Insight into appetite and blood glucose regulation in anorexia nervosa and health:

Examining gastrointestinal changes in starvation and with short-term refeeding

A thesis submitted by

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Dedication

This thesis is dedicated to my daughter, Allegra.

"Well, maybe it started that way. As a dream, but doesn't everything. Those buildings. These lights. This whole city. Somebody had to dream about it first. And maybe that is what I did. I dreamed about coming here, but then I did it."

– Roald Dahl, James and the Giant Peach

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Abbreviations

3-OMG; 3-ortho-methylglucose

AN; Anorexia nervosa

ANOVA; Analysis of variance

ARC; Arcuate nucleus

AUC; Area under the curve

BL; Baseline

BMI; Body mass index

CCK; Cholecystokinin

CDC; Centers for Disease Control and Prevention

CO_2; Carbon Dioxide

CV; Coefficient of variation

DPP-IV; Dipeptidyl peptidase IV

DSM5; Diagnostic and Statistical Manual of Mental Disorders

EBW; Expected body weight

EDE-Q; Eating Disorders Examination Questionnaire

FFQ; Food frequency questionnaire

GI; Gastrointestinal

GIP; Glucose-dependent insulinotropic peptide

GIS; Gastrointestinal Symptom Score

GLP-1; Glucagon-like peptide-1

GOAT; Ghrelin-O-acyl-transferase

HbA1c; Glycated hemoglobin

HC; Healthy control

HOMA; Homeostatic model assessment

iAUC; Incremental area under the curve

ID; Intraduodenal

IV; Intravenous

O_2; Oxygen

OGTT; Oral glucose tolerance test

PP; Pancreatic polypeptide

PYY; Peptide YY

RCADS; Revised Children's Anxiety and Depression Scale

REE; Resting energy expenditure

RMR; Resting metabolic rate

RQ; Respiratory quotient

SD; Standard deviation

SEM; Standard error of the mean

STAI; State Trait Anxiety Inventory

TEF; Thermic effect of feeding

TFEQ; Three-Factor Eating Questionnaire

VAS; Visual Analogue Scale

VCO_2; Volume of carbon dioxide

VO_2; Volume of oxygen

Abstract

Anorexia nervosa (AN) is a mental health disorder characterised by a restriction of energy intake relative to requirements, typically leading to a significantly low body weight, an intense fear of gaining weight or becoming fat, and a disturbance in the way in which one's body weight or shape is experienced (1). Patients frequently experience high levels of anxiety (2), as well as numerous other psychological and medical comorbidities (3), including hypoglycemia (4, 5). Despite severe malnutrition due to chronic dietary restriction, patients with AN also commonly report reduced perceptions of hunger and increased fullness (6, 7), and a high frequency of gastrointestinal (GI) symptoms, including nausea, abdominal pain, bloating and heartburn (8-10), all of which may contribute to the disordered eating behaviour. Current treatment of AN focuses on medical stabilisation followed by nutritional rehabilitation and either individual or family-based psychological therapies (11). However, disordered eating behaviour frequently continues post-treatment, with studies reporting between 30 and 67 % of patients relapsing within two years (12-14), and up to ~50% (13, 15) developing binge eating and/or purging. Hence, there is an urgent need to develop a better understanding of the mechanisms underlying the disturbances in glycemia, appetite and GI symptoms in patients with AN.

The GI tract plays a critical role in the regulation of glycemia and appetite in health, however, its role in mediating changes in patients with AN remains poorly defined. This thesis examined the GI mechanisms involved in appetite and glycemic control in anorexia nervosa, during starvation and with nutritional rehabilitation. The work submitted for this

thesis comprises a mechanistic study in health, as well as the outcomes of a large multi-variable study in patients with anorexia nervosa.

In health, the presence of nutrients in the GI tract results in increased fullness, and decreased hunger and subsequent energy intake. This is mediated by several inter-related changes in GI function, including the slowing of gastric emptying (which prolongs gastric distension and the perception of fullness), and the release of GI hormones including cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY), as well as the suppression of ghrelin. The rate of gastric emptying and the secretion of the incretin hormones, GLP-1 and glucose-dependent insulinotropic peptide (GIP) are also important determinants of postprandial glycemia (16). The study presented in **Chapter 2** generated new knowledge regarding the physiological effects of active GLP-1 and GIP concentrations on responses to fat in healthy lean males. The actions of endogenous GLP-1 and GIP to slow gastric emptying and reduce postprandial glycemia are limited by their rapid degradation by the enzyme dipeptidyl peptidase IV (DPP-IV). We utilised the DPP-IV inhibitor, vildagliptin, to modulate the effects of intraduodenal (ID) fat, examining downstream effects on glycemia, postprandial triglycerides, energy intake and energy expenditure.

Both gastric emptying and GI hormone secretion are sensitive to changes in nutrient exposure (17, 18). For example, a 4-day fast slows gastric emptying in health (17), and a 30% dietary restriction for 12 weeks modifies postprandial GI hormone release in obesity (18). The studies outlined in **Chapters 3 and 4** of this thesis explored the hypotheses that in patients with AN (i) prolonged energy restriction induces pathophysiological changes in the GI nutrient sensing mechanisms involved in appetite regulation and glycemia when

compared with healthy controls (HCs), and (ii) refeeding would rapidly alter GI function leading to improvements in appetite perceptions and postprandial glycemia.

The key findings of the studies were:

1. DPP-IV inhibition in health modulated the effects of ID fat to enhance active GLP-1 and GIP, stimulate insulin and suppress glucagon, thereby reducing glycemia (**Chapter 2**).
2. DPP-IV inhibition also reduced total PYY and PYY(3-36), and increased energy expenditure, without affecting energy intake (**Chapter 2**).
3. In response to a mixed-nutrient test meal, malnourished patients with anorexia nervosa had 'flattened' postprandial glucose, with the reduced initial rise in glucose associated with slower gastric emptying. In addition, plasma insulin was lower, while C-peptide tended to be higher, suggesting increased insulin clearance. Fasting glucagon was also elevated and not suppressed postprandially, and GLP-1 appeared higher, probably due to higher baseline concentrations (**Chapter 3**).
4. After two weeks of refeeding, patients with anorexia nervosa had a greater postprandial rise in glucose, faster gastric emptying, increased 3-OMG and reduced baseline insulin and C-peptide compared with Wk0. While overall glucose responses in patients remained less, there were no significant differences in gastric emptying, baseline glucagon or postprandial insulin compared with HCs (**Chapter 3**).
5. Malnourished patients with anorexia nervosa before a mixed-nutrient test-meal had increased fullness, bloating, anxiety, acyl ghrelin and PYY, and decreased hunger, while postprandially patients also had increased acyl ghrelin, bloating and

anxiety, and decreased hunger. These findings suggested a disconnect between hunger and acyl ghrelin (**Chapter 4**).

6. After two weeks of refeeding, patients with anorexia nervosa had decreased baseline PYY, fullness and anxiety, and increased baseline and postprandial hunger, with these improvements implicating malnutrition in the observed disturbances seen in starved patients (**Chapter 4**).
7. In patients with anorexia nervosa, no improvements were seen in baseline or postprandial acyl ghrelin or bloating, nor in postprandial anxiety with short-term refeeding (**Chapter 4**).

