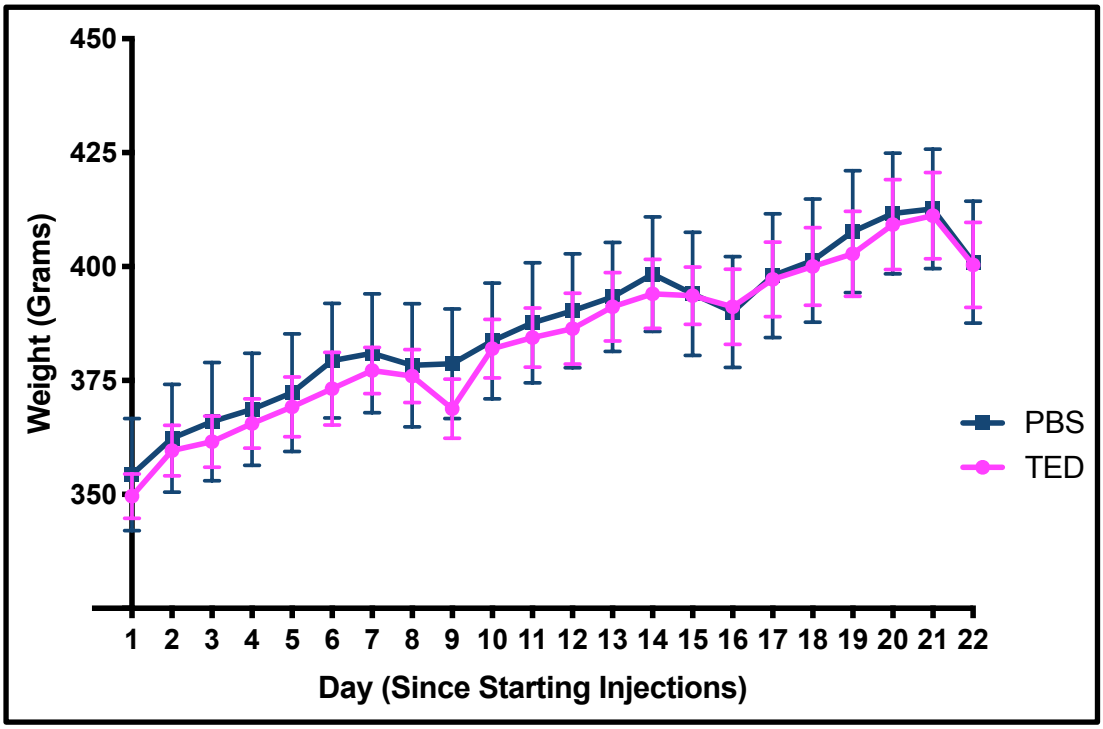



**Figure 168:** Mean total bodyweights throughout study.

**Figure 169** shows how weights, and rate of weight increase, compared between the SC and HFD rats.

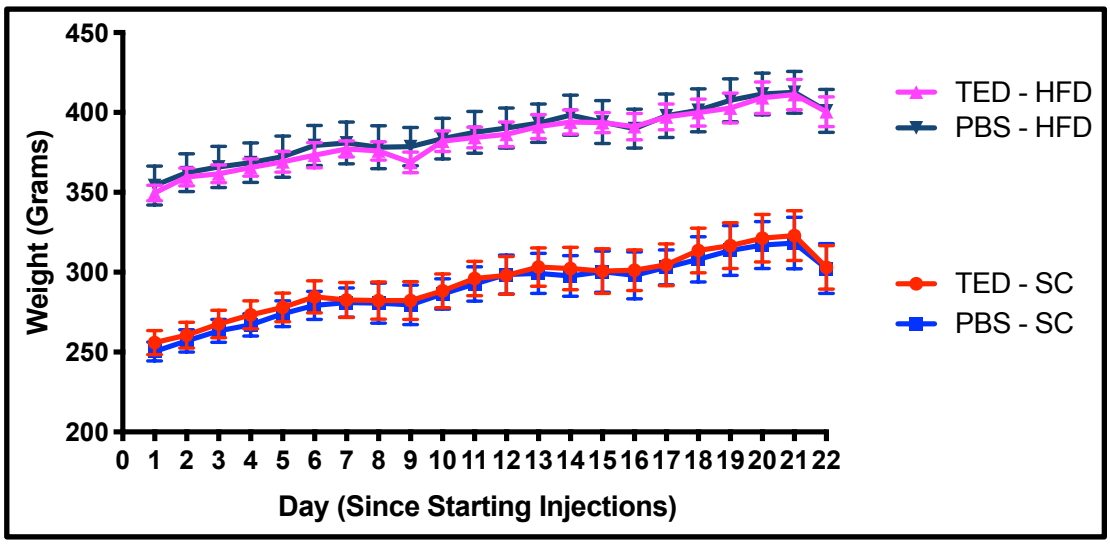



**Figure 169:** Comparison of mean total bodyweights throughout study in standard chow (SC) versus high fat diet (HFD) groups.

There was no difference between weight of the standard chow consumed between the two groups in the HFD study (**Figure 170**).

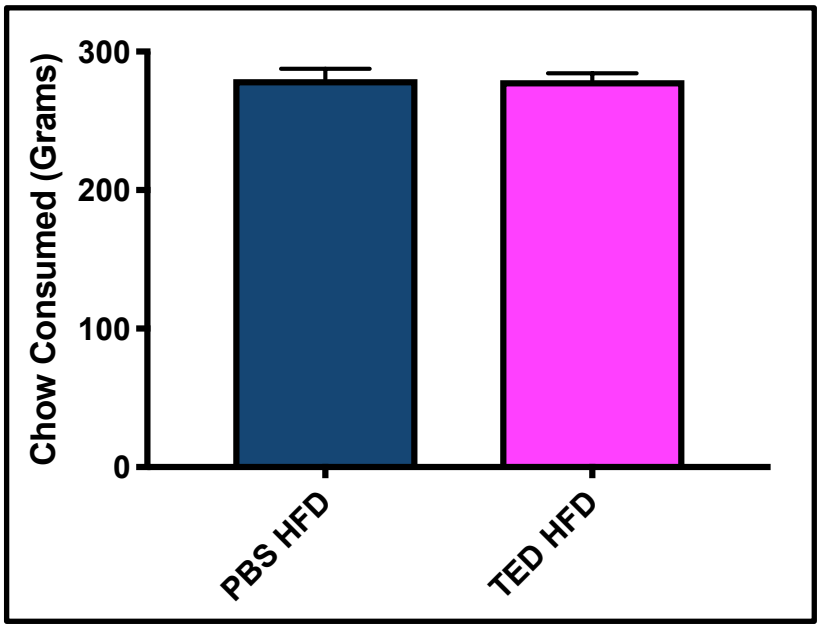



**Figure 170:** Mean total chow consumed.

There was a significant difference in amount of food consumed between the SC and HFD groups (**Figure 171**), however, this is most likely explained by the energy-density of the different types of chow. The HFD chow is more energy-dense and, thus, less needs to be consumed to gain the same number of calories compared to the standard chow.

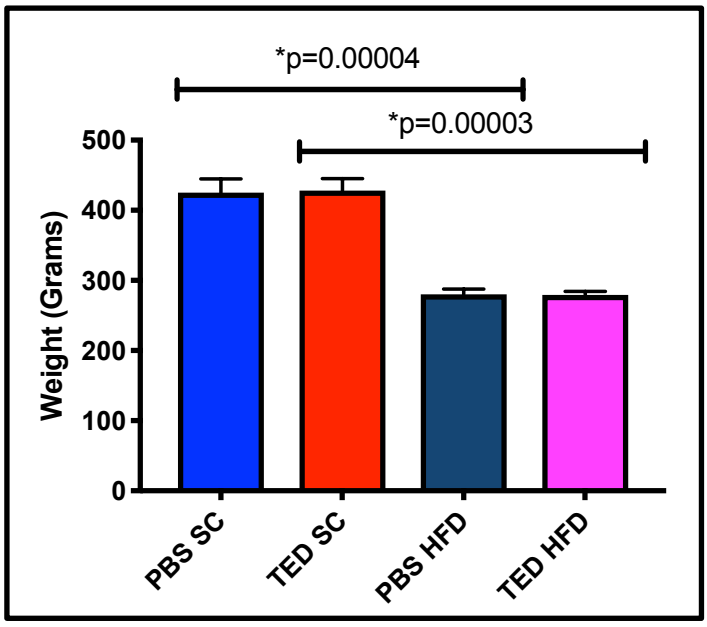



**Figure 171:** Comparison of mean total chow consumed between standard chow (SC) and high fat diet (HFD) groups.

The combined weight of jejunum and ileum was significantly higher (p=0.002) in the TED group (6.36g) compared to the PBS group (4.83g) (**Figure 172**).

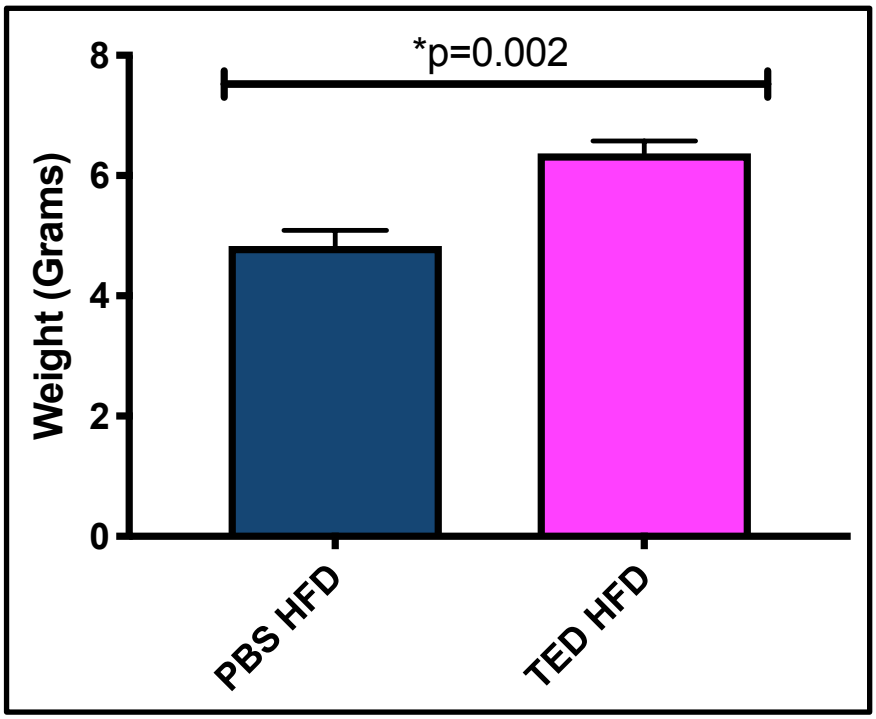



**Figure 172:** Mean combined jejunal and ileal

When weight-adjusted the TED group still had a significantly increased combined jejunal and ileal weight (p=0.003) (**Figure 173**).

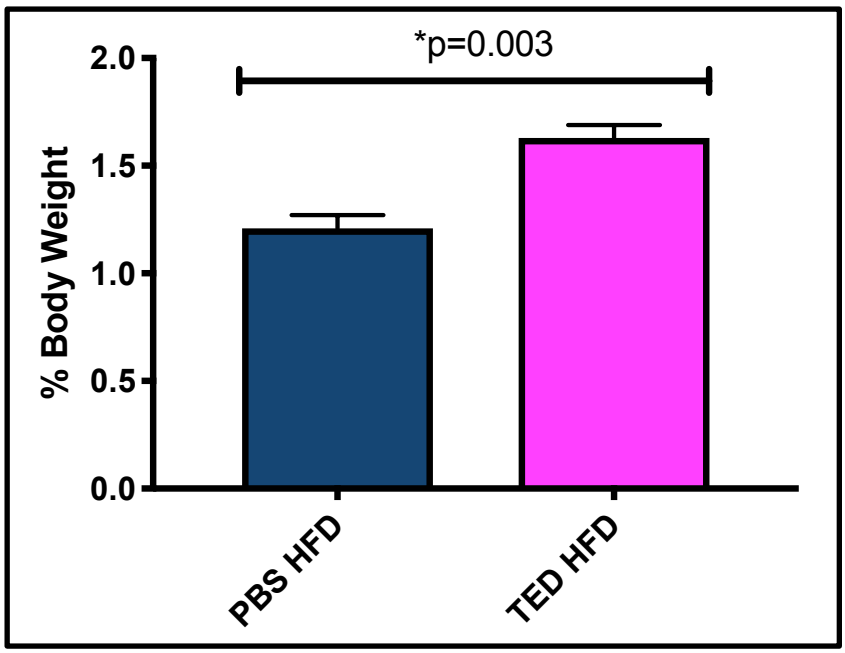



**Figure 173:** Mean combined jejunal and ileal weight, expressed as % bodyweight.

The first 5cm of jejunum was harvested. The TED group were shown to have a significantly heavier first 5cm of jejunum compared to the PBS group (0.37g v 0.30g, p=0.004) (**Figure 174**).

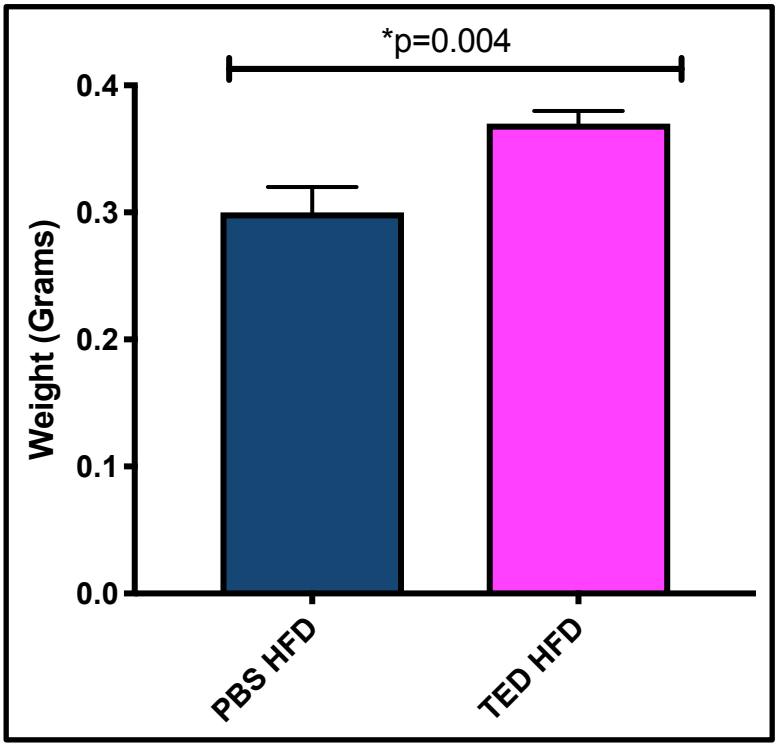



**Figure 174:** Mean 5cm jejunal weight.

Again, when weight-adjusted, the TED group still had a significantly heavier first 5cm of jejunum (p=0.006) (**Figure 175**).

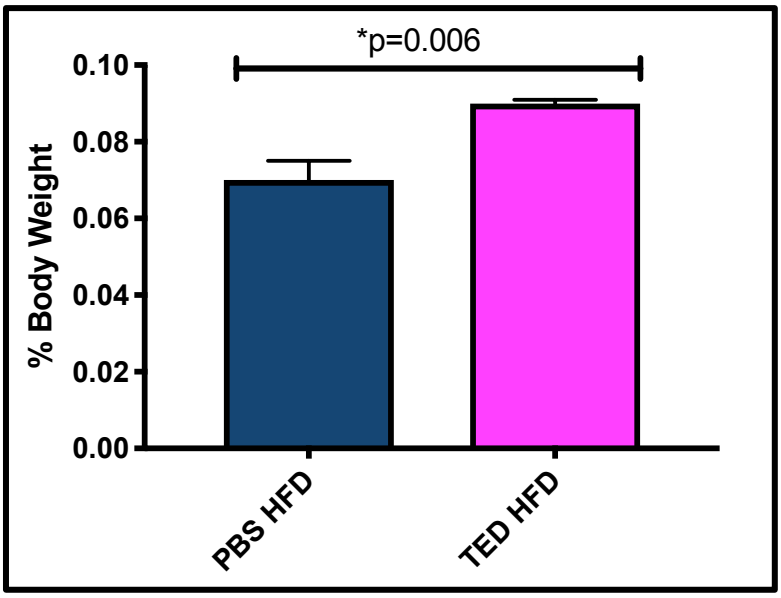



**Figure 175:** Mean 5cm jejunal weight, expressed as % bodyweight.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic GIP hormone analysis, via Luminex. At baseline, GIP levels were not significantly higher or lower in the TED group compared to the PBS group (**Figure 176)**.

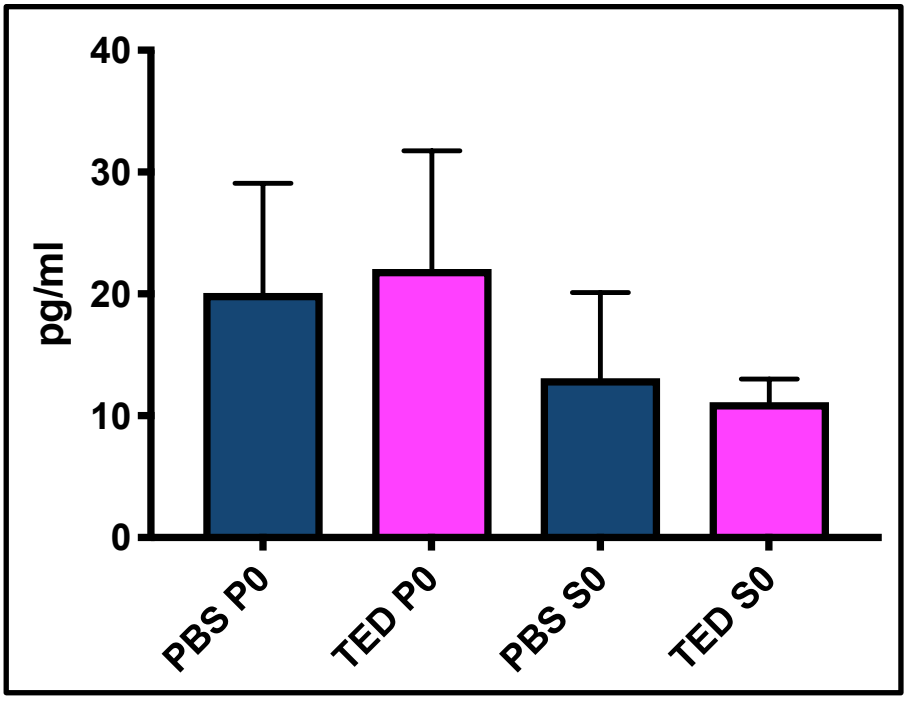



**Figure 176:** GIP levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

This shifted after the duodenal glucose infusion (given over the first 5 minutes of the experiment), so that at 30 minutes, portal and systemic glucose levels in the TED group became significantly higher than portal and systemic glucose levels in the PBS group (portal: 93.6pg/ml v 15.6pg/ml, p=0.003, and systemic: 70.1pg/ml v 9.8pg/ml, p=0.02) (**Figure 177**).

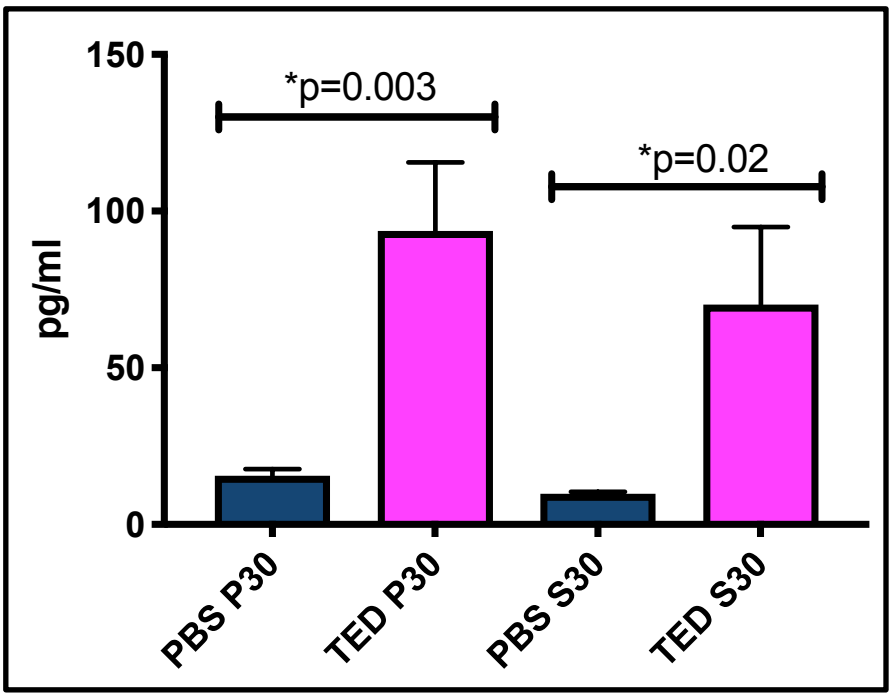



**Figure 177:** GIP levels at 30 minutes, in portal (P) and systolic (S) blood.

This pattern remained the same at the 60-minute time point; both portal and systemic blood glucose levels in the TED group were significantly higher than in the PBS group (portal:78.1pg/ml v 19.6pg/ml, p=0.0008 and systemic: 50.5pg/ml v 15.2pg/ml, p=0.01) (**Figure 178**).

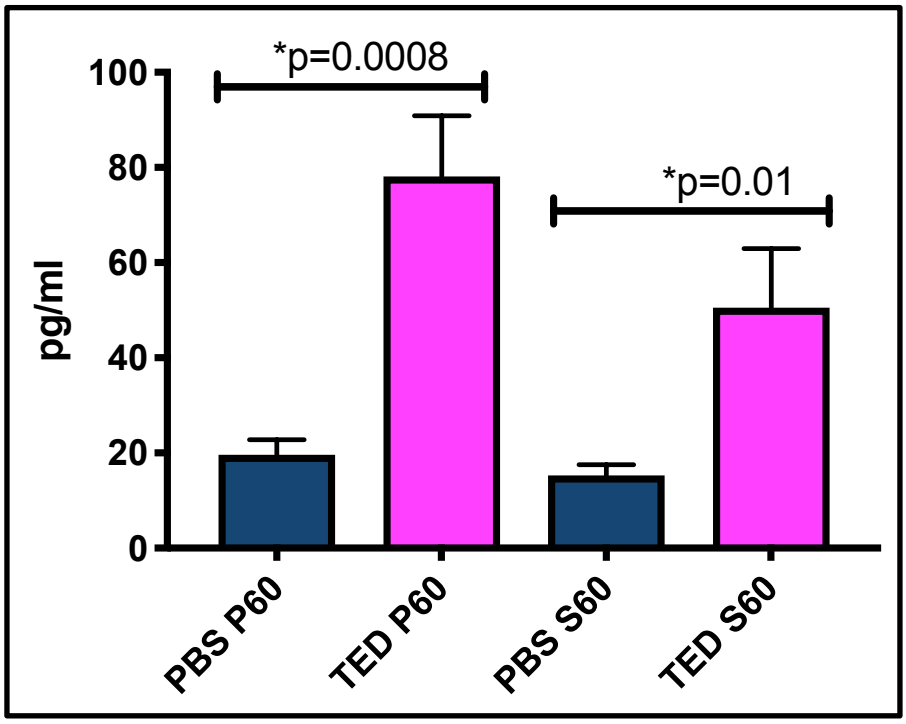



**Figure 178:** GIP levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic GLP-1 hormone analysis, via Luminex. There was no significant difference in GLP-1 levels between groups, portally or systemically, at baseline (**Figure 179**).

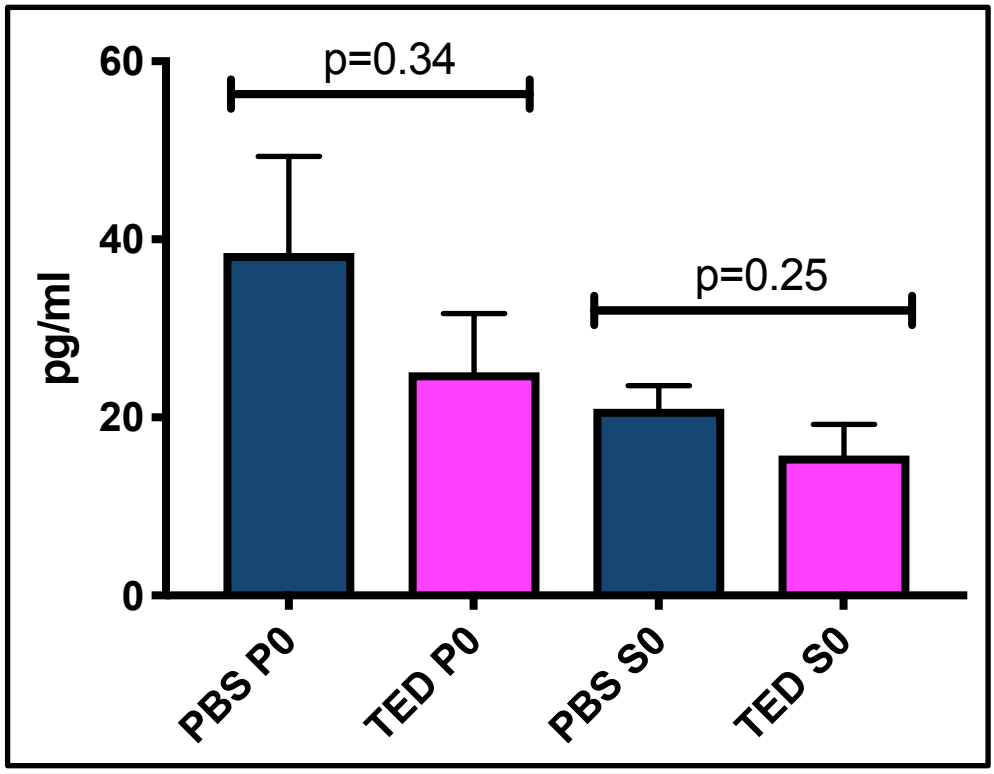



**Figure 179:** GLP-1 levels at baseline (0 minutes), in portal (P) and systolic

At 30 minutes and thus following the duodenal glucose infusion, GLP-1 levels became significantly increased portally within the TED group compared to the PBS group (338.7pg/ml v 165.1pg/ml, p=0.01) (**Figure 180**).

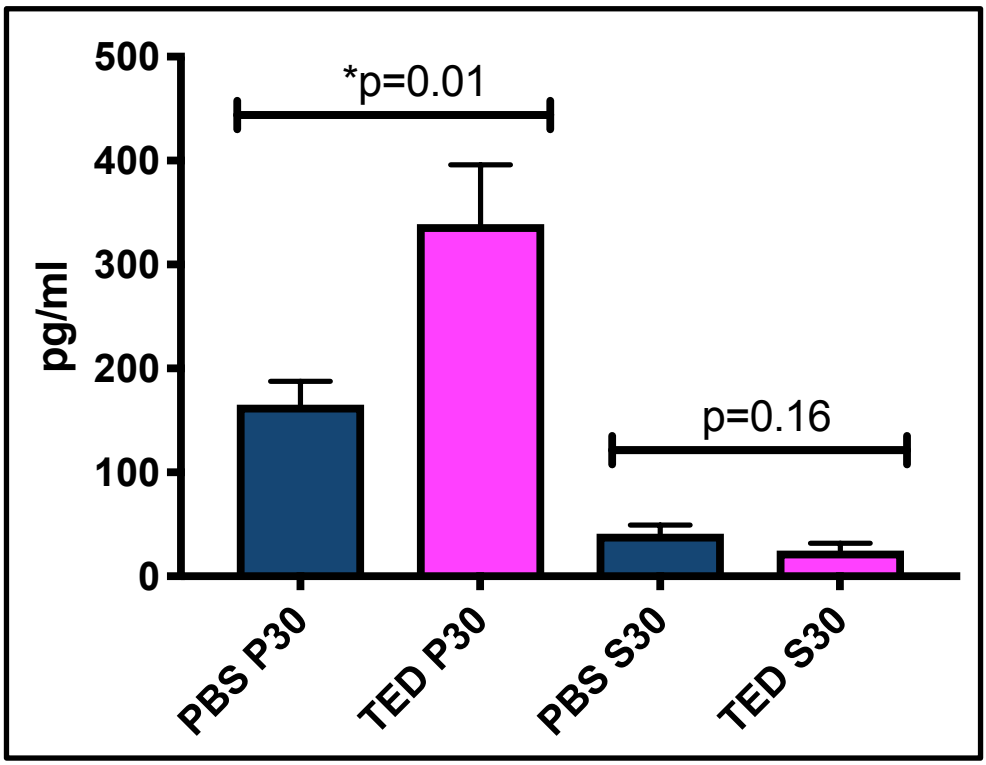



**Figure 180:** GLP-1 levels at 30 minutes, in portal (P) and systolic (S) blood.

This pattern was repeated at 60 minutes, with portal GLP-1 levels in the TED group remaining significantly higher compared to the PBS group (972.4pg/ml v 472.9, p=0.001) (**Figure 181**).

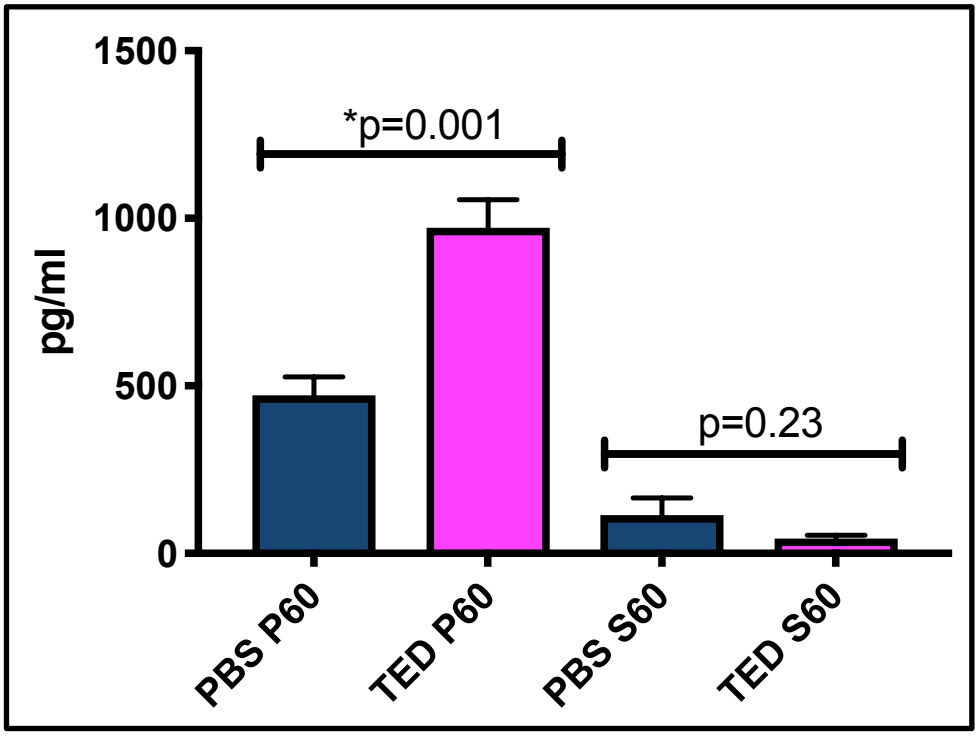



**Figure 181:** GLP-1 levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic glucagon hormone analysis, via Luminex. At baseline, glucagon levels within TED portal blood were already significantly higher compared to PBS portal blood (108.3pg/ml v 49.5pg/ml, p=0.02) (**Figure 182**).

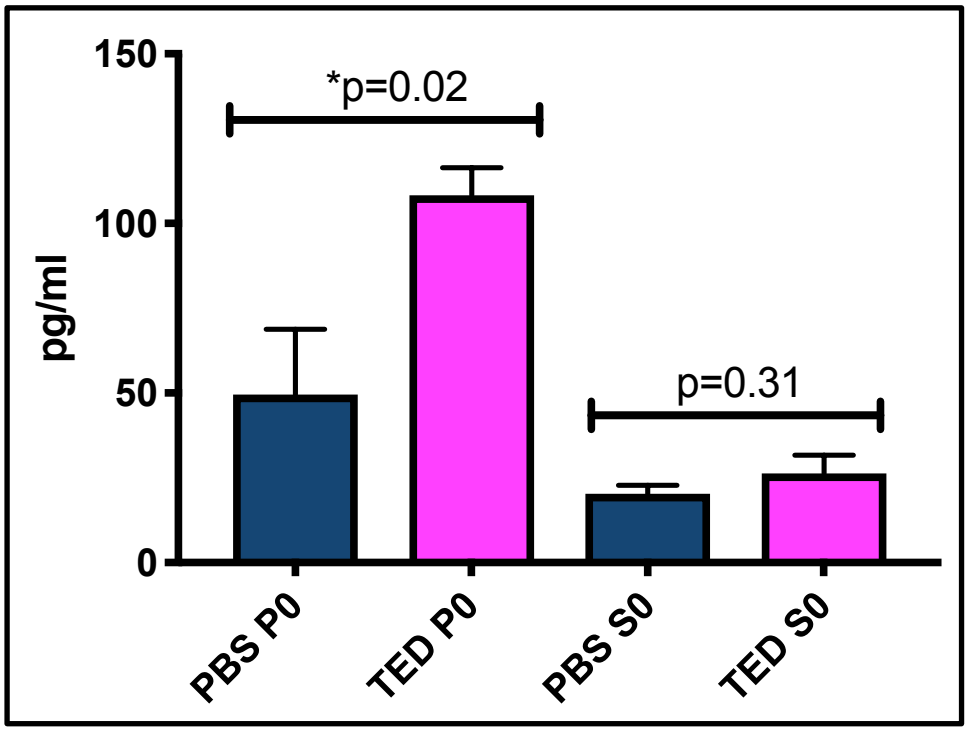



**Figure 182:** Glucagon levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

At 30 minutes, TED systemic blood had significantly higher levels of glucagon compared to PBS (58.9pg/ml v 41.0pg/ml, p=0.03) (**Figure 183**).

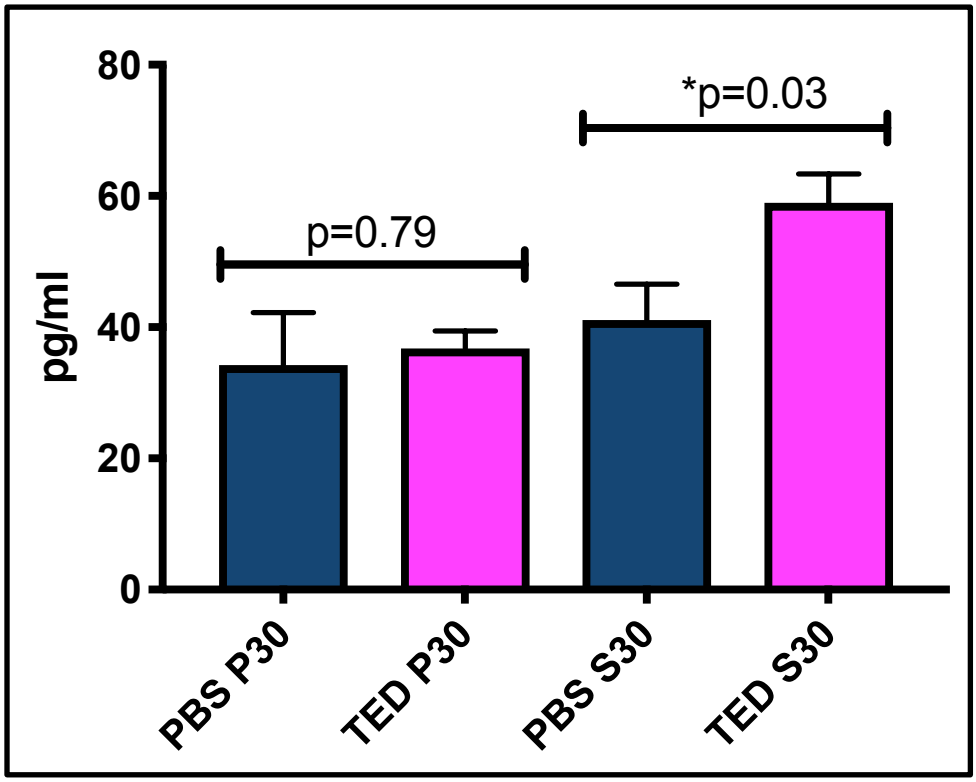



**Figure 183:** Glucagon levels at 30 minutes, in portal (P) and systolic (S) blood.

Finally, at 60 minutes, both portal and systemic TED blood contained higher levels of glucagon than the PBS portal and systemic blood (portal: 287.0pg/ml v 109.5pg/ml, p=0.005, and systemic: 82.4pg/ml v 56.3pg/ml, p=0.01) (**Figure 184**).

