UNIVERSITY OF CAMBRIDGE

Institute of Metabolic Science

The metabolic sequelae of oesophago-gastric resection

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SUMMARY

The metabolic sequelae of oesophago-gastric resection. Geoffrey Roberts

Bypass or resection of the stomach and oesophagus, has long been recognised to result in profound changes in the handling of ingested nutrients. This results in significant morbidity after radical surgery for oesophago-gastric cancer, in particular post-prandial hypoglycaemia, altered appetite, early satiety and noxious post-prandial symptoms.

By profiling and challenging the gut hormone axis in healthy volunteers and patients who had undergone total or subtotal gastrectomy, or oesophagectomy, this thesis explores the possible causative mechanisms for the challenges faced by this patient population.

In the surgical groups, an oral glucose tolerance test (OGTT) resulted in enhanced secretion of satiety and incretin gut hormones (GLP-1, GIP, PYY) and insulin, followed by hypoglycaemia in a cohort of patients. Continuous glucose monitoring of gastrectomy participants over two weeks of normal lifestyle identified an increased incidence of day and night time hypoglycaemia. RNAseq and mass spectrometry based peptidomics of human and murine enteroendocrine cells in the pre- and post-operative populations revealed no significant change in the underlying cellular pathways for nutrient sensing and gut hormone secretion, indicating that the altered hormone secretion is primarily driven by accelerated nutrient transit, rather than adaptive changes in the gut. Finally, specific blockade of the GLP-1 receptor in post-gastrectomy patients using Exendin 9-39 normalised insulin secretion and prevented reactive hypoglycaemia after an OGTT.

In conclusion, profound changes in gut hormone secretion as a result of enhanced nutrient transit after foregut surgery likely underlie the early and late post-prandial symptoms seen in this group, and therapies specifically targeting the gut hormone axis, and GLP-1 in particular, could be the first targeted treatments for post-gastrectomy syndromes.

PREFACE

STATEMENT OF CONTRIBUTION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Acknowledgements and specified in the text.

It is not substantially the same as any other work that I have submitted, and is not being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text.

WORD LIMIT

This thesis does not exceed the prescribed word limit for the Degree Committee for Clinical Medicine and Clinical Veterinary Medicine.

Word count: 48065.

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I inherited the protocols for the core clinical studies from my predecessor in the Gribble-Reimann group, Dr Claire Meek, who also helped me set up the initial studies. The team in R&D at Cambridge University Hospitals NHS Foundation Trust, particularly Katrina Gateley and Sona Kadyan, reviewed, improved and approved my study proposals in a helpful and prompt fashion.

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Finally, and most importantly, my wife and son have been a constant source of support and encouragement, and ensured I didn’t disappear down a rabbit hole of work and kept some balance to my life!

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ABBREVIATIONS

5-HT	5-Hydroxytryptamine (also serotonin)
5-HTR	5-HT receptor
Abx	Antibiotics
AdjR^2	Adjusted R^2
ADM	Adrenomedullin
AEBSF	4-benzenesulfonyl fluoride hydrochloride
AgRP	Agouti-related peptide
ANOVA	Analysis of Variance
ASIC5	Acid/amiloride Sensing Channel 5
AUC	Area under the curve
AVPR1B	Arginine-Vasopressin receptor 1B
bHLH	basic Helix-Loop-Helix
BIS-11	Barratt Impulsiveness Scale 11
BMI	Body Mass Index
BP	Blood Pressure
BRC	Biomedical Research Centre
BSA	Bovine Serum Albumin
BSP	Basic Salivary Protein
CALCR	Calcitonin receptor
cAMP	cyclic Adenosine Monophosphate
CASR	Calcium Sensing Receptor
CCK	Cholecystokinin
CCRC	Cambridge Clinical Research Centre
CDH1	Cadherin 1
cDNA	complementary DNA
CGM	Continuous Glucose Monitor
CH_4	Methane gas
CHGA	Chromogranin A
CHGB	Chromogranin B
COH	Creature of Habit
CPM	Counts Per Million
CRF	Clinical Research Facility
CRHR2	Corticotropin Releasing Hormone Receptor 2 (also UCN3 receptor)
DenDF	Denominator Degrees of Freedom
DI	Disposition Index
DM	Diabetes Mellitus
DMBT	Deleted in Malignant Brain Tumours
DNA	Deoxyribonucleic Acid
DPP4	Dipeptidyl Peptidase 4
DSS	Dumping Severity Score
DTT	Dithiothreitol
EC	Enterochromaffin Cell
EDTA	Ethylenediaminetetraacetic acid

EEC	Enteroendocrine Cell
ELISA	Enzyme Linked Immunoassay
EPAC2	Exchange Protein directly Activated by cAMP 2
EYFP	Enhanced Yellow Fluorescent Protein
FACS	Fluorescence-Activated Cell Sorting
FFAR	Free Fatty Acid Receptor
FFPE	Formalin Fixed Paraffin Embedded
FGF19	Fibroblast Growth Factor 19
FGFR4	Fibroblast Growth Factor Receptor 4
fMRI	functional Magnetic Resonance Imaging
FSC-A	Forward Scatter (FACS)
FSC-W	Pulse width (FACS)
FXR	Farnesoid X receptor
GABA	Gamma-Aminobutyric Acid
GALR1	Galanin receptor 1
GCG	Glucagon
GHRH	Growth Hormone Releasing Hormone
GHRL	Ghrelin
GHSR1a	Growth Hormone Secretagogue receptor 1a
GI	Gastrointestinal
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	GIP receptor
GLN	Glicentin
GLP-1	Glucagon like peptide-1
GLP1R	GLP-1 receptor
GLP-2	Glucagon like peptide-2
GMP	Good Manufacturing Practices
GOAT	Ghrelin O-Acyl Transferase (also known as MBOAT4)
GPBAR1 / TGR5	G-protein Coupled Bile Acid Receptor 1
GPCR	G-protein Coupled Receptor
GRPP	Glicentin related pancreatic peptide
H_2	Hydrogen gas
Hb	Haemoglobin
HbA1c	Glycosylated Haemoglobin percentage
HBGI	High Blood Glucose Index
HBSS	Hanks' Buffered Saline Solution
HCl	Hydrogen Chloride
Hct	Haematocrit
HDGC	Hereditary Diffuse Gastric Cancer
HOMA	Homeostatic Model Assessment
HOMA-B	HOMA Beta cell function
HOMA-IR	HOMA Insulin Resistance
HOMA-IS	HOMA Insulin Sensitivity
HR	Heart Rate
HRA	Health Research Authority
Hypo	Hypoglycaemic episode
IAUC	Incremental area under the curve

IAUC_120	120 minute post OGTT incremental area under the curve
ID	Participant ID
IGI	Insulinogenic Index
INSL5	Insulin-like 5
ISEC	program to calculate Insulin Secretion
ISR	Insulin Secretory Rate
IUPHAR	International Union of basic and clinical Pharmacology
LBGI	Low Blood Glucose Index
LC	Liquid Chromatography
LC-MS/MS	Liquid chromatography - Mass Spectrometry / Mass Spectrometry
LMEM	Linear Mixed Effects Model
Log	Natural log (unless specified)
LTBP4	Latent Transforming Growth Factor Beta Binding Protein 4
MAGE	Mean Amplitude of Glycaemic Excursions
MAP	Mean Arterial Pressure
MBOAT4	Membrane Bound O-Acyl Transferase (also known as GOAT)
MeanSq	Mean of Squares
MLN	Motilin
mRNA	messenger RNA
NEFA	Non-esterified Fatty Acid
NHS	National Health Service
NIHR	National Institute for Health Research
NKA	Neurokinin A
NMU	Neuromedin U
NPW	Neuropeptide W
NPY	Neuropeptide Y
NT	Neurotensin (peptide)
NTR	Neurotensin receptor
NTS	Neurotensin (gene / transcript)
NumDF	Numerator Degrees of Freedom
OCTT	Oro-Caecal Transit Time
Oes	Oesophagectomy
OG	Oesophago-gastric
OGTT	Oral Glucose Tolerance Test
OIT3	Oncoprotein Induced Transcript 3
Osmol	Plasma osmolarity
OXM	Oxyntomodulin
OXTR	Oxytocin receptor
P50	Price at 50% maximum demand on demand-price plot
PBS	Phosphate Buffered Saline
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PCA	Principal Component Analysis
PCR	Polymerise Chain Reaction
PCSK1N	ProSAAS
PFA	Para-Formaldehyde
PIGR	Polymeric Immunoglobulin Receptor
PKA	Protein Kinase A

ppm	Parts per million
PTG	Prophylactic Total Gastrectomy
PYY	Peptide YY
qPCR	quantitative PCR
REC	Research Ethics Committee
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RNAseq	RNA sequencing
RXFP4	Relaxin Family Peptide/INSL5 Receptor 4
RYGB	Roux-en-Y Gastric Bypass
SCFA	Short Chain Fatty Acid (acetate, butyrate, propionate)
SCG2	Secretogranin 2 / Chromogranin C
scRNAseq	Single Cell RNAseq
SCTR	Secretin receptor
SGLT1	Sodium-Glucose Linked Transporter 1
SIBO	Small Intestinal Bacterial Overgrowth
SSC	Side Scatter (FACS)
SST	Somatostatin
SSTR	Somatostatin Receptor
STG	Subtotal / partial Gastrectomy
SumSq	Sum of Squares
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TAC1	Preprotachykinin 1
TFEQ-r18	Three Factor Eating Questionnaire r18 version
TG	Total Gastrectomy
TMB	3, 3', 5, 5'-Tetramethylbenzidine
TPH1	Tryptophan Hydroxylase 1
TRF	Translational Metabolic Research Facility
TRPA1	Transient receptor potential cation channel, subfamily A, member 1
UCN3	Urocortin 3
UEM	Universal Eating Monitor
VAS	Visual Analogue Scale
VGF	VGF (non-acronymic)
VIP	Vasoactive Intestinal Peptide
VMAT	Vesicular Monoamine Transporter
YR	Neuropeptide Y receptor
YSI	Yellow Springs Instrument

SHORT TABLE OF CONTENTS

TABLE OF CONTENTS

1 INTRODUCTION

1.1 BACKGROUND

The long-term sequelae of major surgery, and in particular gastrointestinal cancer surgery, represent a growing but poorly understood burden of disease[1,2]. Within the field of gastro-oesophageal surgery, a loosely linked collection of gastrointestinal and systemic symptoms, termed "dumping syndrome", has been ascribed to the altered flow of nutrients after gastric or oesophageal resection[3]. These include early post-prandial symptoms such as nausea, bloating, malaise, palpitations and dyspnoea, and later symptoms such as restlessness, headache, drowsiness and anxiety which have classically been attributed to hypoglycaemia[4]. Altered dietary preferences, reduced appetite and early satiation are also anecdotally described. While this description of symptoms provides a framework to discuss post-operative recovery and quality of life, it is not supported by a robust pathophysiological understanding of the problem, or indeed any particularly effective treatment options[5].

This history of foregut surgery perhaps explains the limited nature of our understanding of the field, and the opportunities offered not only to the group of patients suffering after gastro-oesophageal resection, but also the understanding of gastrointestinal and metabolic physiology.

Visceral surgery was made possible by the advent of ether anaesthesia and antisepsis in the 19th Century, and Billroth completed the first successful gastric resection for an antral tumour in Vienna in 1881. Surgical treatment of peptic ulcer disease, initially with antrectomy, followed by the development of vagotomy and pyloric drainage procedures, followed Billroth's surgical techniques. While employing different strategies to reduce duodenal acid exposure, all procedures resulted in accelerated delivery of nutrients to the small intestine. The symptomatic complications of gastrectomy were first described in the academic literature by Hertz in 1913[3], however they became the focus of intense study through the mid-20th Century, as peptic ulcer surgery became commonplace. Multiple theories were postulated as to the underlying pathological basis of "dumping syndrome", including altered gastrointestinal innervation, secretion of unknown humoral factors, and systemic hypovolaemia secondary to osmotic fluid transit from the circulation to the small intestinal lumen[6-10]. In practice, research in the field was limited by the lack of specific techniques (i.e. antibodies) for the

identification and analysis of humoral factors, and interest waned as peptic ulcer surgery was rendered obsolete by the development of acid suppressing drugs[11].

Two major developments in the 21st Century have reignited interest in this field. Firstly, long-term survival after gastro-oesophageal resection has significantly improved, as modern peri-operative oncological treatments mean that 5 year survival for surgically treated gastro-oesophageal tumours is approaching 50%[12,13]. The identification of familial cancer syndromes, in particular Hereditary Diffuse Gastric Cancer (HDGC) and causative underlying genetic mutations, has resulted in a small but growing population of people undergoing prophylactic total gastrectomy, often in the third or fourth decade of life, who then have a normal life-expectancy but face the challenges associated with the long-term sequelae of surgery[1,14].

Secondly, the epidemic of obesity and type 2 diabetes mellitus has led to an explosion of interest in the physiological mechanisms controlling food intake and insulin secretion. Presently, the most effective short and long-term treatment for obesity and type 2 diabetes is bariatric, or perhaps better termed “metabolic”, surgery[15]. Interestingly, while metabolic surgery techniques closely mirror those used in gastro-oesophageal resection by accelerating nutrient delivery to the small intestine, the metabolic effects of both surgical approaches (i.e. reduced food intake, negative post-prandial sensations and enhanced post-prandial insulin secretion) could explain many of the negative symptoms experienced by the cohort of patients undergoing gastro-oesophageal resection. A major focus of current research into diabetes and obesity is directed to understanding the physiological basis by which surgery drives weight loss and improved glycaemic control. Indeed, the ultimate goal is to replicate surgery with a cheaper, lower-risk and more widely available alternative, be it pharmacological or endoscopic[16].

Applying knowledge and techniques developed in the bariatric field to patients who have undergone gastrectomy or oesophagectomy could allow novel insights into the challenges experienced by this patient group[17]. Studying the non-bariatric group also presents a major opportunity to examine the effects of gastro-oesophageal surgery without the confounding effects of peri-operative obesity, insulin resistance and highly calorie restricted diets, which underlie many of the controversies surrounding the mechanisms of action of metabolic surgery[18-20].

1.2 GUT HORMONE PHYSIOLOGY

Gut hormones, secreted from the gastrointestinal tract, often under the control of luminal nutrient concentration, are a key mechanism linking food ingestion to local gastrointestinal functions and the systemic response to eating. At least fourteen gut hormones have been described, with actions including control of gut motility, bilio-pancreatic secretion, pancreatic insulin secretion, gastrointestinal mucosal repair, hunger and satiety. This is a field of great interest, with novel gut hormone based therapies for obesity, diabetes, short gut syndrome and cachexia recently described[21-25]. Gut hormones have been demonstrated to act locally on enterocytes or other gut hormone secreting cells, on enteric, vagal or spinal sensory neurons, or in an endocrine fashion after entering the bloodstream[26].

Profound changes in post-prandial gut hormone secretion have been previously linked to the reduced appetite and enhanced insulin secretion seen after metabolic surgery, and to the symptom complex experienced after gastro-oesophageal resection[27,28]. The challenges of studying these heterogeneous groups, and the lack of high quality assays for many gut hormones, means there is at present a limited understanding of the relationship between surgery, eating, gut hormones and symptoms.

1.2.1 Nutrient sensing

Gut hormone secreting enteroendocrine cells (EECs) make up ~1% of intestinal mucosal epithelial cells. Most have a distinct apical–basal (open-type) orientation, with an apical surface facing the intestinal lumen and hormone containing vesicles located towards the basolateral membrane[29]. Studies comparing oral and intravenous nutrient administration have demonstrated that most gut hormones are preferentially secreted in response to luminal nutrients[30,31], and that polymeric macronutrients must be digested into monomers (monosaccharides, free fatty acids and monoacylglycerides or di/tri-peptides and amino acids) before they are capable of triggering gut hormone release[32].

Individual populations of EECs were previously assumed to have a discrete hormone secretory profiles, with for example K cells secreting GIP and L cells GLP-1 and peptide YY (PYY). Recent studies involving confocal imaging and single cell RNA sequencing have identified a more continuous spectrum of EEC

hormone production, with individual cells secreting a range of hormones which varies as much along the gastrointestinal tract as between cells[33].

A variety of mechanisms link luminal nutrient composition to gut hormone secretion, with the final common pathway being cytoplasmic Ca^{2+} dependent vesicular exocytosis[26]. Transcriptomic studies, including single cell RNA sequencing, have identified a range of transporters and receptors in fluorescently tagged murine EECs, including multiple pathways within individual EECs[33,34]. There is compelling evidence that EECs sense luminal glucose using sodium-coupled glucose transporters (SGLT1), with the intake of Na^{+} ions coupled to glucose influx triggering electrical activity and Ca^{2+} entry through voltage-gated Ca^{2+} channels[34]. EECs express a variety of nutrient sensing G-protein coupled receptors (GPCRs), including those responsive to long and short chain fatty acids, monoacylglycerides, amino acids, bile acids and bitter tastants[34,35]. These stimulate gut hormone secretion either by directly increasing cytoplasmic Ca^{2+} through a G_q / phospholipase C / IP_3 pathway, or by modulating ion channel sensitivity and exocytosis through a G_s / cAMP / PKA or EPAC2 pathway[36,37].

It is important to note that the activation of these nutrient sensing mechanisms, and so gut hormone secretion, appears to be linked to the rate of absorption of nutrients. Glucose sensing via SGLT1 directly links the rate of cellular uptake of glucose to gut hormone secretion, and GPCRs for bile and long-chain fatty acids are located on the basolateral membrane, requiring nutrient absorption prior to signalling[38-41].

1.2.2 Enteroendocrine cell differentiation and development

While not a core topic of this thesis, a passing appreciation of the development of EECs is necessary in order to understand their role in gut hormone secretion, and the studies investigating cell level (transcriptomic and peptidomic) EEC function and adaptation.

EECs are one of the four distinct populations of enteric mucosal epithelial cells (the others being enterocytes, goblet cells and Paneth cells). All are derived from crypt-based stem cells, with differentiation under the control of both internal (specific transcription factor patterns) and external (cell-cell signalling) factors[42]. Three basic helix-loop-helix transcription factors (Math1, Neurog3 and NeuroD1) have been identified as necessary and sufficient to drive enteroendocrine differentiation from intestinal stem cells, with knock-out, or villin conditional knock in (i.e. expression in all enteric epithelial cells) demonstrating their role[42]. These bHLH factors are directly inhibited by activity in the NOTCH pathway, by which maturing EECs prevent surrounding cells from following the same differentiation pathway, and limit EEC number to ~1% of all intestinal mucosal cells[43].

1.2.3 The incretin effect

To fully understand the effects of intestinal nutrient sensing on whole body physiology it is necessary to discuss the incretin effect. This refers to the finding that an orally administered glucose load will stimulate approximately double the insulin secretion of an intravenous glucose bolus eliciting the same plasma glucose concentration[30,44,45]. This is mediated by two key hormones, secreted from the gut in proportion to nutrient absorption, glucagon like peptide 1 (GLP-1), and glucose-dependent insulinotropic polypeptide (GIP). It therefore directly links glucose absorption to glucose storage and utilisation, by priming pancreatic beta cells to respond to the associated rise in plasma glucose.

1.2.4 Proglucagon derived peptides

The glucagon gene (GCG) encodes a 180 amino acid preprohormone. Differential post-translational processing (Figure 1-1) produces either a pancreatic (glucagon [amino acids 53-81], glicentin related pancreatic peptide [21-50], major proglucagon fragment [92-178]) or intestinal (glicentin [21-89], oxyntomodulin [53-89], GLP-1 [98-128] and glucagon like peptide-2 [GLP-2, 146-178]) profile of secreted peptides. This arises due to the differential cleavage activity of prohormone convertase (PC) 1/3 in EECs and PC2 in pancreatic alpha cells[46-48]. The shared amino acid sequences, and hence epitopes, of the various proglucagon derived peptides results in significant cross-reactivity of immunoassays for this group of hormones, and has historically hampered research efforts[49].

GLP-1 was initially predicted to be a 37 amino acid peptide, however synthesized GLP-1_{1-37} is biologically inactive, whereas two shorter peptides, GLP-1_{7-37} and GLP-$1_{7-36amide}$ proved highly biologically active at the G_s coupled GLP-1 receptor (GLP1R). GLP-1 acts peripherally and centrally to integrate nutrient ingestion to nutrient disposal by potentiating glucose stimulated insulin secretion from pancreatic beta cells (the incretin effect), suppressing hunger and delaying gastric emptying[44,50-53].

GLP-1 based drugs (either GLP-1 analogues, or inhibitors of the enzyme responsible for GLP-1 degradation, dipeptidyl peptidase 4 [DPP4]) have proven highly effective for the treatment of type 2 diabetes mellitus (T2DM), and have recently been licensed for the treatment of obesity[23]. There is also good evidence from studies using specific GLP1R antagonists in human participants that enhanced post-prandial secretion of GLP-1 is responsible for enhanced insulin secretion and glucose tolerance, and altered eating behaviour, after metabolic surgery[54,55].

GLP-2 is co-secreted with GLP-1, with its major recognised only known function being to promote intestinal regeneration and repair[56]. A GLP2R agonist, Teduglutide, is presently licensed for the treatment of short gut syndrome, and there is interest in its potential ability to reduce bone turn over in osteoporosis[21,57].

Oxyntomodulin and glicentin are co-secreted with GLP-1 and GLP-2, but do not have specific receptors. Glicentin is not presently believed to have a significant physiological role, and is regarded as a cleavage by-product of the other pro-glucagon genes. There are however no published mouse knock-out models, or in vivo infusion experiments to support this reasoning, and changes in plasma glicentin concentration tend to mirror those of other proglucagon peptides, hence extrapolating causation from correlation is impossible. Oxyntomodulin is processed by the cleavage of the N-terminus of glicentin, and is made up of the full-length amino acid chain of glucagon$_{53\text{-}81}$, with 6 additional C-terminus amino acids. It does not have a specific receptor, but acts at both the GLP-1 and glucagon receptors, albeit with lower affinity and in a biased fashion with greater activity on cAMP pathways than GRK2/beta arrestin pathways compared to the cognate ligands[58]. Human and murine studies, including a phase two clinical trial, have demonstrated a major role for oxyntomodulin in energy and weight homeostasis, and GLP-1/glucagon receptor co-agonists are in advanced phases of clinical trials for the treatment of obesity[24,59].

Of the pancreatic profile proglucagon peptides, the classical glucagon peptide (representing amino acids 53-81) carries significant biological activity, with a well-defined role in glucose homeostasis and possible roles in body weight control and the cardiovascular system[60,61]. It has been proposed that intestinal glucagon secretion, perhaps through adaptive changes in EEC function leading to PC2 production in GCG expressing EECs, occurs in patients after metabolic surgery and may contribute to the beneficial effects of surgery[62].

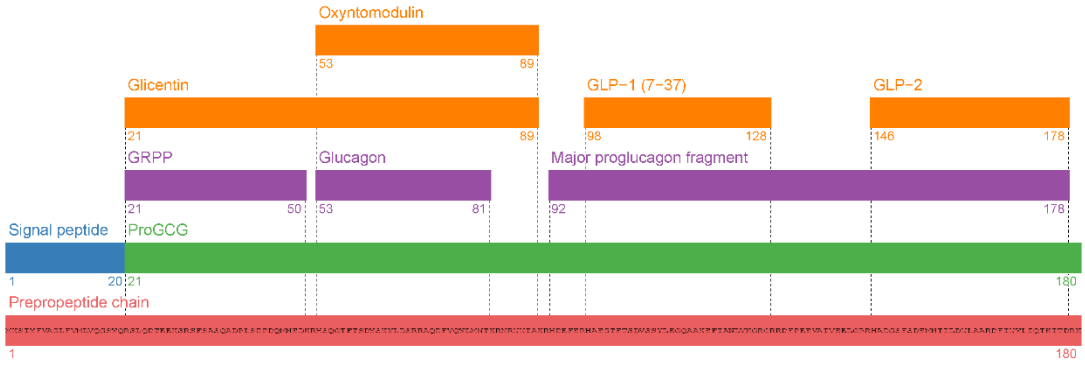

Figure 1-1. Proglucagon derived peptides (orange - intestinal; purple - pancreatic). Note that the entire sequence of pancreatic glucagon is nested within both glicentin and oxyntomodulin (which differs from glucagon by only 8 amino acids).

Figure 1-2. Peptide YY (PYY) peptides.

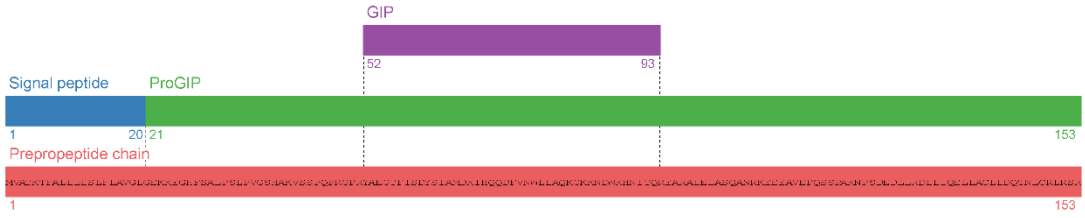

Figure 1-3. GIP peptide and prohormone.

1.2.5 Peptide YY

Peptide YY (PYY), first described in 1980, is a 36 amino acid peptide produced by intestinal L cells, primarily of the ileum and colon[63]. The full length peptide PYY_{1-36} is shortened by 2 amino acids at the N-terminus by DPP4 (Figure 1-2), with the longer and shorter variants having overlapping but distinct receptor specificities and functions[64-68]. The roles of the PYY peptides include signalling satiety, slowing gastrointestinal motility, inhibition of gastric acid secretion and maintenance of fluid homeostasis[69-74].

PYY_{1-36} acts at all neuropeptide Y family receptors (YR1-5), whereas the N-terminal truncated version, PYY_{3-36} acts preferentially at Y2R[65]. PYY colocalises with GLP-1 and other proglucagon derived peptides in cell populations traditionally labelled as "L-cells", with the ratio of PYY:GLP-1 production increasing in a gradient along the gastrointestinal tract[75].

In two double-blind, placebo controlled crossover studies, administration of exogenous PYY to lean or obese human volunteers resulted in a similar reduction in food intake in both groups[73,76]. Animal model data is more nuanced and has yet to adequately differentiate the peripheral effects of PYY on vagal afferents from the central effects of PYY directly on hypothalamic neurons[77-79]. Coupled to the role of PYY in control of meal size is its effect on digestive processes – human and animal models have demonstrated that it has an inhibitory effect on pentagastrin mediated gastric and pancreatic exocrine secretion, and delays intestinal transit[71,72,80].

1.2.6 Glucose-dependent insulinotropic polypeptide (GIP)

GIP, initially described over 40 years ago as a potent suppressor of gastric acid secretion (hence its early name “gastric inhibitory peptide”), has more recently been found to have little or no effect on gastric function, but a major role in the control of pancreatic endocrine secretion[81-83]. It is a 42 amino acid peptide (Figure 1-3), predominantly secreted from the duodenum and proximal jejunum in response to nutrient ingestion[75,84].

GIP has classically been described as being secreted from K cells within the intestinal mucosa, however as with all gut hormones and EECs the distinction between individual hormone secreting cells is now regarded as more nuanced.

While the best described function of GIP is as an incretin hormone, it also appears to modulate glucagon secretion, and may play a role in the control of bone and adipose tissue turnover[85,86]. This is supported by GIP receptor (GIPR) knockout mice demonstrating accelerated reduction in bone density, GIP infusion in humans resulting in reduced plasma markers of bone turnover, and the association in humans of a GIPR missense mutation with increased post-menopausal fracture risk[87,88].

1.2.7 Ghrelin

Ghrelin is the only described orexigenic gut hormone, acting through the growth hormone secretagogue receptor (GHSR1a) on neuropeptide Y / Agouti-related peptide (NPY/AgRP) neurons in the hypothalamus to stimulate feeding[89]. It is an unusual peptide, in that its biological activity is dependent on the modification of its 28 amino acid chain (Figure 1-4) by addition of a fatty acid group (catalysed by the enzyme MBOAT4 / GOAT), typically octanoic acid, to the serine at position 3[90]. While several attempts have been made to ascribe a function to des-acyl ghrelin, at present this is regarded as a biologically inert by-product of acyl-ghrelin synthesis[91].

In contrast to GLP-1, PYY and GIP, ghrelin is largely secreted by closed-type X/A-like enteroendocrine cells (i.e. those without a luminal border), predominantly located in the stomach[92,93]. While the plasma concentration of ghrelin is highest in the fasted state, and it is suppressed by eating, it is likely to be less nutrient responsive than peptides secreted from open-type EECs. This is supported by experimental evidence showing altered ghrelin secretion in response to humoral factors, vagal activity, and cholinergic and adrenergic drugs as well as glucose and fatty acids[94-98].

The mechanisms by which ghrelin stimulates initiation of feeding are a topic of debate, with animal models of vagotomy suggesting the vagus is essential for its actions, but conflicting studies in humans showing that ghrelin may increase food intake after gastrectomy with vagotomy, and central administration of ghrelin being as effective as intra-peritoneal administration in rodents[99-102]. Experimental evidence also exists for ghrelin having a role in glucose homeostasis, both through its actions as a growth hormone secretagogue, and through direct stimulation of pancreatic delta cells to secrete somatostatin and thus suppress islet insulin and glucagon secretion[103-105]. It is particularly of note that mice lacking the ability to produce MBOAT4, and so synthesise acyl-ghrelin, suffer profound hypoglycaemia on prolonged fasting, suggesting a protective effect of ghrelin in the fasted state[106].

1.2.8 Neurotensin

Neurotensin (NT) is a 13 amino acid peptide isolated from the hypothalamus and then small intestine in 1978[107]. The exact physiological role of NT remains uncertain, with evidence accumulating for functions as diverse as gastrointestinal motility, glucose homeostasis and cell proliferation. One key reason for the degree of uncertainty over the actions of NT is that early studies used supra-physiological doses of NT in human and animal studies, whereas later, lower dose studies have yielded conflicting results. It is clear that NT, and Neuromedin N, a differential cleavage product of the NTS gene, are also central neurotransmitters with possible roles in thought disorders, addiction and pain.

The NTS gene, located on chromosome 12, encodes a 170 amino acid prepropeptide, with a 23 amino acid N-terminal signal peptide. Numbering from the first amino acid of the signal peptide, amino acids 24-148 encode "large" Neuromedin N, which contains the 5 amino acids of Neuromedin N at residues 144-148. NT is sited at amino acids 151-163, and there is a short tail peptide[108-110]. Post-translational processing (Figure 1-5) is by prohormone convertases at dibasic residues flanking the active peptide sequences[111].

Luminal fatty acids and alcohols are the most potent secretagogues of NT, with limited roles for saline, glucose and amino acids in human and canine experiments[112,113]. Fat induced NT secretion has been reported to reduce after proximal gastric vagotomy in man, and in healthy volunteers plasma NT concentrations correlated with the volume of oleic acid delivered to the duodenum[114].

Three NT receptors have been isolated: NTR1 and NTR2 are G-protein coupled receptors, NTR3 is a single transmembrane receptor with full homology to gp95/sortilin[115-119]. The exact downstream transduction mechanism of the NT receptors has yet to be adequately described, with conflicting publications suggesting Gi, Gq and Gs responses in different cellular models, and the possibility that NT acts as an inverse agonist or antagonist at NTR2[120,121].

Functional studies of NT action have identified possible effects including delayed intestinal transit[122-124], induction of defaecation[125], stimulation of pancreatic exocrine secretion[126,127], enhanced pancreatic islet function and survival[128-131], and on body weight homeostasis[132,133].

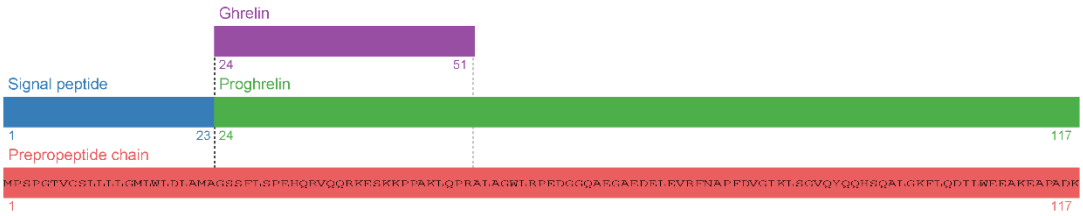

Figure 1-4. Ghrelin peptide sequence. Acyl group is bound to the serine at position 3 of the ghrelin peptide (S26 on this figure which includes the signal peptide).

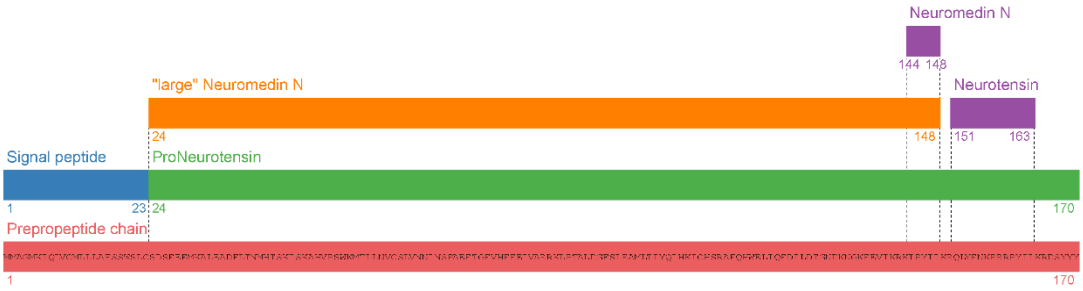

Figure 1-5. Neurotensin peptide sequence. Large Neuromedin U is of uncertain biological significance.

1.2.9 5-HT

Serotonin, also known as 5-hydroxytryptamine (5-HT) is a monoamine neurotransmitter with significant effects throughout the body. It has a key role in central neural signalling, and is of significant interest in the study and treatment of mood and thought disorders. The majority of 5-HT is however produced in the gut, where it is secreted by Enterochromaffin (EC) cells and stimulates intestinal motility, secretion and vasodilatation. Peripheral, non-enteric actions of 5-HT are primarily the result of EC-secreted 5-HT which is carried by platelets to target organs[134].

5-HT is synthesized from L-tryptophan in a two-step process. The first, rate-limiting step, the addition of a hydroxyl group to the aromatic ring of tryptophan is catalysed by tryptophan hydroxylase in ECs[135]. The second step is removal of the carboxyl group, catalysed by aromatic amino acid decarboxylase in the presence of pyridoxal phosphate[136]. Synthesized 5-HT is then packaged into vesicles by vesicular monoamine transporter 1 (VMAT1)[136].

EC cells secrete 5-HT in response to a range of stimuli, including autonomic nervous system activity, mechanical stimulation, GABA and nutrients (carbohydrates, amino acids, fatty acids)[137-141]. Somatostatin exerts a potent inhibitory effect on EC cell 5-HT secretion[142].

At present 5-HT receptors are categorized into 14 different types, of which 13 are G-protein coupled receptors and 1 (5-HT3R) is a ligand gated ion channel, although the latter is a homo- or heteropentamer with five different genes encoding subunits. The G-protein coupled 5-HT receptors are a heterogeneous population, with actions through Gq, Gs, and Gi coupled pathways[143]. Within the gastrointestinal tract, 5-HT primarily acts through 5-HT3 and 5-HT4 receptors, and exerts vasoactive effects through 5-HT2 receptor subtypes[144]. The 5-HT3 receptors are ligand-gated sodium / potassium ion channels, and the 5-HT4 receptor is a predominantly G_s coupled receptor[143].

Multiple ex vivo and in vivo studies examining intestinal motility in the presence of selective 5-HT3 and 5-HT4 receptor agonists and antagonists have demonstrated a clear, additive role for these two receptors in propagating peristalsis[144-147]. It is of interest however that intestinal motility is impaired in Tph2 (i.e. neuronal 5-HT) selective knock out mice to a greater extent than in Tph1 (i.e. EC cell 5-HT)

knockout mice[147]. It has been suggested therefore that 5-HT is sufficient, but not required, for peristalsis, and that 5-HT neurons play a more key role than EC cells.

Luminal secretion of fluid by enterocytes, and local vasodilatation, in response to 5-HT secretion are mediated by a 5-HT3 and 5-HT4 receptor dependent neural circuit, involving local neural secretion of vasoactive intestinal peptide (VIP), and by direct actions through predominantly G_q-coupled 5-HT2 receptors on enterocytes[148-150]. In tandem with local myenteric neural circuits, EC cell secreted 5-HT acts via 5-HT3 receptors on vagal afferents to generate sensations of nausea, satiety and discomfort[151-153].

Aberrant gastrointestinal 5-HT production and signalling have been implicated in several pathological processes. 5-HT secreting neuroendocrine tumours are classically asymptomatic until the presence of metastatic disease in a position where the secretory products bypass the portal circulation and hence first pass hepatic metabolism. Once symptomatic, the systemic effects of excessive 5-HT secretion include diarrhoea, hypotension, tachycardia, facial flushing and bronchoconstriction.

Peripheral plasma 5-HT concentrations are a poor indicator of splanchnic concentrations, as platelets act as a buffering system, and free 5-HT is extensively metabolized during a single passage through the liver[154]. Sample handling is also a significant challenge, as platelet degranulation during plasma preparation is difficult to avoid and significantly elevates the measured levels. Consequently, there is as yet little consensus on a “normal” plasma 5-HT concentration, with analysis necessitating the collection of platelet poor plasma and an extensive review of the literature proposing that “normal” plasma concentrations should be below 1nM[154].

1.2.10 Gut hormones – an integrated view

The gut hormones discussed here represent a small part of an integrated neuro-humoral system linking nutrient ingestion to digestion, nutrient disposal and appetite. Further gut hormones not described in detail above include those controlling secretory functions (secretin, CCK, gastrin), motility (motilin) and the global counter-regulatory hormone somatostatin. Autonomic innervation of the gastrointestinal tract, and the myenteric nervous system, are also regarded as important mediators of gastrointestinal motility and secretion, as well as carrying afferent signals in response to nutrient ingestion.

A key concept within gut physiology is the requirement to match the quantity and composition of nutrients ingested to the appropriate response. This appears to occur through the direct stimulation of gut hormone secretion by luminal nutrients, in a dose-dependent fashion, using either ion channels or GPCR pathways to stimulate cellular depolarisation, a rise in intracellular calcium and vesicular exocytosis.

This must be considered in parallel with an understanding of nutrient flow through the digestive tract. Ingested food passes rapidly to the stomach, which acts as a reservoir in which food is physically homogenised into chyme. The gastric mucosa, while highly specialised for maintenance of a highly acidic lumen, has relatively few nutrient sensing cells. Thus, in the usual state, the exposure of EECs which secrete gut hormones in response to luminal nutrients is more closely related to the rate of gastric emptying than the rate of ingestion.

It is perhaps more apt to consider this a system which monitors the rate of nutrient delivery to the small intestine, which usually represents a small proportion of the total volume of nutrients in the stomach at any given time. The relevance of this becomes clearer when we consider the effect of oesophago-gastric surgery on the rate of nutrient delivery from mouth to small intestine.

1.3 OPERATIONS

Surgery on the stomach and oesophagus encompasses several varying operations, albeit with similar long-term metabolic effects. Ignoring the underlying reason for surgery, these can be loosely classified into two categories: “gastric bypass” and “accelerated gastric drainage”.

1.3.1 Gastric bypass type operations

Within current surgical practice, the standard approach to reconstructing the intestinal tract after a gastric resection, or for bypass of the stomach and proximal small intestine, is a Roux-en-Y procedure. This involves division of the jejunum (commonly 50cm from the Ligament of Treitz), with anastomosis of the distal portion of jejunum to the oesophagus or gastric remnant, and anastomosis of the proximal to distal jejunum approximately 50cm distal to the proximal jejunal anastomosis (Figure 1-6). When performed to treat gastric cancer, this includes resection of all or part of the stomach, division of both vagi and an extensive lymph node resection. A prophylactic total gastrectomy (PTG) in the hereditary diffuse gastric cancer (HDGC) population can be performed with a more limited lymph node resection and preservation of at least the posterior vagus nerve. During Roux-en-Y gastric bypass (RYGB) for obesity, the stomach is not resected, but the proximal stomach is divided, leaving a ~50ml pouch which drains directly to the jejunum.

1.3.2 Accelerated gastric drainage type operations

As gastrectomy with Roux-en-Y reconstruction can be regarded as analogous to RYGB, oesophagectomy is in many ways a similar procedure to sleeve gastrectomy performed for obesity (Figure 1-6). During oesophagectomy, the oesophagus and both vagi are resected from the level of the azygous vein (Ivor-Lewis procedure) or lower neck (McKeown procedure) to include the proximal stomach. The gastric remnant is mobilised based on the vascular pedicles of the right gastric and right gastreoepiploic vessels and tubularised to form a conduit based on the greater curve, prior to anastomosis to the proximal oesophagus. A procedure to enhance pyloric drainage, either a formal pyloroplasty or endoscopic balloon dilatation, is often carried out in light of the impaired post-operative contractility of the stomach. During sleeve gastrectomy, the greater curve of the stomach is resected to leave a tubularised stomach based on the lesser curve, with reduced reservoir capacity and enhanced transit of food.

1.3.3 Historic peptic ulcer procedures

Surgery for peptic ulcer disease involved either resection of a significant acid secreting portion of the stomach (antrectomy) or division of the vagus nerves (vagotomy) to reduce duodenal acid exposure. While no longer commonly carried out, the operations share features of oncologic and metabolic foregut surgery, in that they involve either bypassing or disrupting the pylorus to aid drainage of the denervated / atonic stomach.

The two key common features of all the above procedures are accelerated delivery of ingested nutrients to the small intestine via bypass or disruption of the pylorus, coupled to the reduced or abolished reservoir capacity of the stomach.

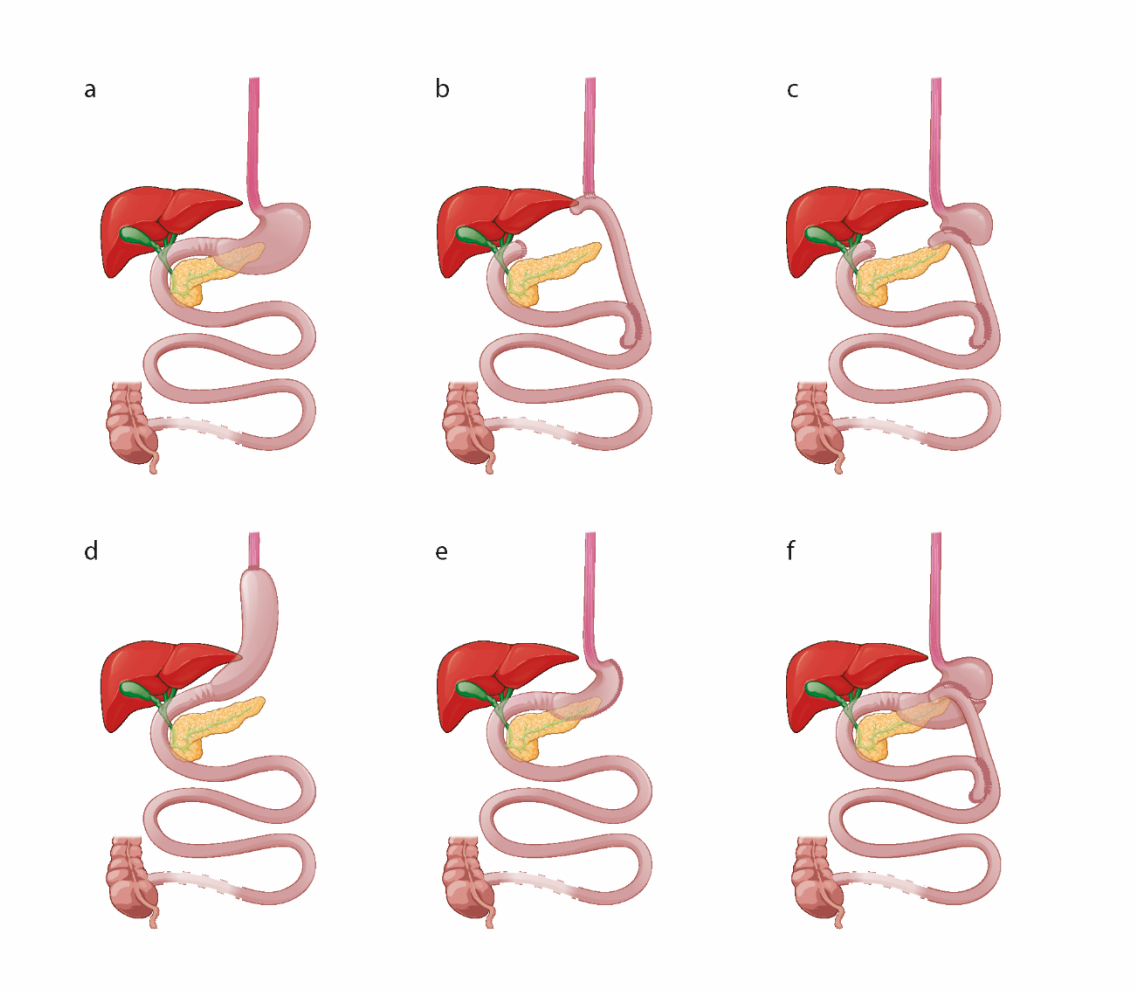

Figure 1-6. Normal and post-operative intestinal anatomy. a - Normal anatomy. b - Total gastrectomy (same as for prophylactic total gastrectomy). c - Subtotal gastrectomy. d - Oesophagectomy with gastric conduit. e - Sleeve gastrectomy. f - Roux-en-Y gastric bypass. Note the similarity in nutrient passage between b, c and f; and between d and e. All procedures result in reduced gastric reservoir size and accelerated delivery of nutrients to the small intestine.

1.4 MECHANISMS OF ACTION OF METABOLIC SURGERY

The epidemic of obesity and diabetes during the last two decades has seen a vast expansion in research into metabolic pathways that may cause the disorder, and hence could be modulated to achieve weight loss and improved glucose tolerance. Despite this, current therapeutic options are extremely limited, with exercise regimes, dietary programs and medications (lipase inhibitors, 5-HTR_{2C} agonists, GLP1R agonists and centrally acting sympathomimetic and GABA-ergic drugs) rarely achieving sustained weight loss of >10%[155]. By contrast, metabolic surgery is highly efficacious in treating obesity, type 2 diabetes mellitus (T2DM) and importantly the late consequences of both conditions, including early death[156]. This is remarkable for two reasons: primarily, obesity and diabetes are whole body conditions, and the fact that surgery on a single organ system, not immediately linked to the pathology, is so effective suggests a significant systemic role for gut derived factors. Secondly, post-operative improvements in glucose handling often precede any significant weight loss, suggesting that specific operative factors act to either enhance insulin secretion or reduce insulin resistance independently of body mass[157]. Elucidating the underlying mechanisms which drive this change has proven challenging, with major confounding factors being the pre-operative obesity and peri-operative dietary restrictions.

While all bariatric operations were initially differentiated as “restrictive”, “malabsorptive” or both, this has proven far too simplistic an approach. Scintigraphic studies of intestinal transit in humans after RYGB, described as both restrictive due to the small gastric pouch and malabsorptive due to the small intestinal bypass, have clearly shown that food rapidly exits the gastric pouch, thus volumetric restriction is unlikely to result in reduced food intake[158]. In humans undergoing long-limb RYGB (i.e. 150cm, rather than the standard 50cm, of small intestine without bilio-pancreatic enzymes), macronutrient malabsorption was responsible for <10% of the initial reduction in energy intake[159]. Animal models of bariatric surgery have also clearly demonstrated that it is possible to increase food intake to cope with physiological stressors such as pregnancy and lactation after metabolic surgery[160]. Thus, it is unlikely that malabsorption of macronutrients is a major contributor to weight loss after metabolic surgery, and systemic neural signals and humoral factors have become key areas of

research. Identification and pharmacological replication of the underlying mechanism of action of metabolic surgery is therefore a highly appealing approach to the management of obesity and type 2 diabetes.

Attempts to understand the underlying causative factors of both reduced energy intake and enhanced glucose handling in patients undergoing metabolic surgery have largely suffered from the confounding effects of pre-operative morbid obesity, post-operative weight loss and peri-operative dietary restriction (patients are restricted to ~1000kcal/day for at least two weeks prior to surgery to facilitate shrinkage of the liver and safe operating around the diaphragmatic hiatus). Several hypotheses are however supported by compelling experimental evidence.

The most robust theory is that enhanced post-prandial secretion of incretin and satiety gut hormones results in increased insulin secretion and early satiation, so glucose handling is improved and meal size is reduced[27]. It has been recurrently shown in post-RYGB patients that post-prandial GLP-1 secretion is enhanced[161], specific blockade of the GLP1R returns glucose and insulin profiles to a pre-operative, diabetic state[162], and in obese and diabetic non-operated patients GLP-1 administration elicits similar effects to surgery, albeit with reduced magnitude[163,164]. In a small group of patients with gastrostomy tubes sited in the gastric remnant after RYGB, administration of a glucose tolerance test to the remnant elicited post-prandial glucose, insulin and gut hormones responses similar to non-operated patients, suggesting that the rapid delivery of nutrients to the jejunum is key[165]. Similar effects are seen with PYY as with GLP-1, and coinfusion of GLP-1, PYY and oxyntomodulin in short term studies has proven an effective weight loss strategy[161,166].

Impaired glucose tolerance in obese patients arises due to both insulin resistance and impaired beta cell function, with diabetes arising when insulin requirements outweigh the secretory capacity[167]. Interestingly, it appears that visceral, and specifically pancreatic and hepatic fat, is a significant factor in both insulin resistance and beta cell impairment, and this can be reduced in the short-term by an extremely low calorie (<600kcal/day) diet, resulting in diabetes resolution prior to significant

reductions in body mass[168,169]. This raises the question as to whether early diabetes remission after surgery is driven by surgical factors, or is largely an effect of the pre-operative diet.

Bile acid circulation and the intestinal microbiome warrant consideration together, due to the complex interaction of bacteria and bile acids. The enteric microbiome, consisting of up to 100 trillion bacteria in >1000 taxa, plays a key role in normal human physiology through multiple mechanisms about which we have only limited understanding[170]. Using high-throughput sequencing techniques to classify bacterial species based on their unique 16S ribosomal RNA signature, it is possible to classify an individual's whole microbiome by abundance of different bacterial species.

Primary bile acids (in humans principally cholic and chenodeoxycholic acid) are synthesized from cholesterol within hepatocytes. Conjugation of the bile acids to either glycine or taurine increases water solubility and reduces toxicity prior to excretion into the biliary system and onwards to the duodenal lumen. While most bile acids reaching the terminal ileum are reabsorbed and circulated to the liver (via the enterohepatic circulation) for excretion back into the biliary system, those that reach the colon are subject to deconjugation and 7α-dehdroxylation by bacterial enzymes, resulting in secondary bile acids (deoxycholic acid and lithocholic acid), which are also absorbed and recirculated[171]. Only a small fraction of bile acids is lost in the faeces and replaced by de novo synthesis. The microbiome therefore both influences, and can be influenced by, the bile acid circulation, resulting in altered plasma concentrations of the various bile acids[172]. As well as acting in a tightly controlled feedback loop on their own synthesis, bile acids have been demonstrated to act on several organ systems, through either the nuclear bile acid receptor (FXR), the G-protein coupled bile acid receptor (GPBAR1 / TGR5) or through stimulation of synthesis of FGF19, which acts at FGFR4[171].

Multiple studies have now correlated specific microbiome patterns with obesity, and shown clear changes in response to metabolic surgery or faecal transplant from lean or post-metabolic surgery humans to obese mice[173,174]. This implies a causative role for the gut microbiome in metabolic disorders, however enthusiasm for this should be tempered both by the high risk of correlation confounding causation when dealing with the large number of data points in a microbiome sequencing

data set, and a recent and elegantly performed study where obese men were randomised to antibiotics or placebo with clear changes in microbiome but no alteration in any metabolic factors[175]. It should also be noted that the microbiome is heavily dependent on diet and bile acid circulation, both of which are altered by metabolic surgery[174].

There is growing interest in the potential metabolic effects of bile acid physiology[171]. Multiple studies have identified differences in the circulating bile acid pool between lean, obese and diabetic patients, and arising due to metabolic surgery, although other studies have directly contradicted this hypothesis and the true role of bile acids, if any, remains unclear[176-179]. Animal models, including FXR knockout mice, and of foregut bile bypass have evidenced a weight and glucose controlling effect of altering bile acid homeostasis[180,181]. Activation of TGR5 on EECs stimulates GLP-1 secretion and may mediate some of these effects[39,182].

It is therefore plausible, if not yet proven, that linked changes in circulating bile acid pools and the enteric microbiome arising due to metabolic surgery are in part responsible for weight loss and enhanced glucose tolerance.

What is clear from the current metabolic surgery literature is the immediate and enduring physiological shift that occurs after surgery. Disentangling the early effects of surgery from the peri-operative diet, and the late metabolic effects from the marked weight loss seen in the majority of patients has proven challenging. A means of addressing this problem would be to study bypass surgery in a lean cohort of patients, so removing any confounding effect of peri-operative diet and pre-operative metabolic disease. While patients undergoing total or subtotal gastrectomy have a different procedure (removal rather than bypass of the stomach), the reconstruction of the gastrointestinal tract is identical and so effects of altered nutrient delivery should be the same.

1.5 HUNGER

The sensation of hunger is a fascinating and challenging topic, particularly with reference to the current epidemic of obesity. More pertinent to this thesis, it is well established that gastric and oesophageal resection profoundly alters eating behaviour[183,184]. While this has been discussed in depth, very few studies have attempted to address the effects of surgery on hunger in a systematic fashion.

Understanding hunger requires appreciation of the complex interplay of central and peripheral factors (including neural and humoral inputs to the central nervous system). Considering what is hunger is surprisingly challenging, and a few published definitions from Blundell et al[185] and Mela[186] are useful:

> "Hunger is a conscious sensation reflecting a mental urge to eat. Can be traced to changes in physical sensations in parts of the body – stomach, limbs or head. In its strong form may include feelings of light-headedness, weakness or emptiness in stomach."
>
> "Satiety is process that leads to inhibition of further eating, decline in hunger, increase in fullness after a meal has finished."
>
> "Satiation is the process that leads to the termination of eating. Also known as intra-meal satiety."
>
> "Appetite refers specifically to qualitative aspects of eating, sensory aspects or responsiveness to environmental stimulation."
>
> "Liking reflects the immediate experience or anticipation of pleasure from the orosensory stimulation of eating a food (hedonic value or "palatability")."
>
> "Desire, or wanting, is the intrinsic motivation to engage in eating a food, now or in the (near) future."
>
> "Preference is the selection of a food over relevant alternatives at the point of choice, including intrinsic and extrinsic factors (for humans, this may include liking and desire, but also consideration of health values, brand, cost, convenience, etc.)."

Various models exist that propose explanations for eating behaviour to combine the experiences of hunger, eating and satiation. The core of all models is the notion that eating behaviour is learned, with input of higher order processes influenced by metabolic processes (e.g. gut hormone or leptin activity through hypothalamic circuits) and sensory processes (appearance, smell, taste and gastrointestinal sensation via vagal afferents)[185].

Detailed investigation of the nature of appetite and satiety in patients after metabolic surgery is pertinent to understanding the mechanisms by which weight loss arises, and key to developing pharmacological techniques to augment or replace surgery. It is unsurprising that far more studies have examined this field in the obese than the lean population, although the methodology remains limited by current techniques and understanding of the central pathways driving hunger and satiation. Studies examining meal size and preference, and questionnaire or task measures of eating behaviour, have demonstrated reduced hunger scores, meal size and reduced preference for high energy foods after metabolic surgery, with enhanced measures of cognitive restraint[187]. Functional imaging of the central nervous system has identified changes in both sensory hypothalamic regions, and those associated with reward behaviour, suggesting that surgery can result in altered central handling of appetite and nutrient based stimuli[188]. Similar studies in lean patients after oesophago-gastric resection are lacking, but comparison with metabolic surgery is illustrative and offers a framework for assessing the problems of post-operative eating behaviour.

1.6 HYPOGLYCAEMIA

Hypoglycaemia is a widely discussed topic within diabetes, and is increasingly recognised to be a complication of both metabolic and resectional foregut surgery. For such an important topic, the definition of hypoglycaemia is itself contentious, with the accepted cut-off of <4mmol/l being of dubious clinical relevance[189,190]. In practice, plasma glucose concentration of <3.3mmol/l is a pragmatic definition of clinically significant hypoglycaemia, as this is the level at which central nervous system effects are noted due to impaired neuronal substrate delivery[191].

The physiological response to hypoglycaemia can be categorised into sympathoadrenal effects and cognitive effects presenting as hunger, tachycardia, sweating, anxiety, irritability, confusion and ultimately loss of consciousness or seizures. Recurrent hypoglycaemia in diabetic patients results in blunting of some of these symptoms and ultimately tolerance of lower plasma glucose levels, however with little or no protection from the cognitive impairment experienced during hypoglycaemia[192]. This is a particularly dangerous position, as lack of symptoms or insight can result in patients undertaking tasks requiring attention and concentration (e.g. driving) despite cognitive impairment, and statutory agencies typically have strict criteria for provision of driving licenses to patients at risk of hypoglycaemia.

Hypoglycaemia occurs after gastrectomy, typically in the post-prandial period, although the incidence is unknown as most studies have relied upon symptomatic measures[193-199]. If recurrent hypoglycaemia in the gastrectomy population results in reduced hypoglycaemia awareness, as in diabetic patients, this could result in both under-reporting of symptoms, and an as yet unrecognised clinical problem.

Within this thesis, the term “reactive hypoglycaemia” refers to hypoglycaemia occurring within 3 hours following a meal, snack or calorific drink.

1.7 CONTINUOUS GLUCOSE MONITORING

Continuous glucose monitors (CGMs) are a relatively new technology by which a wearable sensor measures glucose concentrations in a defined fluid (usually interstitial fluid) at regular intervals (5-15 minutes)[200]. Interstitial fluid glucose concentration typically lags behind plasma glucose by 5-15 minutes. CGMs sense glucose by an enzyme based technique – a glucose oxidase generates an electrical current in proportion to the number of available glucose molecules by release of electrons during oxidation.

Current devices for patient use consist of the worn sensor / transmitter parts and a coupled receiver / monitor. Both alone and in combination with insulin pumps in a closed loop system CGMs have been demonstrated to improve glycaemic control in diabetic patients. Within the range of devices presently available, the Freestyle Libre Pro presents the greatest opportunity in both research and potentially clinical investigation of post-operative patients[201,202]. The two key factors of this device are that it requires no calibration, so not needing finger-prick glucose measuring as well, and that it is blinded, with participants wearing the monitor for two weeks with no immediate feedback.

This technology therefore allows the identification of trends in glucose handling in post-operative patients with the straightforward application of a ~£2 coin sized patch to the arm, and could augment current (primarily lab based) studies.

1.8 SYMPTOMS AFTER GASTRO-OESOPHAGEAL CANCER SURGERY

The term “dumping syndrome” is used in an attempt to provide a diagnosis to patients suffering often disabling symptoms after gastro-oesophageal surgery. In practice, the diagnosis of “dumping syndrome” is based upon soft measures including symptom scores, and represents the categorisation, based on time and symptoms, of a continuum of problems. This distinction has allowed epidemiological studies of the incidence of early (occurring within one hour of a meal) and late (occurring 1-3 hours post-prandial) dumping syndromes using a variety of symptom scores[203]. Despite this, estimation of the prevalence and severity of post-surgical symptoms varies widely between 10 and 75%, in large part due to the lack of a standard symptomatic assessment or objective measures[2,28,204-206]. While there is no well-validated tool to assess severity of symptoms, the most pernicious symptoms suffered are syncope and seizures, secondary to either hypovolaemia or hypoglycaemia, and can have significant impact on personal and professional functioning.

In practice, while the artificial subcategorization of symptoms is helpful for patient education and management, it is likely to be unhelpful to the understanding of the underlying physiology of patients.

1.8.1 Current theories of the mechanism of "early" dumping syndrome

Symptoms historically classified as "early dumping syndrome" can be categorised as gastrointestinal (abdominal pain and bloating, nausea, diarrhoea) and vasomotor (fatigue, "desire to lie down", flushing, palpitations and sweating, with associated tachycardia and hypotension)[5]. It has reasonably been assumed that symptoms occurring within one hour of a meal are directly linked to the delivery of large volumes of undigested food to the small intestine, in a fashion that would not occur with a functionally intact gastric reservoir and pylorus[207-209]. Indeed, instillation of nutrients directly to the jejunum of healthy volunteers at a rate comparable to that after surgery precipitated symptoms similar to those experienced by the post-operative group[8].

Measurement of intestinal transit time has been conducted in several studies of patients undergoing total or subtotal gastrectomy, RYGB or sleeve gastrectomy, with inconsistent results. While all agree that nutrient delivery to the proximal small intestine is accelerated, different studies show accelerated, delayed, or unchanged oro-caecal transit time (OCTT) after surgery[158,207-212]. While there is no supportive evidence, it is reasonable to assume that patients with early post-prandial diarrhoea, particularly of the malabsorptive type, have extremely rapid intestinal transit and delivery of food to the proximal colon.

Less clear is the cause of post-prandial abdominal pain, nausea and vasomotor symptoms. Two early hypotheses were hyperglycaemia secondary to rapid nutrient absorption, or hypovolaemia secondary to osmotic fluid shift from the circulation to the intestinal lumen. Both have been disproven by elegantly conducted experiments – in one, hyperglycaemia due to intravenous glucose did not precipitate symptoms, and in the other intravenous infusion of saline (to maintain plasma volume) during an oral glucose tolerance test did not ameliorate symptoms[8,213]. Similar symptoms can however be precipitated in healthy controls by distension of the jejunum[8], suggesting that jejunal distension by large volumes of food, with or without some osmotic shift of fluid to the lumen, may precipitate symptoms.

Interestingly, the plasma concentration of a variety of humoral factors secreted from the intestine has been shown to rise in parallel with the development of symptoms and changes in splanchnic blood flow[6,214,215]. A study examining haemodynamic factors in post-gastrectomy patients identified significant changes in plasma renin, aldosterone and atrial natriuretic peptide, in keeping with a hypovolaemic state, in response to an oral glucose challenge[216]. This was abolished by administration of octreotide, a somatostatin analogue, suggesting that a humoral factor (and one would assume therefore vasodilatation rather than fluid loss) was responsible for the vasomotor aspects of early dumping syndrome[216]. Several studies, including small randomised controlled trials, have demonstrated a reduction in early dumping symptoms and enhanced appetite with administration of octreotide, supporting the hypothesis that both gastrointestinal and vasomotor aspects are at least in part driven by humoral factors[28,203,217-222].