In summary, the studies described in this thesis have advanced the understanding of GI mechanisms underlying the regulation of glycemia, appetite and energy intake in both healthy individuals and patients with AN. In AN, starvation and reduced nutrient exposure instigate significant pathophysiological changes in the GI mechanisms involved in the regulation of glycemia and appetite, while short-term nutritional rehabilitation and re-exposure to nutrients result in partial improvements in these GI factors. Future research should examine the effects of longer-term refeeding and weight restoration to clarify whether GI function can be fully restored to normal or if persistent disturbances contribute to poor treatment outcomes.

Chapter 1: Introduction

1.1 Anorexia nervosa: Morbidity and medical complications

Anorexia nervosa (AN) is a serious mental health condition characterised by a restriction of energy intake relative to requirements, typically leading to a significantly low body weight, an intense fear of gaining weight or becoming fat, and a disturbance in the way in which one's body weight or shape is experienced (1). AN affects between 1.7 and 3.6% of females (19), and with onset typically during adolescence (20) AN has severe impacts on individuals, families and their surrounding communities. With 20% mortality at 20 years after diagnosis, AN has the highest mortality rate of any psychological illness (21). It is, therefore, not surprising that eating disorders are the second highest mental health diagnosis leading to hospitalisation in young Australian females (22). However, given that disordered eating behaviour frequently continues post-treatment, with studies reporting between 30 and 67 % of patients with AN relapsing within two years (12-14), and up to ~45% (13, 15, 21) developing binge eating and/or purging after treatment, improved treatment strategies are desperately needed. Since inadequate nutrient intake is a key feature of AN, insight into the physiological mechanisms underlying nutrient intake regulation is likely to be pivotal to the development of more effective management strategies.

AN is characterised by chronic dietary restriction, as well as anxiety about weight gain or becoming "fat" (1). Despite severe malnutrition, patients experience reduced hunger and increased fullness (6, 7) and high levels of gastrointestinal (GI) symptoms (8-10) with ~75% of patients with AN meeting criteria for at least one functional GI disorder (9). It is possible that the appetite and GI symptom disturbances contribute to persistently low caloric intake (7, 23). Following nutritional rehabilitation, hunger increases and fullness decreases (6, 7), while GI symptoms are often exacerbated during initial

refeeding and can persist in up to 30% of patients post-treatment (24). Furthermore, caloric intake remains lower after refeeding than in healthy controls (HCs) (23). Patients with AN also experience high levels of pre- and post-meal psychological distress (25) that may affect appetite, since anxiety is known to alter hunger and food intake in health (26, 27). The mechanisms underlying changes in appetite are poorly defined in AN, but given the GI tract is known to play a fundamental role in appetite regulation in health, it is possible that the appetite disturbances in AN are associated with altered GI function. The studies outlined in this thesis therefore investigated the hypothesis that starvation-induced changes in GI function may contribute to the appetite disturbances seen in AN, and that these may be restored with nutritional rehabilitation.

Malnutrition in AN also results in extensive pathophysiological changes to organ function and metabolism resulting in high rates of medical complications (28) including cardiovascular complications (29), reduced bone density (29), abnormal liver function, hypothermia, hypophosphatemia and hypoglycemia (30). Postprandial hypoglycemia (blood glucose ≤3.5 mmol/L) is observed in ~30% of hospitalised patients with AN (5), with several case reports in patients with AN associating hypoglycemia with coma and/or death (4, 31, 32). However, clinically, blood glucose levels are assessed at random times, or with patients in the fasted state, and thus, may not capture the risk for postprandial hypoglycemia. In health, the GI tract plays an important role in the regulation of postprandial glycemia via the slowing of gastric emptying and the stimulation of the incretin hormones GLP-1 and GIP (16), hence, it is feasible that postprandial hypoglycemia in AN may result from disordered GI nutrient sensing following chronic nutrient deprivation. This review will outline the basic GI physiology underlying the regulation of postprandial glycemia, appetite and energy intake in health, followed by

what is currently known about these GI mechanisms in patients with AN both during starvation and after nutritional rehabilitation.

1.2 GI factors regulating postprandial glycemia and appetite in health

The GI tract plays a pivotal role in both glycemic control (16) and appetite regulation (33) in health. The interaction of nutrients with the stomach and small intestine induces a number of changes in GI function, including the release of several GI hormones, including peptide YY (PYY) (34), cholecystokinin (CCK) (35), glucagon-like peptide-1 (GLP-1) (36), glucose-dependent insulinotropic polypeptide (GIP) (37), and the suppression of ghrelin (38) **(Figure 1.1)**. These hormones modulate antral, pyloric and duodenal motility, which slows the rate of gastric emptying, prolongs gastric distension and consequently suppresses subsequent energy intake (39-41). In addition, gastric emptying regulates blood glucose concentrations by moderating the rate at which nutrients enter the small intestine, and the release of the incretin (insulin-stimulating) hormones, GLP-1 and GIP. Although the relationship of GI factors to glycemic control

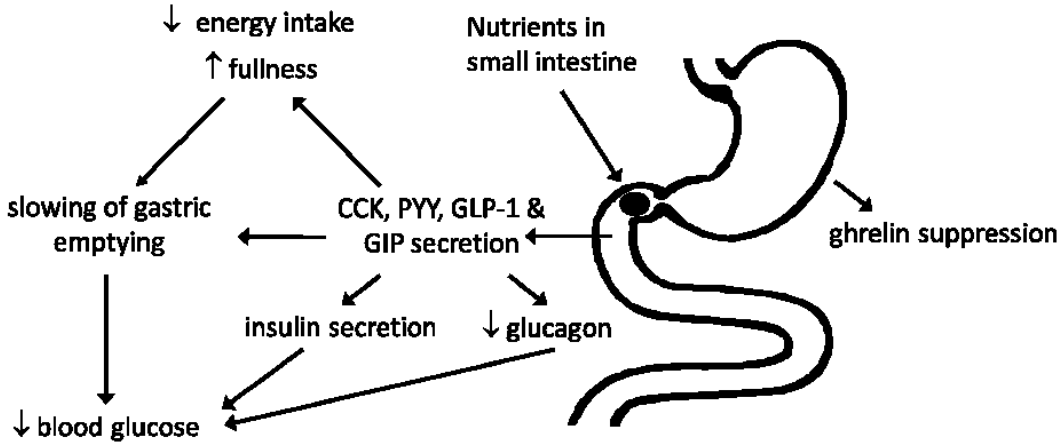

Figure 1.1.
As nutrients enter the GI tract, ghrelin is suppressed and CCK, PYY, GLP-1 and GIP are released, which signals the slowing of gastric emptying and regulates blood glucose and appetite.

and appetite is established in health, it remains unknown whether alterations in these regulatory systems may contribute to the postprandial hypoglycemia, disturbed appetite perceptions and high GI symptom prevalence observed in patients with AN.

1.2.1 Gastric emptying

Gastric emptying refers to the process of gradually transferring a meal from the stomach into the small intestine. It is regulated by tightly coordinated contractile activity, or motor activity, in the proximal and distal regions of the stomach, pylorus (the opening from the stomach into the duodenum) and the duodenum. This activity, in turn, is modified by the constantly changing nature of small intestinal content, with the arrival of nutrients.