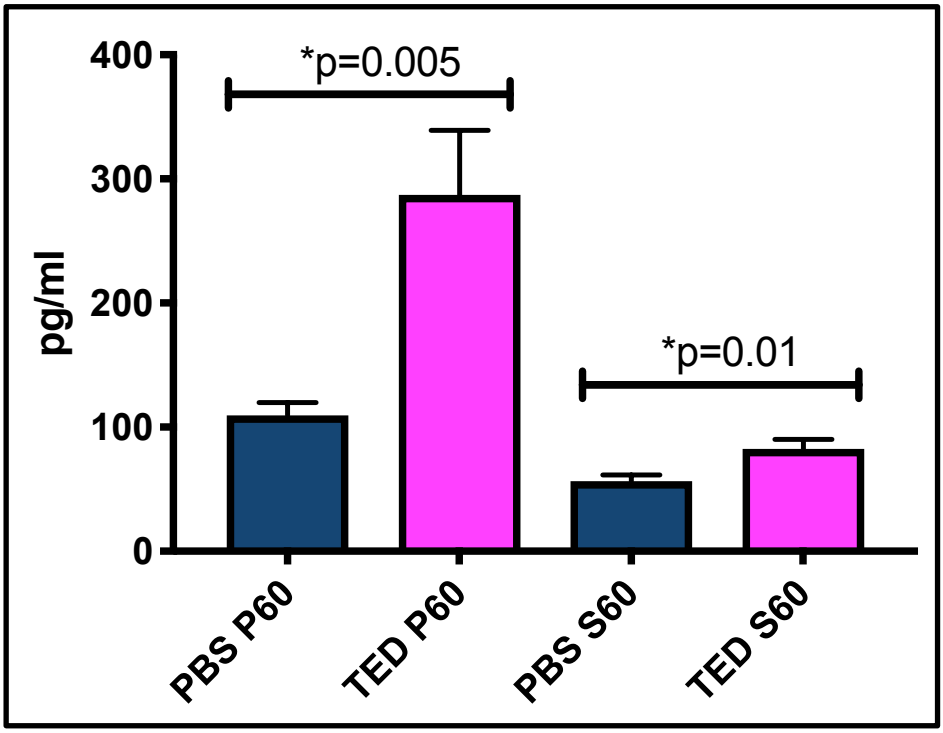



**Figure 184:** Glucagon levels at 60 minutes, in portal (P) and systolic (S) blood.

During the harvest procedure, blood was taken at baseline, 30 and 60 minutes for portal and systemic insulin hormone analysis, via Luminex. At baseline, insulin levels within TED portal and systemic blood were already significantly higher compared to PBS portal blood (portal: 808.4pg/ml v 108.5pg/ml, p=0.0009, and systemic: 266.6pg/ml v 65.9pg/ml, p=0.01) (**Figure 185**).

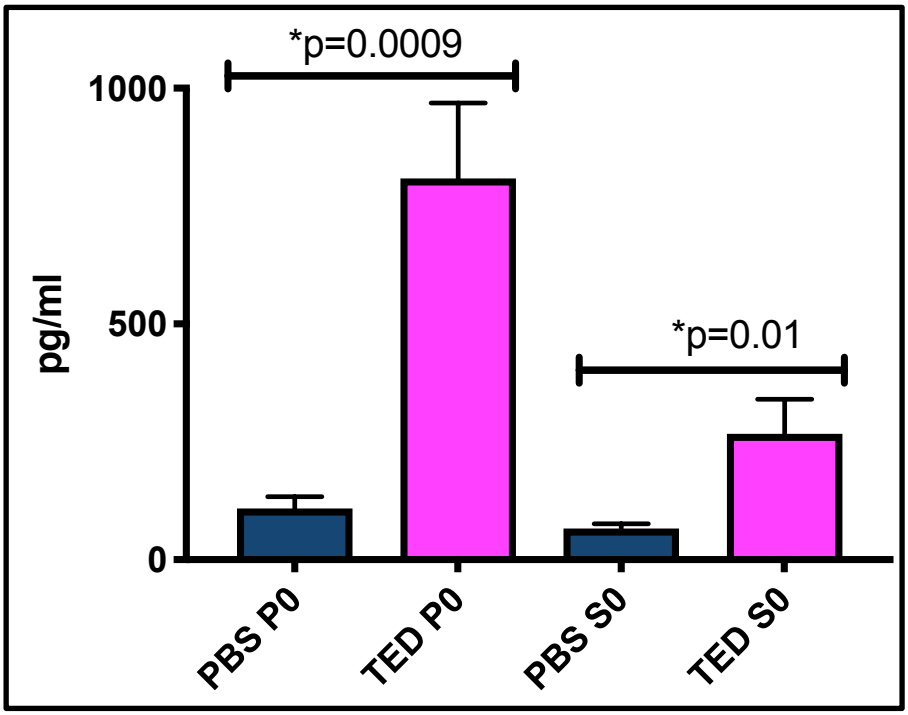



**Figure 185:** Insulin levels at baseline (0 minutes), in portal (P) and systolic (S) blood.

Insulin levels were also significantly higher in TED portal blood at the 30 minutes compared to PBS (1409.9pg/ml v 699.3, p=0.002), but not in TED systemic blood compared to PBS (**Figure 186**).

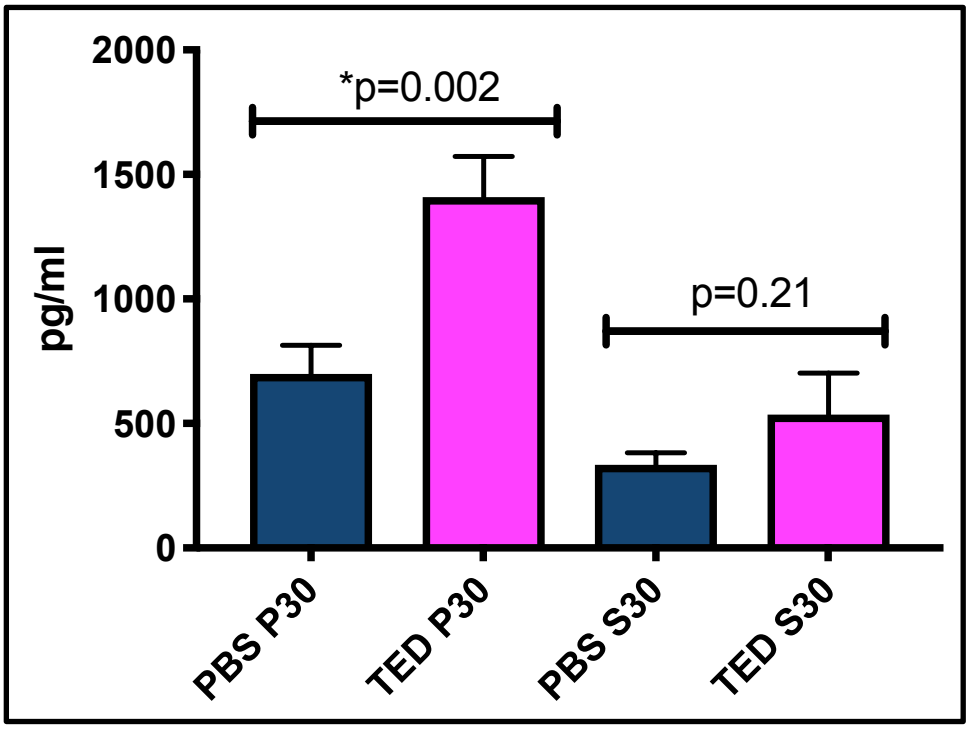



**Figure 186:** Insulin levels at 30 minutes, in portal (P) and systolic (S) blood.

There were no significant differences between the two groups at 60 minutes (**Figure 187**).

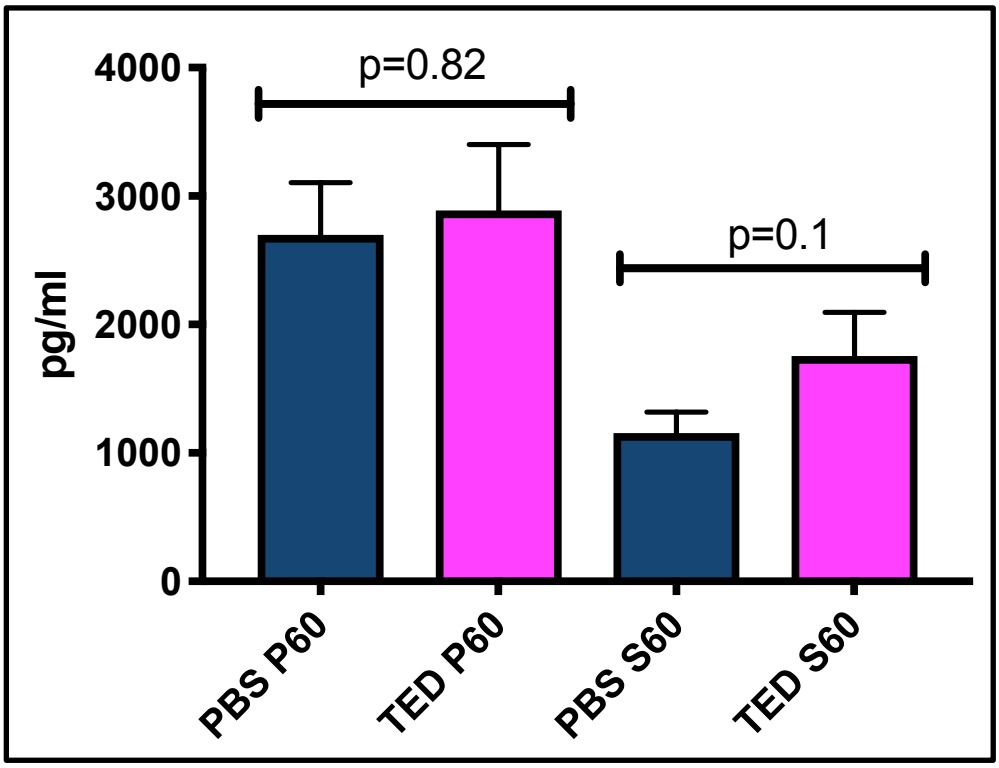



**Figure 187:** Insulin levels at 60 minutes, in portal (P) and systolic (S) blood.

Real-Time PCR was carried out on jejunal mucosa to quantify the following the following genes: SGLT-1, SGLT-3, GLUT-1, GLUT-2, GLP-2R, IGF-1 and T1R1.

Jejunal SGLT-1 expression was found to be significantly higher in the TED group, with a 7.4-fold change (p=0.0002) (**Figure 188**). **Figure 189** provides a direct comparison between SGLT-1 expression in the HFD group versus the SC group.

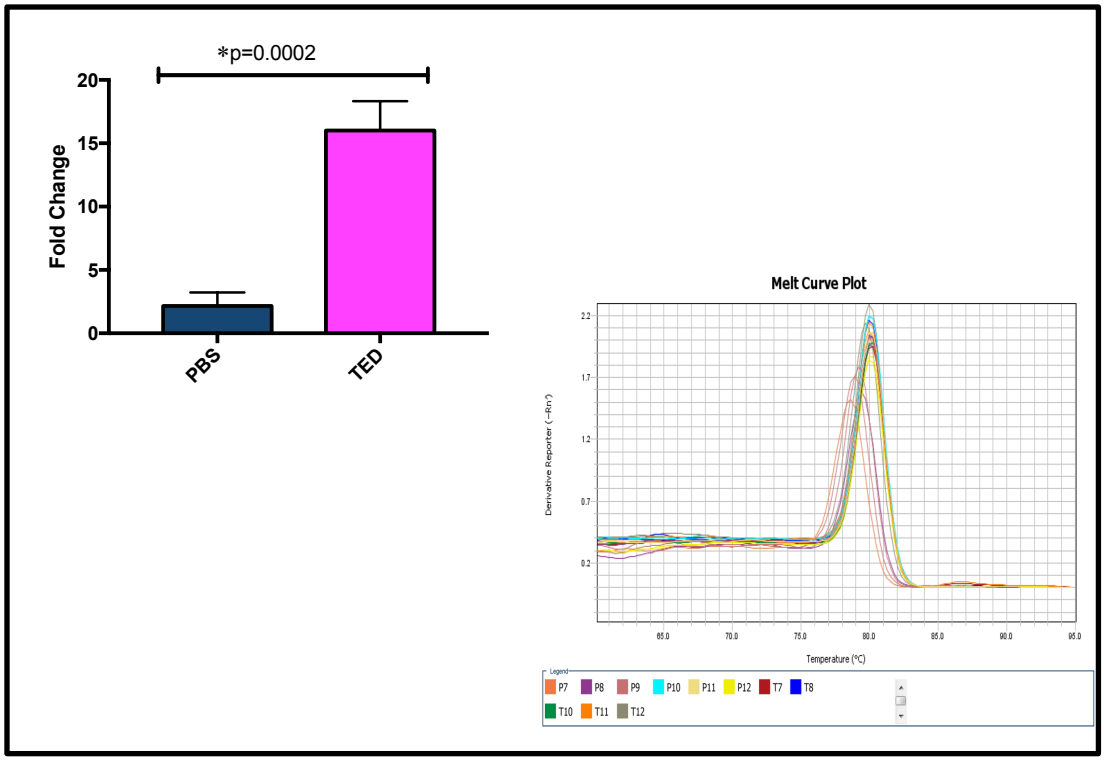



**Figure 188:** Jejunal SGLT-1 expression.

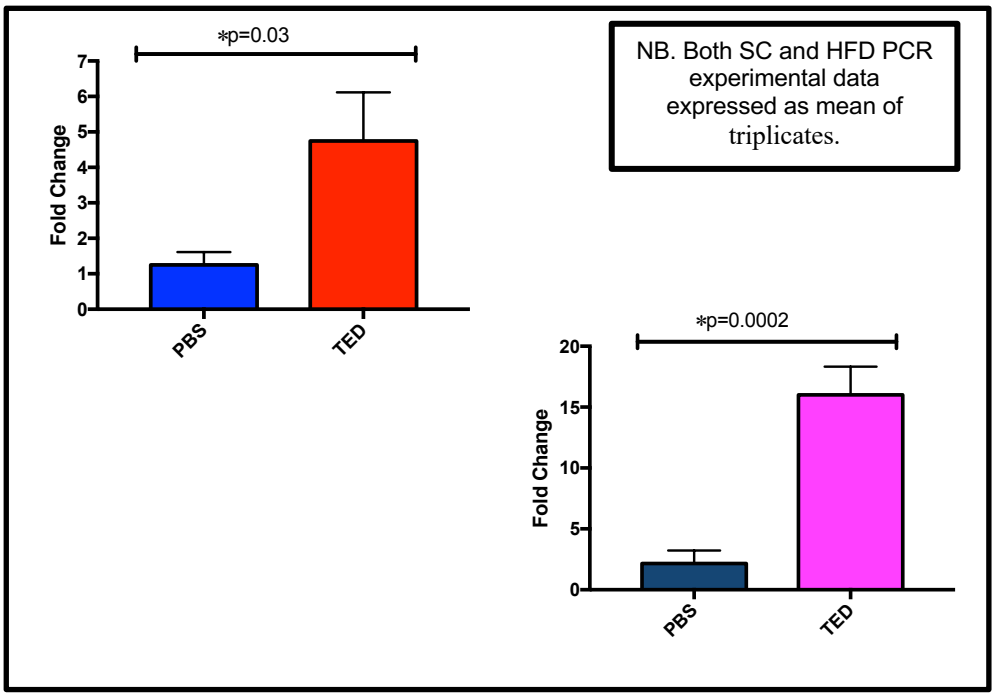



**Figure 189:** Comparison of jejunal SGLT-1 expression between SC and HFD groups.

Jejunal SGLT-3 expression was also found to be significantly higher in the TED group compared to the PBS group, with a 26.2-fold change (p=0.03) (**Figure 190**). **Figure 191** provides a direct comparison between SGLT-3 expression in the HFD group versus the SC group.

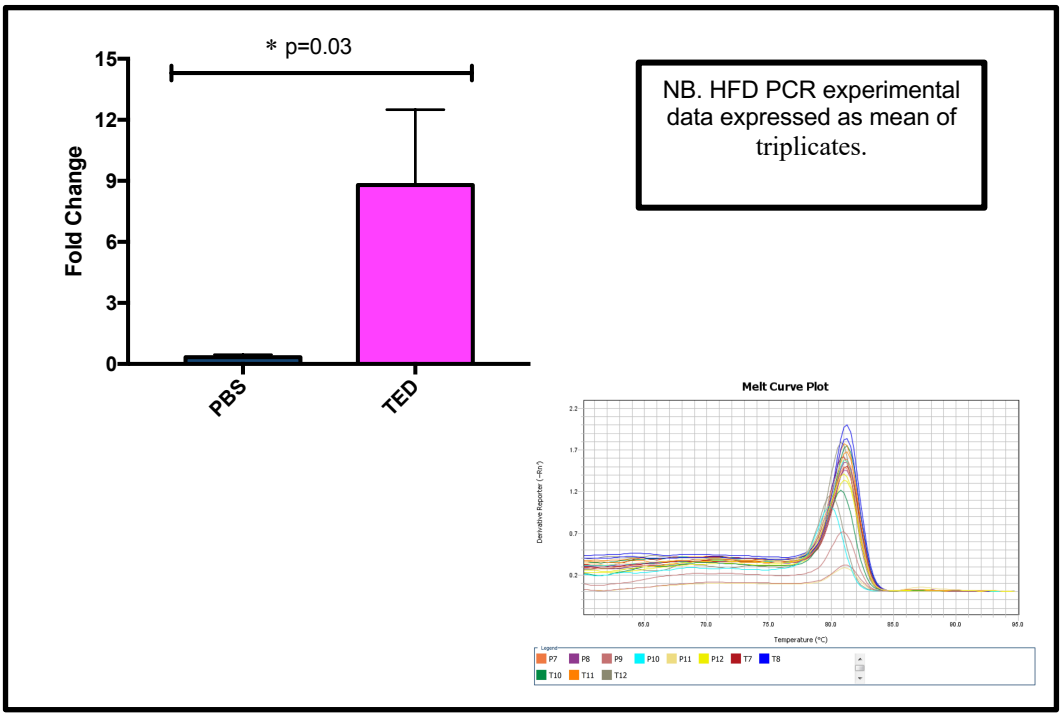



**Figure 190:** Jejunal SGLT-3 expression

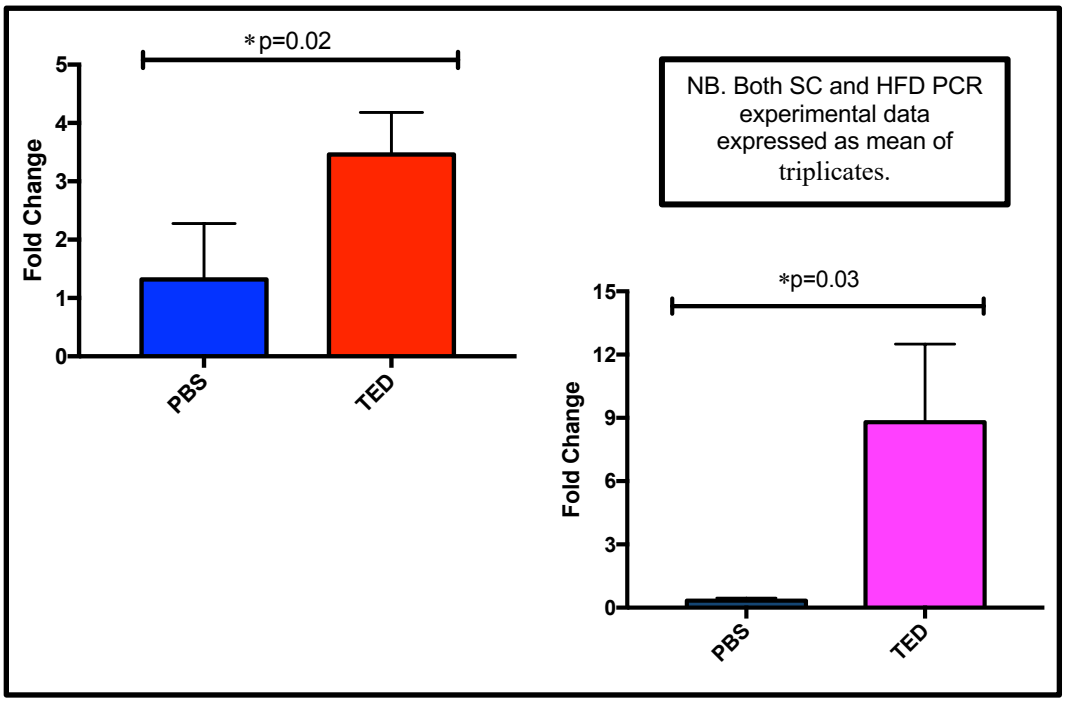



**Figure 191:** Comparison of jejunal SGLT-3 expression between SC and HFD groups.

There were also significantly higher levels of the glucose transporter genes, GLUT-1 and GLUT-2 within the TED group compared to the PBS group. There was a 5.8-fold change of GLUT-1 expression (p=0.02) (**Figure 192**) and a 17.2-fold change of GLUT-2 expression (p=0.03) (**Figure 194**). **Figures 193 and 195** provide direct comparison of SC and HFD group gene expression in GLUT-1 and GLUT-2, respectively.

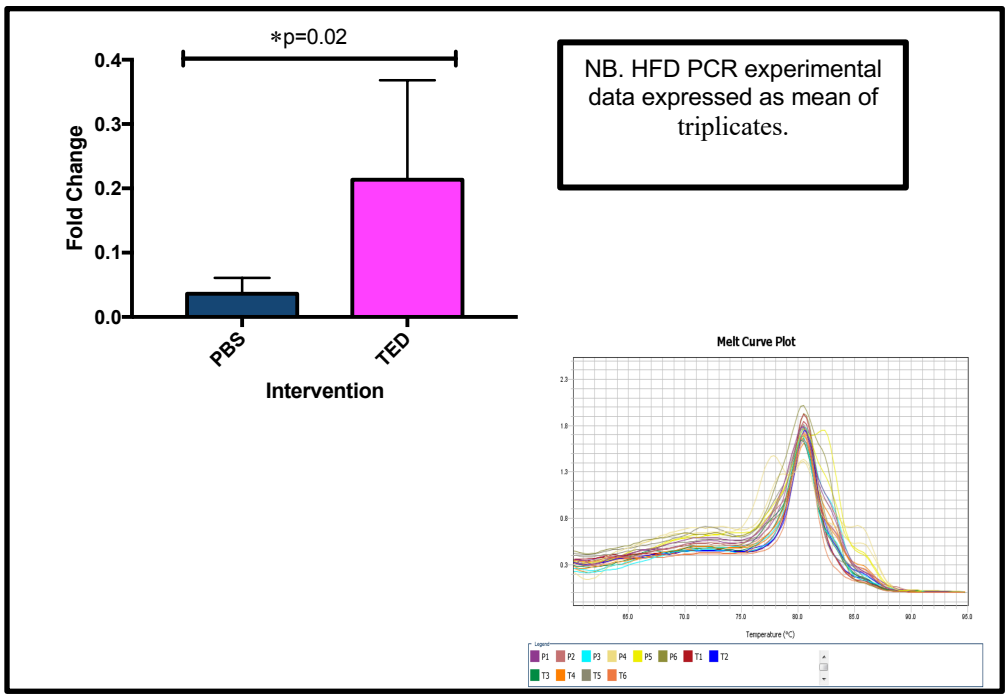



**Figure 192:** Jejunal GLUT-1 expression.

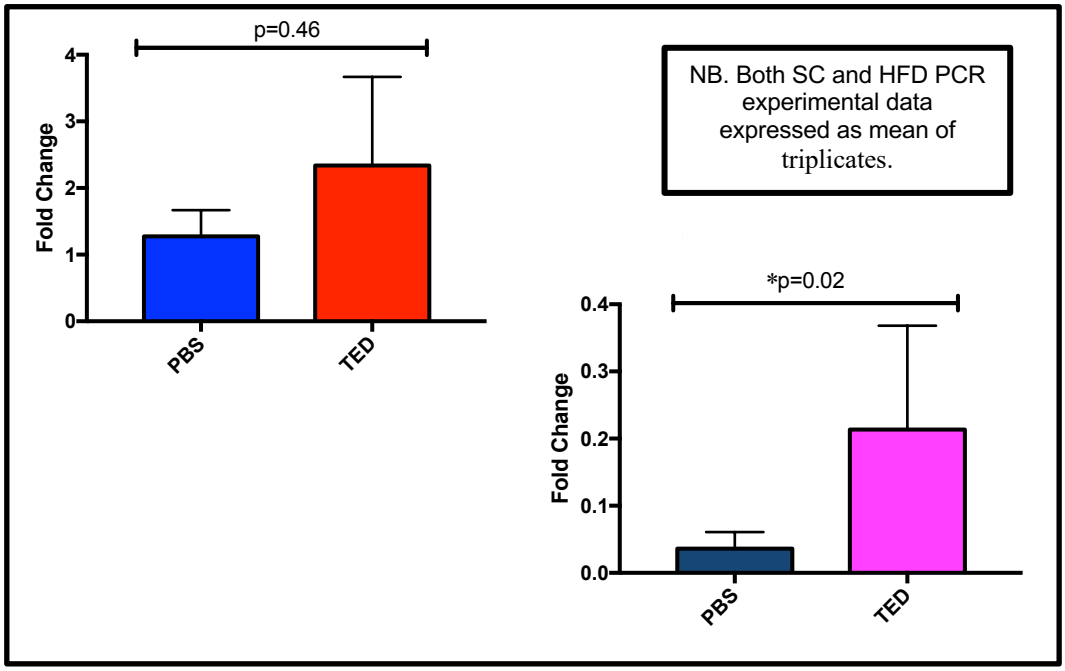



**Figure 193:** Comparison of jejunal GLUT-1 expression between SC and HFD groups.

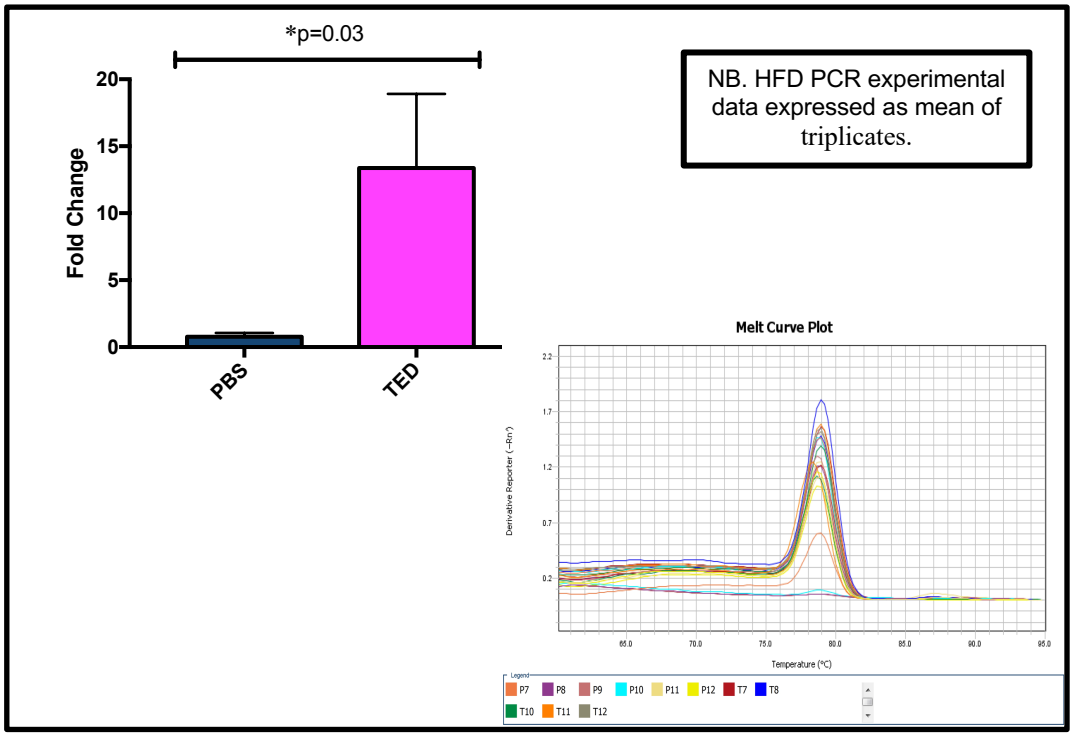



**Figure 194:** Jejunal GLUT-2 expression.

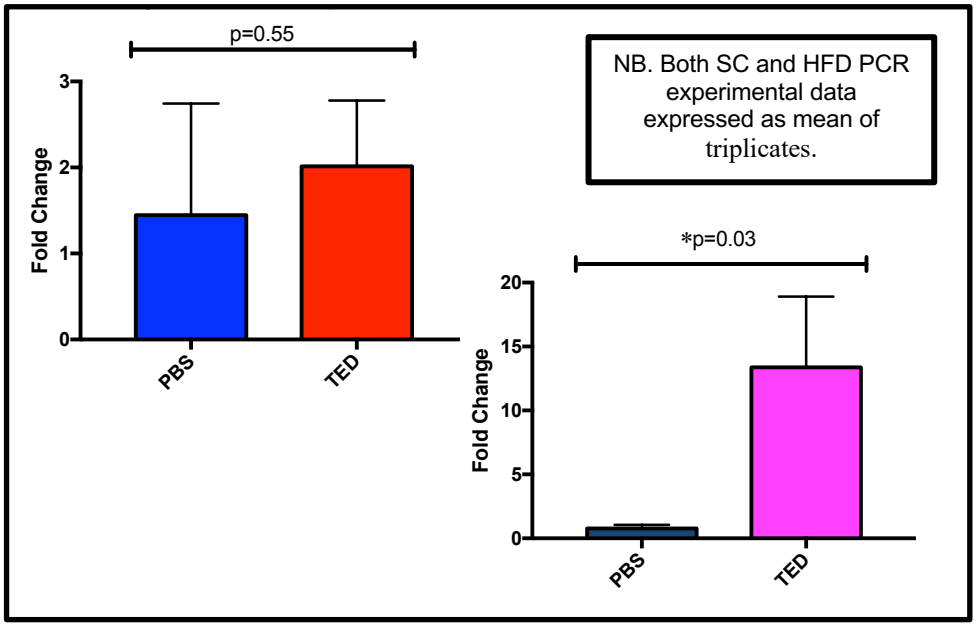



**Figure 195:** Comparison of jejunal GLUT-2 expression between SC and HFD groups.

There was no significant difference between groups concerning GLP-2R (**Figure 196**), IGF-1 (**Figure 198**) or T1R2 (**Figure 200**), although an apparent increase in GLP-2R expression within the TED group was close to reaching significance (p=0.08) (**Figure 199**). **Figures 197, 199 and 201** are positioned for direct comparison.