1.8.2 Current theories of the mechanism of "late" dumping syndrome

Symptoms historically classified as "late dumping syndrome" are primarily related to hypoglycaemia, and include neuroglycopaenic (fatigue, weakness, hunger, confusion) and autonomic symptoms (sweating, tachycardia, tremor, irritability). The cause of post-prandial hypoglycaemia in the gastrectomy population is incompletely understood, and a core theme of this thesis.

Initial studies hypothesised that rapid delivery of carbohydrates to the small intestine caused an early and elevated peak in plasma glucose, exaggerated insulin response for the total ingested meal and subsequent late (1-3 hour) hypoyglycaemia[194]. The concept that an intestinal factor may contribute to pancreatic insulin secretion however predates the description of dumping syndrome[223], and more recent work in the field of metabolic surgery has shown the key role of incretin gut hormones in enhanced post-operative insulin secretion[162].

It is highly interesting that one of the key benefits of metabolic surgery (enhanced insulin secretion) closely matches a key morbidity of gastric and oesophageal cancer surgery. Given the similarity in post-operative intestinal anatomy and nutrient flow, it is likely that similar mechanisms underlie this process in both groups. The stark difference between the groups is the degree of pre-operative obesity and insulin resistance, and indeed the parallel study of lean patients undergoing oesophago-gastric cancer surgery is an exciting model of the effects of gastric bypass without the confounding metabolic syndrome[17,199].

While it is well-accepted that post-prandial hypoglycaemia occurs in the gastrectomy and oesophagectomy cohort, the underlying studies have relied on either laboratory based meal or glucose challenges, or symptom questionnaires[2,5,28,205,224-227]. At present, an objective measure of the incidence and severity of hypoglycaemia in this group while undertaking normal daily activities is lacking, and no allowance has been made for the possibility of asymptomatic hypoglycaemia. There is every reason to suspect that the large swings in plasma seen in the post-gastrectomy / oesophagectomy cohort could result in poor hypoglycaemia awareness as they do in diabetic patients[228]. It is therefore crucial to develop an understanding of what proportion of patients develop hypoglycaemia after gastrectomy,

how frequently this happens and whether autonomic or other warning symptoms become downregulated.

Another key question is whether hypoglycaemia in this cohort is only seen in the post-prandial setting, or whether fasting hypoglycaemia occurs in the setting of the ghrelin deficiency seen after gastrectomy as it does in ghrelin knock-out mice[106]. Both types of hypoglycaemia could represent a major and underestimated problem for the growing population of post-gastrectomy / oesophagectomy patients[193,229].

1.8.3 Small intestinal bacterial overgrowth (SIBO)

The bacterial content of the small intestine should be relatively sterile, however bacterial proliferation can occur in a variety of conditions, with small intestinal bacterial overgrowth (SIBO) present when there are greater than 10^5 colony forming units/ml in enteric contents. According to several case series, the incidence of SIBO in patients after gastrectomy or oesophagectomy ranges between 38% and 100% of patients and likely occurs due to loss of the bactericidal effect of gastric acid, the presence of intestinal blind loops and focally impaired small intestinal flow[230-234]. SIBO has a variable clinical presentation, which can include bloating, flatulence, belching and abdominal pain due to intestinal distension by fermentation by-products; and diarrhoea and malabsorption of micro- and macro-nutrients arising due to bacterial metabolism of ingested nutrients prior to their absorption[235]. These symptoms closely match those of "early" dumping syndrome, and indeed bacterial overgrowth may represent a causative factor in post-gastrectomy symptoms.

The intestinal microbiome is a particularly feverish area of research at present, with research groups claiming links between the gut flora and pathological processes as disparate as obesity, colorectal cancer, dementia and osteoporosis[236-239]. Of the very few studies to develop the hypothesis beyond the point of correlation, those in the field of metabolism have generated conflicting results. While a randomised trial of vancomycin for the treatment of obesity demonstrated a clear change in microbiome, no change was demonstrated in multiple markers of metabolic disease, including energy expenditure, insulin secretion, nutrient handling, adipose tissue function and gut permeability[175]. However, it has been established that metabolic surgery results in an altered faecal microbiome, which, when transplanted to obese mice, results in significant weight loss[173], and faecal transplant from lean to obese humans resulted in improved insulin sensitivity[240].

Considering the possible mechanisms of action of the microbiome, mRNA for G-protein coupled receptors for the bacterial metabolites acetate, butyrate and propionate (short chain fatty acids, SCFAs) has been identified in enteroendocrine cells and in vitro studies of primary cultures of intestinal mucosa from mice and humans have demonstrated gut hormone secretion in response to SCFA

exposure[241-245]. The literature on in vivo effects of SCFAs on gut hormone secretion is limited, and it has not been established whether SIBO results in enhanced gut hormone secretion due to increased exposure of small intestinal EECs to SCFAs, however this could contribute to the altered gut hormone physiology and any downstream effects seen in post-gastrectomy patients.

1.8.4 Current deficits in our knowledge of the post-gastrectomy patient

The published prevalence of "dumping" symptoms is highly variable, depending upon the patient population and investigational tool. While multiple studies have examined the prevalence and severity of symptoms after gastro-oesophageal resection, few studies have attempted to link symptoms and quality of life measures with physiological investigations. While one might expect the altered nutrient flow and gastrointestinal physiology to be relatively constant between patients, it is remarkable that the symptoms experienced in this cohort vary between patients and indeed within individual patients over time. Key questions that should be addressed are:

1. In what way is gut hormone signalling altered after gastrectomy and oesophagectomy?
2. To what extent does this contribute to:
    a. Early post-prandial symptoms?
    b. Altered glucose handling and reactive hypoglycaemia?
3. Does small intestinal bacterial overgrowth impact upon gut hormone signalling and post-prandial symptoms?
4. What is the incidence of hypoglycaemia during normal living, and does it relate to symptoms?
5. How can symptoms be assessed in the post-operative patient, and do other nutrient seeking behaviours (i.e. habits, impulsivity) change after gastrectomy / oesophagectomy?
6. Do functional changes in the small intestinal epithelium contribute to altered gut hormone signalling?

It is widely acknowledged that surgery to bypass, or limit the volume, of the stomach results in profound changes in gastrointestinal endocrine and neural signalling. By objectively characterising these changes it should be possible to generate a clear picture of the physiology of all patients after gastro-oesophageal resection, and then understand the symptoms experienced by some patients from a pathophysiological perspective.

1.9 SUMMARY

It is fortuitous that recent advances in long-term prognosis after oesophago-gastric resection have occurred during the explosion in understanding of the metabolic role of the gut. Approaches to the study and treatment of patients undergoing oesophagectomy or gastrectomy can be adapted from those used to understand obesity and metabolic surgery, and it is possible to move beyond the relatively simplistic physiological studies of the mid-20th Century to consider the orchestrated metabolic response to altered nutrient delivery seen in this group. Conversely, the study of lean patients undergoing surgery which is highly analogous to metabolic surgery for obesity and diabetes offers the potential for significant insights into the mechanism of action of metabolic surgery. At the very least, it should be possible to identify factors which are independent of pre-operative obesity and arise due to surgery.

1.10 SCOPE OF THESIS

This thesis aims to assess the altered nutrient response of patients after total gastrectomy, with inclusion of oesophagectomy and subtotal gastrectomy patients where possible. The goal was to identify nutrient sensing and signalling factors that change due to surgery, whether adaptive changes in the gut or the microbiome contribute to these changes, and how these changes contribute to hypoglycaemia and negative post-prandial symptoms. This can best be considered by testing the following hypotheses:

1. Post-prandial gut hormone and insulin secretion is enhanced after oesophago-gastric resection and is associated with early satiation and noxious post-prandial symptoms.
2. Free-living glucose profile, measured using CGMs, is altered after oesophago-gastric resection, resulting in increased frequency and duration of hypoglycaemic episodes.
3. Eating behaviour is altered by oesophago-gastric surgery.
4. Enhanced post-prandial GLP-1 secretion after gastrectomy results in enhanced insulin secretion, reactive hypoglycaemia and reduced appetite.
5. The gut does not produce and secrete pancreatic type glucagon in response to nutrient intake in patients after foregut bypass.
6. Intestinal microbial fermentation by-products (SCFAs) stimulate gut hormone secretion in the fasting and post-prandial state.
7. The transcriptome and peptidome of human jejunal EECs do not significantly change in response to gastrectomy.

2 METHODS

2.1 ETHICAL APPROVAL

All clinical studies were undertaken in line with the principles of the Declaration of Helsinki[246] and following approval by a NHS research ethics committee and the Cambridge University Hospitals and University of Cambridge research governance offices. Specific ethically approved projects which have supported this thesis are:

Study title	Approving REC	REC reference	Clinicaltrials.gov reference
Assessment of gut peptide levels in patients with motility disorders	East of England – Cambridgeshire and Hertfordshire	13/EE/0195	N/A
The study of human gut hormones	East of England – Cambridge Central	09/H0308/24	N/A
The metabolic consequences of GI surgery	East of England – Cambridge South	16/EE/0338	NCT02836353
Gastrectomy, eating behaviour and GLP-1	East of England – Cambridge East	16/EE/0545	NCT02971631

Table 2-1: Research Ethics Committee approvals.

2.2 RECRUITMENT

Patient participants were recruited for the various studies through either the oesophago-gastric unit at Addenbrooke's Hospital, or the Hereditary Diffuse Gastric Cancer (HDGC) registry, which is kept by Professor Rebecca Fitzgerald at the University of Cambridge. Healthy volunteers were recruited by word of mouth or were relatives of patient participants. All participants received written information relevant to the study in which they participated and gave written, informed consent to the study.

Participants were categorised by operation:

1. Healthy volunteer / control
2. Prophylactic total gastrectomy (PTG) for prevention of diffuse gastric cancer in a carrier of a pathogenic mutation in the CDH1 gene. Performed with limited D1 lymph node resection and preservation of posterior vagus.
3. Total gastrectomy (TG) – D2 total gastrectomy with division of both vagi for treatment of invasive gastric cancer or proximally situated gastrointestinal stromal tumour.
4. Subtotal gastrectomy – resection of the body and antrum of the stomach with Roux-en-Y reconstruction, with or without D2 lymph node resection
5. Oesophagectomy – resection of the oesophagus (Ivor-Lewis, minimally invasive McKeown or left thoracoabdominal approach) with division of both vagi, pyloroplasty and reconstruction with gastric conduit.

2.3 LOCATION

All participant research visits were conducted in the Clinical Research Facility (CRF, pre-August 2017) and Translational Research Facility (TRF, post-August 2017) within the Cambridge Clinical Research Centre (CCRC), Addenbrooke's Hospital, Cambridge, UK. Endoscopy was performed in the Addenbrooke's Hospital endoscopy suite, or the research endoscopy suite in the CCRC. Surgical samples were collected from main theatres at Addenbrooke's Hospital.

2.4 INCLUSION CRITERIA

2.4.1 All

Aged 18 years or older

Male or female

Able to fast overnight

Able to tolerate oral glucose tolerance test

Have capacity to give voluntary informed consent

2.4.2 Surgical participants

Have undergone, or have planned within the next 12 months, surgery including removal of all or part of the stomach or oesophagus; or be a carrier of a genetic mutation conferring a high risk of requiring a future prophylactic gastrectomy.

Be willing and able to tolerate the endoscopy or physiological intervention

2.4.3 Antibiotic study

Surgical participant with proven small intestinal bacterial overgrowth on 50g OGTT (rise in exhaled hydrogen or methane concentration of >20ppm within 45 minutes of ingestion)

2.4.4 Exendin 9-39 study

At least 3 months post completion of treatment for gastric cancer, or prophylactic gastrectomy

Able to understand and retain all information regarding the study and give informed consent.

Willing to receive an infusion of human albumin solution.

2.5 EXCLUSION CRITERIA

2.5.1 All

Recent history of untreated anaemia.

Lack of capacity to read and retain information about the study.

Communication issues which prevent accurate understanding of the study rationale and requirements.

Pregnant or breastfeeding (if participant becomes pregnant during the study, may recommence participation on cessation of breastfeeding)

Active gastric or oesophageal cancer

2.5.2 Endoscopy

Known contraindication to upper gastrointestinal endoscopy

Currently taking medication that would pose a significant risk of haemorrhage after biopsy (e.g. Clopidogrel, Warfarin, Dipyridamole, novel oral anticoagulants, therapeutic dose heparin)

Significant medical comorbidity that may be adversely affected by endoscopy or sedation (e.g. unstable ischaemic heart disease, significant respiratory impairment)

2.5.3 Physiological challenge with antibiotic

Known allergy to the proposed agent

Have received a course of antibiotics in the preceding two months

On medication which interacts with the proposed agent

Previous history of Clostridium difficile colitis

2.5.4 Exendin 9-39 study

Known diagnosis of diabetes.

2.6 ORAL GLUCOSE TOLERANCE TESTS

The night prior to oral glucose tolerance tests (OGTTs), participants consumed a standardised meal. The energy content of the meal was 1/3 of a participant's daily requirements estimated from predicted resting metabolic rate and multiplied by an activity factor of 1.35. Meal composition was 30–35% fat, 12–15% protein and 50–55% carbohydrate by energy[247,248].

OGTTs were prepared by dissolving 55g of glucose monohydrate in 200ml of water, providing 50g of glucose for ingestion. Participants started consuming the glucose solution at time 0 and completed it within 5 minutes.

Participants were assessed prior to, and at time points up to three hours following ingestion of the glucose solution. Assessments were: blood sampling, heart rate, blood pressure, visual analogue scores of hunger, fullness and nausea, Sigstad score and hydrogen/methane breath testing[4].

2.6.1 Blood collection

Blood was collected into several tubes as required by downstream assay, immediately placed on wet ice, processed as per Table 2-2 and snap frozen in 500µl aliquots on dry ice within 30 minutes.

2.6.2 Exhaled hydrogen / methane measurement

Exhaled hydrogen and methane were measured using a Bedfont Gastrocheck, which provides immediate results at the bedside.

Sample	Collection tube	Target assay	Post-phlebotomy
EDTA plasma	EDTA	Peptidomics	Centrifuge 3500g for 10 minutes at 4°C
Lithium Heparin	Lithium heparin	Insulin, glucose	
DPP4 inhibitor / aprotinin / EDTA plasma	2.5ml blood to syringe with 20µl DPP4 inhibitor (EMD Millipore), mixed, immediately transferred to 2.6ml EDTA tube with 1000 units aprotinin	Gut peptides, glucagon	
Serum	Serum	Leptin, fatty acids	Stand for 30 minutes at room temperature then centrifuge 3500g for 10 minutes at 4°C
Platelet poor plasma	EDTA	5-HT	Centrifuge 200g 10 minutes at room temperature, aliquot supernatant then centrifuge 10,000g 2 minutes 4°C
Acidified sample	2.5ml to syringe, transfer immediately to 4ml EDTA tube with 200mM AEBSF	Ghrelin	Centrifuge 3500g for 10 minutes at 4°C, 500µl aliquots immediately treated with 100µl 1M hydrochloric acid

Table 2-2: Blood processing protocols.

2.6.3 Subjective measures and symptom scores

Visual analogue scores were collected by marking a 10cm line between the statements “Not at all” and “Extremely”, following the questions:

1. How hungry are you right now?
2. How full are you right now?
3. How nauseated are you right now?

The Sigstad score was published in 1970 as a tool for clinical diagnosis of “dumping syndrome”[4]. It was developed by studying test then validation cohorts of patients after partial gastrectomy, and correlating the symptom matrix to plasma glucose and volume changes. Despite the shortfalls inherent in using the catch-all term “dumping”, the tool is useful as it has been widely used to quantify the post-prandial symptom burden of patients after foregut resection[220,227,249], and represents a quantitative, albeit subjective, means of classifying the severity of post-prandial symptoms in participants.

Sigstad score was measured using a grid derived from the originally published score as per Table 2-3, with participants giving a simple yes/no answer to each symptom at each timepoint[4].

Symptom	Time 0	15	30	45	60	75	90	120	150	180
Desire to lie or sit down										
Breathlessness, dyspnoea										
Weakness, exhaustion										
Sleepiness, apathy, drowsiness, falling asleep										
Palpitations										
Restlessness										
Dizziness										
Headache										
Feeling of warmth, sweating, pallor, clammy skin										
Nausea										
Abdominal fullness										
Borborygmi (stomach rumbling)										
Eructation (belching)										
Vomiting										
Fainting, syncope, unconsciousness										
Shock										
Total score										

Table 2-3: Sigstad score grid.

2.7 PLASMA ASSAYS

All plasma assays were performed by the Core Biochemistry Assay Laboratory (CBAL) at Addenbrooke's Hospital unless otherwise specified. Total GLP-1, GIP and PYY were assayed using Mesoscale Discovery sandwich ELISAs. Acyl ghrelin was measured using the EMD Millipore sandwich ELISA. Insulin was measured using the Diasorin Liaison sandwich immunoassay. Glicentin, pancreatic Glucagon and $Glucagon_{1\text{-}61}$ were measured using Mercodia sandwich immunoassays. Oxyntomodulin, 5-HT and pancreatic glucagon were measured by mass spectrometry by R Kay, A Koulman and LGC Limited using previously described techniques[250,251]. Nano-mode LC-MS/MS of plasma for an extended peptide profile was conducted by R Kay. Fasting glucose concentrations were measured in plasma using a hexokinase method on the Siemens' Dimension analyser. Bedside plasma glucose was measured with a Yellow Springs Instrument glucose meter (YSI).

2.8 PSYCHOLOGICAL STUDIES

2.8.1 Food attention bias

To attempt to quantify altered food attention in the post-gastrectomy population, a subset of participants completed a computer based attention bias task designed by Hisham Ziauddeen.

Food attention bias was measured before and during OGTT using a dot-probe task[252,253] with a selection of 40 food and 40 non-food images (Figure 2-1). Participants were shown images on a computer monitor (Presentation v14.5, Neurobehavioural Systems) side by side for either 2000ms (to assess "maintained attention") or 500ms (to assess "motivational bias"), immediately followed by a dot behind one image. Participants were instructed to press the arrow key corresponding to the side of the dot, as rapidly and accurately as possible. Each image was shown four times on each side, twice at each duration. Participants undertook the task immediately prior to ingestion of the OGTT, and repeated it at 15 and 90 minutes post-ingestion.

Food bias was measured as the response time when the dot was incongruous with a food image minus the response time when congruous (i.e. a greater value implies faster response when the sides of food and dot correlated and hence food attention bias). Efforts taking >2000ms, <200ms or more than 2 standard deviations from the mean for that task were excluded.

Bias time was calculated for each duration of image, and as an average over all efforts within a task and normalised to a factor (mean non-food response time from that task divided by mean non-food response time for all tasks during that OGTT) to account for altered response times between tasks resulting from fatigue or experience.

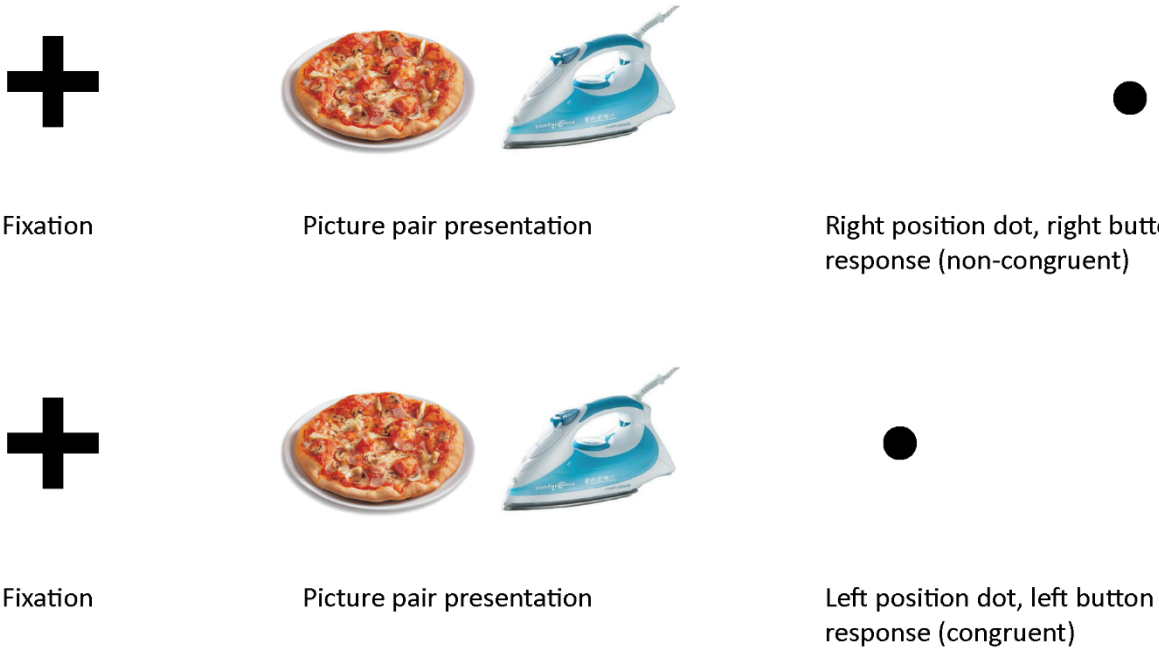

Figure 2-1. Dot probe image pair example. Participant first sees fixation cross, then picture pair presentation for 500ms or 2000ms, then dot. Top row - dot non-congruent with food image, bottom row - dot congruent with food image.

2.8.2 Food motivation

Food motivation was measured using a grip force task designed and analysed by H Ziauddeen[254,255]. A grip force transducer was connected to a laptop computer through a Biopac MP150. The grip force task was programmed in Matlab (MathWorks) using six categories of image: high calorie healthy, high calorie unhealthy, low calorie healthy, low calorie unhealthy, rewarding non-food, unrewarding non-food. Initial calibration was conducted (five efforts) to measure maximum grip strength. Participants were shown each image 30 times and instructed to squeeze the transducer as hard as they wished the item in the image at that time. The outcome measure was the area under the curve of the force-time curve for each effort.

To account for visit effect and fatigue across studies the results were residualised against visit and trial number using a mixed model with visit and trial number as fixed effects and participant ID as random effect. The residuals of this model were used for further analysis, in which the results were normalised against the pre-OGTT results and collapsed into a food vs non-food comparison. Final analysis was by generation of a linear mixed model with treatment, stimulus category (food / non-food) and trial as fixed effects and ID as random effect.

2.8.3 Ad libitum meal administration

Ad libitum meals were administered at a universal eating monitor (UEM)[256]. Participants were served a large serving bowl of pasta with sauce (in order to nullify visual cues of volume of food eaten) on a hidden set of scales. The duration and rate of eating were measured.

2.9 PHYSIOLOGICAL CHALLENGES

2.9.1 Antibiotic treatment

In the absence of the ability to correct the anatomical causes of bacterial overgrowth, the current best treatment is antibiotic therapy, which may need to be repeated as the GI tract is recolonised[257]. In this study, participants identified with SIBO at OGTT and wishing to be treated were given Rifaximin, which is a non-absorbable derivative of Rifampicin, at a dose of 400mg TDS orally for 1 week[257]. Rifaximin was selected following discussion with pharmacy and microbiology at Addenbrooke's Hospital, as there is good evidence supporting its efficacy in SIBO, and as it is non-absorbed it has an excellent side effect profile[257]. Repeat OGTT was performed within 72 hours of completion of the course of antibiotic.

2.9.2 Exendin 9-39

Exendin 9-39 is a peptide antagonist of the GLP-1 receptor which was initially identified in the saliva of the Gila monster (*Heloderma suspectum*)[258]. Its effects during intravenous infusion in humans have been described in studies of healthy volunteers, people with type 2 diabetes and patients after bariatric surgery, with few adverse effects[54,162,224,259-265]. For this study, GMP grade lyophilised synthetic Exendin 9-39 was purchased from Bachem AG (Switzerland), stored at -20 ^{o}C and supplied to the TRF through the pharmacy supply chain of Addenbrooke's Hospital. Unfortunately Bachem ceased routine production of Exendin 9-39 during this study, limiting study numbers to 5 participants.

Exendin 9-39 is highly prone to adhering to plastic fluid bags and tubing used for infusion, and so following dissolution in sterile water it was diluted in 500ml of 1% human albumin in 0.9% sodium chloride for injection in low protein-binding bags (Macoflex N, Macopharma, France) and infused through low protein-binding tubing (Baxter, USA). Participants received a weight based bolus of 7500pmol/kg of Exendin 9-39 over 4 minutes, then an ongoing infusion at 500pmol/kg/min as per Craig et al[224]. Placebo was a rate matched infusion of 1% human albumin in 0.9% sodium chloride for injection.

2.10 CONTINUOUS GLUCOSE MONITORING

Free-living continuous glucose monitoring was undertaken using the Freestyle Libre Pro system (Abbott, Germany). This required application of a sensor to the back of the arm which continuously sampled the interstitial fluid to measure glucose concentration every 15 minutes for two weeks. Participants were blinded to their glucose results. On completion of the two week period, the participant removed the sensor and returned it to the study team in the post.

Continuous glucose monitoring during the Exendin 9-39 infusion study was undertaken using the Dexcom G4 system, due to the higher frequency of reading (every 5 minutes) than the Freestyle Libre Pro. This requires application of a sensor with subcutaneous probe to the abdomen, connection of a transmitter and remaining constantly in range of a receiver device.

Both devices are designed and licensed for home use for glucose monitoring and can be applied without local anaesthesia and with minimal training.

2.11 SYMPTOM / EATING BEHAVIOUR QUESTIONNAIRES

A range of questionnaires were selected to generate a snapshot of symptoms, food preference and eating and other behaviour in the post-surgical cohort and a healthy control group.

2.11.1 Three factor eating questionnaire r18 (TFEQ-r18)

A validated measure of hunger, disinhibition and restraint as applied to eating behaviour, initially developed to differentiate restrained and unrestrained eaters, and further developed using data from the Swedish Obese Subjects study[187,266]. Participants are characterised based on three factors: "cognitive restraint", "uncontrolled eating" and "emotional eating". The tool has been used in obese and general populations, but not to date in a post-gastrectomy population[267].

Part 1 asks participants to rate a series of statements by one of four answers ("Definitely false", "Mostly false", "Mostly true", "Definitely true"):

1. When I smell a sizzling steak or juicy piece of meat, I find it very difficult to keep from eating, even if I have just finished a meal.
2. I deliberately take small helpings as a means of controlling my weight.
3. When I feel anxious, I find myself eating.
4. Sometimes when I start eating, I just can't seem to stop.
5. Being with someone who is eating often makes me hungry enough to eat also.
6. When I feel blue, I often overeat.
7. When I see a real delicacy, I often get so hungry that I have to eat right away.
8. I get so hungry that my stomach often seems like a bottomless pit.
9. I am always hungry so it is hard for me to stop eating before I finish the food on my plate.
10. When I feel lonely, I console myself by eating.
11. I consciously hold back at meals in order not to gain weight.

Part 2 asks four questions with specific answers:

1. How often do you feel hungry? (“Only at meal times”, “Sometimes between meals”, “Often between meals”, “Almost always”)
2. How frequently do you avoid "stocking up" on tempting foods? (“Almost never”, “Seldom”, “Usually”, “Almost always”)
3. How likely are you to consciously eat less than you want? (“Unlikely”, “Slightly likely”, “Moderately likely”, “Very likely”)
4. Do you go on eating binges though you are not hungry? (“Never”, “Rarely”, “Sometimes”, “At least once per week”)

All questions in parts 1 and 2 are scored from 1 to 4. Part 3 asks about eating restraint:

On a scale of 1 to 8, where 1 means no restraint in eating (eating whatever you want, whenever you want it) and 8 means total restraint (constantly limiting food intake and never "giving in"), what number would you give yourself? Please circle a number below:

1 2 3 4 5 6 7 8

I eat whatever I want, when I want it.

I constantly limit my food intake

2.11.2 Food purchasing task

We used a previously published food purchasing task[268,269]. Participants were asked how many mini Snickers bars, or apple slices, they would purchase and eat immediately over a range of prices from £0 to £3. The questionnaire attempts to quantify the amount of work the participant is willing to perform in return for the given reward and is modelled as an inverse sigmoid plot of demand (number of bars / slices) against price. The key measures are the price at 50% maximum demand (P50) and the demand-price elasticity (the gradient of a plot of demand vs price at P50).

2.11.3 Barratt impulsiveness scale

As some recent evidence has suggested that ghrelin may influence impulsive behaviour and plasma ghrelin concentration should be reduced by gastrectomy, impulsiveness was assessed using the Barratt Impulsiveness Scale (BIS-11)[270]. This uses 30 questions, and ascribes behaviour to 6 first order factors (attention, cognitive instability, motor, perseverance, self-control, cognitive complexity) and 3 second order factors (attentional, motor, non-planning). A higher score indicates greater impulsiveness. While developed on a mixed population of psychiatric inpatients, incarcerated convicts and psychology students, this tool has been applied to people with altered eating behaviour, primarily to explore the relationship between binge eating disorder and impulsiveness[271], albeit never in a surgical population.

Participants are asked to give a response to thirty statements from options of “rarely / never”, “occasionally”, “often” and “almost always / always”:

I plan tasks carefully.	I change jobs.
I do things without thinking.	I act “on impulse.”
I make-up my mind quickly.	I get easily bored when solving thought problems.
I am happy-go-lucky.	I act on the spur of the moment.
I don’t “pay attention.”	I am a steady thinker.
I have “racing” thoughts.	I change residences.
I plan trips well ahead of time.	I buy things on impulse.
I am self-controlled.	I can only think about one thing at a time.
I concentrate easily.	I change hobbies.
I save regularly.	I spend or charge more than I earn.
I “squirm” at plays or lectures.	I often have extraneous thoughts when thinking.
I am a careful thinker.	I am more interested in the present than the future.
I plan for job security.	I am restless at the theatre or lectures.
I say things without thinking.	I like puzzles.
I like to think about complex problems.	I am future oriented.

Table 2-4. BIS-11 question list.

2.11.4 Creature of habit questionnaire

To investigate whether oesophago-gastric resection results in altered habitual behaviour, related or unrelated to eating, we used the creature of habit scale[272]. This recently developed tool was designed to identify habitual tendencies that may drive behavioural differences in a general population. Results are subcategorised into those involved in routine behaviour and automatic responses. It consists of a 27 item questionnaire (Table 2-5), to which the respondent answers from five options ("strongly disagree", "mildly disagree", "undecided", "mildly agree", "strongly agree"). The result for each question is scored from 1 ("strongly disagree") to 5 ("strongly agree").

I like to park my car or bike always in the same place.	I tend to like routine.
I generally cook with the same spices / flavourings.	I usually treat myself to a snack at the end of the workday.
When walking past a plate of sweets or biscuits, I can't resist taking one.	In a restaurant, I tend to order dishes that I am familiar with.
I tend to go to bed at the same time every night.	I am one of those people who get really annoyed by last minute cancellations.
I often take a snack while on the go (e.g. when driving, walking down the street or surfing the web).	I often find myself eating without being aware of it.
I quite happily work within my comfort zone rather than challenging myself, if I don't have to.	I usually sit at the same place at the dinner table.
I tend to do things in the same order every morning (e.g. get up, go to the toilet, have a coffee...).	I often find myself running on 'autopilot', and then wonder why I ended up in a particular place or doing something that I did not intend to do.
Eating crisps or biscuits straight out of the packet is typical of me.	I tend to stick with the version of the software package that I am familiar with for as long as I can.
Whenever I go to the kitchen, I typically look in the fridge.	I always follow a certain order when preparing a meal.
I always try to get the same seat in places such as on the bus, in the cinema or in church.	Television makes me particularly prone to uncontrolled eating.
I often find myself finishing off a packet of biscuits just because it is lying there.	I often find myself opening up the cabinet to take a snack.
I normally buy the same foods from the same grocery store.	I am prone to eating more when I feel stressed.
I rely on what is tried and tested rather than exploring something new.	I find comfort in regularity.
I generally eat the same things for breakfast every day.	

Table 2-5. Creature of habit questionnaire.

2.11.5 Dumping severity score

This brief questionnaire was developed to support a clinical trial of long-acting somatostatin analogues in the treatment of "dumping syndrome" and is a potentially useful and quick to administer scoring mechanism for dumping symptoms. It assesses the severity of 14 post-prandial symptoms (eight "early", i.e. < 1 hour; six "late", i.e. > 1 hour post meal) on a four point scale (0 – absent, 1 – mild, 2 – relevant, 3 – severe and interfering with daily activities)[203]:

Early symptoms

1. Sweating
2. Flushing
3. Dizziness
4. Palpitations
5. Abdominal pain
6. Diarrhoea
7. Bloating
8. Nausea

Late symptoms

1. Sweating
2. Palpitations
3. Hunger
4. Drowsiness / unconsciousness
5. Tremor
6. Irritability

2.12 TISSUE COLLECTION

Human jejunal samples were collected from the operating theatre or endoscopy suite at Addenbrooke's Hospital, placed immediately into L-15 media (Sigma-Aldrich, MO, USA) and onto ice.

To sample "normal" jejunum, the discarded offcut of the alimentary limb of the Roux-en-Y reconstruction after total gastrectomy was collected. This is approximately 50cm from the Ligament of Treitz.

To sample post-operative jejunum, samples were collected during upper gastrointestinal endoscopy, either opportunistically during a clinically indicated endoscopy or during a specific research endoscopy. Up to ten biopsies were taken from the apex of the alimentary limb, just distal to the oesophago-jejunostomy, and if possible from the distal end of the biliopancreatic limb just before the entero-enterostomy. These sites correspond to the point at which the jejunum was divided during gastrectomy, and so to the sample collected during surgery.

All endoscopies were performed by a JAG accredited endoscopist, using conscious sedation or pharyngeal anaesthesia as per the participant's preference. Biopsies were taken with a standard 2.8mm biopsy forceps with spike. There were no adverse events during or after an endoscopy for this study.

2.13 TISSUE PROCESSING FOR RNASEQ

2.13.1 Single cell digest and antibody staining

FACS and RNA extraction from fixed human cells followed a modified version of the MARIS protocol[273]. Intestine was rinsed in cold phosphate buffered saline (PBS) and the muscular coat removed. Diced mucosa was digested twice in 0.1% w/v collagenase XI (Sigma-Aldrich) in Hanks' Buffered Saline solution (HBSS) #9394 (Sigma-Aldrich) for 30 minutes each time, shaking vigorously every 10 minutes. Supernatants were triturated, passed through a 50µm filter and centrifuged at 300g. Pellets were resuspended in PBS and fixed in 4% w/v paraformaldehyde (PFA) at 4°C for 20 minutes. PFA-fixed cells were washed twice in nuclease free 1% w/v bovine serum albumin (BSA) in PBS, and if a fluorescence assisted cell sorting (FACS) facility was not immediately available, were suspended in 1% w/v BSA and 4% v/v RNAsin plus RNAse inhibitor (Promega, WI, USA) in PBS at 4°C overnight.

Cells were permeabilised by the addition of 0.1% w/v Saponin (Sigma-Aldrich) to solutions in all steps from this point until after the first wash post-secondary antibody staining.

Primary antibody staining was for one hour in 4% v/v RNAsin, 1% w/v BSA and primary antibodies as indicated in PBS at 4°C. Cells were then washed twice in 1% w/v BSA, 1% v/v RNAsin, and secondary antibody staining was for 30 minutes in 4% v/v RNAsin, 1% w/v BSA, 0.2% v/v donkey anti-goat Alexa 555, and/or 0.2% v/v donkey anti-rabbit Alexa 647 in PBS at 4°C. Cells were washed twice then suspended in 4% v/v RNAsin, 1% w/v BSA in PBS on ice for FACS.

Control populations were generated by omitting all antibodies (unstained population) or omitting the primary antibody only (secondary only population).

2.13.2 FACS

Cell populations were sorted on a BD FACS ARIA III in the Cambridge NIHR BRC cell phenotyping hub or at Institut Cochin, Paris (by P Richards). Gating strategies were designed based on the baseline fluorescence of the control populations. Single cells positive for Alexa 647 but not Alexa 555 (i.e. CHGA/SCG2 +ve / GLP-1 –ve) were classified as GCG- enteroendocrine cells. Single cells positive for both Alexa 647 and Alexa 555 were classified as GCG+ enteroendocrine cells. At least 5000 cells were collected for each positive population. Twenty thousand double negative cells were collected as the negative (i.e. non-enteroendocrine) cell population. Cells were sorted into 2% v/v RNAsin in PBS at 4^{o}C.

2.13.3 RNA extraction

RNA was extracted using the Ambion Recoverall Total nucleic acid isolation kit for FFPE (Ambion, CA, USA) with modifications to the protocol as below. The FACS sorted cell suspension was centrifuged at 3000g for 5 minutes at 4^{o}C and the pellet resuspended in 200µl digestion buffer with 4µl protease and incubated at 50^{o}C for 3 hours. The solution was then stored at -70^{o}C for at least 12 hours prior to further extraction. After thawing, RNA was extracted using the manufacturer's protocol with the exception of performing 2x 60µl elutions from the filter column in the final step.

The RNA solution was concentrated using an RNEasy Minelute cleanup kit (Qiagen, Hilden, Germany). RNA aliquots were diluted to 200µl with nuclease free water. The standard manufacturer's protocol was followed with the exception that 700µl, not 500µl, of 100% ethanol was added to the solution in step two, to generate optimum binding conditions for the PFA fragmented RNA. RNA concentration and quality were analysed using an Agilent 2100 Bioanalyser (Agilent, CA, USA).

2.13.4 Sequencing

cDNA libraries were created using the Clontech SMARTer Stranded Total RNA-Seq – Pico Input Mammalian v1 kit (Takara Bio, USA). RNA input quantity was 5ng and the non-fragmentation protocol was used. The standard manufacturer's protocol was followed with the exception that 175µl of AMPure beads were used for the final bead purification to ensure recovery of the small fragments of RNA arising from PFA fixation. Sixteen PCR cycles were used for amplification.

50 base single-end sequencing was performed using an Illumina HiSEQ 4000 at the CRUK Cambridge Institute Genomics Core.

2.13.5 RNAseq bioinformatics pipeline

Quality control and trimming of adaptors was performed using FastQC[274]. Sequenced transcripts were mapped to the human (GRCh37) genome using TopHat v2.1.0 and raw counts generated using Cufflinks 2.2.1[275-277]. Differential gene expression analysis was performed in RStudio using DESEQ2[278]. Normalised counts per million (CPM) values were generated from the DESEQ2 model. Gene annotation was pulled from the Ensembl dataset held in BioMart[277]. Receptor and ion channel lists were generated from the IUPHAR "targets and families" list[279]. Graphical output used ggplot2 and pheatmap in RStudio[280].

2.13.6 Mouse transcriptome

Corresponding murine transcriptomic data for the human:mouse comparison were provided from an experiment performed by a post-doctoral researcher in the group (P Larraufie). Proximal small intestinal mucosa from three mice from each of two breeds of fluorescent reporter mice (GLU-Venus to label all proglucagon expressing cells [corresponds to GCG+] and NeuroD1 to label all endocrine lineage cells [corresponds to GCG-]) was homogenised and FACS purified (using DAPI and DRAQ5 to gate for intact live cells). RNA was extracted using an RNeasy Micro plus kit (Qiagen) and cDNA libraries generated through amplification by SPIA amplification using the Ovation RNAseq system V2 kit (Nugen, CA, USA), then subsequent fragmentation by sonication.

Sequencing and bioinformatics pipeline were identical to the human protocol.

2.13.7 Human:mouse transcriptomic comparison

Mouse and human data sets were compared using only the 15,507 transcripts present in both datasets, not annotated as ribosomal, mitochondrial or small-nuclear and described with one-to-one homology according to the Ensembl mouse-human homology dataset[277]. Normalised CPM (counts per million) were compared for the human GCG+ population versus the murine GLU-Venus population and the human GCG- population versus the murine NeuroD1 population. Linear models were generated of the log10 CPM of the human vs murine datasets by a total least squares strategy, 99% confidence intervals calculated and the outliers hand searched for relevant genes.

2.14 TISSUE PEPTIDOMICS

2.14.1 Peptide extraction

Small pieces of jejunal mucosa (10-50mg, ~2x2mm) were collected from intra- and post-operative samples (corresponds to ~2x endoscopic biopsy bites) and homogenised in 250µl 6M guanidine hydrochloride using Lysing matrix D (MPBio) on a FastPrep24 homogeniser (4x40s at $6ms^{-1}$). Samples were stored at -70°C and further processed in batches. Proteins were precipitated by adding 80% acetonitrile in water, centrifugation at 12000g at 4°C for 5 minutes, collection of the aqueous phase containing the peptides and then drying on a centrifugal vacuum concentrator, and stored at -70°C prior to analysis.

2.14.2 Mass spectrometry

Samples were extracted using a Waters HLB µElution solid-phase extraction (SPE) plate (Waters, MA, USA) after being resuspended in 500 µl 0.1% v/v formic acid in water. The wells were sequentially washed with 600µl 0.1% v/v formic acid in water then 600µl 5% methanol / 1% acetic acid v/v in water and eluted onto a low protein binding plate with 2x 30µl elutions of 60% methanol / 10% acetic acid v/v in water. The eluate was evaporated under nitrogen. Cysteine-cysteine cross-links were reduced and alkylated by addition of 75µl 50mM ammonium bicarbonate / 10mM dithiothreitol (DTT), incubation for 1 hour at 60^{o}C, then addition of 15mM iodo-acetamide and incubation for 30 minutes in the dark and 30 minutes in ambient light. 1% v/v formic acid in water was added to bring the volume to ~150µl and the sample stored at -20^{o}C until analysis on the mass spectrometer[250].

Analysis and peptide matching was performed by R Kay. Samples were analysed using nano-flow based separation and electrospray approaches on a Thermo Fisher Ultimate 3000 nano LC system coupled to a Q Exactive Plus Orbitrap mass spectrometer (ThermoScientific). Downstream analysis was performed using Peaks 8.0 software (Waterloo, ON, Canada) against the human Swissprot database (downloaded 26th October 2017)[281], with a fixed cysteine carbamidomethylation and variable methionine oxidation, N-terminal acetylation and pyro-glutamate and C-terminal amidation modifications. Manual searches were performed for other modifications. Peptides of interest were quantified by measuring peak areas for selected m/z ranges and retention times corresponding to the peptide sequences and normalised by tissue weight and internal standard (bovine insulin or C_{13} labelled GLP-1/PYY). Internal standard normalisation was by multiplication of all peptide peak areas by a factor calculated as the inverse of the peak area of the internal standard in that specimen divided by the mean value for internal standard across all specimens (i.e. to normalise for variation in peptide extraction). Weight normalisation was by division of all peak areas by the sample weight.

2.15 STATISTICAL ANALYSIS

Summary data are presented as mean +/- standard error of the mean unless otherwise specified. All statistical analyses were conducted in RStudio and images plotted using *ggplot2*[280] and *pheatmap*.

Normality of data was tested using the Shapiro-Wilk test, and non-normal data were log transformed prior to analysis. Comparisons between groups or interventions were analysed using Welch's t-test in an unpaired or paired fashion as appropriate unless otherwise stated.

2.15.1 Power calculations

This study is made up of several smaller projects, some of which are exploratory / hypothesis generating and so had no prior sample size calculation. In particular, the questionnaire, CGM and RNAseq studies were conducted on as large a sample as possible given the resources and participants available.

Review of the current literature on gut hormone secretion after foregut surgery suggested that GLP-1 secretion after an OGTT would be enhanced >10x in the post-operative cohort, which was confirmed by recruiting a pilot participant to an existing study. The magnitude of effect of surgery was large enough to render any sample size calculation meaningless, and it was decided to recruit a cohort of ~15 participants to examine for differences in response within this group.

A priori sample sizes were calculated to allow for significant differences in glucose handling in the physiological challenge studies. As severity of reactive hypoglycaemia was the finding of greatest clinical interest, we regarded nadir blood glucose during an OGTT as the primary outcome measure. Previous studies within the group have shown a between visit correlation in plasma glucose of 0.52, with a standard deviation of 0.5. There was no previous literature on the effect of antibiotics on nadir glucose to use to estimate a treatment effect, however studies on bariatric populations using Exendin 9-39 have demonstrated a moderate treatment effect (nadir glucose 3.9mmol/l +/-0.6 vs 2.6mmol/l +/- 0.6). A modest treatment effect size of 0.5 was estimated for both interventions, based on a change in mean nadir glucose of 0.5mmol/l between visits. To detect this at 90% power and 95% significance in a pair-analysed population required a sample size of 13 for both the Exendin 9-39 and antibiotic studies.

2.15.2 Analysis of OGTT time-course data

All analyses of changes arising during the OGTT were corrected for baseline values, by calculating the incremental change between time 0 and a time of interest, the fold change, or the incremental area under the curve as in Figure 2-2. Area under the curve was calculated using the trapezoid rule.

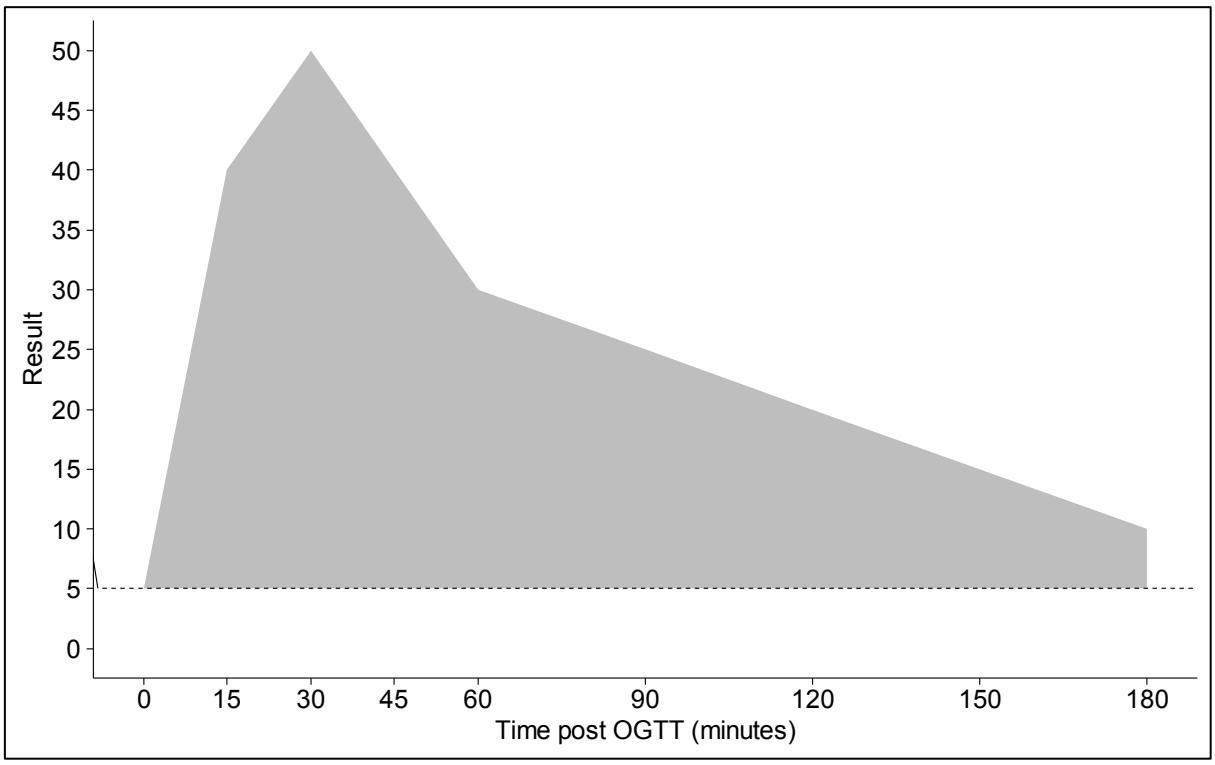

Figure 2-2. Incremental area under the curve (IAUC) is the area of the shaded part of the graph, calculated by subtracting the product of the baseline value and the maximum time from the area under the curve.

2.15.3 Calculation of insulin secretion derivatives

Fasting insulin sensitivity and basal beta cell function were calculated from fasting glucose and insulin samples using HOMA2, which models glucose homeostasis in the fasting state to predict pancreatic beta cell function and whole body insulin sensitivity[282]. Insulinogenic index (IGI) was calculated as the ratio of the 30 minute insulin increment to the 30 minute glucose increment[283]. Disposition index was calculated as IGI / HOMA-IR[284]. Insulin secretory rate (ISR) was calculated using a two-compartment C-peptide deconvolution model, accounting for age, gender and body surface area, using the ISEC program[285]. ISR adjusted for glucose stimulus was calculated as the ratio of the integral of the ISR to the integral of the plasma glucose concentration over a defined time period[286].

2.15.4 Free-living continuous glucose monitoring data analysis

Results from the first 24 hours of wearing the Freestyle Libre Pro were excluded to allow the readings to stabilise. Results were divided into day-time (0800-2200) and night time. Hypoglycaemia was defined as glucose <4.0mmol/l, severe hypoglycaemia <3.3mmol/l, and hyperglycaemia >8mmol/l.

Straightforward measures of glycaemic control were:

1. Percentage of time in normal range, and in each set point outside normal range.
2. Frequency of episodes of hypoglycaemia and severe hypoglycaemia, defined as two consecutive readings outside normal range.

Derived measures of glycaemic control were calculated using the EasyGV calculator[287]:

1. M-value, the mean of the logarithmic transformation of the deviation from a reference value of 5mmol/l for all measurements[288].
2. Mean amplitude of glycaemic excursions (MAGE), where a glycaemic excursion is any change in glucose reading (peak to trough or trough to peak) exceeding 1 standard deviation of the mean glucose reading[289].
3. Low blood glucose index (LBGI) and high blood glucose index (HBGI) are tools for predicting the risk of dysglycaemia in diabetic patients[290]. In order to calculate the LBGI and HBGI, it is necessary to transform plasma glucose readings from their non-symmetrical distribution (i.e. the hyperglycaemic range of 8-33mmol/l is far greater than the hypoglycaemic range of 1-4mmol/l) to a symmetrized distribution in which a euglycaemic value (6.25mmol/l) is given the central value 0. This is achieved by the function:

$f_{(BG)} = (ln_{(BG)})^{1.026} - 1.861$

$r_{(BG)} = 10*(f_{(BG)})^2$

where BG is plasma glucose in mmol/l and:

$rl_{(BG)} = r_{(BG)}$ *if* $f_{(BG)} < 0$ *and 0 otherwise*

$rh_{(BG)} = r_{(BG)}$ *if* $f_{(BG)} > 0$ *and 0 otherwise*

If $x_1, x_2, x_3, \ldots x_n$ is a series of n plasma glucose readings:

$LBGI = \frac{1}{n}\sum_{i=1}^{n} rl(x_i)$

$HBGI = \frac{1}{n}\sum_{i=1}^{n} rh(x_i)$

HBGI and LBGI therefore represent a numerical risk of dysglycaemia, with interpretation in the diabetes population regarding LBGI < 2.5 as low risk, LBGI 2.5-5.0 as moderate risk and LBGI > 5.0 as high risk.

2.15.5 Linear mixed effects modelling

Multivariate analyses were conducted by generation of linear mixed effects models and comparison of the model generated to the null hypothesis of predictor variables having no impact on response variable using a multi-way ANOVA.

Linear mixed effect models were created using the package *lme4*[291] in RStudio, including fixed and random effects as described in the results chapters. Models were examined for normality of residuals and fit of data by four standard approaches:

a. Plot of residuals versus response variable to assess for linearity

   Random scatter of points around y=0 indicates that residuals are not dependent upon the dependent variable and so the linear model is appropriate (vs non-linear transformations).

b. QQ plots of residuals versus a predicted normal distribution

   A close fit to a straight line of y=x indicates that the residuals are normally distributed and so the linear model is appropriate.

c. Plot of fitted versus actual response variable to assess fit of model

   Indicates accuracy of predictive model, and degree of deviation from a line of y=x is the variation in the data not explained by the statistical model.

d. Plot of leverage versus residuals to identify highly leveraged data points

   Highly leveraged data points with large residuals (i.e. points distant from both y=0 and x=0) are outliers and have a greater than average influence on the model, potentially reducing its ability to explain the population level variation in the dependent variable.

In addition, models were sensitivity tested by identifying and excluding any highly leveraged data points, then testing the new model versus the original. As the primary intention of plotting models was to identify predictor variables with significant correlation to the response variable on ANOVA, rather than to develop predictive models, model development by sequential exclusion of factors was not carried out.

Multi-way ANOVA was performed using the *anova* function in RStudio on the best-fitted model.

2.15.6 Principal component analysis (PCA)

Multi-dimensional comparison of datasets was conducted by principal component analysis using FactoMineR in RStudio[292].

2.16 ASSAY SELECTION

While a large number of participants were recruited for glucose tolerance tests, pragmatic decisions were made on the number of samples on which hormone assays were conducted. Gut hormone ELISAs cost £8 to £15 per sample, with a full gut hormone, glucose and insulin panel for a 3 hour OGTT costing up to £1000. It is therefore important to note that not all hormones were measured at all timepoints for all participants. As described, adequate numbers of samples were measured to identify statistically significant differences between cohorts.

2.17 DIVISION OF LABOUR

Unless specified in the methodology of specific chapters, all work was conducted by the author. Specifically, design of studies, managing ethical approvals and recruitment of participants was conducted by the author. Conduct of clinical studies was supported by the nursing staff in the CRF and TRF, including care of participants, administration of trial agents and collection of plasma samples. Endoscopy was performed by the author with assistance from staff in the endoscopy suites at Addenbrooke's Hospital.

Collection and processing of all human tissue samples from the Cambridge participants, including tissue homogenisation, antibody labelling, FACS purification, RNA extraction and generation of cDNA libraries was by the author. Human samples from France were received following RNA extraction, and downstream steps conducted by the author. All murine experiments were performed by a post-doctoral researcher in the Gribble-Reimann laboratory (Pierre Larraufie). Initial processing of RNAseq data to the point of raw transcript counts was conducted by Brian Lam in the Genomics and Transcriptomics Core of the IMS, differential expression analysis and interspecies comparison was conducted by the author. All statistical analyses, including complex modelling, was conducted by the author, with support from Davide Chiarugi (senior bioinformatician in the IMS). This thesis, and all figures included with the exception of the graphical representations of surgery and the human / murine peptidome maps in chapter 9, was produced by the author.

3 GUT HORMONE SECRETION AND GLUCOSE PROFILE AFTER OESOPHAGO-GASTRIC RESECTION

Hypothesis

Post-prandial glucose handling, and secretion of gut and pancreatic hormones is altered by gastrectomy and oesophagectomy.

3.1 INTRODUCTION

The challenges experienced by patients after oesophago-gastric resection are primarily related to nutrient ingestion, be it noxious post-prandial symptoms or reduced appetite and increased satiation resulting in negative energy balance. The physiological response to nutrient ingestion is therefore central to any study of post-surgical physiology. Previous studies have examined various aspects of gut hormone and insulin secretion alongside glucose handling and symptoms in patients after oesophago-gastric resection.

Various mechanisms have been proposed for the altered nutrient handling, glucose profile and symptoms seen in the surgical group, the most compelling of which includes altered gut hormone secretion. Gut hormones, in particular GLP-1, GIP, PYY and ghrelin directly link nutrient ingestion and delivery to the small intestine to systemic factors including insulin secretion and hunger / satiation. Altered gut hormone secretion has also been proposed as the mechanism by which metabolic surgery achieves diabetes remission and weight loss, and suggested as a mechanism underlying both early and late “dumping syndromes”.

In order to characterise the symptomatic and physiological responses to ingested nutrients in the post-operative population, a cohort of healthy volunteers and several groups of post-operative patients were recruited to undergo a 50g oral glucose tolerance test, with sequential measurement of plasma gut and pancreatic hormones and glucose, symptoms and hunger over three hours. An OGTT was selected as the stimulus of choice (over a standardised liquid meal) as it has been previously described to stimulate gut hormone secretion, is easy to standardise and not restricted by taste preference and glucose is described by patients as the most potent stimulus of post-prandial symptoms. In light of this, it was decided to use 50g of glucose, rather than the conventional 75g, to reduce the risk of severe symptoms curtailing study measures.

3.2 METHODS

Participants were recruited from the UK national Hereditary Diffuse Gastric Cancer registry, which is held in Cambridge, and from the oesophago-gastric clinical service at Addenbrooke's Hospital.

OGTTs were conducted as described in chapter 2 on healthy control participants and those who had undergone total or partial gastrectomy, or oesophagectomy. Blood was processed as described and assays were conducted on subsets of timepoints and participants as required to describe the gut hormone profiles of the various groups.

Symptom and physiological measures, plasma and breath samples were collected immediately prior to ingestion of the OGTT and at 15, 30, 45, 60, 90, 120, 150 and 180 minutes post-ingestion. Specifically, the following measures were collected at each timepoint:

1. Blood pressure and heart rate
2. Breath H_2 and CH_4
3. Plasma glucose by YSI
4. Blood for laboratory analysis (processed for EDTA plasma, DPP4/Aprotinin/EDTA plasma, Lithium heparin plasma, serum and AEBSF/HCl plasma [times 0 and 30 only])
5. Symptom measures (Sigstad score and VAS of hunger, fullness and nausea)

Once ethical approval was granted for the study including the dot probe task of food attention, this was administered pre-OGTT, at 15 minutes and at 90 minutes.

For each measure of interest, results are plotted for all groups, with statistical comparison limited to those groups with enough participants for this to be meaningful (i.e. PTG vs control). Between group comparison was conducted using the Welch's T-test (with the input data log-normalised if necessary, indicated in results section).

Multifactorial associations were assessed by constructing linear mixed effects models wherein each timepoint of each OGTT was included as a data point and participant ID was used as a random effect. Models were assessed by reviewing the predictive value of each factor (i.e. F value) and by comparison with the null hypothesis that no model was better fitted than the constructed model, using multi-way ANOVA. Predictive variables significantly associated with model fit on ANOVA (i.e. $p<0.05$) were regarded as associated with the response variable.

3.3 RESULTS

Fifty three participants underwent glucose tolerance tests (17 healthy volunteer / control; 16 prophylactic total gastrectomy [PTG]; 6 total gastrectomy [TG]; 6 oesophagectomy; 4 subtotal gastrectomy [STG]). A further four participants with comorbidities that may influence their metabolic profile were studied (1x PTG with type 1 diabetes; 1x PTG taking long-acting somatostatin analogue; 1x oesophagectomy with type 1 diabetes; 1x oesophagectomy taking high dose steroids for temporal arteritis).

3.3.1 Demographics

Demographics of all participants were as per Table 3-1 and 3-2. Notably, time post-operation ranged from 6 months to 16 years, with median for each group ~3-5 years. The control and PTG groups were younger (39 vs 64 years) than the other post-operative cohorts. The PTG cohort had a marginally lower BMI than the control population (control 25+/-4.1 vs 22.1+/-2.3). No non-diabetic participant had an HbA1c within the diabetic or insulin resistant range, and other blood measures were within the normal range, with the exception of the Hb in the STG group, which was borderline low (mean 110g/dl).

The four comorbid participants were all male and ranged in age from 40 to 65. For the purposes of data analysis, they are considered separately, as their medications or diabetes would likely impact on glucose and insulin homeostasis.

Test	Control	PTG	TG	Oes	STG
Number	17	16	6	6	4
Gender (M:F)	8:9	11:5	3:3	3:3	2:2
Time post op (years; median/range)	NA	3.2 (0.5-14.6)	5.0 (0.9-16.3)	3.4 (1.1-5.3)	2.3 (1.8-8.7)
Age at OGTT (years)	39+/-16	39+/-12	64+/-21	64+/-13	63+/-21
Weight (kg)	74.7+/-18.0	66.9+/-11.8	64.0+/-15.5	59.7+/-17.3	69.5+/-10.5
Height (cm)	171.6+/-9.5	173.6+/-10.9	170.5+/-11.4	158.4+/-8.3	170.5+/-10.0
BMI	25.0+/-4.1	22.1+/-2.3	21.8+/-3.6	23.5+/-5.7	23.8+/-1.6
Waist (cm)	82.8+/-11.2	77.2+/-16.0	83.4+/-16.9	83.8+/-15.7	93.8+/-16.8
Hip (cm)	98.1+/-11.6	89.1+/-15.7	95.6+/-14.1	95.8+/-11.3	100.3+/-8.0
Waist:Hip ratio	0.85+/-0.10	0.86+/-0.07	0.87+/-0.04	0.87+/-0.11	0.94+/-0.20
Hb (g/dl)	139+/-14	140+/-8	127+/-6	129+/-11	110+/-16
ALT	22.1+/-10.5	26.2+/-9.4	29.5+/-18.5	23.5+/-13.9	19.0+/-2.9
Creatinine	69.7+/-15.5	69.6+/-12.2	66.3+/-6.3	65.0+/-8.5	96.3+/-49.6
TSH	1.93+/-1.34	1.71+/-0.98	2.40+/-0.89	1.8+/-1.1	1.6+/-1.5
HbA1c	33.2+/-3.8	35.7+/-2.4	40.0+/-4.5	36.6+/-4.3	34.3+/-6.7

Table 3-1. Participant demographics for all OGTT participants. Values are mean +/- standard deviation unless indicated. Oes – oesophagectomy.

Test	PTG (type 1 diabetes)	PTG (somatostatin)	Oesophagectomy (steroids)	Oesophagectomy (type 1 diabetes)
Number	1	1	1	1
Gender	M	M	M	M
Time post operation (years)	2.8	13.2	3.6	0.5
Age at OGTT (years)	48	65	65	40
Weight (kg)	80.4	66.0	84.2	98.8
Height (cm)	185	178	177.4	184.7
BMI	23.5	20.8	26.8	29.0
Waist (cm)	91	77.5	92.5	103
Hip (cm)	107	91	100	114
Waist:Hip ratio	0.85	0.8	0.92	0.9
Hb (g/dl)	147	127	-	147
ALT	30	21	25	31
Creatinine	70	83	92	62
TSH	3.68	2.13	NA	1.65
HbA1c	56	43	40	65

Table 3-2. Demographics of participants with diabetes or on somatostatin analogues or high dose steroids.

3.3.2 Glucose handling

Plasma glucose was measured using either the Yellow Springs Instrument (YSI) at the point of care, or in CBAL by the hexokinase method on snap frozen lithium heparin plasma, or both, for all participants undergoing OGTT. For those with both YSI and laboratory glucose measures, direct comparison demonstrated them to be highly correlated (Figure 3-1; R^2=0.98), with laboratory glucose predicted by the formula:

Lab glucose = (1.079 * YSI glucose) + 0.121

Laboratory glucose measures were absent for at least some timepoints in eight oral glucose tolerance visits, and YSI measures were absent for sixteen OGTTs, due to a change in methodology of using the YSI during the study. It was therefore decided to generate predicted laboratory glucose values using the available YSI values and the above linear model for all missing laboratory glucose values.