The stomach can be divided into three functional regions: the proximal compartment, including the fundus and proximal corpus (acting as a reservoir for ingested food); the distal compartment, consisting of the distal corpus and antrum (and responsible for the mixing and grinding of solid foods), and the pylorus, which controls the delivery of partially digested food (chyme) into the small intestine at a rate (~1-4 kcal/min in health) that allows for optimal digestion and absorption of ingested nutrients (42). Following meal ingestion, the proximal stomach relaxes, allowing for the meal to be accommodated without discomfort. Contractions in the antrum then grind the solid food into small particles and propel the resulting chyme through the pylorus into the duodenum. Gastric emptying occurs only when the pylorus is open and, as such, tonic and phasic contractions of the pylorus act as a brake to regulate the rate of emptying into a receptive duodenum. Transpyloric flow is regulated by the integration of motor activity in the proximal and distal stomach, pylorus and duodenum **(Figure 1.2, p.22)**.

Gastric emptying can be assessed using scintigraphy (the 'gold-standard' technique of detecting a radioactive tracer to observe an organ or its function), ^{13}carbon-breath tests, ultrasound and magnetic resonance imaging. Patterns of gastric emptying vary with the nature (i.e. liquid or solid), macronutrient composition and caloric content of the ingested meal. Nutrient-containing liquids empty from the stomach in a linear fashion, driven mainly by slow, tonic contraction of the proximal stomach. In contrast, non-nutrient liquids empty relatively rapidly and exponentially from the stomach. The emptying of solids is characterised by an initial 'lag phase', which reflects the time required to initially grind food into small particles (1-2 mm in size) by antral contractions and to mix chyme with gastric secretions, after which time gastric emptying commences and follows a linear pattern. The arrival of nutrients in the small intestinal lumen then activates hormonal (e.g. CCK, GLP-1 and PYY) and neural feedback signals, which reinforce the stimulation of pyloric contractions and further contribute to the slowing of gastric emptying (24).

The retention of food in the stomach causes gastric distension and activates mechanosensitive vagal afferents. These changes prolong the sensation of fullness and inhibit further food intake (34). As gastric emptying progresses, gastric distension diminishes, and feedback arising from the presence of nutrients in the small intestine, including the release of a number of appetite-regulating hormones (particularly GLP-1, PYY and CCK), and the suppression of ghrelin, leads to signalling to the brain to also induce satiety. Released from the small intestine in response to food, GLP-1 and GIP regulate postprandial blood glucose via their effects on insulin and glucagon release, as well as the slowing of gastric emptying by GLP-1 (16) (**Figure 1.3)**.

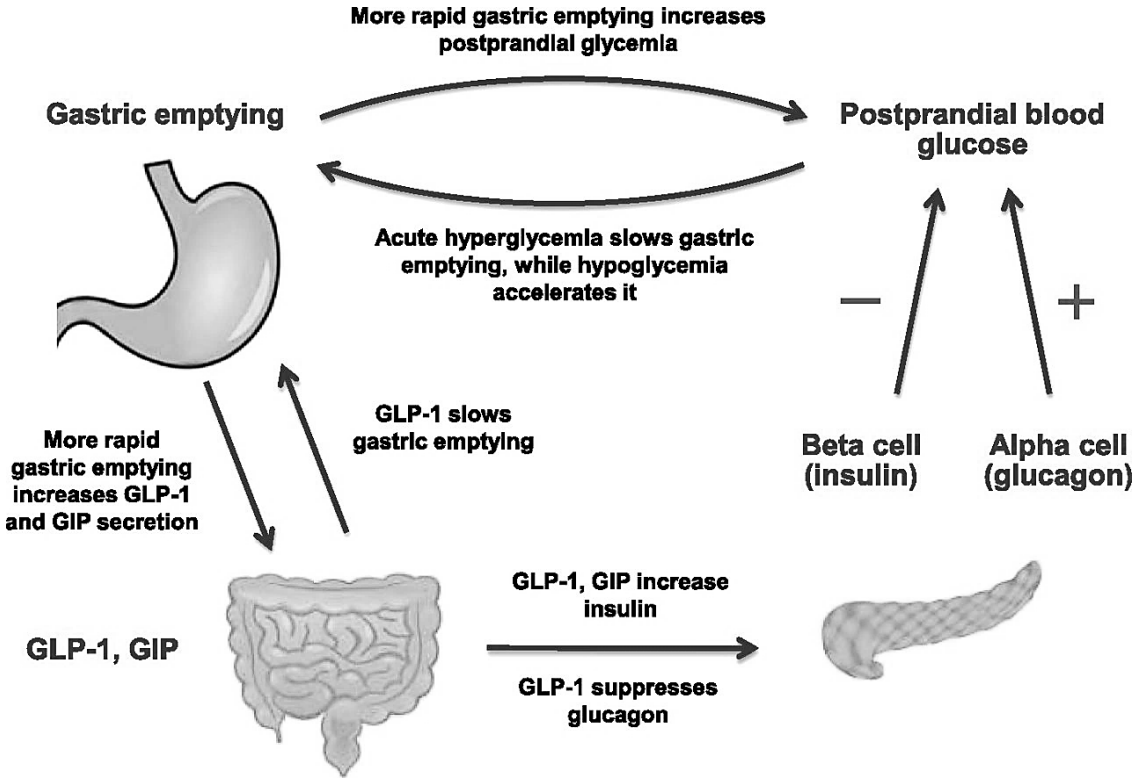

Figure 1.3.
Summary of the interdependent relationships of gastric emptying, postprandial glycemia and incretin hormone secretion (adapted from Marathe et al. (16), American Diabetes Association [Diabetes Care, American Diabetes Association, 2013]. Copyright and all rights reserved. Material from this publication has been used with the permission of American Diabetes Association).

1.2.2 Incretin hormones

GLP-1 and GIP are incretin hormones, stimulating glucose-dependent insulin secretion from the pancreas (43, 44) **(Figure 1.3)**. GLP-1 is secreted from enteroendocrine L-cells located predominantly in the ileum and colon (45), but also in the duodenum (46). GIP is synthesised in K-cells located predominantly in the duodenum and upper jejunum (47). Both GLP-1 and GIP are released upon food intake, most potently by fat (48), to facilitate the absorption and utilisation of ingested nutrients. GLP-1 initially rises within 15 minutes, before food has reached the distal ileum or colon (49), potentially due to either a 'cephalic phase' response to food detected in the mouth, or localised release from the

more recently identified duodenal L-cells (50). Studies have also implicated CCK (released early from the proximal small intestine) in stimulating GLP-1 release, suggesting a proximal-distal small intestinal feedback loop may be involved in stimulating early GLP-1 secretion (51). The later peak in GLP-1 concentrations is most likely stimulated by the presence of nutrients in the distal ileum (41).