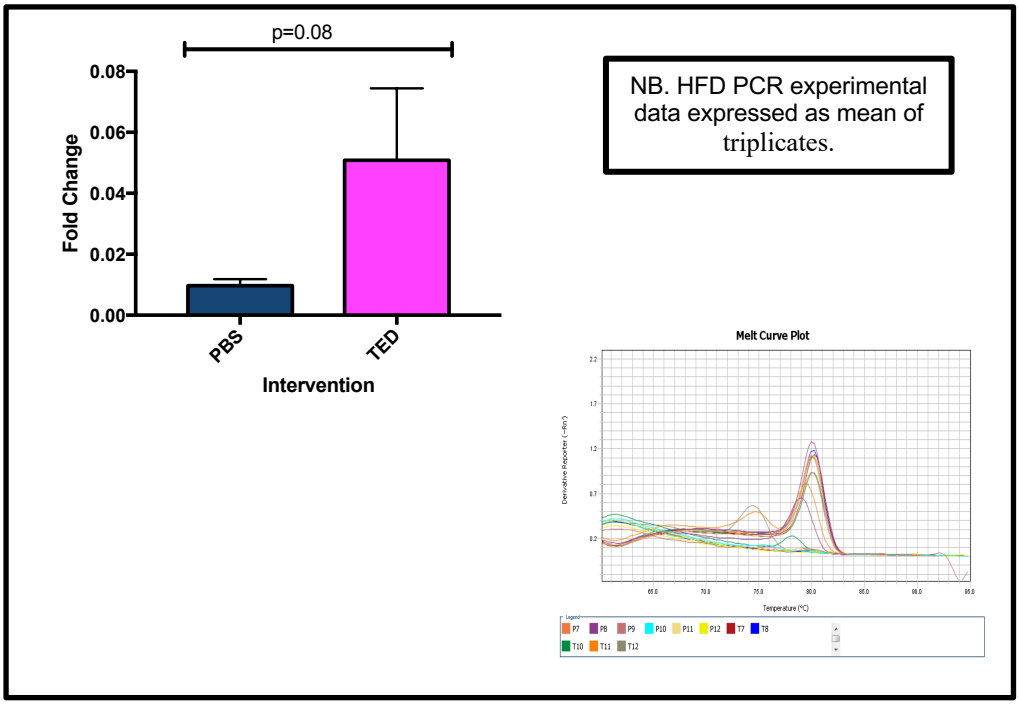



**Figure 196:** Jejunal GLP-2R expression

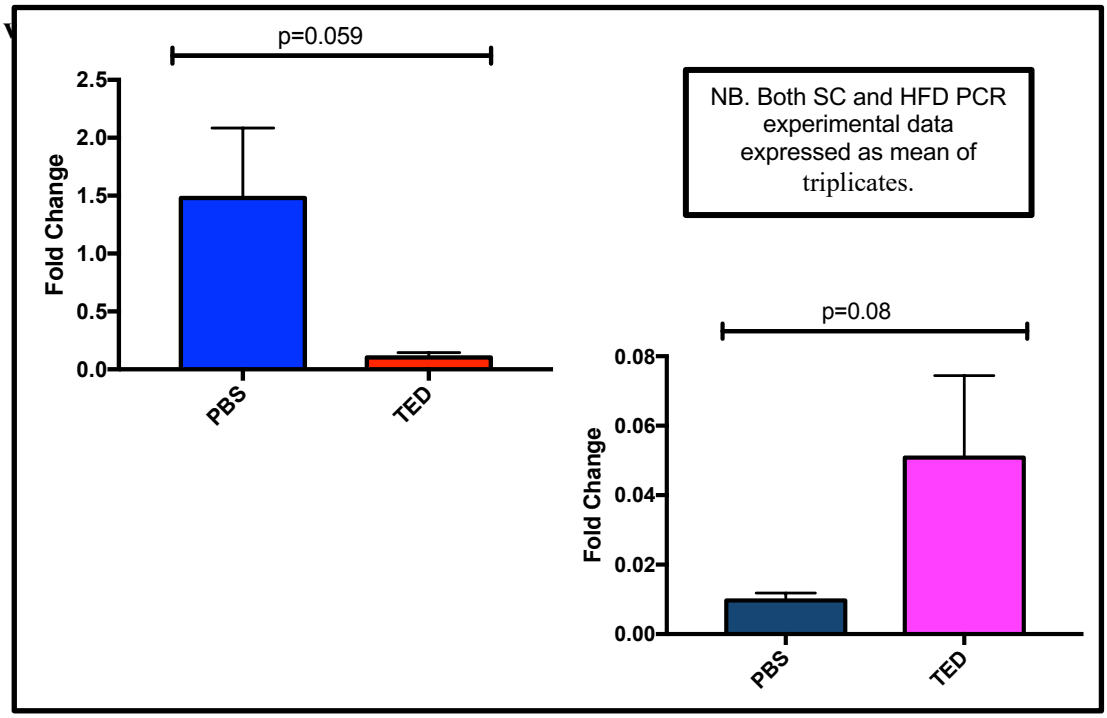



**Figure 197:** Comparison of jejunal GLP-2R expression between SC and HFD groups.

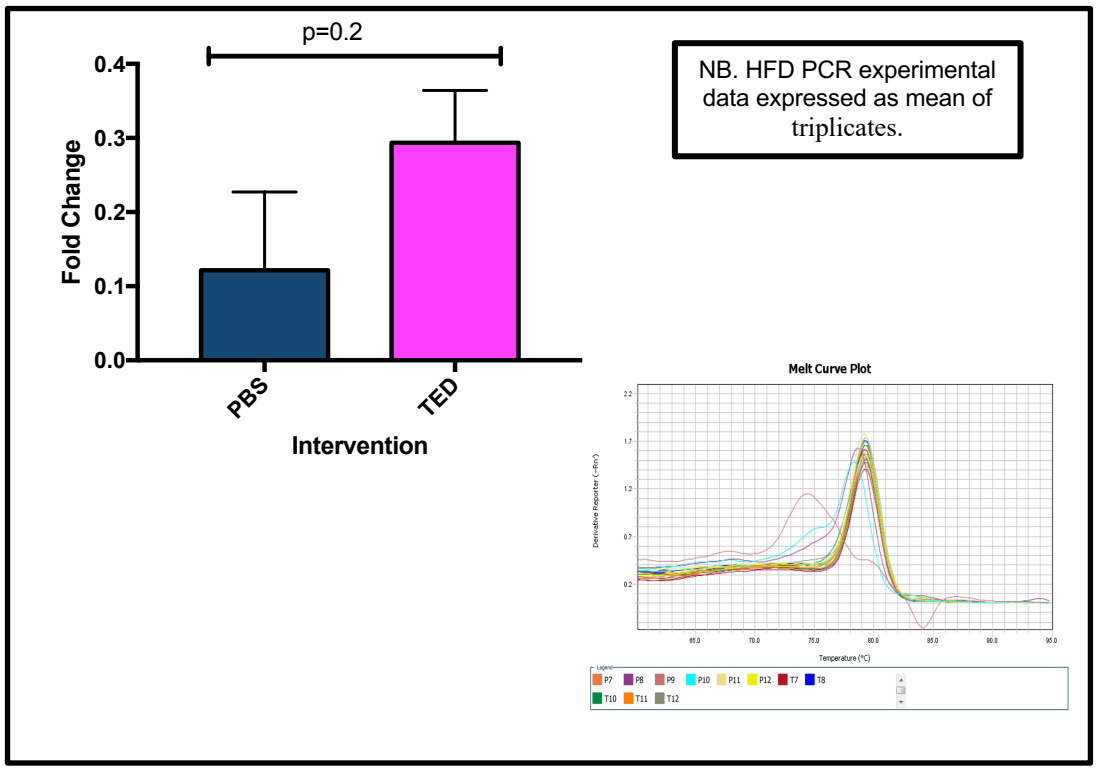



**Figure 198:** Jejunal IGF-1 expression.

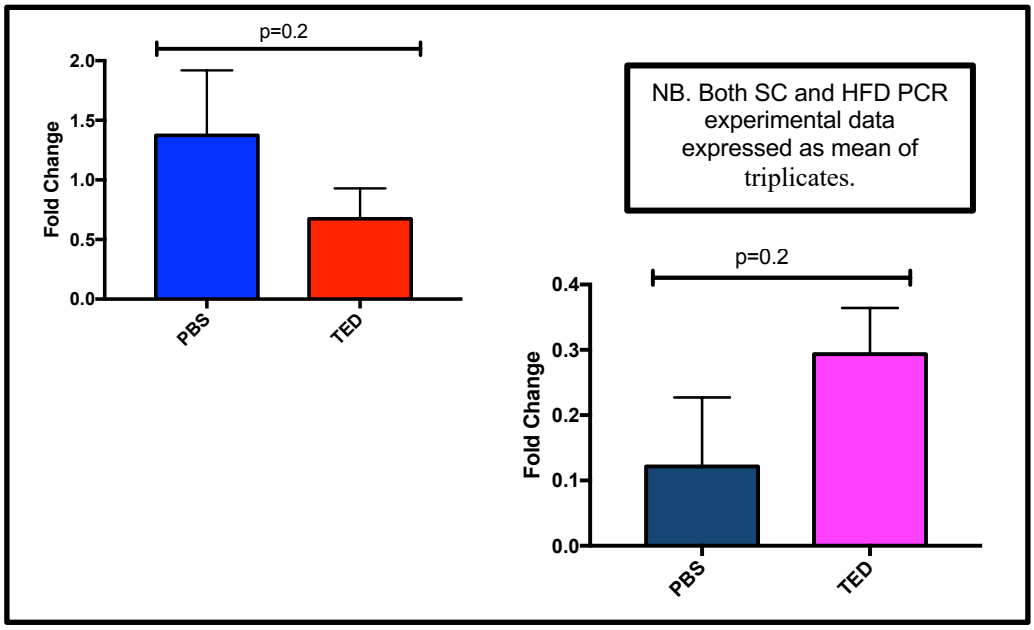



**Figure 199:** Comparison of jejunal IGF-1 expression between SC and HFD groups.

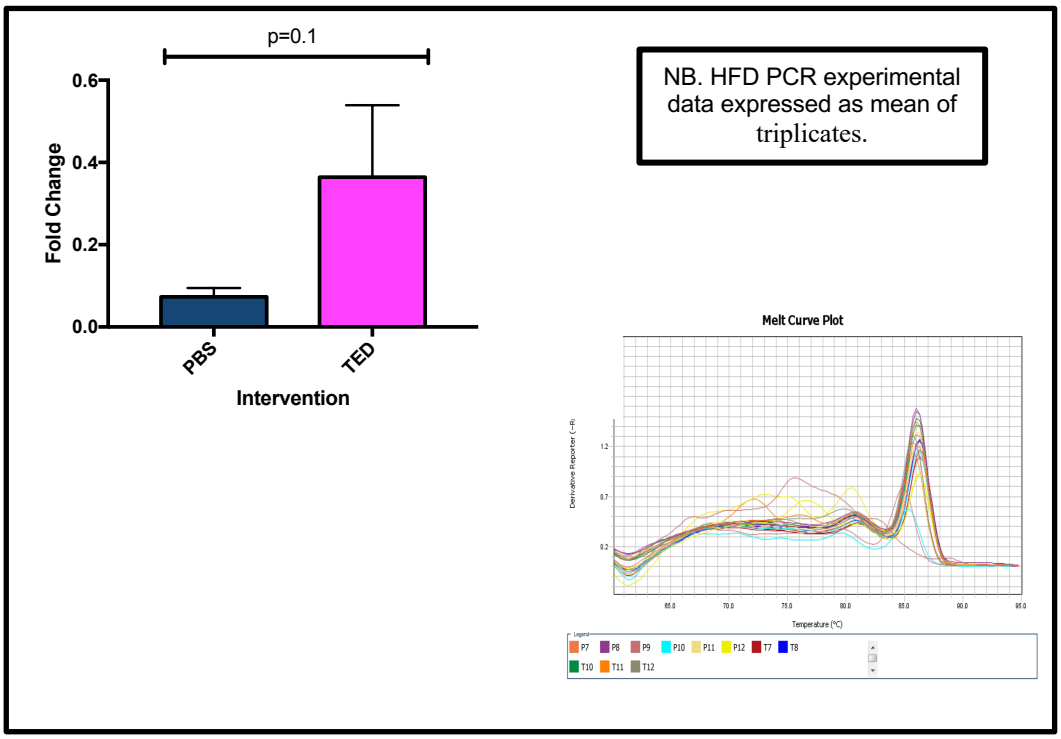



**Figure 200:** Jejunal T1R2 expression.

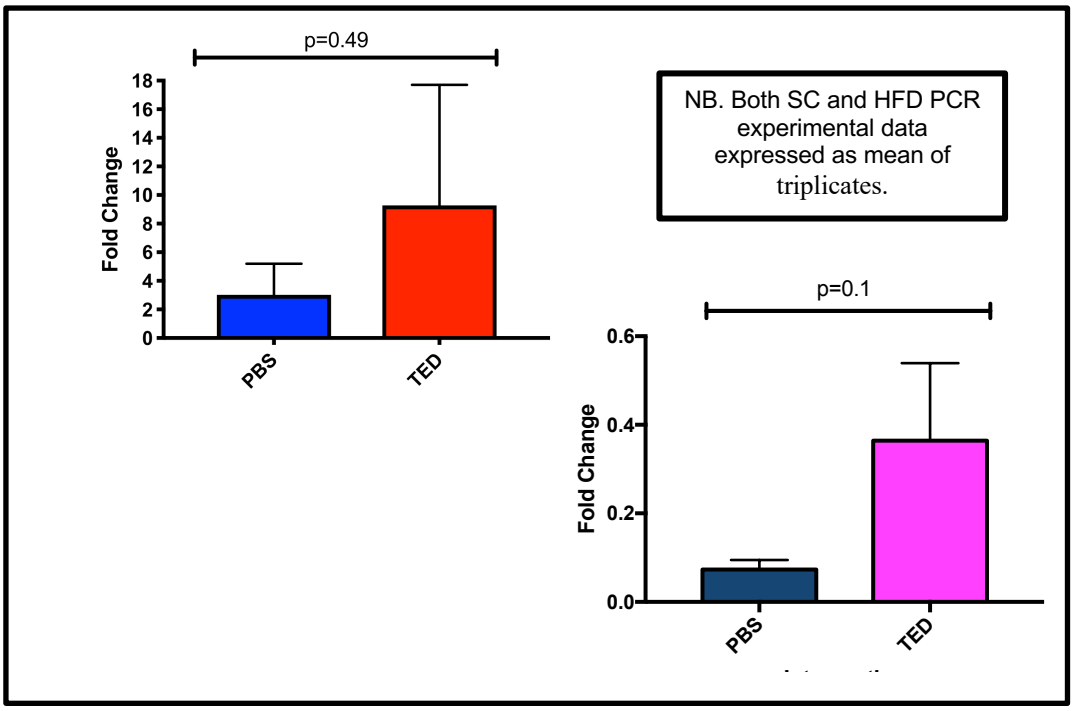



**Figure 201:** Comparison of jejunal T1R2 expression between SC and HFD groups.

*3.7.4 Discussion*

**CASLO PEPTIDE, HIGH FAT DIET, n=6**

Differences in fasting blood glucose levels between rats in this HFD study compared to rats in the SC study were reassuring. Blood glucose levels were all significantly higher at 0 minutes in the HFD group in the baseline, 1-week, 2-week and harvest GGT compared to the SC group, suggesting that a pathological, less glucose-tolerant, model had been successfully generated. It was decided that it would be unnecessary to carry out morphological assessment within this HFD study as the peptide had already been successfully validated within the SC study. Additionally, the significantly heavier mean combined jejunum and ileum and mean weight of the first 5cm of jejunum within the TED group suggested that there was a significant increase in intestinal hypertrophy within this group.

In terms of food intake, rats within Experiment 3 (HFD TED versus HFD PBS) consumed identical amount of food. Once again, this is a surprising result given that GLP-2 is known to cause appetite suppression. However, comparing food intake between Experiments 2 and 3, HFD rats ate significantly less food compared to their SC counterparts. This could be explained by the energy-density of the different types of chow. HFD chow is more energy-dense and, thus, less needs to be consumed to gain the same number of calories compared to the standard chow. Interestingly, and as with the SC experiment, there was no significant difference in total body weight between the TED and PBS groups in the HFD experiment. It is possible that the 22 days of GLP-2 injections were not enough to see a noticeable difference i.e. perhaps differences in weight would become more apparent over months rather than weeks, as repeated daily GLP-2 injections altered rat physiology. It is also possible that the body

composition of the rats in the GLP-2 group had in fact changed, with increased intestinal weight (as shown in section 3.7.3), but that this was cancelled out by a reduction in body fat. Analysis of rat body composition was not carried out; however, it would be interesting to use MRI scanning in future experiments to assess this further.

In terms of glycaema, glucose levels at the 1- and 2-week OGTTs revealed interesting data. Work in this lab had previously shown that a possible mechanism for improved glucose homeostasis, following RYGB, was an increased utilisation of glucose, with a decreased absorption of glucose. Data gained from this experiment does not corroborate a reduced glucose absorption mechanism, with the trend being towards increased glucose absorption within the pathological model. Indeed, the OGTTs/harvest GTTs demonstrated that glucose was being absorbed at a much quicker rate compared to the PBS group. This corresponds with the significant increase in glucose transporter and sensor expression observed via PCR experiments in both the SC and HFD studies, and increased GLUT-2 expression within the HFD study. However, by the 60-minute time point something pivotal seems to have occurred within the pathological HFD group – either absorption slows down, or, intestinal utilisation increases, as both portal and systemic blood glucose levels significantly reduce. This pattern was not seen within the SC healthy group i.e. glucose levels in the SC TED group remained closer in value to the glucose levels within the SC PBS group. Additionally, the HFD experiment PCR data revealed an upregulation of GLP-2R proteins compared to the PBS group (albeit, not significantly so). This is in complete opposition to what was observed in the SC experiment, with higher levels of GLP-2R within the control group compared to the TED group

(p=0.059). As with Experiment 2, Experiment 3 provided no evidence for a link between chronic GLP-2 administration and IGF-1 or T1R2 upregulation.

Luminex data revealed a significant increase in insulin levels in the HFD experiment TED group compared to the SC experiment TED group. This data has important implications. On first inspection it may simply appear that the HFD rats are pathological and, thus, less insulin sensitive (i.e. requiring higher amounts of insulin to be secreted so as to effectively reduce systemic glucose levels). However, this may not be the full story because within the HFD experiment, where all rats are pathological, the TED group were found to have significantly higher systemic insulin levels compared to the PBS group. This suggests that GLP-2 could modulate hepatic and pancreatic insulin sensitivity. This could be explained by the upregulation of glucose transporters seen within the small intestine, along with the increased surface area of intestinal mucosa, leading to more efficient and effective glucose absorption, which is carried to the liver via the portal vein, warranting a quicker and more pronounced hepatic response.

Luminex data also revealed a significant increase in glucagon secretion – within the portal circulation at baseline, within the portal circulation at 30 minutes, and within the systemic circulation by 60 minutes. These findings are corroborated by another group who found that GLP-2 administration markedly increases glucagon concentrations during both fasting and post-prandial states (Meier, Nauck et al. 2006). Clearly, if GLP-2 causes increased glucagon secretion, this could go some way to explaining the higher peak glucose levels seen in TED injected rats. However, glucagon secretion was not significantly increased within the TED SC (Experiment 2)

rats, which suggests that, within the confines of this study, if glucagon secretion is upregulated following chronic TED injections, it is only seen within the pathological state.

The changes in glycaemic behaviour, along with the PCR and Luminex data could suggest that a pathological state is more receptive to the effects of GLP-2 and that TED has a positive effect on glucose homeostasis, in terms of maximising glucose utilisation, within a pathological model, but not to any great extent within a healthy one. Additionally, chronic administration of the peptide demonstrated the greater effect. This certainly seems to fit with the data gleaned from Aim 1, with all experiments carried out in healthy animals, and with only minimal effects on glucose observed during the acute infusion duration. Once again, it was disappointing to obtain data that showed only non-significant changes in glucose utilisation and absorption within the TED group, with both tending towards an increase.

Following on from Aim 2, it was decided that it would be useful to investigate, in more detail, the effect of RYGB on hypertrophy and glucose homeostasis, and whether similar physiological outcomes would be seen in operated rats compared to Teduglutide injected rats.

# CHAPTER 4

## 4.1 AIM 3: RYGB VERSUS SHAM SURGERY

- Assess effects of RYGB on glucose tolerance/homeostasis and on intestinal hypertrophy.
- Assess RYGB GLP-2R expression/location and use mass spectroscopy to carry out metabolic profiling in RYGB rats v SHAM (control) rats.

### *4.1.1 Overview*

- Healthy, SD rats underwent either RYGB or SHAM procedure.
- Both groups weighed daily, and food intake measured weekly.
- 2 and 3-week 120-minute OGGT performed.
- 4-week 60-minute OGTT performed at harvest, with tissues taken for GLP-2 and GLP-2R quantification.

### *4.1.2 Hypothesis*

- Improved glucose homeostasis post-RYGB is mediated by GLP-2-hypertrophic effect on small intestine, and specifically on the Roux limb.

### *4.1.3 Materials and Methods*

*Animals*

Animal studies were performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. 26 male SD rats (200-220g, Charles River) were acclimatised for one day under a 12:12 light: dark cycle (lights-on at 07.00) with *ad libitum* access to standard rat chow (Purina 5053). At

19.00 the evening before planned surgery, rats were weighed, and placed into 1 of 2 groups – RYGB group or SHAM (control) group, so that the mean total weight was similar in each group and was not significantly different. Random glucose levels were also taken and, again, rats were placed in a group so that mean random glucose levels between groups were as matched as possible. All chow was removed, rats were placed on a wire tray (to prevent consumption of faeces), and they were given water *ad libitum.* Surgery began at 09.00 the next morning, with two operations being carried out per day. RYGB and SHAM operations were alternated to control for diurnal variation. These two procedures are described below.

*Surgery*

**Anaesthesia/General Preparation**

- Rat was placed into a sealed, Perspex box.
- Isoflurane 1.5-2.5% in $O_2$ used to induce and maintain anaesthesia.
- Rat transferred to an operating table and its abdomen shaved, and then prepped with Betadine and Alcohol.
- Penicillin IM given as a prophylactic antibiotic.
- Heat pad placed under the rat to maintain normothermia.
- Sterilised surgical instruments collected from autoclave machine.

**RYGB Procedure (Figure 202A):**

- Midline laparotomy performed.
- Stomach mobilised, with division of attachments to the liver and spleen. Lesser sac entered.
- Stomach divided to create gastric pouch and gastric remnant, using linear stapler (Covidien Endo GIA; 2.0-2.5mm staples).

- Duodenal-jenunal junction/Ligament of Treitz identified, and 16cm measured distally (Biliopancreatic limb).
- Jejunum divided at this point and the mesenteric peritoneum divided to the mesenteric root (whilst preserving mesenteric vasculature).
- 1cm gastrotomy created in the greater curvature of the gastric pouch, with a swab used to control contamination.
- Distal part of jejunum then used to create a side-to-end gastro-jejunal anastomosis with 6.0 PDS, interrupted sutures.
- 10cm then measured from anastomosis distally along jejunum (Roux limb)
- Jejuno-jejunal end-to-side anastomosis created at this point (consisting of biliopancreatic limb and Roux limb), to form common limb. 6.0 PDS, interrupted sutures used.
- Haemostasis achieved.
- Lavage of peritoneal cavity using warmed normal saline.
- Interoperative fluid replacement with 15ml normal saline, subcutaneously.
- Fascia closed with 3.0 Vicryl, continuous sutures.
- Skin closed with 3.0 Vicryl, subcuticular continuous sutures.

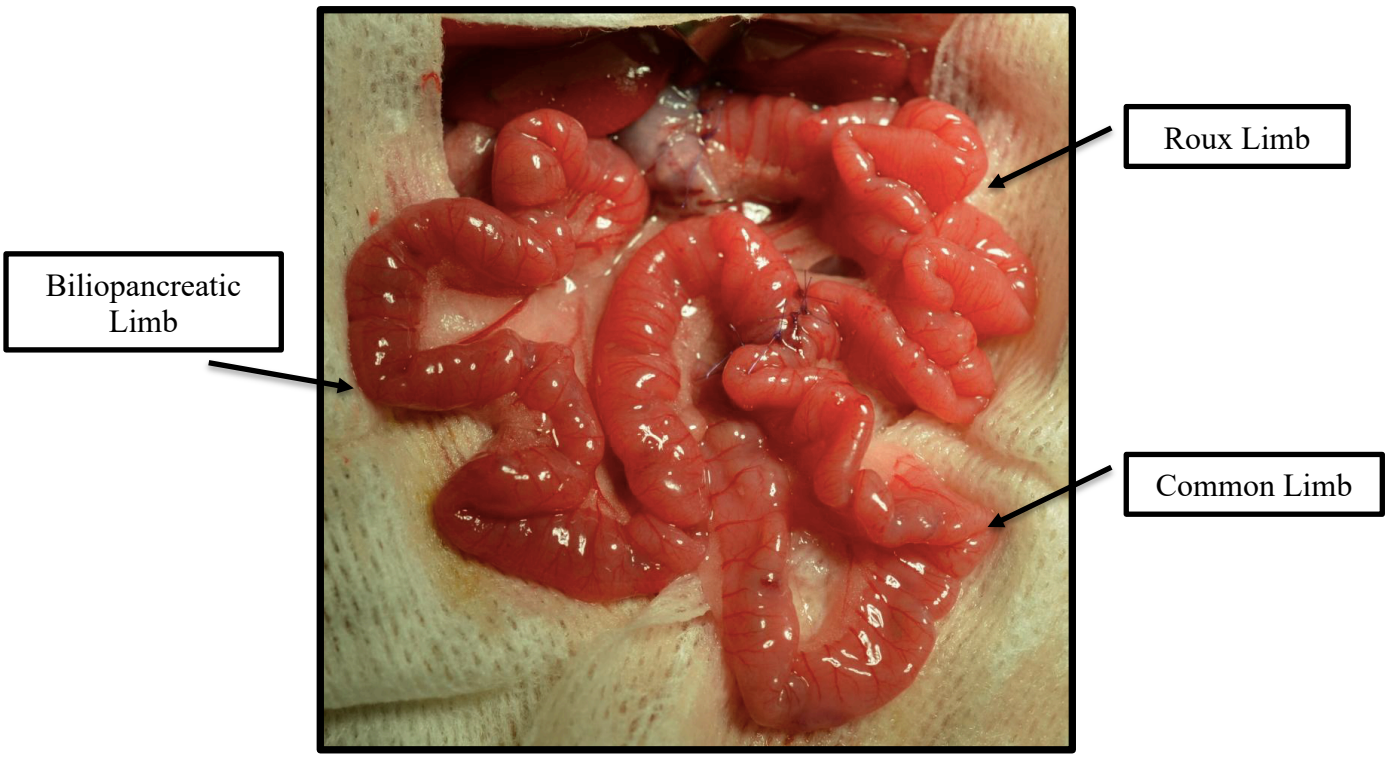



**Figure 202A:** RYGB operative setup.

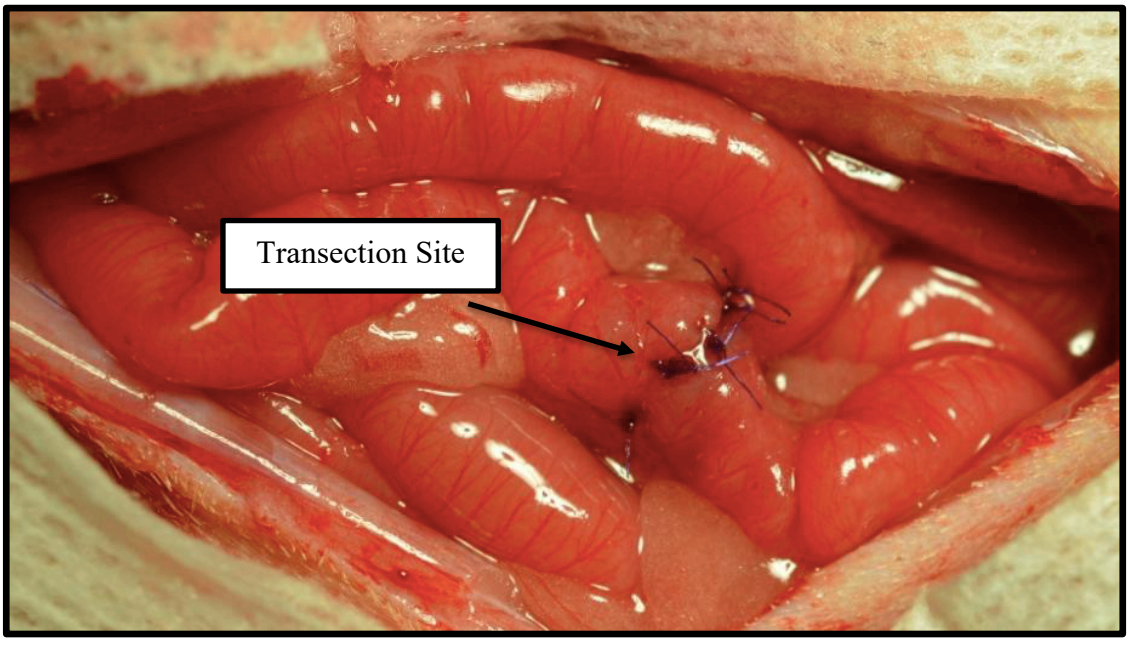



**Figure 202B:** SHAM procedure operative setup.

**SHAM Procedure (Figure 202B):**

- Midline laparotomy performed
- Duodenal-jenunal junction/Ligament of Treitz identified, and 16cm measured distally.
- Jejunum divided at this point.
- Re-anastomosis of the divided jejunum using 6.0 Vicryl, interrupted sutures.
- Haemostasis achieved.
- Lavage of peritoneal cavity using warmed normal saline.
- Interoperative fluid replacement with 15ml normal saline, subcutaneously.
- Fascia closed with 3.0 Vicryl, continuous sutures.
- Skin closed with 3.0 Vicryl, subcuticular continuous sutures.

**Post-operative Procedure**

- Recovery within a Tupperware box with air holes in the lid, lined with a blanket, and a heat lamp positioned above.
- Rat given analgesia once conscious and maintaining sternal recumbency – 0.1ml Buprenorphine, subcutaneously.
- Rat inspected every 12 hours; activity, incision and pain documented on rodent surgery report card.
- Rat given regular analgesia – Buprenorphine 0.05-0.1ml x 4 doses over the next 48 hours
- Oral intake: water *ad libitum* over the next 24 hours, then gel diet (DietGel® Recovery, Clear $H_20$ – see below*) for 5 days.
- Rat kept on metal tray for 24 hours, then swapped back to usual bedding.
- If rat showing signs of distress, for further Buprenorphine +/- euthanasia.
- Rat restarted on solid food (standard chow) on day 6 post-op.

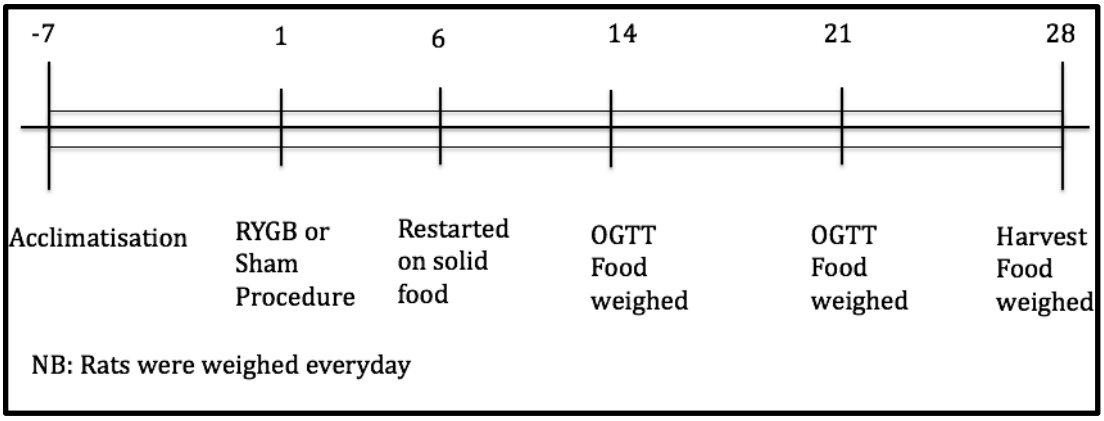



**Figure 202:** Experiment timeline.

*OGTT*

OGTT was performed as described as above (section 3.4.1) and was performed at 2- and 3-weeks post-surgery (**Figure 202**). The decision was made not to carry out an OGTT at 1 week as it was felt that rat recovery could be compromised by subjecting them to an OGTT early into their post-operative recovery.

*Harvest Protocol*

Harvest was carried out at 4 weeks. Portal and systemic venous blood was sampled as in section 2.4.1, before and after an intestinal glucose infusion, and over a 60-minute time-frame. GTT involved giving a 25% glucose solution infused over 5 minutes into either the Roux limb or into the corresponding segment (16cm distal to Duodeno-jenunal junction) in SHAM rats. Whole small intestine was weighed. Additionally, the first 5cm segments of Roux limb, BP limb, common limb and terminal ileum, and their equivalent counterparts within the SHAM rats, were weighed.

*Within the lab, the post-operative diet of choice for the first 5 days had consisted of a liquid diet (CVS Liquid Nutrition, chocolate nutrition). However, veterinarians within the research building had recommended DietGel® Recovery (**Figure 203**).




**Figure 203:** Dietgel® Recovery.

DietGel® Recovery is a nutrient fortified water gel that aids in the recovery of post-surgical, weak or impaired rodents and has been proven to increase survival rates in compromised animals (**Figure 204**).

It is formulated from purified ingredients that provide 60% water, calories and electrolytes. Thus, the decision was made to use this post-operative diet.

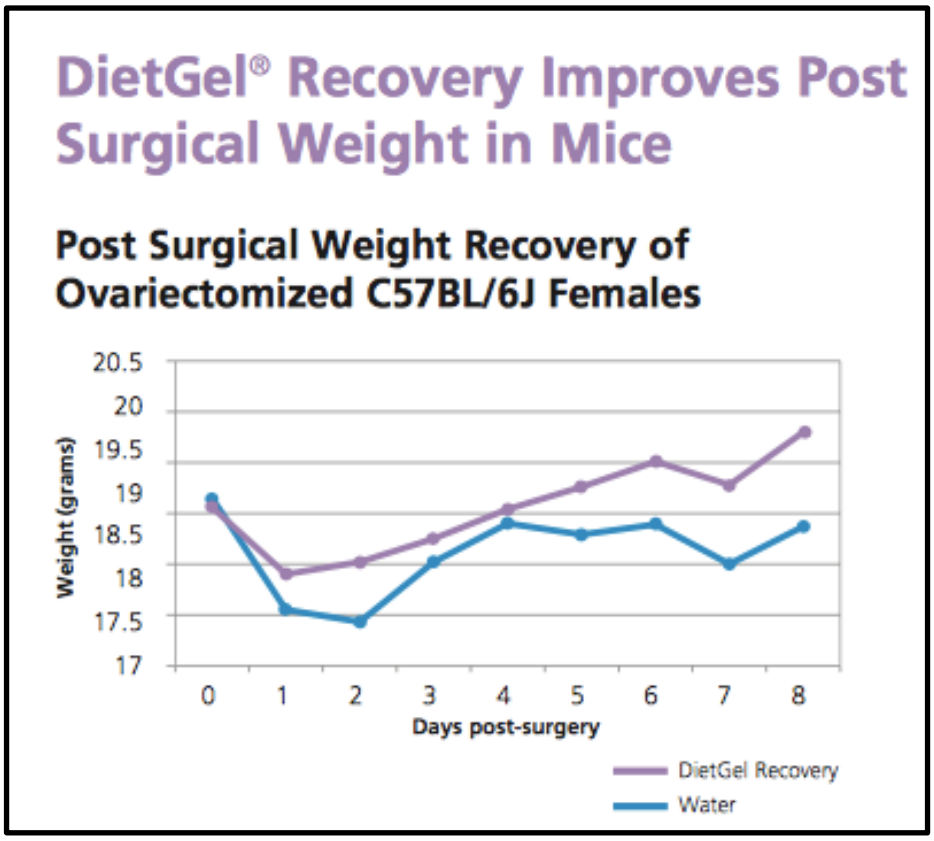



**Figure 204:** Dietgel® Recovery improves post-surgical weight in mice.

*4.1.4 Results*

Statistical analysis was carried out as described in section 2.4.1. Out of a total of 13 bypassed rats, 9 survived the initial surgery to make it through to the end of the 4-week study period. All 13 SHAM rats survived.

The first OGTT (carried out 2 weeks post-surgery in order to give the rats time to recover from their operation) revealed some interesting results. For example, somewhat unexpectedly, the baseline glucose level within the RYGB group was significantly higher than in the SHAM group (92.7mg/dl v 76.7mg/dl, p=0.01). Glucose levels remained significantly higher in the RYGB group at the 30-minute time point (172.4mg/dl v 146.6mg/dl, p=0.049). Glucose levels in the RYGB group were seen to be higher at the 60- and 90-minute time points, although not significantly. At 120 minutes, both groups had a very similar mean glucose level (109.67mg/dl v 112.2mg/dl) (**Figure 205**).

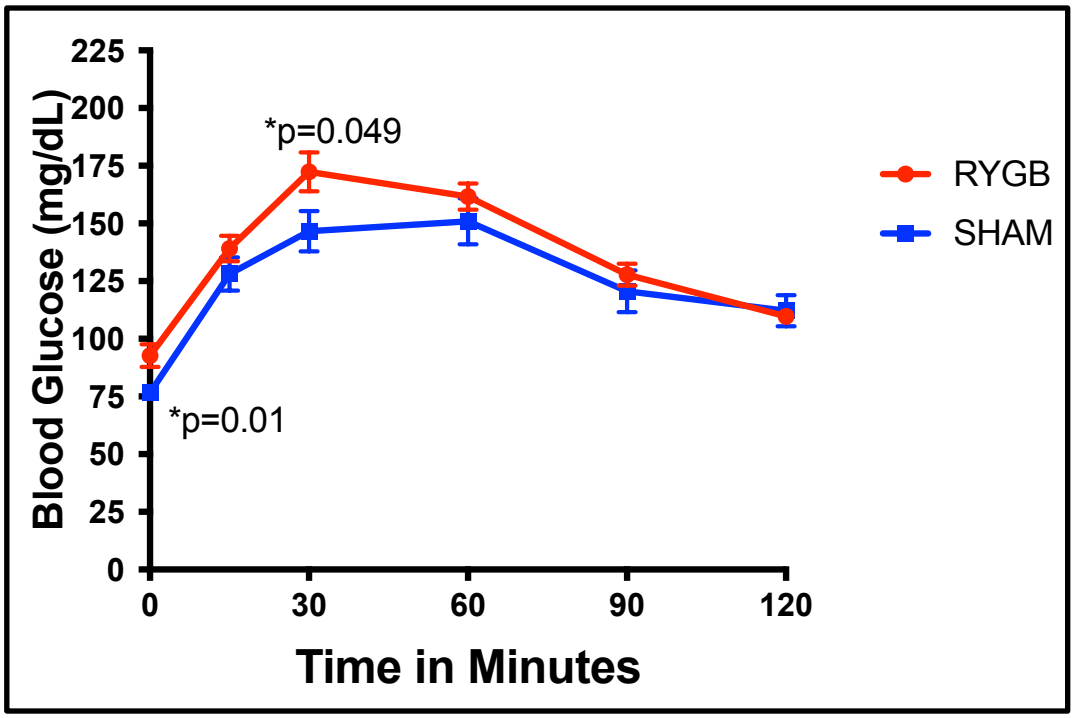



**Figure 205:** Oral glucose tolerance test 1 (2 weeks post-surgery).

The second OGTT (carried out 3 weeks post-surgery) revealed a similar trend to that of the 2-week OGTT, in that the RYGB group had higher blood glucose levels throughout the procedure which again, was unexpected. This time, the increase at baseline and 30-minutes was not significant, however, by 60 minutes, significance was reached (175.89mg/dl v 153.15mg/dl, p=0.01). Unlike the 2-week OGTT, glucose levels at 120-minutes were noticeably different, with the RYGB group still trending higher and just missing out on significance (121.2mg/dl v 108.46mg/dl, p=0.07) (**Figure 206**).