The time of nadir glucose varied from 45 minutes to 150 minutes post OGTT. As the timepoint mean was therefore a poor indicator of glucose handling within a group, the individual participant plots were plotted to demonstrate the range of glucose curves within groups (Figure 3-2). Statistical analysis of peak, nadir and rate of drop of glucose from peak value was conducted from the relevant values for each participant and therefore varies in timing between participants.

There was no significant difference between prophylactic (PTG) and radical total gastrectomy (TG) participants in any glucose measure except nadir glucose during the OGTT. 10/16 PTG participants experience a hypoglycaemia <4mmol/l, versus only 1/6 TG, with mean nadir glucose being significantly different between the groups (3.8+/-0.2mmol/l vs 4.6+/- 0.2mmol/l; p=0.01).

Compared to the control group (Figure 3-3), the prophylactic total gastrectomy group experienced a greater peak glucose (11.87+/-0.62mmol/l vs 9.06+/-0.52mmol/l; p=0.002) and steeper fall in glucose from its peak value (measured as [glucose at T_{peak} – glucose at $T_{peak+30}$]/30; 0.14+/-0.01mmol/l/min vs 0.055+/-0.007mmol/l/min; $p=2*10^{-7}$). There was no significant difference between mean lowest glucose between these groups. Of the ten PTG participants developing hypoglycaemia, the lowest

plasma glucose for four was <3.3mmol/l, versus only 6/17 healthy volunteers having hypoglycaemias (of whom one was <3.3.mmol/l).

There was no significant difference between peak glucose ($p>0.4$) and rate of fall of peak glucose ($p>0.2$) between the any of the three gastrectomy groups. Of the four partial gastrectomy patients studied, two experienced hypoglycaemia <4mmol/l during the OGTT, of whom one had a lowest glucose <3.3mmol/l.

There was no significant difference in peak ($p=0.13$), nadir ($p=0.34$) or rate of decline from peak glucose ($p=0.38$) between oesophagectomy and control groups.

The study protocol was only stopped once in order to treat a hypoglycaemic episode, in a participant with a history of unexplained post-operative seizures whose plasma glucose dropped from 9.9mmol/l to 3.3mmol/l in 30 minutes (and to 2.45mmol/l by the time they ingested a glass of orange juice). All other hypoglycaemic episodes were self-limiting and plasma glucose normalised within 15-30 minutes without intervention.

Of the ten prophylactic total gastrectomy participants with nadir plasma glucose <4mmol/l, only 3 had significant symptoms as measured by Sigstad score at the time of the hypoglycaemia. Of the four with nadir glucose <3.3mmol/l, only two had any symptoms at the time of the hypoglycaemia.

Association between measured factors and risk of severe hypoglycaemia in the post-operative groups (nadir glucose <3.3mmol/l) was investigated by logistic regression, using group, time post-op, age, gender, BMI, IAUC120 insulin and IAUC120 GLP-1 as predictor variables. None was strongly predictive for the occurrence of severe hypoglycaemia during OGTT.

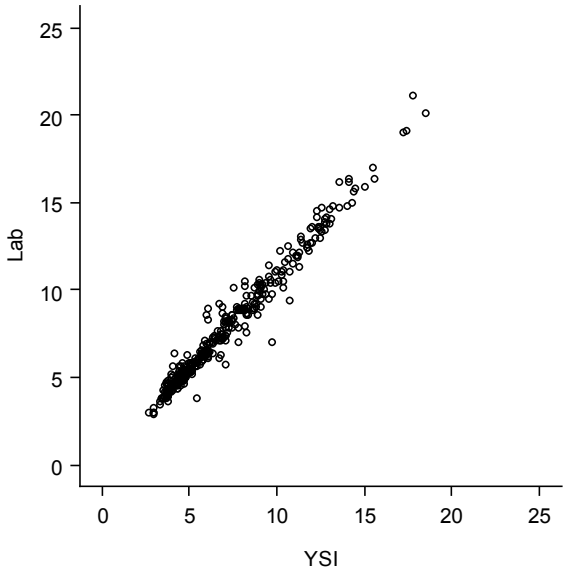

Figure 3-1. Laboratory versus YSI plasma glucose (mmol/l) results for all matched samples collected during OGTT in mmol/l. R^2=0.98.

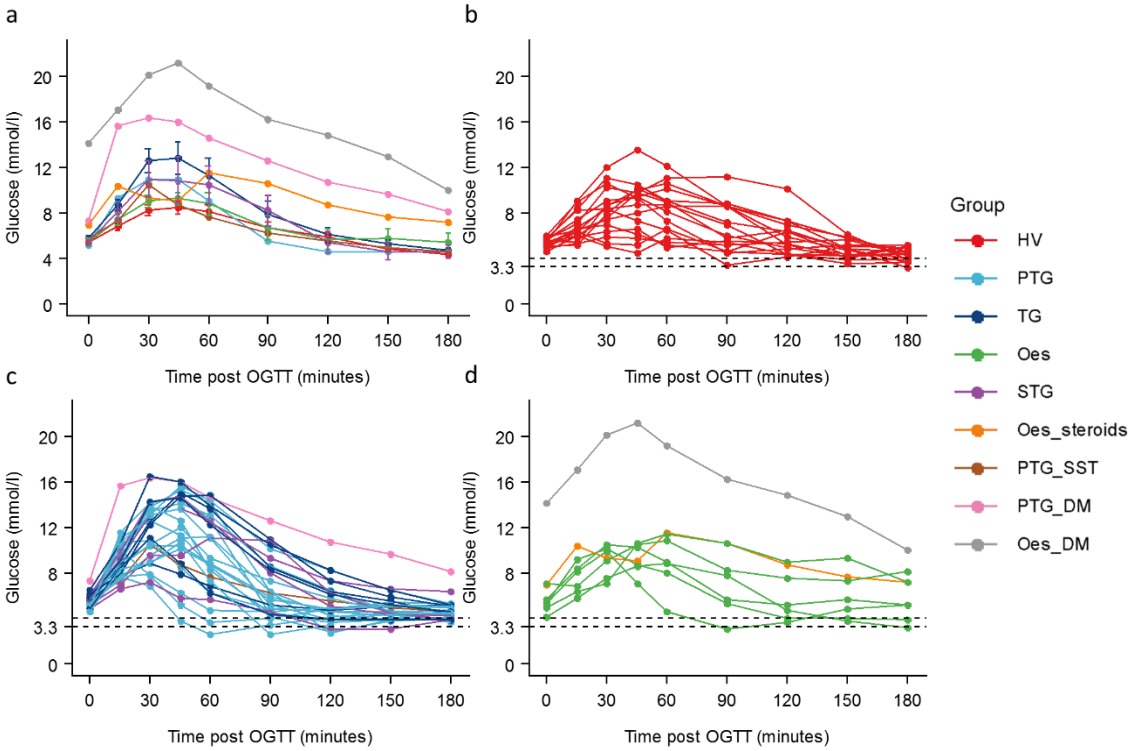

Figure 3-2. Plasma glucose concentration for 3 hours following 50g OGTT. a - Mean +/- standard error for all groups. b - all healthy volunteers. c - all gastrectomy participants. d - all oesophagectomy participants. SST – participant taking somatostatin analogue. DM – participant with type 1 diabetes.

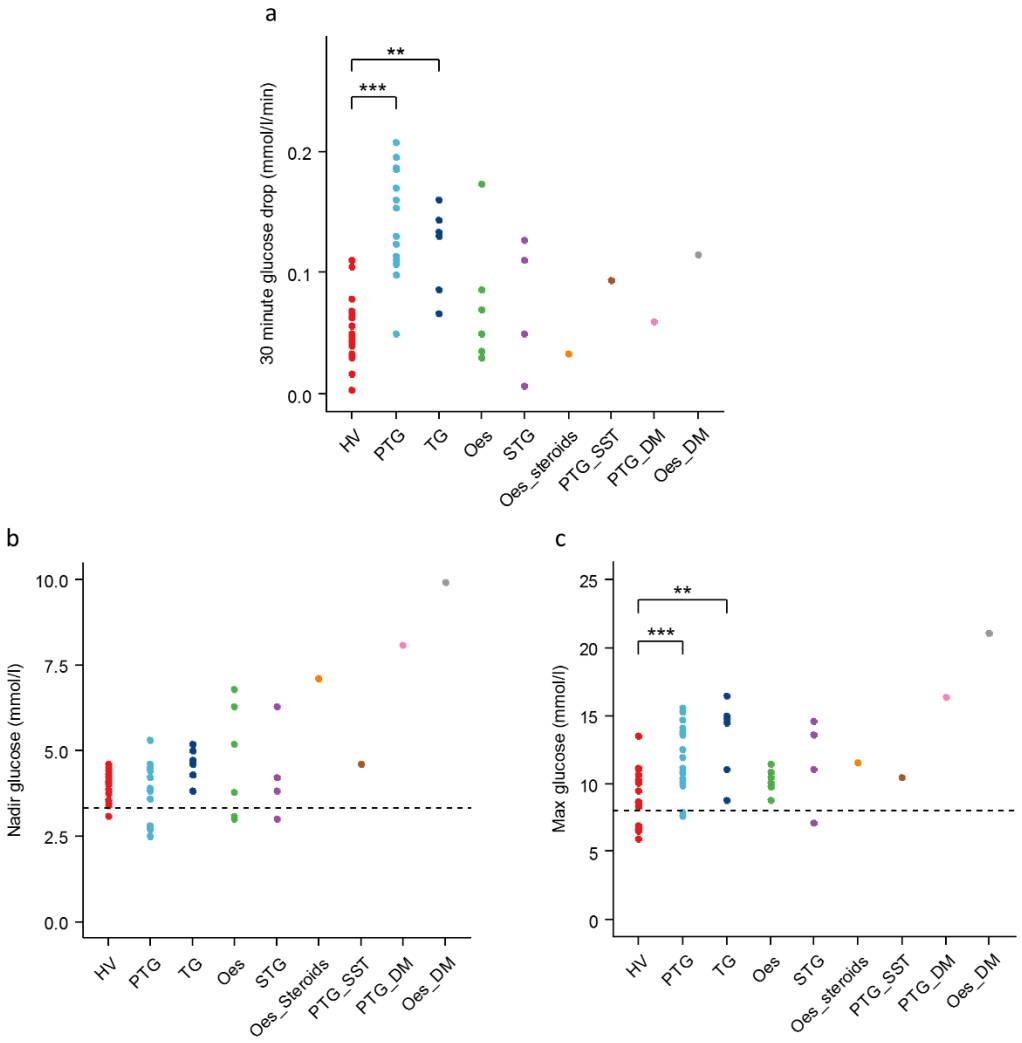

*Figure 3-3. Key glucose handling measures during OGTT in individual participants by group. a - rate of decline of glucose from peak to glucose 30 minutes later. b - nadir glucose (dashed line at 3.3mmol/l). c - maximum glucose (dashed line at 8mmol/l). ** - p<0.01. *** - p<0.001. Welch's T-test (two-sided).*

3.3.3 Insulin secretion

Plasma insulin was assayed during OGTT for 13 control, 17 PTG (including one taking a long-term somatostatin analogue), 6 TG, 5 oesophagectomy (including one taking high dose prednisolone) and 3 STG participants. The results of participants treated with somatostatin analogues and steroids were analysed separately. As it became apparent from the first studies that plasma insulin concentration normalised by 120 minutes post OGTT, plasma insulin for the 150 and 180 minutes timepoints was not measured for later participants (Figure 3-4) and comparisons of total insulin secretion used the incremental area under the curve to 120 minutes.

Overall insulin secretion was presented as the 120 minute incremental area under the curve (IAUC_120) for each group, as a measure of total insulin secretion during the OGTT. IAUC_120 values were non-parametrically distributed within groups and so were log transformed for statistical analysis, with log-transformed values normally distributed on Q-Q plots and Shapiro-Wilk testing.

While insulin IAUC_120 in the control group was relatively closely matched between participants (24600+/-5600pmol/l/min), it was far more variably distributed within the post-operative groups (PTG 45300+/-9900; TG 39100+/-13000; oesophagectomy 48000+/-22000; STG 54000+/-18400). Despite this variability, IAUC120 for insulin was significantly greater in the PTG group than controls ($p=0.03$), although not for STG, TG or oesophagectomy versus controls.

Excluding the outlier in the control group (with IAUC_120 of 88000pmol/l/min, compared to 29600pmol/l/min for the next highest control), 9/16 PTG, 3/6 TG, all three STG and 2/4 oesophagectomy participants had IAUC_120 values greater than the highest control. Examining the individual insulin secretory profiles over the course of the whole OGTT supports this finding, that a subset of post-operative participants have an enhanced insulin response to the OGTT.

3.3.4 Insulin sensitivity

Fasting plasma insulin concentration was significantly lower in the PTG (22.2+/-1.8pmol/l; p=0.02)) but not TG (25.2+/-7.7pmol/l; p=0.11), STG (40.3+/-18.1pmol/l; p=0.87) or oesophagectomy (24.5+/-8.7; p=0.14) groups than in controls (44.0+/-8.2pmol/l), although the small sample sizes limit conclusions that could be drawn from the TG and STG results. Multi-way ANOVA of a linear mixed effects model of fasting insulin, including operative group, age, BMI and gender as predictor variables, identified BMI as the primary predictor of fasting insulin (p=0.03), suggesting that the reduced fasting insulin in the PTG group was primarily driven by the lower BMI in this group.

Fasting insulin sensitivity and beta cell function were derived from the HOMA2 model[282]. This tool assumes steady state insulin / glucagon secretion and hepatic gluconeogenesis in the fasting state and provides a predicted measure of beta cell function and hepatic insulin sensitivity from fasting plasma glucose and insulin measures. The output values are in percent, where 100% is the "normal" value from validation studies. The use of this "normal" value was however inappropriate due to the considerable variability in insulin assay calibration, and so the comparison of groups, using the same assay and laboratory, was used here.

From Figure 3-4f it is apparent that HOMA-IS (insulin sensitivity) was bimodally distributed in the control group used in this study, with the majority of total gastrectomy patients having a greater HOMA-IS value than the lower control group. Direct comparison reveals a marginally significantly greater insulin sensitivity in the PTG than control group (PTG 191.2+/-9.3% vs control 137.0+/-22.5; p=0.04). Again, multi-way ANOVA of a linear mixed effects model constructed to compare contributory factors to HOMA-IS identified the primary driver of this difference as the reduced BMI in the PTG group.

Thus, while superficially it appeared that fasting insulin concentration was reduced, and sensitivity enhanced, after PTG, the results were confounded by the lower BMI of the PTG group, which was a more plausible explanation than any operative effects in the fasting state.

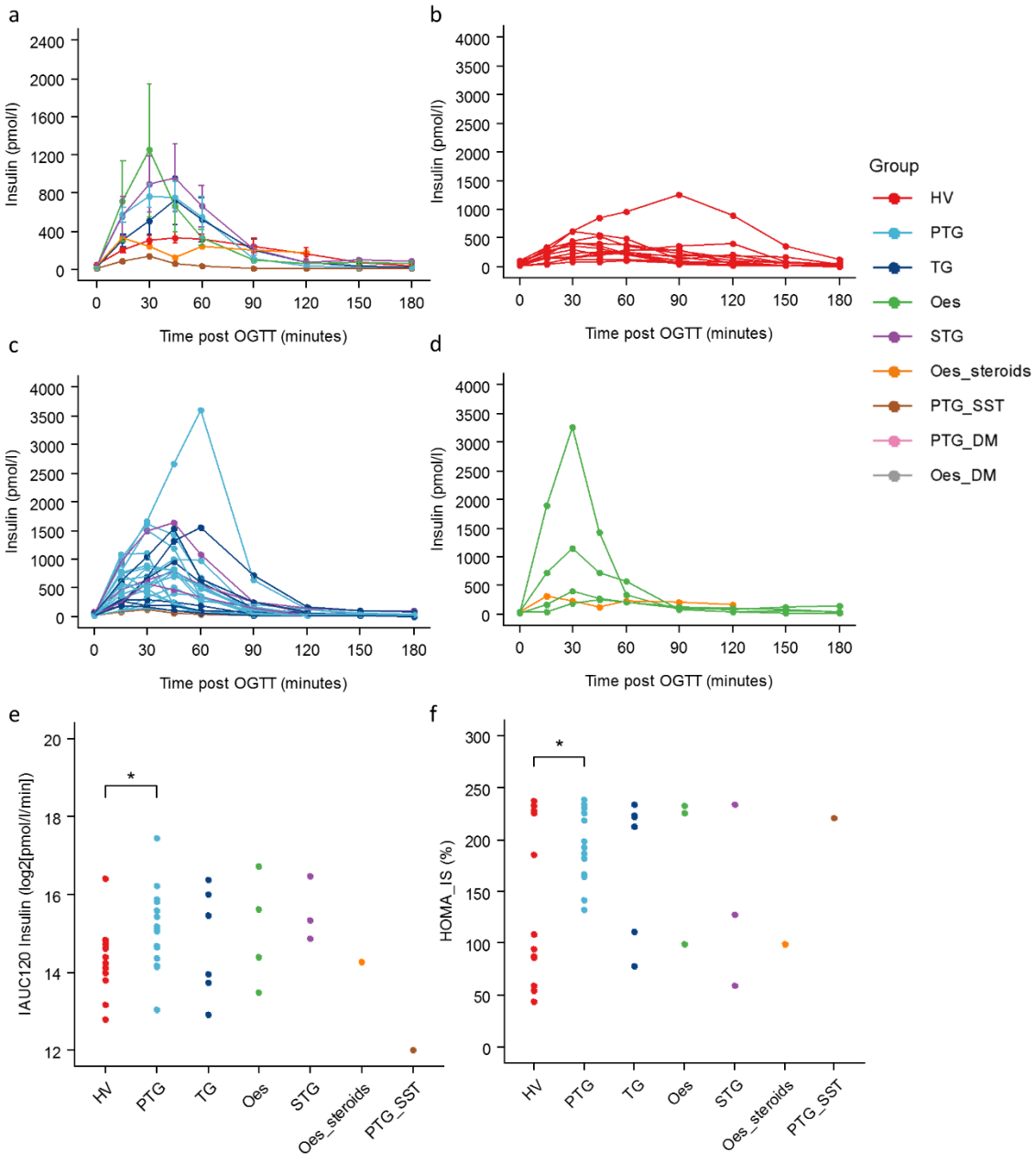

*Figure 3-4. Insulin secretion during OGTT. a-d - plasma insulin concentration over 3 hours following OGTT. a - secretory profile by group (mean +/- standard error). b - individual healthy volunteer traces. c - individual gastrectomy traces. d - individual oesophagectomy traces. e – 120 minute integrated area under the curve by group. f – HOMA-IS score by group. * - p<0.05. Welch's T-test (two-sided).*

3.3.5 Glucagon like peptide 1 (GLP-1) secretion

Plasma GLP-1 was assayed during OGTT for 13 control, 18 PTG (including one with type 1 diabetes mellitus and one on a long-term somatostatin analogue), 6 TG, 7 oesophagectomy (including one with type 1 diabetes mellitus and one taking high dose prednisolone) and 3 STG participants.

Fasting concentration of GLP-1 was not significantly different between any of the groups studied, but peak plasma GLP-1, measured at 15-30 minutes post-OGTT, was markedly greater for all post-operative groups (range of means 102-222pg/ml) than controls (24.7+/-4.2pg/ml).

By statistical comparison using Welch's t-test, GLP-1 IAUC_120 was significantly higher in the PTG (8900+/-1200pg/ml/min; $p=3*10^{-6}$) and TG (6800+/-1300; p=0.004) than control groups (400+/-200). Values for the partial gastrectomy (4600+/-1100pg/ml/min; p=0.06) and oesophagectomy (5700+/-2500pg/ml/min; p=0.12) groups trended to greater GLP-1 secretion than in the control group without reaching statistical significance. Examining the individual participant plots, it was clear that there was significant variation in GLP-1 secretion within groups, with some participants demonstrating profiles similar to the control group, and others with peak concentrations >10x greater than any control (Figure 3-5).

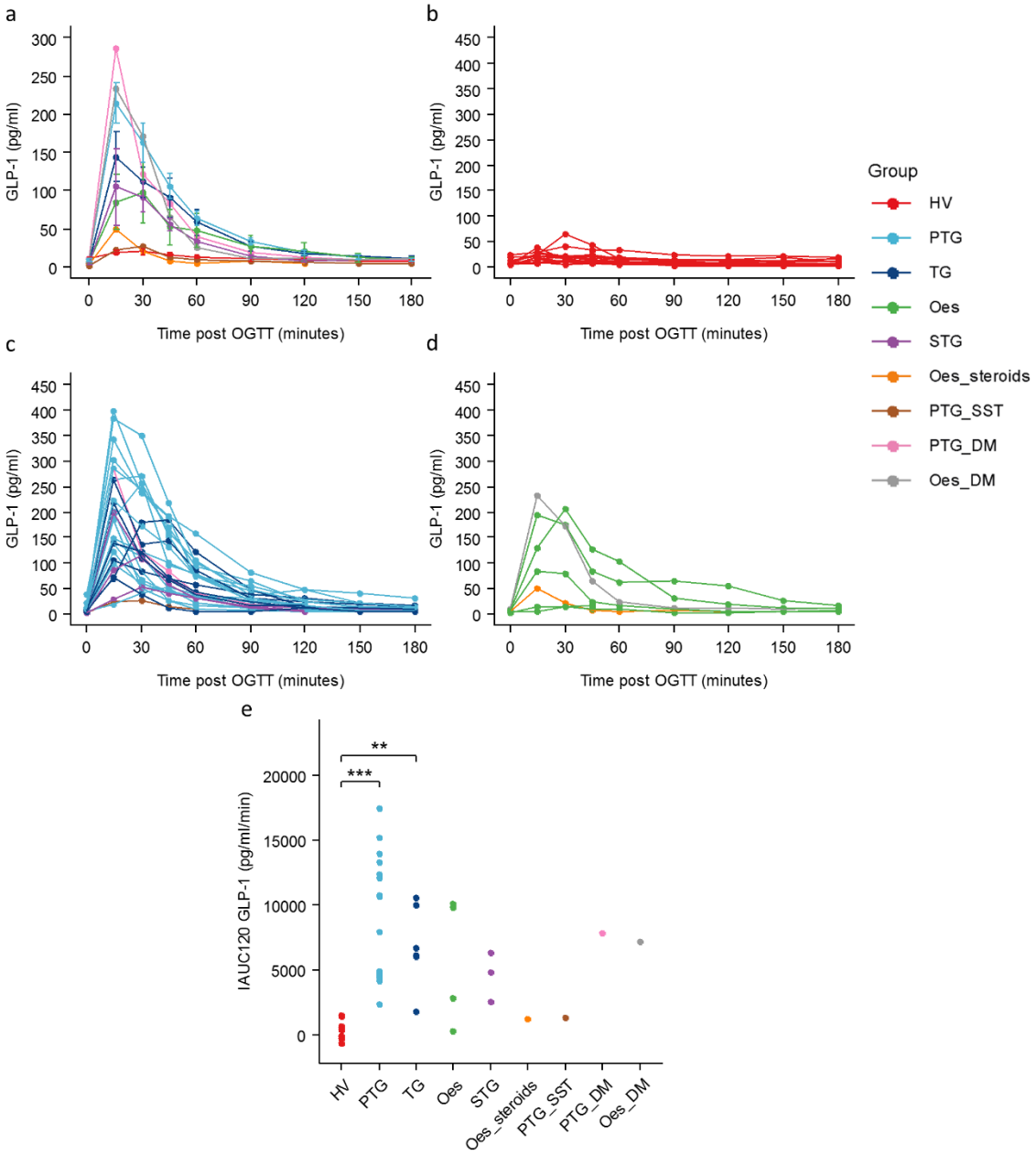

*Figure 3-5. GLP-1 secretion during OGTT. a-d - plasma GLP-1 concentration over 3 hours following OGTT. a - secretory profile by group (mean +/- standard error). b - individual healthy volunteer traces. c - individual gastrectomy traces. d - individual oesophagectomy traces. e – 120 minute integrated area under the curve by group. ** - p<0.01. *** - p<0.001. Welch's T-test (two-sided).*

3.3.6 Glucose-dependent insulinotropic peptide (GIP) secretion

Plasma GIP was assayed during OGTT for 10 control, 13 PTG (including one with type 1 diabetes mellitus and one on a long-term somatostatin analogue), 1 TG and 5 oesophagectomy (including one with type 1 diabetes mellitus) participants.

GIP IAUC_120 was not significantly different between control, PTG or oesophagectomy groups. However, this measure of total secretion after OGTT ignores the clear difference in time course of GIP secretion evident from the individual participant plots (Figure 3-6). Following PTG, the peak GIP was significantly earlier (15 minutes rather than 30 minutes) and greater (298.7+/-36.8pg/ml; p=0.005), and trended to being greater in the oesophagectomy group (211.9+/-20.0) than in the control group (164.2+/-15.2).

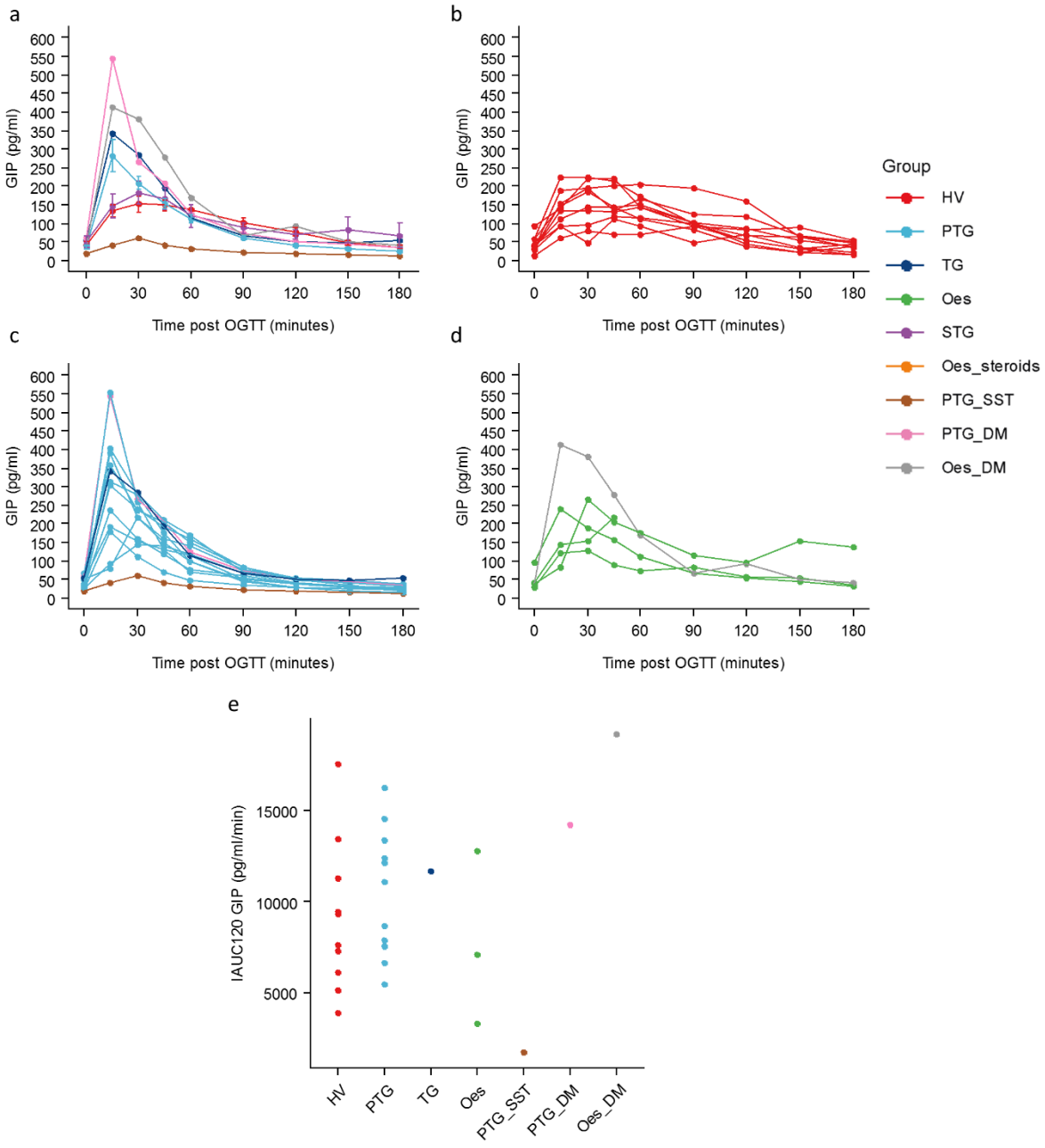

Figure 3-6. GIP secretion during OGTT. a-d - plasma GIP concentration over 3 hours following OGTT. a - secretory profile by group (mean +/- standard error). b - individual healthy volunteer traces. c - individual gastrectomy traces. d - individual oesophagectomy traces. e – 120 minute integrated area under the curve by group.

3.3.7 Peptide YY (PYY) secretion

Plasma PYY was assayed during OGTT for 11 control, 15 PTG (including one with type 1 diabetes mellitus and one on a long-term somatostatin analogue), 5 TG, 6 oesophagectomy (including one with type 1 diabetes mellitus and one taking high dose prednisolone) and 3 STG participants.

PYY IAUC_120 was significantly greater for the PTG (10000+/-1300pg/ml/min; $p=2*10^{-6}$) and TG (9000+/-2300pg/ml/min; p=0.02) than the control groups (150+/-440pg/ml/min). The IAUC_120 values for the oesophagectomy (6300+/-8500pg/ml/min) and partial gastrectomy (8500+/-3000) groups were greater than for the control group, but did not reach statistical significance.

Examining the individual PYY plots, it was clear that at least a subset of the oesophagectomy and partial gastrectomy groups displayed enhanced post-OGTT PYY secretion. In all groups, when enhanced, the PYY secretory profile was more prolonged than that seen for GLP-1 and GIP perhaps arising due to the more distal secretory profile for PYY than GIP and GLP-1.

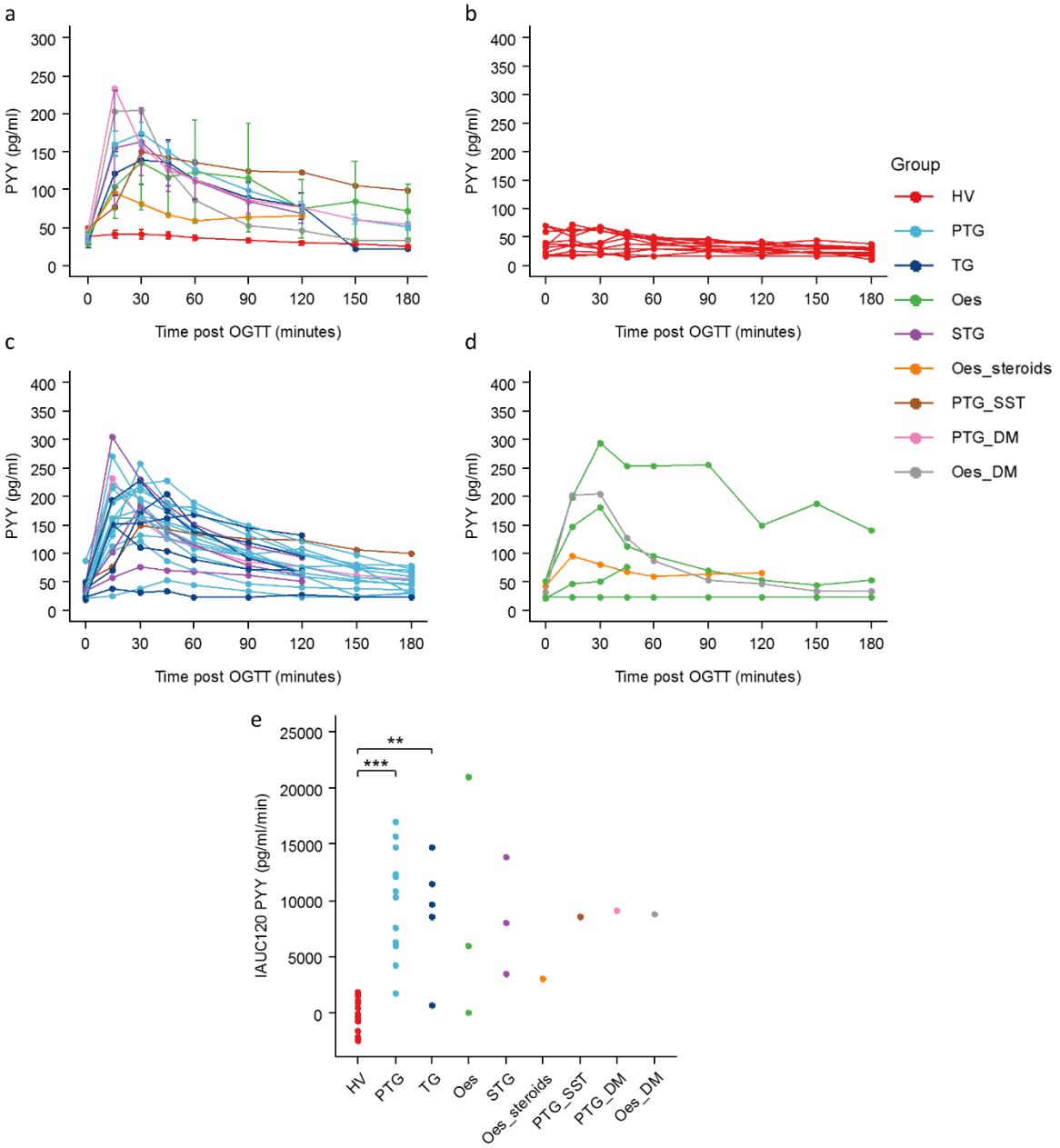

*Figure 3-7. PYY secretion during OGTT. a-d - plasma PYY concentration over 3 hours following OGTT. a - secretory profile by group (mean +/- standard error). b - individual healthy volunteer traces. c - individual gastrectomy traces. d - individual oesophagectomy traces. e – 120 minute integrated area under the curve by group. ** - p<0.01. *** - p<0.001. Welch's T-test (two-sided).*

3.3.8 Acyl ghrelin profile

Fasting acyl ghrelin was measured for 11 control, 14 PTG (including one with type 1 diabetes mellitus and one on a long-term somatostatin analogue), 7 oesophagectomy (including one with type 1 diabetes mellitus and one taking high dose prednisolone) and 4 STG participants. Acyl ghrelin was also measured at 30 minutes post OGTT in all the above participants with the exception of one post-oesophagectomy participant (Figure 3-8).

In healthy volunteers, plasma acyl ghrelin concentration was significantly reduced from fasting to 30 minutes post OGTT (678+/-118pg/ml vs 482+/-85; p=0.001). The concentration of acyl ghrelin was below the lower limit of detection for the assay (60pg/ml) for all gastrectomy samples bar one, for which the fasting concentration was 74pg/ml (the corresponding 30 minute sample was undetectably low). Fasting plasma ghrelin concentration in the oesophagectomy and partial gastrectomy groups were within the range seen in healthy volunteers, and the low participant numbers make it impossible to accurately conclude any effect of these operations on acyl ghrelin secretion.

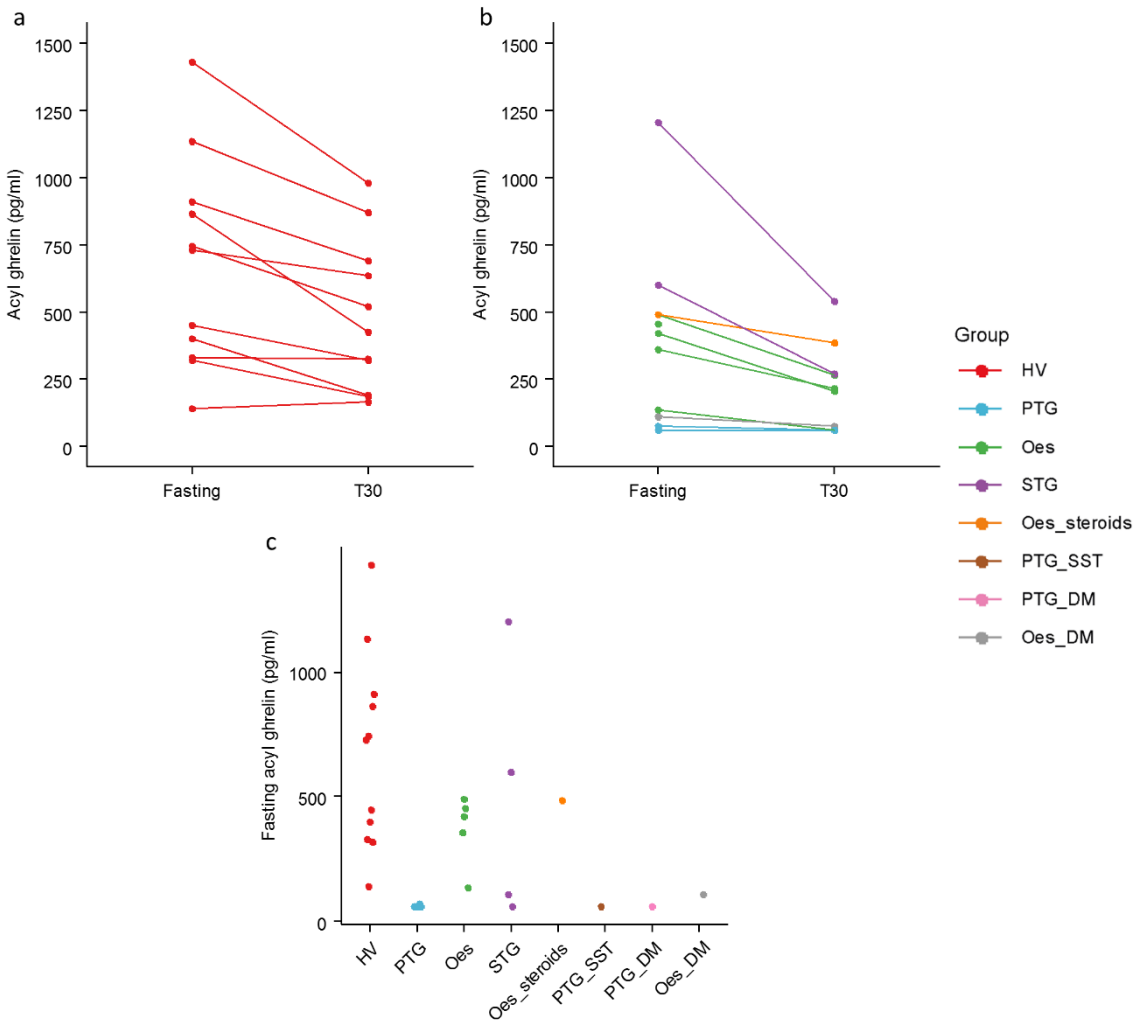

Figure 3-8. Plasma acyl ghrelin concentration in healthy volunteers (a) and post-operative participants (b) at time 0 and 30 during OGTT and fasting levels across all groups (c). Samples with undetectable concentrations of acyl ghrelin assigned a value corresponding to the lower limit of detection of the assay (60pg/ml).

3.3.9 Plasma 5-HT

It was hypothesized that the symptom complex seen post-prandially in patients after gastrectomy is driven in part by excessive secretion of 5-HT due to overstimulation of Enterochromaffin cells by rapid delivery of nutrients to the small intestine. 5-HT was measured in platelet poor plasma by LC-MS from 5 control and 5 PTG participants from specimens collected over 3 hours after a 50g OGTT.

The lowest measurable concentration of 5-HT detected was 4.3nM, with sixteen specimens having no detectable 5-HT (Figure 3-9). As this was above the speculative upper limit of normal from historical studies (1nM), in both control and gastrectomy samples, it was presumed that platelet activation had occurred during collection of the plasma, resulting in the assay measuring both free and platelet bound 5-HT. This would therefore not be an accurate measure of free and hence metabolically active 5-HT, and so not advance the hypothesis for the role of 5-HT in post-operative symptoms. Within this limitation, there was also no significant difference in the trends in 5-HT concentration between control and gastrectomy samples.

It was decided not to pursue this avenue further due to the lack of a good quality method for collecting platelet poor plasma, and focus on other gut hormones.

3.3.10 Leptin

Leptin was measured in the fasting state for 10 participants after PTG (including 1 diabetic and 1 on somatostatin), 7 controls, and 1 after oesophagectomy. There was no significant difference in serum leptin between the PTG and control groups, and in keeping with the current literature on leptin, it was closely correlated to BMI (R^2 = 0.72; Figure 3-9).

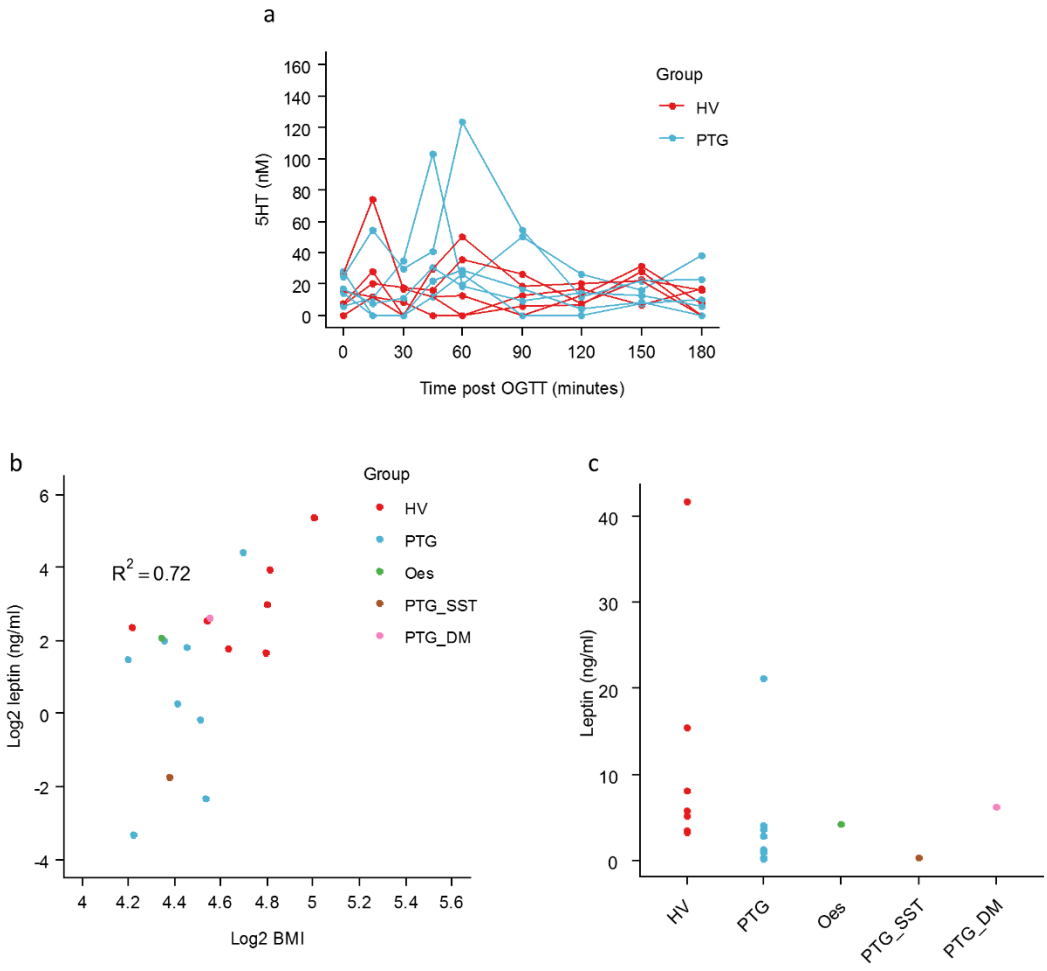

Figure 3-9. a - Plasma 5-HT concentration over 3 hours post OGTT. b - Log-log plot of serum leptin versus BMI. c - plot of serum leptin concentration by group.

3.3.11 Heart rate and blood pressure

Heart rate and blood pressure were measured for all participants during OGTT. Notably, in the total gastrectomy (PTG and TG) group heart rate evidenced a small but significant rise which was not apparent in the control group. This was >10 beats per minute for 8 of the 16 PTG participants, however there was no association between haematocrit rise, Sigstad score or heart rate rise on chi squared test.

While a similarly heterogeneous response was seen for mean arterial pressure (MAP), the trend was similar in direction and magnitude for post-operative and controls, and there was no correlation between change in blood pressure and symptoms.

	Control	PTG	P value (Welch's T-test)
15 minute delta HR (%)	-3.50+/-1.64	29.7+/-4.53	$2*10^{-6}$
15 minute delta MAP (%)	-4.01+/-1.68	-3.46+/-3.74	0.89

Table 3-3. Haemodynamic measures after OGTT. MAP - mean arterial pressure.

3.3.12 Haemoglobin and haematocrit

Haemoglobin and haematocrit were measured for all timepoints post OGTT in 12 PTG, 3 TG, 9 control, 1 STG and 1 oesophagectomy participants. Notably, while both values dropped from fasting to the 15 minute timepoint in control participants, both rose in the gastrectomy populations. From this point, both trends were closely matched.

The percentage change in both values was significantly different between populations:

	Control	PTG	P value (Welch's T-test)
15 minute delta Hct (%)	-2.63+/-0.62	3.63+/-1.37	$8*10^{-4}$
15 minute delta Hb (%)	-2.05+/-0.39	3.19+/-0.91	$1*10^{-4}$

Table 3-4. 15 minute delta for circulatory measures

As historically two diagnostic criteria for dumping syndrome have been a Sigstad score of at least 7 and a 3% rise in haematocrit value, the association between the presence of these two values was tested and found to be lacking (p=0.87, Chi squared test). Thus while 9 of the 15 total gastrectomy participants studied had a diagnostically significant rise in Hct, this did not result in a matched symptomatic response, indeed only two participants with a delta Hct >3% had a corresponding Sigstad score >7.

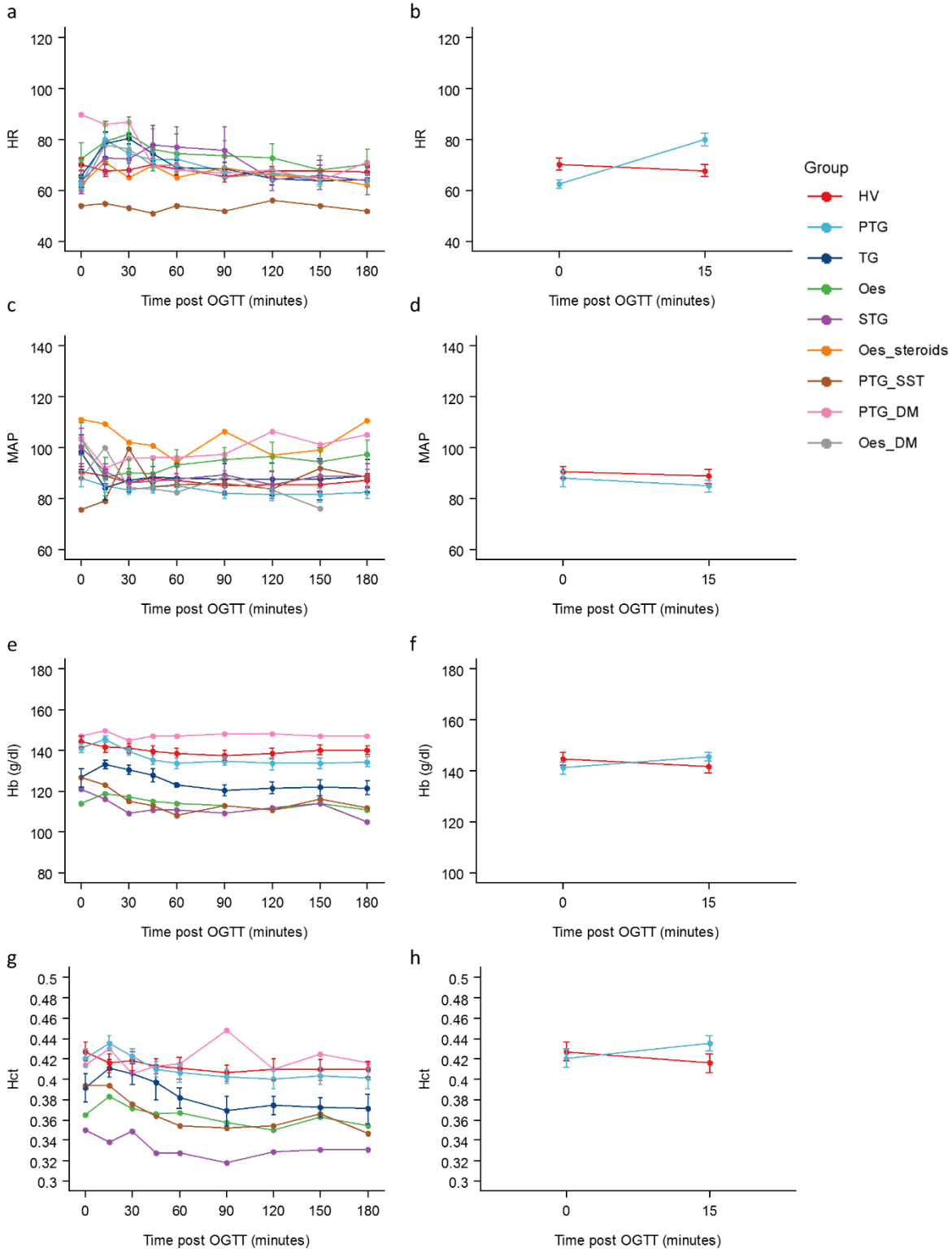

Figure 3-10. Haemodynamic factors after OGTT. a,c,e,g - 3 hour traces by group (mean +/- standard error). b,d,f,h - absolute change at from T0 to T15 in control and PTG groups (mean +/- standard error). a,b - heart rate (beats per minute). c,d - mean arterial pressure (mmHg). e,f – haemoglobin (g/dL). g,h – haematocrit.

3.3.13 Sigstad score

Symptom severity during OGTT was measured using the Sigstad score for 17 control, 18 PTG (including 1 with type 1 diabetes and 1 taking a somatostatin analogue), 6 TG, 8 oesophagectomy (including 1 on high dose steroids and 1 with type 1 diabetes) and 4 STG participants. Total Sigstad score summed across all 9 timepoints was used as a measure of total symptom severity during the OGTT, with a maximum possible score of 261. Excluding one outlier with a total Sigstad score of 83, the total score for the control group ranged from 0 to 23 (Figure 3-11).

Fifteen participants after total gastrectomy (ten after PTG, five after TG) had a total Sigstad score greater than 23, ranging to a maximum of 85. Interestingly, 7 did not suffer symptoms any worse than a healthy volunteer, demonstrating the variability in response to gastrectomy which is explored later in this chapter. Examining the individual participant time course plots for each gastrectomy participant, it was clear that the classical description of a bimodal distribution of symptoms was not represented in this data set. Of the 7 participants with a total score of <23, two have peak scores of 7 or greater, which historically has been regarded as a diagnostic cut off for dumping syndrome. Of those with a total score of >23, one did not have a peak score of >7.

In the subtotal gastrectomy group, the distribution of total Sigstad score appeared greater than in the control group, but the picture was more nuanced for the oesophagectomy group.

Investigation of factors correlated with Sigstad score by ANOVA of a linear mixed effect model incorporating operative group, age, gender, BMI, time post OGTT, glucose, insulin, PYY, GLP-1 and GIP as fixed effects and participant ID as a random effect, identified male gender, group (PTG and TG) and time post OGTT to be highly correlated with Sigstad score (Table 3-5, Figure 3-12). Insulin and gut hormone concentrations were not correlated with symptoms by this measure.

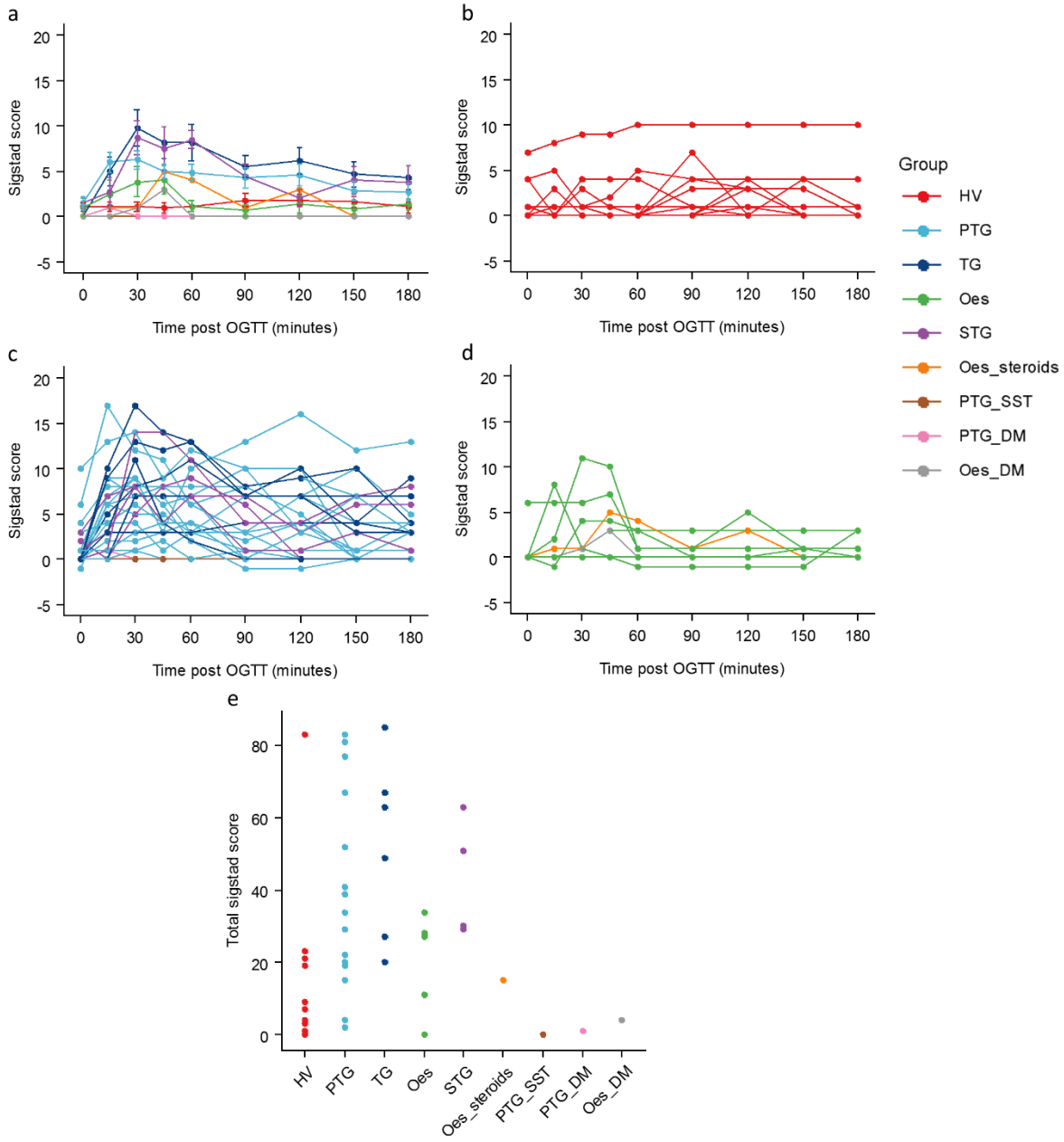

Figure 3-11. Sigstad score during OGTT. a - scores by group over 3 hours (mean +/- standard error). b,c,d - individual participant Sigstad score traces over 3 hours (controls, gastrectomy, oesophagectomy respectively). e - total Sigstad score for each participant by group.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	3.08	0.62	5	16.01	3.30	0.03
Age	0.85	0.85	1	13.35	4.55	0.05
Gender	1.49	1.49	1	12.65	8.00	0.01
BMI	0.00	0.00	1	14.27	0.00	0.95
Time	2.94	0.49	6	151.68	2.63	0.02
Glucose	0.05	0.05	1	169.82	0.29	0.59
Insulin	0.03	0.03	1	172.83	0.16	0.69
GLP1	0.40	0.40	1	172.98	2.15	0.14
PYY	0.09	0.09	1	159.40	0.51	0.48
GIP	$5*10^{-4}$	$5*10^{-4}$	1	170.61	$3*10^{-3}$	0.96

Table 3-5. ANOVA table for factors associated with Sigstad score at any given timepoint by linear mixed effects model with ID as random variable. NumDF – numerator degrees of freedom; DenDF – denominator degrees of freedom.

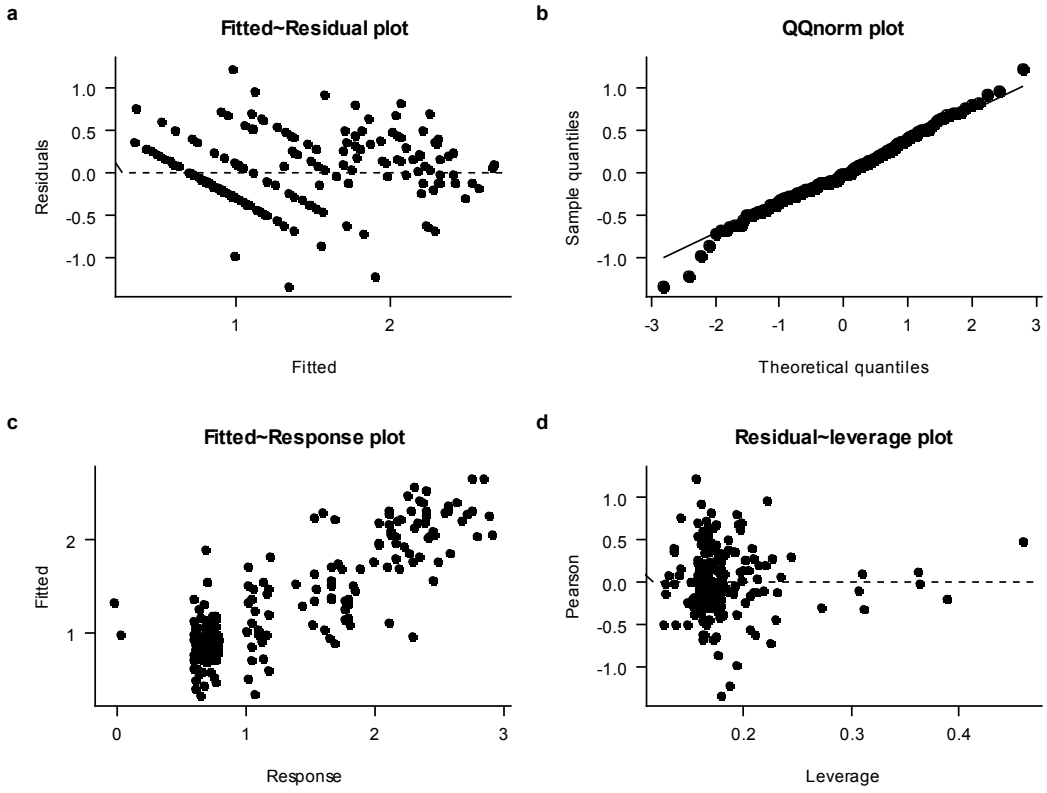

Figure 3-12. Diagnostic plots for Sigstad score linear mixed effects model. a - fitted-residual plot was homogenous around y=0, indicating the model accurately reflects trends in the data. b - QQ plot of residuals versus normal distribution, indicating residuals follow a normal distribution and the assumption of linearity in the model was correct. c - plot of actual Sigstad score versus model prediction, demonstrating the predictive value of the model. d - Residual - leverage plot identified only seven points with a higher degree of leverage, with datapoints with higher residuals having relatively smaller leverage.

3.3.14 Hunger, fullness and nausea

Hunger, fullness and nausea were measured during OGTTs using visual analogue scales for 17 control, 18 PTG (including 1 with type 1 diabetes and 1 taking a somatostatin analogue), 6 TG, 8 oesophagectomy (including 1 on high dose steroids and 1 with type 1 diabetes) and 4 STG participants. Examining the trend across the whole OGTT by the integrated area under the curve, there was no notable difference between groups, with the exclusion of a subset of participants after total and subtotal gastrectomy with greater nausea scores (Figure 3-13).

Examination of the individual traces for the PTG participants (Figure 3-14) revealed two clear patterns for all three factors, with a subset of ~6-8 participants having an appropriate response to the glucose (i.e. suppressed hunger, increased fullness and minimal nausea), while the rest experienced no significant change in hunger or fullness during the OGTT, and a significant rise in nausea scores. However, comparison (by Chi squared test) of the subgroups with aberrant response to the OGTT showed no correlation between response or otherwise with nausea, fullness or hunger.

ANOVA of linear mixed effects models was used to investigate correlation between hunger, fullness and nausea scores and possibly correlated effects (demographics, operative groups, gut hormone concentrations). Hunger was negatively correlated with time post-OGTT, and positively (but weakly) with plasma PYY concentration ($p=0.001$). Fullness was only associated with time post-OGTT, and nausea with time post-OGTT and positively with GLP-1 ($p=0.004$) and negatively with GIP ($p=7*10^{-5}$) concentrations (Table 3-6, Table 3-7, Table 3-8; Figure 3-15, Figure 3-16, Figure 3-17).