GLP-1 appears to play an important role in food intake regulation. Exogenous GLP-1(7-36) administration has been shown to stimulate pyloric contractions, inhibiting transpyloric flow (52, 53), and potently slows gastric emptying (52, 54, 55). Although this may maintain postprandial gastric distension and enhance fullness, there are inconsistent reports regarding the effect of GLP-1 on desire to eat and energy intake. Several studies showed that intravenous infusion of physiological doses of GLP-1 reduce meal size in health (56, 57), however, another study failed to inhibit eating (58). Furthermore, supraphysiological doses reduced meal size in one study (57), but not in another (59). In addition, the GLP-1 receptor antagonist, exendin (9-39), did not increase meal size under various experimental conditions (60, 61). However, longer-term administration of GLP-1-receptor agonists (e.g. liraglutide, exenatide) are associated with reduced appetite and weight loss in obesity and Type 2 diabetes (62, 63). The latter studies suggest that the effect of GLP-1 on food intake is likely to be influenced by its rapid inactivation to its inactive form, GLP-1(9-36), by the enzyme, dipeptidyl peptidase-IV (DPP-IV). DPP-IV also cleaves the active form of GIP(1-42) into its inactive metabolite GIP(3-42), but GIP appears to be less susceptible to breakdown than GLP-1, with 40% of GIP remaining intact and bioactive compared to 20% of GLP-1 following intravenous infusion (64, 65).

GIP plays an important role in the regulation of blood glucose concentrations, with its significance demonstrated by the impaired glucose tolerance and defective glucose-dependent insulin secretion that results from GIP receptor antagonism (66, 67). GIP stimulates glucagon at basal glucose concentrations (68), and increases gastric emptying (69). In contrast, GLP-1 suppresses glucagon secretion (70, 71)**.** However, the dominant mechanism by which exogenous GLP-1 improves postprandial glycemia appears to be by slowing the rate of gastric emptying **(Figure 1.3)**, since postprandial insulin levels are reduced, rather than enhanced, by its administration (72, 73). Since DPP-IV rapidly converts GLP-1 and GIP to their inactive forms, a therapeutic strategy to improve blood glucose control has, therefore, been the development of inhibitors of DPP-IV predominantly to raise endogenous active GLP-1, but also GIP concentrations (74). Furthermore, DPP-IV inhibitors have also been reported to augment postprandial lipid mobilization and fat oxidation (75). Given GLP-1 and GIP may mediate changes not just in appetite and glycemia, but also metabolism and energy expenditure, the study described in **Chapter 2** examined whether dipeptidyl peptidase IV (DPP-IV) inhibition would enhance plasma active incretin concentrations and modulate the glycemic, gut hormone, triglyceride, energy expenditure, and energy intake responses to intraduodenal fat infusion.

1.2.3 Insulin

The cleavage of proinsulin results in the secretin of equimolar amounts of insulin and C-peptide from pancreatic β-cells in response to increased blood glucose and amino acids following meal ingestion. Because C-peptide is not taken up by other tissues (e.g. liver, other organs) it can be used as a marker of insulin secretion. Insulin's primary role is to stimulate cellular glucose uptake and lower blood glucose concentrations **(Figure 1.3)**. It

does this in three key ways: by signalling to insulin-sensitive peripheral cells (e.g. skeletal muscle) to increase glucose uptake; by promoting glycogen production in the liver (glycogenesis); and by inhibiting the secretion of glucagon – a hormone that acts to increase blood glucose during hypoglycemia. Cell signalling occurs through insulin binding at insulin receptors on target tissues (e.g. skeletal muscle, adipose tissue), triggering glucose uptake via translocation of the GLUT4 glucose transporter. Insulin also has other actions, including stimulating fat synthesis, promoting triglyceride storage and promoting protein synthesis.

The role of insulin in appetite and energy intake regulation remains unclear. In healthy participants, postprandial insulin has been associated with decreased hunger and increased satiety (76), as well as lower subsequent energy intake (77), suggesting a possible role in short-term appetite regulation. However, using a euglycemic clamp to hold glucose concentrations steady, intravenous (IV) infusions of insulin had no effect on appetite or subsequent *ad libitum* meal intake, suggesting that physiological levels of insulin are unlikely to affect appetite or energy intake without changes in blood glucose concentrations (78). Further research is needed to clarify the effect of changes in glucose on insulin's appetite-regulating effects and the mechanisms underlying this interaction.

1.2.4 Glucagon

Glucagon is a 29-amino acid glucoregulatory peptide secreted from the α-cells of the pancreas when blood glucose concentrations fall below the normal range during the fasted state. Binding to glucagon receptors on the liver, it stimulates the breakdown of stored hepatic glycogen (glycogenolysis) and the production of hepatic glucose (gluconeogenesis) to return blood glucose to the normal range **(Figure 1.3)**. Glucagon

increases during hypoglycemia and long-term fasting, as well as in response to increasing levels of amino acids (79) and fatty acids (80). During and following a meal, when postprandial blood glucose is elevated, glucagon secretion is suppressed. Since the slowing of gastric emptying (81) and suppression of food intake (82-84) only seem to occur with exogenous glucagon at much higher than physiological levels seen after eating, there is currently inadequate evidence to support a major role for glucagon in appetite regulation.

1.2.5 Peptide YY

PYY is synthesised by endocrine L-cells located predominantly in the ileum and colon (85), where it is co-located with GLP-1 (86). Following nutrient ingestion, it is secreted as PYY(1-36) and cleaved by DPP-IV to PYY(3-36) – the major circulating form of the hormone (34, 87). Approximately 15 minutes after food intake, plasma PYY concentrations begin to increase, reaching a plateau within two hours (85). High protein meals result in higher PYY concentrations when compared with high-fat and high-carbohydrate meals, and this is associated with reduced hunger in both obesity and health (34).

PYY(3-36) has an important role in regulating GI function and energy intake. Studies using exogenous infusion of PYY have demonstrated inhibition of gastric emptying in animals (88, 89) and humans (90, 91), as well as reduced gastric secretion in humans (92). The two forms of PYY have been shown to have opposing effects on food intake: PYY(1-36) is orexigenic (93) and PYY(3-36) is anorexigenic (94). Exogenous PYY(3-36) administration in humans has been shown to reduce hunger ratings (94, 95) and perceived ability to eat (96), as well as subsequent energy intake (95-97). However,

exogenous PYY(3-36) infusions have additionally been associated with increased nausea (91, 95, 96), which may, at least in part, explain the effect on gastric emptying and energy intake suppression (91). In contrast, exogenous PYY(1-36) infusion has not been observed to induce nausea (91, 96), and while one study found no impact on ratings of prospective consumption to eat using an infusion rate of 0.8pmol/kg/min (91), another following a higher dose infusion (1.6pmol/kg/min) found increased premeal hunger and lower satiety levels (96). Although there are no human studies with PYY receptor antagonists, the anorectic effects of PYY_{3-36} are inhibited by a receptor antagonist in mice (98), suggesting that PYY(3-36) plays a physiological role in regulating energy intake. While exogenous PYY(3-36) administration studies suggest PYY(3-36) is anorexigenic, antagonist studies are needed to clarify the role of endogenous PYY in the regulation of energy intake in humans.

1.2.6 Cholecystokinin

CCK is primarily produced by endocrine I cells of the duodenal and proximal jejunal mucosa, with a small proportion also being produced by neurons in the GI tract and brain (99). It is secreted soon after the start of a meal, with fat and protein shown to potently stimulate its release, and carbohydrate to a lesser extent (35). CCK exists in multiple molecular forms ranging from 8 to 58 amino acids, and has a range of effects, including pancreatic enzyme release (35), the stimulation of gallbladder (35) and pyloric contractions (100) and slowing of gastric emptying (101, 102). Exogenous CCK administration has been observed to increase plasma PYY concentrations and the frequency of pyloric pressure waves, whilst reducing the number and amplitude of both antral and duodenal pressure waves (103). This suggests that CCK, and possibly PYY, may mediate the effect of small intestinal nutrient exposure on gastric motility and is

supported by the findings that the CCK-1 receptor antagonist, loxiglumide, attenuates the inhibitory effect of fat on gastric emptying (104).