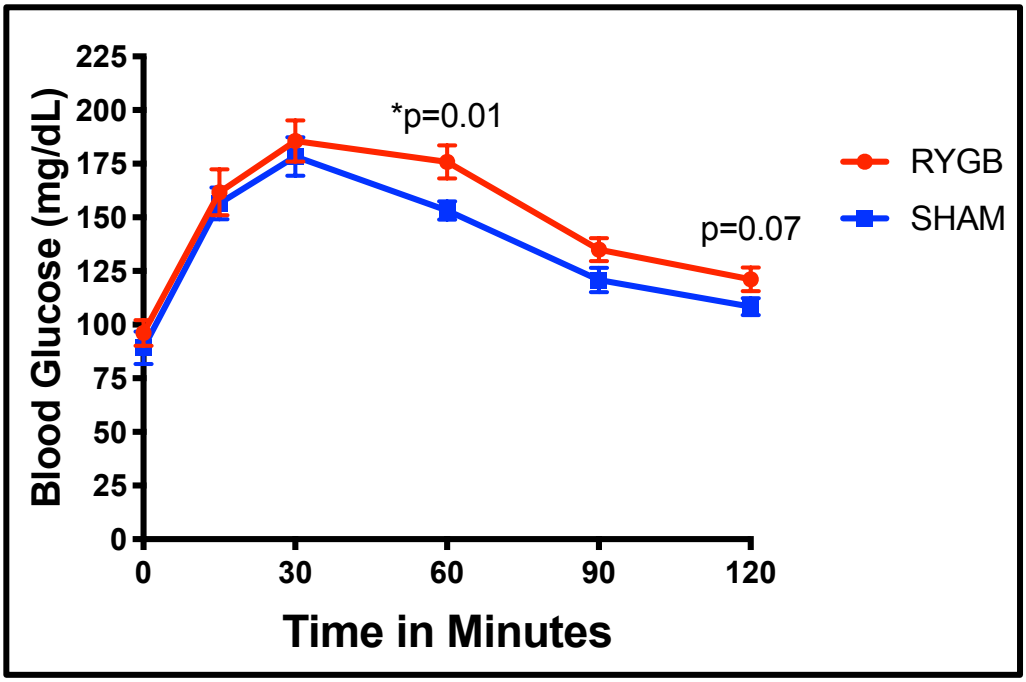



**Figure 206:** Oral glucose tolerance test 2 (3 weeks post-surgery).

Harvest blood glucose levels, following the usual interoperative duodenal glucose infusion over the first 5 minutes, also showed a convincing pattern. Both portal and systemic blood glucose levels were found to be significantly higher at baseline (portal: 166.0mg/dl v 126mg/dl, p=0.008, and systemic: 188.8mg/dl v 138.5mg/dl, p=0.02). They were also found to be significantly higher at the 15-minute time point (portal: 399.3mg/dl v 294mg/dl, p=0.01, and systemic: 271.0mg/dl v 225.0mg/dl, p=0.02).

However, from 30 to 60 minutes, both portal and systemic glucose levels within the RYGB group were on a par with the SHAM blood glucose levels (**Figures 207 and 208**).

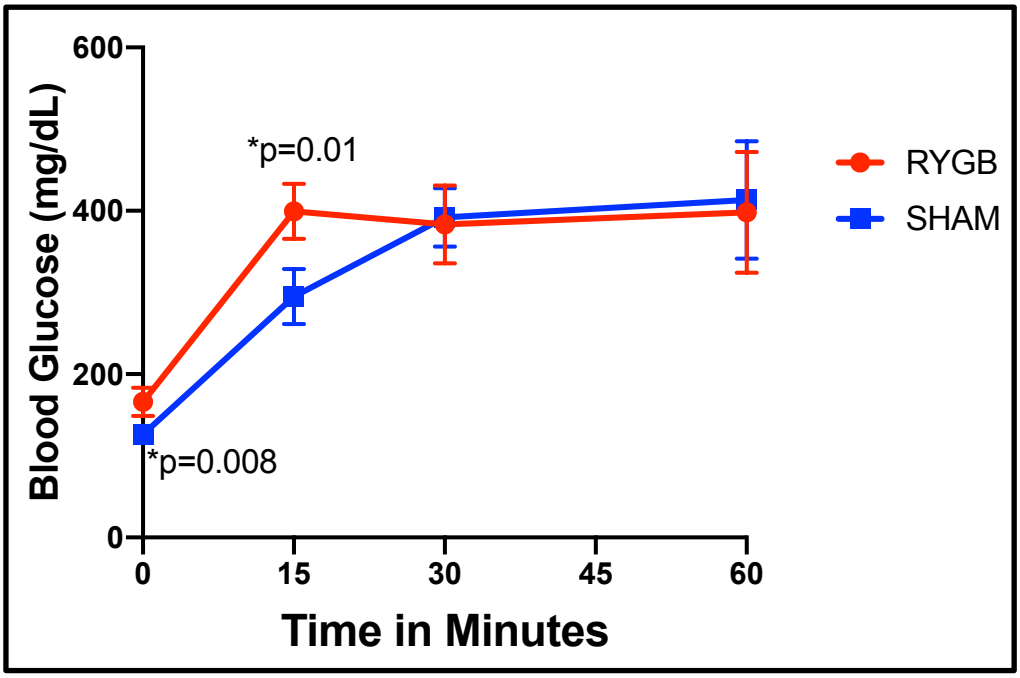



**Figure 207:** Mean portal glucose levels at harvest GTT (day 28).

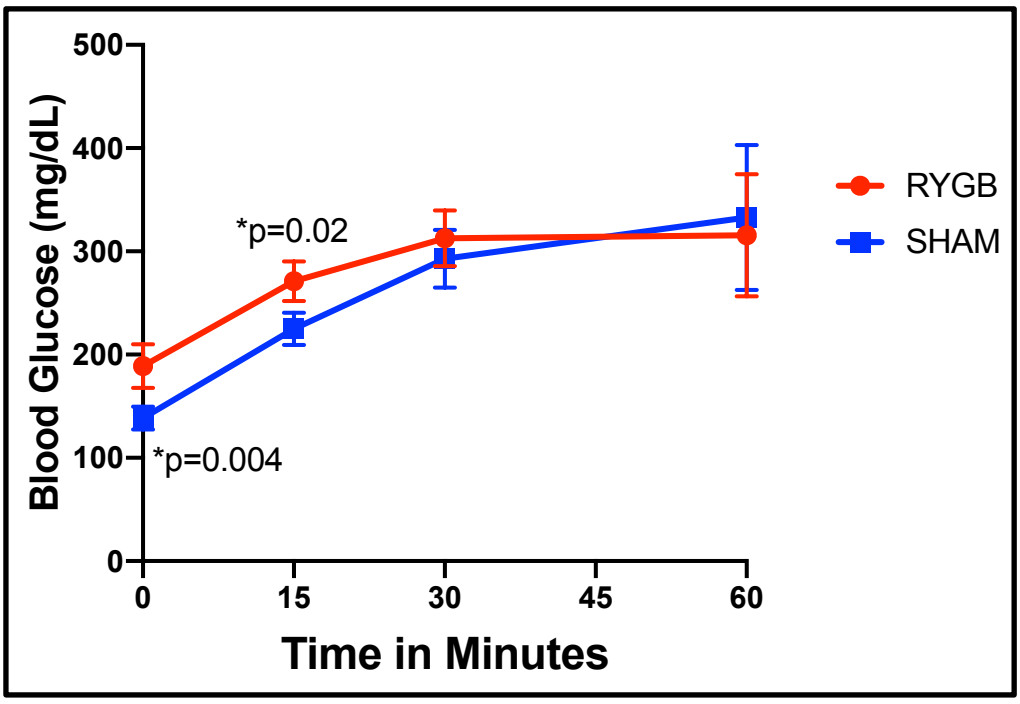



**Figure 208:** Mean systemic glucose levels at harvest GTT (day 28).

$G_{PS}$ was significantly more negative in the RYGB group compared to the SHAM group at baseline (-22.8mg/dl v -12.2mg/dl, p=0.007), however, this then dramatically switched at 15 minutes, with the RYGB group $G_{PS}$ becoming significantly more positive than the SHAM group (128.2mg/dl v 69.8mg/dl, p=0.03). This then changed again at the 30-minute time point, with the RYGB $G_{PS}$ becoming less positive than the SHAM group (not significantly so). Both gradients finished at a similar point at 60 minutes (**Figure 209**).

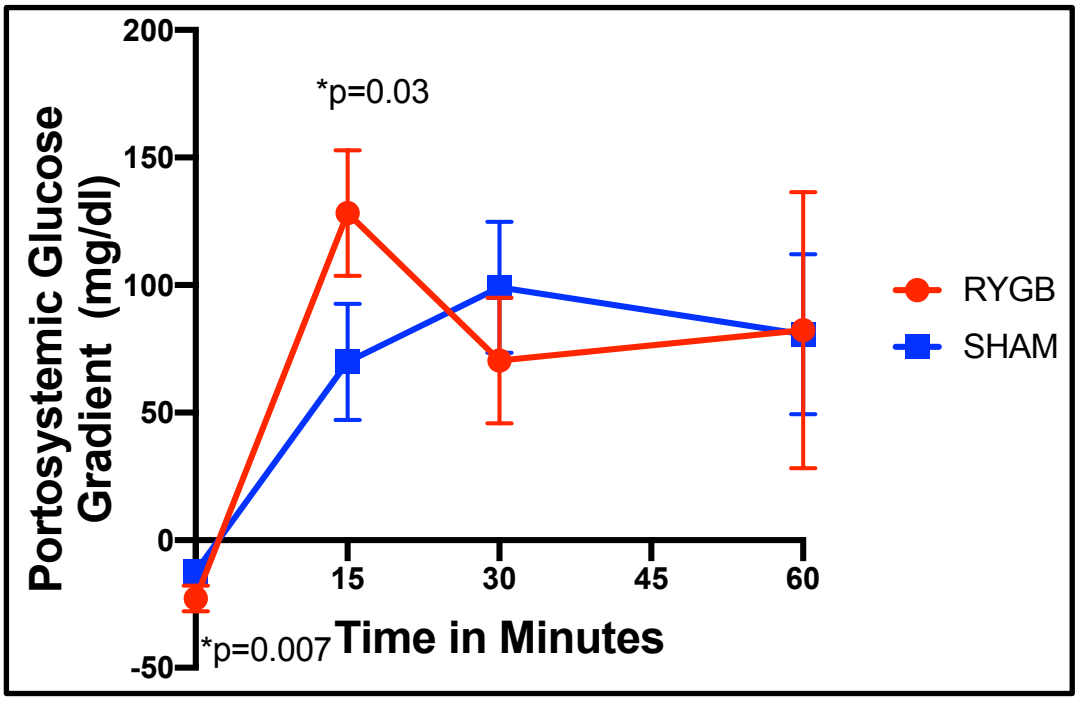



**Figure 209:** Porto-systemic glucose gradients at harvest GTT (day 28).

Baseline glucose utilisation was significantly increased in the RYGB group (116.6mg/dl v 62.6mg/dl, p=0.007) (**Figure 210**).

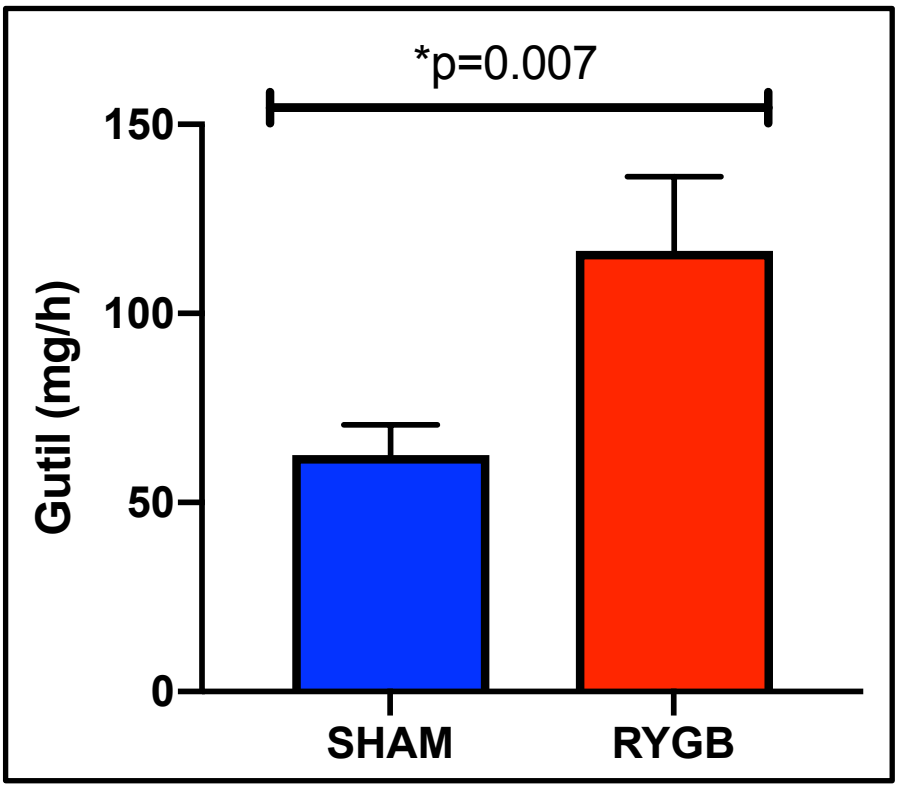



**Figure 210:** Intestinal glucose utilisation.

There was no significant difference in glucose absorption over the duration of the experiment, between the two groups (**Figure 211**).

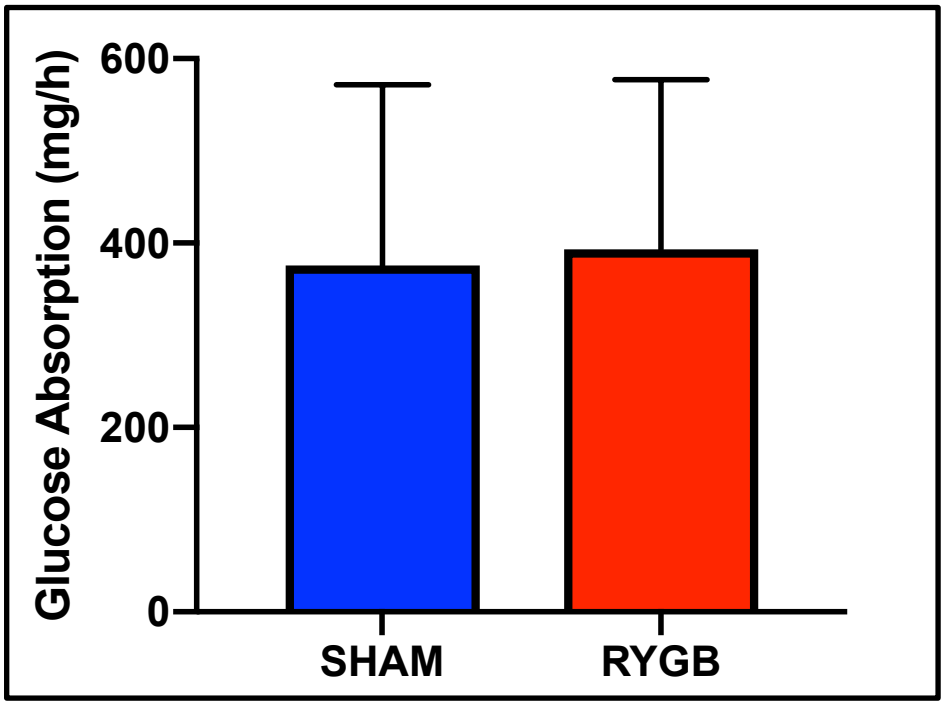



**Figure 211:** Intestinal glucose absorption.

Mean total body weight between the groups was comparative at baseline, however, from as early on as day 2, RYGB rats had a noticeably lower body weight. On post-operative day 6, the RYGB group had a significantly lower bodyweight than the SHAM group (204.2g v 224.7g, p=0.02), and this significant difference carried on all the way through to the last day of the study, day 28 (278.0g v 319.2g, p=0.003) (dips due to overnight fasting for OGTT/harvest GTT) (**Figure 212**).

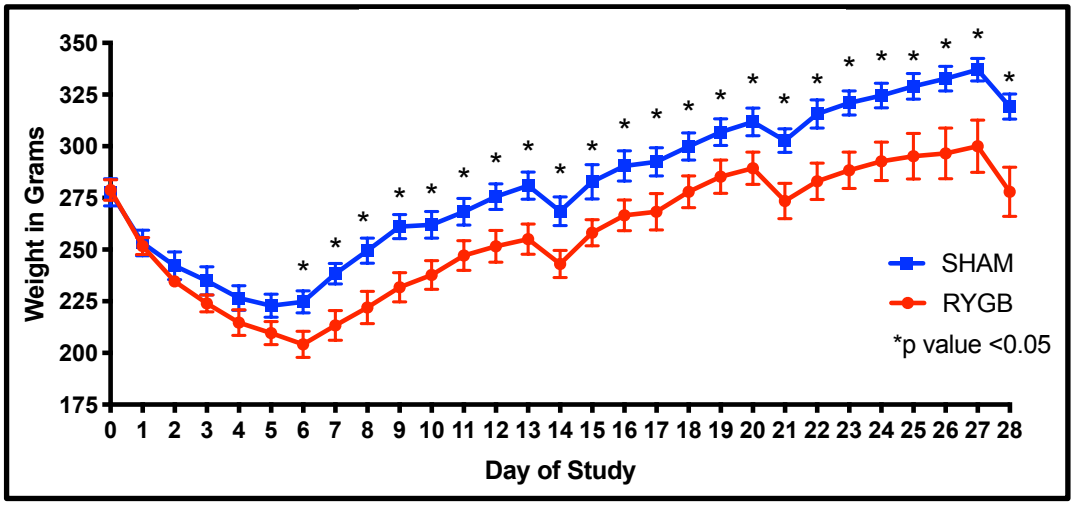



**Figure 212:** Mean total bodyweights throughout study.

Although RYGB consumed less chow over the study period, there was no significant difference demonstrated (438.8g v 474.6g, p=0.24) (**Figure 213**).

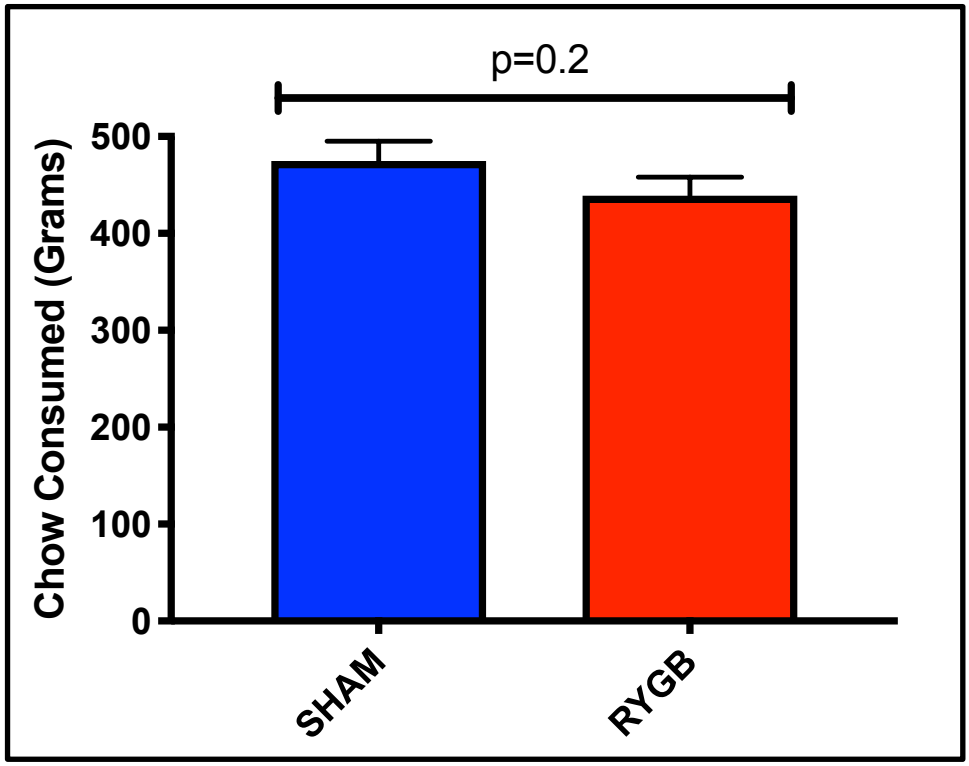



**Figure 213:** Mean total chow consumed.

The mean total weight of small intestine within the RYGB compared to the SHAM group was not found to be significantly higher (**Figure 214**).

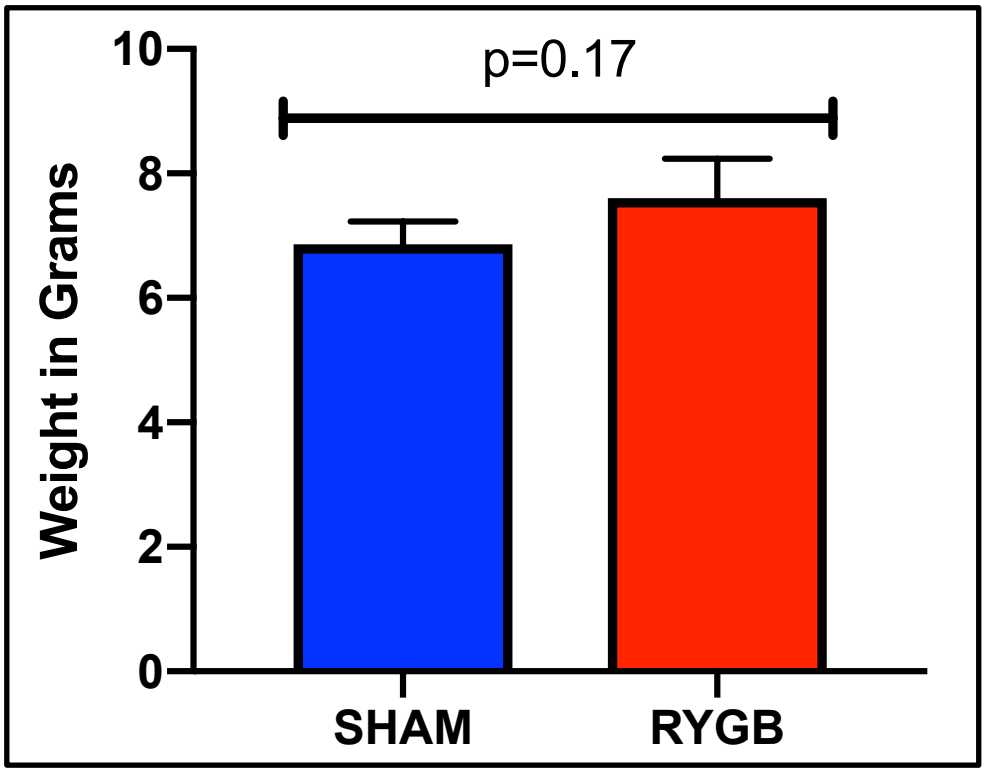



**Figure 214:** Mean total weight of small intestine.

However, when the data was analysed so that the intestinal weight was calculated as a % of total body weight i.e. taking account of the size of the rat (given that the RYGB rats were significantly lighter for most of the study), the difference between the two groups became significant, with total small intestinal weight significantly greater in the RYGB group (2.74% v 2.18%, p=0.01) (**Figure 215**).

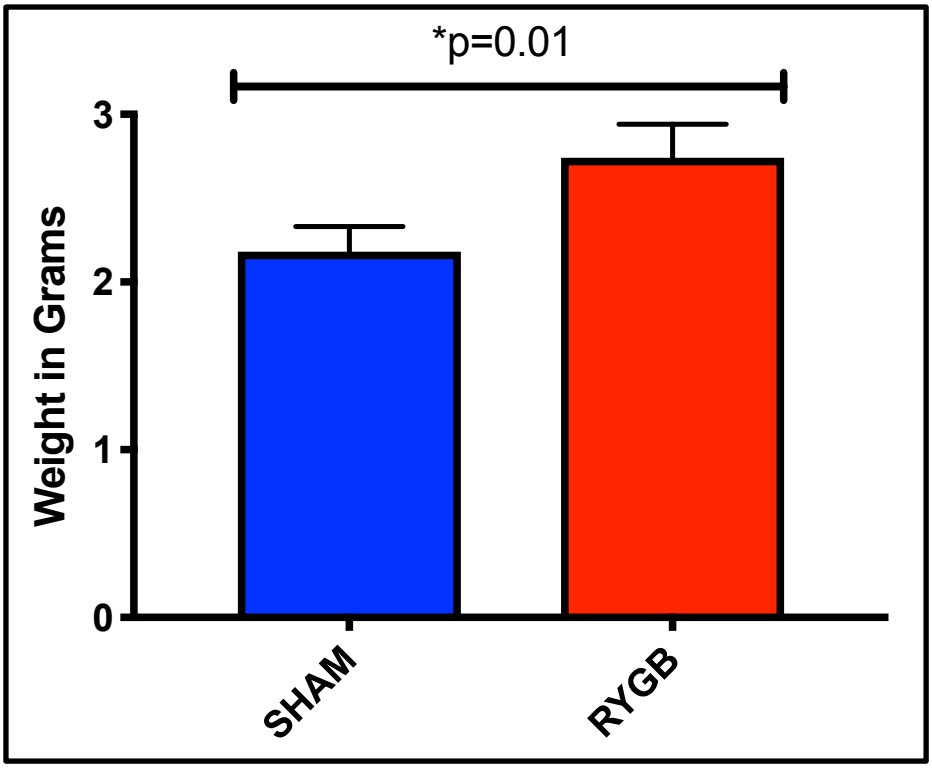



**Figure 215:** Mean total weight of small intestine, expressed as % bodyweight.

When the first 5cm of each type of limb segment (Roux, biliopancreatic, common), along with the last 5 cm of terminal ileum, were weighed and then calculated as a % of total body weight, a significant increase in weight in the Roux limb in RYGB rats, (0.30% v 0.19%, p=0.001) (**Figure 216**), and in the common limb in RYGB rats, (0.24% v 0.15%, p=0.01) (**Figure 217**), was observed.

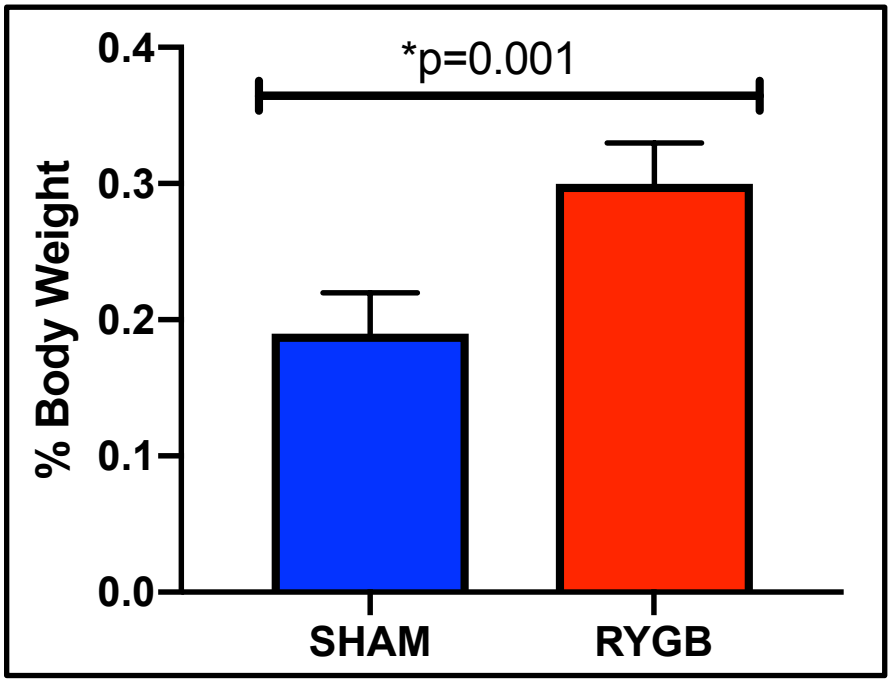



**Figure 216:** Mean 5cm weight of Roux limb, expressed as % bodyweight.

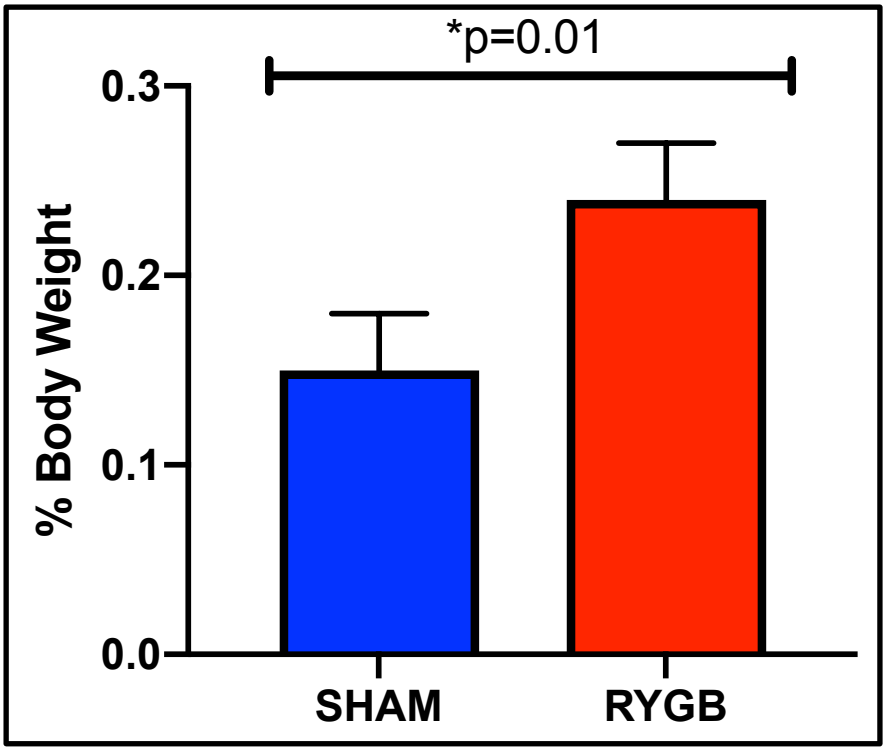



**Figure 217:** Mean 5cm weight of common limb, expressed as % bodyweight.

**Figure 218** shows how each 5cm intestinal segment (as % of total body weight) compares between the RYGB and SHAM groups.

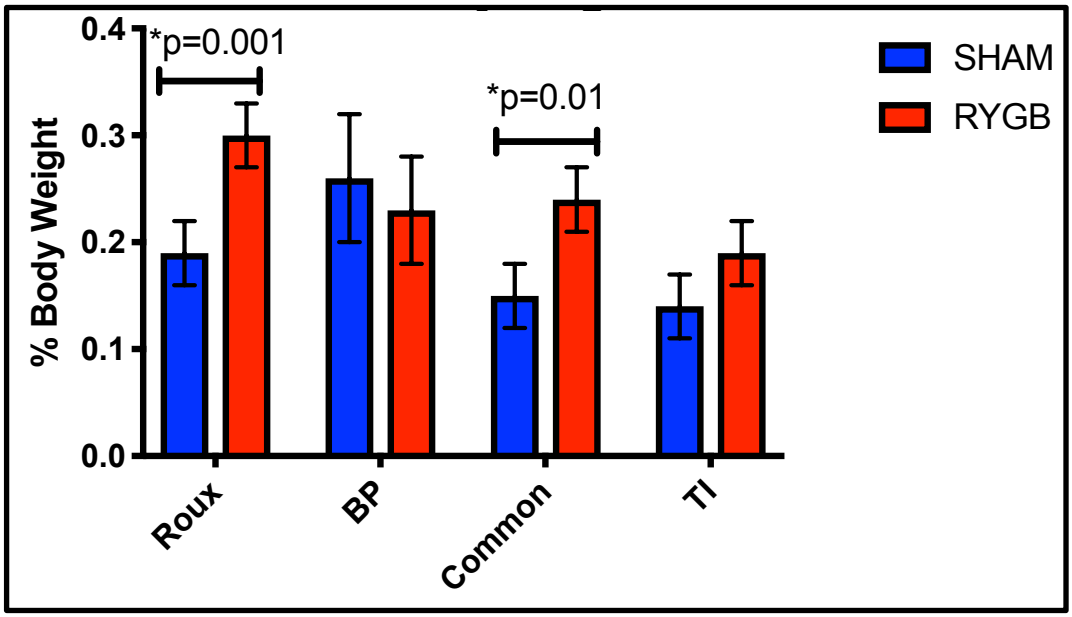



**Figure 218:** Comparison of mean weights of 5cm intestinal segments (as % of total body weight) between the RYGB and SHAM groups.

*4.1.5 Discussion*

Operative success of the procedures was confirmed by monitoring rat weights daily for 4 weeks. There was a consistently significant difference in weight between the bypassed rats and the SHAM rats, with the SHAM rats weighing more. None of the RYGB rats developed diarrhoea or any other symptoms that might be indicative of malabsorption. Interestingly, there was no difference in food intake. Part of the success of RYGB, in terms of its effect on weight loss, used to be attributed to it being a 'restrictive' procedure. However, it is now known that there is far more to the procedure than patients simply not being able to consume as much food, with the success of the procedure being linked to increased energy expenditure (Werling, Fändriks et al. 2015). The food intake data appears to go some way towards corroborating this concept.

Mean total weight of small intestine (represented as a percentage of rat bodyweight to account for the fact that bypassed rats became significantly lighter than their SHAM counterparts) was found to be significantly higher in RYGB rats compared to SHAM rats. This supports work carried out by other lab members (**Figure 11**). These findings appear to go some way in supporting the concept that GLP-2 mediated hypertrophy occurs most within the Roux limb, given that the Roux limb was found to be the heaviest and was significantly heavier in the RYGB group compared to SHAM. BP limb was the next heaviest, but there was no significant difference between the RYGB and SHAM groups. Next heaviest was the common limb, again significantly heavier in the RYGB group. Finally, there was no significant difference in terminal ileum 5cm weight between the two groups.

As confirmed by the histology, this aim confirmed successful demonstration of a RYGB healthy rat model and that morphological changes were taking place, with the degree of intestinal adaptation dependent on the rearrangement of intestine. However, it was particularly intriguing that bypassed rats had significantly higher peak blood glucose levels at the 2- and 3-week OGTTs (**Figures 205 and 206**), especially at baseline. This was replicated in the 4-week harvest intra-operative enteral glucose infusion, where both portal and systemic blood glucose levels were higher in the RYGB group. Data obtained from identical experiments carried out subsequently (by other members of the lab) have also revealed this trend. Clearly it contradicts what might be expected from RYGB – that is, lower fasted baseline glucose levels, as this is what is demonstrated in bypassed diabetic humans. One possible explanation for this might be that a healthy rat model was used. It is possible that, as with Aim 2 (Teduglutide experiments), benefits are only conferred when a pathological model is used. Accordingly, it was decided that RYGB would be conducted within a pathological model (Aim 4), adopting the high fat diet used within Aim 2 to cause a decrease in glucose tolerance.

Unfortunately, time did not allow for GLP-2 ELISA or GLP-2R PCR experiments. Nevertheless, both the blood plasma and mucosal tissue are available for lab successors to conduct studies upon in order to add additional findings to these studies. Additionally, plasma has been sent for lipid and protein analysis by mass spectroscopy and results are still awaited.