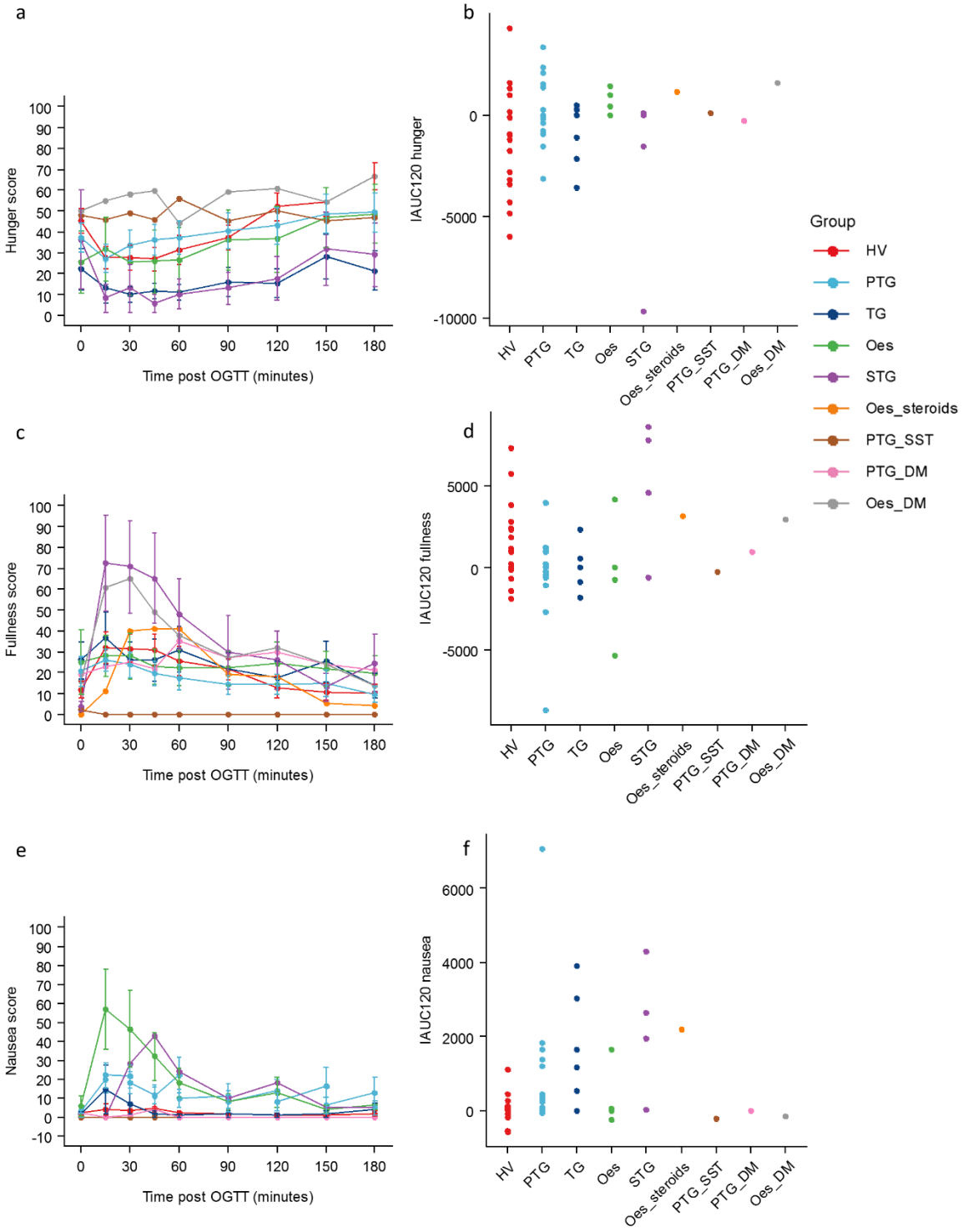

Figure 3-13. Hunger, fullness and nausea scores (out of 100) over 3 hour OGTT for all groups. a,c,e - group profiles over 3 hours (mean +/- standard error). b,d,f - incremental area under the curve for each score over 120 minutes post-OGTT.

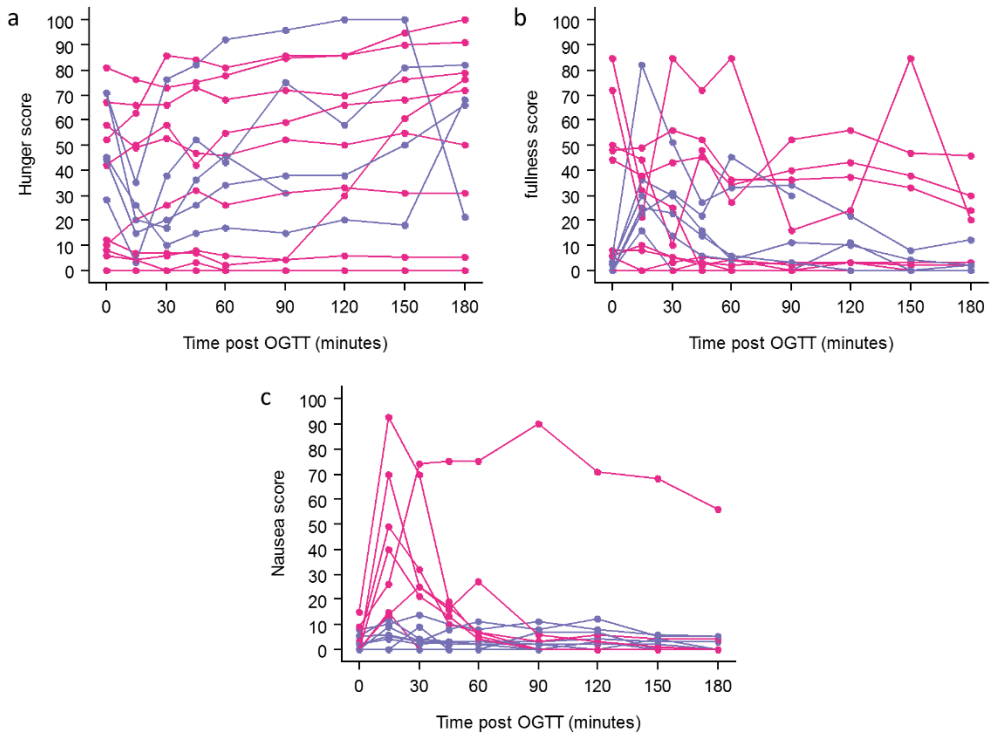

Figure 3-14. Hunger (a), fullness (b) and nausea (c) scores over 3 hours post-OGTT for individual PTG participants. Purple - appropriate (i.e. reduced hunger, increased fullness, minimal nausea). Pink - inappropriate response. Individual plots with an appropriate response in one measure did not closely correlate with an appropriate response to other measures.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	993.49	198.70	5	21.60	1.37	0.27
Age	60.48	60.48	1	19.19	0.42	0.53
Gender	8.54	8.54	1	19.03	0.06	0.81
BMI	260.38	260.38	1	19.67	1.80	0.19
Time	7207.37	1201.23	6	153.51	8.31	$8*10^{-8}$
Glucose	26.28	26.28	1	160.19	0.18	0.67
Insulin	11.10	11.10	1	158.29	0.08	0.78
GLP1	3.33	3.33	1	159.46	0.02	0.88
PYY	1570.02	1570.02	1	163.20	10.86	0.001
GIP	36.01	36.01	1	156.79	0.25	0.62

Table 3-6. ANOVA table for factors associated with hunger score at any given timepoint by linear mixed effects model with ID as random variable.

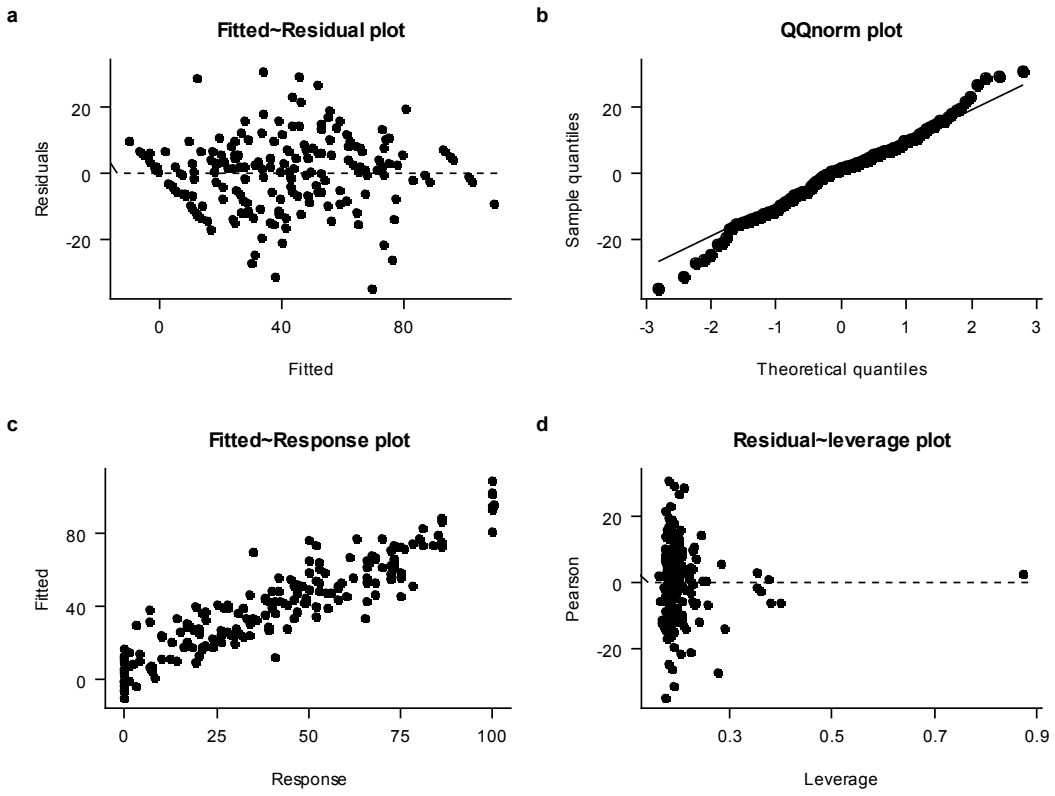

Figure 3-15. Diagnostic plots for hunger score linear mixed effects model. a - fitted-residual plot was homogenous around y=0. b - QQ plot of residuals versus normal distribution. c - plot of actual hunger score versus model prediction. d - Residual - leverage plot identified seven highly leveraged data points.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	2.38	0.48	5	21.95	1.10	0.39
Age	0.51	0.51	1	19.43	1.19	0.29
Gender	0.43	0.43	1	19.25	0.99	0.33
BMI	1.03	1.03	1	19.94	2.38	0.14
Time	11.04	1.84	6	153.86	4.26	0.0005
Glucose	0.01	0.01	1	160.98	0.01	0.91
Insulin	0.03	0.03	1	159.00	0.06	0.80
GLP1	0.005	0.005	1	160.17	0.01	0.92
PYY	0.62	0.62	1	164.09	1.43	0.23
GIP	0.12	0.12	1	157.37	0.29	0.59

Table 3-7. ANOVA table for factors associated with fullness score at any given timepoint by linear mixed effects model with ID as random variable.

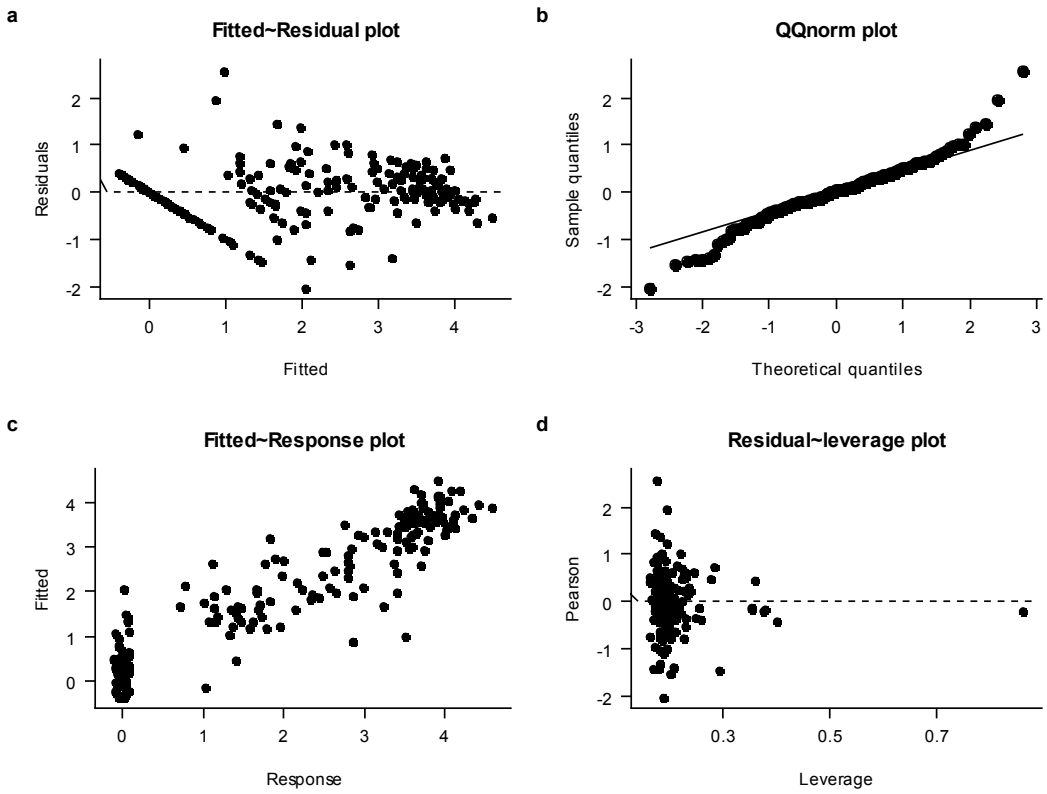

Figure 3-16. Diagnostic plots for fullness score linear mixed effects model. a - fitted-residual plot was homogenous around y=0. b - QQ plot of residuals versus normal distribution. c - plot of actual fullness score versus model prediction. d - Residual - leverage plot identified seven highly leveraged data points.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	241.59	48.32	5	22.56	0.71	0.62
Age	10.17	10.17	1	18.90	0.15	0.70
Gender	79.41	79.41	1	17.96	1.16	0.30
BMI	143.65	143.65	1	20.15	2.10	0.16
Time	1079.93	179.99	6	157.85	2.63	0.02
Glucose	128.61	128.61	1	170.91	1.88	0.17
Insulin	46.29	46.29	1	172.90	0.68	0.41
GLP1	579.97	579.97	1	172.99	8.48	0.004
PYY	2.84	2.84	1	163.71	0.04	0.84
GIP	1141.06	1141.06	1	171.31	16.68	$7*10^{-5}$

Table 3-8. ANOVA table for factors associated with nausea score at any given timepoint by linear mixed effects model with ID as random variable.

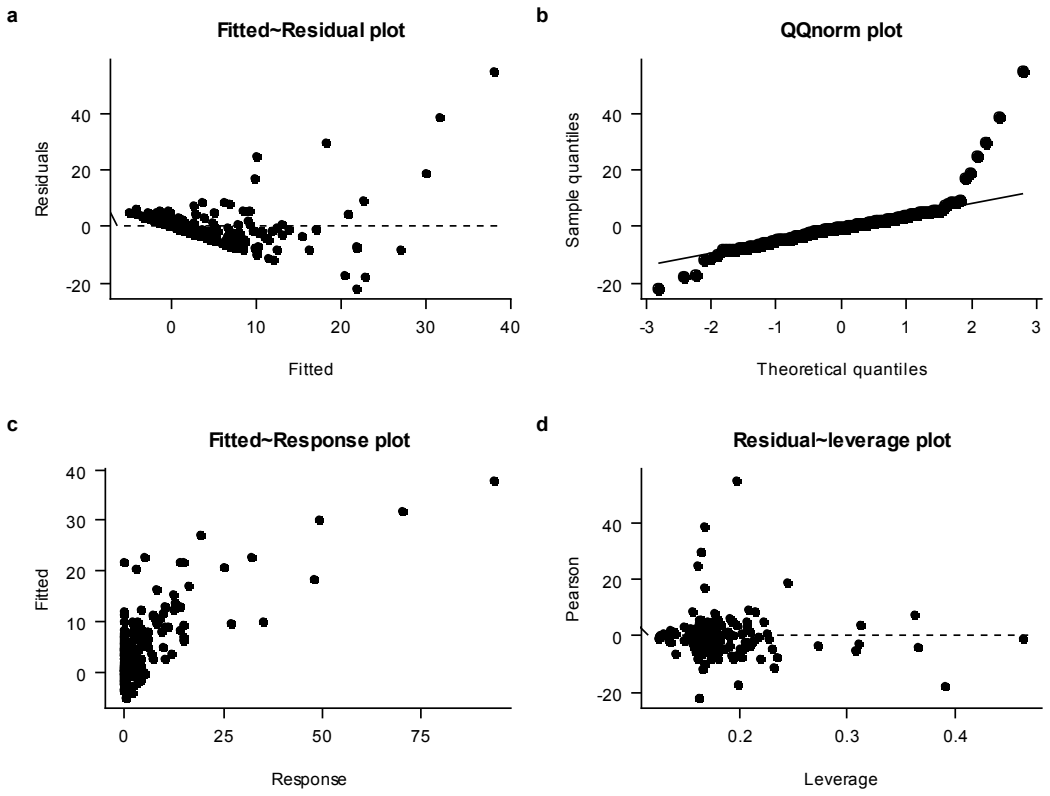

Figure 3-17. Diagnostic plots for nausea score linear mixed effects model. a - fitted-residual plot was moderately homogenous around y=0. b - QQ plot of residuals versus normal distribution – note poor fit at higher values. c - plot of actual nausea score versus model prediction. d - Residual - leverage plot identified nine highly leveraged data points.

3.3.15 Food attention bias

Food attention bias was measured for 13 participants after total gastrectomy (PTG and TG), 6 after oesophagectomy, 7 controls and 4 after partial gastrectomy. Of these, results were available in only the fasting state for 1 control, 2 oesophagectomy and 1 total gastrectomy participants (due to the participants being too symptomatic to engage with the computer tasks after the OGTT), with results also available at 15 and 90 minutes post OGTT for all other participants.

Plotting the normalised mean response time for each group individually demonstrated the sizeable inter-individual variability in this task, both in the fasting state and in the response to the OGTT (Figure 3-18). There was no clear difference in fasting or post OGTT food attention bias between groups, and no clear trend for response to the OGTT within or between groups. This represents either the fact that food motivation was a highly heterogeneous factor that was not significantly altered by surgery, or that the task failed to elicit a consistent response and so the results are meaningless. The fact that 4/23 post-operative participants were unable to engage with the task after the OGTT due to symptoms is also of concern and may have skewed the results.

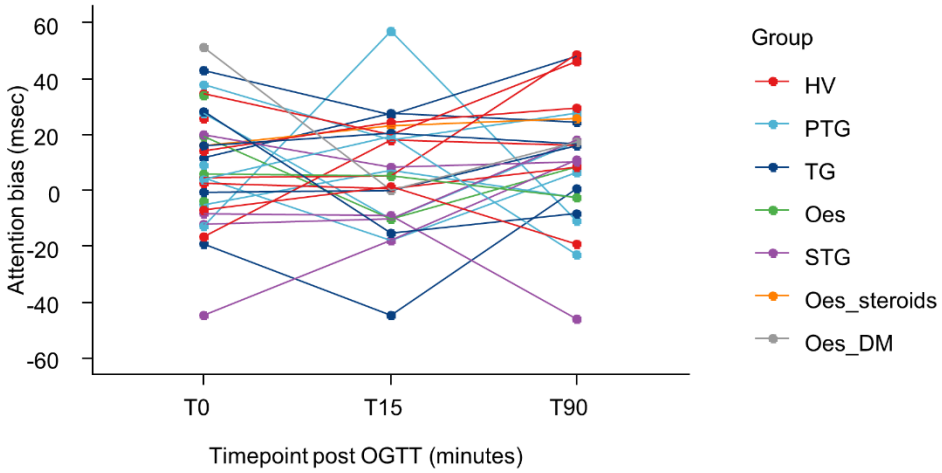

Figure 3-18. Food attention bias by timepoint for all participants, measured by dot probe paradigm.

3.3.16 Plasma peptidomics

LC-MS/MS was used to identify multiple peptides in the plasma of five control and five PTG participants pre- and at 30 and 90 minutes post OGTT. A total of 27 peptides were identified, although the biological relevance of these peptides is unclear. Of note, several GIP, proglucagon and PYY peptides demonstrated enhanced post-prandial secretion in the PTG cohort. Pancreatic glucagon concentration did not increase at 30 minutes in any samples (control or post-gastrectomy). Interestingly, neurotensin appeared to have a greater post-OGTT response in the PTG group, which has not previously been described.

Two peptides were identified that had different fasting plasma concentrations between control and PTG participants, and were derived from the PIGR and DMBT1 sequences. PIGR is a secreted immunoglobulin receptor, and DMBT1 a potential tumour suppressor gene. Neither have functions ascribed to secreted peptide components, and the concentrations were not altered by OGTT, suggesting they are not secreted by EECs.

Ultimately, the peptidomic approach demonstrated here has not identified novel gut peptides, but could potentially be useful as a multiplexed approach to measuring plasma peptides in the future.

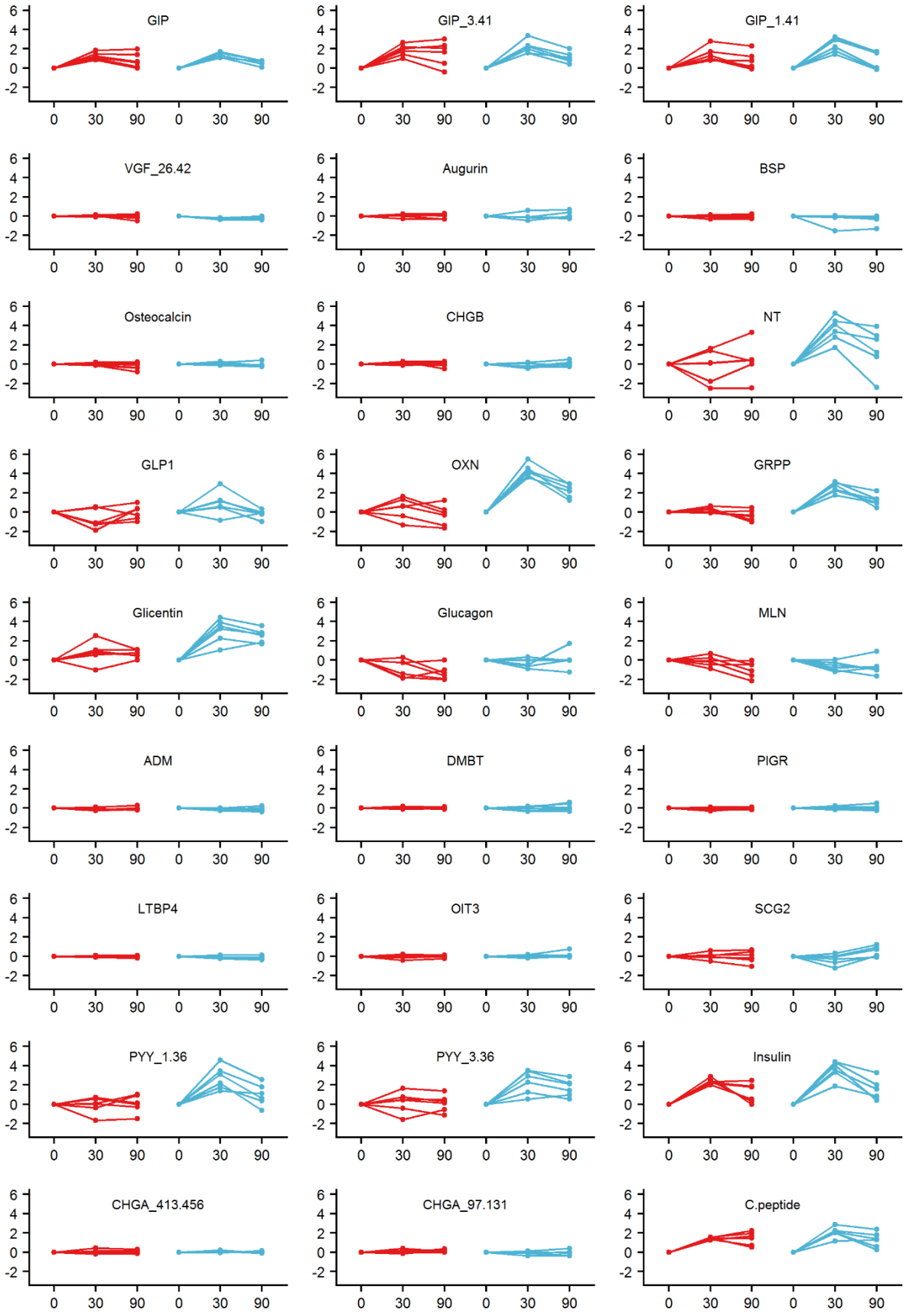

Figure 3-19. Plasma peptides identified by LC-MS/MS from plasma collected in the fasting state and at 30 and 90 minutes post-OGTT. Red - control. Blue - PTG. Values are natural log of the fold change in concentration from the fasting value.

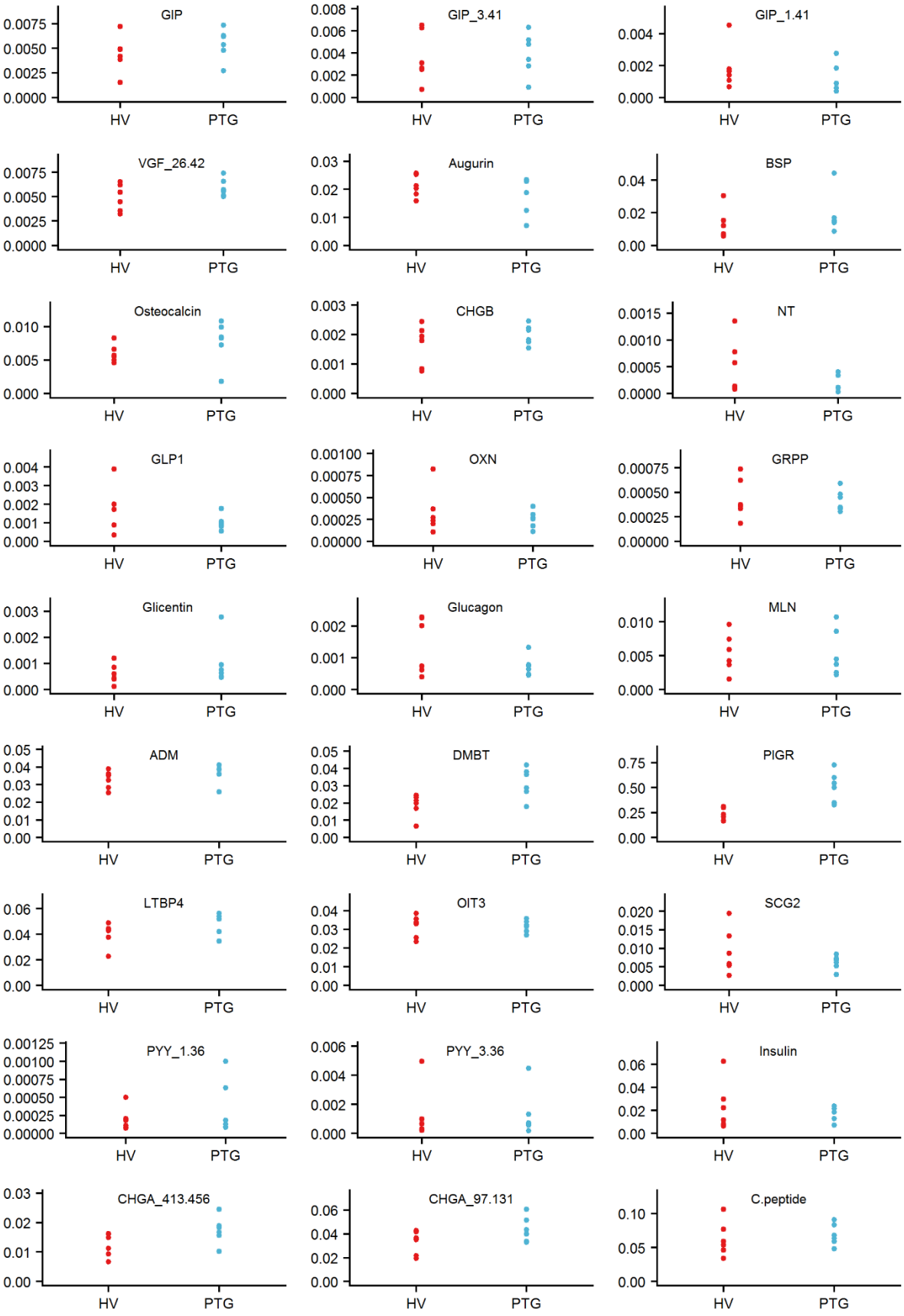

Figure 3-20. Fasting concentrations of plasma peptides identified by LC-MS/MS by group. Concentrations are measured in peak area and are not comparable between peptides.

3.3.17 Comorbid participants – type 1 diabetes, somatostatin analogues, steroids

Recruitment of participants with type 1 diabetes, or taking somatostatin analogues or steroids allowed a limited investigation of the modifying effect of these conditions on gut hormone concentrations. While somatostatin suppressed secretion of all peptides (with the intriguing exception of PYY), diabetes and steroids appeared to have little objective effect (although as would be expected, the participant taking steroids was more insulin resistant and had a higher glucose curve). Given the variability in symptom and other subjective measures, the limited numbers in the diabetic / SST / steroid groups makes it impossible to comment on any changes in softer measures.

3.4 DISCUSSION

This chapter described an integrated approach to symptoms, glucose handling, gut hormone physiology and indices of eating behaviour in patients after oesophago-gastric cancer surgery. Patients after resection, in particular those after total gastrectomy, are at risk of a range of symptoms, and reactive hypoglycaemia. There was significant variability in symptom response, and in glucose handling, with some patients experiencing few detrimental effects of surgery, while others experienced profound hypoglycaemia and / or highly unpleasant symptoms.

Plasma glucose profile was highly variable between individuals, even within groups. While peak glucose was significantly different between those after gastrectomy (PTG, TG or STG) and controls on a groupwise comparison, nadir glucose was not. However, roughly half of the post-gastrectomy group experienced a nadir glucose below 4mmol/l, with 4/24 experiencing hypoglycaemia in the neuroglycopaenic range <3.3mmol/l. Subjectively, this appeared to occur with few or no autonomic symptoms, and fewer than half of gastrectomy participants were symptomatic using the Sigstad score at the time of hypoglycaemia. The altered glucose profile, in particular the presence of reactive hypoglycaemia, was not as apparent in the oesophagectomy group, although work from other units has shown the profile herein described in the gastrectomy population was akin to that seen after oesophagectomy, and it is likely the variable phenotype and small sample size seen masked the effect in this study.

The elevated peak glucose is readily explained by the rapid delivery of nutrients to the absorptive small intestine in the post-operative group in the absence of a gastric reservoir. The significantly heightened insulin secretion in the post-operative group is sufficient to explain the more rapid decline from peak glucose seen in the post-gastrectomy groups, although it remains unclear as to why only a subset of participants experienced hypoglycaemia. Attempting to model nadir glucose from the factors available, it was impossible to find a statistically significant individual factor, although the factors contributing the greatest, in order of F-value, were PYY secretion, fasting glucose, insulin secretion, GIP secretion and BMI. The PTG group, in which 10/16 participants experienced hypoglycaemia during

the OGTT, did have a marginally greater HOMA-IS score, and so fasting insulin sensitivity (albeit driven by BMI rather than other physiological factors), suggesting that this may be a factor influencing the risk of severe hypoglycaemia.

The normalisation of plasma glucose after hypoglycaemia was reassuring, and reflects the short half-life of endogenously secreted insulin and the ability of the beta cell to rapidly cease insulin secretion, likely combined with pancreatic glucagon secretion. It is crucial to note that this population therefore is different in risk profile to the diabetic population administering exogenous insulin with an extended half-life, whose risk is prolonged hypoglycaemia due to overdosing insulin. The post-operative group appears to be more at risk of rapid onset/offset hypoglycaemia, with a short period of severe but self-limiting hypoglycaemia. While this is reassuring, there are at least five patients in the UK who have experienced hypoglycaemic seizures after prophylactic total gastrectomy (out of a population of ~100).

Secretion of nutrient sensing gut hormones GLP-1, GIP and PYY was significantly enhanced after all surgical procedures. Notably, GIP had an earlier and more elevated peak after surgery, then rapidly dropped, in keeping with the rapid passage of the glucose through, or bypass of, the primary GIP secreting mucosa in the proximal jejunum. The ~10-20x elevation in post-OGTT GLP-1 and PYY secretion following gastrectomy or oesophagectomy likely reflects the rapid delivery of high concentrations of glucose to the primary sites of their secretion in the distal small intestine and proximal colon. An interesting question that is addressed later in this thesis is whether post-operative adaptive changes in the small intestinal mucosa could also drive changes in gut hormone secretion.

The stimulation of pancreatic beta cells by the combined effect of hyperglycaemia and elevated GLP-1 and GIP concentrations was the likely mechanism driving the hyperinsulinaemia seen in the post-operative groups. GIP was the only factor in this study which appeared to correlate with both nadir glucose and risk of hypoglycaemia (plasma glucose <4mmol/l) in linear models examining either timepoint specific factors, or overall exposure by incremental area under the curve. It is therefore unclear from this study what factors may differentiate those post-operative patients at risk of hypoglycaemia from those not at risk. From this dataset it could be speculated that the combined

effects of insulin secretion, fasting glucose and BMI are important, and it is quite plausible that variation in population insulin sensitivity (for which BMI and fasting glucose could be regarded as poor surrogate markers) is of significance. While this has not been addressed by this study, future work, including formal measures of insulin sensitivity by euglycaemic clamp experiments, could be of use.

Plasma acyl ghrelin was unmeasurably low after total gastrectomy (and therefore at least 10x lower than in controls), however was detectable within ranges seen in control participants after oesophagectomy and partial gastrectomy. This implies that while the primary source of circulating acyl ghrelin is the stomach, preservation of only part of the stomach is adequate for circulating levels, and the part that is preserved appears unimportant – the proximal stomach is preserved after partial gastrectomy, the distal after oesophagectomy. The attempt to generate an objective measure of food behaviour through the computerised food-attention dot probe task did not generate useful data. The possible reasons for this are: there was no difference between the groups; there was such inter-person variability in response that a much larger group would be required to identify any difference (explaining the heterogeneous data collected); or the test, in these circumstances, does not adequately measure the variable of interest to the exclusion of confounding factors. While not formally measured here, discussion with participants identified that the subjective sensation of hunger, and in particular the reward sensation of eating when hungry, was profoundly altered by surgery. It is plausible that some of the subjective changes in hunger arise as a result of ghrelin deficiency, although this will remain speculation until GHSR1a agonists are trialled in this group.

The variability in response to OGTT was also manifest in visual analogue scores of hunger, fullness and nausea, and symptom scores. Despite the profound, and population level changes, seen in satiety and hunger hormones (GLP-1, GIP, PYY, ghrelin), division of the vagi and removal of the gastric reservoir, only a subset of participants experienced abnormal subjective responses to the OGTT. As with nadir glucose, it was not possible to identify significant factors contributing to this variability by modelling the outcome on available factors from this study. Interestingly, early post-prandial symptoms did not correlate well with a rise in haematocrit. Historically, the Sigstad score, and a 3% rise in haematocrit

have been used as diagnostic criteria for “dumping syndrome”, however the discordance in these measures represents a fundamental weakness of this approach.

While the hypothesis that rapid nutrient delivery to the small intestine stimulated 5-HT over-secretion through similar mechanisms to those driving gut hormone over-secretion was elegant, it proved beyond the specificity of current techniques to test. Significant work would be required to optimise protocols for the collection and analysis of platelet poor plasma and this represents an opportunity for future work. Similarly, measurement of leptin was interesting, although the finding that leptin primarily correlated with BMI, rather than being differentiated across study groups, supports the current state of knowledge that leptin is an adipokine, rather than a gut hormone.

The lack of correlation of the subjective response to OGTT with a range of objective measures, including gut hormones, insulin, glucose and cardiovascular measures, does not advance current knowledge any further as to the cause of negative symptoms in a subset of post-operative patients. All post-operative participants had significantly deranged physiology, in particular with elevated gut hormone and insulin secretion, which should be regarded as the norm after oesophago-gastric resection. It could be speculated that the presence or absence of symptoms in the post-operative population arises as a result of variation in the adaptation to what are essentially noxious signals that in the healthy state mediate sensations of over-eating (indeed exogenous administration of high doses of GLP-1 can drive similar symptoms), and in the subjective response to these signals.

The difference in measures between the prophylactic total gastrectomy, and total gastrectomy, groups is of interest as these are highly similar procedures. There was a higher incidence of hypoglycaemia in the PTG group, which was associated with a lower fasting insulin and hence greater insulin sensitivity. However, multi-factorial analysis of these populations identified that the lower BMI in the PTG population was the primary driving factor for insulin sensitivity, not operative group. There was no significant difference in gut hormone secretion profile or symptoms between the PTG and TG groups. Of note, the two main differences between the PTG and TG groups were age and BMI. Multifactorial analysis of these populations identified BMI, rather than operative group, to be the key factor

associated with hypoglycaemia and insulin sensitivity. Given that the TG group was also older than the PTG group, and both insulin secretory capacity and sensitivity decline with age, this likely was also a factor in the lower incidence of hypoglycaemia in the TG group.

While this study provides a large set of data with which to explore correlations between physiological factors and objective sensations, it was ultimately an observational study. Hence conclusions as to exact relationships between findings remain correlative rather than causative. There was also a degree of variability within groups, representing the heterogeneous population undergoing surgery. The sample size was small for oesophagectomy and partial gastrectomy populations, as the primary focus of the study was the total gastrectomy population, and the small dataset from this group could be regarded as pilot data for future work on those specific populations.

In summary, oesophago-gastric resection results in profound changes in the physiological response to an OGTT, the degree of which does not correlate well with any subjective measures of negative symptoms, satiety or hunger. However, individual gut hormone receptor modulating drugs represent promising avenues for future research into targeted treatments for post-operative symptoms, hypoglycaemia and loss of appetite.

4 FREE-LIVING GLUCOSE PROFILE AFTER OESOPHAGO-GASTRIC RESECTION

Hypothesis

Hypoglycaemic episodes occur more frequently and to a greater severity in patients after gastrectomy or oesophagectomy.

4.1 INTRODUCTION

Continuous glucose monitoring is becoming standard practice in the care of diabetic patients. The several devices on the market work by similar principles – a reader/transmitter is affixed to the skin of the patient, with a small subcutaneous cannula for sampling of interstitial fluid every 5-15 minutes; this transmits to a receiver, on the screen of which real-time glucose and trends are shown.

While potentially revolutionary in the field of diabetes, the technology also represents an opportunity to take the study of glucose profile in post-surgical patients into the real world, with observation of glucose trends while living normally. Multiple studies have demonstrated aberrant glucose handling in gastrectomy and oesophagectomy patients following standardised tests using glucose solutions, ice creams or standardised liquid meals, however the relevance of this data is unclear when patients are eating their usual diet. Key questions are:

1. Do participants suffer post-prandial hypoglycaemia, and if so to what severity?
2. Is hypoglycaemia entirely related to ingestion of food, or does it occur in the fasting state (i.e. overnight)?

To address this, a cohort of post-operative patients and healthy controls were fitted with continuous glucose monitors for two weeks and instructed to make no lifestyle modifications.

4.2 METHODS

Freestyle Libre Pro continuous glucose monitors were applied to all participants recruited to visit Addenbrooke's hospital following approval of this study. The sensor/transmitter was applied to the back of the arm as per standard instructions, and reader kept by the investigator. Blinding of the participant was essential for two reasons. Firstly, it prevented lifestyle changes arising from knowledge of glucose concentrations. Secondly, as it was hypothesized that the participant would suffer hypoglycaemic episodes, and there is no current "normal" profile for plasma glucose in the post-surgical population, it avoided unnecessary concern should the monitor elicit an alarm for hypoglycaemia which would be of worry in the diabetic population but of no immediate concern for the participant.

In this chapter the term "glucose" refers to glucose as measured by the CGM device, which is interstitial fluid glucose as a proxy for plasma glucose. The calculations used to generate the output from the device mean the two values are essentially equal and can be, for the purposes of this chapter, regarded as interchangeable, particularly with regards to "normal" reference ranges.

4.3 RESULTS

CGM data were collected from 11 participants after PTG, 6 after TG, 17 controls, 4 after oesophagectomy and 4 after STG. Groupwise comparisons were performed on the derived variables described above (MAGE, M-value, HBGI, LBGI and frequency / percent outside range) by linear modelling and multi-way ANOVA.

Linear models were constructed using group (PTG vs TG vs control vs oesophagectomy vs STG), age, gender and BMI on the whole data set. To assess effect of time after surgery, further models were generated using group, age, gender, BMI and time post-op on data from post-operative participants only. Multi-way ANOVA was performed on each model to identify statistically significant factors.

It was not possible at this stage to build models including OGTT outcomes (particularly gut hormone and insulin secretory profiles and HOMA scores), as these data were only available for a small set of participants.

4.3.1 Demographics

	Control	PTG	TG	STG	Oesophagectomy
Age (years)	40+/-3	42+/-4	64+/-8	63+/-10	60+/-7
Gender (M:F)	10:7	9:2	3:3	2:2	2:2
Height (cm)	172.1+/-2.5	177.6+/-2.6	170.5+/-4.7	170.5+/-5.0	162.7+/-2.3
Weight (kg)	69.7+/-3.4	80.0+/3.0	63.4+/-6.3	69.5+/-5.3	67.6+/-5.8
BMI	23.4+/-0.8	22.5+/-0.9	21.8+/-1.5	23.8+/-0.8	25.6+/-2.3
Time post-op (years)	NA	3.7+/-0.9	6.2+/-2.4	3.8+/-1.6	3.7+/-1.0
CGM days	9.8+/-0.8	9.9+/-0.9	9.4+/-2.1	12.8+/-0.1	12.2+/-0.5
CGM nights	10.0+/-0.8	10.3+/-1.0	9.7+/-2.1	12.6+/-0.2	12.4+/-0.6

Table 4-1. Demographics of CGM study participants. Mean +/- standard deviation.

Participant groups were well matched. The monitors were designed with a maximum life of 14 days, however ~50% fell off prior to the 14 day cut off, so an average of 10 days data was collected per participant.

4.3.2 Measures of glycaemic variability

The M value and MAGE score are both measures of glycaemic variability, with increasing values representing greater deviation from an idealised norm (for M-value, 5mmol/l; for MAGE, 1 standard deviation outside the mean range). They therefore assess the tendency of glucose values to vary in either direction and are a useful measure of total aberrant glucose handling in the surgical group.

Post-operative groups had greater measures of glycaemic variability, with the two total gastrectomy groups significantly more variable than the STG and oesophagectomy groups (Table 4-2, Figure 4-1).

Regressing the M value by group, age, gender and BMI generated a reasonably well fit model ($p=3.9*10^{-5}$, adjusted $R^2=0.5$), with M value primarily correlated to group (in the order TG > PTG > STG > Oesophagectomy > Control; ANOVA $p=4.6*10^{-6}$). A similar picture was seen with the MAGE regression ($p=3.4*10^{-7}$, adjusted $R^2=0.63$), with group being the only significant factors on ANOVA ($p=2.9*10^{-8}$), albeit with PTG more strongly associated with increased MAGE than TG.

Regressing M value and MAGE in the post-operative groups and adding time since surgery as a factor to the model resulted in a slightly poorer fit of model (M value: p=0.029, adjR^2=0.37; MAGE: p=0.036, adjR^2=0.35). This new model did however show longer time since surgery to be associated with an increase in both M value and MAGE (ANOVA p=0.013 and p=0.047 respectively).

	Control	PTG	TG	STG	Oesophagectomy
M value	1.11+/-0.18	3.89+/-0.43	8.28+/-1.7	3.87+/-1.94	2.67+/-1.20
MAGE	2.28+/-0.11	4.96+/-0.28	6.83+/-0.90	4.48+/-0.86	3.58+/-0.76

Table 4-2. Measures of glycaemic variability by participant group. Mean +/- standard error.

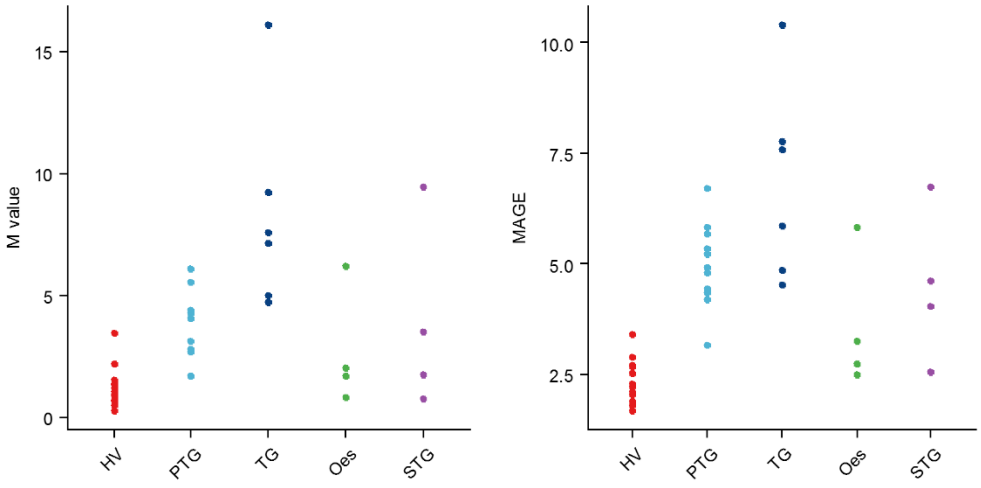

Figure 4-1. Measures of glycaemic variability while wearing CGM for individual participants by group.

4.3.3 Measures of hypoglycaemia

Hypoglycaemia was assessed using the low blood glucose index (LBGI) as a validated measure (albeit in diabetic patients) of risk of hypoglycaemic events, and by straightforward measures of frequency and percentage time spent hypoglycaemic (glucose <4mmol/l) or severely hypoglycaemic (<3.3mmol/l).

All measures of hypoglycaemia were at least marginally elevated in the PTG and TG groups compared to controls (Table 4-3, Figure 4-2). Interestingly, even healthy volunteers experienced a degree of hypoglycaemia <4mmol/l, but this was markedly less prevalent at a cut off of 3.3mmol/l. The most notable variation in prevalence of hypoglycaemia was detected by the LBGI. While the incidence of hypoglycaemic episodes was relatively similar across groups (~one severe episode per 3 days and ~one severe episode per 2-3 days), the percentage time <3.3mmol/l (day and night) was greater in the surgical groups. Groups and variables were compared by linear regression and multi-way ANOVA.

Regressing LBGI on the whole dataset generated a relatively well fitted model ($p=0.0001$, adj$R^2=0.47$), with group as the only significant factor (TG > PTG > STG > Oesophagectomy > Control, ANOVA $p=3.4*10^{-5}$). Excluding controls and adding time post-op as a factor resulted in similarly well fitted model ($p=0.011$, adj$R^2=0.46$), which preserved group ($p=0.003$) in the above order as significant, however did not show any significant role for time since surgery or any other factor. The LBGI value for the TG group was 7.37+/-1.16 and so would be categorised as “high risk”, and for the PTG group was 4.35+/-0.37, indicating “moderate risk”.

Day and night-time glucose control was starkly different, due to the large spike and rapid decline seen in glucose after eating. Modelling uncomplicated measures of hypoglycaemia (frequency of hypoglycaemic episodes and percentage time below normal range) revealed reasonably well fit models for the day-time (0800-2200) values, but on inclusion of night-time values the models were not representative of the data (i.e. $p>0.05$, adj$R^2<0.2$). Data are therefore discussed for day and night time hypoglycaemia episodes separately.

Including the control population, linear models of number of hypoglycaemic episodes per day (p=0.03; adjR^2=0.22), severe hypoglycaemic episodes per day (p=0.045; adjR^2=0.19) and percentage time <3.3mmol/l per day (p=0.03; adjR^2=0.21) were adequately fitted to the data. Significant predictors of day-time hypoglycaemia (as % time) in the model and by ANOVA were group (TG > PTG > STG > oesophagectomy > control; p=0.03 to 0.04) and female gender (p=0.028 to 0.03). Removal of control participants and inclusion of time since surgery neither improved the fit of the model nor revealed any further contributing factor.

Attempts to model incidence or duration of nocturnal hypoglycaemia, either <4mmol/l or <3.3mmol/l by linear regression were poorly fitted (p~0.4, adjR^2<0.15).

	Control	PTG	TG	STG	Oesophagectomy	P value
LBGI	2.56+/-1.41	4.35+/-0.37	7.37+/-1.16	2.75+/-0.31	3.38+/-1.15	$3*10^{-5}$
Hypos per day	1.23+/-0.38	2.31+/-0.35	2.96+/-0.42	0.94+/-0.45	2.02+/-0.95	0.04
Severe hypos per day	0.18+/-0.09	0.10+/-0.59	0.19+/-0.73	0.16+/-0.14	0.29+/-0.39	0.04
Hypos per night	1.03+/-0.27	1.79+/-0.29	1.72+/-0.38	0.75+/-0.30	1.29+/-0.60	0.26
Severe hypos per night	0.28+/-0.12	0.59+/-0.14	0.73+/-0.17	0.14+/-0.08	0.39+/-0.24	0.16
Day-time % time <4mmol/l	7.44+/-2.80	9.22+/-1.80	18.45+/-3.55	3.42+/-1.67	8.53+/-4.73	0.10
Day-time % time < 3.3mmol/l	0.77+/-0.36	1.14+/-0.33	3.19+/-1.10	0.98+/-0.56	2.23+/-1.02	0.03
Night-time % time < 4mmol/l	16.15+/-5.61	22.12+/-6.15	42.31+/-8.35	4.48+/-2.00	12.09+/-6.85	0.05
Night-time % time < 3.3mmol/l	2.50+/-1.26	2.88+/-0.64	8.37+/-2.47	0.64+/-0.47	2.74+/-1.82	0.05

Table 4-3. Measures of hypoglycaemia. LBGI - low blood glucose index. Mean +/- standard error. P value was the likelihood that the group of the participant did not contribute to variation in the outcome measure on a linear mixed effects model.

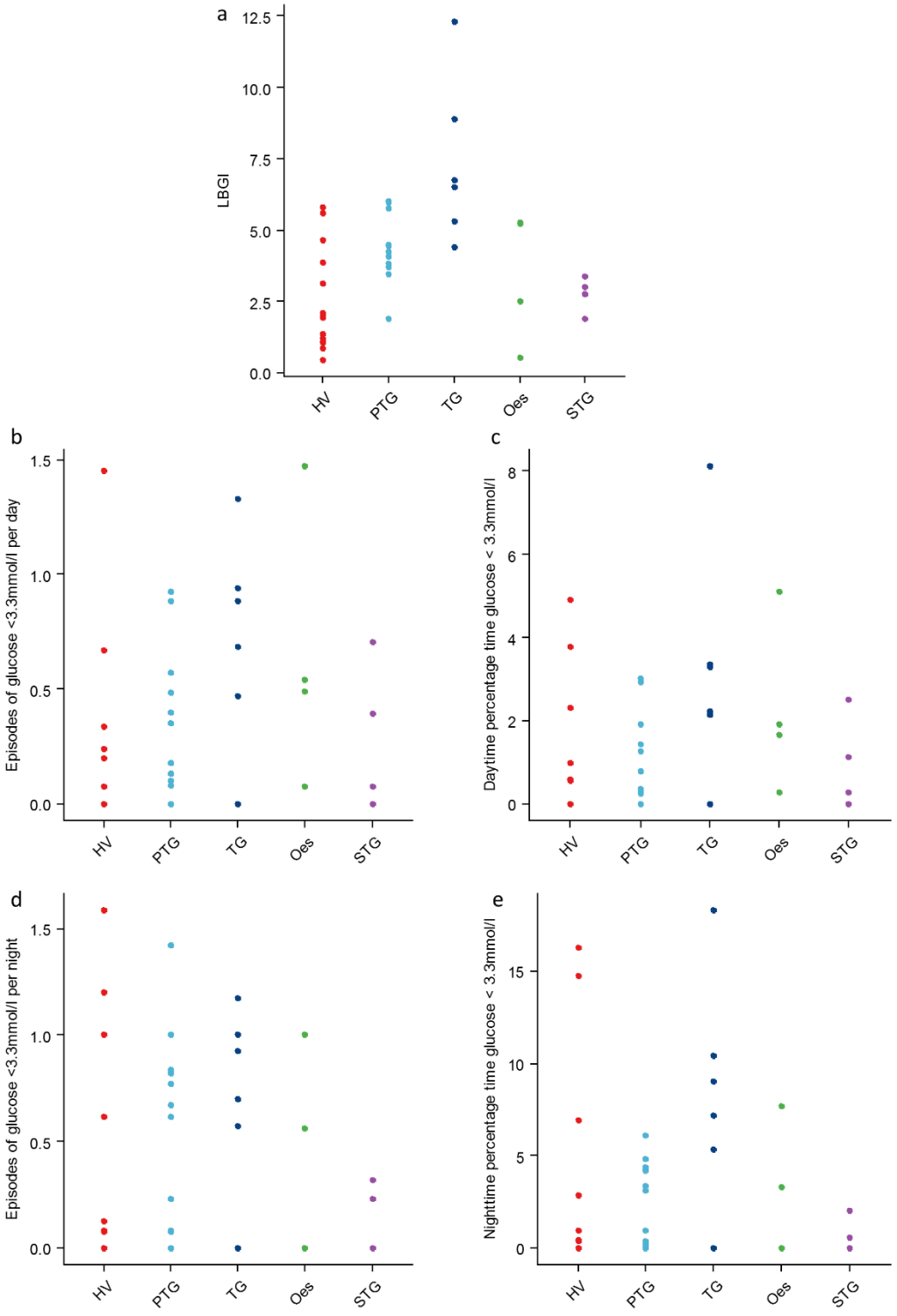

Figure 4-2. Measures of CGM hypoglycaemia for individual participants by group. a - low blood glucose index. b - day-time episodes of glucose <3.3.mmol/l (episodes per day). c - day-time percentage time glucose <3.3mmol/l. d - night-time episodes of glucose <3.3.mmol/l (episodes per day). e - night-time percentage time glucose <3.3mmol/l.

4.3.4 Measures of hyperglycaemia

Hyperglycaemia was measured using the HBGI, and as percentage time >8mmol/l.

All post-operative groups had markedly elevated measures of hyperglycaemia (Table 4-4, Figure 4-3). Linear regression including controls resulted in a reasonably well fit model ($p=8*10^{-5}$; adjR^2=0.48) for HBGI but not for percent time over 8mmol/l (either by day or night; p>0.1, adjR^2<0.25). For HBGI, the only significant factor was group, with TG > PTG > STG > oesophagectomy > control. Removal of the control population and addition of time since surgery rendered the HBGI model less representative and did not improve the time percent model.

	Control	PTG	TG	STG	Oesophagectomy	P value
HBGI	0.72+/-0.11	3.25+/-0.45	5.94+/-1.45	2.79+/-1.05	1.75+/-0.89	0.04
Day-time % time > 8mmol/l	2.89+/-1.03	15.52+/-2.13	21.51+/-4.31	13.62+/-5.78	8.54+/-5.02	$2*10^{-5}$
Night-time % time > 8mmol/l	0.08+/-0.04	0.23+/-0.06	0.32+/-0.17	0.43+/-0.23	0.07+/-0.03	0.04

Table 4-4. Measures of hyperglycaemia. Mean +/- standard error. P value is the likelihood that the group of the participant did not contribute to variation in the outcome measure on a linear mixed effects model.

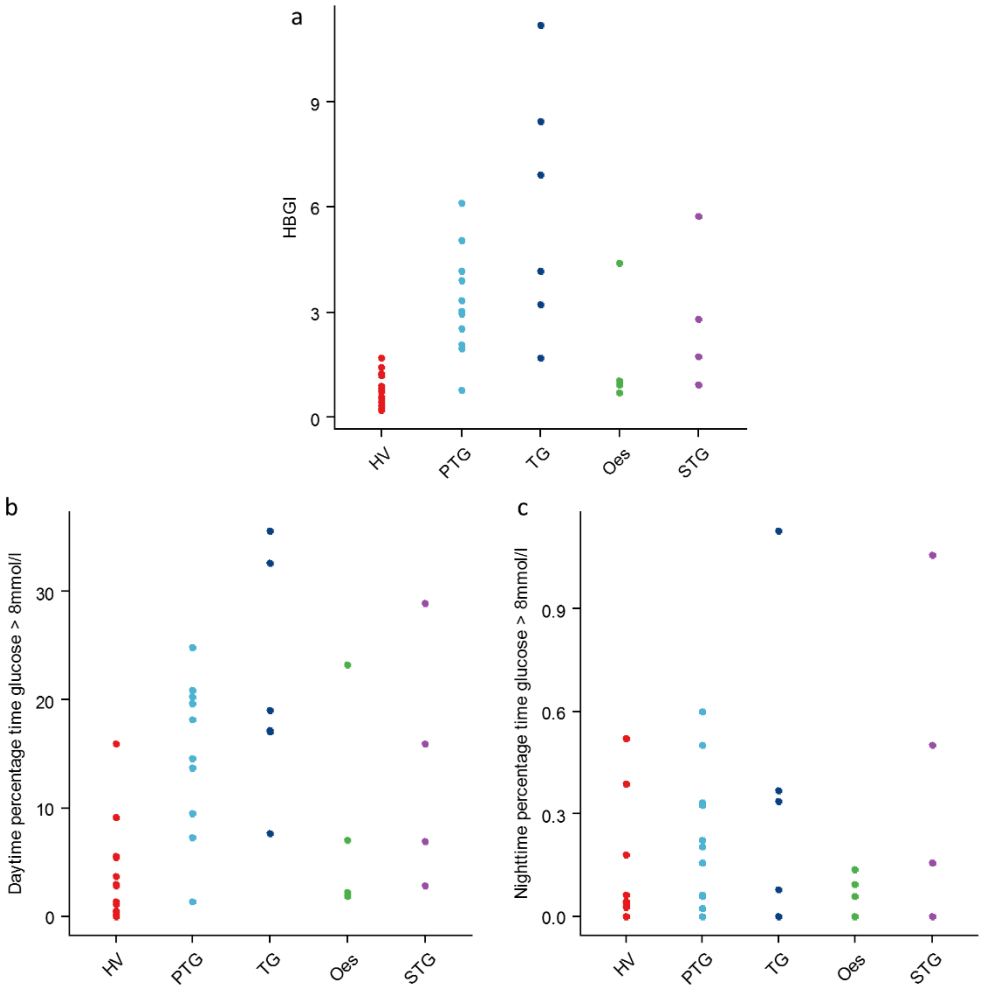

Figure 4-3. Measures of CGM hyperglycaemia for individual participants by group. a - high blood glucose index. b – day-time percentage time over 8mmol/l. c – night-time percentage time over 8mmol/l.

4.4 DISCUSSION

This chapter is the first systematic description of free-living glucose profiles in patients after oesophago-gastric resection. CGM measures validated in the diabetic population demonstrate clear differences between post-operative groups and healthy controls, for measures of glycaemic variability as well as hyper and hypoglycaemia. These "free-living" data support previously published evidence of highly altered glucose handling from laboratory studies, and the findings from chapter 3 of this thesis. Minor hypoglycaemic episodes appear as common in the control population as the post-operative groups, however examining the incidence of severe hypoglycaemia <3.3mmol/l showed a greater effect for surgery.

Patients after TG and PTG appeared to have the greatest risk of dysglycaemia, with up to 10% of nocturnal time spent <3.3mmol/l, whereas those after STG and oesophagectomy did not have measures significantly different from the control population.

Glycaemic profile differed between day and night time. While the glucose profile was relatively flat overnight, it was marked by post-prandial peaks and troughs during the day time. There were fewer episodes of severe hypoglycaemia, and less time <3.3mmol/l during the day than at night time, although day and night time profiles were not statistically compared.

As with symptoms and hypoglycaemia during OGTT, CGM findings were heterogeneous, with some participants having frequent, severe hypoglycaemic episodes and others appearing no different from the control group. Linear regression models using available demographic factors (age, BMI, gender) were an adequate fit to most dependent variables, but only showed a significant effect for operation group (and female gender on rate of hypoglycaemic episodes) on outcome. Identification of factors contributing to this variability would be highly relevant to treatment of post-operative hypoglycaemic episodes.

An interesting further step would be to compare diet and symptoms to free-living glucose profile. OGTT data suggested that hypoglycaemia was largely asymptomatic, and symptoms are unrelated to nadir

glucose in a highly controlled environment. If this is also apparent in the real world, the management of symptoms may require considerations beyond rectifying blood glucose. Asymptomatic hypoglycaemia, if a frequent occurrence after gastrectomy, would represent a new and challenging problem for this patient group.

Attempts to expand the linear model in this data set to include gut hormone and insulin secretion during OGTT, insulin sensitivity and risk of hypoglycaemia during OGTT were limited by the small sample size. Thus while it could be hypothesized that other patient factors contribute to aberrant glucose handling, this study was inadequately sized to address this question.

In summary, continuous glucose monitoring confirms OGTT findings of a risk of post-prandial hypoglycaemia in gastrectomy patients, but not those after oesophagectomy. Factors influencing the risk of dysglycaemia are not apparent from this data set, but future, larger studies could include measures of insulin sensitivity and secretion. This also represents a possible tool for future investigation and management of post-gastrectomy patients, with CGMs used to stratify patients by risk of hypoglycaemia and target management accordingly.

5 SYMPTOMS AFTER OESOPHAGO-GASTRIC RESECTION

Hypothesis

Post-prandial symptoms are common, and behavioural traits associated with feeding, habituation and impulsivity are altered after gastrectomy or oesophagectomy.

5.1 INTRODUCTION

Hunger, satiety and eating behaviour are often described as profoundly altered by oesophago-gastric resection. Anecdotally, a majority of patients will describe either complete loss of the sensation of hunger, or a significant change in the way they sense a need to eat, with several participants recruited to this study describing a need to eat by the clock, rather than by internal signalling of nutritional need. In contrast to this, some patients describe either a completely unchanged relationship with food, or in the case of two participants a constant and insatiable hunger that is psychologically debilitating.

Relatively few measures exist to quantify the eating behaviour of humans, and the data on this topic in post-operative patients are limited. While metabolic surgery studies in humans and animals have identified objective measures of altered behaviour, or even central processing of nutrient stimuli on fMRI, no similar work has yet been conducted in the oesophago-gastric resection group.

The purpose of this overall project was to quantify objective and physiological changes arising due to surgery, however the opportunity was taken to administer a raft of straightforward, validated eating behaviour tools to as many participants as could be recruited. This would serve as a pilot dataset to guide development of in depth psychological studies, and potentially identify interesting behaviour traits within the study group.

5.2 METHODS

A panel of online questionnaires was uploaded to a centrally hosted qualitative research tool (Qualtrics), allowing remote completion of questionnaires, anonymously, in approximately 15 minutes. Symptom profile was assessed using the dumping severity score (DSS)[217]. Possible changes in habitual and impulsive behaviour were assessed using the Barratt Impulsivity Scale 11 (BIS-11) and Creature of Habit (COH) tools[270,272]. Food motivation and reward were assessed using the three factor eating questionnaire r18 (TFEQ18) and a food purchasing task[187,269]

All participants recruited for an OGTT or CGM were asked to complete the questionnaire panel, and as many post-operative patients and healthy controls were recruited as possible through attendances at clinic or research team contacts. The numbers of participants to this study are therefore greater than those undergoing OGTT, however demographic data for this set are limited to age and gender.