It is well established that CCK stimulates pyloric contractions, and that pyloric stimulation is associated with suppression of energy intake (105). CCK is also an independent determinant of energy intake, with exogenous CCK administration shown to suppress food intake (106-109), reduce hunger(109) and increase perceived fullness, while reducing the desire to eat (58, 103). Infusion of CCK also increases pre-meal anxiety (109). Studies using CCK antagonists to block the effects of endogenous CCK demonstrate increased hunger (110, 111) and greater caloric intake (111), as well as attenuation of the effects of intraduodenal fat on fullness and food intake (112). In contrast, another study failed to show an effect of food intake after a carbohydrate-rich meal (113), potentially suggesting differences between macronutrients in their ability to stimulate CCK's satiety effects. Nevertheless, current evidence suggests CCK may have a moderate effect on energy intake and appetite regulation and play an indirect role through its modulation of the secretion of two other appetite-regulating peptides, PYY and ghrelin (114, 115).

1.2.7 Ghrelin

Ghrelin is a 28 amino-acid peptide with an acyl side-chain, n-octanoic acid, known for its appetite-stimulating properties in both animals (116) and humans (117, 118). Ghrelin signals to the brain via the autonomic nervous system, to adjust food intake and energy expenditure (119, 120). It is produced in the oxyntic glands of the gastric fundus next to the stomach lumen (121), and to a lesser extent in the small intestine and pancreas, where it is encoded by the preproghrelin gene. The enzyme, ghrelin-O-acyl-transferase

(GOAT), attaches a fatty acid side-chain (preferably C8 or C10) to the unacylated form of ghrelin (desacyl ghrelin) to create acyl ghrelin (121-123), which is the active form responsible for the metabolic effects. Ghrelin and GOAT share similar tissue expression profiles, with the highest expression in the pancreas and stomach in humans (124, 125).

Ghrelin has been shown to stimulate gastric emptying and hunger (118), and to increase food intake (117) and body weight (126). Ghrelin levels rise during fasting prior to meal ingestion and decrease postprandially (127). Moreover, the magnitude of the decrease is proportional to the caloric load and macronutrient content, with lipids being the least effective at suppressing ghrelin (128, 129). However, while ghrelin appears to stimulate hunger, the effects appear short-lived and do not stimulate longer term food intake, which is supported by the lack of chronic anorexigenic effect of ghrelin antagonists (130). Alternatively, it has been proposed that ghrelin's role is to prepare an organism for incoming food to metabolise and store energy efficiently (131, 132). This is supported by growing evidence suggesting the GOAT-ghrelin system may act as a nutrient sensor (131). With prolonged fasting in mice, GOAT transcript levels decrease, resulting in increased desacyl ghrelin levels.

Ghrelin appears to have an even more complex role in food intake and metabolism regulation, including, stimulating gut motility and gastric acid secretion (116, 133), modulating taste sensation and reward-seeking behaviour (134-136), stress and anxiety (137-140) and regulating glucose metabolism (141, 142). Both ghrelin and its receptor are expressed in pancreatic islets, and human studies demonstrate that ghrelin administration increases plasma glucose and decreases insulin (143-145). Continuous ghrelin infusion has been shown to suppress glucose-stimulated insulin secretion and

impair glucose tolerance (146). Growth hormone secretagogue receptors that bind ghrelin are also found in pancreatic α-cells, and may help ghrelin directly stimulate glucagon secretion (147). The mechanisms underlying ghrelin's role in glucose metabolism and appetite regulation is a rapidly increasing area of research, in the ongoing search for effective treatments for diabetes, obesity, GI motility disorders, cachexia and AN.

Taken together, there is substantial evidence demonstrating that a complex interaction of GI factors contributes to both blood glucose and food intake regulation in health. Given the disturbances in blood glucose and food intake observed in AN, it is plausible that changes in underlying GI function may also be present.

1.3 Effects of changes in nutrient exposure on GI function and impacts on glycemic and appetite regulation

In AN, chronic caloric deprivation and reduced nutrient exposure could potentially alter GI sensitivity to nutrients, which may underlie the disturbances observed in glycemia and appetite regulation. This section outlines the research in health and obesity that supports this hypothesis, demonstrating the acute GI changes that occur with altered nutrient exposure, followed by an overview of the effects of both starvation and refeeding on GI function in AN.

1.3.1 Effects of nutrient deprivation and excess in health and obesity

Both energy excess (148) and energy restriction (149) alter GI function, which has implications for appetite and glycemic regulation. Changes in GI sensitivity to nutrient deprivation or excess have been shown to impact on gastric emptying. In health, dietary changes have been shown to profoundly influence gastric emptying, with accelerated emptying of fat observed after two weeks on a high-fat diet (148, 150), and markedly slowed emptying of an oral glucose load observed after an acute 4-day fast (17). In rats, after a 65% energy-restricted diet for four weeks, gastric emptying duration was significantly prolonged compared with free-feeding controls, and normalised after returning to a non-energy restricted diet (152). These changes appear to be mediated by diet-induced alterations in small intestinal nutrient sensing mechanisms that result in altered GI hormone secretion, with consequent effects on gastric emptying, as well as the regulation of appetite and blood glucose. For example, after only 4 days on a 70% caloric restriction in obese males, fasting ghrelin was higher, while following intraduodenal lipid infusion there was a greater increase in PYY and a greater suppression of ghrelin when compared with baseline (149). Additionally, in a longer study, 12 weeks of 30% energy

restriction increased the effects of intraduodenal fat to stimulate PYY responses and suppress energy intake in obese males (18). In contrast, an overall decrease in PYY, CCK and insulin and increase in ghrelin and hunger were observed after 10 weeks of dietary restriction on a very low calorie diet for weight loss in obesity, and these defects were maintained at one-year follow-up (153). These differing results may have been due to greater dietary restriction on a very low calorie diet, no control group and the potential for gastric emptying of the oral mixed-nutrient test-meal to confound the postprandial hormone concentrations recorded (153), while both former studies observed hormonal changes directly in response to an intraduodenal infusion, using the most potent hormone stimulus, fat (18, 149).

Overall, there is convincing evidence that both acute and prolonged changes in nutrient exposure result in adaptations in the GI mechanisms involved in the control of appetite and glycemia. Since patients with AN experience chronic nutrient deprivation, it is feasible that malnutrition-induced changes in gastric emptying and GI hormones may underlie the disturbances in glycemia and appetite.