# CHAPTER 5

## 5.1 AIM 4: (I) GLP-2R ANTAGONISM

## (II) ALTERED BILIOPANCREATIC LIMB LENGTH

- Use of GLP-2R antagonism to demonstrate reversal of improvements in glucose homeostasis post-RYGB in HFD rats (**EXPERIMENT 1**).
- Alter BP limb length in RYGB to modulate glucose homeostasis (**EXPERIMENT 2)**.

## 5.2 EXPERIMENT 1 – GLP-2R ANTAGONISM

### *5.2.1 Overview*

**Assess the role of chronic subcutaneous GLP-2 inhibition on intestinal glucose utilisation post RYGB**:

- SD fats fed HFD to become glucose intolerant (pathological model) and to undergo RYGB.
- To receive GLP-2 inhibitor (GLP-2 Receptor Antagonist) or saline (PBS) control.
- Assess impact on intestinal morphology and glucose homeostasis at 4 weeks, using systemic and portal catheterisation technique, with duodenal glucose infusion equivalent to OGTT.
- Collect intestine and liver segments for PCR.

*5.2.2 Hypothesis*

**It is hypothesised that chronic subcutaneous GLP-2 inhibition will:**

- Reduce the morphological changes usually seen after RYGB including a reduction in intestinal mucosal hypertrophy
- Reduce intestinal glucose utilisation and absorption
- Reduce improvements of hyperglycaemia
- Reduce rodent weight loss

## 5.3 VALIDATION PROTOCOL 1

*5.3.1 Materials and Methods*

*Teduglutide*

Teduglutide was once again purchased from CASLO (as described in section 3.5).

*Teduglutide Receptor Antagonist*

Given the success of the Teduglutide peptide received from CASLO, the decision was made to order a GLP-2 Receptor Antagonist (GLP-2RA) from the same company. This particular peptide had previously been used with success by other groups (Baldassano, Rappa et al. 2015) (Baldassano, Amato et al. 2016). CASLO provided GLP-2 (3-33) with the following peptide sequence:

"Asp-Gly-Ser-Phe-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Ala-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp".

The theoretical weight for GLP-2 (3-33) is 3357.97g/mol. The peptide which was provided by CASLO had a weight of 3557.07g/mol (**Figure 219**), which was

reassuring. Nevertheless, it was felt that a validation experiment needed to be carried out in order to confirm the functionality of the peptide, despite the groups above choosing not to perform one.

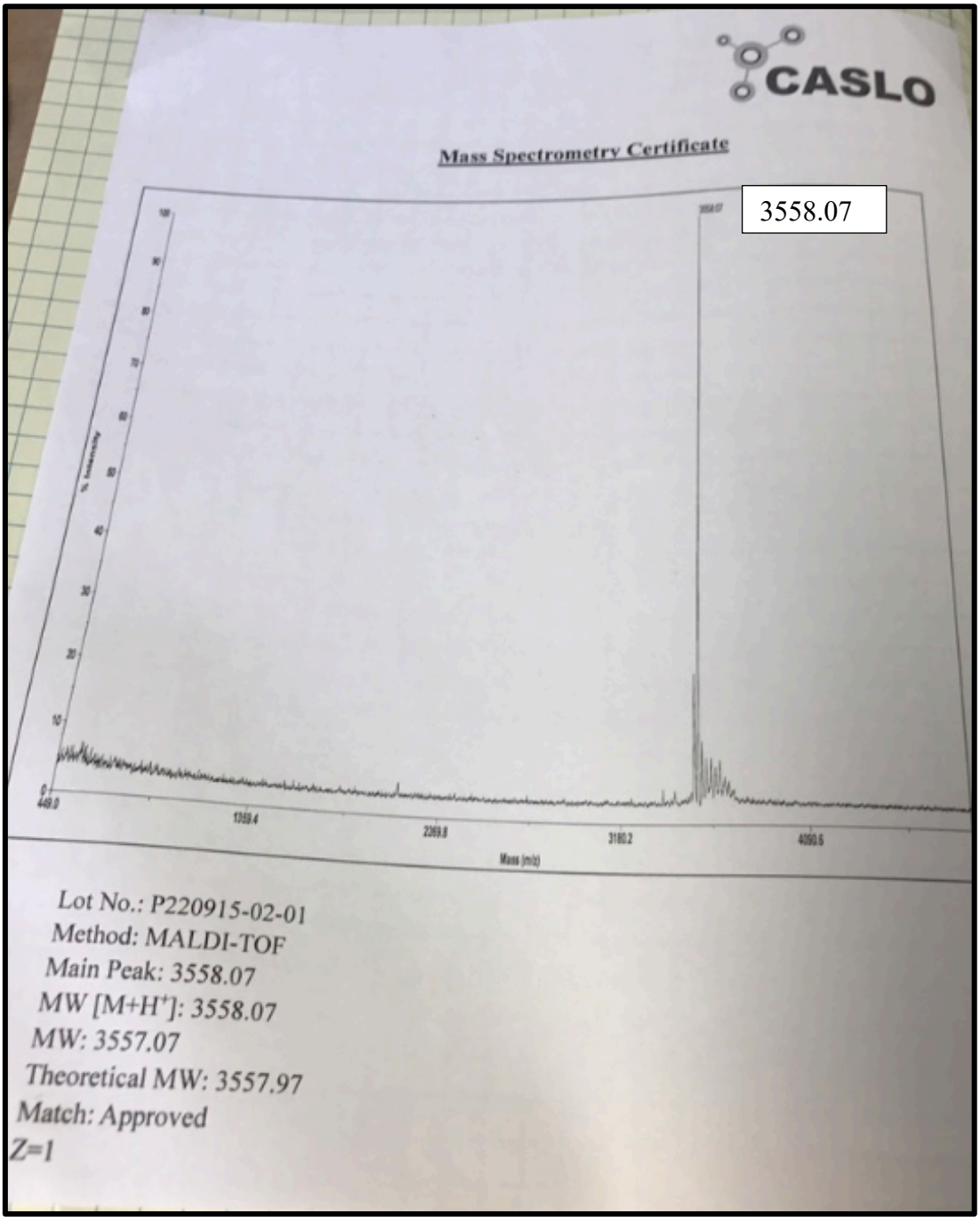



**Figure 219:** CASLO laboratory certificate of GLP-2RA analysis, showing a mass spectroscopy peak at 3558.07g/mol.

*Animals*

Animal studies were once again performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. Nine male SD rats (200-220g, Envigo) were acclimatised in the same was as above (section 3.4.1) and fed standard chow (Purina 5053). The nine rats were divided in to three weight-adjusted groups, all of which received daily subcutaneous injections for a two-week period:

- Group 1 received 0.4mg/kg Teduglutide (dissolved in 200µl PBS).
- Group 2 group received 0.4mg/kg Teduglutide (dissolved in 200µl PBS), *plus* 0.4mg/kg GLP-2 Receptor Antagonist (dissolved in 200µl PBS).
- Group 3 group received 200µl PBS alone.

Rats were weighed daily, and food weighed weekly, as per experiment timeline (**Figure 220**).

*Harvest Protocol*

*Catheter Placement*

Portal and systemic catheters were placed in the usual way (section 2.4.1). Blood glucose levels were taken at baseline only. Duodenal infusion was *not* given.

*Weighing of Tissue*

The jejunum and ileum were removed from the abdomen en-block and weighed. The first 5cm of jejunum was then removed and weighed.

*Termination of Experiment*

Each experiment was ended in the usual way; by puncturing the diaphragm with scissors and cutting the heart in two, allowing exsanguination under anaesthesia.

*Statistical Analysis*

Statistical analysis was carried out using a 2way ANOVA and Dunnett's Multiple Comparisons Test, where the two experiment groups (TED and TED + GLP-2RA) were directly compared to the control group (PBS)**.**

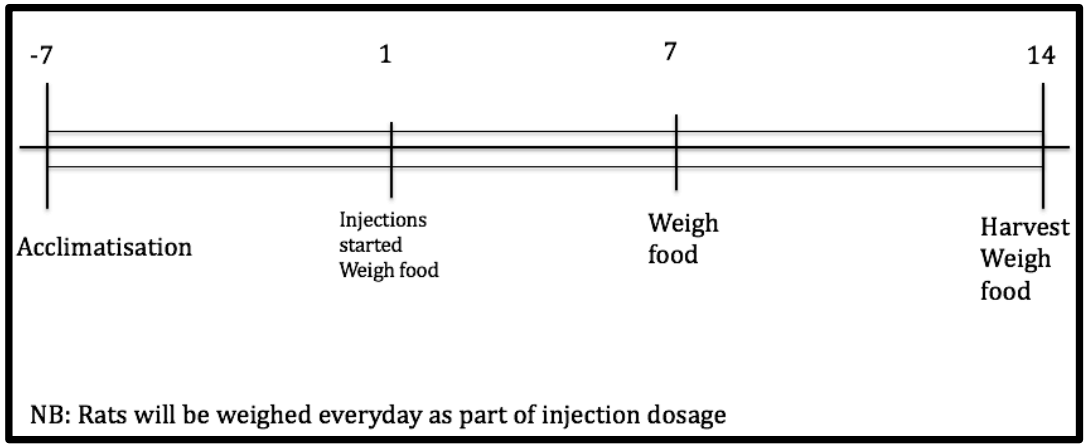



**Figure 220:** Validation Protocol 1 – Experiment timeline.

*5.3.2 Results*

Statistical analysis was carried out as in per section 5.3.1. All 9 rats survived the 2-week study period and the harvest procedure (thus, n=3 in each group). At harvest, blood had been taken for glucose levels from both catheters in the usual way. Portal blood glucose levels were highest in the TED group, followed by the TED + GLP-2RA group and then the PBS group. Unfortunately, there was a large degree of variation within each group (**Figure 221**). A similar pattern was seen within the systemic blood, with data once again showing a high degree of variation (**Figure 222**).

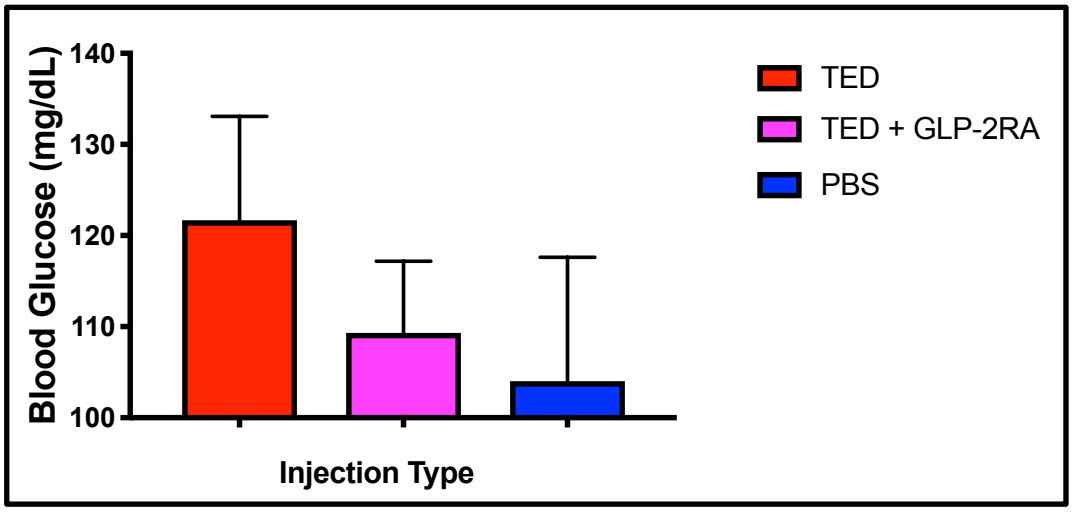



**Figure 221:** Validation Protocol 1 - Mean baseline portal glucose levels at harvest (day 14).

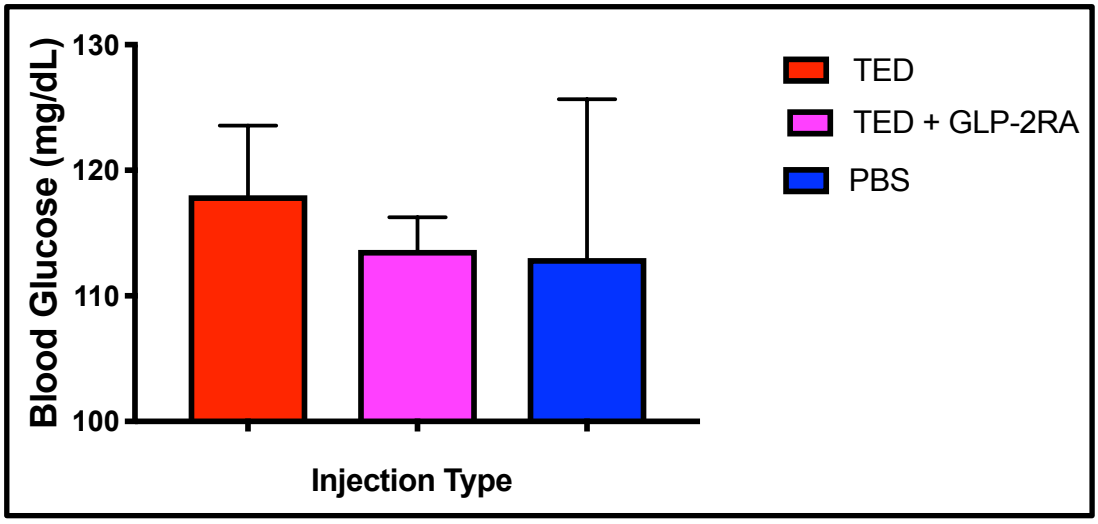



**Figure 222:** Validation Protocol 1 - Mean baseline systemic glucose levels at harvest (day 14).

Interestingly, the only positive $G_{PS}$ was within the TED group – the other two groups had a negative $G_{PS}$, with the PBS group $G_{PS}$ being more negative than the TED + GLP-2RA $G_{PS}$, although not significantly so (**Figure 223**).

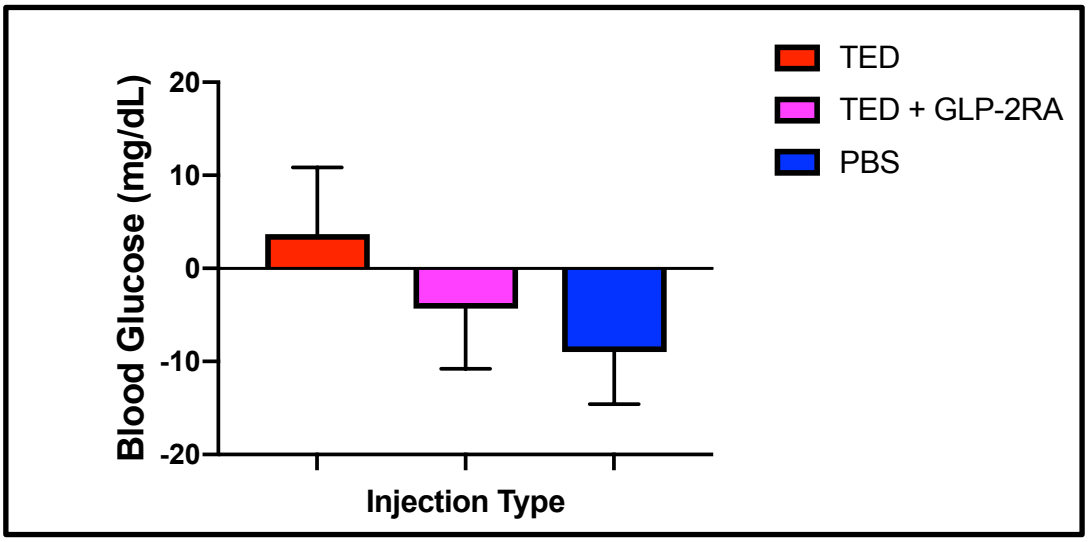



**Figure 223:** Validation Protocol 1 - Mean porto-systemic glucose gradients at harvest (day 14).

In terms of body weight, there was no significant difference between the TED group and the PBS control group, or between the TED + GLP-2RA group and the PBS control group on any day of the study (**Figure 224**). In terms of the amount of food consumed, there was no significant difference between the two experiment groups with the control group at the end of week 1 or week 2 (**Figure 225**).

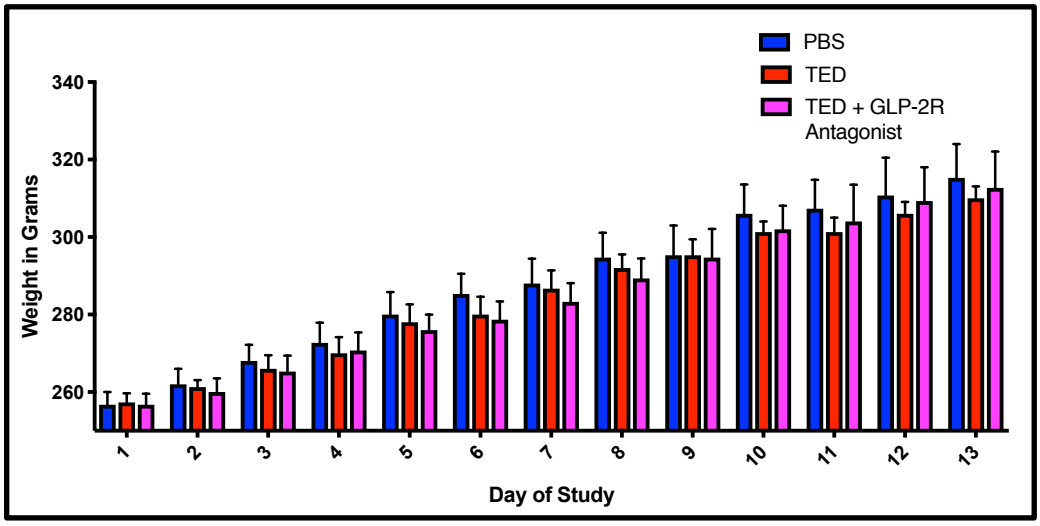



**Figure 224:** Validation Protocol 1- Mean total bodyweights throughout study.

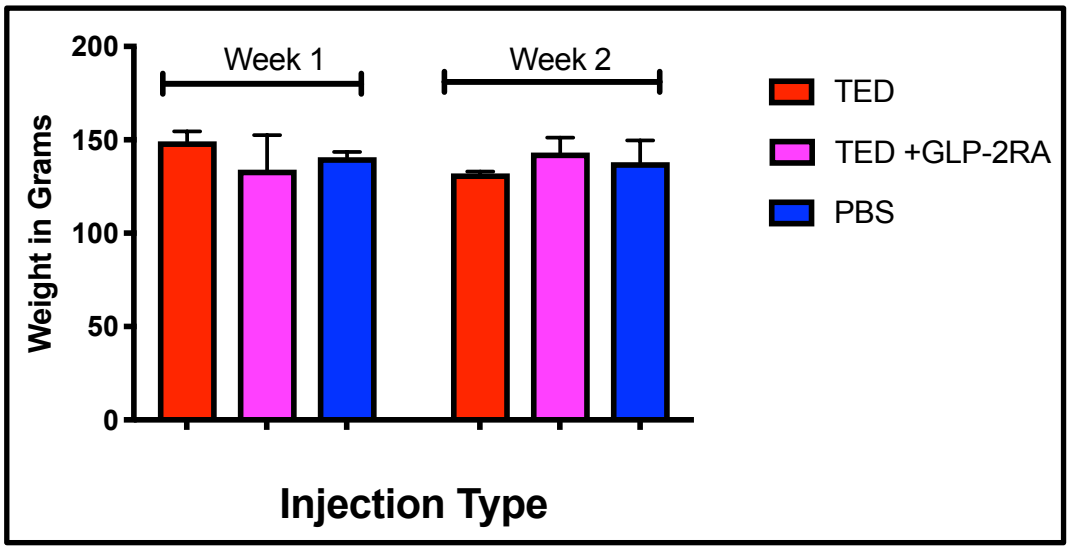



**Figure 225:** Validation Protocol 1 - Food intake during week 1 and week 2**.**

The mean combined jejunal and ileal weight of both experiment groups was found to be significantly heavier when each was compared to the PBS control group (TED: 6.13g v 5.07g, p=0.04 and TED + GLP-2RA: 6.47g v 5.07g, p=0.009) (**Figure 226**).

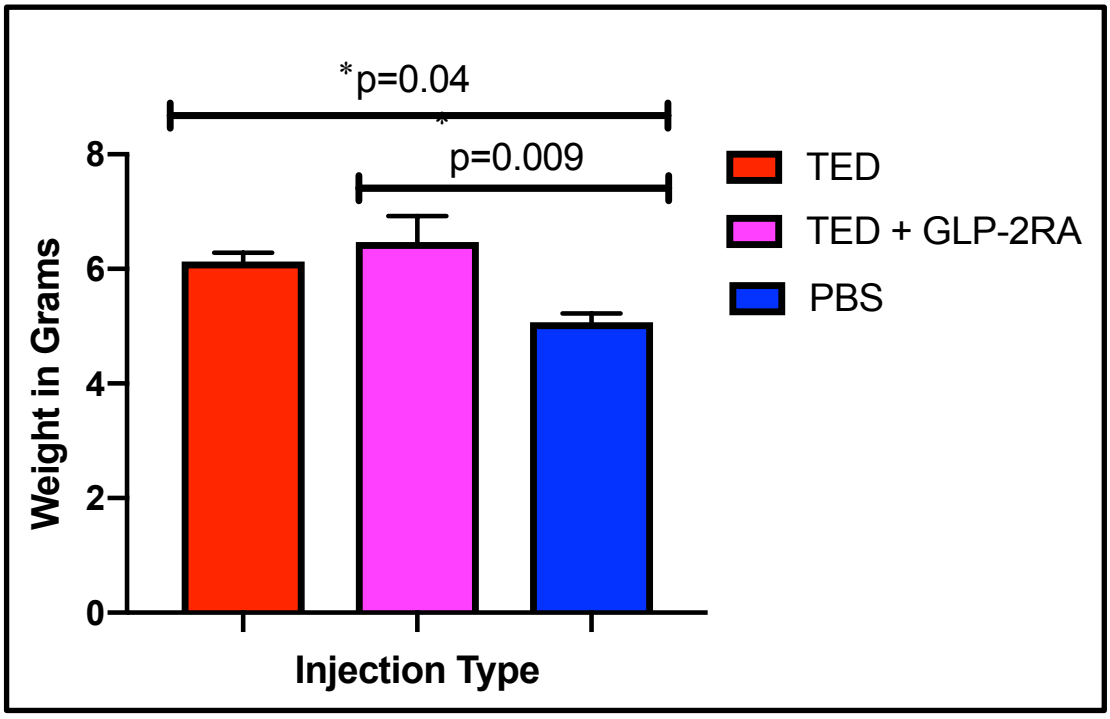



**Figure 226:** Validation Protocol 1– Combined jejunal and ileal weight.

Additionally, the first 5cm of jejunum of both experiment groups (groups 1 and 2) was found to be significantly heavier when each was compared to the PBS control group (TED: 0.34g v 0.24g, p=0.0001 and TED + GLP-2RA: 0.35g v 0.24g, p=0.0001), which corroborates the combined jejunal and ileal weight data (**Figure 227**).

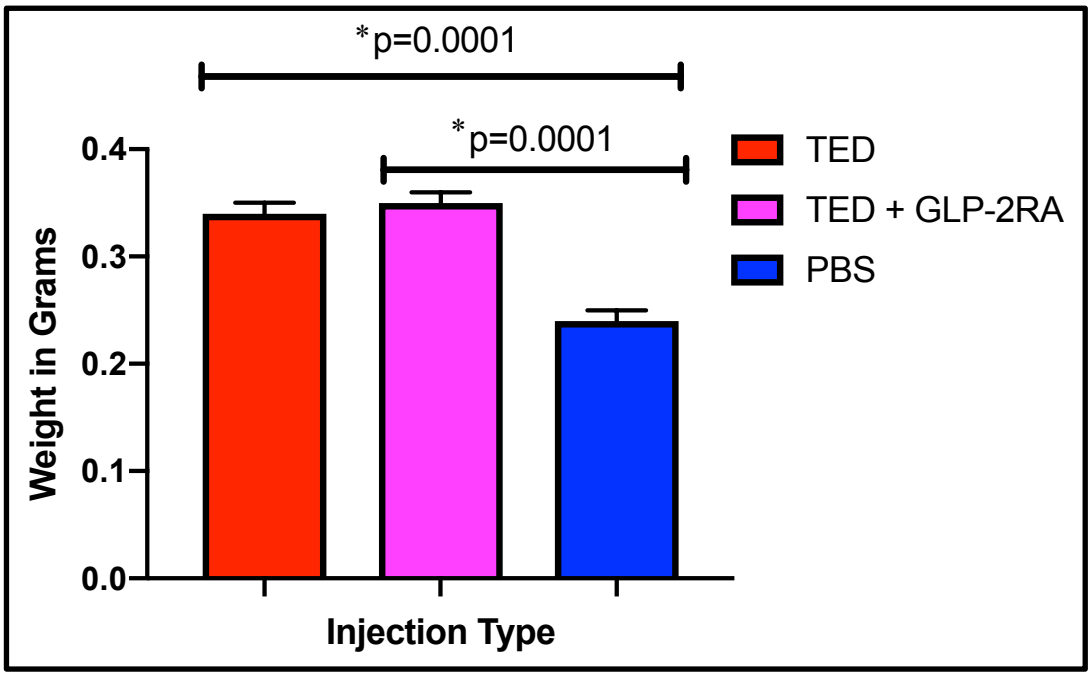



**Figure 227:** Validation Protocol 1 – 5cm jejunal weight.

*5.3.3 Discussion*

From the results of the Validation Protocol, i.e. the increased intestinal weight within the TED + GLP-2RA group, it appears that the GLP-2RA functioned more as an agonist than an antagonist. This is interesting as another group found that GLP-2RA may also act as a weak agonist at pharmacological concentrations (Thulesen, Knudsen et al. 2002). This could suggest that dosing was not optimal. An alternative explanation could be that, once again, experiments were carried out within a healthy model. A study carried out by Baldassano et al, and which had guided the decision in the use of this CASLO GLP-2RA peptide, had used HFD rats (Baldassano, Amato et al. 2016). Thus, it was felt that it was still appropriate to carry out GLP-2RA experiments at the same dose used within the Validation Protocol, but within a pathological model, that is, rats fed a HFD for 7 weeks.

## 5.4 EXPERIMENT 2 - ALTERED BILIO-PANCREATIC LIMB LENGTH

### *5.4.1 Overview*

Assess the glycaemic effects of altering BP limb length, with concurrent GLP-2RA injections in pathological Sprague-Dawley (SD) rats, fed a high fat standard diet.

### *5.4.2 Hypothesis*

It is hypothesised that BP limb length modulates the anti-diabetic effect of RYGB. Additionally, it is hypothesised that these changes may be inhibited or reduced with the concurrent use of a GLP-2RA, indicating a role for GLP-2 in improving glucose tolerance.

### *5.4.3 Materials and Methods*

#### *Teduglutide Receptor Antagonist*

The results from the Validation Protocol seem to suggest that, in a healthy model at least, the GLP-2RA was working as more of an agonist. However, given the encouraging results gained from using a pathological model within Aim 2, the decision was made to use it in a pathological rat model with this experiment to see if TED conferred a positive on glucose intolerance. The GLP-2RA used was from the same batch previously provided by CASLO.

#### *Animals*

Animal studies were performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. 14 male SD rats (200-220g, Envigo) were acclimatised for one week under a 12:12 light: dark cycle (lights-

on at 07.00) with *ad libitum* access to standard chow (5053, Purina). All rats were then switched to HFD (D12492i, Research Diet) for 7 weeks.

*Surgical Groups*

At 7 weeks, rats were divided into three weight-adjusted groups:

Group 1: RYGB surgery with a BP limb length of 20cm (control) – n=5

Group 2: RYGB surgery with a BP limb length of 40cm – n=4

Group 3: RYGB surgery with a BP limb length of 40cm *plus* daily GLP-2RA subcutaneous injections from day of surgery – n=5

Two BP limb lengths were fashioned: 16 cm or 36 cm from the ligament of Treitz to JJ anastomosis so, with a duodenal length of 4cm, the overall BP length was 20cm (Group 1) and 40cm (Groups 2 and 3), respectively. Total small intestinal length was 90cm. Apart from the BP limb length modifications described, all rats underwent RYGB surgery as described above (section 4.1.3). Three operations were carried out per day, with rotation of groups in order to account for diurnal variation. Post-operatively, all rats were fed Dietgel® Recovery for 5 days and switched back to HFD from day 6.

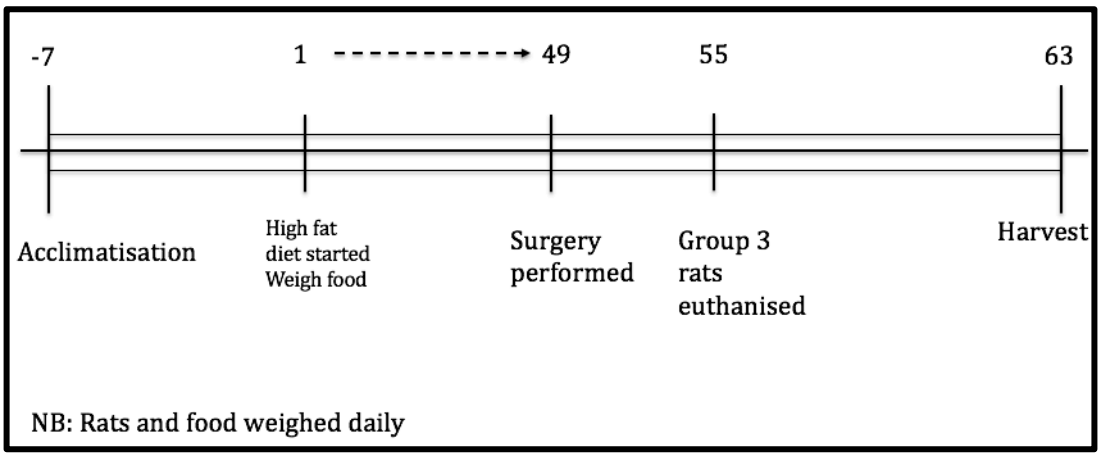



**Figure 228:** Experiment timeline.

*Harvest Protocol*

*Catheter Placement*

Portal and systemic venous blood was sampled as in section 2.4.1, before and after an intestinal glucose infusion, and over a 60-minute time-frame. Harvest GTT involved giving a 25% glucose solution infused over 5 minutes into the Roux limb.

Blood analysis, morphological preparation, weighing of tissue and termination of experiment was carried out as in section 2.4.1.

*5.4.4 Results*

Statistical analysis was carried out as per section 2.4.1 and section 5.3. All 14 animals survived the initial RYGB surgery. However, at day 6 post-surgery, something unexpected happened – 3 of the GLP-2RA daily injection group rats (out of 5) were moving noticeably slower than the other two rats in the group, as well as compared to rats in the other two groups. It was observed that there was an absence of normal behaviour, and the concern was that they were in pain.

Signs that a lab rat are in pain include depression (with reluctance to move or difficulty in getting up), recumbency (for longer than usual), abnormal appearance (head down, tucked abdomen, hunched, facial distortion, or pallor), along with vocalisation (when palpated for example). Buprenorphine was administered, and the rats were flagged up to the animal facility vet. However, by day 7 post-surgery it was clear that the rats were very unwell and so, as per experimental protocol, the decision was made to euthanise them. The following was found at post-mortem:

**Rat 1** – Enlarged gastric pouch with what appeared to be a sealed perforation, along with ischaemic bowel from the GJ anastomosis down to large bowel.
**Rat 2** – Enlarged gastric pouch, with multiple adhesions, but no evidence of perforation or bowel ischaemia
**Rat 3** – Normal post-surgical abdomen – no reason found to explain post-operative demise.

Given the amount of work that goes into feeding, operating on, injecting and caring for lab rats, this type of outcome is both frustrating and upsetting. The difficult

decision to euthanise the two other rats in the group at day 7 was made as, even though they were perfectly well post-op, there was no longer a reason to keep them alive. Fortunately, all rats within the other two groups survived until harvest day, at the 2-week time point (BP limb length 20cm, n=5 and BP limb length 40cm, n=4).

At harvest, blood was taken for glucose levels from both catheters in the usual way. Portal blood glucose levels were matched at baseline between the two groups, but then significantly lower in the 40cm BP limb length at the 15-, 30- and 60-minute time points, with the most significant difference seen at 60 minutes (391.0mg/dl v 473.1mg/dl, p=0.003) (**Figures 229 and 230**).

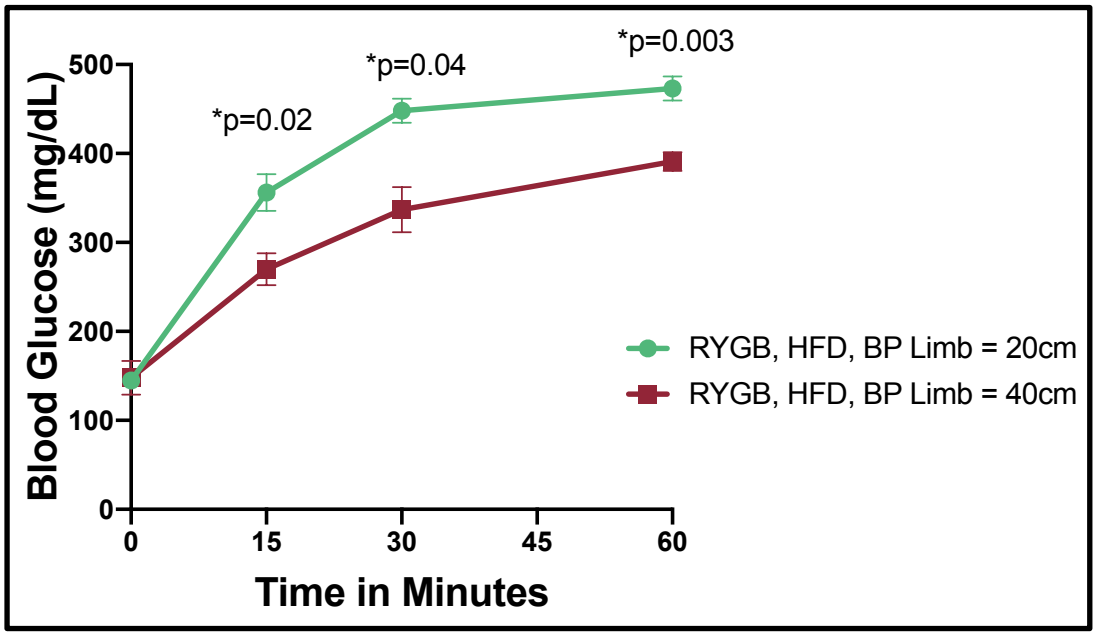



**Figure 229:** Mean portal glucose levels at harvest GTT (day 14).

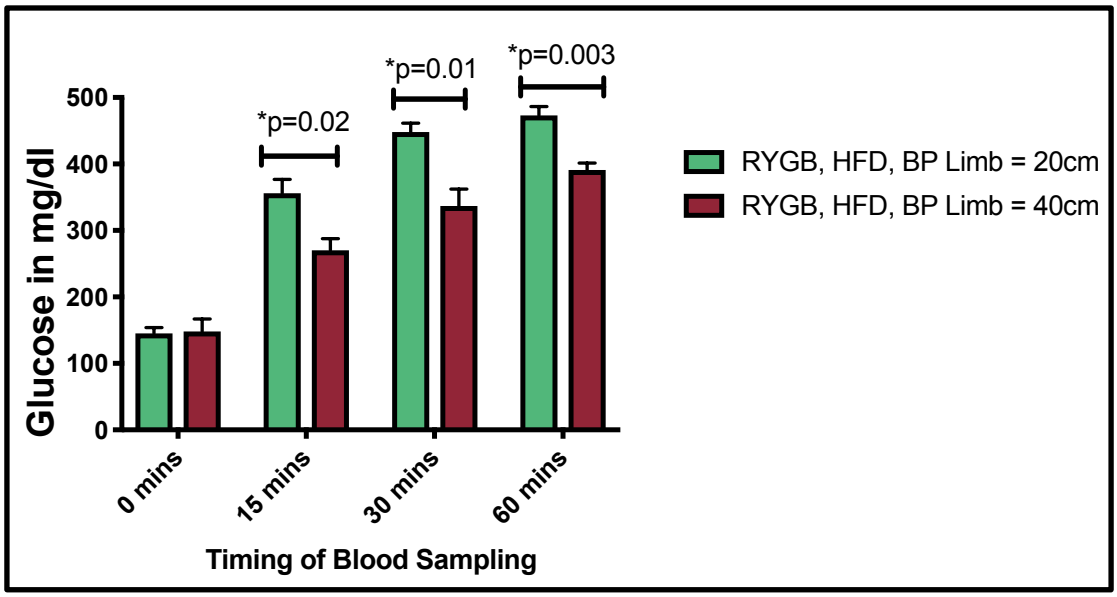



**Figure 230:** Mean portal glucose levels at harvest GTT (day 14), displayed in bar-chart format.

Systemic blood glucose levels were also matched at baseline and followed a very similar pattern to the portal blood glucose data, with significant difference between the two points at 15, 30 and 60 minutes. Again, the most significant difference was seen at the 60-minute time point (312.1mg/dl v 438.2mg/dl, p=0.004) (**Figures 231 and 232**).