Analysis was conducted on the raw scores, and the summative factors described in the publications introducing the questionnaires. Principal component analysis of all data points (i.e. for each individual question in each participant) collected, and of all individual points within each questionnaire, was conducted to identify trends in the data. Multi-way ANOVA was used to statistically compare groups, and plots of behaviour factors within questionnaires, and total questionnaire scores were used to visualise group comparisons.

5.3 RESULTS

Questionnaire data were collected from 65 participants. PCA of all questionnaire data for all participants did not differentiate participant groups on any axis.

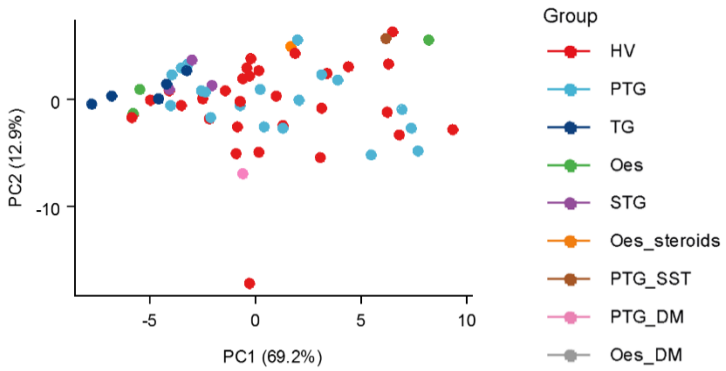

Figure 5-1. Principal component analysis of all questionnaire data points.

5.3.1 Demographics

	HV	PTG	TG	Oes	STG	Oes steroids	PTG SST	PTG DM
Number	31	20	5	3	3	1	1	1
Age (years)	44+/-16	45+/-14	72+/-7	64+/-16	64+/-26	65	67	45
Gender (M:F)	19:12	13:7	3:2	1:2	2:1	M	M	M
Time post-op (years)	NA	6.2+/-4.4	3.8+/-3.0	2.7+/-2.9	1.3+/-0.7	3	14	3

Table 5-1. Demographics of participants recruited to questionnaire study. Age and time post op are mean +/- standard deviation. Oes – oesophagectomy.

5.3.2 Symptoms

The DSS subgroups symptoms by whether they are experienced symptoms early (< 1 hour; 8 symptoms) or late (> 1 hour; 6 symptoms) after eating, with each symptom scored from 0 (absent) to 3 (severe). Early DSS was therefore scored out of 24, and late DSS out of 18.

PCA of all points in all participants clearly separated post-operative groups from healthy volunteers on the first component (Figure 5-2). Multi-way ANOVA of early and late DSS showed only post-operative group (i.e. versus control) to be highly significantly associated with DSS ($p=2*10^{-6}$). DSS_{early} and DSS_{late} were highly correlated (R^2=0.7).

Examination of just the participants after PTG did not identify any significant association between age, gender or time post-surgery and DSS (early or late).

It is perhaps more instructive to view the DSS as a categorical measure of "normal" post-prandial experience. In the control group, the greatest DSS_{early} score was 8, and DSS_{late} was 3. Regarding these as a rough cut-off for whether a post-operative participant experienced abnormal post-prandial symptoms, 20 (67%) participants after PTG or TG were above the DSS_{early} value, and 18 (60%) above the DSS_{late} value.

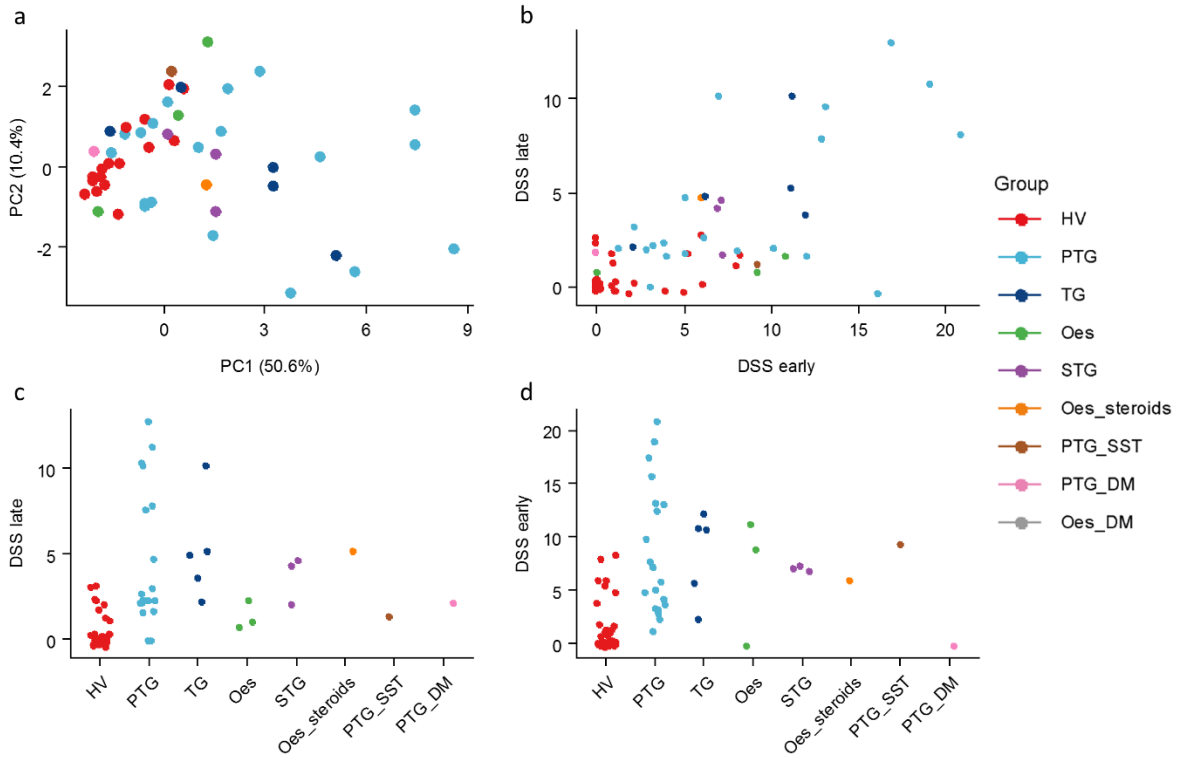

Figure 5-2. Dumping severity score questionnaire results. a - PCA using all individual question answers for all participants. b - correlation of early and late DSS results. c - DSS (late) scores for participants by group. d - DSS (early) scores for participants by group. Note that data points apparently <0 in plots c and d are at 0, with the apparent position an artefact of "jittering" the data to demonstrate multiple identical data points.

5.3.3 BIS-11

PCA of all data points, and individual examination of the six first order factors (attention, cognitive instability, perseverance, motor, self-control and cognitive complexity) failed to identify any difference between operative groups and healthy volunteers for BIS-11 responses (Figure 5-3).

5.3.4 Creature of habit

PCA of all data points, and direct comparison of the total scores across groups failed to identify any difference between operative groups and healthy volunteers for COH responses (Figure 5-4).

5.3.5 TFEQ-r18

PCA of all data points for the TFEQ-r18 separated healthy volunteers from all operative groups on the second dimension, but not first or third dimensions (Figure 5-5). Multi-way ANOVA of each of the three factors making up the TFEQ-r18 identified significantly reduced scores for cognitive restraint and emotional eating ($p=0.03$ for both) but not for uncontrolled eating, in post-operative groups compared to healthy volunteers.

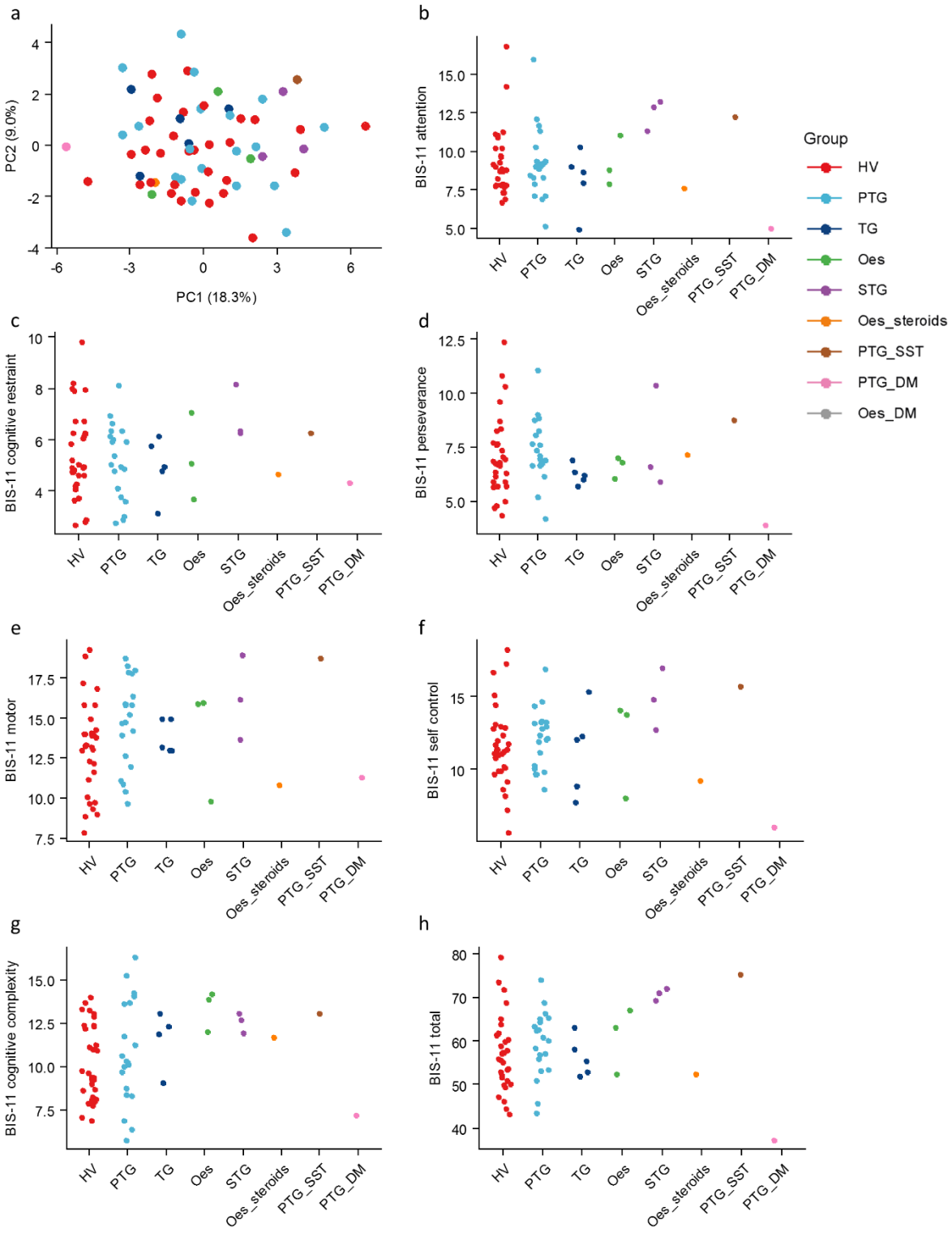

Figure 5-3. Barratt Impulsiveness Scale (BIS-11) measures for all participants by group. a – PCA using all individual question answers for all participants. b-h – summative measures by first order factors of BIS-11. b – attention. c – cognitive restraint. d – perseverance. e – motor. f – self-control. g – cognitive complexity. h – total BIS-11 score.

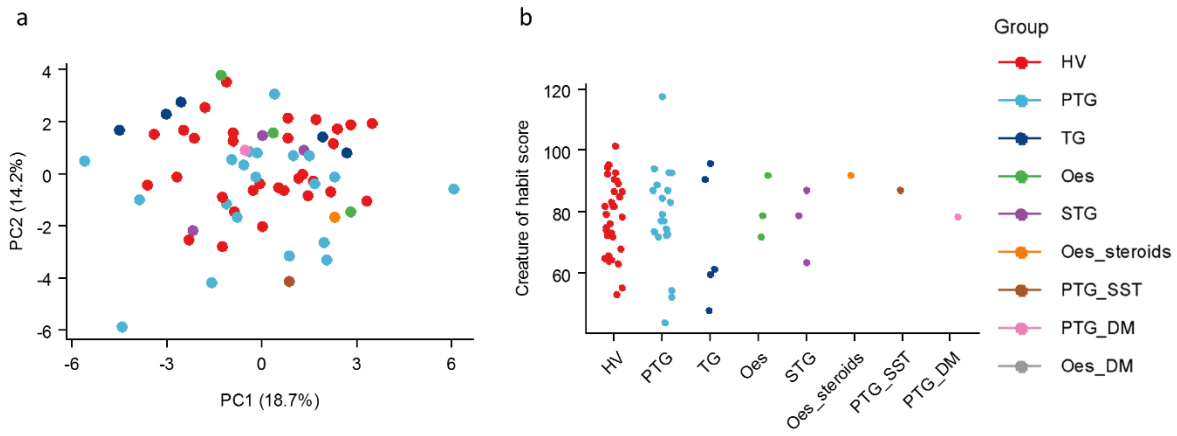

Figure 5-4. Creature of habit measures for all participants by group. a – PCA using all individual question answers for all participants. b – total creature of habit scores by group.

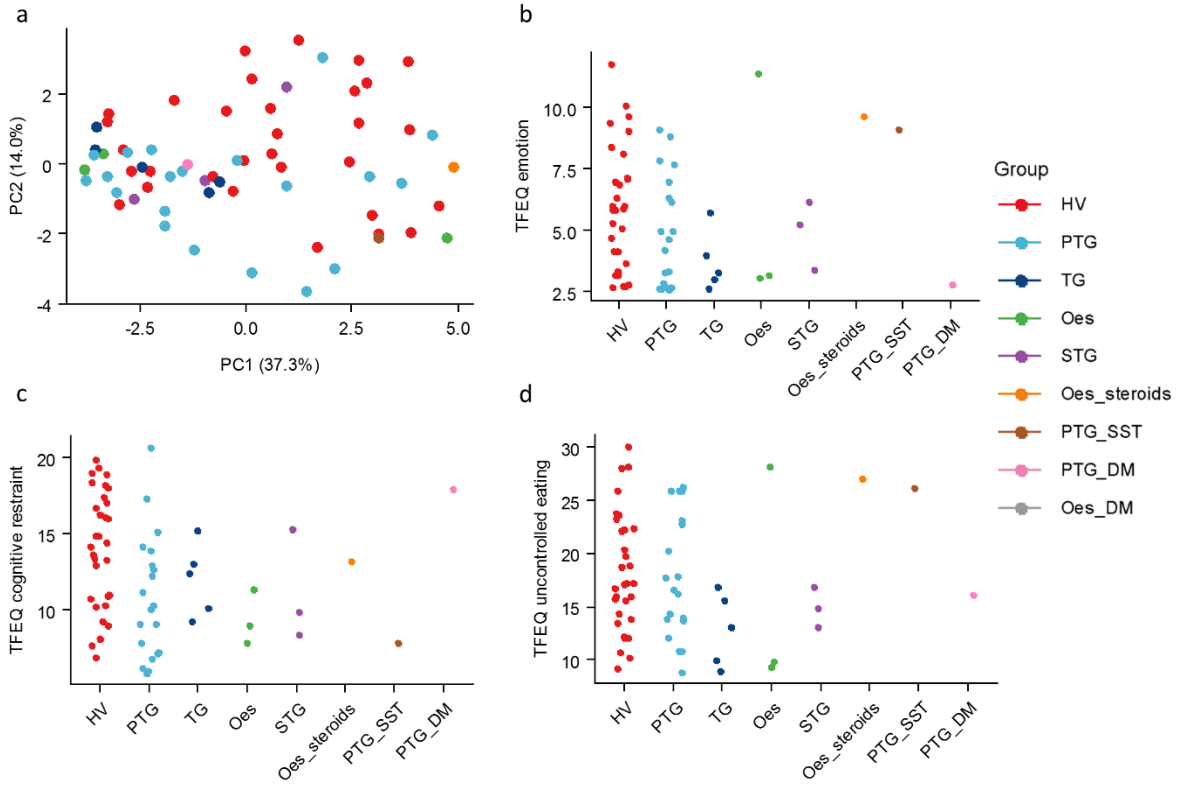

Figure 5-5. Three factor eating questionnaire results for individual participants by group. a – PCA using individual question scores for each participant. b-d – individual factors assessed by TFEQ. b – emotional eating. c – cognitive restraint. d – uncontrolled eating.

5.3.6 Food purchasing tasks

Participants completed food purchasing tasks for apple slices and mini snickers bars. Responses were plotted as a demand-price curve, with demand normalised to the amount purchased when free (giving a range of demand from 0 to 1) and a logistic regression fitted to the resulting inverse sigmoid curve (Figure 5-6). The key outcome measures (price at 50% max demand [P50] and demand elasticity [the slope at P50]) were calculated for each participant from the individually fitted regression model.

There was no significant difference between groups for P50 or demand elasticity as measured by multi-way ANOVA for either apple slices or snickers bars.

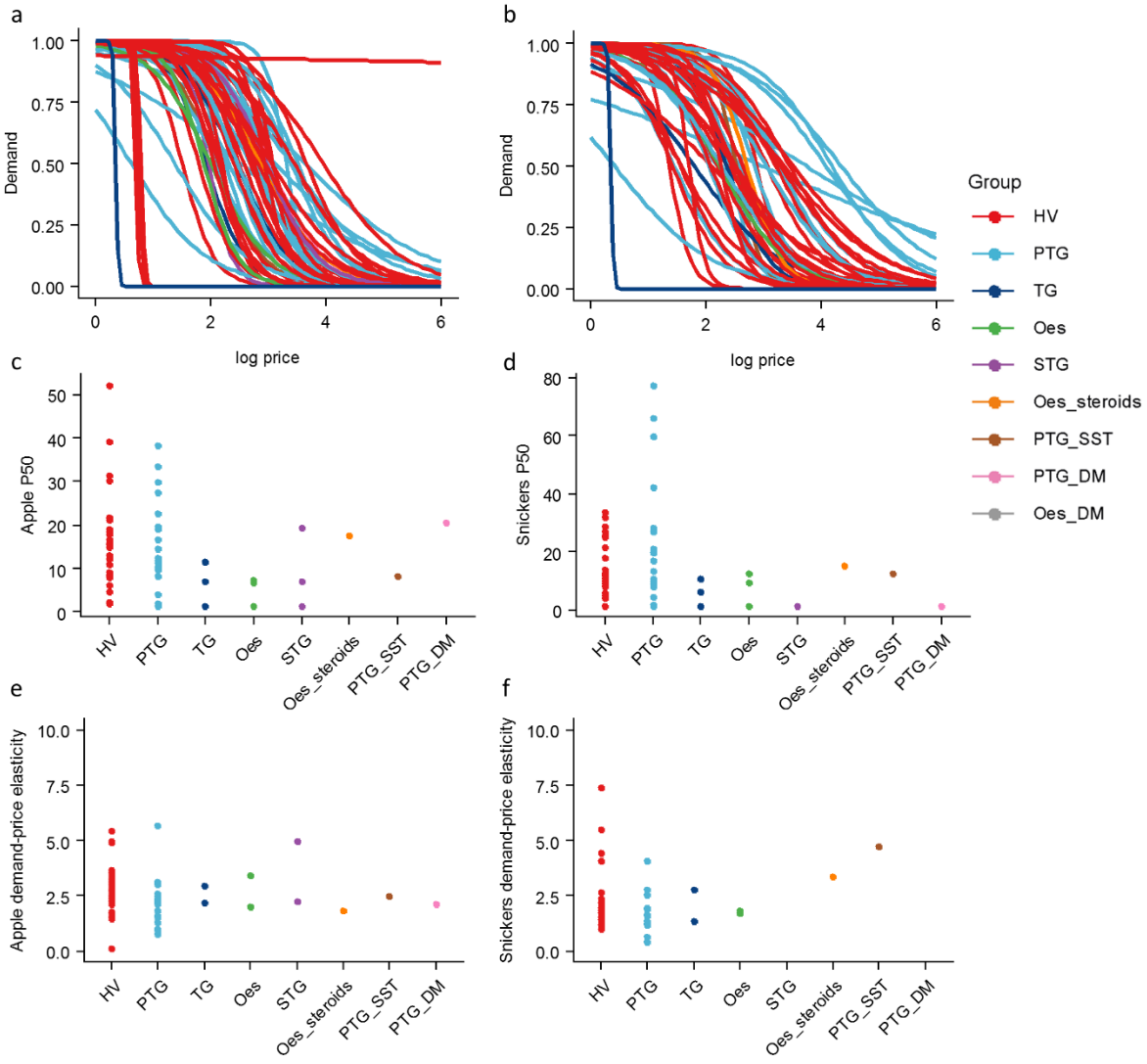

Figure 5-6. Food purchasing task results. a,c,e – apple slices. b,d,f – mini-snickers bars. a,b – demand-price curve for all participants. c,d – P50 – price of commodity at 50% of demand for individual participants by group. e,f – demand-price elasticity – maximum gradient of demand-price curve.

5.4 DISCUSSION

The effect of oesophago-gastric surgery on eating and associated behaviours, despite being long-recognised, remains poorly characterised. This study attempted to characterise post-operative patients from the perspective of post-prandial symptoms, eating behaviour, food motivation and habitual and impulsive behaviour. Post-operative groups were significantly different from controls for measures of post-prandial symptoms and eating behaviour, but not for any indices of food motivation, or habitual / impulsive behaviour.

Unsurprisingly, groupwise analysis of post-prandial symptoms using a previously validated symptom score clearly differentiated post-operative patients from controls. Further analysis of the symptom data identified a wide variation in symptom scores within the post-operative group, which was not associated with age, gender or time since surgery. Using a cut off value corresponding to the upper value experienced by healthy volunteers, 60-67% of patients after TG or PTG could be classified as suffering an increased burden of post-prandial symptoms, or “dumping syndrome”. The severity of early and late symptoms was highly correlated, indicating a possible link in either susceptibility to symptoms or causative mechanisms.

The only other factor that was found to be significantly different between groups was the TFEQ-r18, and within this primarily the measures of cognitive restraint and emotional eating were reduced in post-operative patients. The effect was however subtle, with only minimal difference notable when plotting these factors across groups, and no difference on any measure of food motivation when measured in a validated purchasing task. DSS and TFEQ scores were also poorly correlated, indicating no clear link between altered eating behaviour and significant symptomatology.

While a compelling hypothesis that altered gut hormone secretion, and in particular ghrelin concentration given its link to impulsive behaviour in animal models, may result in changes to impulsive and habitual behaviour, this was not borne out by this study.

This study was limited by the demographic measures available, and lack of repeat measures within participants. A larger data set, both in participant numbers, details and completion of questionnaires on multiple occasions could potentially identify more subtle changes in behaviour. Also, comparison with “harder” measures, for instance glucose and gut hormone profiles during OGTT, could further stratify the symptomatic findings in this study.

In summary, post-operative patients can be characterised by severity of symptoms using a simple questionnaire tool, but measures of behaviour used in this study did not detect significant operative effects. Clinically, the DSS could be a useful tool to objectively identify the subset of patients with significant symptoms and potentially quantify those symptoms.

6 GLP-1 RECEPTOR BLOCKADE AFTER TOTAL GASTRECTOMY

Hypothesis

Enhanced post-prandial secretion of GLP-1 in the gastrectomy population is a significant contributing factor to reactive hypoglycaemia, early satiation and negative symptoms.

6.1 INTRODUCTION

GLP-1 has been demonstrated to have a major potentiating effect on glucose stimulated insulin secretion in animal models and humans with and without type 2 diabetes[293]. There remains considerable controversy over the relative importance of the multiple physiological changes resulting from metabolic surgery, including enhanced gut hormone secretion, for weight loss and improved glycaemic control[18,294]. However, several studies have clearly demonstrated that elevated GLP-1 secretion is a major stimulus of elevated insulin secretion and glucose handling in the post-prandial state after RYGB[55,162,295-297]. For the small but significant group of patients who suffer disabling reactive hypoglycaemia after metabolic surgery (estimated to be 1-5%), a small study demonstrated that GLP-1 receptor (GLP1R) blockade with a specific antagonist (Exendin 9-39) prevented hypoglycaemia after an OGTT[224]. A modified Exendin 9-39 preparation with an extended half-life has now entered phase 2 clinical trials for the treatment of post-bariatric hypoglycaemia (PBH) and may enter the market in the next few years[298].

It was hypothesized that the enhanced post-OGTT insulin response and reactive hypoglycaemia seen in post-gastrectomy participants was driven by a combination of the elevated peak plasma glucose concentration and higher plasma GLP-1 concentrations. As many of the side-effects of GLP-1 therapy for type 2 diabetes (nausea, vomiting, anorexia, tachycardia, headache, dizziness) match those described within the complex of “early dumping” syndrome, it was also hypothesized that GLP-1 was responsible for negative post-prandial symptoms in post-gastrectomy patients.

In order to test these hypotheses, a randomised, placebo-controlled, double-blinded crossover study was designed, wherein Exendin 9-39 was administered as an intravenous infusion to human participants who had previous evidence of reactive hypoglycaemia after total gastrectomy.

6.2 METHODS

Ethical approval was granted by the NHS HRA and the Cambridge East Research Ethics Committee. The study was registered with clinicaltrials.gov (NCT02971631).

Participants were recruited from previous research studies and clinical follow-up at Addenbrooke's Hospital. All had clinical or biochemical evidence of post-prandial hypoglycaemia. All were screened for anaemia, and hepatic and renal dysfunction prior to recruitment and gave written consent following provision of a detailed information leaflet and discussion with the research team.

The study required two overnight stays on the Translational Research Facility (TRF); four of five participants consolidated this to one two-night stay with study interventions on consecutive days (previous studies using Exendin 9-39 have demonstrated no ongoing effect of the agent after 12 hours)[299].

Participants were admitted at 5pm and provided with a weight standardised meal, following which they were permitted only water prior to the study the next day. A Dexcom G4 continuous glucose monitor was sited in the evening to allow it to stabilise and calibrate prior to study interventions. The following morning, participants were woken at 0700 to allow study interventions to commence at 0730.

Exendin 9-39 or placebo infusion was prepared on the morning of the infusion by the nursing staff on the TRF and infused into a venous cannula. Participant and investigator were blinded to the infusion contents.

One cannula was sited in each ante-cubital fossa of the participant (one for infusion, one for blood sampling). Following collection of baseline bloods and symptom scores, Exendin 9-39 was given as a bolus (7500pmol/kg) over four minutes followed immediately by infusion at 500pmol/kg/minute, starting at T-40 minutes. Placebo bolus and infusion were at the same rate as for Exendin 9-39.

Figure 6-1 is a flow chart of participant recruitment and study interventions. Pre-OGTT cognitive measures started at T-30 minutes and were completed by T-5, when further blood samples and VAS

and symptom scores were collected prior to administration of a 50g OGTT at T0. Blood samples and VAS and symptom scores were collected every 15 minutes for two hours, with cognitive measures conducted between T15-45 and T90-120 to coincide with “peak GLP-1” and “baseline GLP-1” concentrations based on previous studies. At T130, the participant was served an ad libitum meal on a universal eating monitor (UEM) and instructed to “eat as much as they wished”. Following the meal, blood samples were collected, cognitive tasks were repeated then the infusion was stopped. VAS scores of hunger, fullness and nausea were completed at the time of stopping the infusion, and every 30 minutes for at least 4 hours prior to discharge.

Initial power calculations suggested 13 participants would be required to reach significance on the primary outcome measure, however following a change in production policy at Bachem it was not possible to source a second batch of Exendin 9-39 at GMP grade and so the study was restricted to five participants.

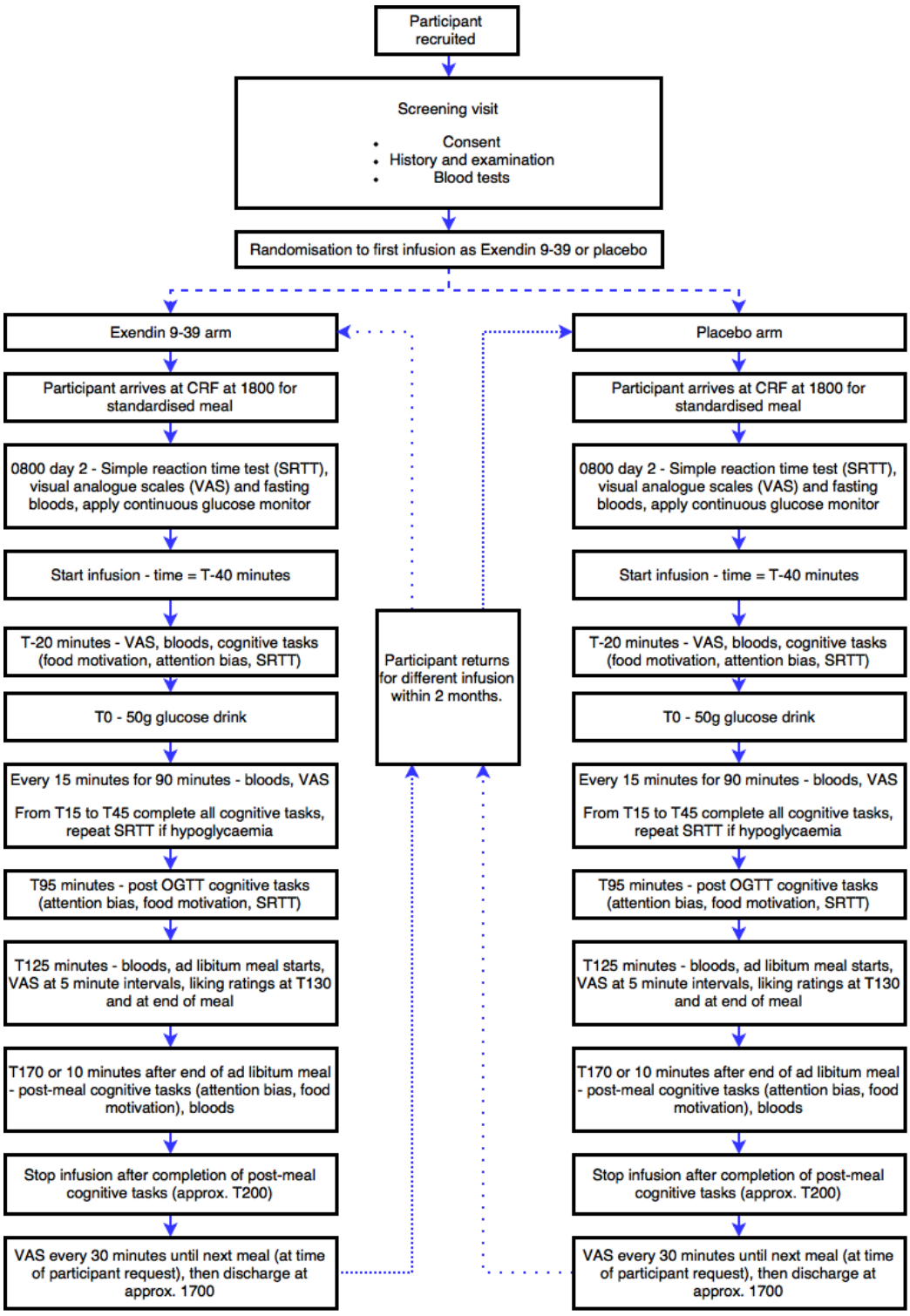

Figure 6-1. Study protocol.

6.2.1 Outcome measures

The primary outcome measure was lowest plasma glucose (nadir glucose) during 2 hours after a 50g oral glucose tolerance test (OGTT), however in order to maximise the findings from this intensive study, measures of hormone secretion, eating behaviour, symptoms and meal size were included.

Study measures (Table 6-1) were blood collection, visual analogue scores (hunger, fullness and nausea), Sigstad score of symptoms, cognitive computer tasks (simple reaction time task, dot-probe food bias and grip strength food reward) and an ad libitum meal on universal eating monitor.

Measure	Times relative to OGTT (minutes)
Blood sampling	-40 (pre-infusion), -5, 15,30,45,60,75,90,105,120, post-meal (~180)
VAS	-40 (pre-infusion), -5, 15,30,45,60,75,90,105,120, post-meal (~180) and every half hour for four hours post end of infusion
Sigstad score	-40 (pre-infusion), -5, 15,30,45,60,75,90,105,120
Dot-probe task	-25, 15, 90, post-meal
Grip strength task	-15, 30, 105, post-meal
Simple reaction time task	-30, 115
Ad libitum meal	130

Table 6-1. Exendin study measures / timepoints.

6.2.2 Analysis

Insulin secretory measures were calculated as described in chapter 2. Given the low number of replicates, measures are presented for each participant, and statistical comparison conducted using two-way paired T-tests on either area under the curve (AUC), or incremental area under the curve (IAUC) values as representative of secretory profile throughout the 2 hour OGTT. Comparison with non-operative controls was by unpaired two-way t-test against results from the 13 control participants from chapter 3.

ISR (log transformed to better fit a linear model) was investigated by a linear mixed effects model using the package *lme4* with glucose, GLP-1 and infusion (and their interactions) as fixed effects and ID as random effect[291]. Log transformed hunger score (for the 120 minutes of the OGTT) was modelled using *lme4* with glucose, GLP-1 and infusion as fixed effects and ID as random effect, excluding participant 1 who scored 0 on all hunger VAS charts for both infusions.

6.3 RESULTS

No adverse events were reported.

6.3.1 Participant demographics

All participants had undergone prophylactic total gastrectomy at least six months prior to recruitment, had a stable weight and normal renal and hepatic function and non-diabetic HbA1c. None were taking medication. Data from the 13 healthy volunteers from chapter 3 for whom insulin and glucose data were available were used for comparison.

Age	39.2+/-7.1
Gender (M:F)	4:1
Height (cm)	179.6+/-4.5
Weight (kg)	70.5+/-3.2
BMI	22.1+/-1.8
Waist (cm)	84+/-3.3
Hip (cm)	99.9+/-3.1
Waist:hip ratio	0.84+/-0.03

Table 6-2. Infusion participant demographics. Mean +/- standard deviation.

6.3.2 Glucose

All five participants experienced hypoglycaemia following the OGTT while receiving placebo, in four the nadir glucose was <3.3mmol/l, in the fifth it was 3.85mmol/l (Figure 6-2). Nadir glucose was greater in all participants while receiving Exendin 9-39, although in two it was still <4mmol/l (3.6 and 3.9mmol/l).

Interestingly, the rate of glucose decline between peak and nadir glucose was only marginally greater during placebo than Exendin 9-39 (Table 6-3).

The time of peak glucose only changed between infusions for one participant (delayed from 15 to 30 minutes), while the time of nadir glucose was unchanged at 120 minutes for one participant, earlier for two participants and delayed for two participants.

The CGM data were unreliable, with frequent drop-outs of the reading during the rapid fluctuation in glucose immediately post-OGTT, and on the decline in glucose. This is in keeping with the primary use of CGMs as measures of trends of glucose, used to guide close glycaemic control in diabetic patients, not the measurement of rapid changes in a research setting.

Glucose measure (mmol/l)	Placebo	Exendin 9-39	Control	P value: Placebo vs Exendin	P value: Placebo vs control	P value: Exendin vs control
Pre-infusion	4.7+/-0.1	4.8+/-0.1	NA	0.4	NA	NA
Infusion fasting	4.9+/-0.1	5.1+/-0.1	5.4+/-0.1	0.02	0.003	0.06
Peak	9.1+/-0.2	9.5+/-0.9	9.0+/-0.6	0.6	0.89	0.72
Nadir	3.0+/-0.3	4.4+/-0.3	4.8+/-0.2	0.008	0.0004	0.22
Delta peak-nadir	6.2+/-0.3	5.1+/-0.7	4.2+/-0.6	0.09	0.008	0.35
Peak-nadir rate of drop (mmol/l/min)	0.08+/-0.01	0.07+/-0.01	0.08+/-0.01	0.06	0.90	0.64

Table 6-3. Glucose handling measures. Placebo vs Exendin comparisons are paired T-tests (two-way), comparisons with control participant data are unpaired two-way T-tests. Mean +/- standard error.

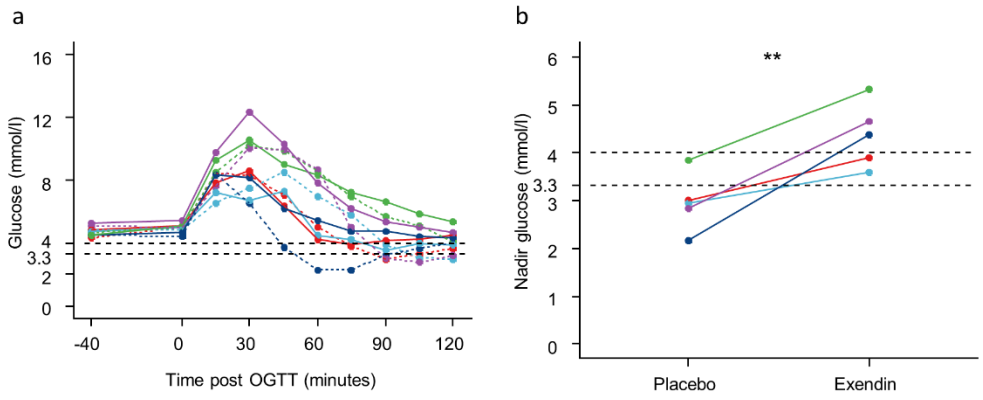

*Figure 6-2. Glucose profile for participants during Exendin 9-39 infusion by participant. a – full time-course plasma glucose concentrations for all participants (Exendin 9-39 infusion is denoted by solid line, placebo by dashed line, each participant is one colour). b – nadir plasma glucose during each infusion by participant. ** - p<0.01, paired t-test.*

6.3.3 Insulin secretion

Infusion of Exendin 9-39 reduced all measures of insulin secretion compared to placebo after OGTT, with the post-OGTT insulin plot matching that of healthy volunteers after a matched OGTT (Table 6-4, Figure 6-3). Incremental areas under the curve over 120 minutes (IAUC_120) for insulin and C-peptide were significantly lower during Exendin 9-39 infusion than placebo. Insulin secretory rate (ISR), normalised for plasma glucose concentration and integrated over the 120 minutes of the OGTT was significantly lower during Exendin 9-39 infusion. Two other derivative measures of insulin secretion (insulinogenic index and disposition index) were non-significantly reduced during Exendin 9-39 infusion and not compared with healthy volunteer results.

Plotting ISR by GLP-1 (Figure 6-4) for both infusions across all time points demonstrated a greater effect of GLP-1 on ISR during placebo than Exendin 9-39 infusion, with some correlation of GLP-1 and insulin secretion even in the Exendin 9-39 group (although GLP-1 and glucose were also closely correlated).

Examination of a linear model of all timepoint results from 0 to 120 minutes of log(ISR) by plasma GLP-1 and glucose concentrations, time post-OGTT and infusion identified glucose and infusion as predictive for ISR, with glucose concentrations positively correlated with ISR and Exendin 9-39 infusion suppressing ISR (Figure 6-5, Table 2-1). The overall model was remarkably well fitted, and ANOVA of the model against the null hypothesis that the predictive variables were not associated with ISR confirmed the strong association between glucose and infusion type, and ISR.

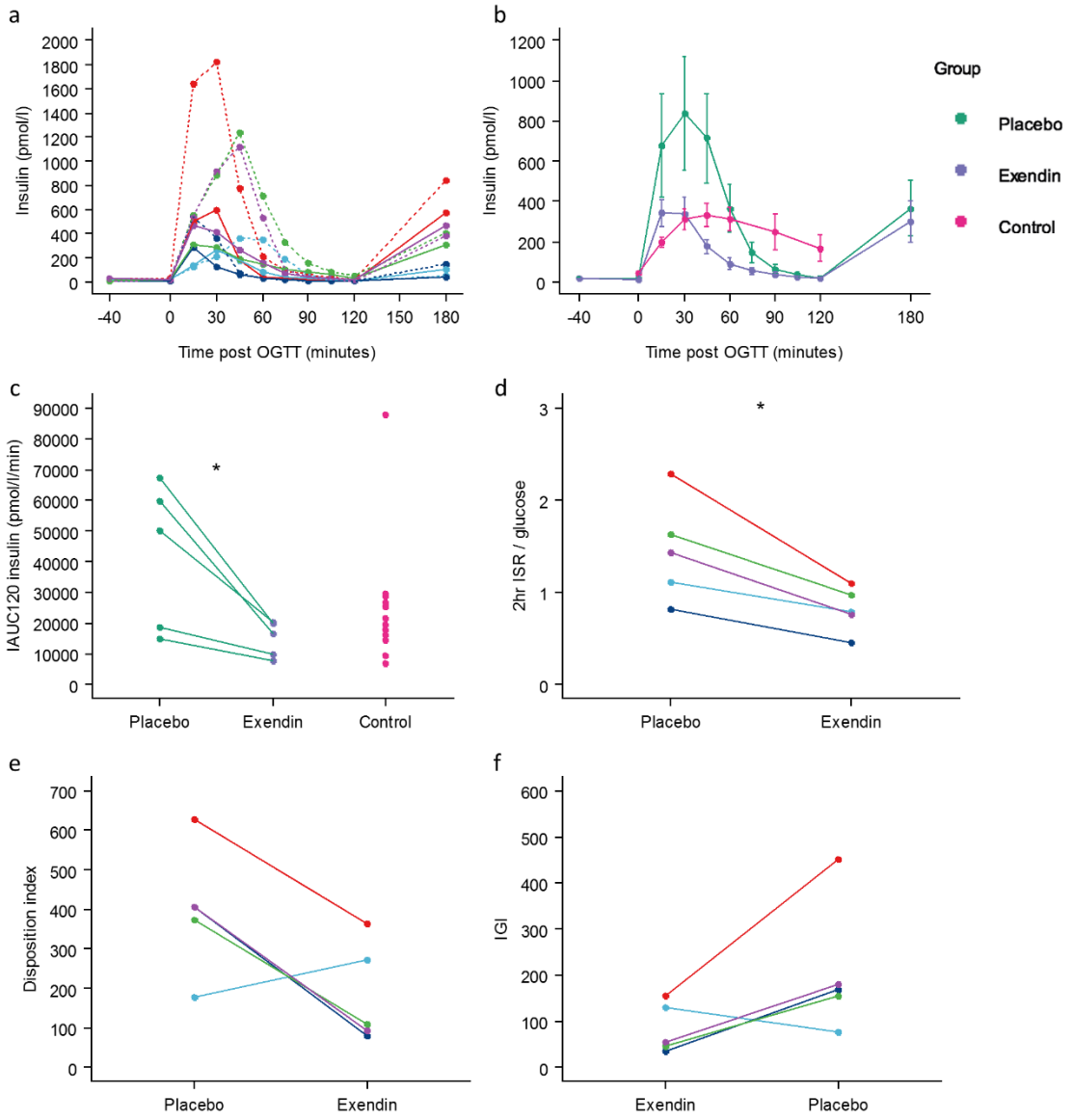

*Figure 6-3. Insulin secretory measures during Exendin 9-39 / placebo infusion. Infusion from time 0 to time 120, meal at time 120. a – individual participant plasma insulin concentration by timepoint, solid line denotes Exendin infusion, dashed denotes placebo. b – mean +/- standard error of group plasma insulin concentration by timepoint. c – IAUC_120 for insulin secretion during OGTT by group. d – 2 hour integral insulin secretory rate normalised by integral plasma glucose concentration. e – insulin disposition index. f – insulinogenic index. * - p<0.05.*

Measure	Placebo	Exendin 9-39	P value
IAUC120 insulin (pmol/l/min)	41000+/-10400	14500+/-2600	0.03
IAUC120 C-peptide (pg/ml/min)	261500+/-52400	136300+/-23200	0.02
ISR/glucose	1.46+/-0.25	0.81+/-0.12	0.01
IGI	206.0 +-83.5	63.9 +-24.5	0.09
DI	397.6+-71.4	183.1 +-56.9	0.05

Table 6-4. Insulin secretory measures. Comparison is by paired t-test (two-way).

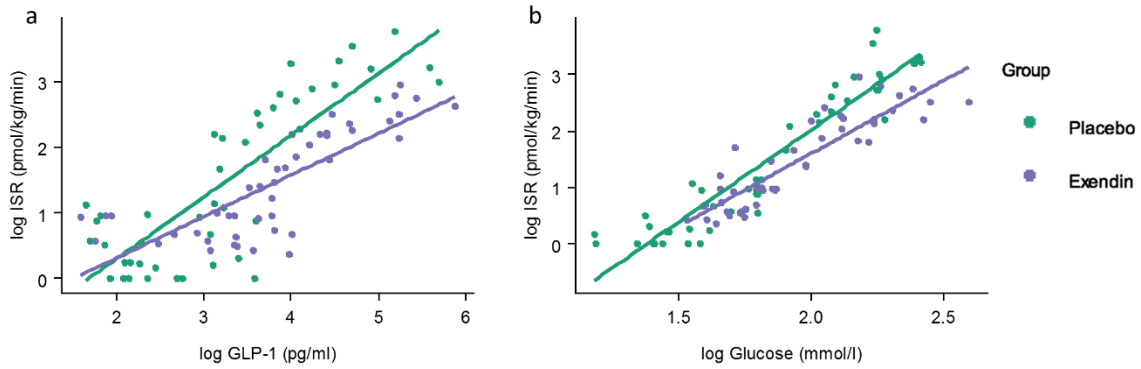

Figure 6-4. Insulin secretory rate by plasma GLP-1 (a) and glucose (b) concentration as log-log plots. Fitted lines are simple linear models of the presented data by group, demonstrating enhanced ISR at high GLP-1 / glucose in placebo group versus Exendin 9-39 group.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Glucose	10.19	10.19	1	74.99	75.64	$6*10^{-13}$
Infusion	0.83	0.83	1	76.14	6.13	0.02
GLP1	0.03	0.03	1	77.94	0.19	0.67
Time	2.13	0.27	8	75.24	1.98	0.06

Table 6-5. ANOVA table for linear mixed effects model of factors contributing to log(ISR), with ID as random effect, after OGTT during infusion of Exendin 9-39 or placebo.

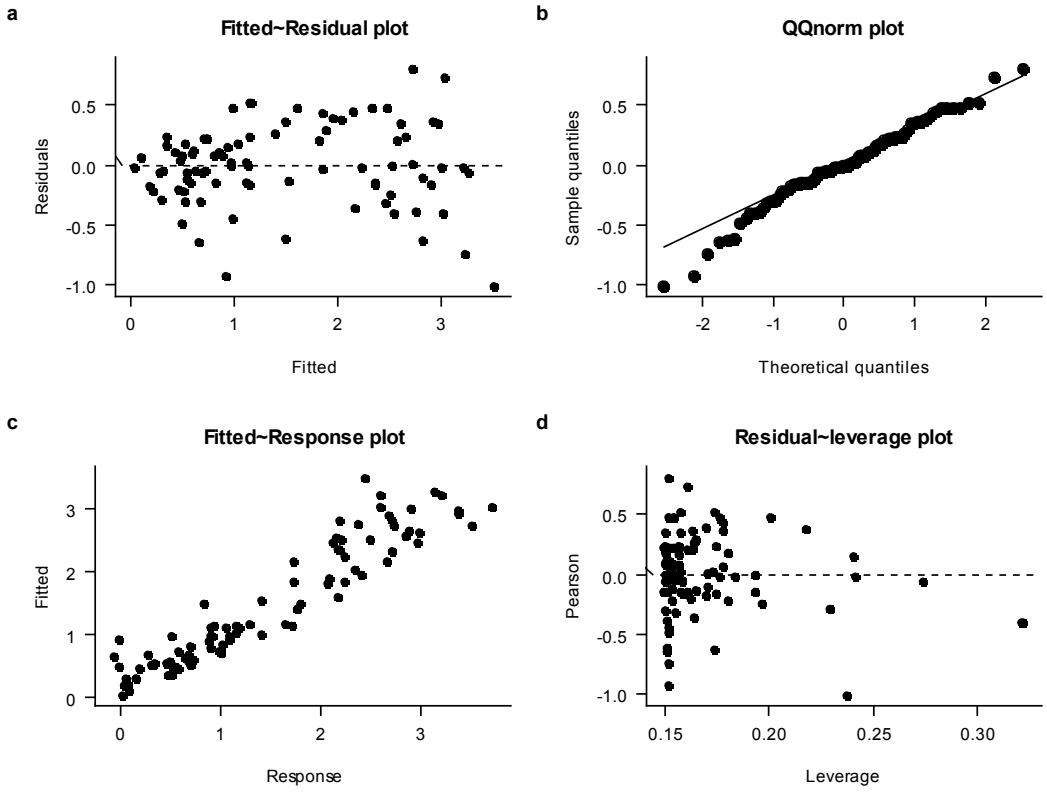

Figure 6-5. Model diagnostic plots for linear mixed effects model of log(ISR) by GLP-1 and glucose concentrations, time post-OGTT and infusion, with ID as random effect.

6.3.4 Glucagon secretion

Plasma glucagon was assayed using the modified (high glicentin, see chapter 7) protocol at T0 and T30 to assess any effect of GLP-1 blockade on glucagon secretion / suppression (Figure 6-6d). The delta glucagon was -4.9+/-1.1pg/ml during placebo and +1.7+/-1.4 (p=0.03) during Exendin 9-39 infusion (i.e. plasma glucagon concentration rose despite hyperglycaemia with GLP1R blockade).

6.3.5 Gut hormone secretion

IAUC_120 for GLP-1 (Figure 6-6) during placebo infusion was significantly lower than during Exendin 9-39 infusion (6025+/-1704pg/ml/min vs 8430+/-991; p=0.046), as was that for PYY (8386+/-2435pg/ml/min vs 14342+/-1522; p=0.04).

IAUC_120 for GIP was however reduced during Exendin 9-39 infusion compared with placebo (9707+/-1357pg/ml/min vs 6640+/-1050pg/ml/min; p=0.02), implying that GLP-1 suppresses secretion of PYY and GLP-1, but stimulates secretion of GIP.

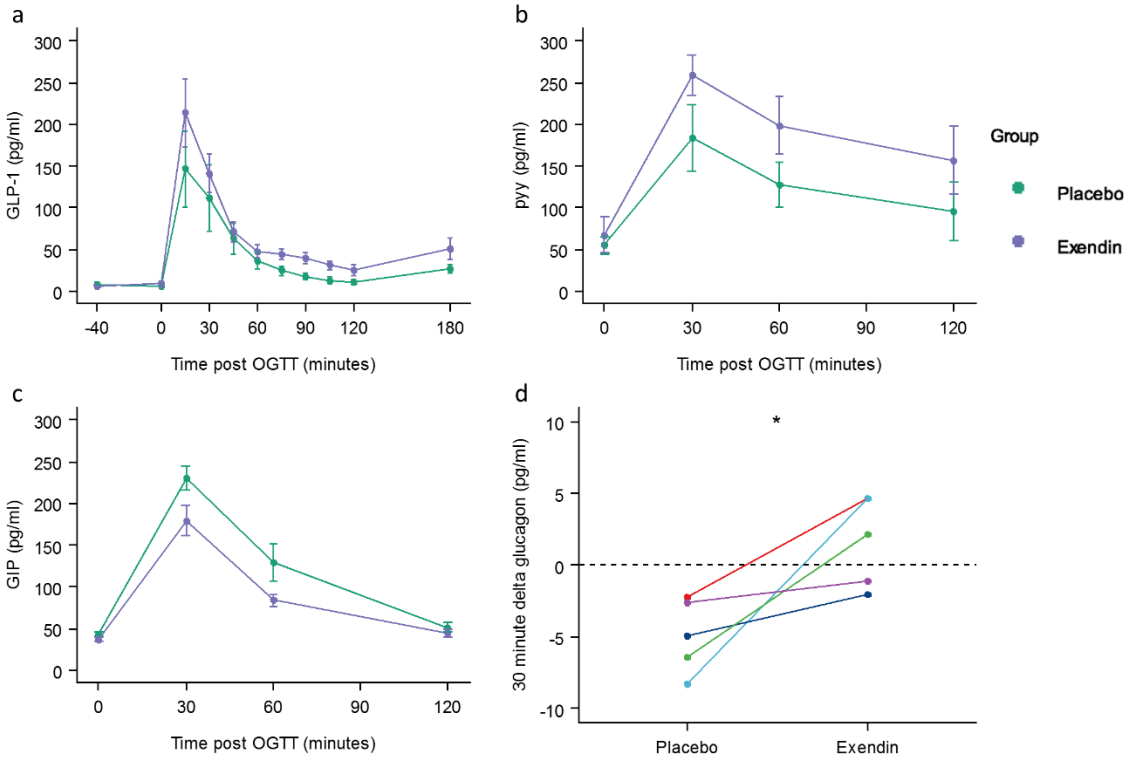

*Figure 6-6. Gut hormone and glucagon profiles during Exendin 9-39 or placebo infusion. Infusion started at T-40, OGTT at T0. a-c – group mean +/- standard error, d – individual participant results. a – plasma total GLP-1 concentration by group (mean +/- standard error). b – plasma PYY concentration by group. c – plasma GIP concentration. d – change in plasma glucagon concentration from T0 to T30 for each participant by infusion. * - p<0.05.*

6.3.6 Symptom and visual analogue scores

Hunger was measured out of 100 on a VAS at each timepoint during OGTT. The effect of infusion on the hunger score was examined by generation of a linear mixed effects model, including infusion, GLP-1 concentration, time post-OGTT and glucose concentration as fixed effects, and ID as random effect (excluding the one participant who scored 0 for hunger throughout both OGTTs). The model was reasonably well fitted, with a small number of highly leveraged datapoints and residuals following a roughly normal distribution (Figure 6-8). The only significant predictor of hunger score was infusion, with Exendin 9-39 associated with significantly increased hunger score (p=0.0009, Table 6-6).

Noxious symptoms were rare, with fullness and nausea scores not significantly different between infusions (Figure 6 7). Examining Sigstad score by time for all participants identified one participant with significant symptoms resulting from the OGTT during placebo infusion who was asymptomatic during Exendin 9-39 infusion. The effect was not as dramatic for the other four participants, however a linear mixed effects model of Sigstad score including infusion, GLP-1, glucose and time post-OGTT as fixed effects and ID as random effect did identify Exendin 9-39 infusion as significantly reducing symptoms (p=0.03 on multi-way ANOVA).

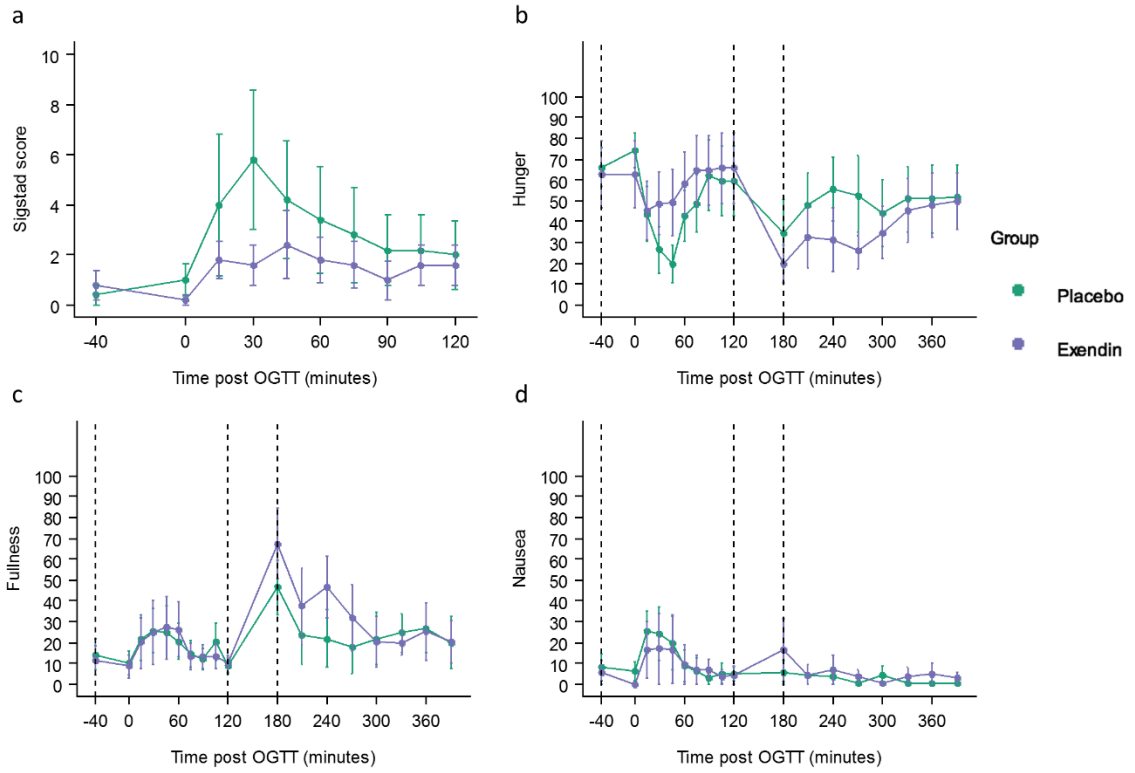

Figure 6-7. Cognitive measures during and after Exendin 9-39 infusion or placebo. Mean +/- standard error. a – Sigstad score. b,c,d – hunger, fullness, nausea scores out of 100. Infusion commenced at T-40, OGTT at T0, end infusion and meal at T120 and end of meal at T180.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Infusion	3488.08	3488.08	1	59.63	12.18	0.0009
GLP1	3.40	3.40	1	55.27	0.01	0.91
Time	3062.00	382.75	8	57.00	1.34	0.24
Glucose	361.08	361.08	1	43.12	1.26	0.27

Table 6-6. Coefficient table for linear mixed effects model of factors contributing to hunger score, with ID as random effect, after OGTT during infusion of Exendin 9-39 or placebo.

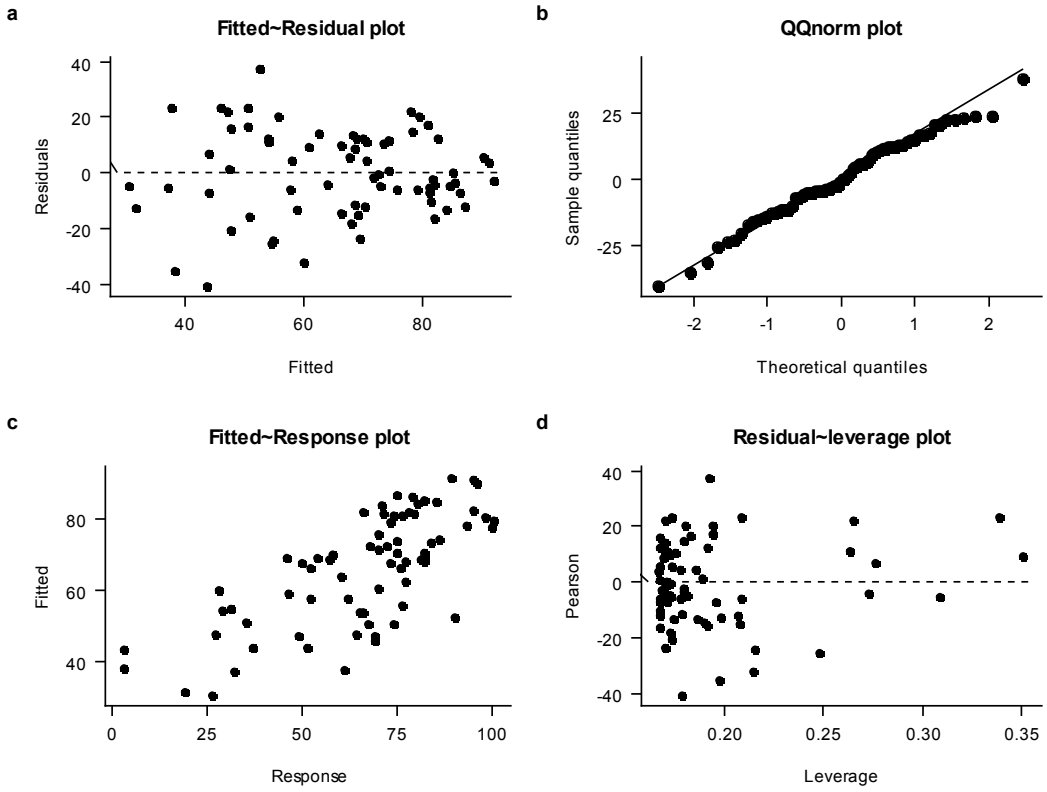

Figure 6-8. Model diagnostic plots for linear mixed effects model of hunger score by GLP-1 and glucose concentrations, time post-OGTT and infusion, with ID as random effect – plots a-c demonstrate normally distributed residuals and reasonable predictive value of the model, however d indicates several highly leveraged datapoints, indicating a need for caution when interpreting the model.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Infusion	37.39	37.39	1	76.25	4.84	0.03
Age	22.61	22.61	1	2.97	2.93	0.19
GLP1	7.09	7.09	1	76.50	0.92	0.34
Glucose	0.01	0.01	1	68.78	0.00	0.97
Time	44.54	5.57	8	74.67	0.72	0.67

Table 6-7. ANOVA table for linear mixed effects model of Sigstad score dependent upon infusion, age, GLP-1 and glucose concentrations and time post-OGTT.