1.3.2 Changes in GI function in AN

1.3.2.1 Gastric emptying in AN

The rate of nutrient delivery to the small intestine may play an important role in GI hormone release, glycemia and appetite perceptions in AN. Scintigraphy and ultrasound studies using solid and semi-solid, mixed-nutrient or protein-only meals have consistently demonstrated delayed gastric emptying in patients with AN compared with HCs (154-161). In contrast, results of studies examining liquid gastric emptying are less consistent, with some studies finding no difference in liquid emptying compared to HCs

(155, 157, 158), and others reporting it is delayed in AN (154, 159). This may indicate that the motor mechanisms that govern gastric emptying of solids may be selectively compromised in AN, although this hypothesis requires investigation. Delayed gastric emptying has been reported in both restrictive and binge/purge subtypes of AN (160). Since only some studies report a correlation between the magnitude of the delay in gastric emptying and duration of illness (162) or body weight (6, 161, 162), it remains unclear whether body weight, or perhaps the extent of recent dietary restriction, is driving the changes in gastric emptying. This is particularly hard to determine due to inconsistencies in study design including patient characteristics (e.g. the inclusion of outpatients or inpatients) and differences in nutritional status (e.g. inclusion of patients prior to refeeding vs. those who had commenced nutritional rehabilitation).

Previous studies have reported that refeeding rapidly and markedly improves gastric emptying in AN. Gastric emptying is significantly faster after 2-4 weeks of refeeding (154), and appears to improve further towards the healthy range within 2-6 months (160, 163). However, to date, only one study has evaluated temporal changes in the longer-term using a 2D ultrasound technique, finding that slowed gastric emptying improved in a small sample of patients with AN (restricting sub-type) (n=11) after 4 weeks of nutritional rehabilitation, and was significantly faster after 22 weeks (160). Thus, more research is needed to clarify the extent of refeeding required for gastric emptying to improve. Comprehensive studies on the mechanisms underlying delayed gastric emptying in AN are limited. However, several studies, including some case studies, have

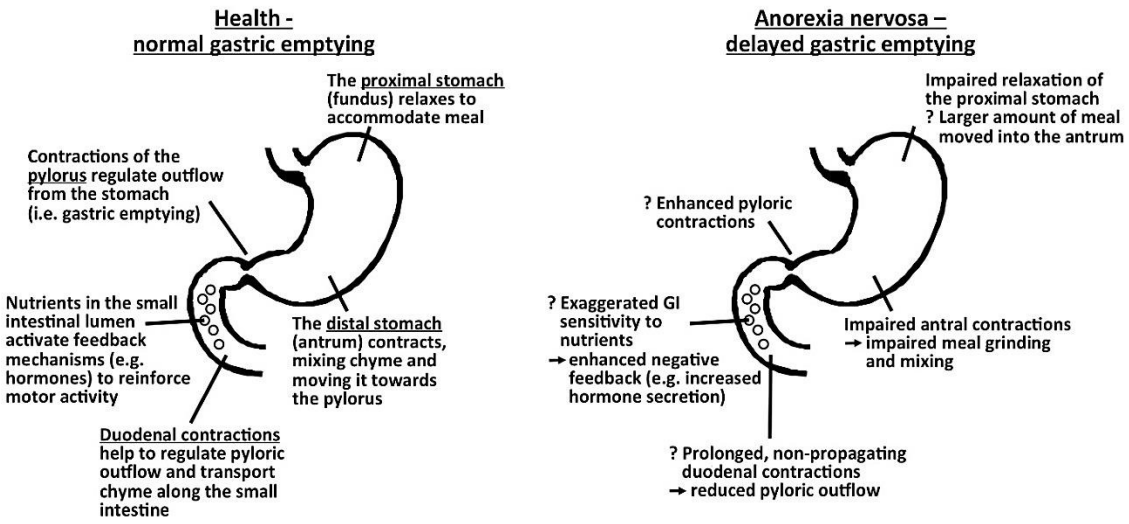

Figure 1.2.
Patterns of motor activity in the stomach and small intestine that regulate gastric emptying in health (left) and possible disturbances underlying delayed gastric emptying in anorexia nervosa (right) (Reprinted by permission from Springer Nature: Springer Nature, Encyclopedia of Eating and Feeding Disorders, Heruc et al., 2017 (24)).

reported disturbances in GI motor function (**Figure 1.2**). These include increased episodes of gastric dysrhythmias (i.e. disturbances in gastric myoelectrical activity that underlie the initiation of coordinated GI contractions) (155), impaired gastric accommodation (which compromises accommodation of the meal in the stomach) (9) and impaired antral contractility (155, 162), as well as prolonged, but non-propagating, contractions in the duodenum (164). However, the role of disturbances in pyloric motor function, as a key regulator of gastric emptying in health, as well as enteric nerve function, remain to be characterised.

It is also possible that feedback signalling induced by the arrival of nutrients in the small intestine, which modulates these motor and other GI functions, may be disturbed. Several studies have reported increased fasting and/or postprandial plasma concentrations of GI hormones, including CCK and PYY, in patients with AN compared with HCs, and these are discussed in the following sections. The effects of these hormones to slow gastric emptying could be enhanced, either via exaggerated release or through an enhanced sensitivity, brought about by physiological adaptations in small intestinal nutrient sensing mechanisms induced by chronic undernutrition.

Research has not yet looked at gastric emptying and hormone responses to food in the same patient sample to help clarify the mechanisms underlying delayed gastric emptying, and their relationship to disturbed appetite perceptions. In addition, delayed gastric emptying in patients with AN has frequently been associated with early satiety, bloating and abdominal distension (165-167). Since such GI symptoms can lead to difficulties in refeeding and weight restoration (165), increased understanding of the relationship between GI hormones and gastric emptying may help modify treatment strategies to improve symptom management and treatment compliance, and consequently their success. Moreover, despite the strong relationship between gastric emptying and blood glucose regulation, research has not concurrently assessed these variables, which may provide insight into the mechanisms underlying postprandial hypoglycemia in AN.

1.3.2.2 Incretins in AN

Since gastric emptying is delayed in patients with AN, nutrient delivery to the small intestinal enteroendocrine cells will be altered with consequences for the secretion of GI hormones. There has been limited research on the incretin hormone, GLP-1 in AN.

Investigations by Tomasik *et al*. indicated that both basal and postprandial GLP-1 were significantly lower in adolescents with AN than HCs (168-170). However, contrasting results have also been found, with average GLP-1 concentrations over 24 hours being reported to be significantly higher in patients with AN than those who were constitutionally thin, and trending towards higher than controls (171). Similarly, there is limited and conflicting research of changes in GIP in patients with AN, reporting higher (169), or lower (172), fasting and postprandial GIP concentrations, although patients in the latter study had already commenced treatment, resulting in difficulty distinguishing between the effects of starvation and refeeding on GIP (172). Given the inconsistent results thus far, further research is needed into any role GLP-1 and GIP might play in regulating glycemia and food intake in AN. Moreover, there is currently no research examining fasting or postprandial GLP-1 or GIP responses in patients with AN after nutritional rehabilitation.

1.3.2.3 Insulin in AN

In untreated patients with restrictive AN, research has consistently demonstrated lower fasting insulin (173-179) as well as attenuated responses following oral (179) or intravenous (173, 175) glucose tolerance tests and oral mixed-nutrient test-meals (177) compared with HCs. Two other studies also found a shift in insulin profile and longer time to peak, however, this may have been a consequence of patients consuming the mixed-nutrient test-meal over a 50 minute duration and likely prolonged gastric emptying (180) or small sample size (181). In addition, despite the observed lower fasting and postprandial insulin concentrations, no difference has been shown in insulin secretion (through examination of co-secreted C-peptide) between untreated patients with AN and controls (175). Therefore, the changes in plasma insulin are likely to be due to an

increased metabolic clearance rate of insulin in untreated patients with AN compared with controls (175, 178, 182). Furthermore, there is also literature supporting increased insulin sensitivity in patients with AN using euglycemic clamp (178), minimal-model-derived (174) and homeostatic model assessment (HOMA) (176, 183) measures.