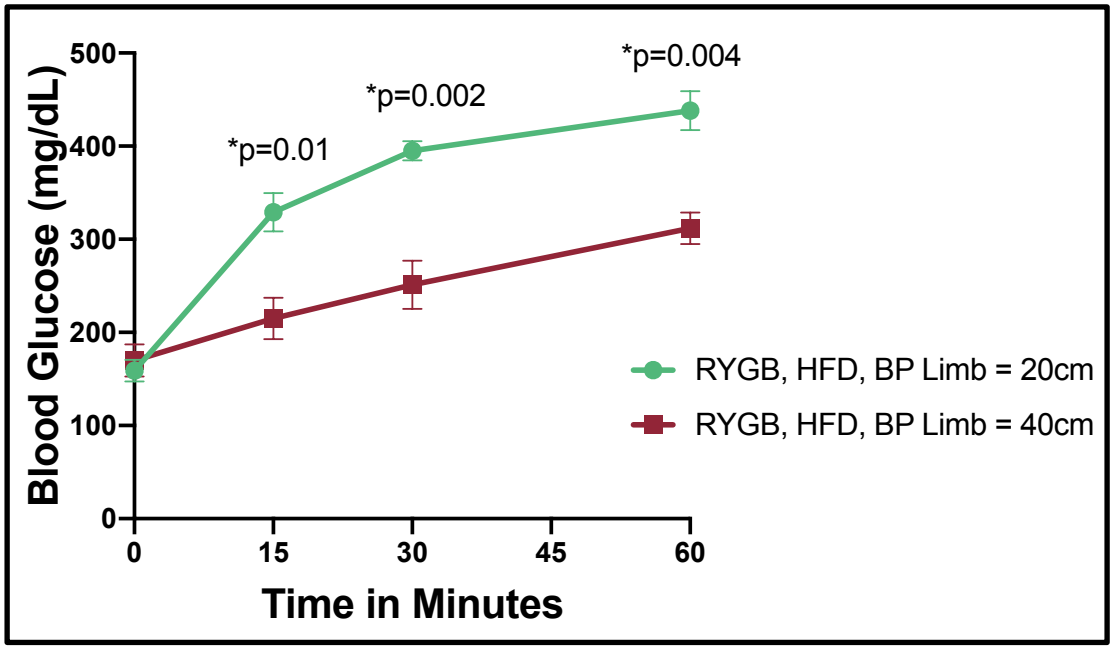



**Figure 231:** Mean systemic glucose levels at harvest GTT (day 14).

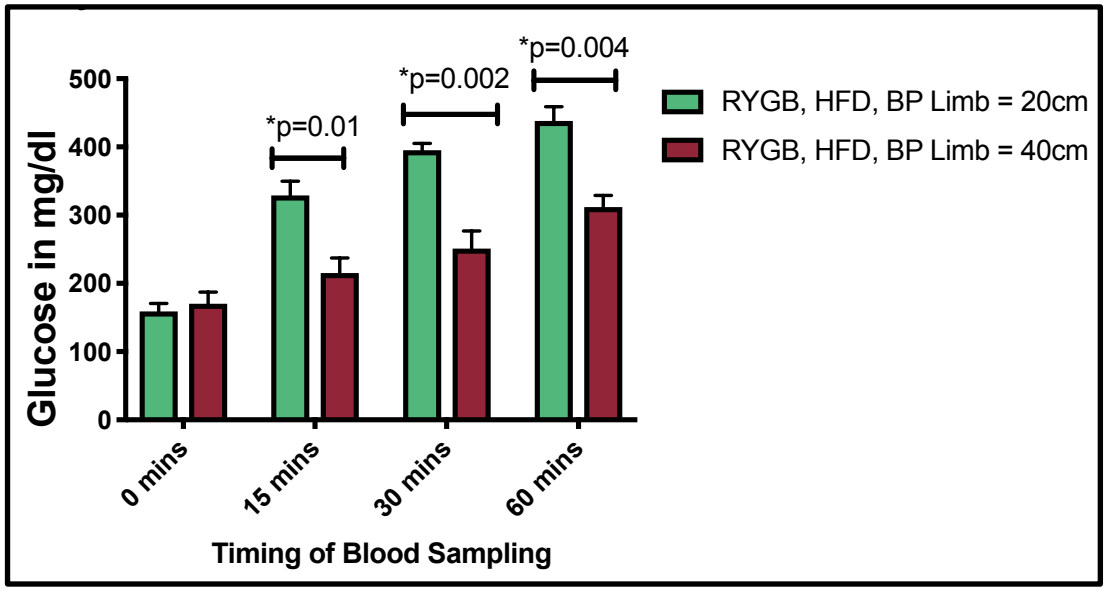



**Figure 232:** Mean systemic glucose levels at harvest GTT (day 14), displayed in bar-chart format.

$G_{PS}$ was significantly more positive in the 40cm limb length group compared to the 20cm BP limb length group, and at 15-, 30- and 60-minute time points, with the most significant difference seen at 60 minutes (79.3mg/dl v 35.1mg/dl, p=0.01) (**Figures 233 and 234**).

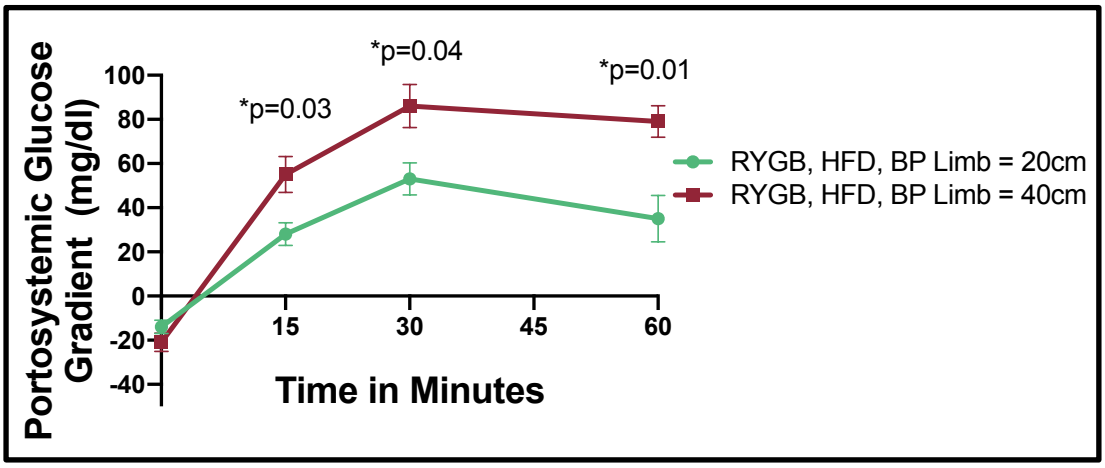



**Figure 233:** Mean porto-systemic glucose gradient at harvest GTT (day 14).

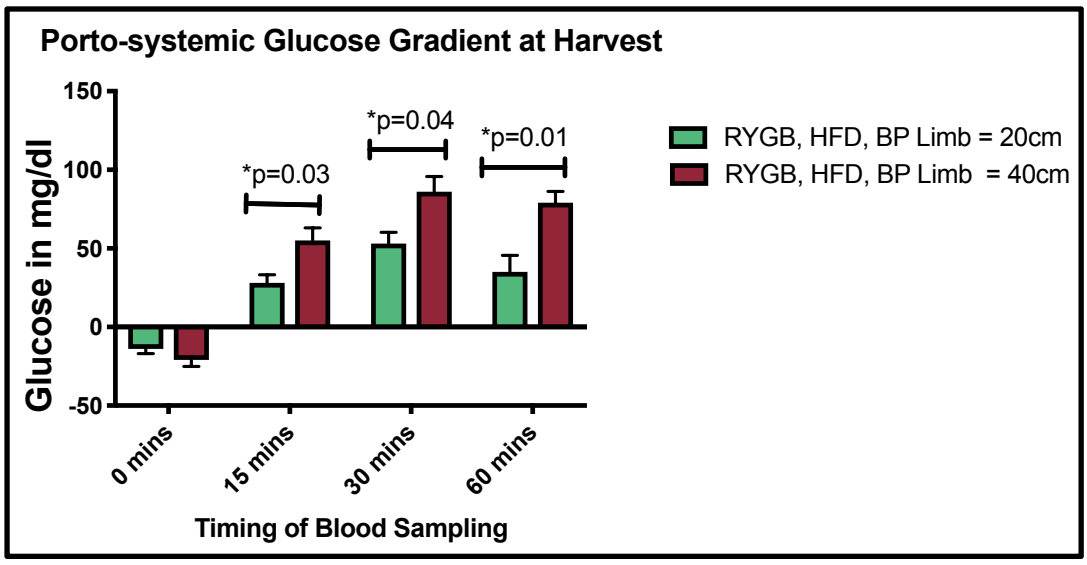



**Figure 234:** Mean porto-systemic glucose gradient at harvest GTT (day 14), displayed in bar-chart format.

There was no significant difference in baseline glucose utilisation between the two groups (**Figure 235**).

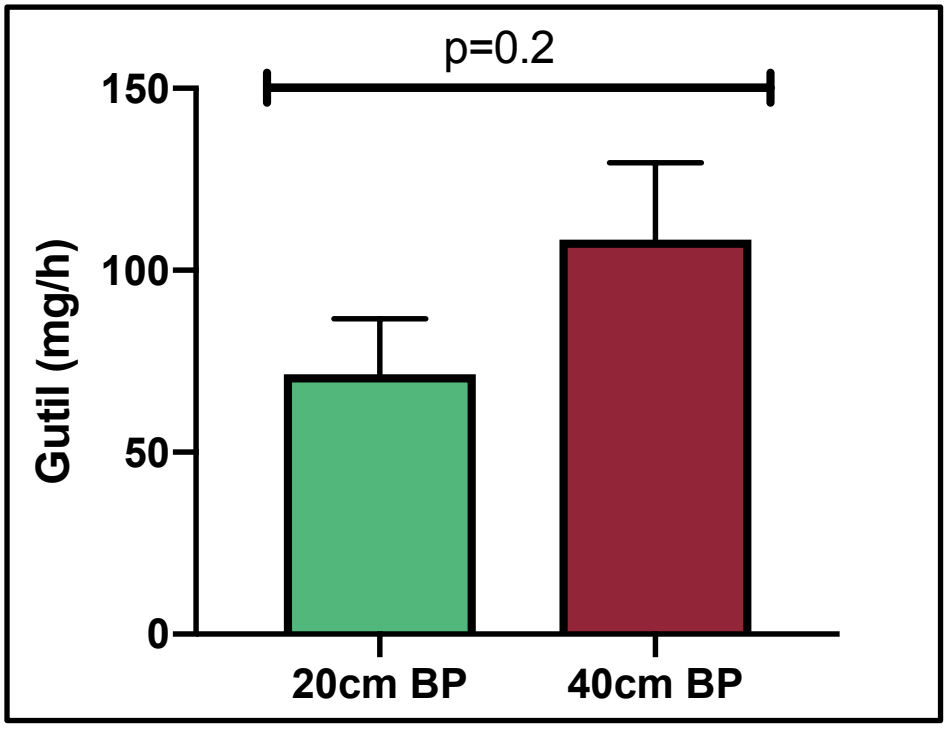



**Figure 235:** Intestinal glucose utilisation.

Glucose absorption was found to significantly increased in the 40cm BP limb length group (329.3mg/h v 178.7mg/h, p=0.02) (**Figure 236).**

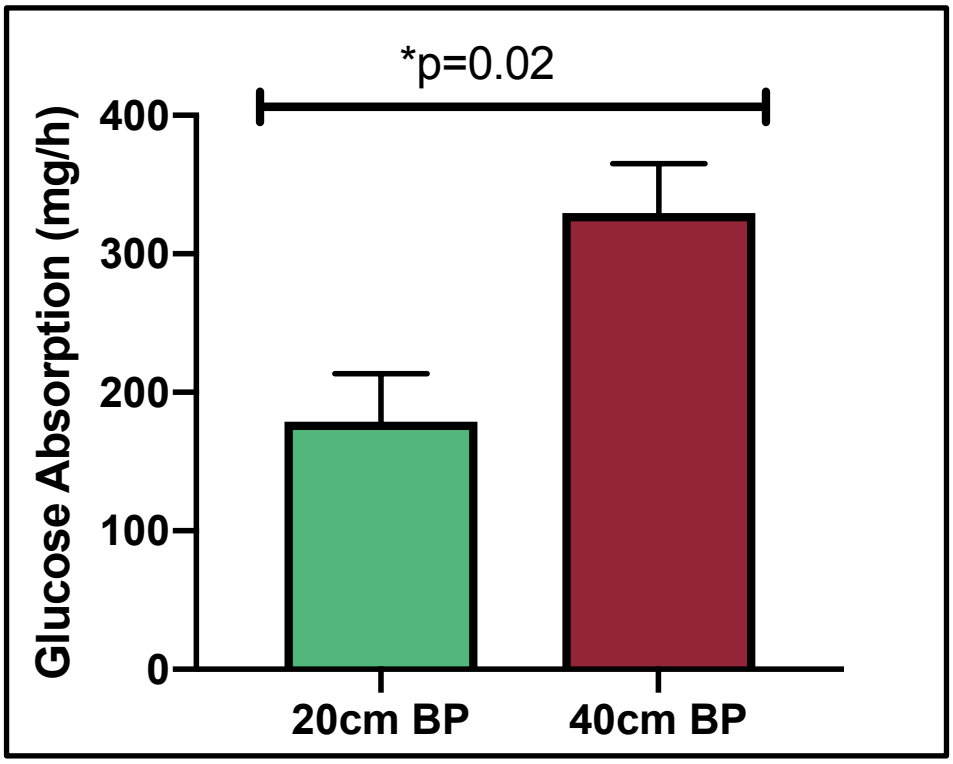



**Figure 236:** Intestinal glucose absorption.

Looking at mean rat weight across the three groups, from day 2 post-op there was already a noticeable (although, not significant) difference in body weight between the 20cm BP limb length (control) group and the two 40cm BP limb length groups. From day 3, the two 40cm BP limb length groups also seemed to be separating out, with the group receiving the daily GLP-2RA injections weighing less than those not being injected. As mentioned above, the 40cm BP limb length + GLP-2RA group was removed from the study at day 7. Up and until day 7 there was no significant difference between the control group (20cm BP limb length) and either the 40cm BP limb length group or the 40cm BP limb length + GLP-2RA injection group. By day 13, however, there was a significant difference between the two remaining groups, with the 40cm BP limb length group weighing significantly less than the 20cm BP limb length group (352.5g v 383.0g, p=0.04) (**Figure 237**).

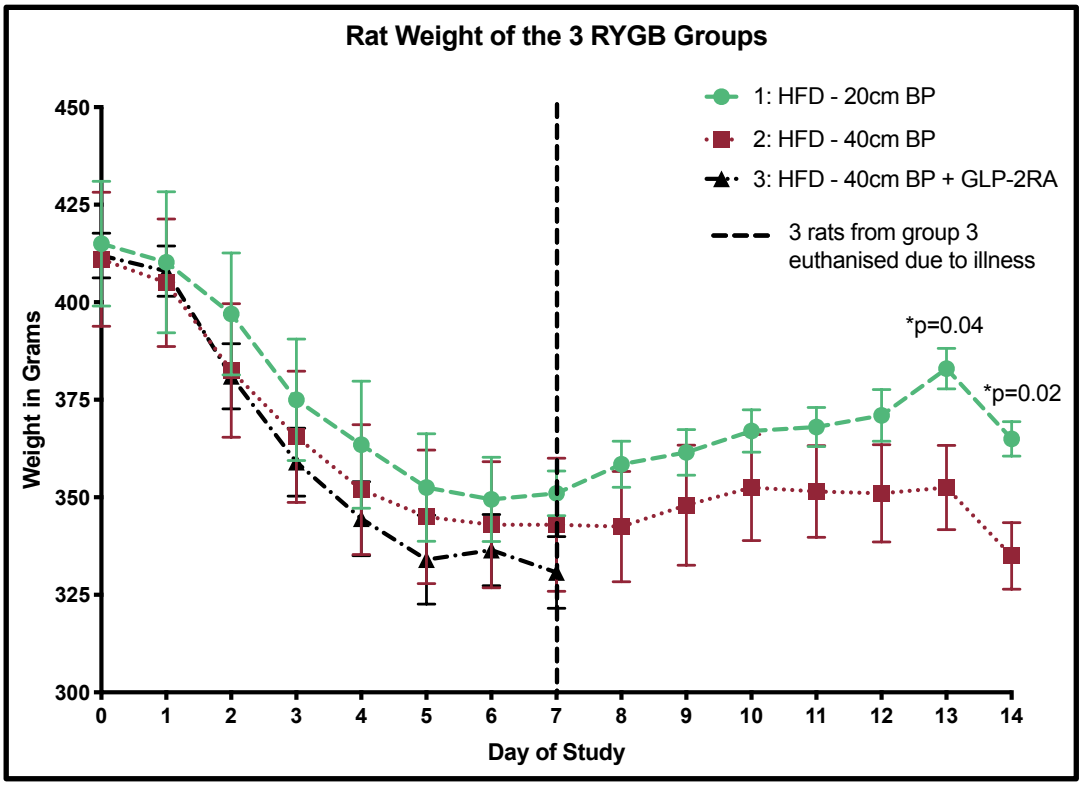



**Figure 237:** Mean rat bodyweights throughout the study.

The mean total weight of small intestine within the 40cm BP limb length group compared to the 20cm group was not found to be significantly higher (**Figure 238**). However, when the data was analysed so that the intestinal weight was calculated as a % of total body weight i.e. taking account of the size of the rat (given that the 40cm group rats weighed significantly less at harvest) the difference between the two groups became significant, with total small intestinal weight significantly greater in the 40cm group (2.37% v 1.72%, p=0.008) (**Figure 239**). Thus, all further analysis is presented as weight-adjusted data.

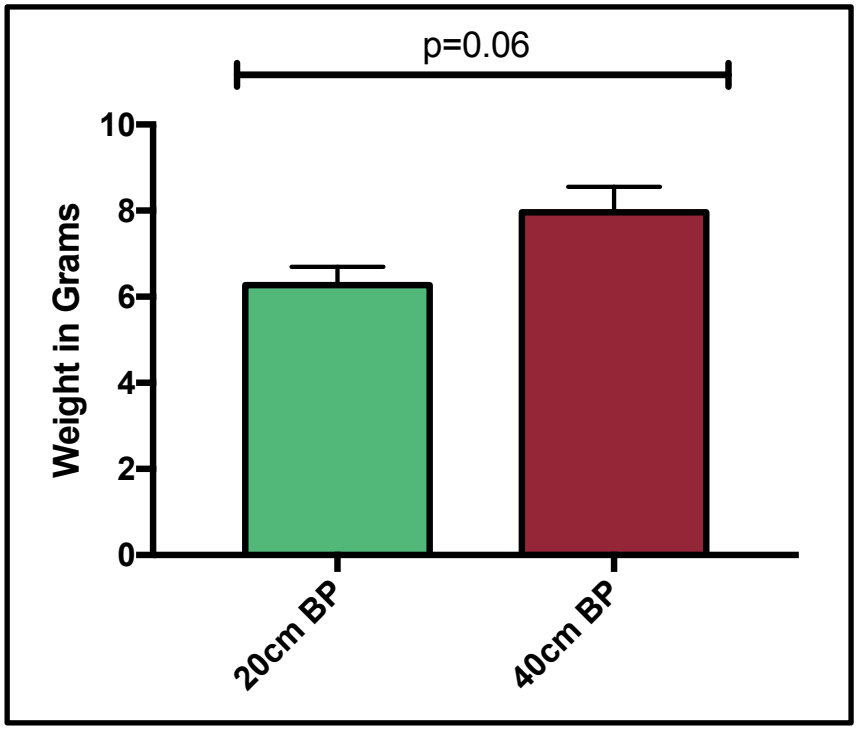



**Figure 238:** Total small intestinal weight.

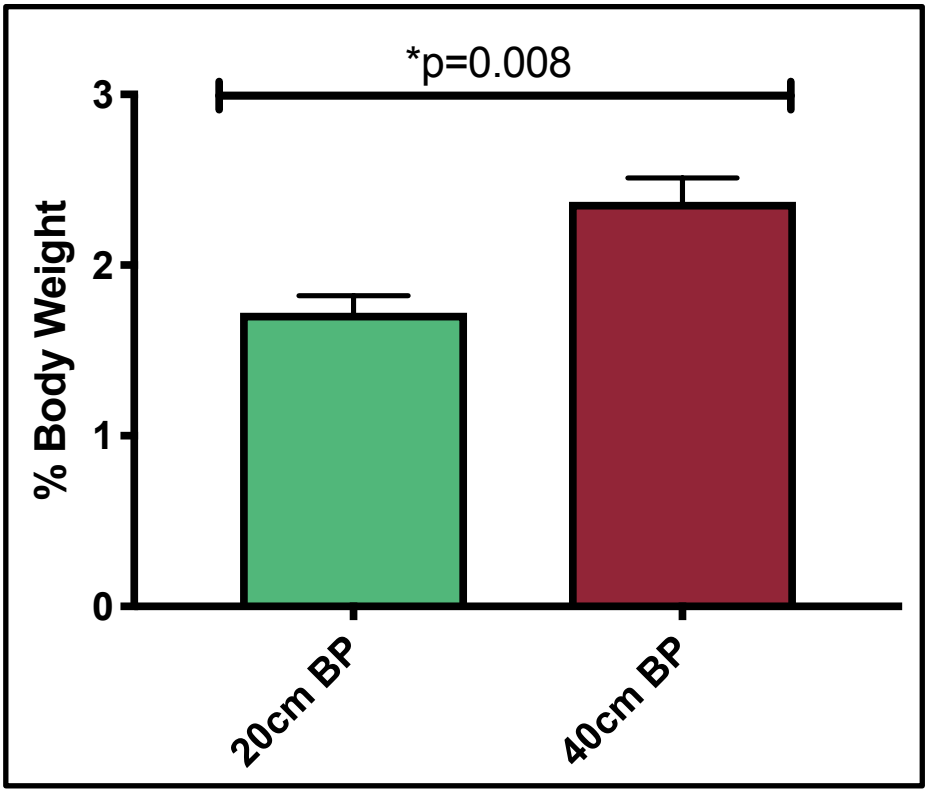



**Figure 239:** Total small intestinal weight, expressed as % bodyweight.

When the first 5cm of each type of limb segment (Roux, biliopancreatic, common), along with the last 5 cm of terminal ileum, were weighed and calculated as a % of total body weight, there was a significant increase in weight in the Roux limb in 40cm BP limb length rats (0.39% v 0.24%, p=0.03) (**Figure 240**) and in the BP limb (0.27% v 0.14%, p=0.01) (**Figure 241**).

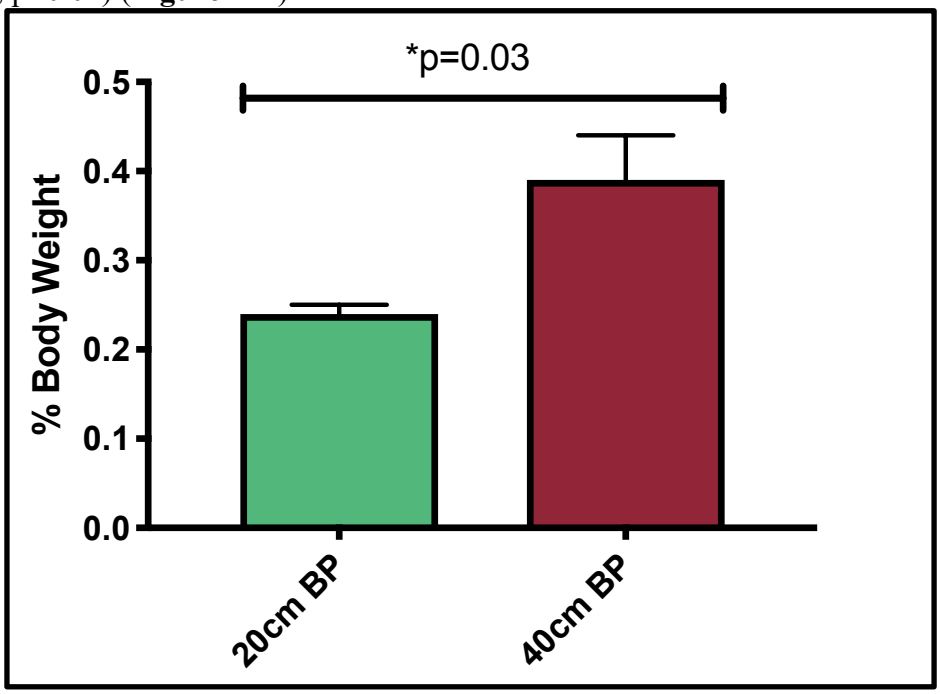



**Figure 240:** 5cm Roux limb weight, expressed as % bodyweight.

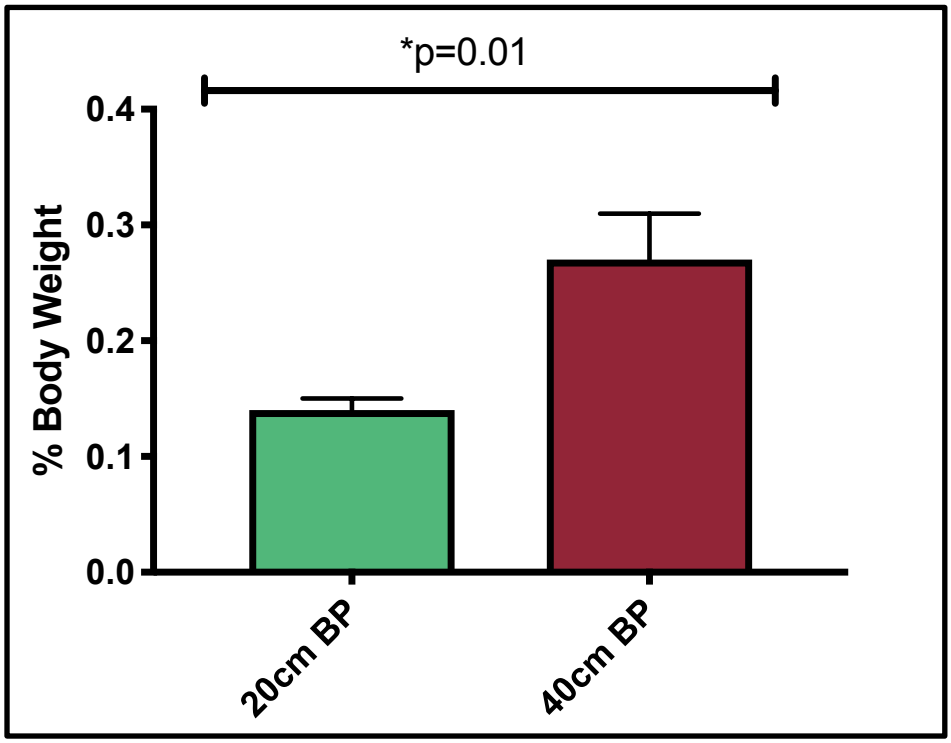



**Figure 241:** 5cm BP limb weight, expressed as % bodyweight.

Statistical significance was just missed regarding the common limb (0.23% v 0.16%, p=0.07) (**Figure 242**). There was no difference observed regarding the terminal ileum (**Figure 243**).

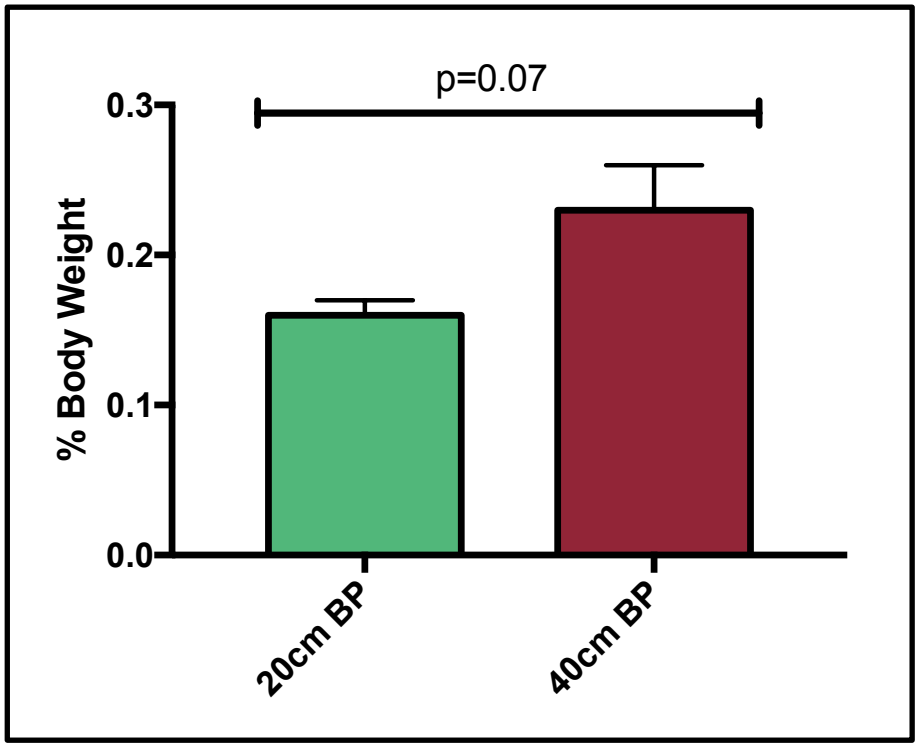



**Figure 242:** 5cm common limb weight, expressed as % bodyweight.

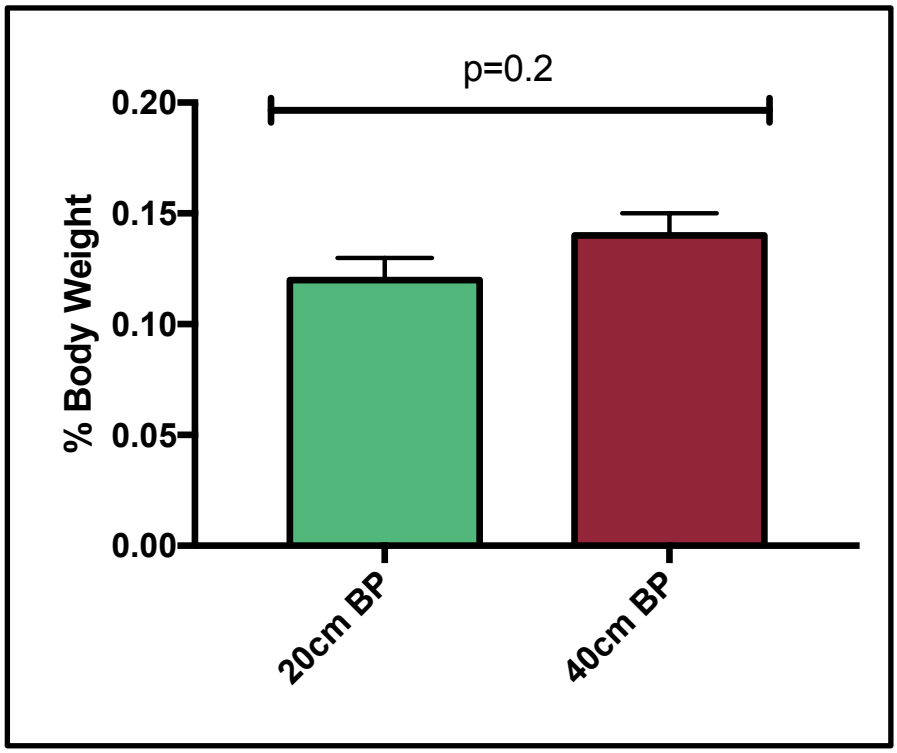



**Figure 243:** 5cm terminal ileum weight, expressed as % bodyweight.

**Figure 244** shows how each 5cm intestinal segment (weight-adjusted) compares between the 40cm BP limb length group and the 20cm BP limb length group.

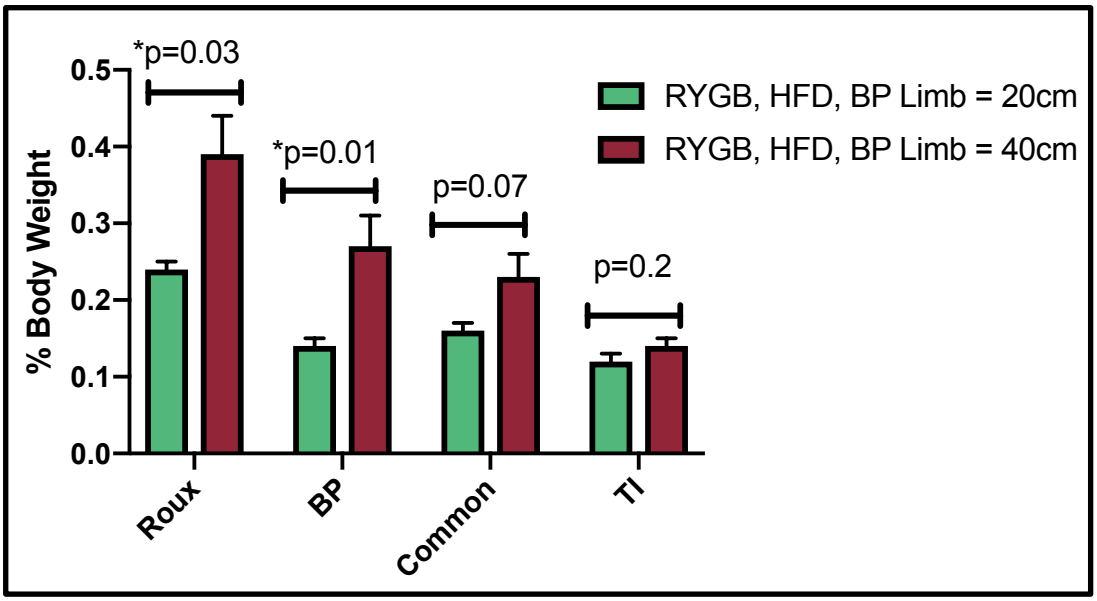



**Figure 244:** Comparison of 5cm intestinal segment weights, expressed as % bodyweight.

*5.4.5 Discussion*

Results from Experiment 1 (the first part of Aim 4), which aimed to study the effects of GLP-2RA on RYGB, in terms of glycaemia and hypertrophy, were disappointing. The Validation Protocol showed that the GLP-2RA was having the opposite effect to the desired one. Nevertheless, this had been within a healthy model. Interestingly, all rats injected with the GLP-2RA within the Validation Protocol survived. The decision was made to enter the main experiment to see if a HFD pathological model altered effects of the GLP-2RA peptide.