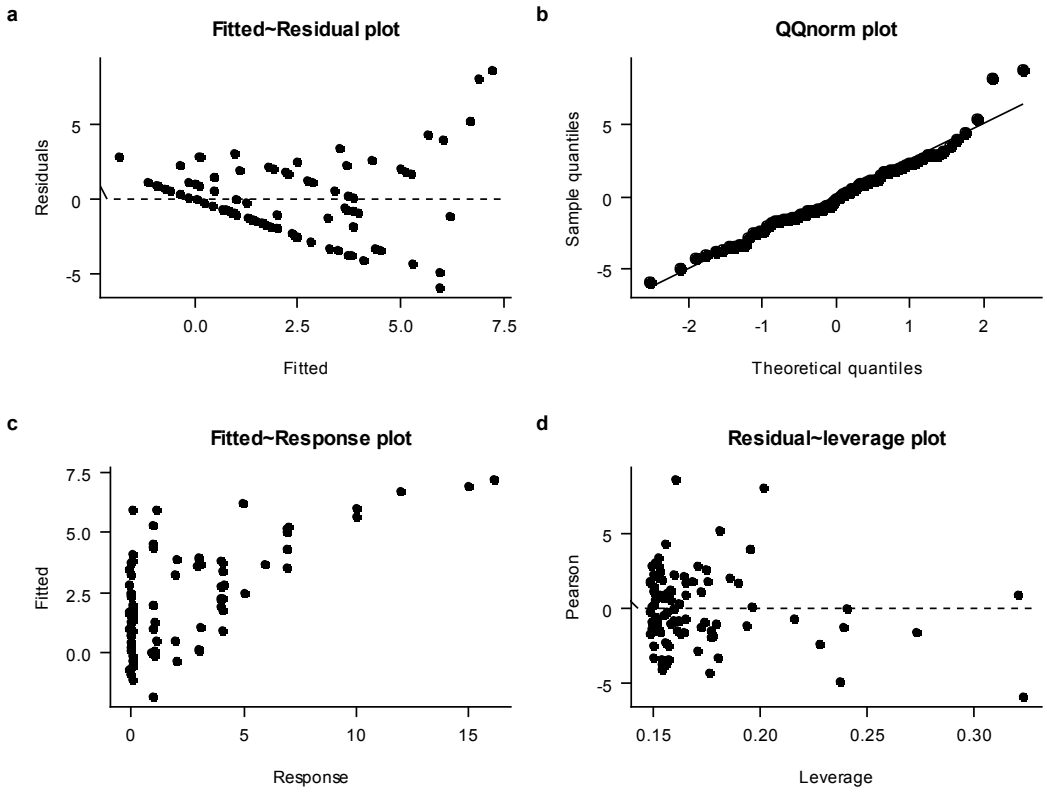

Figure 6-9. Model diagnostic plots for linear mixed effects model of Sigstad score by GLP-1 and glucose concentrations, age, time post-OGTT and infusion, with ID as random effect.

6.3.7 Non-esterified fatty acids and plasma osmolality

Exendin 9-39 infusion did not change plasma osmolality or serum non-esterified fatty acids in the fasting state or following the OGTT (Figure 6-10a,b).

6.3.8 Cognitive measures

There was no significant difference in grip strength or food bias arising due to the OGTT, or between infusions (Figure 6-10c,d).

6.3.9 Ad libitum meal

There was no significant difference between infusions for total amount of meal eaten or rate of consumption of meal (Figure 6-10e,f).

6.3.10 Exendin 9-39 concentration

Plasma Exendin 9-39 concentration reached a level of ~400ng/ml by the time of administration of the OGTT, which continued to slowly rise to ~550ng/ml by the end of the infusion (Figure 6-10g).

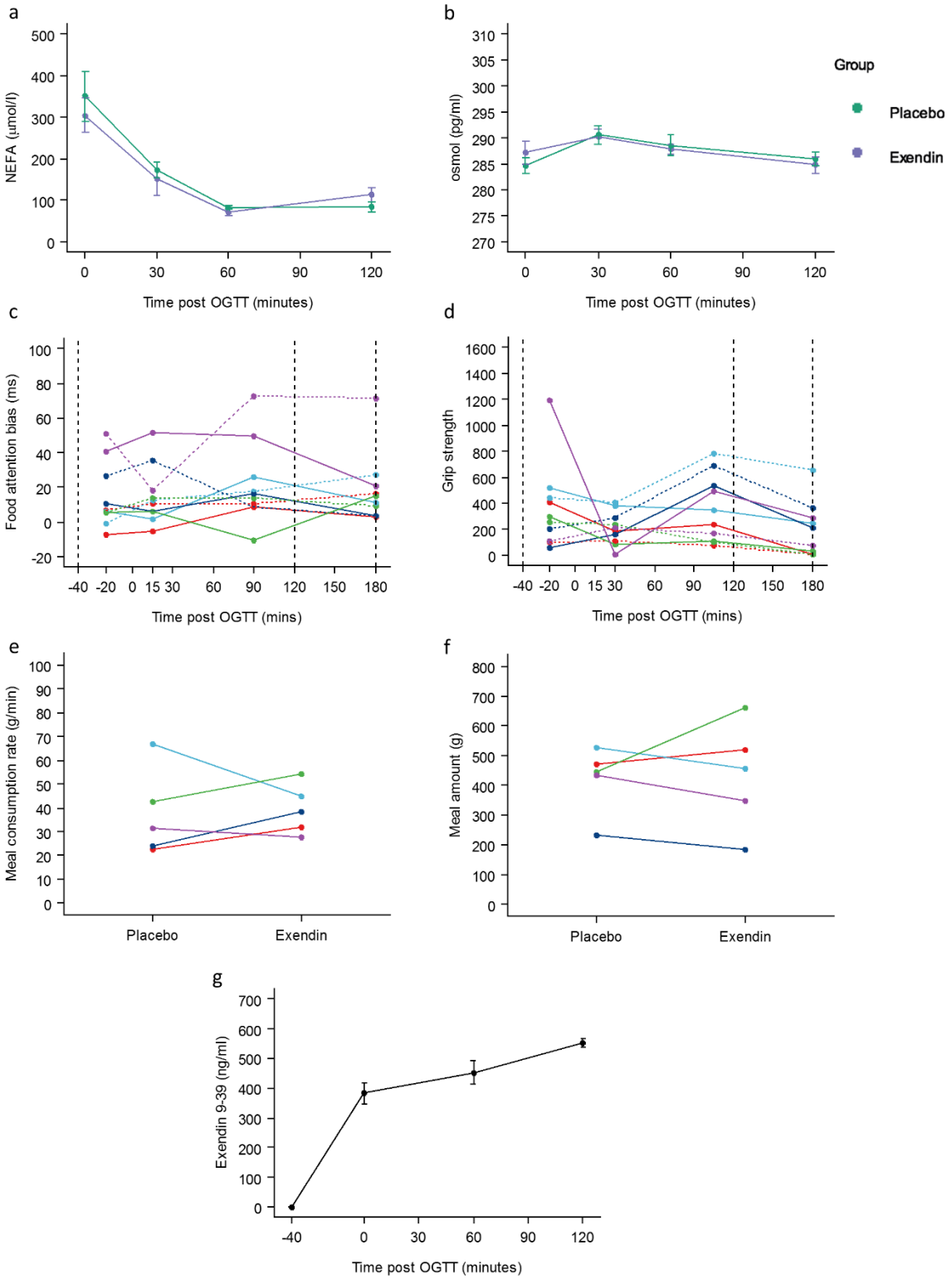

Figure 6-10. a – serum non-esterified fatty acids (NEFAs) during OGTT by infusion (mean +/- standard error). b – plasma osmolality during OGTT by infusion (mean +/- standard error. c,d – individual participant results over OGTT and ad libitum meal for food attention bias (c) and (infusion start at T-40, OGTT at T0, meal at T120, end infusion at T180; solid line Exendin 9-39 infusion, dashed line placebo; each participant is one colour). e,f – ad libitum meal consumption rate (e) and total amount consumed (f) by infusion (each colour is an individual participant). g – plasma Exendin 9-39 concentration during infusion (mean +/- standard error).

6.4 DISCUSSION

This study demonstrated a clear role for elevated post-prandial GLP-1 secretion in glucose handling and insulin secretion, and a possible role in post-prandial symptoms following gastrectomy. While unavoidably underpowered, the pairwise design and analysis, with significant treatment effect, allowed identification of statistically significant findings from five participants.

The plasma concentration of Exendin 9-39 achieved was approximately 1/3 of that previously published in bariatric studies, however this study measured Exendin 9-39 using a highly specific LC-MS method, whereas previous studies have used immunoassays, so this difference was unrepresentative. Exendin 9-39 concentration was ~1000x greater than measured plasma GLP-1 concentration, which has previously been demonstrated to be necessary for complete blockade of the GLP-1 receptor, despite Exendin 9-39 and GLP-1 having similar affinity to the GLP-1 receptor in vitro[300,301]. Given the highly significant effect of Exendin 9-39 infusion on insulin secretion in this study, it can be assumed that the current protocol achieved adequate GLP1R blockade.

The primary outcome measure, nadir glucose during the OGTT, was significantly greater during Exendin 9-39 infusion, supporting the hypothesis that GLP-1 is a key causative factor of reactive hypoglycaemia after gastrectomy. This was not accompanied by an increase in peak glucose, and 120 minute glucose was below the diagnostic cut-off of 11.1mmol/l for diabetes for all participants during Exendin 9-39 infusion. Indeed, infusion of Exendin 9-39 resulted in an insulin profile similar to that of non-gastrectomy, non-diabetic controls, rather than total suppression of insulin secretion. Modelling of ISR by glucose, GLP-1 and infusion showed a clear relationship between ISR, glucose and infusion of Exendin 9-39.

This is in keeping with the known mechanism of action of GLP-1, as a potentiating factor for glucose mediated insulin secretion, and indeed the higher peak plasma glucose in the gastrectomy group (due to rapid delivery of the OGTT to the absorptive small intestine) appeared to stimulate a similar degree of insulin secretion, compared with that achieved by GLP-1 and glucose together in control participants. It is unclear exactly to what degree the slightly higher GIP concentrations of gastrectomy

patients compared with controls impacts on insulin secretion, however it is clearly less important than GLP-1 given the magnitude of effect of Exendin 9-39.

GLP1R blockade resulted in a small but significant increase in secretion of GLP-1 and PYY, which are known to be cosecreted[302]. Previous studies, and transcriptomic work presented later in this thesis, have failed to identify GLP-1 receptors on GLP-1 secreting cells, pointing to a more complex feedback mechanism than direct inhibition. The small but significant decrease in GIP secretion during infusion of Exendin 9-39 also suggests a degree of interaction between GIP and GLP-1 secretory pathways.

Glucagon secretion following OGTT in patients after Roux-en-Y reconstruction of the foregut is a topic of considerable debate, and will be discussed in more detail later in this thesis. The data presented here suggest that while glucagon secretion is appropriately suppressed by hyperglycaemia in gastrectomy patients, GLP1R blockade reverses this effect and results in a small rise in plasma glucagon after OGTT. This is in keeping with previous reports wherein GLP-1 is a potent inhibitor of glucagon secretion[303].

The small participant numbers rendered examination of the secondary behavioural and symptomatic measures challenging. Only two participants met the historic diagnostic criteria for “dumping syndrome” of a Sigstad score of 7 during OGTT with placebo infusion, and notably in the participant with the greatest symptoms (max score 16) this was no longer apparent during Exendin 9-39 infusion. Modelling of Sigstad score by the available variables found Exendin 9-39 infusion to be significantly associated with a reduction in symptoms during the OGTT. Similarly, Exendin 9-39 appeared to increase hunger scores when assessed by multi-way ANOVA across the whole time course of the OGTT.

Asking the participants to eat their post-OGTT lunch on a universal eating monitor was a superficially appealing, and straightforward, addition to a study which was complicated and expensive to run. Two factors however contributed to this being a futile approach. Firstly, the variability in plasma glucose at the end of the OGTT resulted in most participants starting the meal with hypoglycaemia during the placebo infusion, but not during the Exendin 9-39 infusion. The hypothesis that GLP1R blockade would result in an increased volume and rate of meal consumption was therefore confounded by the strong

stimulus to eat provided by hypoglycaemia. Secondly, the ad libitum meal was provided as a bowl of pasta which was so large as to remove any visual cues of amount of food eaten. All participants however argued that their ability to sense that they have eaten an appropriate amount of food requires visual cues of meal size. As the UEM should have assessed subconscious cues of eating control, and all participants were concentrating on visually estimating meal size, it is unsurprising that the results were highly varied.

Despite the small numbers in this study, it clearly demonstrated a key role for GLP-1 in post-gastrectomy hypoglycaemia, and suggested that it may also contribute to post-prandial symptoms and reduced appetite. This is the first study to identify this in patients following gastrectomy for malignancy, and matches previous findings in metabolic surgery patients, both in studies examining the role of GLP-1 in resolution of diabetes following surgery, and the causes of post-bariatric hypoglycaemia. There are presently no GLP1R antagonists licensed for therapeutic use, however a modified subcutaneous version of Exendin 9-39 is in phase two trials in the USA, and other companies have expressed interest in developing GLP1R antagonist drugs for the treatment of post-bariatric hypoglycaemia, which would potentially be of use in the post-gastrectomy group.

7 POST-PRANDIAL PLASMA GLUCAGON$_{33-61}$ AFTER FOREGUT BYPASS

Hypothesis

Ingestion of glucose does not stimulate secretion of pancreatic type glucagon after gastrectomy with Roux-en-Y reconstruction.

7.1 INTRODUCTION

While enhanced post-prandial secretion of GLP-1 is well-established in patients after gastrectomy and RYGB, considerable debate remains over the secretion and functional role of other pro-glucagon derived peptides[62,199,304]. Several groups have published studies suggesting that the plasma concentration of pancreatic type glucagon$_{33-61}$ (henceforth referred to as simply "glucagon") paradoxically rises in response to a meal or glucose tolerance test following RYGB[305-307]. While this seems unlikely given the potency with which hyperglycaemia and elevated GLP-1 concentration suppress pancreatic alpha cell secretion, authors of these studies suggested that adaptive changes in enteroendocrine cells may result in expression of PC2 and so cleavage of proglucagon (notionally destined to produce GLP-1 / GLP-2 / oxyntomodulin / glicentin) to glucagon, resulting in enhanced post-prandial glucagon secretion. Indeed, one group has published evidence of circulating plasma glucagon after total pancreatectomy as evidence of extra-pancreatic glucagon synthesis[306].

The controversy over the importance of glucagon for weight loss and improved metabolic parameters after metabolic surgery is further complicated by emerging evidence that glucagon receptor activity enhances glucose handling and promotes weight loss in the obese[166]. Human and animal studies administering glucagon alongside other gut peptides (e.g. GLP-1, PYY), oxyntomodulin (a glucagon – GLP-1 receptor co-agonist) or synthetic GLP-1/glucagon co-agonists have all demonstrated enhanced efficacy over GLP-1 administration alone[24,166]. It is therefore a compelling argument that adaptive changes in the gut after metabolic surgery could result in glucagon secretion in response to nutrient ingestion, so contributing to the beneficial effects of surgery.

Simply measuring concentrations of plasma or tissue proglucagon peptides is not as straightforward as it may superficially appear. The history of "intestinal glucagon immunoreactivity" and understanding of the multiple proglucagon derived peptides closely matches the development of progressively more specific assays for cleavage products of the proglucagon prohormone[308,309]. Specifically, the initial description of "enteroglucagon" in the mid 20th Century was superseded as the biologically active peptides GLP-1, GLP-2 and oxyntomodulin, as well as glicentin, were identified in the gut[46-48]. In

parallel, the pancreatic derived peptide classically described as “glucagon” was thought to be specifically located to pancreatic alpha cells.

Glicentin corresponds to amino acids 21-89 of the proglucagon prohormone, oxyntomodulin amino acids 53-89 and pancreatic glucagon$_{33\text{-}61}$ to amino acids 53-81 (the large print numbers refer to the sequence including the 20 amino acid N-terminal signal peptide, the small print numbers traditionally used to label the peptide exclude the signal peptide)[49].

Two different pathways for post-translational processing of proglucagon were described: an intestinal pathway wherein PC1/3 cleaves at amino acid positions 69, 78 and 126 for gut peptides, and a pancreatic pathway wherein PC2 cleaves at positions 33 and 61 to generate glucagon[48,310,311]. It however remained a challenge to differentiate glucagon from oxyntomodulin and glicentin, as the entire amino acid sequence of glucagon is present within both oxyntomodulin (including a shared N-terminus) and glicentin, resulting in significant cross-reactivity to classical immuno-assay based methods.

Recently developed sandwich ELISAs for pancreatic glucagon have claimed a low degree of cross-reactivity based on the development of highly specific antibodies. The use of these assays to examine post-prandial plasma glucagon concentrations in patients after metabolic surgery discovered a paradoxical rise in glucagon concentrations, despite hyperglycaemia and elevated GLP-1 concentrations, which would both be expected to significantly inhibit pancreatic alpha cell activity[62].

Mass spectrometry represents a promising avenue for investigation of closely related peptides. As it does not require the use of immunological techniques, and can differentiate peptides with only a single amino acid difference based on mass and charge characteristics, the technique is far more specific than immunoassays. This improved specificity comes at a cost of slightly reduced sensitivity and so a greater lower level of detection, however well-established techniques are able to quantify glucagon concentrations in the range expected after gastrectomy[312].

Several studies have also previously proposed the existence of a novel proglucagon cleavage product glucagon$_{1\text{-}61}$ corresponding to amino acids 21-81 (i.e. the N-terminus of glicentin and C-terminus of glucagon), particularly in people with renal impairment and after total pancreatectomy[306,307]. The same cautions regarding assay cross-reactivity exist for glucagon$_{1\text{-}61}$ as for glucagon. As with oxyntomodulin and glicentin, glucagon$_{1\text{-}61}$ has no clearly identified cognate receptor, however has been demonstrated to have some biological activity at the glucagon receptor[305].

The studies proposing elevated post-prandial pancreatic glucagon secretion in RYGB patients have used sandwich ELISA assays which have been demonstrated to have a low degree of cross-reactivity to physiological concentrations of glicentin and oxyntomodulin but have not been assessed in the presence of high concentrations of these proglucagon products[62].

It was hypothesized that the up to 20 fold elevated plasma GLP-1 concentrations seen post-prandially in RYGB patients compared to controls would be associated with similarly elevated concentrations of glicentin and oxyntomodulin, potentially leading to a false positive result on a glucagon ELISA. As patients undergoing gastrectomy with Roux-en-Y reconstruction have an analogous intestinal reconstruction to those after RYGB, and similar GLP-1 secretory profile, plasma GLP-1, glicentin and oxyntomodulin, and glucagon using two different ELISA protocols and mass spectrometry, were assayed after a 50g OGTT in this group and a group of healthy controls.

7.2 METHODS

Plasma samples (fasting and 30 minutes post OGTT) from a subset of participants described in chapter 3 were further analysed for glucagon, oxyntomodulin, glicentin and glucagon$_{1\text{-}61}$.

Glucagon was measured in DPP4 / aprotinin treated EDTA plasma using a Mercodia sandwich ELISA with two different protocols (Table 7-1). The "standard" protocol was performed as per the manufacturer's instructions. The "specific" protocol was provided by the manufacturer following discussion about the possibility of glicentin / oxyntomodulin cross-reactivity. In summary, the specific protocol added an extra conjugate buffer incubation and wash step following the overnight incubation. Glucagon was also measured in lithium heparin plasma using a commercially developed LC-MS protocol by LGC limited[251].

Glicentin and glucagon$_{1\text{-}61}$ were measured in DPP4 / aprotinin treated EDTA plasma using newly developed sandwich ELISAs provided for evaluation by Mercodia.

Oxyntomodulin was measured in EDTA plasma by LC-MS following acetonitrile precipitation (performed by R Kay). GLP-1 was measured in DPP4 / aprotinin treated EDTA plasma using the Mercodia and Mesoscale Discovery sandwich ELISA kits.

Glucagon assay cross-reactivity to oxyntomodulin and glicentin was measured by applying several concentrations of each peptide to the glucagon assay and processed using both protocols.

Peptide concentrations are presented here in molar rather than mass concentrations to allow direct comparison of assays on a molecule by molecule basis.

Statistical analysis was by paired t-test.

Standard protocol	Specific protocol
Prepare microplate wells for the number of samples and calibrators	Prepare microplate wells for the number of samples and calibrators
Add 100µl Conjugate buffer (plus antibody) to 25µl of sample or calibrator	Add 100µl Conjugate buffer (plus antibody) to 25µl of sample or calibrator
Incubate on a plate shaker (700-900rpm) overnight (18-22hrs) at 2-8^{o}C	Incubate on a plate shaker (700-900rpm) overnight (18-22hrs) at 2-8^{o}C
Wash 6x with 700µl/well Wash buffer 1x solution	Wash 6x with 700µl/well Wash buffer 1x solution
Omit	Add 200µl Conjugate buffer to each well
Omit	Incubate on a plate shaker (700-900rpm) for 1 hour at 2-8^{o}C
Omit	Wash 6x with 700µl/well Wash buffer 1x solution
Add 200µl Substrate TMB to each well	Add 200µl Substrate TMB to each well
Incubate for 15 minutes at room temperature	Incubate for 15 minutes at room temperature
Add 50µl Stop solution to each well	Add 50µl Stop solution to each well
Read optical density at 450nm within 30 minutes	Read optical density at 450nm within 30 minutes

Table 7-1. Glucagon assay protocols.

7.3 RESULTS

Plasma was analysed from ten PTG participants and eight controls (only seven for LC-MS of glucagon due to limited availability of plasma), all previously described in chapter 3.

The subsets included here of control and gastrectomy groups were closely matched for gender, age and waist:hip ratio, but the gastrectomy group had a slightly lower body mass index. All participants had a glycosylated haemoglobin (HbA1c) level within the non-diabetic range, and none were taking regular medication.

7.3.1 Oxyntomodulin, glicentin and standard glucagon concentrations

Using the standard glucagon ELISA protocol, plasma concentration of glucagon appeared to rise in response to glucose ingestion in the PTG group, despite the associated hyperglycaemia described in chapter 3 (Figure 7-1a). Plasma GLP-1 and "standard" glucagon concentrations were highly correlated at the 30 minute time point in the gastrectomy group (Figure 7 1b), an unexpected finding as GLP-1 has been described to inhibit pancreatic glucagon secretion. Plasma glicentin concentrations were highly correlated with measured "standard" glucagon concentrations at the 30 minute timepoint in the gastrectomy group (Figure 7 1c), but not at T0 or in controls, which suggested that the highly elevated glicentin concentrations at this time may have contributed to the "standard" glucagon assay result.

Fasting glicentin concentrations, measured by ELISA, were low in both groups, but markedly elevated in gastrectomy subjects after OGTT, reaching ~400pmol/l at 30 minutes (Figure 7-1d; p=0.00009 vs controls). This was confirmed to be glicentin by separate analysis of the same samples by LC-MS/MS, although the lack of an internal standard means these results can only be presented as relative peak area (Figure 7-1g).

Plasma concentrations of glucagon1-61 were below the detection limit in most fasting samples, and averaged ~5pmol/l at 30 minutes in the gastrectomy group (Figure 7 1e). Plasma oxyntomodulin concentrations were below the lower detection limit (~10pmol/l) in all fasting samples and in all but one sample at 30 minutes in the control group, but averaged ~46 +/- 10pmol/l at 30 minutes in the gastrectomy group (Figure 7-1f).

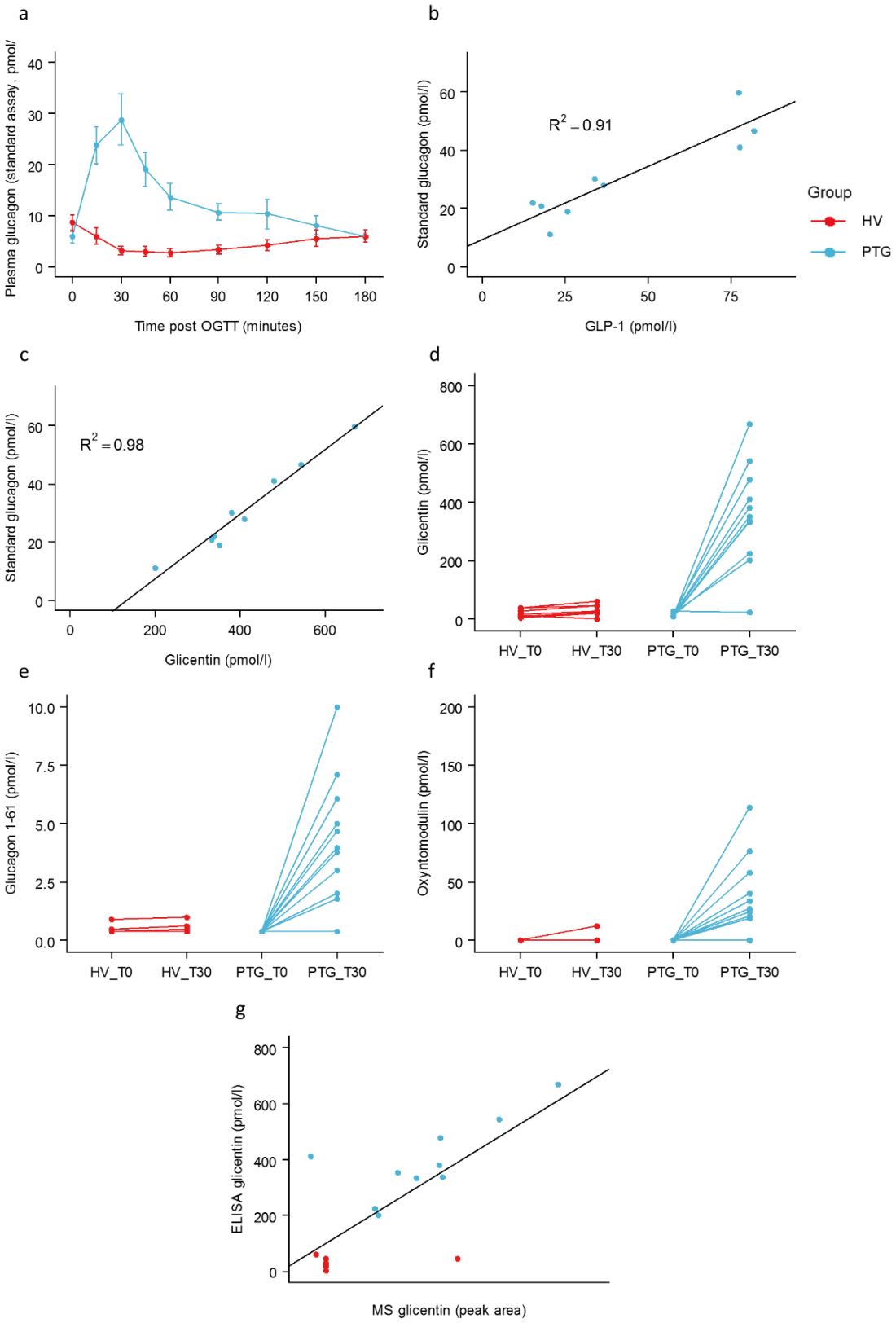

Figure 7-1. Proglucagon peptide concentrations in PTG (blue) and control (red) participants during 50g OGTT. a – plasma glucagon$_{33-61}$ concentration during OGTT as measured by standard assay protocol (mean +/- standard error). b – correlation of GLP-1 and standard glucagon assay results at T30 post-OGTT. c – correlation of glicentin and standard glucagon assay results at T30 post-OGTT. d – plasma glicentin measured by ELISA pre- (T0) and 30 minutes (T30) post OGTT by group. e – plasma glucagon$_{1-61}$ measured by ELISA pre- and 30 minutes post OGTT by group. f – plasma oxyntomodulin measured by LC-MS/MS pre- and 30 minutes post-OGTT by group. g – 30 minute post-OGTT glicentin measured by ELISA (y axis, pmol/l) versus glicentin measured by LC-MS/MS (x axis, peak area).

7.3.2 Glucagon assay cross-reactivity

Using the “standard” glucagon assay protocol, measured cross-reactivity for glicentin and oxyntomodulin was low at concentrations typically found in control subjects, but ~6.6% at the high concentrations found in the gastrectomy group (Table 7-2). No relevant cross-reactivity was observed using the “specific” glucagon assay protocol.

7.3.3 Modified glucagon assay and glucagon LC-MS results

Analysis of 0 and 30 minute samples using a modified glucagon immuno-assay protocol (the “specific” assay) and separately by LC-MS showed a close match for all three assay results in the fasting state, and in control subjects at the 30 minute timepoint. In gastrectomy subjects 30 minutes post-OGTT, both the specific and mass-spec glucagon assays showed that glucagon concentrations fell at 30 minutes in gastrectomy as well as control subjects, contradicting the “standard” glucagon assay result (Figure 7-2).

Comparison of the “standard” glucagon assay result, and the sum of the “specific” glucagon assay result and predicted glicentin and oxyntomodulin cross-reactivity demonstrated that the initially described rise in plasma glucagon at the 30 minute timepoint in the gastrectomy group can be entirely explained by assay cross reactivity, particularly to the highly elevated glicentin concentrations.

Peptide	Peptide concentration, pM	Standard glucagon assay cross-reactivity, %	Specific glucagon assay cross reactivity, %
OXM	226	11	1.7
OXM	22.6	6.6	Not measurable
GLN	295	8.9	Not measurable
GLN	99	3.8	Not measurable
GLN	29.5	Not measurable	Not measurable

Table 7-2 Glucagon assay cross-reactivity. OXM – oxyntomodulin. GLN – glicentin.

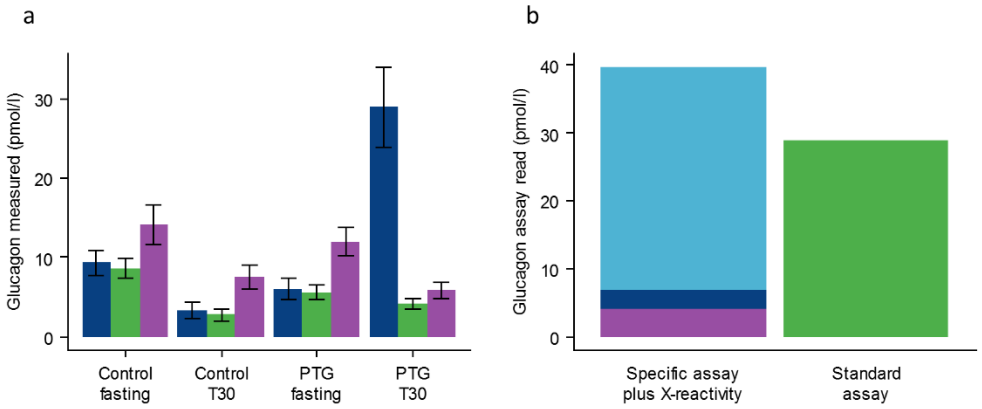

Figure 7-2. Glucagon assay results. a – plasma glucagon measured during OGTT for control and PTG participants (blue – standard assay protocol, green – specific assay protocol, purple – LC-MS/MS). b – glucagon assay cross reactivity in PTG T30 samples only – Standard assay – standard assay result only; Specific assay plus X-reactivity – summed mean specific glucagon assay result (purple), standard glucagon assay read due to cross-reactivity from measured oxyntomodulin (dark blue), standard glucagon assay read due to cross-reactivity from measured glicentin (light blue).

7.4 DISCUSSION

Plasma concentrations of oxyntomodulin, glicentin and GLP-1 correlated well, in keeping with the current model of intestinal handling and secretion of the proglucagon prohormone (i.e. cleavage to GLP-1 / GLP-1 / oxyntomodulin / glicentin). More surprising (but in keeping with recent studies using the same assay in examination of metabolic surgery patients) was the finding that plasma glucagon correlated with GLP-1 and glicentin concentrations following OGTT in the gastrectomy cohort. If correct, this could be another highly interesting humoral factor implicated in the response to foregut bypass, and require a significant shift in the current understanding of glucagon synthesis, secretion and action.

Repeat measurement of plasma glucagon using a more specific assay protocol, and by LC-MS was consistent with the standard assay in the fasting state, and non-operated controls, however these other techniques demonstrated suppression, not stimulation, of plasma glucagon concentrations at the 30 minute timepoint after OGTT in gastrectomy patients.

In order to investigate these discrepancies, the specific and standard glucagon assays were run using selected glicentin and oxyntomodulin standards, within the ranges seen in plasma after gastrectomy. While these findings matched the manufacturer's cross-reactivity data for concentrations seen in non-operated controls, the far greater concentrations of oxyntomodulin and particularly glicentin in gastrectomy patients resulted in assay cross-reactivity approaching 10%.

Using the predicted cross-reactivity measures for each sample, it was shown that the summed specific glucagon, and oxyntomodulin and glicentin cross-reactivities, were not significantly different from the standard glucagon assay read for 30 minute samples in the gastrectomy cohort. Thus, the post-prandial rise in plasma glucagon described in this group can be entirely explained by assay cross-reactivity to previously undescribed rises in glicentin and oxyntomodulin, rather than the more exotic possibility of intestinal glucagon secretion.

The combination of these findings with the current models of pancreatic alpha cell function and intestinal proglucagon cleavage support the hypothesis that recently published findings of post-prandial glucagon secretion after metabolic surgery are likely to be heavily influenced by false positive assay results and should be interpreted with caution.

This study was limited by being performed entirely on lean participants with no history of obesity or diabetes. It is possible that the underlying pathology in the metabolic surgery group could result in altered intestinal proglucagon handling, and so post-prandial glucagon secretion, and it would be sensible to repeat this study in a relevant cohort of obese patients to address this concern. Another approach, described in chapter 10, would be to examine post-operative intestinal mucosal specimens for the presence of the glucagon peptide, or RNA transcripts supporting pancreatic type glucagon synthesis (i.e. PC2).

8 ENTERIC MICROBIAL ACTIVITY AND GUT HORMONE SECRETION

Hypothesis

Small intestinal bacterial overgrowth enhances post-prandial gut hormone secretion.

8.1 INTRODUCTION

While good quality evidence indicates the role of nutrient sensing in EEC secretion and links food ingestion to plasma gut hormone concentrations, the role of the enteric microbiome is less certain. Both the degree of variety within the microbiome and specific microbial taxa have been linked to downstream metabolic functions, including body weight homeostasis and insulin secretion[173,313]. In vitro studies have linked local concentrations of short chain fatty acids (acetate, butyrate, propionate) to gut hormone secretion via FFAR2 and FFAR3[241,242,244,245]. Published in vivo studies in man directly linking the microbiome to circulating gut hormone concentrations are limited, although one well designed randomised controlled study showed no effect of altering the microbiome in humans with antibiotics on a variety of metabolic factors[175].

The altered flow of nutrients and gut hormone secretory profile in patients after oesophago-gastric resection, with high incidence of aberrant small intestinal microbial growth, presented a unique opportunity to study the relationship between bacterial activity and gut hormone secretion in man. First, exhaled hydrogen and methane concentrations were measured during all OGTTs as a surrogate for bacterial metabolism and correlated with gut hormone profiles. Secondly, those post-operative participants with evidence of small intestinal bacterial overgrowth (SIBO, rise in H_2 or CH_4 >20ppm within 60 minutes of the OGTT) were offered treatment and repeat OGTT to assess for changes in glucose handling and gut hormone secretion.

8.2 METHODS

All participants undergoing OGTT as described in chapter 3 had breath hydrogen and methane concentrations measured at the same timepoints at which blood was collected. Participants with symptoms and breath test measures consistent with SIBO were offered a course of antibiotics followed by repeat OGTT within 48 hours of completing treatment.

OGTTs were conducted as described above, with exhaled hydrogen and methane measured at the bedside using a Bedfont Gastrocheck. Antibiotic treatment was with a 7 day oral course of Rifaximin at a dose of 300mg TDS.

Linear mixed effects models were generated to study the association between gut hormone secretion and exhaled H_2/CH_4 concentration in the fasting state and at each timepoint in each participant. Operative group (including controls), age, gender, BMI, timepoint post OGTT (as a factor) and H_2/CH_4 concentration were included as fixed effects. Participant ID was included as a random effect. Associations with plasma concentrations of PYY, GLP-1 and GIP were studied, with the natural log transformed concentration of gut hormones used, as this generated a better fit to the model.

Model formulas were:

Log(Response variable [PYY/GIP/GLP-1]) ~ Operation Group + Age + Gender + BMI + Time + CH_4 + H_2 + (1|ID).

8.3 RESULTS

8.3.1 Demographics

The population for the complete dataset was the same as that in chapter 3. Antibiotics were given to ten post-operative participants (2 after PTG, 4 after TG, 3 after STG and 1 after oesophagectomy).

Gender (M:F)	5:5
Time post operation (years; median, range)	3.2 (0.9-8.7)
Age at OGTT (years)	58+/-19
Weight (kg)	65.2+/-13.5
Height (cm)	170.2+/-11.2
BMI	22.3+/-2.8

Table 8-1. Antibiotic study participant demographics. Mean +/- standard deviation.

8.3.2 Breath test results

There was a rise of >20ppm in H_2 or CH_4 within 45 minutes after OGTT in 13 participants after gastrectomy, 2 after oesophagectomy and 1 healthy volunteer. The individual traces for H_2 concentration following OGTT were highly variable, with some participants evidencing a peak concentration >200ppm, whereas others had no noticeable increase in exhaled H_2 over the course of two hours. Notably, exhaled H_2 increased after OGTT more frequently than CH_4 (which only rose in 1 participant), however baseline exhaled CH_4 was present in most participants, indicated the presence of methanogenic species (i.e. *Archaea*) in these participants.

In the absence of matching intestinal transit data, it was unclear to what extent the high levels of exhaled H_2 and CH_4 indicate SIBO rather than accelerated delivery of nutrients to the caecum. Using a cut off of 45 minutes (based on previously published studies of oro-caecal transit time after gastrectomy) allowed identification of a cohort of participants with potentially treatable SIBO for recruitment to the antibiotic intervention. Results from the whole data set were used to model the relationship between breath H_2 / CH_4 and plasma gut hormone concentrations in the fasting and fed states.

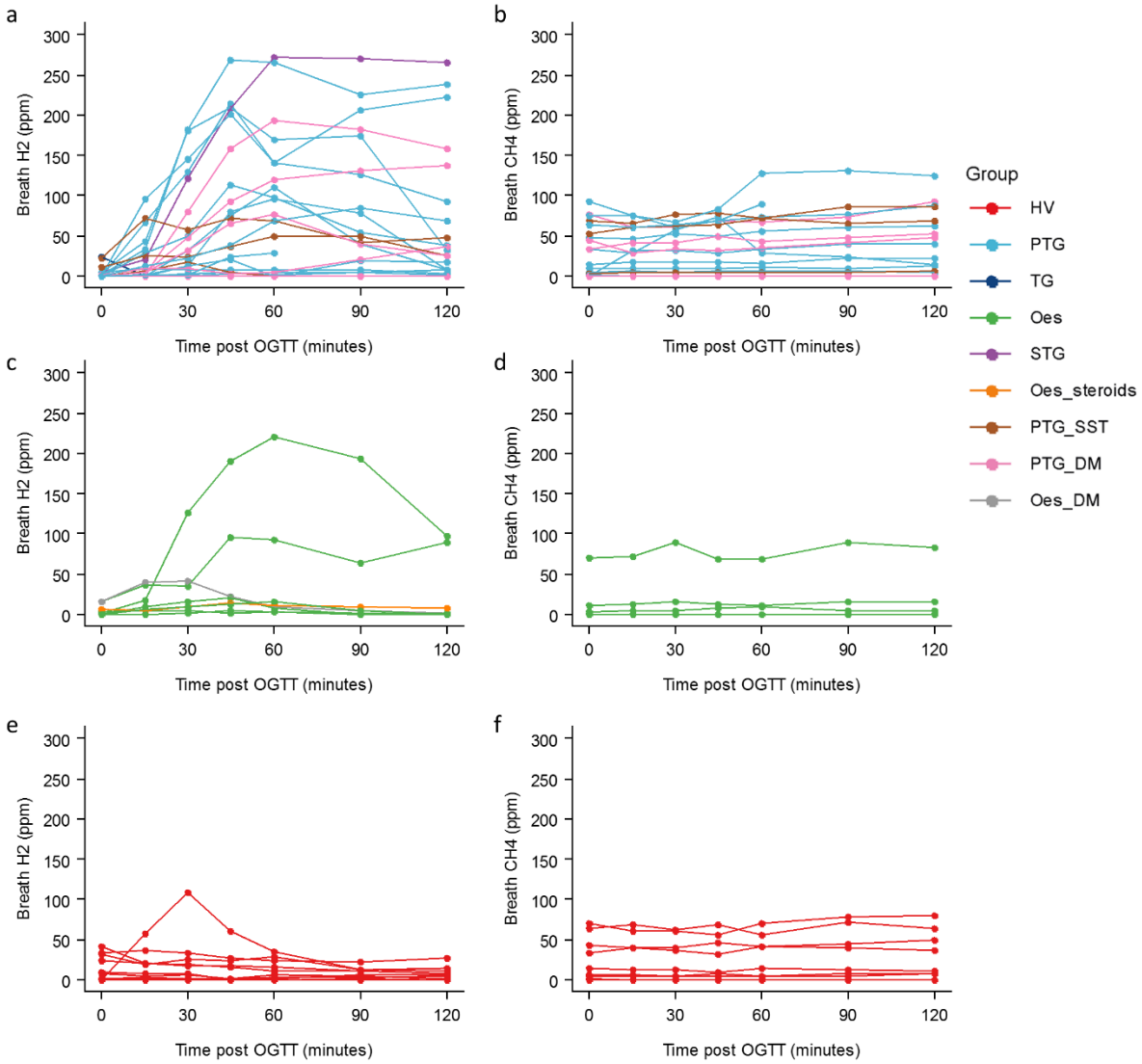

Figure 8-1. Breath test profiles for all participants (first visit only) during 50g OGTT for gastrectomy participants (a,b), oesophagectomy participants (c,d) and healthy volunteers (e,f). Hydrogen (a,c,e) and methane (b,d,f) concentrations in parts per million in exhaled breath.

8.3.3 Fasting gut hormone and breath H_2 and CH_4 correlation

Linear mixed effects models were generated to compare fasting gut hormone concentrations (including all study visits, hence two visits for all antibiotic participants, but allowing for variation due to participant in the model) to the variables described above in the fasting state. All 3 models were adequately fitted when log transformed values were used for gut hormone concentrations. Repeat modelling, by subtraction of factors or use of a generalised model format with log-normal distribution did not improve the predictive power of the models so the initial model was compared to the null hypothesis that the predictive variables did not correlate with the response variable (i.e. plasma GLP-1, PYY or GIP) by multi-way ANOVA (Figure 8-2).

Male gender ($p=0.01$) and fasting breath CH_4 ($p=0.01$) concentration were correlated with fasting plasma PYY concentrations (Table 8-2). None of the tested factors were significantly associated with fasting GIP or GLP-1 concentrations.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	0.74	0.08	9	20.99	0.94	0.51
Age	0.38	0.38	1	17.81	4.37	0.05
Gender	0.71	0.71	1	17.67	8.16	0.01
BMI	0.0034	0.0034	1	26.37	0.04	0.85
CH4	0.62	0.62	1	29.44	7.06	0.01
H2	0.0004	0.0004	1	32.02	0.00	0.95

Table 8-2. ANOVA table for association of the natural log of fasting plasma PYY concentration and operative group, age, gender, BMI and breath test results.

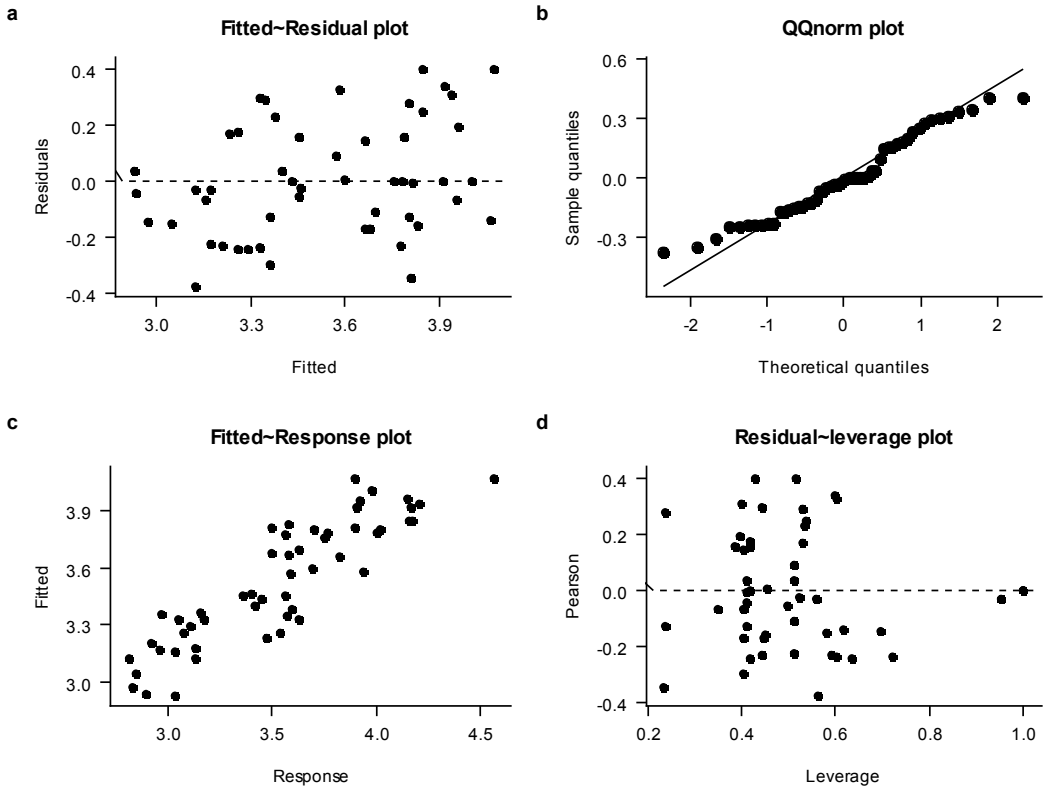

Figure 8-2. Diagnostic plots for linear mixed effects model of log(fasting PYY) predicted by group, age, BMI, gender, CH_4 and H_2 with ID as random effect.

8.3.4 Post-OGTT gut hormone secretion correlated to exhaled hydrogen concentration

Linear mixed effect models were generated to compare plasma gut hormone concentrations to the variables described above across the full time course of the OGTTs. For all 3 models, use of the log transformed gut hormone concentration was a better fit of model than the untransformed values. Using the log-transformed values, the fitted values were well correlated to the actual values for gut hormone concentrations at low values, but were skewed at higher (>200) values. There were no significant outliers on residual-leverage plots of the GLP-1 and PYY models, although several individuals were highly leveraged in the GIP model. Sequential exclusion of highly weighted individuals, and different factors, did not improve the predictive value of the model when compared to the original model by ANOVA. The original model containing all available factors was therefore used to compare to the null hypothesis that breath hydrogen / methane concentrations are not correlated with plasma gut hormone concentration after OGTT. This allowed generation of statistical comparisons, but not a strongly predictive model to predict gut hormone concentrations from the available predictor variables.

Of interest, breath H_2 ($p=6*10^{-12}$) and CH_4 ($p=0.01$), as well as operation group and time post OGTT were strongly associated with log(PYY) concentration (Table 8-3, Figure 8-3).

Operation group, age, timepoint post OGTT and breath H_2 concentration ($p=0.004$) were strongly associated with log(GLP-1) concentration, with breath CH_4 concentration not significant (Table 8-4, Figure 8-4).

Group, time and breath H_2 concentration were significantly associated ($p<0.05$) with log(GIP) concentration, with no association with CH_4 concentration ($p=0.9$), however the GIP model was poorly fitted with multiple highly leveraged data points, rendering this finding less persuasive than for GLP-1 and PYY (Table 8-5, Figure 8-5).

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	5.31	0.59	9	27.60	5.82	0.0002
Age	0.01	0.01	1	25.05	0.06	0.80
Gender	0.11	0.11	1	25.11	1.09	0.31
BMI	0.05	0.05	1	26.78	0.47	0.50
Time	40.57	6.76	6	307.17	66.66	$9*10^{-53}$
CH_4	0.64	0.64	1	246.44	6.32	0.01
H_2	5.20	5.20	1	324.34	51.21	$6*10^{-12}$

Table 8-3. ANOVA table for linear mixed effects model correlating log(PYY) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

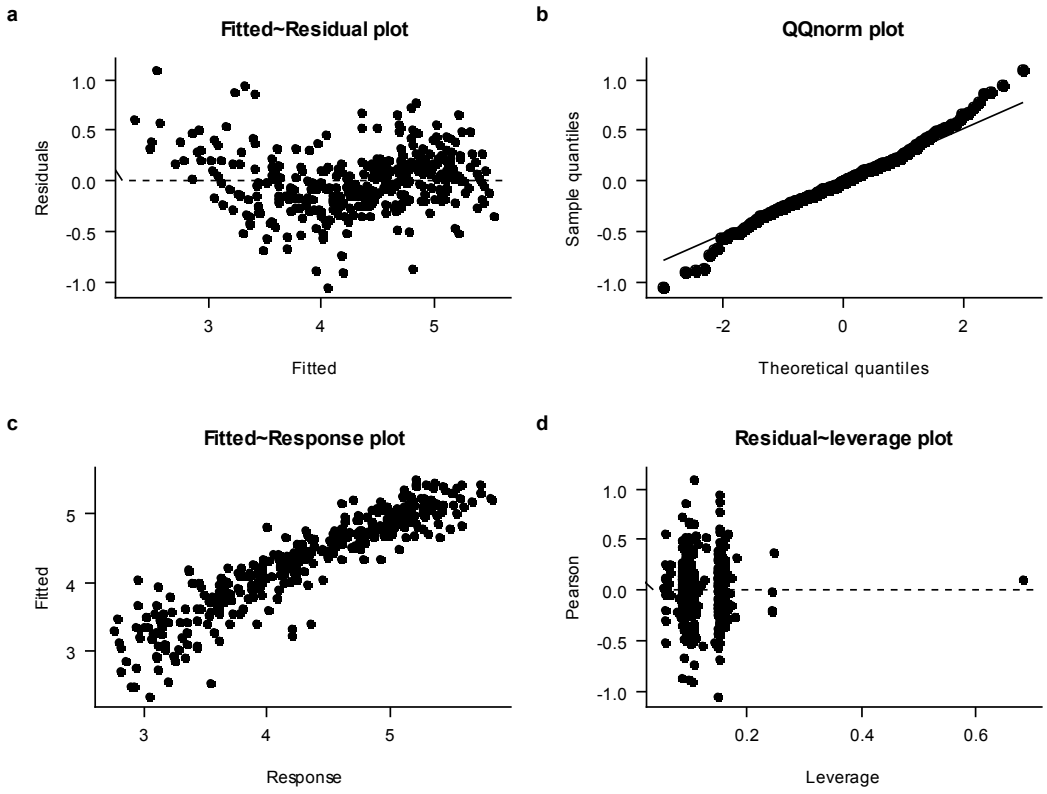

Figure 8-3. Diagnostic plots for linear mixed effects model of log(PYY) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	22.28	2.48	9	29.51	8.23	$5*10^{-6}$
Age	2.82	2.82	1	26.53	9.36	0.01
Gender	0.67	0.67	1	26.03	2.22	0.15
BMI	0.002	0.002	1	28.13	0.01	0.94
Time	262.92	43.82	6	319.90	145.66	$2*10^{-88}$
CH_4	0.75	0.75	1	165.32	2.51	0.12
H_2	2.53	2.53	1	345.74	8.41	0.004

Table 8-4. ANOVA table for linear mixed effects model correlating log(GLP-1) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

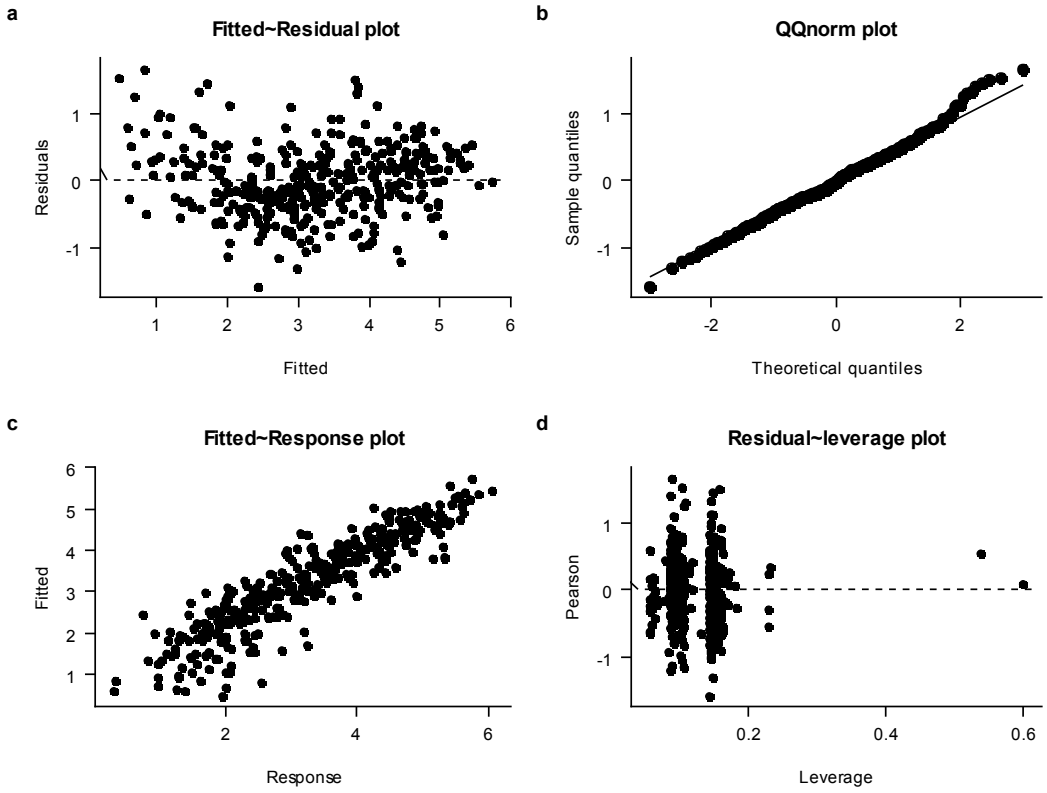

Figure 8-4. Diagnostic plots for linear mixed effects model of log(GLP-1) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
Group	2.12	0.35	6	18.93	2.76	0.04
Age	0.29	0.29	1	17.32	2.23	0.15
Gender	0.0004	0.0004	1	16.89	0.003	0.96
BMI	0.01	0.01	1	17.58	0.09	0.77
Time	69.14	11.52	6	170.42	89.81	$4*10^{-50}$
CH_4	0.0006	0.0006	1	45.89	0.00	0.95
H_2	1.54	1.54	1	174.12	12.02	0.0007

Table 8-5. ANOVA table for linear mixed effects model correlating log(GIP) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

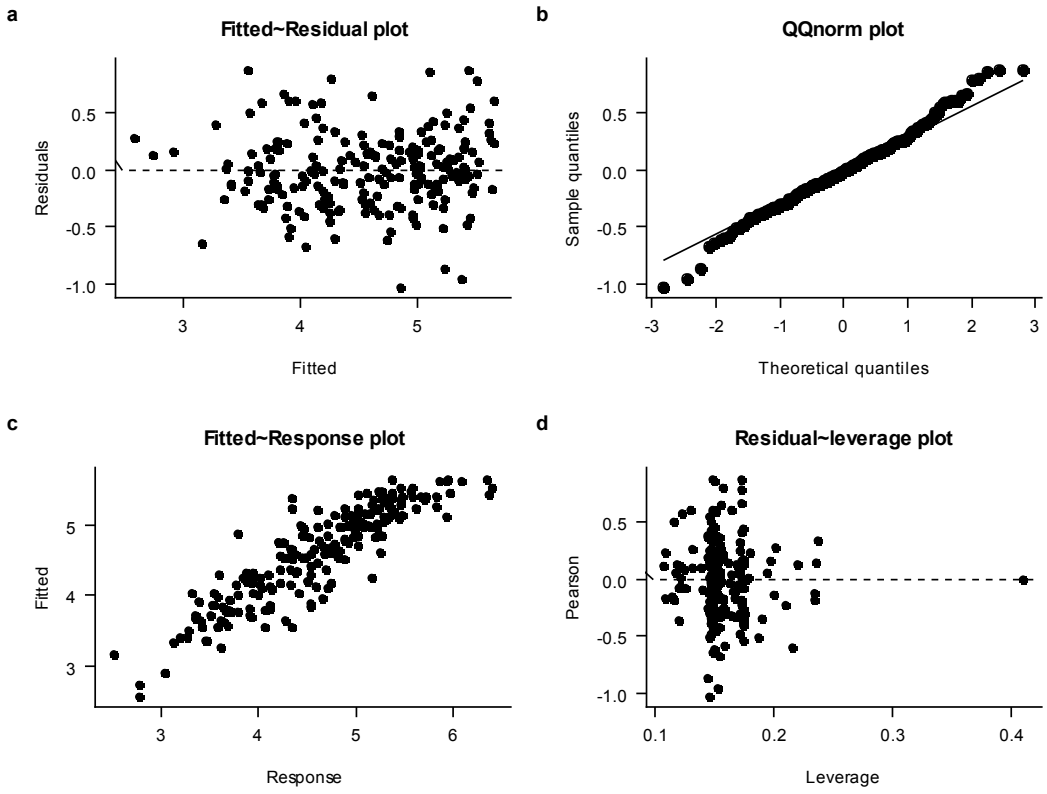

Figure 8-5. Diagnostic plots for linear mixed effects model of log(GIP) concentration during OGTT with group, age, gender, BMI, time, CH_4 and H_2 with ID as random effect.

8.3.5 Results of antibiotic treatment

Ten participants were treated with antibiotics, of whom 4 showed objective evidence of reduction in SIBO on repeat breath testing (Figure 8-6, Figure 8-7). As it is unclear whether complete resolution of SIBO is feasible in this population, and the primary outcome measure of interest was dependent on reduction in small intestinal bacterial activity, objective improvement in SIBO was defined as >25% reduction in the incremental area under the curve for H_2 or CH_4 concentration from pre-OGTT to 45 minutes.

The primary outcome measure of the study, nadir plasma glucose during OGTT, was unchanged after antibiotic treatment for the whole group (p=0.32) or the 4 successfully treated participants (p=0.85) on paired t-test (Figure 8-8).

Gut hormone secretion (GLP-1 and PYY) during OGTT, as measured by IAUC_120, was unchanged by antibiotic treatment in either the whole group, or the treatment successful group (p>0.2).

The relationship between antibiotic treatment and GLP-1 and PYY concentration was further examined by generation of linear mixed effects models with the natural log of the gut hormone as the response variable; age, operation group, visit (pre- or post-antibiotics), gender, BMI, time post-OGTT, treatment success, CH_4 and H_2 as fixed variables; and participant ID as random variable. Neither model identified visit (i.e. whether antibiotics had been taken or not) or antibiotic success as significantly contributing to the prediction of GLP-1 or PYY concentration, and ANOVA of the models against the null hypothesis that visit or antibiotic success were not predictive of gut hormone concentration was not significant (p>0.5 for all measures, Table 8-6Table 8-7). Both models were also poorly fitted to the data, suggesting that the input variables did not adequately explain variation in GLP-1 or PYY concentrations (Figure 8-9, Figure 8-10).

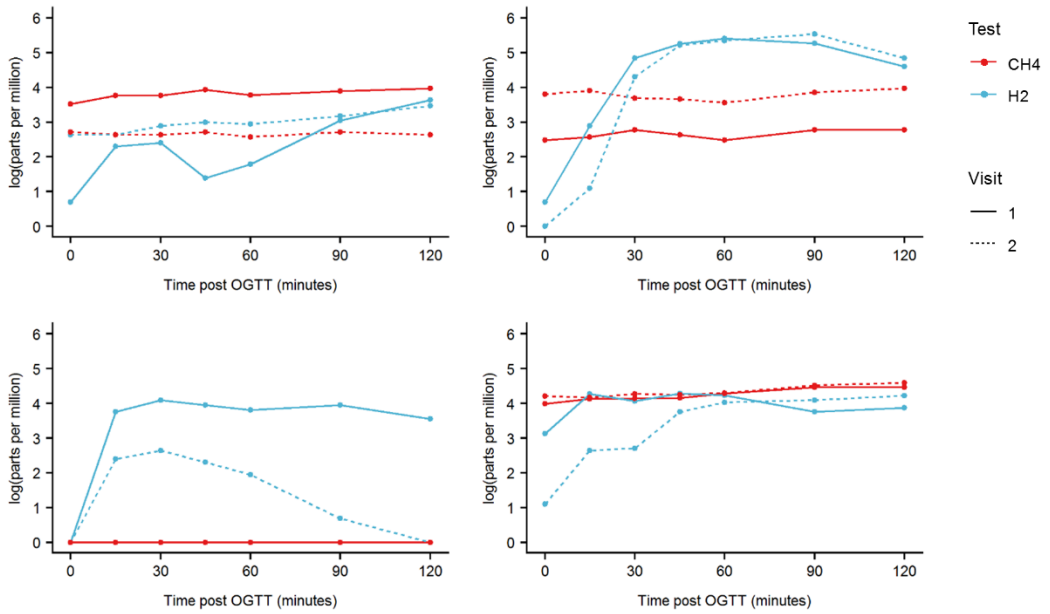

Figure 8-6. Breath test profiles for the 4 participants with successful antibiotic treatment, defined as a reduction of >25% in the 45 minute incremental area under the curve for either H_2 or CH_4. Note that successful treatment reduced but did not abolish the early rise in breath H_2. Visit 1 pre-antibiotics, visit 2 post antibiotics.

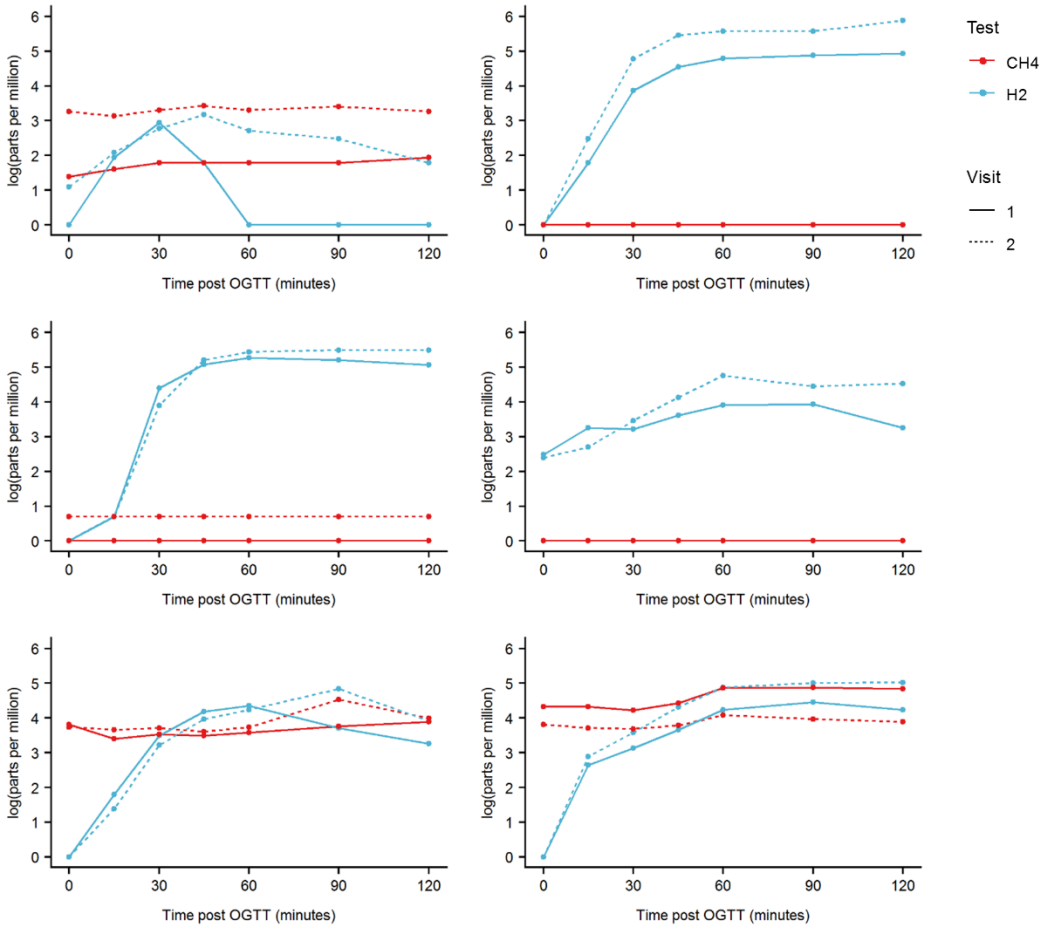

Figure 8-7. Breath test profiles for the 6 participants with unsuccessful antibiotic treatment. Visit 1 pre-antibiotics, visit 2 post antibiotics.