Studies examining insulin after nutritional rehabilitation have yielded conflicting results. One study found that fasting and postprandial insulin responses remained lower in patients with AN than HCs after refeeding (179), while in contrast, a study showed fasting and postprandial responses increased with refeeding to HC levels (177). Both studies had sample sizes >20, followed patients up in an inpatient setting for 3-6 months, and refeeding had successfully increased weight. However, the former study used a 50g oral glucose tolerance test and the latter study used an oral mixed-nutrient meal, potentially contributing to the differing results and suggesting further research is required. In contrast, a third study found no difference in postprandial insulin responses between patients with AN before treatment and HCs, while observing reduced insulin responses after 2 and ~6 weeks of nutritional rehabilitation (180). It is possible that since the meal was being consumed during the first 50 minutes of the 120-minute blood sampling period, the post-meal-consumption sampling duration may have been too short to capture the full insulin response. However, nutritional rehabilitation studies have shown a normalisation of insulin receptor binding in patients with AN with weight gain (184), as well as reduced insulin sensitivity (185).

1.3.2.4 Glucagon in AN

Early research demonstrated that fasting glucagon was elevated in starvation (186), which is expected when nutrient intake is low and the body requires glucagon-stimulated glycogenolysis and gluconeogenesis to maintain blood glucose and supply energy. However, studies in patients with AN are inconsistent, with higher but not significantly different (173), not different (155, 187) and even lower fasting glucagon being reported compared with HCs (179, 188). Differing results are likely due to patients having varied nutritional intake prior to assessment (155), commencing refeeding prior to assessment (179, 187) and small sample size (155, 187). Research has found significantly higher postprandial glucagon in response to glucose ingestion in patients with AN than HCs and following 6-months of nutritional rehabilitation and weight gain, neither fasting nor postprandial glucagon differed from HCs (179).

1.3.2.5 PYY in AN

One small study found no difference in fasting PYY between patients with AN and healthy weight controls (172), nor postprandial differences when averaging concentrations in response to the test-meal over 3 hours. However, this may have disguised any temporal changes in PYY concentrations. Furthermore, the study had a small sample size and the test-meal calorie-content varied between subjects, with the volume consumed adjusted for body weight. In addition, growth hormone and Tanner staging were not controlled for in the adolescent population, despite evidence that fasting PYY is lowest, and nadir growth hormone levels highest, for Tanner stages 2-3 in adolescent girls (189). Although another study reported blunted average PYY in patients with AN measured every 4 hours over a 24-hour period compared to HCs and the constitutionally thin, blood-sampling time-points did not correspond to meal times,

making conclusions on the relationship between food intake and PYY response difficult (171). In contrast to the above studies, a larger study found fasting PYY was significantly higher in girls with AN than in controls, and was predicted by BMI, fat mass, resting energy expenditure, growth hormone and total tri-iodothyronine (190). These results were supported by another study that found significantly higher fasting, and a trend for higher postprandial, PYY in patients with AN compared to HCs (191).

Overall, total PYY was examined in the above studies, making it difficult to ascertain the concentration of orexigenic PYY(1-36) compared to anorexigenic PYY(3-36). Two studies have specifically examined PYY(3-36), finding significantly higher fasting and postprandial levels in AN than in controls (177), as well as higher circulating concentrations (192). Differences in assays and variable blood processing techniques may impact these outcomes. In addition, with research finding both higher (193) and lower (194) serum DPP-IV levels in AN, it remains unclear whether specific differences between PYY(1-36) and PYY(3-36) concentrations might explain the reduced hunger ratings experienced in AN.

Studies of the effect of refeeding on PYY have been inconsistent. One study in patients with AN who were first assessed within 3 days of admission, found elevated fasting total PYY did not improve after 6-19 weeks of nutritional rehabilitation and varied rates of weight gain (195). However, two other studies observed reductions in fasting PYY(3-36) toward HC levels following ~3 months of inpatient (177) and ~12 months of outpatient refeeding and weight gain (190), respectively. The former study also found that after an oral mixed-nutrient test-meal, elevated postprandial PYY concentrations also reduced closer to HC levels following treatment (177). However, the postprandial rise may have

been due to the high fasting levels rather than an increase in the magnitude of response to the meal. Taken together, further research is needed following standardised nutritional intakes and consistent refeeding periods to clarify the effect of refeeding on PYY.

1.3.2.6 CCK in AN

Variable fasting levels of plasma CCK have been found in studies of AN. Compared to HCs, three studies showed increased fasting levels (196-198), two studies found no difference (199, 200), and three studies found decreased fasting levels (169, 170, 201). Inconsistencies may be due to varied diagnostic criteria, BMI and fasting duration (201), and gender-related developmental factors (169, 170).

Four studies found significantly higher postprandial CCK in patients with AN compared with HCs (169, 170, 196, 197), with another study finding concentrations in four patients with AN were double that of HCs (199). However, two studies found no postprandial difference in CCK release between anorexic subjects and controls (155, 200). The significant variability in results of mixed meal studies for both fasting and postprandial CCK in AN may be due to the known difficulties in assaying plasma CCK. Moreover, different test-meal nutrient compositions may contribute to the inconsistent findings, with fat and protein known to be stronger stimuli of CCK release than carbohydrate (35).

Four studies have examined the effects of refeeding on CCK concentrations in AN. One study of T-lymphocyte levels found reduced levels at baseline and no improvement after 4 months of treatment, however, it is unclear whether T-lymphocyte values (rather than plasma) indicate changes in GI function (202). Although an additional study found no difference between untreated and weight-recovered patients with AN in either fasting or

post-prandial CCK, results may be influenced by individual differences not controlled for in the between-subject design and the relatively small sample size (200). In the third study, fasting CCK was elevated in inpatients with AN before treatment and reduced after ~5 months of slow nutritional rehabilitation and partial weight restoration (198). Finally, in the fourth small study (n=4), after an oral liquid mixed-nutrient test-meal, plasma CCK concentrations peaked earlier and higher in patients with AN than HCs, and although the magnitude of the postprandial response tended to be higher in AN, no significant difference was found (199). After two weeks of undescribed nutritional rehabilitation, time-to-peak and peak plasma CCK were no longer different from HCs.

1.3.2.7 Ghrelin in AN

Elevated fasting plasma total ghrelin levels have been found in AN when compared with HCs (172, 177, 181, 190, 203-207). Moreover, nutrient-induced suppression of ghrelin is attenuated in AN, when compared with HCs (172, 177, 181, 204). It has been suggested that the basal elevation of this orexigenic peptide may result from the body's attempt to increase body weight, and that AN may be associated with a state of ghrelin resistance (205). However, earlier research did not compare orexigenic acyl and anorexigenic desacyl ghrelin levels. With improved assays, three studies have now analysed desacyl ghrelin separately in AN, all finding fasting desacyl ghrelin significantly higher than in HCs (203, 208, 209). Interestingly, two studies also found increased acyl ghrelin levels in AN compared with controls (203, 208), but one found no significant difference (208). Combined, these results support the hypothesis that patients with AN may experience anorexigenic effects from desacyl ghrelin via its known inhibitory effect on orexigenic acyl ghrelin, causing them to reduce their food intake.