Within a pathological HFD model, and post-RYGB, GLP-2RA injected rats did not fare as well as those in the Validation Protocol study, as 3 out of the 5 rats had to be euthanised at day 7 post-op. This clearly could have been due to chance, after all, RYGB in a rat is a precarious procedure. Additionally, these rats became unwell 12-24 hours after restarting solid chow, and so arguably were at greatest risk of either obstruction (secondary to anastomotic stenosis) or anastomotic leak. However, it is also possible that the peptide had a deleterious effect on the rats, or that, because GLP-2RA injected rats within the Validation Protocol also received concurrent TED, they were protected from potential harmful effects of GLP-2RA injection. Other studies have looked at a potential protective role of GLP-2 in obese animals. Genetically obese mice were found to have islet dysfunction, endotoxaemia and hepatic inflammation when endogenous GLP-2 signaling was blocked (Cani, Possemiers et al. 2009). Additionally, mice fed a HFD who were chronically treated with GLP-2RA (also purchased from CASLO Laboratory) were found to have aggravated glucose metabolism, with GLP-2RA increasing glucose intolerance, insulin levels and reducing sensitivity to insulin, thus providing evidence for a

protective role for endogenous GLP-2 (Baldassano, Rappa et al. 2015). Interestingly, this group did not choose to validate the peptide, solely relying on purity, mass, sequence and HPLC analysis provided by CASLO. Significantly, they did not report any adverse outcomes within the GLP-2RA injected mice.

In order to gain as much data as possible (in the limited time available), BP limb length studies within HFD fed rats were performed. It was hoped that use of the GLP-2RA to demonstrate blockade of any limb length benefit could have been studied, however, due to the failure of response (and with potential adverse outcomes in a HFD model) observed in Experiment 1 of this aim, the decision was taken not to use the peptide in Experiment 2.

Experiment 2, BP limb length studies, was proposed because although RYGB is an extremely effective intervention for those with obesity and T2D, some patients do not achieve remission, whilst others relapse. It was hypothesised that increasing intestinal limb lengths of RYGB may improve outcomes. Additionally, there is limited data available to help establish optimum length of intestinal limbs within an RYGB model. One randomised controlled trial showed that longer BP and Roux limbs in T2D superobese patients led to a higher rate of T2D remission (Pinheiro, Schiavon et al. 2008). In this study, lengths of both Roux and BP limbs were varied at the same time, hence making it difficult to ascertain the anatomical basis. Additionally, a case report showed that revising BP limb length in recurrent T2D led to remission (Kao, Lo et al. 2012). Additionally, T2D relapse and weight regain have resulted in different types of surgical revision following RYGB, and these include shortening the common limb (Caruana, Monte et al. 2015), lengthening the BP limb (Chaux, Bolaños et al. 2015),

and lengthening the Roux limb (Spivak 2015). Indeed, even for primary RYGB, representing a much larger patient group, intestinal limb lengths have not been standardised. This group is varied, including diabetic and non-diabetic patients, with a wide BMI range and multitude of comorbidities. This heterogeneity suggests that customising the surgery based on patient characteristics could improve overall outcomes (Pal, Rhoads et al. 2018).

Studies have suggested that longer Roux limb in super obese patients leads to a modest weight loss advantage but this is short-lived (Stefanidis, Kuwada et al. 2011). The role of BP limb and impact on weight loss and diabetes remission is however less well studied. Therefore, this experiment sought to identify physiological changes within rats bypassed with differing BP limb lengths. Rats in the 40cm group showed a significantly improved glycaemic profile from 15 to 60 minutes within the harvest GTT. Additionally, these rats had significantly heavier total small intestinal weight, with the Roux limb and BP limb segments significantly heavier in the 40cm BP limb length. Work carried out by other members of this lab had shown similar results but had demonstrated it within a healthy SD rat model (Pal, Rhoads et al. 2018). In 20cm and 40cm BP RYGB rat models, they found a significant increase in Roux limb weight at 2 and 4 weeks compared to SHAM control, but no increase in BP limb weight in the RYGB groups. In fact, they found a tend towards a decrease in BP limb weight in the RYGB groups compared to SHAM. These findings were echoed by the work shown within Aim 3 of this body of work (**Figure 218**). In SD fed rats, bypassed using 20cm BP limb lengths, the BP limb weight was no different to the comparative site within SHAM rats. The only method variable between the work done by Pal et al, the work in Aim 3 and the work in this chapter is the model of rat used i.e. a pathological rat model was used in this aim, with a healthy rat model used in the other

two studies. These findings indicate that a pathological model could be responsible for the histological changes observed.

It was encouraging to see that (a) the RYGB HFD model was successful in terms of the significantly reduced bodyweights and significantly increased Roux limb histological measurements within the 40cm BP limb length group and that (b) the glycaemic benefits of lengthening BP limb also appeared to be present within a pathological HFD model. Pal et al suggested that early post-RYGB, longer BP limb length reduces intestinal glucose absorption and that, later, increased glucose utilisation, Roux limb hypertrophy, and increased hormone secretion are the sustained effects of a long BP limb. In Experiment 2, glucose absorption was found to significantly increase within the longer BP limb group, which is in opposition to colleague data. However, glucose utilisation was found to have increased in the longer limb group, although not significantly. These results go some way to supporting data gained from Aim 2, Experiment 2 (HFD TED studies), which showed a trend towards both glucose absorption and utilisation and Aim 3 (RYGB versus SHAM), which showed significantly increased glucose utilisation.

# CHAPTER 6

## 6.1 AIM 5: ACUTE PROXIMAL JEJUNAL EXCLUSION

- Assessment of physiological changes following acute proximal-jejunal exclusion to mimic RYGB in a non-RYGB surgical model.

### *6.1.1 Overview*

Roux-en-Y gastric bypass (RYGB) alters intestinal anatomy so that undigested nutrients are delivered directly to the distal jejunum. This modification, along with exclusion of the foregut and its luminal sensors, is thought to contribute to improvements in glucose homeostasis. The effect of infusing a glucose bolus into proximal and distal jejunal segments was assessed in order to evaluate the subsequent impact on glucose absorption. Using the lab catheter technique, both fasted and postprandial portal and systemic venous blood was sampled, and from glucose values measured, a portosystemic gradient calculated. Key gastrointestinal hormones were also measured in order to identify how hormonal secretion may alter depending on location of glucose infusion, and thus impact glucose homeostasis.

### *6.1.2 Hypothesis*

Acute proximal jejunal exclusion confers anti-diabetic effects of RYGB in a non-RYGB model.

### *6.1.3 Methods and Materials*

#### *Animals*

Animal studies were performed in accordance with protocols prospectively approved by the Harvard Medical Area Standing Committee on Animals. 14 male SD rats (200-220g, Envigo) were acclimatised for one week under a 12:12 light: dark cycle (lights-on at 07.00) with *ad libitum* access to standard chow (5053, Purina).

#### *Infusion Groups*

Rats were then divided in to two groups:

- Group 1: Rats received a glucose bolus into proximal jejunum, just distal to the duodeno-jejunal junction - a point in the jejunum equivalent to the biliopancreatic limb in a RYGB rat model.
- Group 2: Rats received a glucose bolus into mid-jejunum, 26cm from the DJ junction - a point equivalent with the Roux limb in a RYGB rat model.

#### *Catheter Placement*

Portal and systemic catheters were positioned in the usual way (see section 2.4.4). The jejunal catheters were placed as in **Figure 245** and secured in place with silk suture.

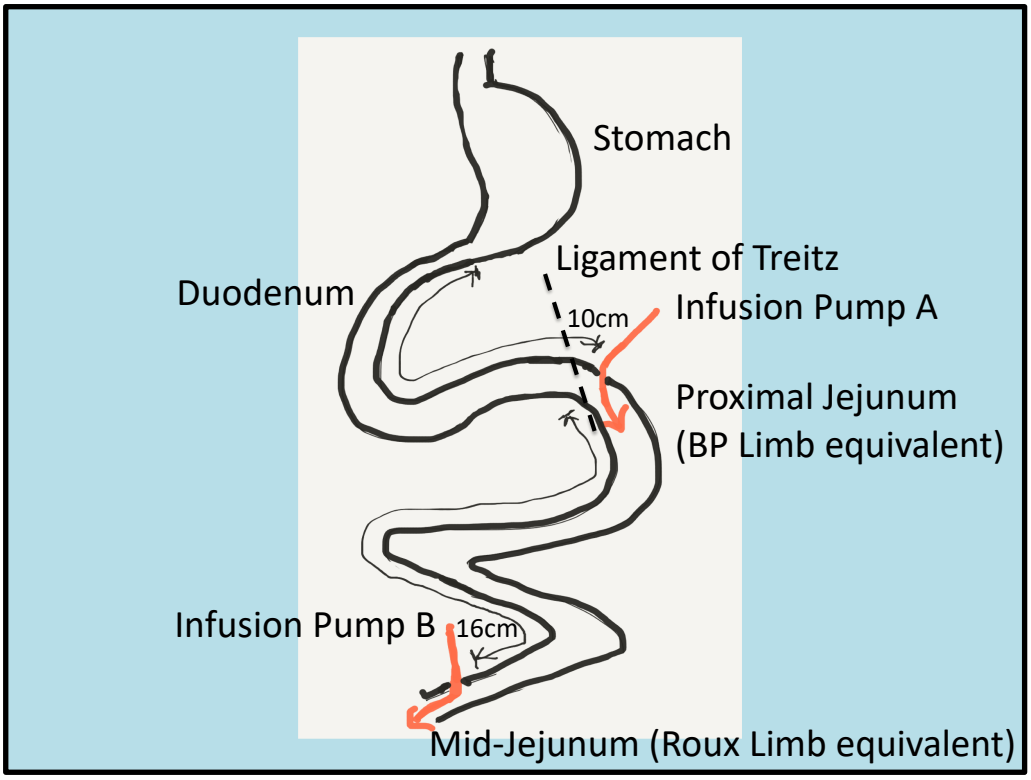



**Figure 245:** Diagrammatic representation of the anatomical setup.

*Blood Analysis*

Portal and systemic venous blood was sampled at baseline, and over a period of 120 minutes after an intestinal glucose infusion (glucose bolus of 2mg/g, given over 5 minutes). Glucose and gastrointestinal hormone (GLP-1, GIP, glucagon, insulin and C-peptide) levels were measured. Portosystemic glucose ($G_{PS}$) gradient was calculated ($G_{PS} = G_P - G_S$, where $G_P$ is portal glucose level and $G_S$ is systemic glucose level at a given time point), and this was used to demonstrate changes in intestinal glucose utilisation and absorption.

*Harvest Protocol*

*Luminex*

Two EMD Millipore Corporation Rat Metabolic Magnetic Bead Panel 96-well plate were purchased. Prior to the harvest procedure taking place DPP-IV Inhibitor and Aproptonin was added to each eppendorph. Luminex was carried out as described in **Appendix 2**.

*6.1.4 Results*

Statistical analysis was carried out as per section 2.4.1. Peak portal glucose levels and peak systemic glucose levels were both lower during mid-jejunal infusion compared to proximal jejunal infusion, with peak portal just missing out on significance (423.1mg/dl v 330.8mg/dl, p=0.06) (**Figure 246**).

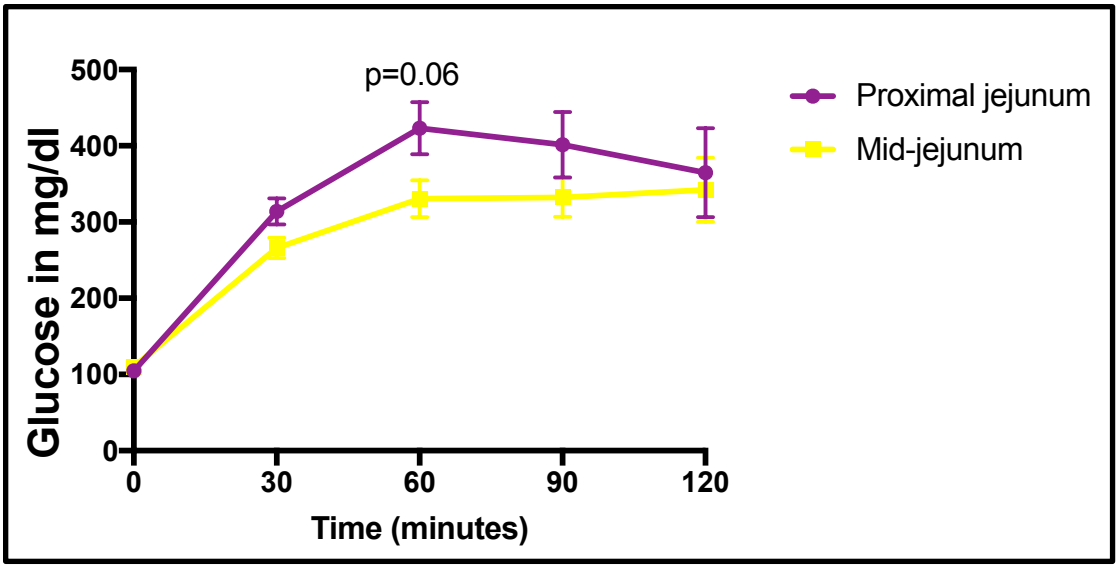



**Figure 246:** Mean portal glucose levels at harvest GTT.

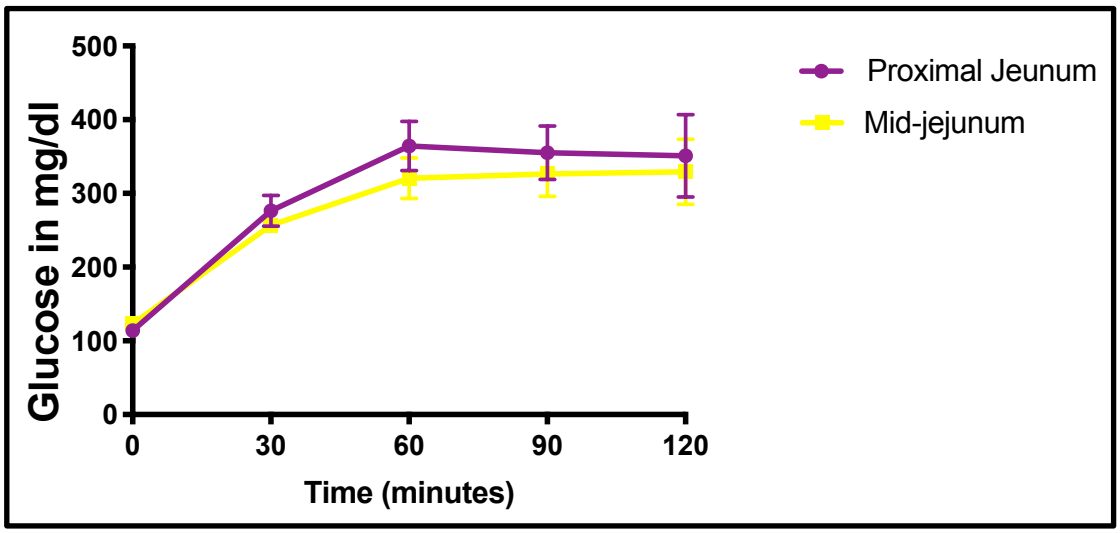



**Figure 247:** Mean systemic glucose levels at harvest GTT.

Difference in portosystemic gradient between the two groups reached significance at 60 minutes and 90 minutes (p=0.01 in both cases) (**Figure 248**).

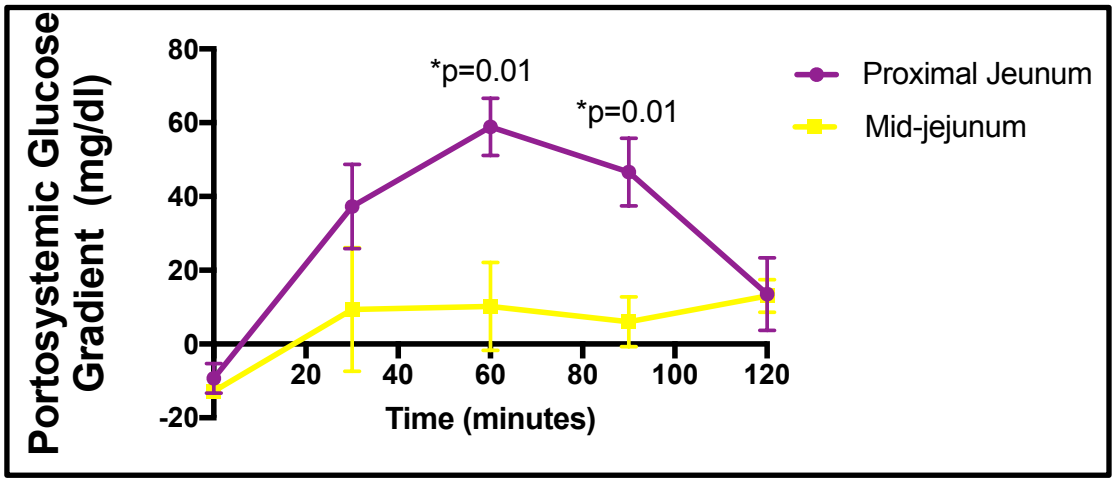



**Figure 248:** Mean porto-systemic glucose gradient at harvest GTT.

There was no significant difference in baseline glucose utilisation between the two groups (**Figure 249**).

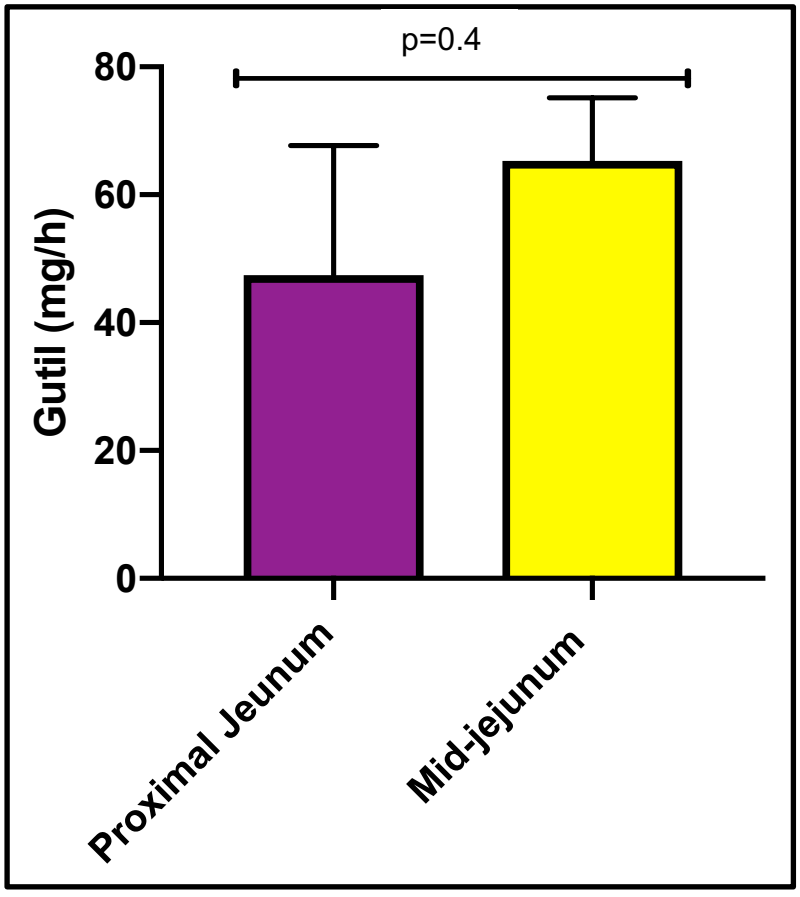



**Figure 249:** Intestinal glucose utilisation.

Glucose absorption was found to be significantly increased in the proximal jejunal group (374.2mg/h v 84.3mg/h, p=0.02) (**Figure 250).**

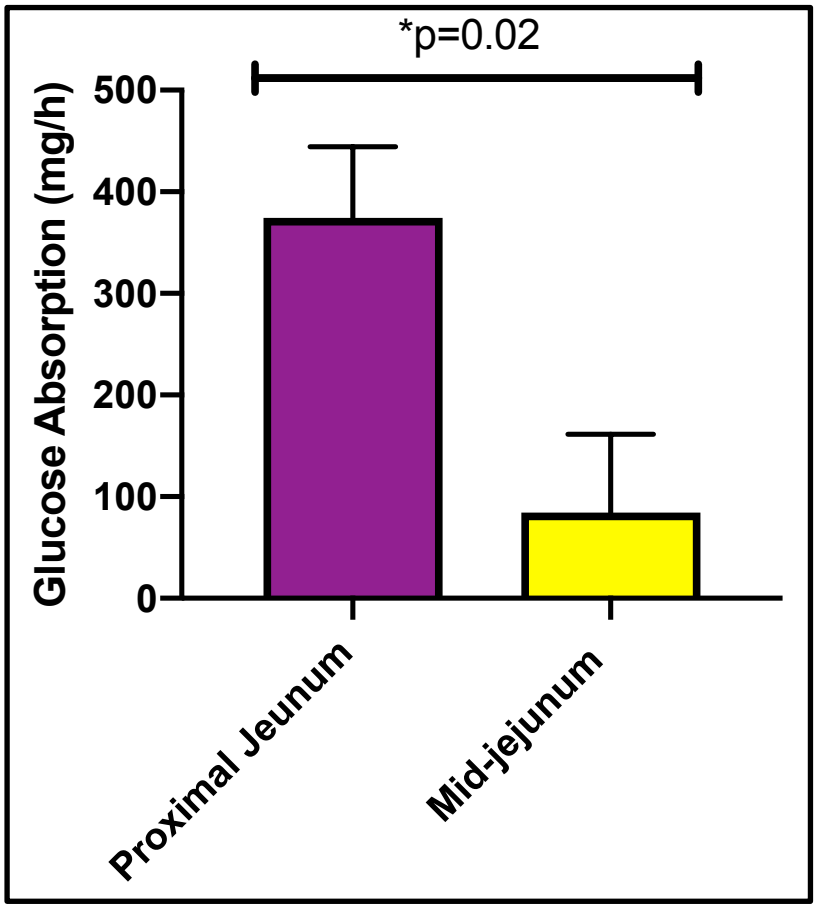



**Figure 250:** Intestinal glucose absorption.

Insulin portal vein levels were significantly lower (p=0.02) in the mid-jejunal infusion group at 60 minutes, compared to the proximal jejunal infusion group (**Figure 251**).

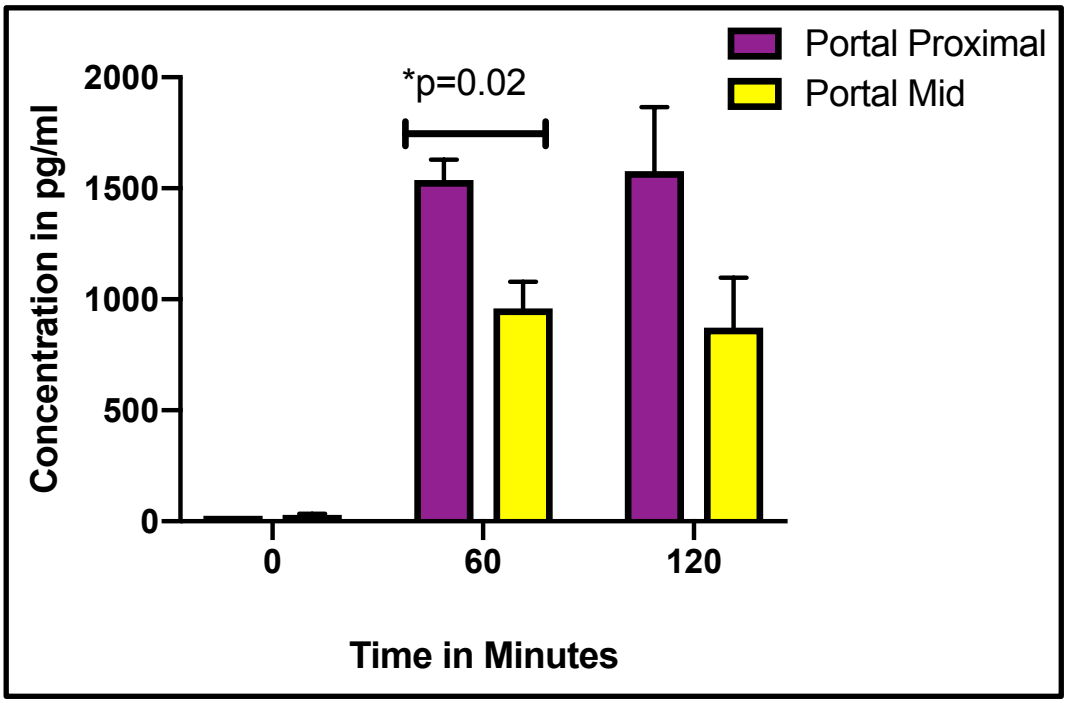



**Figure 251:** Portal insulin levels.

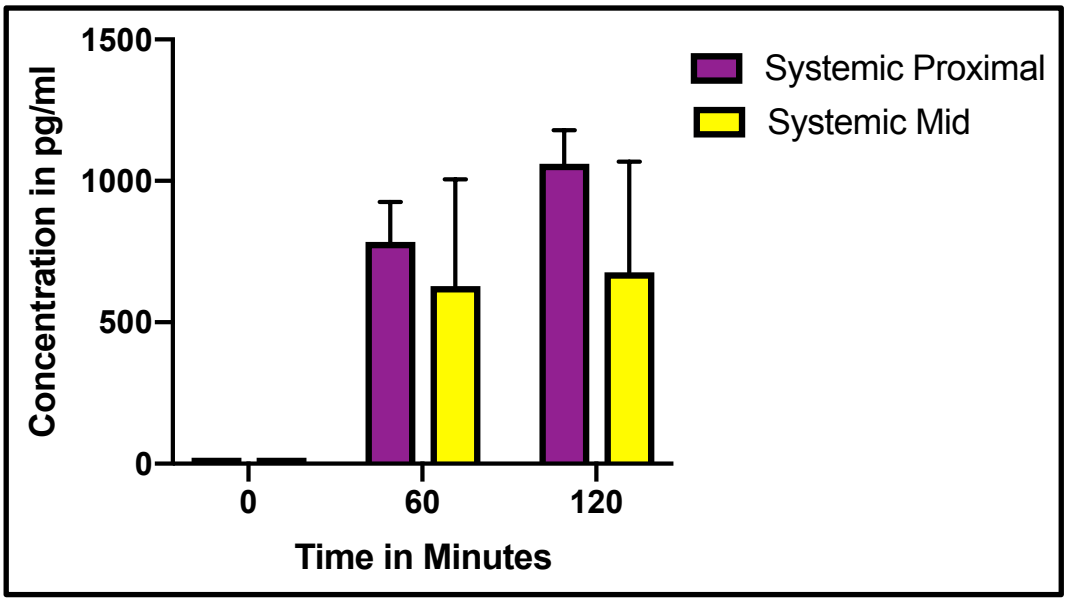



**Figure 252:** Systemic insulin levels.

Portal and systemic C-peptide levels were both significantly lower in the mid-jejunal infusion group and both 0 and 60 minutes (**Figures 253 and 254**).

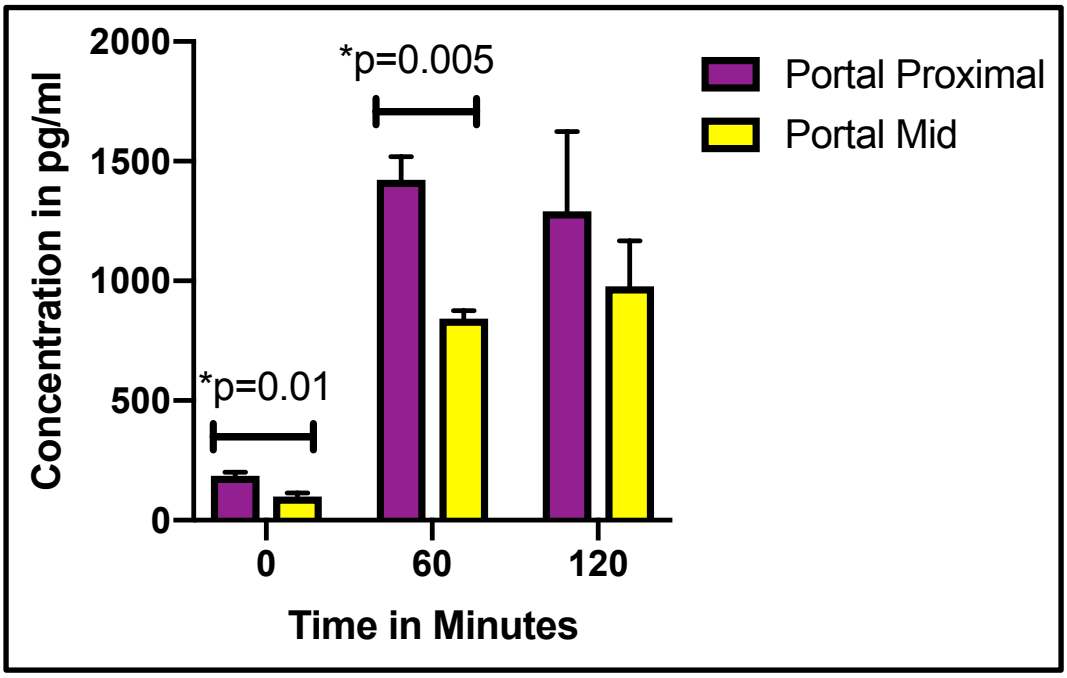



**Figure 253:** Portal C-peptide levels.

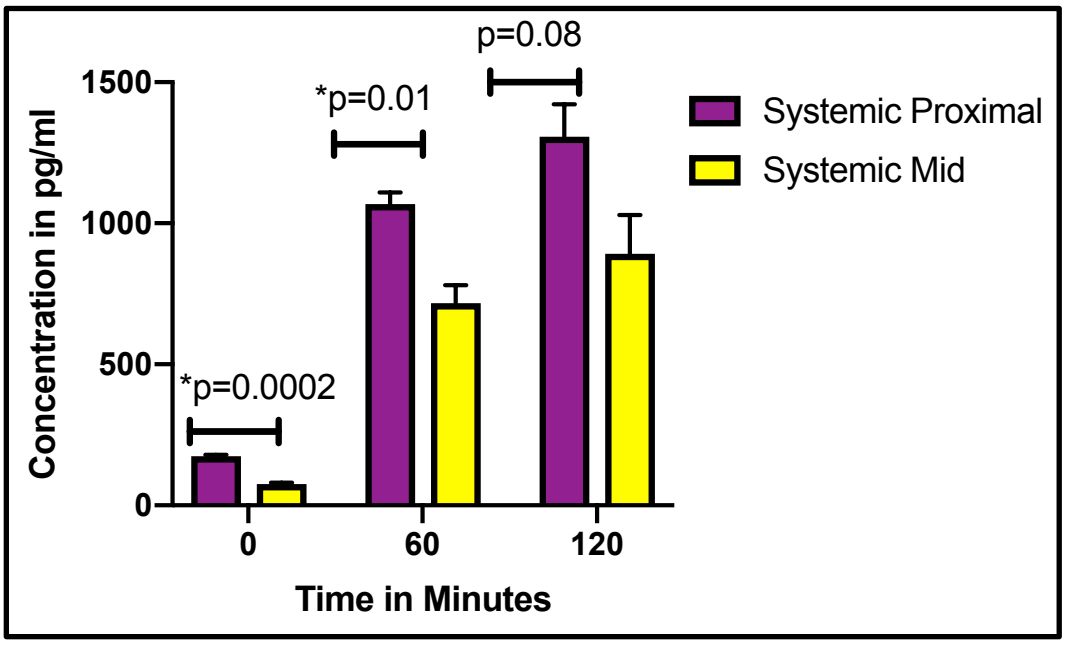



**Figure 254:** Systemic C-peptide levels.

Although GLP-1, GIP and glucagon all followed this trend, that is, levels of each hormone were lower in the mid-infusion group, there was no significant difference between the two groups, in either the portal or systemic blood (**figures 255, 256, 257, 258, 259 and 260**).