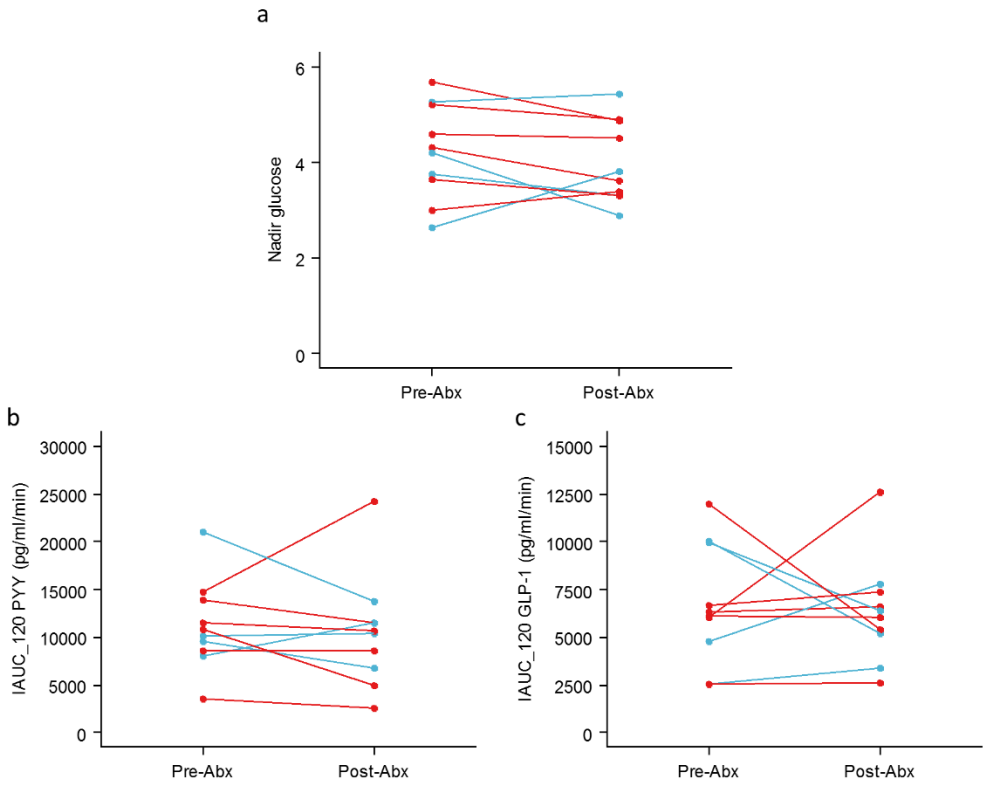

Figure 8-8. Changes in gut hormone and glucose profile during OGTT after successful (blue) and unsuccessful (red) antibiotic treatment. a – nadir glucose (mmol/l). b – incremental area under the curve of PYY. c – incremental area under the curve of GLP-1.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
H2	7.27	7.27	1	124.24	6.11	0.01
Time	9.40	9.40	1	124.78	7.90	0.01
CH4	0.49	0.49	1	20.65	0.41	0.53
Group	3.86	1.29	3	2.00	1.08	0.51
Visit	0.002	0.002	1	123.20	0.002	0.97
Age	3.03	3.03	1	1.91	2.55	0.26
Gender	0.86	0.86	1	2.08	0.73	0.48
BMI	1.39	1.39	1	2.44	1.17	0.38
Rx	0.61	0.61	1	2.11	0.51	0.55

Table 8-6. ANOVA table for linear mixed effects model correlating log(GLP-1) concentration in antibiotic treated participants, during OGTT with group, age, gender, BMI, time, antibiotic treatment, antibiotic treatment success, CH_4 and H_2 with ID as random effect.

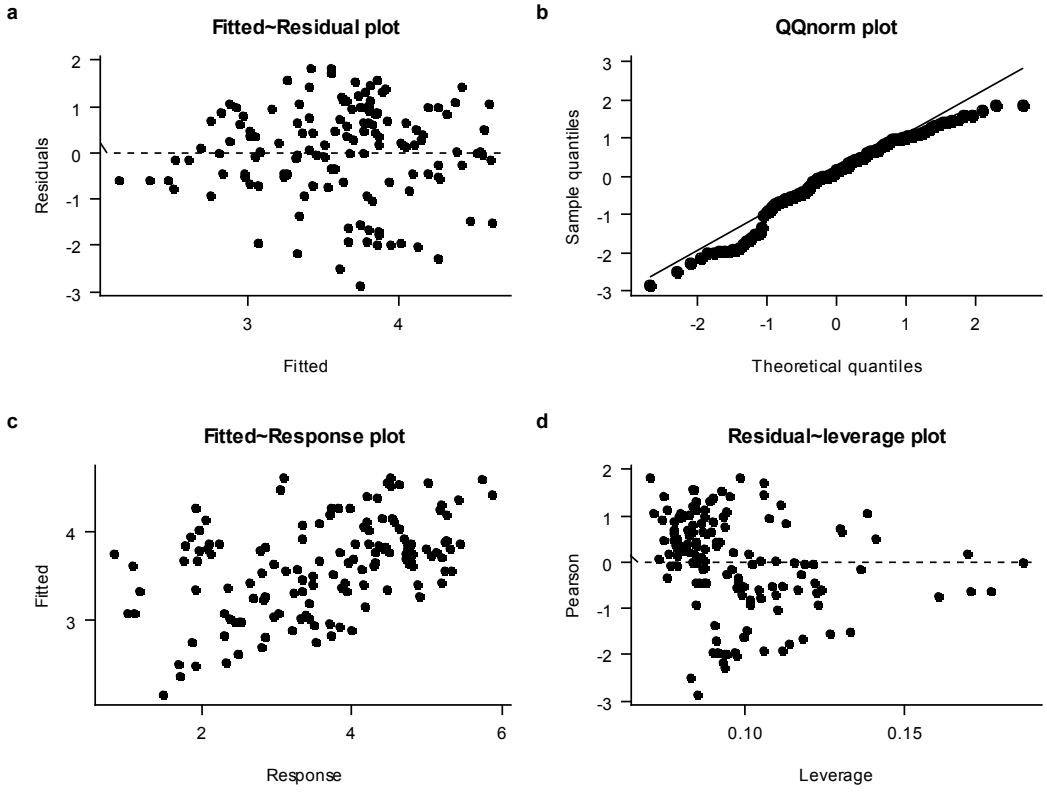

Figure 8-9. Diagnostic plots for linear mixed effects model predicting log(GLP-1) with age, operation group, visit (pre- or post-antibiotics), gender, BMI, time post-OGTT, treatment success, CH_4 and H_2 as fixed variables; and participant ID as random variable. The fitted-response plot is notable for the poor predictive nature of the model, and the residual-leverage plot shows multiple data-points are highly leveraged with large residuals, signifying a poorly predictive model.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	P value
H2	5.54	5.54	1	125.00	18.13	0.00004
Time	0.18	0.18	1	125.00	0.60	0.44
CH4	0.21	0.21	1	125.00	0.70	0.41
Group	1.60	0.53	3	125.00	1.75	0.16
Visit	0.03	0.03	1	125.00	0.10	0.75
Age	1.05	1.05	1	125.00	3.45	0.07
Gender	4.95	4.95	1	125.00	16.21	0.00
BMI	0.92	0.92	1	125.00	3.00	0.09
Rx	0.01	0.01	1	125.00	0.05	0.83

Table 8-7. ANOVA table for linear mixed effects model correlating log(PYY) concentration in antibiotic treated participants, during OGTT with group, age, gender, BMI, time, antibiotic treatment, antibiotic treatment success, CH_4 and H_2 with ID as random effect.

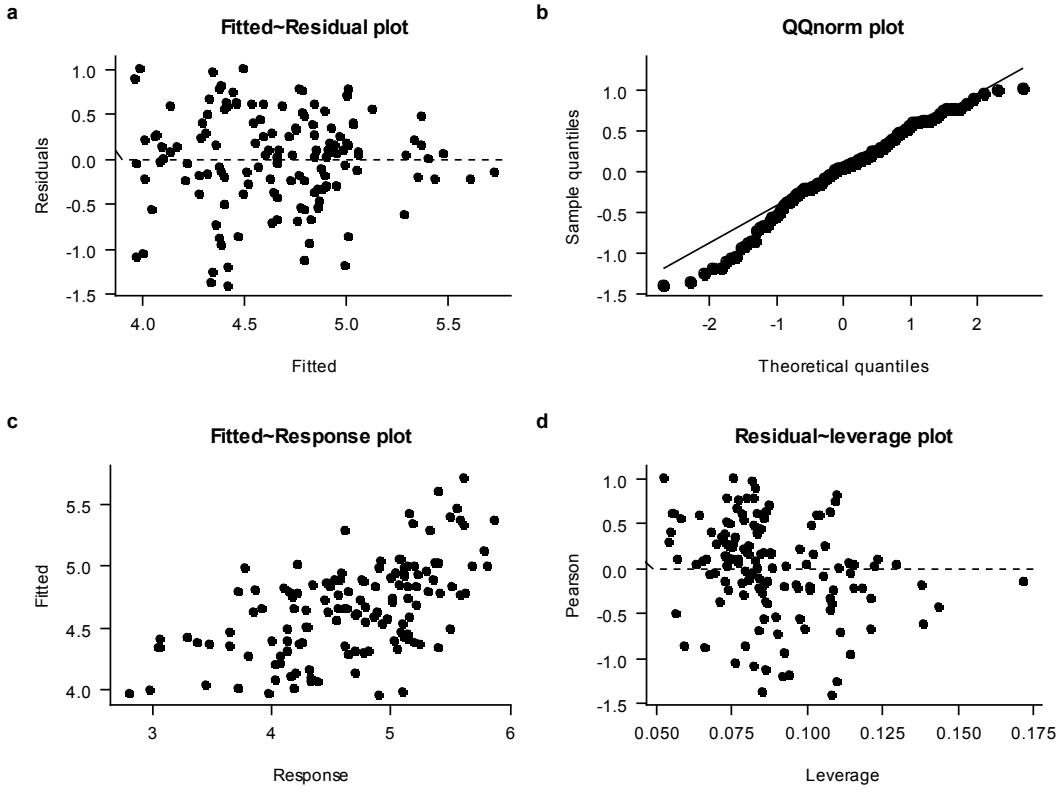

Figure 8-10. Diagnostic plots for linear mixed effects model predicting log(PYY) with age, operation group, visit (pre- or post-antibiotics), gender, BMI, time post-OGTT, treatment success, CH_4 and H_2 as fixed variables; and participant ID as random variable. The fitted-response plot is notable for the poor predictive nature of the model, and the residual-leverage plot shows multiple data-points are highly leveraged with large residuals, signifying a poorly predictive model.

8.4 DISCUSSION

This dataset provides an interesting indication of the possible relationship between enteric microbial activity and gut hormone secretion. The correlation of surrogate markers of microbial activity and plasma gut hormone concentrations, even allowing for the effects of operative group and time post OGTT, indicates a possible link between the enteric microbiome and enteroendocrine cell activity.

This link appeared stronger for PYY than GIP or GLP-1, in both the fasting and fed state, which fits with published data on the relationship between microbial fermentation by-products (short chain fatty acids [SCFAs]) and PYY secretion[241]. Murine in vitro and in vivo studies have demonstrated increased colonic PYY cell numbers, and fasting plasma PYY concentration, after treatments to enhance SCFA signalling (in vivo, no absorbable fermentable carbohydrate, in vitro FFAR2/3 agonists), with results supported by specific agonists and receptor knockout mice[241,242,244,245].

The fact that a correlation between PYY and CH_4 was apparent in the fasting samples was the most striking, as this was definitely not due to nutrient stimulus of colonic mucosa at the same time as feeding the microbiome, and suggests that the previously described mechanisms by which bacterial metabolites stimulate EECs in vitro are active in vivo.

Disappointingly, it proved difficult to recruit to the antibiotic study, with the majority of participants not meeting diagnostic criteria for SIBO and so not eligible, or not willing to have a repeat OGTT due to symptoms during the first OGTT. Treatment efficacy was also poor, with only 4 out of 10 participants having any notable reduction in early breath test readings, and only one having objective resolution of SIBO. The numbers in this study are therefore limited, and no statistically significant differences were discovered, examining either the whole set of 10 participants, or the subset of 4 with successful reduction of SIBO. Indeed, the primary outcome measure (nadir glucose during OGTT) and gut hormone secretion during OGTT (by IAUC_120 or linear mixed effects model) varied independently of antibiotic treatment or efficacy. Examining the raw data suggests that further development of this study is unlikely to be fruitful, and a different approach to assessing the role of the microbiome on gut hormone secretion will be necessary. Future studies could build on previously conducted experiments

wherein gut microbiota were manipulated with antibiotics or faecal transplantation, in tandem with improved understanding of the functional microbiome arising from novel sequencing techniques[175,314]. In practice, the effect size is likely to be minimal compared to the highly elevated gut hormone secretion arising due to accelerated intestinal transit after surgery. While any role played by small intestinal bacteria in gut hormone secretion in the post-gastrectomy population is likely to be academically interesting, it is unlikely to play a significant metabolic role above and beyond that described in chapter 3.

This study provides observational, correlative evidence of a link between plasma PYY, GLP-1 and GIP concentrations and exhaled hydrogen. The numerical association between measured breath H_2/CH_4 and intestinal SCFA concentration has not been described and introduces a significant degree of uncertainty to these results. Indeed, the pathway utilised by *Archaea sp.* to produce CH_4 uses H_2 as a substrate, which may further confound our findings in participants with significant CH_4 production. Thus, while pathways linking enteric SCFA and hence bacterial activity to gut hormone secretion support these findings, this study can only provide a limited forwards step in this field.

One particular challenge of using breath tests in patients after upper GI resection is accelerated intestinal transit. It is unclear how rapidly ingested glucose reaches the caecum in this patient group, with some small studies proposing that it occurs in under 1 hour, while others argue that all glucose should be absorbed in the small intestine. This has implications for the interpretation of breath test results, and in this study a rise in exhaled H_2/CH_4 concentration any later than 45 minutes was regarded as a false positive. While this has implications for the antibiotic intervention, it remains of interest for the modelled dataset of gut hormone secretion vs H_2/CH_4, as in practice the majority of bacterial activity is colonic. Thus, whilst this study does not demonstrate any particular difference in gut hormone secretion due to SIBO, it does link whole gut microbial activity to plasma PYY and GLP-1 concentration.

From a practical point of view, SIBO is an underdiagnosed condition in the post-operative groups studied here. The exact mechanisms linking symptoms to small intestinal bacterial load are poorly understood, however could theoretically include altered gut hormone secretion.

The dichotomy of these results – that CH_4 and H_2 concentration (and by association enteric microbial activity) were linked to plasma gut hormone concentrations, yet reduction in CH_4 and H_2 concentration by treatment of SIBO did not alter gut hormone concentrations – is difficult to explain. Two possibilities are that the association of breath test results and gut hormone concentrations was a false positive related to the correlation of both gut hormones and breath tests with time (although unlikely given the fasting model results) or that the antibiotic study was underpowered to detect a difference. Given the small effect of antibiotic treatment on breath test measures, it is likely this study was underpowered, and a future project involving treatment of SIBO in a far larger population, possibly using a more effective antibiotic regimen, could potentially build on these findings.

In summary, plasma PYY concentration has been correlated to a surrogate marker of enteric microbial activity by linear mixed effects modelling of the response to an OGTT. Intervention with antibiotics to treat SIBO did not identify any subsequent change in gut hormone or glucose homeostasis, however the study as a whole suggests that current literature linking SCFAs to gut hormone secretion is likely correct.

9 TRANSCRIPTOMIC AND PEPTIDOMIC PROFILING OF JEJUNAL EECS

Hypothesis

Purification of human EECs, and novel peptidomic analysis of mucosal homogenates, will identify novel receptors and secreted peptides. Human and murine enteroendocrine profiles are highly analogous at the cellular level.

9.1 INTRODUCTION

The gut hormone system is a rapidly developing field, with significant translational potential as demonstrated by the various GLP-1 based therapies for diabetes and obesity. A better understanding of the pathophysiological role of gut hormone secretion would be of interest in both gastrointestinal and metabolic disorders.

Laboratory investigation of EECs is challenging, as they represent only ~1% of intestinal epithelial cells and are therefore difficult to isolate and enrich. Several transgenic mouse models have been developed in which fluorescent marker expression is dependent on endocrine lineage genes, allowing identification and purification of EECs by fluorescence microscopy and flow cytometry. There are no highly effective techniques for identifying human EECs, so the study of EEC function at the cellular level (e.g. electrophysiology, calcium imaging, transcriptomics) is heavily reliant on animal models.

Detailed examination of the human EEC transcriptome and peptidome is of interest for two reasons. Firstly, it is essential to validate the various mouse models against human data, to ensure that the findings will be transferable. Secondly, even one or two receptors or peptides of relevance in humans but not identified in mice or vice versa could be of significant physiological and therapeutic relevance.

An in depth examination of human EECs at the transcriptomic and peptidomic level could identify key nutrient sensing and secretory pathways, and potentially identify novel hormones. In this study, human EECs were purified by antibody staining a fixed single cell suspension of human jejunal mucosa and fluorescent activated cell sorting, with RNA then extracted for high throughput sequencing. Peptides were also extracted from homogenates of human intestinal mucosa to identify the patterns of peptide concentration throughout the human gut. Comparison of these data with matched murine studies was conducted to validate currently used animal model of EEC function.

9.2 METHODS

The transcriptomic profiling of human EECs was a collaborative project with Paul Richards and Raphael Scharfmann at the Institut Cochin, Paris. Human tissue samples collected in Cambridge were processed entirely by me. RNA from 8 tissue samples collected in Paris, each digested and sorted into 3 cell populations, was transported to Cambridge for creation of the cDNA library and sequencing. For the French samples, specimens were processed to the point of extraction of RNA in Paris, with creation of the cDNA library and sequencing analysis performed by me in Cambridge, supported by P Larraufie and B Lam.

Mouse RNAseq data was provided by P Larraufie, and I performed the interspecies analysis.

Mouse peptidomics was performed by P Larraufie, and generation of the interspecies peptidomic comparisons was in collaboration with P Larraufie.

Human small intestinal specimens from Paris and Cambridge were collected from the operating theatre and processed as in chapter 2.13. Primary antibody staining was with 1% v/v goat anti-GLP-1 (Santa Cruz, Dallas, TX, USA; sc7782), 2% v/v rabbit anti-CHGA (Abcam, Cambridge, UK; Ab15160) and 0.25% v/v rabbit anti-SCG2 (Abcam, Ab12241).

Peptide extraction, mass spectrometry and peptidomic analysis was performed as in chapter 2.14.

9.2.1 Attempts to FACS purify unfixed human jejunal mucosa

The protocol described above requires the cells to be fixed in 4% PFA prior to permeabilisation, antibody staining and FACS purification. The PFA step, while theoretically essential for the preservation of cell architecture following permeabilisation of the plasma membrane, results in cross-linked and degraded proteins and nucleic acids. This reduces the sensitivity and specificity of downstream high-throughput sequencing, and renders LC-MS/MS peptidomics impossible. Two approaches to purifying human EECs without PFA were therefore trialled. One further approach, using ethanol and methanol as fixatives rather than PFA, was trialled by my predecessor in the group without success.

9.2.1.1 Permeabilisation without fixation

The protocol above was followed, with the omission of the PFA step. This has been described in a similar study wherein myocytes were transiently permeabilised for addition of peptides to the cytoplasm and shown to recover after removal of saponin from the culture medium[315]. In my study, following addition of saponin, the cells clumped into a single pellet, rather than a suspension, and it was impossible to generate a single cell suspension for FACS. This was attempted twice then abandoned as an approach.

9.2.1.2 Primary antibody staining against cell surface epitopes

A more promising approach would be to identify highly and uniquely expressed cell surface epitopes on EECs. Examination of the RNAseq data set (described below) identified two promising markers (GPR142 and CDHR3) which were highly enriched in EECs and for which validated antibodies were available to their cell surface epitopes.

These antibodies were trialled in three human jejunum specimens. Samples were digested to single cells using EDTA / trypsin (serial 5 minutes incubations in 30mM EDTA / 1mM DTT and subsequent incubation in trypsin for 10 minutes) or collagenase as above and analysed in parallel. Antibody staining was in 10% v/v fetal bovine serum (FBS), 0.05% v/v Y-27632 Rock inhibitor (Sigma) in HBSS (#9394 from Sigma). Primary antibody concentrations for GPR142 (Ab170329, Abcam, rabbit) and CDHR3 (sc240463, Santa Cruz, goat) ranging from 1:10 to 1:500 were used. Secondary antibody staining was with 1:500 v/v donkey anti-rabbit Alexa 555 and donkey anti-goat Alexa 555. The collagenase digests were divided between warm (37^{o}C, 15 minutes per incubation) and cold (4^{o}C, 60 minutes per incubation) protocols. The EDTA digests were performed at 4^{o}C. Samples were sorted on a FACS Aria III. Primary (goat and rabbit IgG plus secondary antibodies) and secondary (secondary antibodies only) controls were performed.

The EDTA digest resulted in no positive signal on flow cytometry. The collagenase / warm antibody protocol resulted in a significant false positive read on flow cytometry, and no clear true positive. The collagenase / cold antibody protocol, once optimised, produced a positive population in the 555

channel, of approximately 1% of parent cells as would be expected for EECs. These cells, and a negative comparator population of 20,000 cells, were collected in buffer RLT+ and beta-mercaptoethanol and the RNA extracted using an RNeasy Micro Plus kit. qPCR using probes to GCG, GIP, CHGA, SCG2 and CCK, with beta actin as control, did not reveal any difference between the positive and negative RNA from this sort. As the fixed methodology was working well and generating RNA of adequate quality for high-throughput sequencing, this pilot study was halted.

9.3 RESULTS

9.3.1 Tissue and RNA quality

Tissue was obtained from 11 human participants recruited from sites in Cambridge and Paris (Table 9-1). Jejunum samples were collected from all participants, and paired ileum and jejunum were obtained from 2 transplant donors. Tissue pieces were digested, paraformaldehyde (PFA)-fixed and stained for Chromogranin A (CHGA) and Secretogranin 2 (SCG2) as general markers for EECs, and for GLP-1 as a marker of the EEC-subpopulation known as L-cells. By flow cytometry (Figure 9-1a,b,c), pooled cell populations were collected that were (i) positive for CHGA, SCG2 and GLP-1 (henceforth named GCG+), (ii) positive for CHGA and SCG2 but negative for GLP-1 (henceforth named GCG-), and (iii) negative for all 3 markers (i.e. non-endocrine lineage cells, henceforth named negative). The GCG+ (L-cell) population represented ~0.2% of all single cells examined, and the ratio of GCG+ to GCG- cells was ~1:5.

For the cross-species comparison, unfixed murine EEC populations from the upper small intestine of the mouse strain GLU-Venus (n=3) to identify Gcg-expressing L-cells (Figure 9-1e,f,g), and of NeuroD1-Cre/EYFP mice (n=3) to identify the total EEC population (Figure 9-1i,j,k) were collected. NeuroD1 is a well characterised EEC transcription factor found in the same cells as Chga in a previous single cell analysis of murine EECs[316]. GLU-Venus positive cells represented ~0.2% of singlets, and NeuroD1 positive cells ~0.6% of singlets.

RNA extracted from the purified fixed human cell populations had RIN values of only 2-3, with most RNA fragments being 25-500 bases in length (Figure 9-1d), but it was hypothesised that the short fragments might be sufficient for RNA sequencing using random primers. After RNA sequencing there were high proportions of unmatched reads (up to 50% in some samples), likely due to the PFA modified input RNA, but in all cases sequencing was achieved to a depth of at least 8.5×10^5 matched reads per sample. RNAs from murine cell populations purified without fixation had RIN values of >7 (Figure 9-1h,l) and were sequenced to $>5 \times 10^6$ aligned reads per sample.

ID	Source	Age	Gender	BMI
R3	France – RYGB	N/A	N/A	>40
R4	France – RYGB	24	F	47
R5	France – RYGB	31	F	43
R6	France – RYGB	46	M	41
D1	France – donor	27	M	24
D2	France – donor	26	F	21
HGH006	UK – TG	57	M	34
HGH007	UK – TG	22	M	29
HGH008	UK – TG	23	M	28
HGH010	UK – TG	64	M	27
HGH018	UK – TG	61	M	37

Table 9-1. Demographics of tissue donors for RNAseq samples.

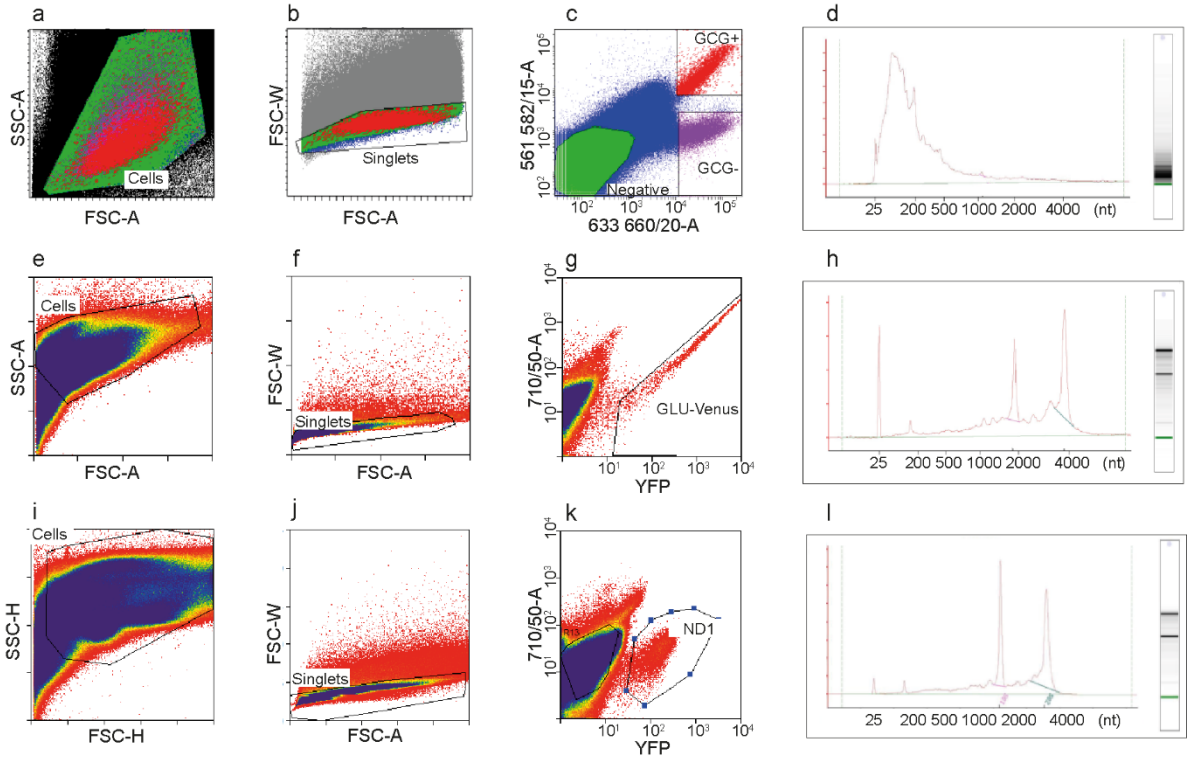

Figure 9-1. (a,b,c) FACS sorting of human GCG+, GCG- and negative cells. Cells were sorted by side scatter (SSC), forward scatter (FSC-A) and pulse width (FSC-W) to collect single cells. CHGA/SCG2 staining was detected by 633nm excitation, 660/20nm emission; GLP-1 staining was detected by 561nm excitation, 582/15nm emission. (e,f,g) FACS sort of murine GLU-Venus cells using gating as labelled. (i,j,k) FACS sort of murine NeuroD1-Cre/ROSA26-YFP cells using gating as labelled. (d,h,l) Agilent Bioanalyzer 2100 trace of RNA quality from human GCG+ (d) and murine GLU-Venus (h) and NeuroD1 (l) cell populations.

9.3.2 Transcriptomics of human EEC populations

9.3.2.1 Whole transcriptome analysis

Individual RNA sequencing data were collected from GCG+, GCG- and negative cell populations from each of 11 human jejunum samples and 2 human ileum samples. Principle component analysis (PCA) of the top 500 differentially expressed genes across all samples showed separation of the EECs (GCG+ and GCG-) from negative cells on the first component, and of GCG+ cells from GCG- cells on the second component (Figure 9-2a). Pairwise analysis of key genes differentiating the cell populations from jejunum was performed using a DESEQ2 model, and the normalised results for the 500 most differentially expressed transcripts are presented in a heatmap (Figure 9-2b) which displays clear transcriptomic differences between the GCG+, GCG- and negative cells. PCA did not demonstrate clustering of samples by BMI of the donor.

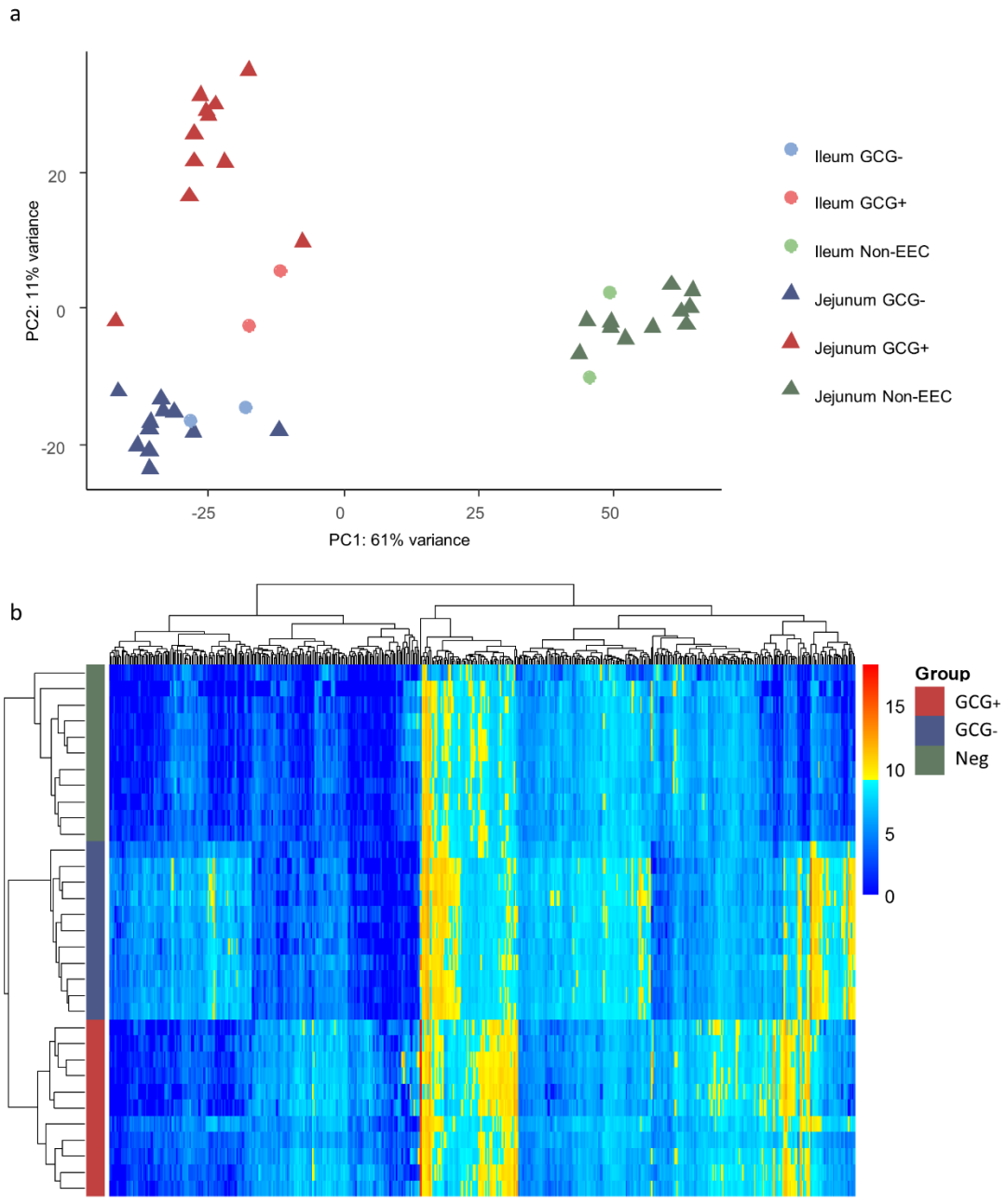

Figure 9-2. Transcriptomic distinction between cell populations from human small intestine. (a) Principal component analysis plot of all human samples (n=3 cell populations from each of 11 jejunal and 2 ileal tissue samples), differentiated by cell population and anatomical site. (b) Pairwise analysis of key genes differentiating the cell populations from jejunum was performed using a DESEQ2 model, and the normalised results for the 500 most differentially expressed genes are presented in a heatmap (n=3 cell populations per each of 11 participants), y axis – cell population identified by coloured bar, x axis – genes.

9.3.2.2 Gene subgroup analysis

Both human EEC populations expressed a wide range of hormonal transcripts (Figure 9-3a,c,e). As expected from the use of GLP-1 antibodies to purify GCG+ cells, the hormonal transcript showing the strongest differential expression in GCG+ vs GCG- samples was *GCG* itself (Figure 9-3c). Consistent with previous findings in mice, a range of additional hormonal transcripts was identified in human GCG+ samples, including *GIP* (glucose-dependent insulinotropic polypeptide), *CCK* (cholecystokinin), *NTS* (neurotensin), *PYY* and *SCT* (secretin) as well as *MLN* (motilin) – a hormone produced by human but not mouse[33,317-320]. Compared with GCG+ cells, GCG- cells had higher expression of *SCT*, *CCK, NTS*, *MLN*, *GHRL* (ghrelin) and *SST* (somatostatin), together with *TPH1*, the enzyme responsible for serotonin biosynthesis in enterochromaffin cells. EECs also expressed the putative gut hormones *UCN3* (urocortin 3), *PCSK1N* (ProSAAS) and *NPW* (neuropeptide W), as well as lower levels of RNAs encoding several peptides not classically described as gut hormones such as *VGF*, *GHRH* and *ADM*.

Transcripts of ~50 GPCRs were either enriched ($p<0.1$) over the negative population, or expressed at > 100 CPM in one or both EEC populations (Figure 9-3b,d,f). Multiple GPCRs previously implicated in post-prandial gut hormone secretion in mice were highly expressed in human EECs compared with negative cells, including the fat-sensing receptors *FFAR1*, *FFAR2*, *FFAR3*, *FFAR4* and *GPR119*, the amino acid sensing receptors *CASR* and *GPR142*, the butyrate and isovalerate sensing *OR51E1*[141], and the bile acid receptor, *GPBAR1*, as well as the zinc receptor *GPR39*, and the alpha-ketoglutarate receptor *OXGR1*. Human EECs differentially expressed a number of hormonal receptors compared with negative cells, including *SSTR1*, *SSTR2*, *SSTR5*, *GIPR*, *AVPR1B*, *OXTR*, *CRHR2*, *RXFP4*, *GLP1R*, *NPY1R*, *CALCR*, *GALR1* and *SCTR*. Two opioid receptors, *OPRK1* and *OPRD1*, not previously described as active in the gastrointestinal tract were identified in EECs, alongside the oestrogen receptor *GPER1* and prostaglandin receptors *PTGDR2* and *PTGER3*. At least four orphan GPCRs were differentially expressed in human EECs: *GPR148*, *GPR160*, *GPR173* and *GPR179*, hinting to as yet undescribed pathways that may control gut hormone secretion.

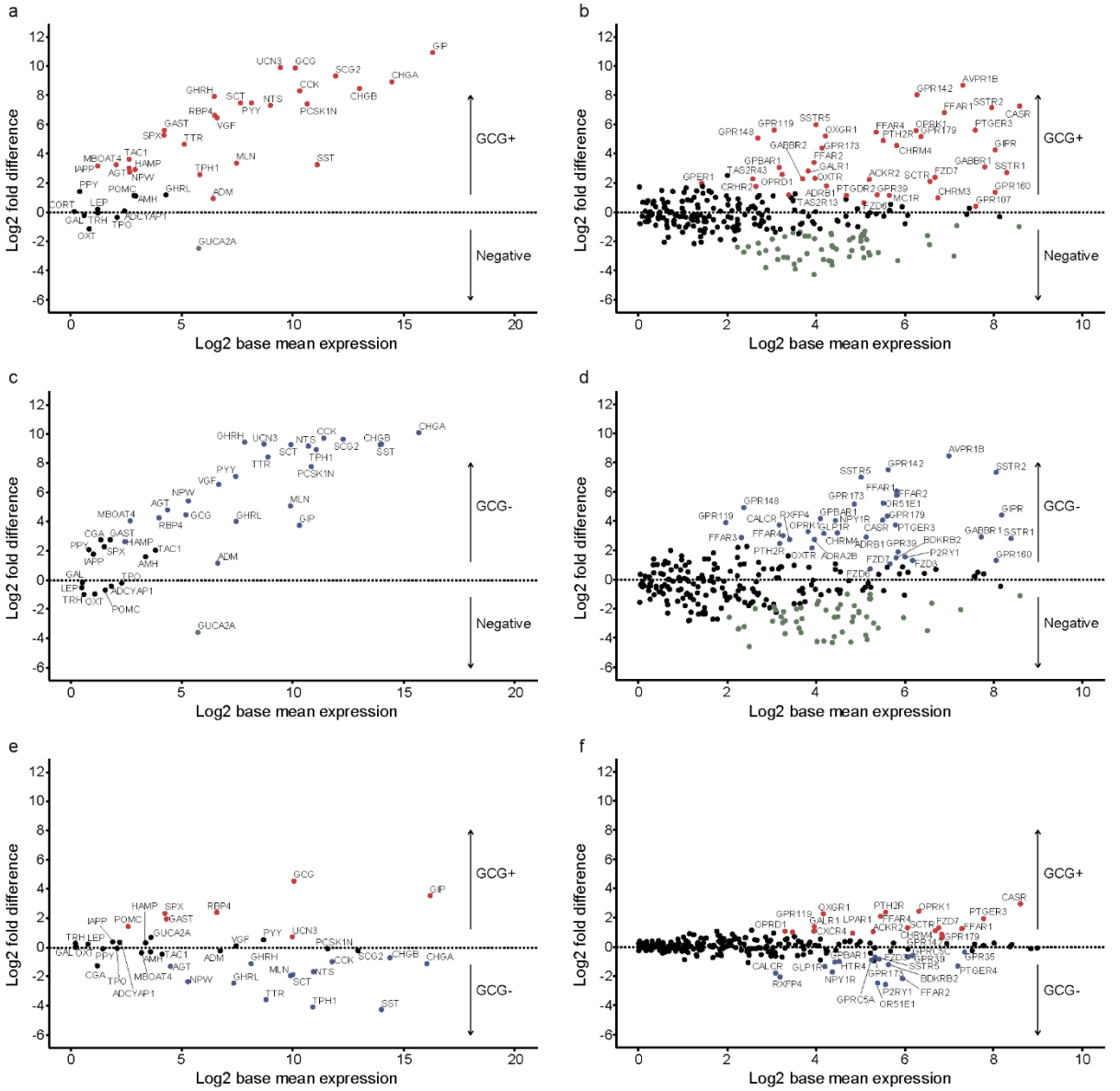

Figure 9-3. Transcripts enriched in human EECs. Enrichment vs expression plots for human jejunum EEC populations. Enrichment is presented as the log2 fold difference between the cell populations indicated, and expression is presented as the log2 base mean normalised expression extracted from the DESEQ2 model. (a,b) GCG+ vs negative, (c,d) GCG- vs negative, (e,f) GCG+ vs GCG-. Hormones and granins are shown in a,c,e; receptors and ion channels are shown in b,d,f. Red – enriched (adjusted P≤0.1 in DESEQ2 model) in GCG+; Blue – enriched in GCG-; Green – enriched in negative cells.

Known endocrine transcription factors highly enriched (> 16x) in both EEC populations versus the negative cell population included *INSM1, RFX6, NEUROD1, ISL1, PAX6, ARX, NKX2.2, BEX1, FEV, MYT1, ETV1, FOXA2, ST18* and *ARNT2* (Figure 9-4a,c,e). Transcription factors without previously known endocrine roles, which were enriched >8x in both EEC populations, included *TSHZ2, RFX2, ONECUT3, APBB1* and *DUSP26*.

Transcription factors distinguishing the EEC populations and enriched >4x in GCG+ vs GCG- cells included: *L3MBTL4* and *HLF,* not previously known to play endocrine roles, *PLAGL1* and *TCF4*[321,322], which have known roles in pancreatic development, and *POU5F1*[323], an early developmental gene. Those enriched >4x in the GCG- population included: *PAX4*[324], a known EEC transcription factor; pancreatic factors *HHEX, MNX1, FOXQ1* and *NR4A3*[325-329]; the EEC factor *NEUROG3*[42]; and *HR* for which there is no known endocrine role.

Transcripts for a range of ion channel subunits were enriched in human EECs (Figure 9-4b,d,f), consistent with previous reports that murine L-cells and enterochromaffin cells are electrically active. Human GCG+ and GCG- EECs were particularly enriched for the voltage-gated calcium channel subunits *CACNA1A* (P/Q-type), *CACNA1C* and *CACNA1D* (L-type), and *CACNA1H* (T-type)[330], and the voltage-gated sodium channel subunits *SCN3A* and *SCN8A*. *TRPA1*, previously described to stimulate GLP-1 and 5-HT secretion in mouse, was enriched in both EEC populations[331,332], as was the amiloride/acid sensitive ion channel *ASIC5*, and the K_{ATP} channel subunits *KCNJ11* and *ABCC88*[333].

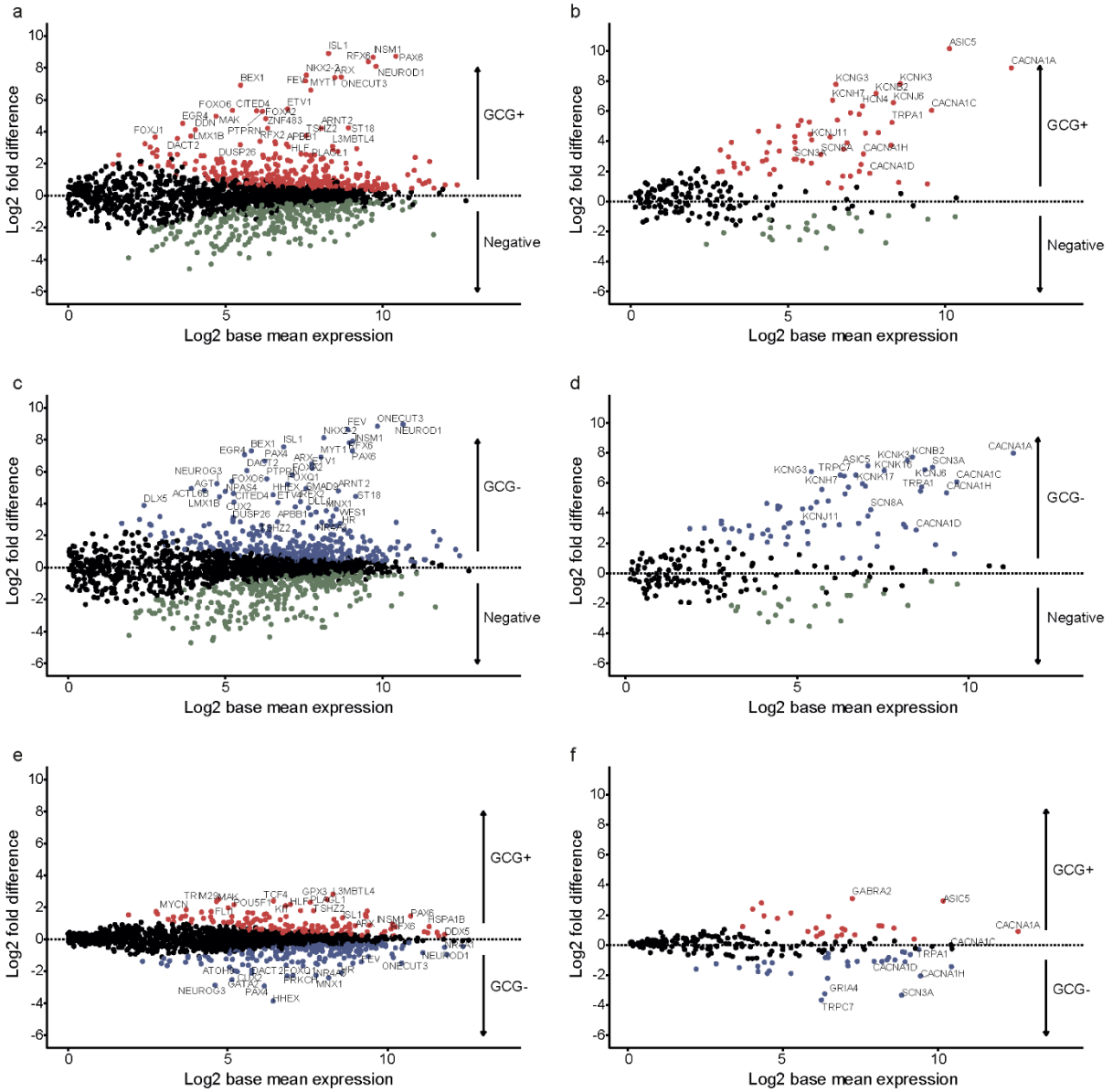

Figure 9-4. Differential vs mean expression plots for human jejunum EEC populations. Differential expression is presented as the log(2) fold difference between the cell populations indicated, and expression is presented as the log(2) base mean normalised expression extracted from the DESEQ2 model. (a,b) GCG+ vs negative, (c,d) GCG- vs negative (e,f), GCG+ vs GCG-. Transcription factors are shown in a,c,e; ion channels are shown in b,d,f. Red – enriched (i.e. adjusted P≤0.1) in GCG+; Blue – enriched in GCG-; Green – enriched in negative cells. Key genes are labelled.

9.3.3 Comparison of human jejunum vs ileum EEC transcriptomes

PCA of the 3 cell populations from each of the 2 matched human jejunum and ileum samples separated EECs from negative cells on the first component and the anatomical location of GCG+ and GCG- cells on the second component (Figure 9-5a). This distinction was driven by a low number of differentially expressed genes, 279 in GCG+ cells and 120 in GCG- cells, when analysed pairwise using a DESEQ2 model. Notable differentially expressed transcripts that were higher in jejunal than ileal EECs (GCG+ or GCG-) included *GIP*, *CCK*, *SST*, *MLN*, *SCT*, *GHRH*, *ASIC5*, and *TRPA1*; whereas transcripts higher in ileal than jejunal EECs included *GCG*, *NTS* and *TAC1* (Figure 9-5b,c).

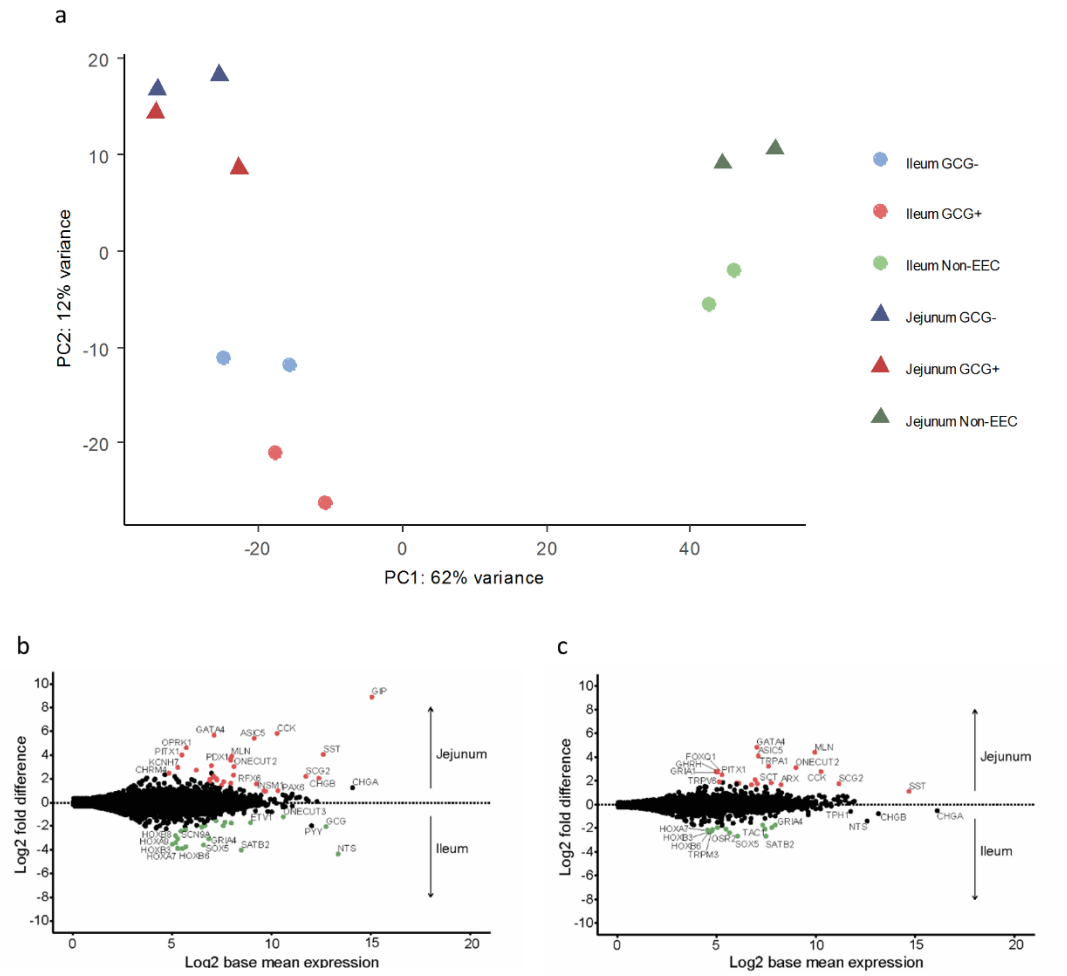

Figure 9-5. Comparison between EECs from human jejunum and ileum. (a) Principal component analysis plot of matched human jejunum vs ileum samples, labelled by cell population and anatomical location (n=3 cell populations from both anatomical regions from each of 2 participants). PCA separated EECs from negative cells on the first component and the anatomical location of GCG+ and GCG- cells on the second component. This distinction was driven by a low number of differentially expressed genes, 279 in GCG+ cells and 120 in GCG- cells, when analysed pairwise using a DESEQ2 model. (b,c) Enrichment vs expression plots for GCG+ (b), and GCG- (c), cell populations. Enrichment is presented as the log2 fold difference between the cell populations indicated, and expression is presented as the log2 base mean normalised expression extracted from the DESEQ2 model. Red – enriched (adjusted P≤0.1 in DESEQ2 model) in jejunum; Green – enriched in ileum.

9.3.4 Comparison of human vs mouse EEC transcriptomes

Human jejunal GCG+ cells were compared with murine upper small intestinal GLU-Venus cells, representing the GLP-1 secreting L-cell populations from each species (Figure 9-6). Only genes with 1:1 homology annotated in Ensembl were included, and genes annotated as ribosomal, mitochondrial and small-nuclear were excluded, giving a total of 15,507 genes for comparison. Log-log plots of normalised gene expression indicated a strong correlation between the two species (R^2=0.73). Of the 354 genes lying outside the 99% confidence interval of the linear model of human versus mouse, notable genes more highly expressed in human than mouse GCG+ cells included *GIP*, *CHGA*, *ASIC5*, *GIPR*, *GPR142*, *SCTR*, *PTH2R*, *CHRNA5* and *OPRK1*; whereas genes more highly expressed in mouse GCG+ cells included *Gpr174*, *Gpr171*, *Ghr*, *Grpr*, *Ptger1*, *Cnr1*, *Insl5*, *Gpr22* and *Ghrl*.

Human jejunal GCG- cells, representing the wider murine EEC population (although depleted of L-cells) were compared with murine NeuroD1-positive cells, revealing a strong correlation between these human and murine EEC populations (R^2=0.74; Figure 9-7). Of the 380 genes lying outside the 99% confidence interval of the linear model for this comparison, notable genes more highly expressed in human than murine EECs included *GIPR*, *SCTR*, *GHRH*, *OPRK1*, *PTH2R* and *TRPV6*, and in murine compared with human cells included *Ghrl*, *Tac1*, *Iapp*, *Gpr22*, *Gast*, *Ptger1*, *Grpr* and *Ghr*.

Species-comparative data for subgroups of genes in L-cells and the total EEC population, separated by their annotated roles as hormones, GPCRs, ion channels and transcription factors, are represented in Figure 9-6 and Figure 9-7, in which the colour code additionally indicates whether the genes were more than four-fold enriched and significantly likely to be differentially expressed based on the DESEQ2 model (adjusted p≤0.1) in EECs compared with negative cells in one or both species.

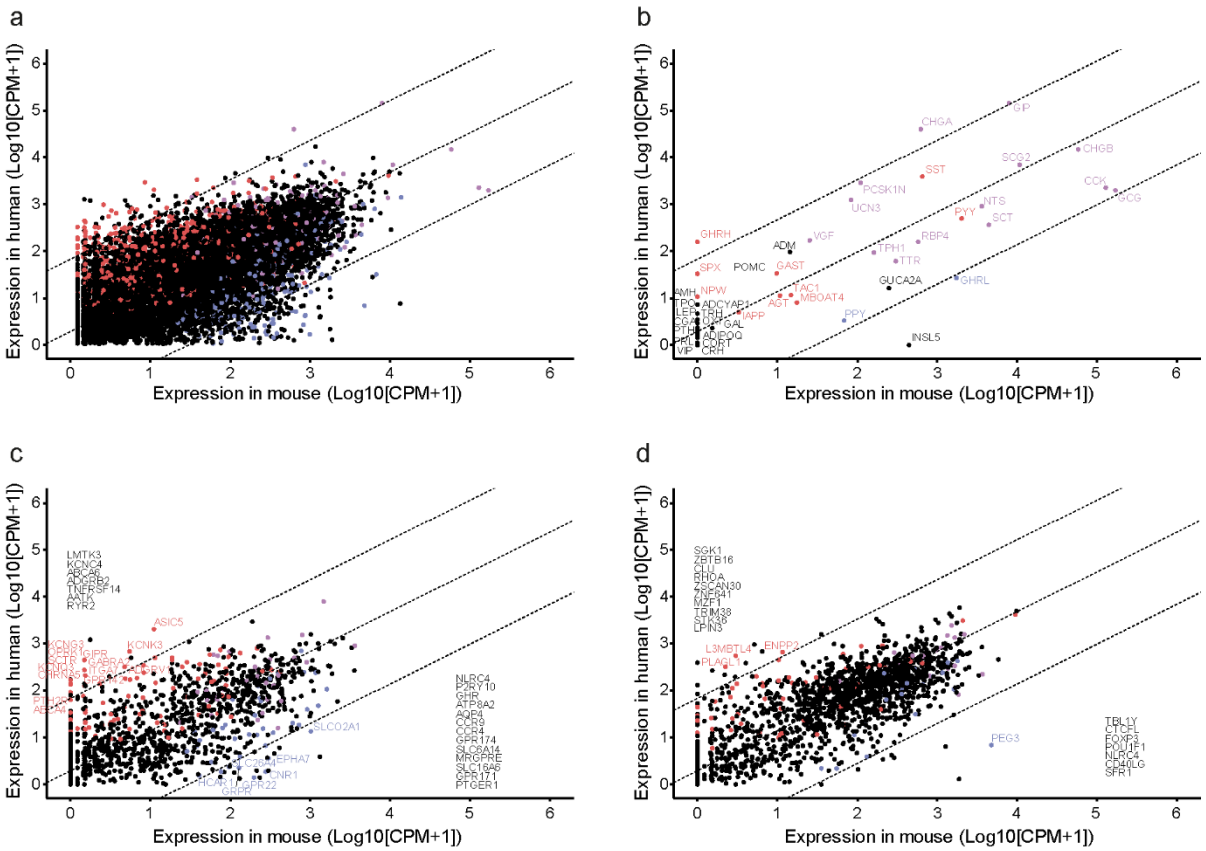

Figure 9-6. Comparison between human and mouse L-cells. Human versus mouse jejunal L cell gene expression (log10 normalised counts per million +1; n = 11 humans, n = 3 mice). (a) all genes with 1:1 homology between species, excluding mitochondrial, ribosomal and small nuclear transcripts (n=15,507). (b) Hormones. (c) GPCRs and ion channels. (d) Transcription factors. Dashed lines are linear regression and 99% confidence interval. Each dot represents normalised CPM+1 for one gene. Red – enriched (>4x fold change) and differentially expressed (adjusted P<=.1) for human GCG+ vs Negative cell populations, but not murine GLU-Venus vs Negative cell populations in relevant DESEQ2 model. Blue – enriched and differentially expressed for murine GLU-Venus vs Negative cell populations, but not human GCG+ vs Negative cell populations. Purple – enriched and differentially expressed in both murine GLU-Venus and human GCG+ cells versus relevant negative cell populations. Black – not enriched and differentially expressed in either human GCG+ or murine GLU-Venus cells versus relevant negative cell populations. All genes are labelled in b, and genes outside the 99% CI are labelled in c and d, with those not differentially expressed or enriched in either population listed along the axis.

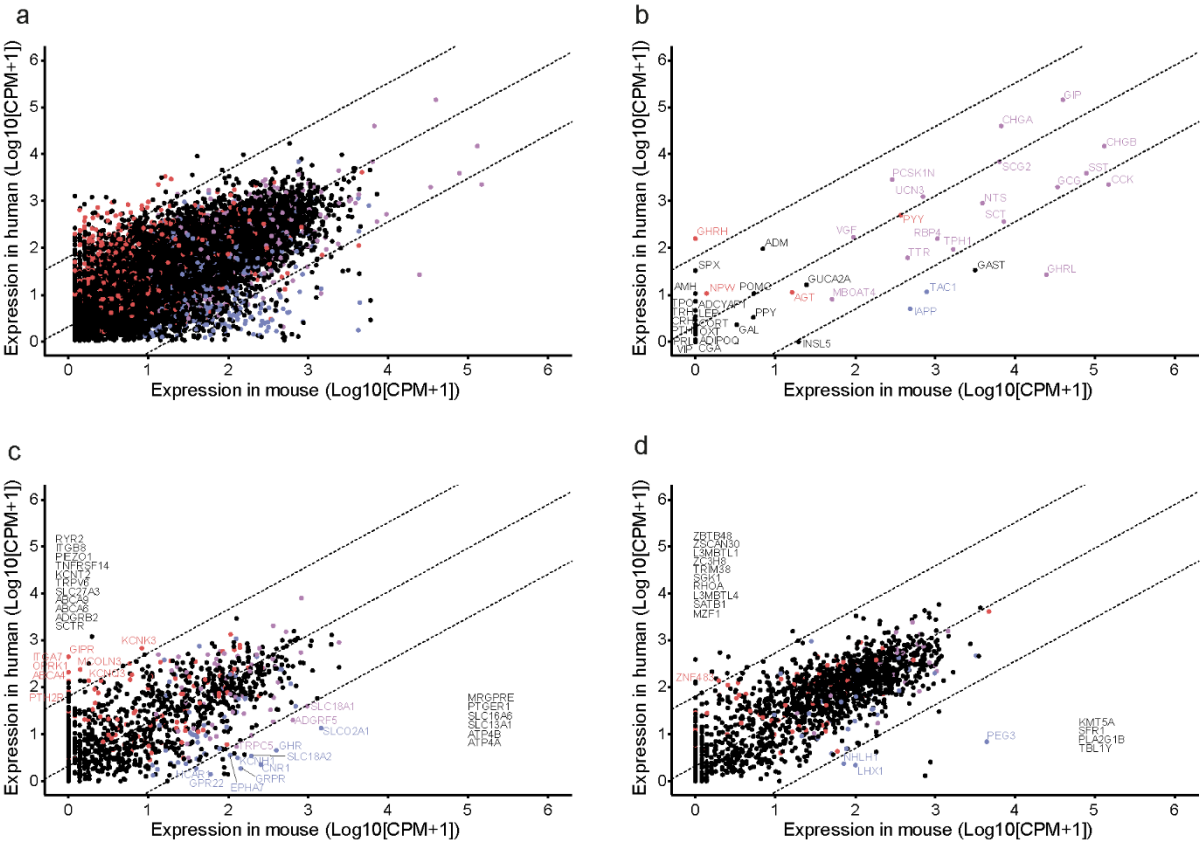

Figure 9-7. Comparison between human GCG- and mouse NeuroD1 cells. Human versus mouse jejunal EEC (GCG-) gene expression (log10 normalised counts per million +1; n = 11 humans, n = 3 mice). (a) all genes with 1:1 homology between species, excluding mitochondrial, ribosomal and small nuclear transcripts (n=15,507). (b) Hormones. (c) GPCRs and ion channels. (d) Transcription factors. Dashed lines are linear regression and 99% confidence interval. Each dot represents normalised CPM+1 for one gene. Red – enriched (>4x fold change) and differentially expressed (adjusted P<=.1) for human GCG- vs Negative cell populations, but not murine NeuroD1 vs Negative cell populations in relevant DESEQ2 model. Blue – enriched and differentially expressed for murine NeuroD1 vs Negative cell populations, but not human GCG- vs Negative cell populations. Purple – enriched and differentially expressed in both murine NeuroD1 and human GCG- cells versus relevant negative cell populations. Black – not enriched and differentially expressed in either human GCG- or murine NeuroD1 cells versus relevant negative cell populations. All genes are labelled in b, and genes outside the 99% CI are labelled in c and d, with those not differentially expressed or enriched in either population listed along the axis.

9.3.5 Peptidomic analysis by LC-MS

In the first instance, tissue samples were analysed by nano LC-MS/MS and compared between human jejunum (n=4) and mouse mid-small intestine (n=4). Peptides were assigned to their parental proteins by Peaks software, and were found to include known EEC prohormones, granins and enteric neuronal signalling peptides as well as peptides derived from a variety of house-keeping proteins. Of the 463 and 705 different proteins matched in human and mouse respectively, 234 were common between the two species, and showed good correlation (R^2 = 0.54, Figure 9-8a). To identify candidate EEC-derived peptides, the analysis was restricted to peptides originating from genes that in the transcriptomic analysis showed >4-fold higher expression in at least one EEC sample compared with the corresponding negative cell population (Figure 9-8b). The known gut hormone genes and members of the chromogranin family were mostly common to mouse and human, but motilin and ghrelin were found in human but not mouse jejunum, which is interesting, as the transcriptomic data identified *GHRL* to be preferentially expressed in mouse over human jejunum. A few peptides were derived from proteins not previously known to have signalling roles, but further studies will be needed to determine whether these exhibit bioactivity or simply reflect peptides released from EECs during tissue degradation. To search for novel candidate peptide hormones, the transcriptomic data were searched for unannotated transcripts that had a base mean value >100 and were >16 fold more highly expressed in EECs than control cells. This analysis identified *MIR7-3HG*[334], *C1orf127* and *C6orf141*, but corresponding peptides were not detected in the LC-MS/MS data.

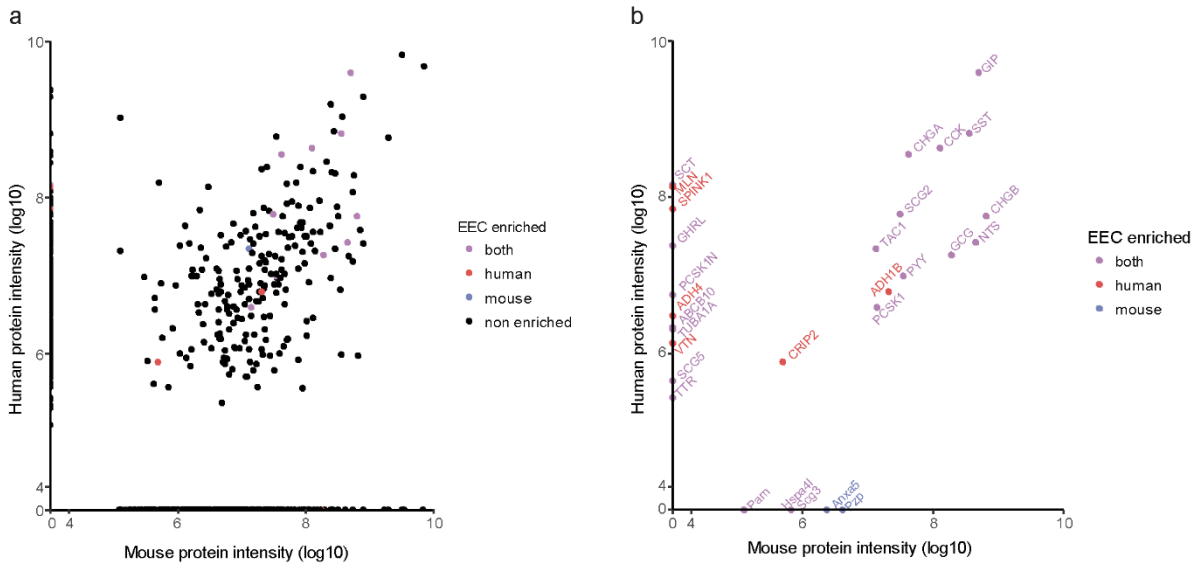

Figure 9-8. Comparison between human and mouse jejunum peptidome. Tissue samples were analysed by nano LC-MS/MS and compared between human jejunum (n=4) and mouse mid-small intestine (n=4). Peptides were assigned to their parental proteins by Peaks software, and protein intensity was calculated by Peaks v8.0 for all proteins detected corresponding to genes with 1:1 homology between human and mouse for jejunum mucosal homogenates. (a) All proteins, (b) proteins from genes enriched in at least one of the species from the human and the mouse transcriptome datasets. Enrichment was defined as adjusted P<=.1, fold difference >4 and base mean expression >50 from DESEQ2 model. Colours indicate in which species the mRNA for the genes was enriched. Courtesy of P Larraufie.

A longitudinal LC-MS/MS analysis of known bioactive peptides along the length of the mouse and human GI tract was performed by taking sequential samples at 5cm intervals from the stomach to the rectum in mice (n=4 for each location), and human biopsies from the stomach (n=5), duodenum (n=9), jejunum (n=2), ileum (n=4), proximal colon (n=3), sigmoid colon (n=5) and rectum (n=3). Most EEC peptides were identifiable in their known bioactive forms but fragments of pro-CCK mostly lacked the C-terminally active 8 amino acids, perhaps reflecting an extraction artefact as CCK is known to be highly labile under different conditions and our method was not optimized for very small peptides such as CCK-8. A robustly present peptide from the CCK prohormone, CCK_{21-44}, was used as a surrogate of CCK production. Peptides were depicted in separate heatmaps for mouse and human (Figure 9-9), divided into their origin from EEC prohormones, granins and non-EECs (likely reflecting enteric neural peptides). The observed longitudinal profiles broadly mirror historical immuno-staining patterns[75] but additionally provide details of the exact peptide sequences and their post-translational modifications that cannot be deduced from antibody staining. The human and mouse profiles were similar for most EEC peptides, with the notable exceptions that CCK, NTS and SCT extended into the colon only in mice, and that Gastrin and Ghrelin could be detected in human but not mouse proximal small intestine. Of the non-EEC peptides, NMU and galanin were found along the full length of mouse small and large intestine but in human NMU was largely restricted to the small intestine and galanin to the distal small intestine and colon/rectum.

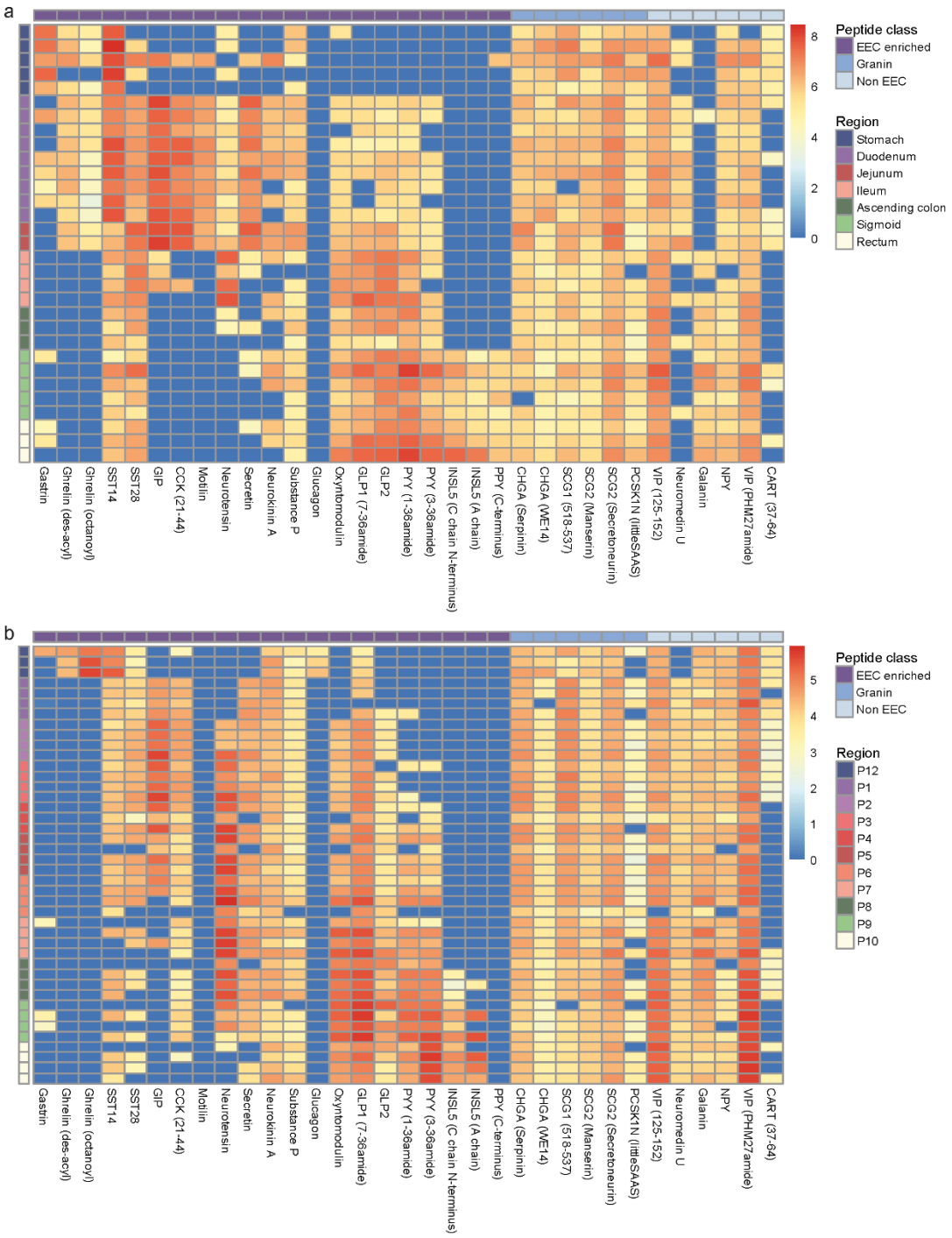

Figure 9-9. Longitudinal profiles of gut peptides along the human and mouse gut. Heatmap of gut peptide quantification normalised by tissue weight (log10 scale) along human (a) and mouse (b) GI tract. Sequential samples were taken at 5cm intervals from the stomach to the rectum in mice (n=4 for each location), and human biopsies were analysed from the stomach (n=5), duodenum (n=9), jejunum (n=2), ileum (n=4), proximal colon (n=3), sigmoid colon (n=5) and rectum (n=3). Blue indicates not detected in the sample. Rows: Samples ordered from proximal to distal and colour coded by their region of origin. Mouse regions: P12: stomach lesser curvature, P1 to P7: small intestine from proximal to distal, sampling every 5 cm, P8 to P10: large intestine (proximal, mid and distal). Columns: peptide (using the human name if different between human and mouse), classified by origin (purple: classical EEC hormone peptides; medium blue: peptides from granins; light blue: enteric neuron bioactive peptides). Courtesy of P Larraufie.

9.4 DISCUSSION

By RNA sequencing of fixed, FACS-purified cells from the human gut, this study reports the transcriptome of human L-cells and the wider EEC population, and a between-species comparison showing a strong similarity with matching cells from the mouse. LC-MS based peptidomic analysis revealed longitudinal gradients of a range of EEC peptides, including their sequences and post-translational modifications, along the length of the human and mouse GI tract. The amalgamated transcriptomic and peptidomic picture provides a unique insight into the sensory apparatus and hormonal secretory profile of human EECs, and the extent to which the mouse is a valid model for studying the human enteroendocrine system.

The study was limited by many of the same challenges that have consistently hindered RNA and peptidomic analyses. As it proved impossible to retrieve RNA from unfixed, permeabilised cells, or adequately purify EECs based on surface epitopes, it was necessary to rely on good quality internal epitopes to purify human EECs for transcriptomics. This however required cell fixation and permeabilisation prior to cell separation by flow cytometry. The available RNA extraction and sequencing kits were only just technically capable of dealing with PFA-modified RNA and although the resulting high throughput sequencing consequently had a lower than desired mapping rate the read depth was sufficient for detection of low abundance mRNAs such as those encoding receptors and ion channels. The transcriptomes of human L-cells and GCG- EECs mapped robustly onto the homologous RNAseq data from freshly purified matching murine cell populations. Genes without a 1:1 homology between species or that were not protein encoding were excluded from the comparative analysis, but can be examined in the raw deposited datasets. An alternative transcriptomic approach that would also not depend on the availability of genetically labelled models is single cell RNA sequencing of large numbers of dissociated cells, as recently applied to murine intestinal organoids[316]. Although EECs accounted for only a small percentage of the total cell count, they were readily identifiable from their transcriptomic signatures. This approach could be extended to human organoids or fresh human tissue, but the potential influence of prolonged organoid culture conditions on the cell transcriptome

still needs to be established and the outputs of most single cell RNA sequencing methods are currently limited by a read-depth that precludes reliable quantification of low-abundance transcripts.

Although jejunal samples from 11 humans with varying BMI were analysed, it was not possible to detect any effect of BMI on the EEC transcriptome. Tissue samples, were, however, collected and sorted in both France and the UK where pre-operative dietary intakes were likely quite different. As the high BMI samples largely originated from the French site and the statistical model accounted for the donor location when constructing the models of enriched transcripts, the study was insufficiently powered to detect small differences attributable to obesity. It is therefore not possible from this study to exclude the possibility that altered EEC function might arise as a result of obesity or variation in dietary intake, or contribute to the causation of obesity in a subset of people.

GPCRs that are strongly and specifically expressed in EECs underlie some of the key sensory roles of these cell types and deserve consideration as candidate drug targets for enhancing gut hormone secretion as a therapy for type 2 diabetes and obesity. The GPCR repertoire of human EECs largely mirrored their well-studied murine counterparts, including expression of receptors for amino acids (*CASR*, *GPR142*), triglyceride digestion products (*FFAR1*, *FFAR4*, *GPR119*) and bile acids (*GPBAR1*), as well as for hormones such as somatostatin (*SSTR1*, *SSTR2*, *SSTR5*), GIP (*GIPR*) and arginine vasopressin (*AVPR1B*). Interestingly, *GPR142* was highly expressed in human EECs, supporting current studies looking to exploit its ability to stimulate both insulin and incretin hormone secretion[335].

RNAseq analysis revealed expression in human and mouse EECs of genes encoding known gut peptides, as well as several putative hormones previously described in the gut or elsewhere. Of the hormone-encoding genes with a 1:1 homology between species, there was a good correlation between the expression profiles of mouse and human. Motilin was expressed in human but not murine EECs, consistent with previous literature[320]. The peptide extraction protocol combined with nano-LC-MS analysis enabled identification of the exact peptide sequences biosynthesised in human and mouse intestinal mucosa, including post-translational modifications, for peptides from ~8-10 to a maximum length of 65 amino acids. From the proglucagon gene it was possible to detect multiple processed and

pre-processed products, including GRPP, oxyntomodulin, GLP-$1_{7\text{-}36amide}$, GLP-$1_{7\text{-}37}$, GLP-$1_{1\text{-}37}$, $IP_{131\text{-}142}$, IP-GLP2 and GLP-2. Intact (pancreatic-type) glucagon was detected in samples from the mouse stomach, but was undetectable in the remainder of the intestine and colon from both species, conflicting with recent suggestions that the small intestine can secrete intact glucagon[336], but consistent with findings from chapter 7 that post-prandial glucagon concentrations were not altered following gastric bypass surgery in lean subjects despite dramatic increases in GLP-1[199]. Whether the gut might adapt to produce glucagon in pathological conditions such as obesity or type 2 diabetes cannot be concluded from this data. In addition to the set of well-recognised gut hormones, LC-MS also identified some additional peptides encoded by EEC-enriched genes, including peptides derived from PCSK1N, chromogranins and secretogranins. Whether any of these have specific physiological roles or are simply inactive by-products of enzymatic processing of the contents of secretory vesicles, requires further evaluation.

Mapping of gut hormone production along the GI tract length has previously been performed using immuno-staining methods and extraction/immuno-assays for specific peptides[75,337]. Immuno-staining relies on the detection of all antigenic sequences binding to a primary antibody and is a reliable method for localising cells producing any given prohormone but cannot generally distinguish whether the prohormone was processed or unprocessed, or post-translationally modified. Nevertheless, immunostaining methods have resulted in longitudinal maps of EEC sub-types producing different prohormones[75]. This LC-MS method provides a surprisingly robust mirror of these immuno-staining maps, whilst assigning an exact peptide sequence to each identified peptide, clearly distinguishing e.g. oxyntomodulin from glucagon, and $PYY_{1\text{-}36}$ from $PYY_{3\text{-}36}$, despite these peptide pairs differing only by extensions of a few amino acids at the C and N-terminus, respectively. The method also benefits from being unbiased, providing MS-based identification of all peaks triggered from the liquid chromatography separation, rather than analysing only a sub-group of specified peptide sequences. Interestingly, acylated as well as non-acylated ghrelin was identified from the human jejunum despite the previous finding that plasma acylated ghrelin levels were undetectable in humans after total gastrectomy[199]. It was surprising to find high levels of $PYY_{3\text{-}36}$ as well as $PYY_{1\text{-}36}$ in tissue homogenates,

suggesting that dipeptidyl-peptidases (DPP) are active within L-cells themselves, whereas GLP-$1_{7\text{-}36amide}$ was much more abundant than GLP-$1_{9\text{-}36amide}$, indicating they had not been subject to DPP4 cleavage. Why GLP-1 but not PYY seems protected from DPP-mediated processing in L-cells, despite both peptides being located in the same vesicular pool (unpublished work from our group), remains unclear.

9.5 SUMMARY

Using novel transcriptomic and peptidomic approaches this study has validated the use of mice as a model of enteroendocrine physiology, and generated a gut hormone map of the gastrointestinal tract. Central to the core of this thesis, the human transcriptomic data identifies known and putative gut hormones that are known to, or may be differentially secreted after oesophago-gastric resection. The description of the GPCR-ome, as well as key ion channels and transcription factors should allow more detailed examination of the mechanisms underlying gut hormone secretion. A key question is whether the altered gut hormone secretion seen after upper gastrointestinal bypass, either for cancer or obesity, is driven simply by altered nutrient delivery to unchanged EECs, or whether functional adaptations in hormone production, nutrient sensing and hormone secretion occur. Ultimately, a detailed transcriptomic and peptidomic description of the human enteroendocrine system is a powerful tool for the investigation of gastrointestinal and metabolic pathology, and for identifying novel therapeutic targets.

10 Effect of gastrectomy with Roux-en-Y reconstruction on the transcriptome and peptidome of the jejunum

Hypothesis

Adaptive changes in EECs at the transcriptomic and peptidomic contribute to the altered gut hormone secretory profile identified after gastrectomy with Roux-en-Y reconstruction.

10.1 INTRODUCTION

Having demonstrated the role of altered gut hormone secretion in post-gastrectomy patients, it would be of interest to identify the physiological mechanism underlying these changes. In vitro and in vivo studies have demonstrated a dose-response correlation for EEC secretion, with exposure to higher concentrations of nutrients resulting in secretion of greater concentrations of gut hormones. Gastrectomy, oesophagectomy (with the exception of patients with a failed conduit), RYGB and sleeve gastrectomy all result in rapid delivery of higher concentrations of nutrients to the nutrient-sensing small intestine. An obvious factor driving enhanced gut hormone secretion in these groups would therefore be elevated exposure to nutrients, both in concentration and volume, resulting in greater stimulation of EECs.

Adaptive changes within the intestinal tract could also contribute to changes in gut hormone secretion, with possibilities including altered cell number, hormone processing and composition, nutrient receptors and ion channels. Previous studies by Rhee and Nergard have identified changes in EECs from endoscopic biopsies collected during small bowel enteroscopy after RYGB[338,339]. Rhee identified increased numbers of EECs (particularly those immunoreactive for GLP-1, PYY, CCK and GIP) within the enteric mucosa, with qPCR identifying reduced expression of *GHRL*, *SCT* and *GIP*, unchanged expression of *PYY*, *CCK* and *NTS*, and increased expression of *GCG* mRNA in whole mucosal homogenates. Nergard identified similar changes to Rhee with respect to EEC cell density, but conflicting results from qPCR of gut hormone mRNA transcripts.

The above two studies demonstrated both the feasibility of assessing the post-operative small intestinal mucosa. Recruitment for a non-clinically indicated endoscopy can be challenging, with participant numbers low. Endoscopic biopsies are small, and collection of adequate cell numbers requires multiple biopsies, increasing the length of procedure and risk of complications. Following tissue collection, the use of antibodies has well documented shortcomings, particularly when trying to assess cell populations that make up <1% of all mucosal cells, with findings highly sensitive to small

changes in staining efficacy. Similarly, examining RNA from whole tissue homogenates effectively dilutes any change in EEC transcriptome by a factor of 100, potentially masking any subtle changes.

Returning to the controversy surrounding post-bariatric intestinal glucagon production, two published studies now suggest that intestinal adaptation results in altered handling of the proglucagon prohormone in post-operative L-cells and intestinal secretion of pancreatic glucagon[62,306]. Antibody and RNA based methodologies are inadequate to investigate this suggestion further (GLP-1 and glucagon result from post-translational modification of the same prohormone and so mRNA, and antibodies differentiating the two are poorly specific). LC-MS/MS can however identify single amino acid differences in peptides, and could potentially differentiate pancreatic glucagon from glicentin and oxyntomodulin in intestinal biopsies.

In order to address the question of intestinal adaptation further, endoscopic biopsies were collected from the apex of the alimentary limb of several patients after total gastrectomy, and from jejunum collected during gastrectomy for comparison. From some participants, single cell digests were fixed, antibody stained, FACS purified for EECs and RNA sequenced to identify transcriptomic changes in EECs resulting from gastrectomy. From the rest, whole tissue homogenates were processed for LC-MS/MS based peptidomics, with multiple peptide fragments quantified. While it would have been preferable to undertake peptidomics on FACS purified EECs, the fixation required for purification rendered the tissue unsuitable for LC-MS/MS and, as described above, a non-fixation method for purification of EECs has yet to be developed.

10.2 METHODS

10.2.1 Endoscopy

Participants were recruited either from post-operative patients undergoing planned endoscopies for clinical reasons, or to undergo an endoscopy specifically to collect tissue for this study. Endoscopy was performed by JAG accredited endoscopists in the Addenbrooke's endoscopy unit, or Cambridge Clinical Research Centre endoscopy unit, under pharyngeal anaesthesia or procedural sedation (as selected by the participant). Biopsies were collected from the jejunum <10cm distal to the oesophago-jejunal anastomosis with spiked 2.8mm forceps and immediately placed into cooled L-15 medium. Where possible for the RNAseq project, the endoscope was passed to the entero-enterostomy and samples were also collected from the bilio-pancreatic limb of the reconstruction For the RNA sequencing project 10 biopsies were collected, for the peptidomics project 2 biopsies were sufficient.

10.2.2 RNA sequencing

Jejunal biopsies were processed as in chapter 2.13. Primary antibody staining was with 2% v/v rabbit anti-CHGA (Abcam, Cambridge, UK; Ab15160) and 0.25% v/v rabbit anti-SCG2 (Abcam, Ab12241), resulting in the purification of a single CHGA/SCG2 positive population representing all EECs from the background cell populations. Working within the limits of cell numbers (~1pg of RNA from each sorted cell, >1000pg RNA required for library preparation; ~200,000 cells per biopsy sample) it was not possible to collect an adequate number of GLP-1 positive cells for sequencing, hence the decision to study all EECs as a single population.

As the specific question of interest was whether adaptive changes occur in EECs after gastrectomy / Roux-en-Y, only the positive cell populations were sequenced. Comparison of populations was by a DESEQ2 model, with the factor of interest coded as the group (i.e. pre-op or post-op).

10.2.3 Peptidomics

Peptide extraction, mass spectrometry and peptidomic analysis was performed as in chapter 2.14. Two biopsies were sufficient for extraction of peptides and LC-MS/MS.

10.3 RESULTS

For RNAseq, specimens were collected from five post-operative patients and four patients during gastrectomy for baseline comparison. Unfortunately the RNA extraction and cDNA library preparation steps did not result in adequate libraries for sequencing for one pre- and one post-op sample, or for either of the samples which were collected from the biliopancreatic limb during endoscopy (sampling at this site was limited by participant tolerance and challenges reaching the site, resulting in smaller and poorer quality samples).

For peptidomics, specimens were collected by endoscopy from the apex of the alimentary limb of post-operative patients, and from seven patients during gastrectomy for baseline comparison.

10.3.1 FACS

Purification of EECs for RNAseq was performed as described in chapter 2.13 and as for chapter 8. Using smaller amounts of tissue however resulted in unique challenges. With this technique, the negative populations in the fully antibody treated samples all evidenced a greater degree of baseline fluorescence than controls made of cells treated with no antibody, or primary or secondary antibodies alone. Gating was therefore best performed by review of the fully antibody stained cells to identify the ~0.2% highly fluorescent population. This did not represent a problem in the study described in chapter 8, as the tissue samples sizes were large enough to allow up to a million antibody treated cells to be analysed to identify the appropriate gating position for positive and negative cell populations prior to cell collection.

For the endoscopy study, with smaller sample sizes, it was not possible to waste this many cells and still collect enough to extract a useful amount of RNA. Gating was therefore extrapolated from settings that had previously worked on samples treated with the same protocol, with minor modification if the baseline population appeared to have shifted. Unfortunately, for three of the four endoscopy specimens this resulted in a degree of contamination of the positive fluorescent cell population with negative cells, which was only apparent on completion of the sort.

10.3.2 Comparison of pre- and post-operative EEC transcriptome

Comparison of the entire transcriptome by DESEQ2 of the four endoscopic and three anatomically matched operative samples identified ~450 differentially expressed transcripts and clear separation of pre- and post-operative specimens on principal component analysis. Review of the genes apparently enriched in the post-operative samples identified multiple Paneth cell type transcripts (*LYZ*, *DEFA4*, *DEFA6*, *SOX9*) and *NOTCH1*, which suppresses EEC differentiation. Due to the contamination identified on review of the FACS data, it was concluded that it would not be possible to accurately comment upon changes in gene expression that may be simply due to the presence of non-EECs in the post-op samples.

To enable a limited examination of the data, the normalised expression data were restricted to genes known to be enriched in EECs (identified from the study in chapter 8), allowing comparison of expression of 3319 transcripts. PCA of this dataset showed that the four post-operative specimens clustered, along with one pre-operative specimen. The two remaining pre-operative specimens were distinct from this cluster (one on PC1, one on PC2) but not in a contiguous fashion.

Within the EEC enriched dataset, as EECs would be effectively diluted and so transcript numbers reduced in the post-op samples, only gene enrichment, not depletion, could be reliably discussed. Forty five transcripts were differentially expressed and enriched in post-operative samples ($p \leq 0.1$ in DESEQ2 model). Within these 45 genes, several transcripts implicated in cell turnover and differentiation (in particular of pancreatic islet or enteroendocrine lineages) stand out as potentially significant. Several factors implicated in *NOTCH* pathway signalling were enriched after gastrectomy, including *FOXP2*, *BCL6*, *HES6* and *NEURL1B*. Other enriched transcripts included endocrine specific (*PDX1*, *SPRY4*) or general (*ZNF703*) transcription factors, and other transcripts involved in cell proliferation (*STMN1*, *EPHB3*, *RARRES1*, *TCEAL9* and *IGFBP2*). Transcripts of genes encoding gut hormones, nutrient receptors, ion channels or those involved in vesicular packaging / exocytosis were not enriched in the post-operative cohort.

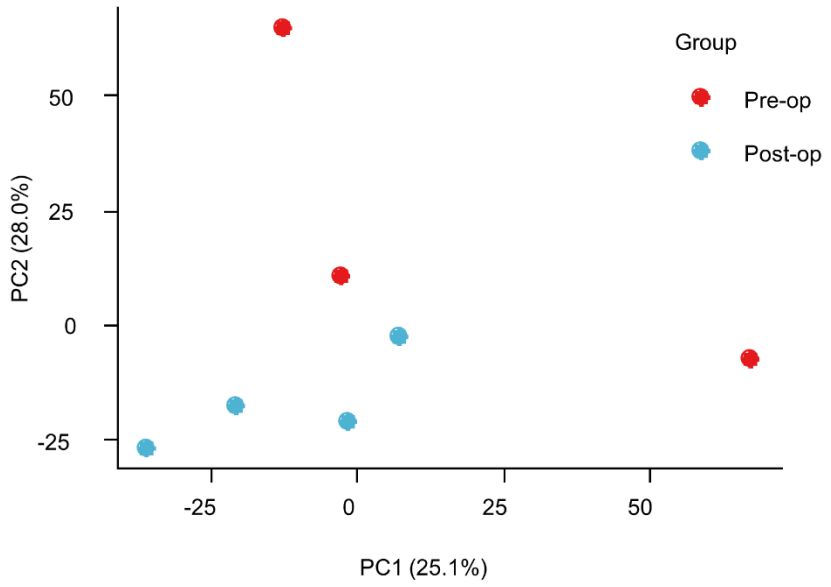

Figure 10-1. PCA of RNAseq results of FACS purified EECs from jejunum biopsies collected during gastrectomy (pre-op) and in participants after gastrectomy (post-op), including only genes known to be enriched in EECs. Addition of the third principal component did not improve segregation of the groups.

10.3.3 Comparison of pre- and post-operative jejunal peptidome

Twenty four secretory peptides were identified and quantified in homogenates of jejunal mucosa from pre- and post-operative patient samples. Peak area was normalised by tissue weight and internal standard (C_{13} labelled GLP-1 and PYY) to allow comparison of each peptide between samples.

PCA of the samples by all measured peptides did not differentiate pre- and post-operative samples (Figure 10-2). The one outlier identified on PCA was not brought closer to the cluster by analysis following different normalisation techniques (using one or other of the internal standards alone, or by weight alone). Examination of results for each peptide and comparison by multi-factorial ANOVA did not reveal a significant difference between pre- and post-operative groups for any individual peptide. Within this, the results for motilin visually appeared to be trending towards a greater value in the post-operative group, and outlying results within those for several peptides (e.g. SST14, SST28, PSCK1N, TAC1 substance P) raise the possibility that the within group variance was large enough to make the sample size too small to identify statistically significant changes in peptide concentration (Figure 10-3).

The results for all samples were specifically searched for the presence of pancreatic type glucagon, which was not identified in any pre- or post-operative specimens.

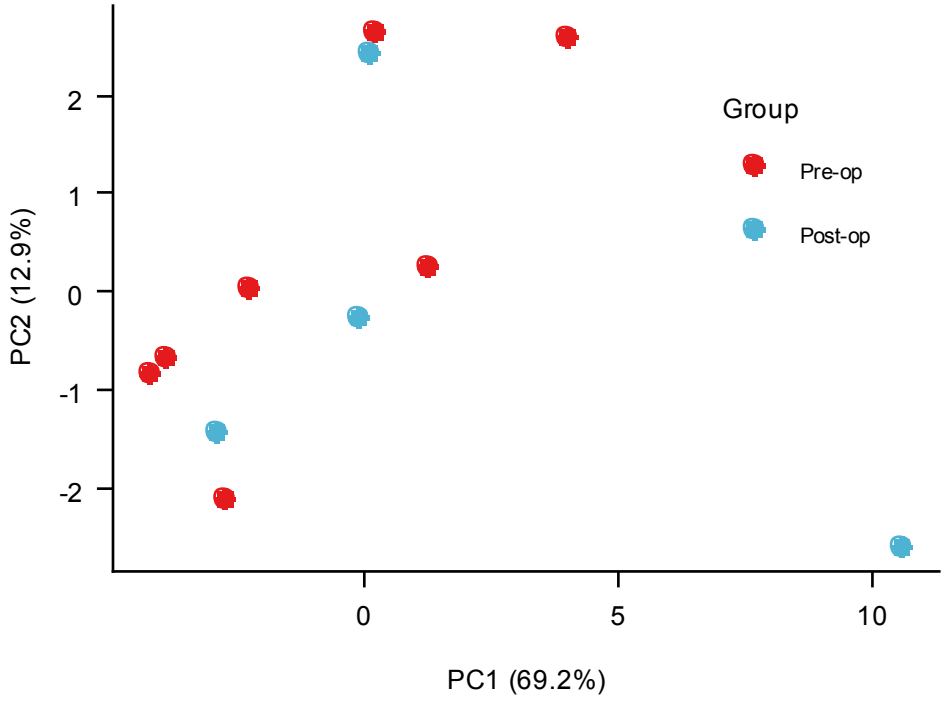

Figure 10-2. PCA of human jejunum homogenate peptide concentrations measured by LC-MS/MS for all measured peptides, coloured by whether collected from pre- or post-operative tissue.

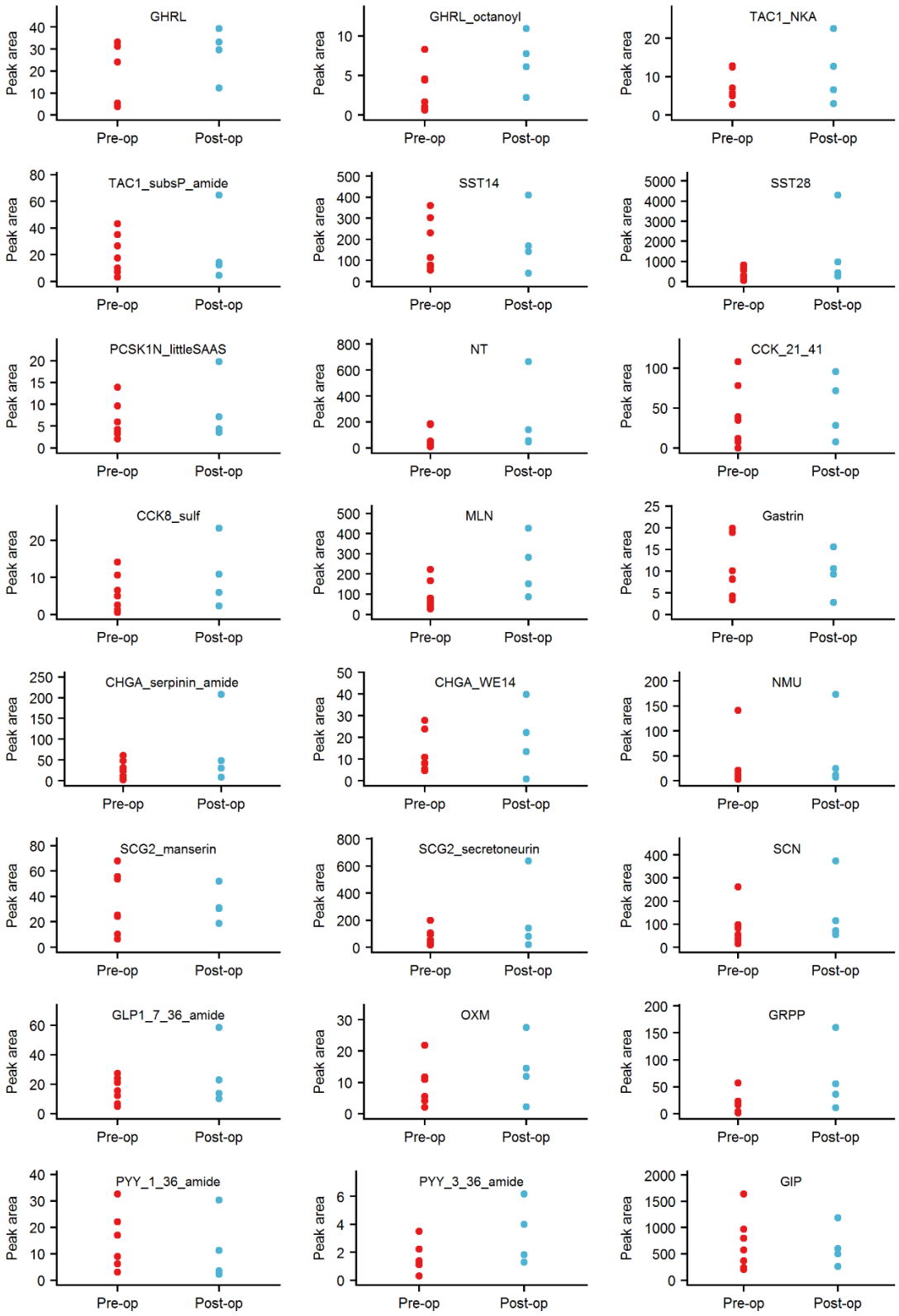

Figure 10-3. Individual sample peptide concentrations from pre- and post-operatively collected jejunum biopsies measured by LC-MS/MS. Concentrations are measured as relative peak area (normalised by weight and internal standard) and so are comparable between groups within peptides, but between peptide comparison is not valid. GHRL – ghrelin, NKA – neurokinin A, SST – somatostatin, NT – neurotensin, CCK – cholecystokinin, MLN – motilin, CHGA – chromogranin A, NMU – neuromedin U, SCG2 – Secretogranin 2, SCT – secretin, OXM – oxyntomodulin, GRPP – glicentin related polypeptide.

10.4 DISCUSSION

This small study, developing on the techniques described in chapter 8 and using smaller and more difficult to access tissue samples, quantified the transcriptomic and peptidomic profile of the jejunal mucosa after total gastrectomy with Roux-en-Y bypass.

Within the limitations of the technique (low RIN quality RNA, contaminated FACS sorts), there was no clear difference in the expression level of the majority of EEC enriched transcripts in purified EECs collected from post-operative samples when assessed en masse by PCA. Of the small number of enriched genes, most have been previously described to have roles in *NOTCH* signalling and therefore likely EEC differentiation, or cell turnover. Previous studies using different techniques (whole mucosa qPCR or immunohistochemistry) have identified architectural changes, including EEC number, within the mucosa. The limited transcriptomic data set presented here is compatible with previously published data suggesting changes in EEC cell number, in the presence or absence of altered mucosal thickness) in the alimentary limb after Roux-en-Y gastric bypass[338,339]. Of note, transcripts of genes implicated in gut hormone secretion (mRNAs for hormones, nutrient receptors or secretory machinery) were not differentially expressed between groups.

There was however no major difference in mucosal concentrations of a panel of 24 secretory peptides measured in whole mucosa homogenates before and after gastrectomy, either in individual peptides or analysed en masse by PCA. Together, these data suggest any adaptive change in gut hormone processing or secretion in the jejunum is limited, in particular in comparison to the ~10x changes described in plasma concentrations of GLP-1 and PYY after OGTT.

These two small studies are however limited by size and contamination of the RNAseq samples. As described in chapter 8, purification and RNA extraction from EECs is a novel technique using technology at the edge of sequencing capabilities. It is however promising that adequate tissue can be collected for both sequencing and peptidomics from endoscopic biopsies. This study also does not assess changes beyond the apex of the alimentary limb of the Roux-en-Y reconstruction. It is clear that in the post-operative group nutrient and bile exposure is altered along the entirety of the intestinal tract,

and changes in the more distal small intestine, or the bilio-pancreatic limb may have profound effects on nutrient sensing and gut hormone secretion.

Future work could build on this methodology, to improve the accuracy of FACS purification and by increasing sample numbers to better characterise the within group variance in peptidome and transcriptome. Organising these samples within the availability of tissue and current ethical approvals was the correct way to pilot these methods, however future studies could improve on this by collecting matched pre- and post-operative samples for both RNAseq and peptidomics from a small subset of individuals, hence reducing within participant variance and so increasing the power to detect small changes.

Further techniques to add to these results could include histological examination of specimens (including immunohistochemistry for cell distribution and number, as well as general architecture of the mucosa) and single cell RNAseq techniques. While prohibitively expensive at present (i.e. requiring sequencing of ~100,000 cells to sequence 1000 EECs), scRNAseq of samples from matched pre- and post-operative samples could identify shifts in the ecology of EECs with greater sensitivity than bulk RNAseq on fixed samples.

In summary, despite the small sample sizes and technical limitations of this post-operative study, major shifts in the transcriptome and peptidome of the jejunal mucosa have not been identified to occur due to Roux-en-Y reconstruction. Smaller changes may be detected by more detailed techniques, or greater sample sizes, but are unlikely to explain the magnitude of change in gut hormone secretion in post-op patients, which is likely primarily driven by delivery of highly concentrated nutrients to the small intestine.

11 CONCLUSIONS

This thesis examines the altered eating behaviour and post-prandial symptoms seen after oesophago-gastrectomy and describes associated and potentially causative changes in gut hormone physiology. Using oral glucose tolerance tests, symptom questionnaires and CGMs to assess symptoms and hypoglycaemia in the patient group revealed a heterogeneous picture, with some patients experiencing neither symptoms nor hypoglycaemia, some experiencing one but not the other, and some both. Gut hormone secretion is however significantly altered in all post-operative patients examined – enhanced post-prandial incretin and satiety hormone secretion and reduced or absent fasting plasma acyl ghrelin. There was no clear association between physiological measures (gut hormones or haemodynamic measures) and symptom scores, despite historical descriptions of "dumping syndrome" including change in haematocrit as a diagnostic criteria.

It would be of interest to develop these findings further by quantifying changes in plasma concentration of other gut hormones (e.g. NT, MLN, CCK, SCT and SST), 5-HT, and bile acids arising due to surgery, as potential contributing factors to a model of post-operative gastrointestinal physiology. Within this concept, it must be noted that plasma concentration is only a surrogate marker for activity of many of these factors, which act locally and are largely metabolised before entry to the systemic circulation. Division of the vagus, as part of most operations described here, likely also has a significant effect on post-operative gastrointestinal physiology, although it is not assessed in this thesis. Indeed to do so would require disambiguation of effects arising from the profound changes in gut hormone secretion, which would be particularly challenging.

CGM measures indicated a greater degree of glycaemic variability and low blood glucose index in post-operative groups, particularly those after total gastrectomy. This is both confirmation of previous laboratory findings of reactive hypoglycaemia, and potentially a novel method for assessing glycaemic profile in post-operative patients.

While not a focus of this work, actively observing biochemically significant hypoglycaemia in a number of post-operative patients highlighted the fact that autonomic symptoms of hypoglycaemia appear to be blunted, with those having severe hypoglycaemia often demonstrating some slurred speech, but

no tachycardia, sweating or irritability. Future studies should include formal measures of hypoglycaemia awareness, as this may be a significant but unrecognised problem. There is at present no literature on the risks associated with hypoglycaemia unawareness in this group, or the increasing number of patients reporting post-prandial hypoglycaemia after metabolic surgery, however the problem carries a major burden of morbidity, and significant lifestyle challenges in the diabetic population.

Behavioural assessments including validated measures of habit, impulsiveness and food attention, failed to identify a difference between post-operative groups and controls, despite long-standing and well-described subjective changes in appetite and satiation after gastrectomy and oesophagectomy. The one positive finding from behavioural assessments was from the TFEQ-r18, which identified reduced measures of cognitive restraint and emotional eating. This is interesting, in that patients often self-report a degree of cognitive control over portion size in order to limit post-prandial symptoms, although the questions measuring cognitive restraint in the TFEQ-r18 are more focused on deliberate avoidance of high energy foods for weight control than on symptom control. The reduction in emotional eating is also of interest – anecdotally, and in a fashion yet to be formally tested, many post-gastrectomy patients describe either a total absence of a sense of hunger, or a significantly altered sense of hunger. This manifests both as a loss of reward on eating, and in a need for some to eat “by the clock”. While not formally assessed in this study, the surprisingly nuanced concept of “hunger” deserves further attention in this patient group, which in itself may offer insights into the pathways controlling hunger in man.

The dumping severity score did identify and quantify symptoms in post-operative patients compared to controls, and may represent a simple and quick means of screening the post-operative population for those who would benefit most from intervention.

Currently, somatostatin analogue treatments for post-gastrectomy symptoms work by suppression of all humoral factors including gut hormones and insulin. A more elegant, and potentially less comorbid approach, would be to identify critical humoral mediators and specifically suppress them. Specific

blockade of GLP-1 in post-gastrectomy patients prevented post-OGTT hypoglycaemia in 5/5 patients studied, and was associated with reduced post-OGTT satiation and symptoms. This study also identified that GLP-1 suppresses GLP-1, glucagon and PYY secretion (previously described) and enhances GIP secretion in man (a novel finding).

Use of highly specific techniques to assay glucagon, with reduced cross-reactivity, clarified that plasma pancreatic glucagon is not increased by ingestion of glucose after foregut bypass, but that classically used assays are highly cross-reactive to the elevated plasma glicentin found in this population. Furthermore, pancreatic glucagon was not identified in jejunal mucosal homogenates collected from post-operative patients, suggesting that recent literature proposing intestinal glucagon production in post-operative patients may be adversely influenced by assay cross-reactivity.

Correlation of gut peptides and measures of enteric microbial activity, in particular PYY in the fasting state, is supportive of current theories implicating the microbiome in glucose and body weight homeostasis. The study designed to investigate this by treating SIBO and reassessing gut hormone profiles was limited by poor efficacy of treatment and small sample size, but may be worth investigating further. At present, this can be regarded at best as correlative, but in vitro data from previous work identifies mechanisms which could explain the findings discussed here.

It is likely that the altered gut hormone profile described in general here is primarily driven by accelerated delivery of highly concentrated nutrients to the small intestine. Transcriptomic and peptidomic profiles of human EECs closely matched those of mice and confirmed the presence of nutrient sensing receptors and channels, without any obvious adaptive changes when post-operative samples were examined. Previous studies have however identified structural changes in the jejunal mucosa after RYGB, which was not assessed here. Given the small study numbers, complexity of methods used to assess tissue and contaminated FACS sorts in this study, it is possible that optimisation of the techniques and more participants may reveal subtle changes in EEC populations arising due to surgery.

All the above findings, in particular the gut hormone and insulin profiles and the effects of GLP1R blockade are highly similar to those described in studies of metabolic surgery. Certainly, the altered gut hormone response is the same and so independent of pre-operative obesity. It is also clear that gastrectomy in a lean population does not increase fasting insulin sensitivity independently of BMI, suggesting that surgical factors are largely irrelevant in insulin sensitivity.

11.1 CLINICAL RELEVANCE

These findings are of significant interest to the care of patients after gastrectomy, particularly those undergoing PTG at a young age. Considering people undergoing highly similar surgery for obesity receive lifelong endocrinology follow-up, there is a strong argument that follow up after oesophago-gastric resection should follow a similar protocol.

To extrapolate from this thesis, it is possible to identify patients with a significant symptom burden by simple questionnaire, and with reactive hypoglycaemia using CGMs. It is yet unclear whether loss of hypoglycaemia awareness is common, although the episodes of recurrent hypo- and hyperglycaemia seen in the CGM arm of this study could contribute to loss of autonomic symptoms. This is potentially a major clinical problem, with patients driving with little education of the problem and how to obviate associated risks. Use of a glucose monitor, and avoidance of precipitating factors could be a useful approach. Knowledge is also however limited within the medical profession, with the post-gastrectomy population still relatively small, and so those with severe post-gastrectomy problems presenting rarely.

Presently, there are limited treatment options for those with significant symptoms after gastrectomy (having excluded SIBO, pancreatic exocrine insufficiency and bile acid malabsorption). Historical descriptions of “dumping syndrome” are not supported by this study, and it could be more constructive to regard all post-operative patients as having significantly altered gastrointestinal physiology, with patients exhibiting variation in symptoms along a spectrum of their ability to adapt to the altered nutrient response. It could be helpful to regard post-gastrectomy symptoms in three categories: loss of appetite / altered eating behaviour to the point of inability to maintain weight; early post-prandial symptoms, possibly due to highly elevated gut hormone concentrations; and late post-prandial symptoms arising due to reactive hypoglycaemia. There is significant overlap in these categories, and it could be argued that all patients experience a continuum of symptoms of varying severity.

Novel potential treatments include Exendin 9-39 or other GLP1R antagonists, which could offer a highly targeted approach to treating the side effects of excess GLP-1 secretion, and in this study appeared to prevent significant hypoglycaemia and symptoms, and reduce early satiation. Ghrelin receptor

agonists are also in development, albeit not aimed at post-gastrectomy patients, however it would be interesting to see what effect, if any, replacement of ghrelin has in this group that effectively has near complete absence of circulating ghrelin. Given the current level of understanding of the actions of ghrelin, improved hunger and reduced incidence of hypoglycaemia are both possible.

While not discussed at all in this thesis, micronutrient deficiency is quite possible after gastrectomy, even in the presence of supplementation. There is no standard protocol for monitoring of vitamin and mineral levels in post-gastrectomy patients, however the protocol given in appendix 1 is now in routine use in Addenbrooke's Hospital, having been developed during work on this thesis.

Finally, patient education is at present the best means to reduce the effect of post-prandial symptoms and risks associated with hypoglycaemia. Patient facing literature should discuss the mechanisms by which surgery changes nutrient sensing and processing, discuss early and late symptoms and provide guidance on diagnosis and treatment of hypoglycaemia.

11.2 FUTURE STUDIES

The ultimate goal within this patient group should be to develop a large scale randomised trial of a novel therapy for severe post-prandial symptoms and reactive hypoglycaemia in post-gastrectomy / oesophagectomy patients. Several smaller scale studies could provide the necessary physiological background to identify possible therapies and identify the therapeutic indications.

11.2.1 Identification of incidence and severity of hypoglycaemia unawareness

A combination of OGTTs with validated measures of hypoglycaemia unawareness, and CGM studies including symptom and dietary measures could identify which patients are at risk from hypoglycaemia and confirm the prevalence of clinically significant hypoglycaemia.

11.2.2 GLP-1 blockade in symptomatic post-gastrectomy patients

The Exendin 9-39 study described here was limited by the need to learn how to use the peptide safely (hence restricting early patients to those with few symptoms) and the availability of the peptide. Recruiting a greater number of participants, including those with significant symptoms, to a similar study would identify how critical GLP-1 is as a possible causative factor in early post-prandial symptoms, as well as later hypoglycaemia.

11.2.3 GLP-1 blockade early post-gastrectomy

Patients struggle with early satiation, nausea and reduced appetite immediately after surgery, and take months to years to adapt and return to a relatively normal quality of life. A study using Exendin 9-39 and a test meal in the early post-operative period (e.g. 2 weeks) would identify whether some or all of these symptoms can be attributed to excess GLP-1 secretion.

11.2.4 Ghrelin replacement

As discussed above, the use of a ghrelin receptor agonist, presently in development for cancer cachexia and frailty, in the post-gastrectomy patient group would be both an elegant means to investigate the physiological role of ghrelin in man, and to investigate a potential means for improving appetite in post-operative patients.

11.2.5 Further detailed assessment of post-operative enteric adaptation

Increasing the number of peptidomic samples (both plasma and biopsy, and from the same patient pre- and post-operation) would significantly increase the power of the study to detect changes in hormone profile. Novel techniques, such as single cell RNAseq, while currently prohibitively expensive, could provide a highly sensitive assessment for adaptive change in all epithelial cell populations, not just EECs, and guide future clinical approaches.

11.3 SUMMARY

Oesophago-gastric resection results in a remarkably consistent physiological response to nutrient ingestion, with enhanced secretion of enteric and pancreatic hormones, and disposition of ingested glucose. There is however significant variability in both symptoms and glucose handling, reflecting heterogeneity in the patient response to the underlying physiological changes. Classically, patients with a significant symptomatic burden have been labelled as suffering from “dumping syndrome”. It is clear that this is an inadequate term to encompass the many problems faced by this patient group, which may not even present symptomatically (specifically hypoglycaemia unawareness and absence of hunger). It can reasonably be argued that the range of symptoms experienced by patients represents differences in functional adaptation, and in underlying insulin sensitivity and baseline insulin secretory capacity (as a key step in hypoglycaemia). Dumping syndrome is therefore an inadequate term, and its use should be replaced by a combination of objective (particularly hypoglycaemia risk) and subjective measures to identify clinically relevant hypoglycaemia, and key quality of life limiting symptoms that can be managed as best as possible. Included in this, new technologies such as continuous glucose monitoring could enhance the clinical assessment of post-operative patients.

This thesis has found no evidence of underlying functional adaptations within enteroendocrine cells after gastrectomy, supporting the hypothesis that the enhanced gut hormone secretion seen in this group is primarily driven by accelerated delivery of higher concentrations of ingested nutrients to the midgut.

Most excitingly, it appears that GLP-1 alone is a key metabolic factor in the aetiology of reactive hypoglycaemia, early satiation and negative post-prandial symptoms. This therefore represents a novel and highly specific therapeutic target for the range of problems experienced by patients after oesophago-gastric resection.

To summarise, this thesis identifies key metabolic factors which could be responsible for the significant clinical and symptomatic morbidity experienced after foregut resection, and proposes both an evolution of how this patient group is assessed, and a novel therapeutic direction.

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APPENDIX 1 – MICRONUTRIENT MONITORING AFTER GASTRECTOMY

GUIDELINES FOR MICRONUTRIENT SUPPLEMENTATION AND MONITORING AFTER TOTAL AND SUBTOTAL GASTRECTOMY

KEY MESSAGES

- Vitamins and minerals are likely to be poorly absorbed after gastrectomy
- All patients should take a daily multivitamin and mineral tablet
- After total gastrectomy, all patients should receive 12 weekly vitamin B12 injections
- It is possible that micronutrient deficiency could develop even with supplementation
- All patients should be regularly screened for micronutrient deficiencies as outlined below

SUMMARY

All patients who have undergone total or partial gastrectomy (excluding sleeve gastrectomy) should receive daily oral multivitamin and mineral supplementation and intramuscular vitamin B12 supplementation every 12 weeks. Micronutrient status should be monitored as per the schedule below:

Test	**Frequency**
U&Es (including magnesium)	Six monthly for two years, then annually
Creatinine	Six monthly for two years, then annually
Liver function tests	Six monthly for two years, then annually
Full blood count	Six monthly for two years, then annually
Ferritin	Six monthly for two years, then annually
B12 (pre-injection)	Annually
Folate	Six monthly for two years, then annually
Calcium	Six monthly for two years, then annually
Vitamin D	Six monthly for two years, then annually
INR	Six monthly for two years, then annually
Vitamin A	Annually
Vitamin E	Annually
Zinc	Annually
Copper	Annually
Selenium	Annually

SCOPE

Departmental – pertinent to oesophago-gastric surgery and gastroenterology.

PURPOSE

Guidelines for micronutrient supplementation and monitoring in the gastrectomy population, to facilitate prevention and early detection of micronutrient deficiency.
These guidelines are not intended to be used in the bariatric surgery population.

DEFINITIONS

Gastrectomy – total or subtotal gastrectomy with Roux-en-Y reconstruction or equivalent, NOT sleeve gastrectomy.

INTRODUCTION

The long-term metabolic and nutritional consequences of gastrectomy are unclear, as there have been no good quality long-term studies. This document is based on guidelines published for the bariatric surgical population, which have been amended to reflect the different nutritional challenges experienced by the gastrectomy population[1-4]. The few long-term studies of micronutrient deficiency after upper gastrointestinal surgery support this approach[5,6].

The clinical presentation of micronutrient deficiency can be subtle, and may include non-specific visual and neurological symptoms. The presence of unexplained non-specific symptoms in the post-gastrectomy patient should instigate prompt biochemical assessment and consideration of referral to a specialist in nutrition or clinical biochemistry.

A comprehensive set of guidance on the management of long-term symptoms in this patient group can be accessed at: http://fg.bmj.com/content/early/2016/10/14/flgastro-2016-100714[7].

Within this document, key recommendations are highlighted in bold, above rationale for the guidance in normal script.

This document is intended to guide the hospital team and general practitioner caring for a patient who has undergone total or partial gastrectomy. Within the prophylactic total gastrectomy group, this includes patients undergoing surgery in their 20s, who may manifest micronutrient deficiencies years or decades later.

All patients should have testing as below every six months for the first two years post-surgery, then annually thereafter. Micronutrient deficiencies can still occur many years after the index surgery, although the interval of testing may be extended if the patient has experienced many years of stable results (albeit with a low threshold for a full micronutrient assessment in the presence of any symptoms). Replacement of micronutrient deficiencies in this population can be challenging, and carry its own risks, and consideration should be given to seeking specialist support from local nutrition services and / or the specialist dietitians within the Cambridge Oesophago-Gastric Centre.

It is worth noting that some drug formulations, particularly enteric coated tablets, are poorly absorbed in this population, as there is no reservoir in which the tablets can be dissolved, and intestinal transit is rapid.

RESPONSIBILITIES

This document has been produced with input from the departments of surgery (Mr Geoffrey Roberts, Mr Richard Hardwick), Gastroenterology (Dr Dunecan Massey, Professor Rebecca Fitzgerald), Clinical Biochemistry (Dr Claire Meek, Professor Fiona Gribble, Dr Anita Sarker) and Dietetics (Miss Nicola Sunderland, Mrs Sam Grimes).

It has been externally peer reviewed by Dr J Andreyev, Consultant Gastroenterologist, The Royal Marsden Hospital.

It has been reviewed by the oesophago-gastric cancer specialist MDT.

GUIDANCE

ROUTINE SUPPLEMENTS IN THE POST-GASTRECTOMY PATIENT

All patients after gastrectomy are recommended to receive a daily multivitamin and mineral tablet (including Vitamin B complex), and at least three monthly Vitamin B12 intramuscular injection regardless of nutritional state. Simple multivitamin tablets without minerals are insufficient. Over the counter A-Z multivitamin and mineral supplements are normally adequate.

PERSISTENT MALABSORPTION AND DIARRHOEA

Patients with persisting malabsorption may have another underlying cause and we recommend a low threshold for screening for pancreatic exocrine insufficiency, coeliac disease, bile acid malabsorption and small intestinal bacterial overgrowth in refractory cases of micro or macro-nutrient deficiency.

Steatorrhoea may be underdiagnosed in the post-gastrectomy group, and result in multiple, refractory, vitamin and mineral deficiencies. The primary causes are pancreatic exocrine insufficiency, bile acid malabsorption and small intestinal bacterial overgrowth. We recommend that patients with steatorrhoea be investigated with faecal elastase and hydrogen / methane breath tests and, if these are negative, a SeHCAT scan.

GENERAL BIOCHEMISTRY

Annual creatinine, urea, electrolytes (including magnesium) and liver function tests should be performed.

Patients are at risk of dehydration and protein energy malnutrition. Unfortunately there is no single biochemical test which can give an indication of global nutritional status. All biochemical measurements need to be interpreted in the light of clinical factors, including the patient's weight, any recent weight change and the presence of any inflammatory or infective processes, which will increase nutritional requirements. Albumin can be regarded as a (poor) surrogate marker of chronic malnutrition in the absence of acute illness (i.e. when the C-reactive protein and white cell count are both normal).

ANAEMIA

To identify and prevent anaemia, annual testing for Full Blood Count, ferritin, transferrin saturation, B12 (pre-injection) and folate should be performed. All pregnancies in this patient group should be regarded as at higher risk for neural tube defect and the mother should be treated with high dose folate commencing prior to pregnancy.

Absorption of iron, folate and B12 is significantly impaired after gastrectomy[8]. Many patients may have anaemia due to a deficiency of more than one nutrient. Regardless of the mean corpuscular volume, all patients presenting with anaemia should have a full assessment of haematinics, vitamin B12 and folate, with supplementation as indicated. Oral iron replacement should be with chewable or soluble tablets, as non-dissolvable forms can pass intact through the entire intestinal tract[9].

The high risk of folate malabsorption after gastrectomy means that all women considering pregnancy should be treated as at risk of a neural tube defect, and treated accordingly with high dose folate[10]. Women should be encouraged to have planned pregnancies with a pre-pregnancy nutritional assessment. It should also be noted that some multivitamin preparations contain high levels of Vitamin A and may be inappropriate in the pre-conception and pregnant period.

BONE DENSITY

All patients should have annual testing for calcium and vitamin D.

It is possible that patients are at risk of osteoporosis due to impaired absorption of calcium and vitamin D after gastrectomy. Limited studies indicated the risk may be as high as 71% in the post-menopausal female population[11-16]. This is an area of clinical uncertainty and will benefit from further research.

There is at present insufficient evidence to support routine DXA screening for osteoporosis in this patient group, however this may change in the future. We recommend a low threshold for DXA scanning in this population, and opportunistic screening should be considered in all post-gastrectomy patients over the age of 50 or post-menopause (whichever comes earlier).

FAT SOLUBLE VITAMINS

All patients should have annual testing for vitamins A, E and D, and an INR check which provides an assessment of the adequacy of vitamin K stores.

The degree and severity of fat malabsorption in patients after a gastrectomy is unclear, although recent literature suggests it is a notable risk and carries the added complication of fat soluble vitamin malabsorption[17].

Patients with deficiencies should be given supplementation and appropriate dietary advice. A low threshold for interval testing should be applied if the patient has steatorrhea or non-specific visual or neurological symptoms.

VITAMIN C

Testing for possible vitamin C deficiency should be performed after consultation with a clinical biochemist.

The incidence of Vitamin C deficiency in bariatric surgery patients is up to 34%[18] and Vitamin C malabsorption is possible in the post-gastrectomy group. Early diagnosis and treatment of deficiency may prevent clinical manifestations, however accurate measurement is challenging and requires specialist input. Routine testing is not recommended.

ZINC AND COPPER

All patients should have annual testing for zinc and copper levels and supplementation, if necessary, should be at a ratio of 8-15mg of Zinc to 1mg of Copper. Supplementation of Zinc or Copper without the other must be avoided.

Zinc and copper are primarily absorbed in the proximal small intestine, which is partly bypassed after a gastrectomy[19]. Deficiency of either should be considered in the post gastrectomy population. Zinc and copper compete for the same transporter for absorption from the intestine into the blood, and excessive zinc consumption, for example during supplementation, can precipitate copper deficiency. Copper deficiency presents insidiously but can cause neurological abnormalities, gait disturbance and anaemia. Absorption of supplemented zinc and copper may be poor.

SELENIUM

All patients should have selenium levels checked annually.

There is no evidence to support routine testing for selenium levels in the absence of chronic diarrhoea or other evidence of malabsorption[20]. However, the absence of long-term data in the gastrectomy cohort limits our recommendations, and unpublished data suggest a not inconsiderable incidence of selenium deficiency in the post-gastrectomy population.

THIAMINE

There is no evidence for routine testing for thiamine levels, however if a patient suffers significant weight loss or has symptoms suggestive of Wernicke / Korsakoff syndrome they should be promptly admitted to hospital for IV thiamine replacement.

MONITORING COMPLIANCE WITH AND THE EFFECTIVENESS OF THIS DOCUMENT

These are guidelines, representing best available evidence, and are intended to guide both local practice and support GPs following up patients who have undergone gastrectomy. It is envisaged that these guidelines will be circulated to GPs at the point of discharge of patients from routine outpatient follow up, and it will therefore not be possible to formally assess compliance in all patients, given our national catchment area for prophylactic gastrectomy.

For local patients, compliance will be assessed by internal audit on a two-yearly basis, responsibility resting with the oesophago-gastric MDT.

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