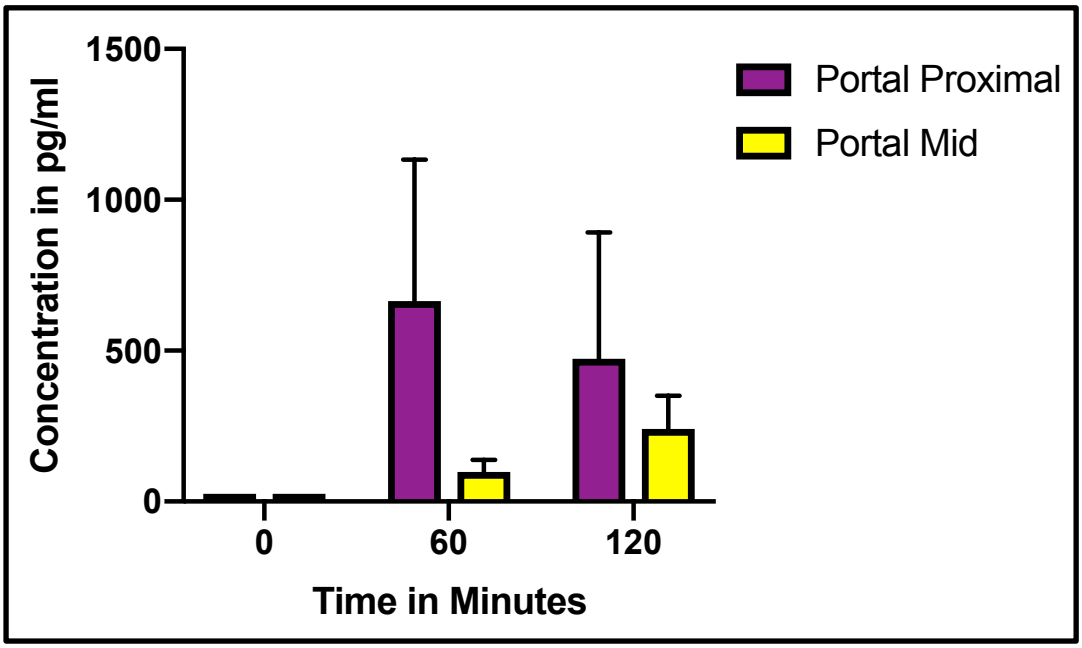



**Figure 255:** Portal GLP-1 levels.

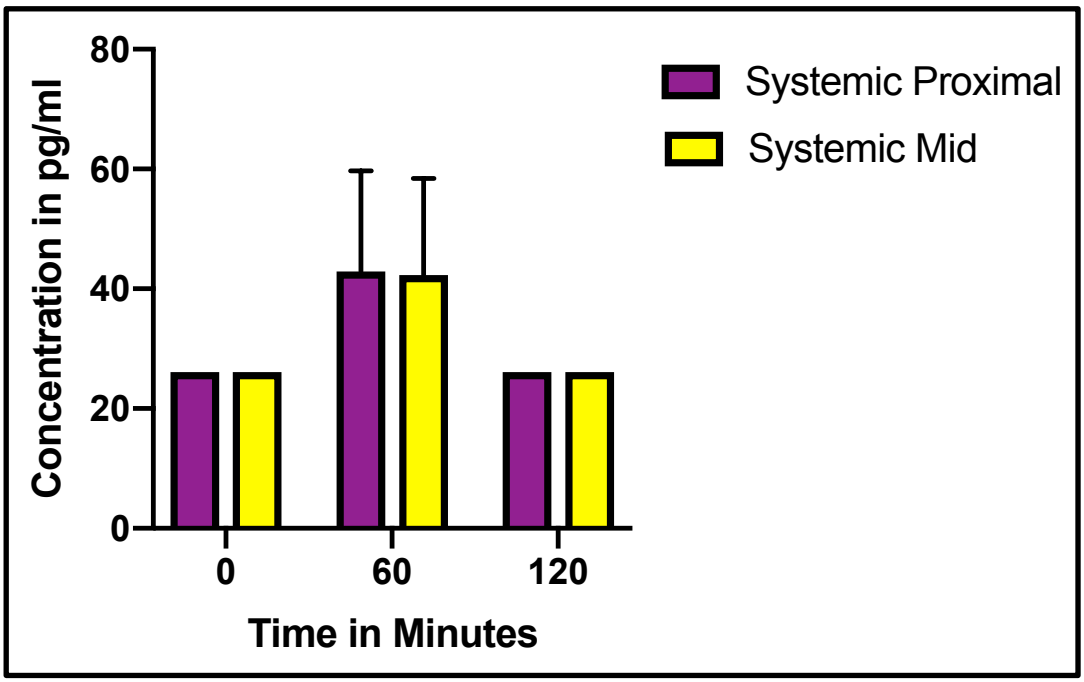



**Figure 256:** Systemic GLP-1 levels.

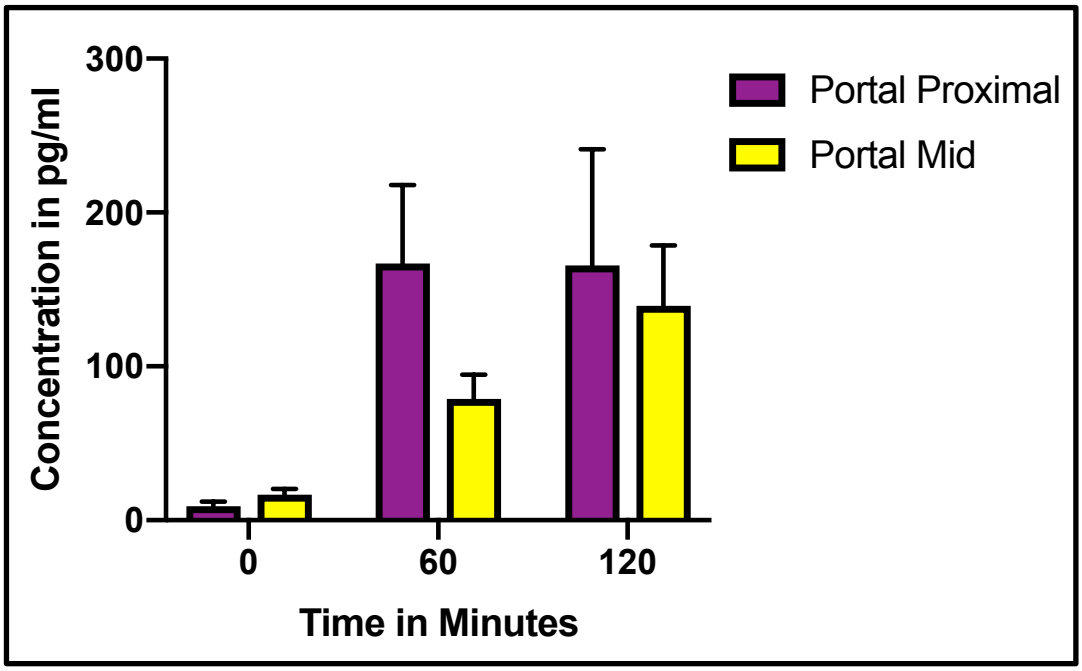



**Figure 257:** Portal GIP levels.

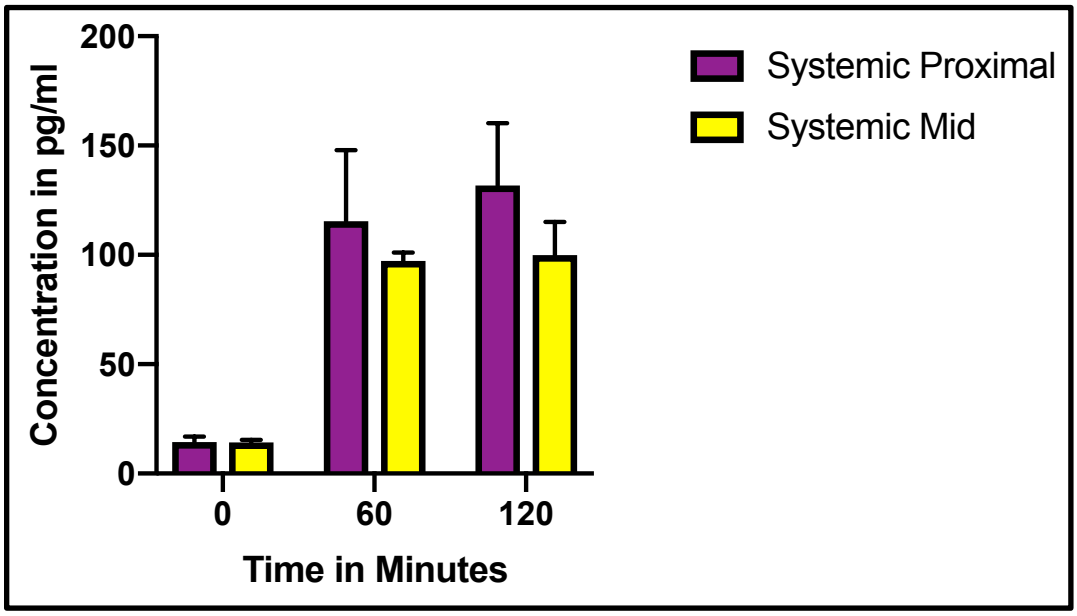



**Figure 258:** Systemic GIP levels.

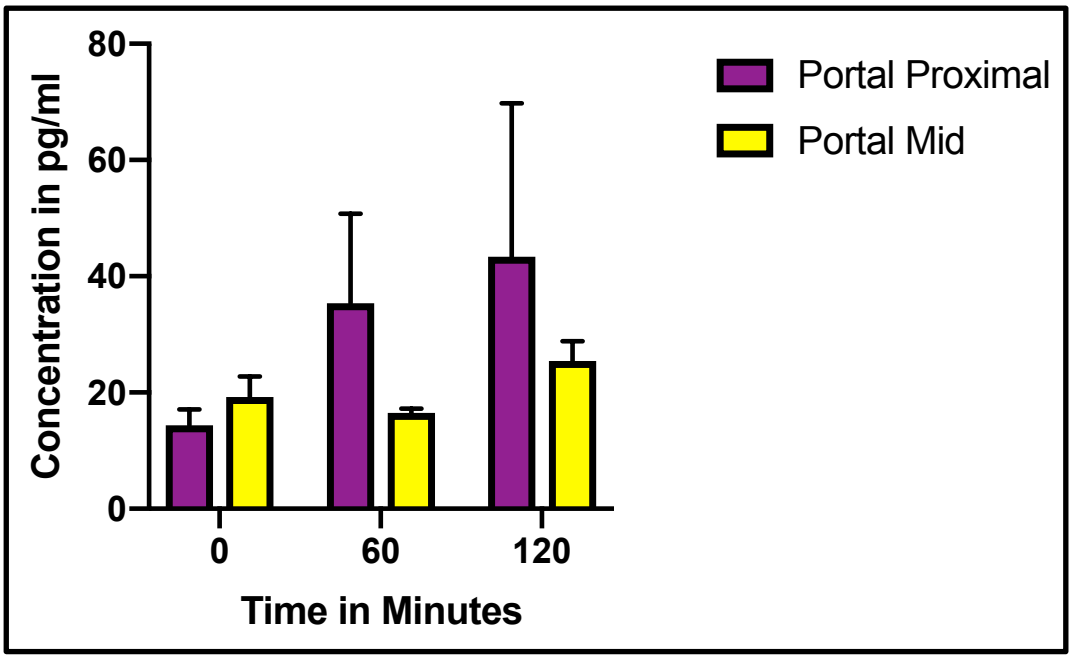



**Figure 259:** Portal glucagon levels.

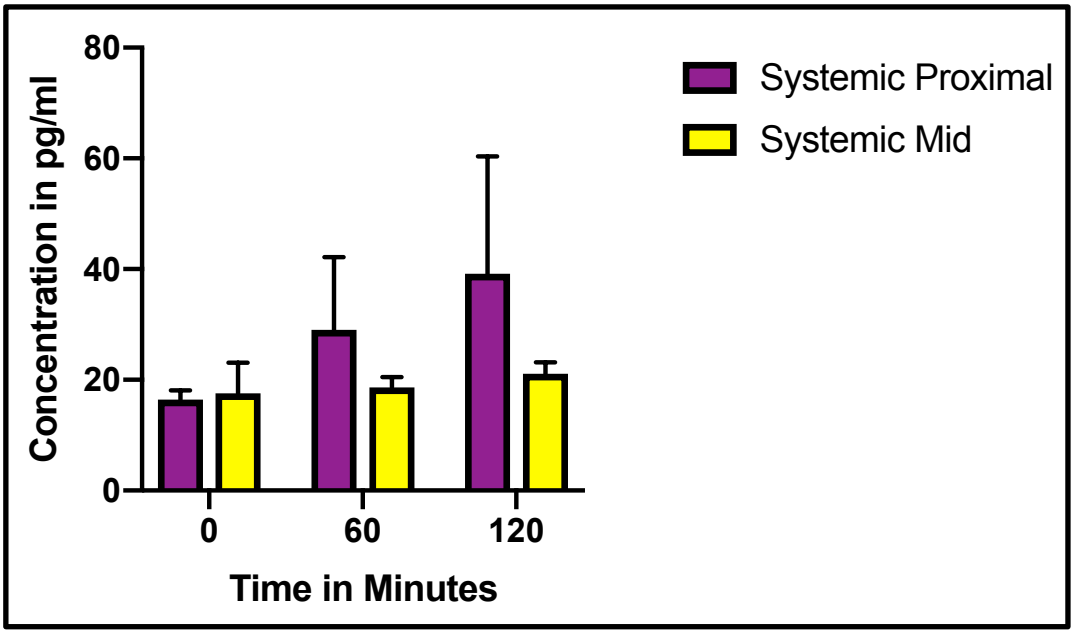



**Figure 260:** Systemic glucagon levels.

*6.1.5 Discussion*

RYGB alters intestinal anatomy so that undigested nutrients are delivered directly to the jejunum. This small, side-study aimed to replicate RYGB physiology using a non-RYGB model. It is worth noting that total adult rat small intestine length is approximately 95cm. Thus, at point A, there is approximately 80% of small intestine available for glucose absorption. At point B, there is approximately 65% small intestinal surface area available. With only a difference of 15% between the two points, it could be argued that this would not be enough to see significantly less glucose absorption through reduced small intestine absorptive surface availability alone.

Results showed that proximal jejunal exclusion led to a reduction in $G_{PS}$ gradient and thus decreased glucose absorption, with an associated decrease in insulin levels and C-peptide levels, but without altering incretin (GLP-1, GIP) hormone levels. This is in contrast to Aim 2 (chronic Teduglutide injection studies) which, by the end of the harvest protocol, showed a significant increase in GIP levels in both healthy and pathological rat models, and a significant decrease in GLP-1 levels in the healthy model, with a significant increase in GLP-1 levels in the pathological model. GLP-2 levels were not analysed, although this would certainly be a focus for further work, particularly so as to be able to compare to GLP-2 levels demonstrated in Aim 2.

This modest experiment has shown, in an intact rat model, that isolating the proximal small intestine and its luminal glucose sensors, from the nutrient stream, appears to lead to a significant decrease in intestinal glucose absorption capacity, thus appearing to satisfy the original hypothesis – that isolating the proximal small bowel goes some

way to conferring the anti-diabetic effects of RYGB. This is in opposition to data gained from Aim 2, 3 and 4, which all demonstrated increased glucose absorption. It is difficult to explain why this variation in intestinal glucose absorption exists. Potentially, it could be due to the acute nature of the experiments, versus the chronic experiments carried out within the other aims.

Nevertheless, this study provides an important result because, despite the mechanism (whether it is reduced glucose absorption or another, as yet undefined, mechanism), it suggests a potential role for non-operative and less-invasive therapeutic options in the treatment of T2D; treatments which can mimic the anatomical changes created by surgery. Indeed, devices such as the Endobarrier (a duodenal-jejunal bypass sleeve) have already been employed with varying degrees of success (van Rijn, Roebroek et al. 2019).

# CHAPTER 7

## 7.1 CONCLUSION

The intriguing weight-independent anti-diabetic effect of RYGB is well-known and yet the mechanisms underlying this metabolic benefit remain elusive. Though several mechanisms have been proposed, the interaction amongst these mechanisms is also unclear. It is likely that major anatomical changes after surgery lead to multiple physiological changes which in combination lead to changes in glucose homeostasis. The series of experiments conducted within this thesis aimed to identify some of these physiological changes. The data obtained contributes modest, yet interesting, insights into a possible role for GLP-2.

Aim 1 suggested that GLP-2 may potentially have useful effects on glucose handling, in terms of lowering peak glucose levels. The results were difficult to reproduce within an acute administration model and were minimal at best. Thus, a chronic administration model was designed, using a degradation-resistant GLP-2 analogue, Teduglutide. Aim 2 revealed that Teduglutide appears to have the most potent effect on glycaemia when administered chronically. Additionally, this effect was most evidently seen within a pathological model, with upregulation of glucose transporter/sensor genes (SGLT-1, GLUT-1, GLUT-2 and SGLT-3), and increased insulin and GIP secretion. Furthermore, increased intestinal hypertrophy was seen within the pathological group (compared to the healthy group), suggesting GLP-2 was conferring a greater effect on intestinal mucosa. Analysis of rat body composition was not carried as part of this aim; however, it would be interesting to use MRI scanning in future experiments to assess this further.

Aim 3 didn't necessarily add any further information in terms of GLP-2 mechanistic role, however, it served to help create a successful RYGB rat model; the bypassed rats became significantly lighter in weight compared to the SHAM rats. Additionally, the procedure resulted in reaffirming data from other studies which had shown an increase in intestinal hypertrophy, with the Roux limb showing greatest adaptation. Aim 4 was unable to demonstrate any real effect in terms of GLP-2 inhibition, which was frustrating. However, it did show that increasing BP limb length in an RYGB pathological rodent model resulted in lowering blood glucose levels compared to a shorter BP limb length model. Finally, Aim 5 showed the benefits of foregut exclusion upon glucose homeostasis, by forgoing any anatomical rearrangement but, instead, infusing an 'undigested' glucose meal straight into the mid-jejunum (equivalent to the Roux limb). Within the confines of the experiment, acute proximal jejunal exclusion appeared to confer anti-diabetic effects of RYGB using a non-RYGB model.

This thesis set out to explore mechanisms behind improved glucose handling observed following RYGB surgery, and to investigate a potential role for GLP-2 in mediating this. Although GLP-2 has been implicated in influencing gastrointestinal functions, such as reduction of gastric emptying and gut motility (Baldassano, Amato et al. 2016), increasing mesenteric blood flow, facilitating nutrient absorption and enhancing gut barrier function (Drucker and Yusta 2015), it is known mainly for its trophic properties. It is only recently that GLP-2 has been connected to glucose metabolism. Although it would be ambitious to claim that the studies within this thesis demonstrate GLP-2 contributing towards improvements in glycaemic control, they do seem to suggest that GLP-2 causes a glucose-related effect in animals treated with it, either acutely or chronically. Additionally, these effects were more dramatic within a

pathological animal model. Furthermore, PCR experiments revealed significant upregulation of glucose transporter/sensor genes within the pathological model. Given that pathological animals displayed significantly increased small bowel hypertrophy compared to healthy GLP-2 treated rats, it is possible that Teduglutide may convey a beneficial effect to hyperglycaemic animals. This is supported by work performed by Baldassano et al. They demonstrated that endogenous GLP-2 may act as a protective factor against dysregulation of the glucose metabolism that occurs in HFD mice (Baldassano, Amato et al. 2016).

A large amount of time was spent trying to obtain a signal from the Teduglutide peptides purchased and, thus, hindered further work that might have helped with understanding potential mechanisms at play. Fortunately, a large number of blood and tissue samples were taken from these experiments and stored within the lab, paving the way for other colleagues to continue this work. For example, concerning Aims 2-5, blood was collected for quantification of GLP-2 levels at all time points, in all harvest experiments. Carrying out a GLP-2 ELISA on this stored blood might help to elucidate how exogenous GLP-2 injections affect endogenous GLP-2 production, and how glucose levels vary with GLP-2 levels. Blood was also taken from Aim 3 (RYGB versus SHAM) in order for blood plasma to be sent for lipid and protein analysis via mass spectroscopy. Results are still awaited from these studies.

Additionally, mucosal scrapings were taken from stomach, duodenum, jejunum, terminal ileum, colon, liver, omentum and pancreas in Aim 2, and from Roux limb, BP limb, common limb, terminal ileum, colon, liver, omentum and pancreas in Aims 3 and 4. This allows further PCR experiments to be carried out, particularly in the

delineation of the location of GLP-2R, which is still the cause of much research debate (El-Jamal, Erdual et al. 2014). Furthermore, it would be useful to run PCR specifically on liver samples collected from these experiments. Measurement of hexokinase activity, to assess conversion of glucose into glucose-6-phosphate, signalling intestinal glucose utilisation, and measurement of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK), to assess gluconeogenic pathways, would certainly be a priority for future work. It would be reassuring to see an increase in expression of the former and a reduction in expression in the latter as this could suggest a decrease in intestinal gluconeogenesis. This would help corroborate the theory that the biochemical changes seen post-RYGB are largely driven by increased metabolic action of the intestine as it undergoes hypertrophy of its mucosa.

A further concept that was discussed when planning the studies within this project, and which may be of use to carry out in the future, was the use of FDG radiolabelling to assess glucose utilisation. Using this technique, other studies have shown that the Roux limb of RYGB-treated rats exhibit reprogramming of intestinal glucose utilisation in order to meet increased energy requirements (Saeidi, Meoli et al. 2013). Data from Aim 2 corroborated the findings from Saeidi et al – that is, an increase in GLUT-1 upregulation (a trend in the SC group and a significant increase in the HFD group within these studies), and that the increased glucose is then likely directed towards metabolic pathways that mediate tissue growth. This is an important finding as rats in Aim 2 had not undergone RYGB. The implication might be that, despite lack of anatomical arrangement, there were still changes in glucose metabolism

pathways and that these pathways could be exploited to devise non-operative, less-invasive, treatment modalities.

The original literature review that was carried out to guide this body of work did not reveal any rat GLP-2 administration studies (either acute or chronic) that measured blood glucose as a primary or secondary endpoint. A literature search conducted more recently has revealed a study that examined the effect of 1 week administration of exogenous GLP-2 on blood glucose in healthy rats, in the presence of either distal small bowel resection or SHAM transection (Lai, de Heuvel et al. 2017). They found that exogenous GLP-2 attenuated resection-induced increases in blood glucose (and more so than in the SHAM transection group). Additionally, they saw a significant increase in SGLT-1, GLUT-2 (in resected animals), which echoes findings from Aim 2 within this thesis. Interestingly, exogenous GLP-2 decreased GLP-2R mRNA in resected rats compared to transected rats, and this was also shown in Aim 2, within Experiment 2 (CASLO Teduglutide administration within healthy rats). A further study by Wismann et al studied the effect of 7-day administration of a GLP-1/GLP-2 co-agonist, versus Teduglutide monotherapy in healthy *mice* (Wismann, Pedersen et al. 2018). They showed that the GLP-1/GLP-2 co-agonist significantly improved glucose tolerance, as well as reducing food intake and increasing intestinal volume, compared to Teduglutide alone. Furthermore, Teduglutide monotherapy was not found to show any beneficial effects on metabolic outcomes, i.e. glucose levels or food intake, within this 7-day study. This suggests that the effects of Teduglutide are highly diet dependent, given that other studies, including those within this thesis, have demonstrated glucose reducing effects in HFD, obese mice (Baldassano, Amato et al. 2016).

This thesis set out to prove a link between increased intestinal hypertrophy and the improved glycaemic control seen following RYGB surgery, and that this potential link could be the gut hormone, GLP-2. One of the questions to come out of this thesis is whether the mechanism might involve a reduction in luminal glucose absorption (as suggested by other members within this lab), or, a possibility that increased luminal glucose absorption is then followed by increased glucose utilisation, which the majority of studies within this project seem to suggest. As with the experiments conducted within this thesis, other studies looking at intestinal glucose metabolism have also shown inconsistent results, with both increased and decreased intestinal glucose utilisation and absorption being reported.

Although only modest data was obtained from the studies carried out in this thesis, there is statistically significant data to suggest that GLP-2 reprogrammes intestinal glucose metabolism, rendering the intestine a major site for glucose disposal, and thus contributing to the improvement in glycaemic control following RYGB. Ultimately, the mechanisms underlying the potential impact of GLP-2 upon glucose metabolism are yet to be established, and further studies are required to delineate these effects and to prove definitive links between GLP-2 mediated bowel hypertrophy and improved modulation of glucose.

# LIST OF APPENDICES

## Appendix 1

*ELISA Method*

An EMD Millipore Corporation GLP-2 96-well plate ELISA kit was purchased. Prior to the harvest procedure taking place, DPPI4 inhibitor was added to each eppendorph (0.1mcl per 0.1ml of blood) in order to prevent the breakdown of GLP-2. The plasma gained from the harvest procedure was allowed to thaw on ice after being removed from a -80°C freezer. Portal and systemic plasma GLP-2 levels were measured at 0, 30 and 60-minute time points in duplicate using the Millipore rat metabolic hormone panel (catalogue number EZGLP2-37K), Millipore, Darmstadt, Germany). The ELISA was carried out according to manufacturer instructions, however, the following summarises the main steps involved.

**1 – GLP-2 Standard and Quality Controls Preparation**

- The GLP-2 Standard was reconstituted with deionised water. Serial dilutions were made using assay buffer.
- GLP-2 Quality Control 1 and 2 were reconstituted with deionized water

**2 – Assay Procedure**

- Diluted Wash Buffer was used to wash each well in the 96-well plate
- Assay buffer was added to blank wells and sample wells
- Matrix Solution was added to blank wells, Standard wells and Quality Control wells

- GLP-2 Standards were added in ascending order of concentration to the appropriate wells.
- Quality Controls 1 and 2 were added to the appropriate wells
- The plasma samples were added in duplicate to the remaining wells
- The plate was sealed and incubated at room temperature for 2 hours on a plate shaker at 150rpm.
- The plate sealer was then removed, contents decanted and each well washed 3 times with diluted Wash Buffer
- Detection antibodies were then added to all wells.
- The plate was resealed and incubated at room temperature for 1 hour on a plate shaker at 150rpm.
- The plate sealer was then removed, contents decanted and each well washed 3 times with diluted Wash Buffer
- Enzyme Solution was then added to each well.
- The plate sealer was then removed, contents decanted and each well washed 3 times with diluted Wash Buffer.
- Substrate solution was then added to each well, the plate re-sealed and the plate placed on a plate shaker for 20 minutes. Blue colour was observed in the wells of the GLP-2 Standards and the plasma-sample wells.
- The plate sealer was then removed, and Stop Solution added, with the plate shaken by hand. The blue colour was observed turning to yellow.

- A plate reader was then used to read absorbance at 450nm and 590nm within 5 minutes.

**3 – Calculations**

- The average concentration of GLP-2 from each duplicate well was calculated using EXCEL.

**4 - Interpretation**

- The Standard dilutions were confirmed to be within the expected range.
- The Quality Controls 1 and 2 were confirmed to be within the expected range.
- The differences between duplicate results of all samples fell within a 15% coefficient variant.

# Appendix 2

*Luminex Method*

An EMD Millipore Corporation Rat Metabolic Magnetic Bead Panel 96-well plate was purchased. Prior to the harvest procedure taking place Aproptonin was added to each eppendorph (0.1mcl per 0.1ml of blood) in order to prevent the breakdown of glucagon. The plasma gained from the harvest procedure was allowed to thaw on ice after being removed from a -80°C freezer. Portal and systemic plasma GLP-2 levels were measured at 0, 30 and 60-minute time points in duplicate using the Millipore rat metabolic hormone panel (catalogue number RMHMAG-84K), Millipore, Darmstadt, Germany). The procedure was carried out according to manufacturer instructions, however, the following summarises the main steps involved.

**1 - Preparation of Plasma Samples**

Samples were thawed completely and mixed well by centrifuging at 3000G for 5 minutes.

**2 – Preparation of Reagents for Immunoassay**

*A – Preparation of Antibody-Immobilised Beads*

Each antibody-bead vial was vortexed for 1 minute. 150µl from each antibody bead vial was added to a Mixing Bottle and the final volume brought up to 3.0ml with Bead Diluent. The mixed beads were vortexed well.

*B – Preparation of Quality Controls*

Quality Control 1 and Quality Control 2 were reconstituted with 250 µl deionized water. The vial was inverted several times in order to mix, and then vortexed. The

vial was allowed to sit for 5-10 minutes, vortexed and the controls then transferred to appropriately labelled polypropylene microfuge tubes.

*C – Preparation of Wash Buffer*

The 10X Wash Buffer was brought to room temperature and mixed to bring all salts into solution. 60ml of 10X Wash Buffer was then diluted with 540ml deionized water.

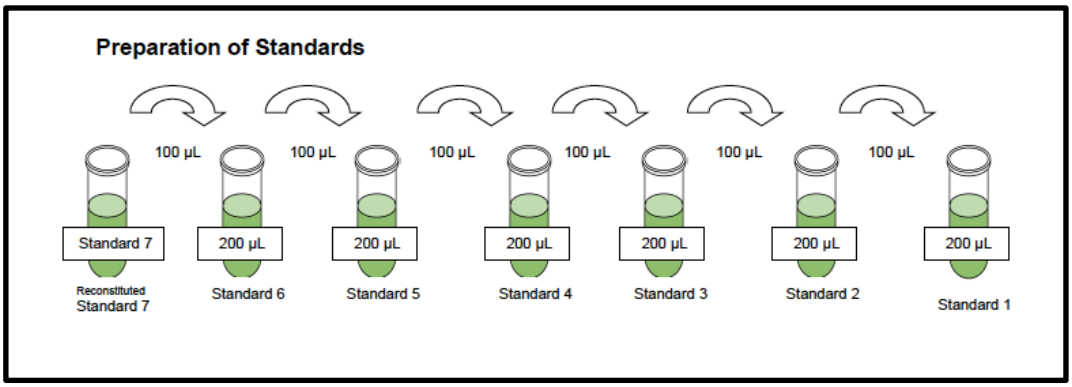
*D – Preparation of Serum Matrix*

1.0mL deionized water was added to a bottle containing lyophilized Serum Matrix and the two mixed together. 10 minutes was allowed for complete reconstitution.

*E – Preparation of Rat Metabolic Hormone Standard*

- The Rat Metabolic Hormone Standard was reconstituted with 250µl deionised water, the vial inverted several times to mix and then vortexed for 10 seconds. The vial was allowed to sit for 5-10 minutes, vortexed and the standard transferred to appropriately labelled polypropylene microfuge tube (Standard 7)

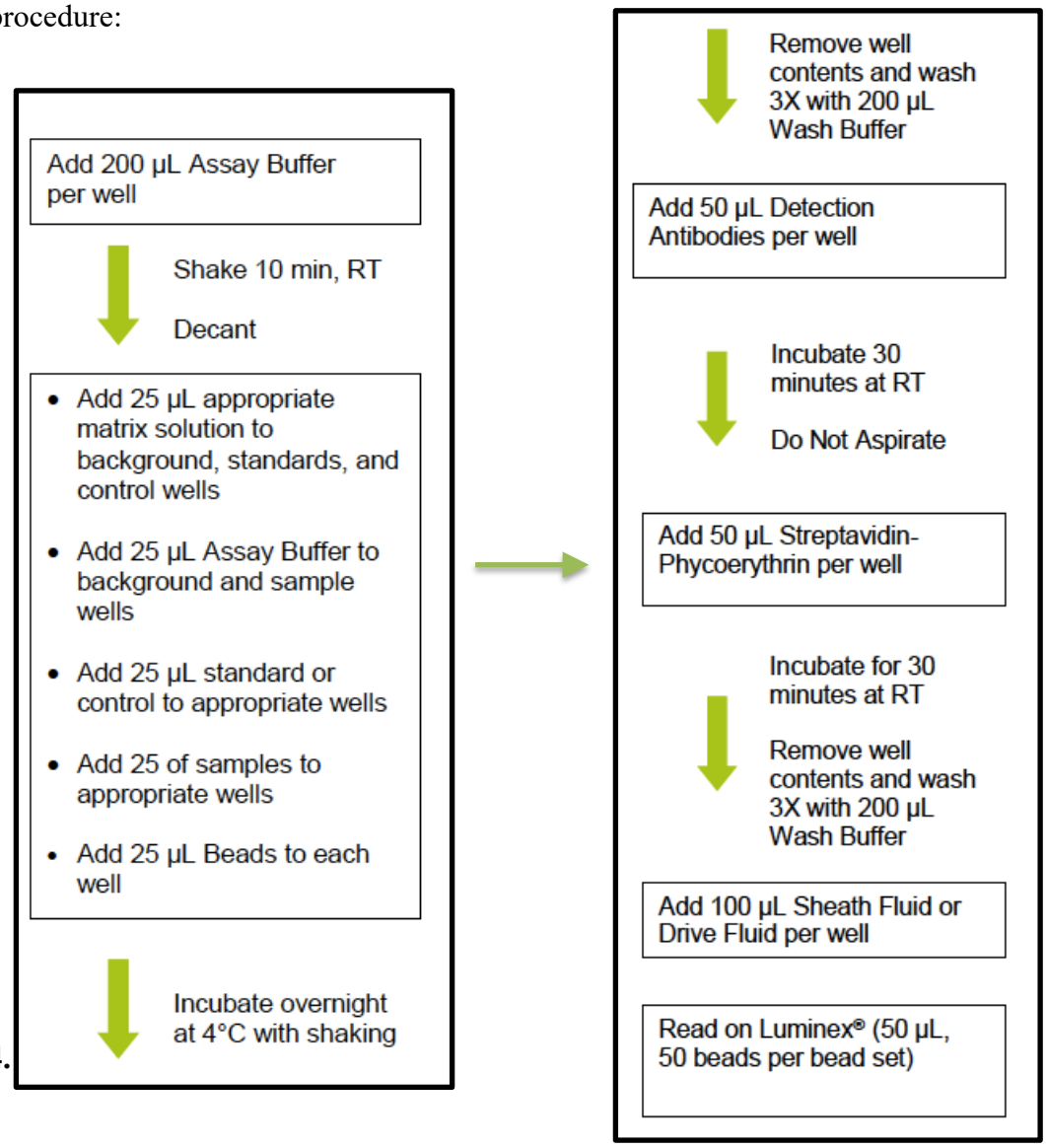
- Preparation of Working Standards: 6 polypropylene microfuge tubes were labelled “Standard 6,” “Standard 5,” “Standard 4,” “Standard 3,” “Standard 2,” and “Standard 1.” 200µl Assay Buffer was added to each of the six tubes. 3 times serial dilutions were performed by adding 100µl of the “Standard 7” to the “Standard 6” tube, mixed well and then 100µl of the “Standard 6” transferred to the “Standard 5” tube, mixed well and then 100µl of the “Standard 5” transferred to “Standard 4” tube etc (fig)




### 3– Immunoassay Procedure

All reagents were allowed to warm to room temperature (20-25°C) before use in the assay. The following flow-chart (protocol reference) summarises the immunoassay procedure:

4.



Data generated from the Luminex machine was then sent to Terry Whitehead (Technical Applications Scientist, MilliporeSigma) for analysis using Milliplex Analyst.

# Appendix 3

*PCR Method*

A Qiagen Rneasy Minikit (Cat No./ID: 74104) was purchased. The following summarises the three steps of PCR procedure:

**1. RNA Isolation**

- Jejunal mucosa samples were removed from the -80°C freezer.
- 30mg of each sample tissue was weighed and added to 600µl of RLT Buffer (a lysis buffer, plus the addition of β-mercaptoethanol).
- Each sample was then homogenised, with the homogeniser cleaned with RNase free water in between samples.
- All samples were then centrifuged for 6 minutes at 14000rpm (on Eppendorf centrifuge 5417R).
- The supernatant was then pipetted and added to 550µl ETOH.
- 700µl of each sample was then transferred to an RNeasy Mini spin column, placed within a 2ml collection tube.
- Samples were then centrifuged at 11000rpm for 15 seconds, and the flow-through discarded.
- Any remaining sample (from the supernatant and ETOH mixture) was then transferred to the spin column and re-centrifuged.
- DNA-digestion was then carried on the column:
- 350µl of RW1 was added to each column and centrifuged at 8000G for 15 seconds. Follow-through was discarded.
- 10µl of DNase 1 stock solution (consisting of RNase free water and DNase) was added to 70µl of RDD and mixed by inversion. The entire 80µl of the

DNase 1 incubation mix was then added to the column, and this then incubated at room temperature for 15 minutes.

- 350µl of RW1 was added to each column and the columns centrifuged at 8000G for 15 seconds. Follow-through was discarded.
- 500µl of RPE Buffer (consisting of RPE and 100% ETOH) was added to each column, and the columns centrifuged at 8000G for 2 minutes. Follow-through was discarded.
- The columns were then added to new 2ml collection tubes and centrifuged at 8000G for 1 minute (to dry the column membrane).
- Columns were then placed in a fresh 1.5ml collection tube and 35µl RNase free water added.
- Samples were then centrifuged at 8000G for 1 minute to elute the RNA.
- RNA was then quantified using a Nano-drop (Thermo Scientific™NanoDrop™ 2000 Spectrophotometer) - 2µl of RNA was placed on to the Nanodrop and its purity (260/280 >2.05) used to calculate the ng/µl of RNA present.

**2. cDNA Synthesis**

- Each component was mixed and briefly centrifuged before use
- The following were then combined in a 2ml tube:
    - 2µg RNA (volume calculated by Nanodrop)
    - 1µl Primers (50 µM oligo $(dT)_{20}$)
    - 1µl 10mM dNTP mix
    - DEPC-treated water (to make up to final volume of 10µl)

- A C100 Thermal Cycler (Thermofisher) was then set up with the following parameters:
    a. Incubate at 65°C for 5 minutes
    b. Hold at 4°C for 5 minutes
    c. Incubate at 50°C for 50 minutes
    d. Terminate reaction 85°C for 5 minutes
    e. Hold at 4°C for 5 minutes
    f. Incubate at 37°C for 20 minutes
    g. End
- During step a, cDNA synthesis mix was made. It consisted of the following:
    - 2µl of 10x RT Buffer
    - 4µl 25mM $MgCl_2$
    - 2µl 0.1M DTT
    - 1µl RNase Out (40U/µl)
    - 1µl Superscript III RT (200U/µl)
- During step b, add 10µl of cDNA synthesis mix to each reaction. Mix gently.
- During step e, add 1µl of RNase H. Incubate for 20 minutes at room temperature.

**3. Realtime PCR Using SYBR Green**

- Primers were designed using PrimerQuest® Tool (Intergrated DNA Technologies). All primers were purchased from ThermoFisher Scientific).
- Primers were diluted using RNase free water.

- A 11.5µl volume (per RNA sample) of SYBR Green Mastermix was made using the following components, and pipetted to the bottom of a 384 PCR well plate:
    - 6.25µl of 2x SYBR Green PCR Master Mix
    - 1.25µl Forward Primer (10µM)
    - 1.25µl Reverse Primer (10µM)
    - 2.75µl $H_2O$
- 1µl cDNA was then added to each well containing Mastermix
- The PCR plate was then covered with an adhesive cover and products analysed on a QuantStudio™ 12K Flex Real-time PCR System (Thermo Fisher Scientific).
- The following parameters were used for primer optimisation:

| | HOLD | CYCLE (40 cycles) | |
|---|---|---|---|
| | | Denature | Anneal/Extend |
| Temp. | 95.0 °C | 95.0 °C | 60.0 °C |
| Time | 10 min | 15 sec | 1 min |
| Volume | 50 µL | | |

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