The GOAT-ghrelin system is activated by a lipid-rich environment and GOAT gene expression is reduced with fasting (210), both potentially supporting elevated desacyl ghrelin in the malnourished AN state. Given the generally inadequate intake of dietary fat in AN, it may be hypothesised that GOAT gene expression may be downregulated in this condition. Moreover, recent research has found a genetic variation in GOAT is associated with AN (211) and future studies into the GOAT-ghrelin system's involvement are warranted.

Recently, interest has grown in the role of ghrelin in recovery from malnutrition and AN. Studies have suggested that significantly higher pre-treatment fasting total ghrelin returns towards normal levels with nutritional rehabilitation (177, 205, 206). Moreover, Koyama *et al.* showed that the elevated pre-treatment fasting desacyl ghrelin observed in n=5 patients with AN reduced towards that of controls after just one week of refeeding (208). The researchers proposed that as body weight was stable during that week, adequate rest and improved energy intake for weight maintenance (rather than the inadequate intake and weight loss prior to admission) may have played a role in improving desacyl ghrelin, and after eight weeks of refeeding and weight gain, desacyl ghrelin had reduced even closer to that of controls, thus supporting the possibility that GI nutrient sensitivity may change with reduced food intake in AN and improve with nutritional rehabilitation.

1.3.2.8 Limitations of AN research and future directions

Several studies have examined GI hormone release in AN. Significantly lower fasting (173-179) and postprandial insulin (173, 175, 177, 179, 180), higher fasting (177, 191) and postprandial (177) PYY and higher fasting ghrelin (203, 208, 209) have been consistently observed in AN in comparison with HCs. Reduced insulin might be related to the postprandial hypoglycemia observed in AN (5), though its relationship to the incretin hormones and gastric emptying remains unexplored. Likewise, the elevated PYY could explain the increased satiety observed in these patients, and these changes, may contribute to, or potentiate, the reduced desire to eat and disordered eating behaviour. However, despite higher ghrelin, patients with AN generally report reduced hunger. Data on GLP-1 (168, 170, 171), GIP (169, 172), glucagon (155, 173, 179, 187, 188) and CCK (155, 170, 196, 197, 200, 201) concentrations in AN are less consistent, with variations in study populations, design and findings. The variability observed in GI hormone concentrations is likely to reflect the use of different assays, timing of blood sampling, differing test meals, effects of oral nutrient exposure on GI function and varied patient characteristics, including exposure to repeated treatment episodes, mixed age groups, unknown reproductive hormone levels and mixed diagnoses. More rigorous study designs are therefore needed in GI research in AN to reduce the effects of such inconsistencies on study outcomes.

Additionally, although studies have consistently shown delayed gastric emptying in patients with AN compared with HCs (154-156), none have investigated GI hormone responses whilst simultaneously quantifying gastric emptying, which could provide insights into mechanisms underlying glycemic and appetite disturbances in AN. Furthermore, delayed gastric emptying in patients with AN has frequently been

associated with early satiety, bloating and abdominal distension (165-167). Since such symptoms can lead to difficulties in refeeding and weight restoration (165), increased understanding of the relationship between GI symptoms, hormones and gastric emptying may assist in modifying treatment strategies to improve symptom management and compliance with treatment.

1.3.3 Conclusions

To date, research has shown that patients with AN have marked disturbances in the GI mechanisms involved in the regulation of appetite and glycemia, and that these may improve toward HC levels with nutritional rehabilitation. However, although several studies have examined GI function following 3-12 months of nutritional rehabilitation, with uncertainty of nutritional intake and treatment compliance in those in outpatient settings, few studies have examined early responses within the first few weeks of treatment. Consequently, the duration of refeeding necessary to normalise GI function remains unclear, with potential implications for the management of GI-related medical complications – hypoglycemia, appetite disturbance and frequent GI symptoms. The present study therefore aims to examine postprandial changes in GI hormone responses during early refeeding in AN.

1.4 Rationale and hypotheses for studies of GI function in AN

The research outlined in this review suggests that GI responses are disturbed in starved patients with AN and that increased nutrient exposure may lead to a normalisation of GI responses. The studies in **Chapters 3 and 4** examined postprandial changes in GI hormone responses in starvation and following short-term refeeding in AN. Since changes in ghrelin have been observed in patients with AN as soon as one week after

hospital admission (208), we aimed to capture changes in GI function early in refeeding. Characterisation of the mechanisms behind AN-related glucoregulatory and appetite disruption may not only help with management of nutritional rehabilitation in this condition, but may also lead to an improved understanding of the mechanisms involved in GI food intake and blood glucose control.

The studies outlined in **Chapters 3 and 4** accordingly, aimed to determine the effects of starvation and short-term refeeding on GI function in adolescents with AN in response to an oral mixed-nutrient test-meal. More specifically, in comparison with HCs, we hypothesised that in patients with AN:

i. Gastric emptying will be slowed, blood glucose, insulin and C-peptide responses attenuated, and GIP and GLP-1 responses enhanced in the starved state; and with two weeks of refeeding, restored towards levels seen in HCs (**Chapter 3**).
ii. Fasting and postprandial PYY, CCK and ghrelin responses will be enhanced, fullness, GI symptoms and state anxiety increased, and hunger decreased in the starved state; and with increased nutrient exposure from short-term refeeding, GI hormone responses will reduce towards levels seen in HCs, with reduced GI symptoms, fullness and anxiety and increased hunger (**Chapter 4**).

Chapter 2: Effects of dipeptidyl peptidase IV inhibition on glycemic, gut hormone, triglyceride, energy expenditure and energy intake responses to fat in healthy males

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Overall percentage (%)	50%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	15/10/2018

2.1 Abstract

Fat is the most potent stimulus for GLP-1 secretion. The aims of this study were to determine whether DPP-IV inhibition would enhance plasma active incretin concentrations, and modulate the glycemic, gut hormone, triglyceride, energy expenditure and energy intake responses to ID fat infusion. In a double-blind, randomized, placebo-controlled cross-over design, 16 healthy lean males received 50 mg vildagliptin (V), or matched placebo (P), before ID fat infusion (2 kcal/min, 120 min). Blood glucose, plasma insulin, glucagon, active GLP-1 and GIP and PYY(3-36) concentrations, resting energy expenditure and energy intake at a subsequent buffet-meal (t=120-150 min) were quantified. Data are presented as areas under the curve (AUC 0-120 min, means±SEM). Vildagliptin modestly decreased glycemia (P:598±8 vs. V:573±9 mmol/L.min^{-1}, P<0.05) during ID lipid. This was associated with increased insulin (P:15964±1193 vs. V:18243±1257 pmol/L.min^{-1}, P<0.05), reduced glucagon (P:1008±52 vs. V:902±46 pmol/L.min^{-1}, P<0.05), markedly enhanced active GLP-1 (P:294±40 vs. V:694±78 pmol/L.min^{-1}) and GIP (P:2748±77 vs. V:4256±157 pmol/L.min^{-1}), and reduced PYY(3-36) (P:9527±754 vs. V:4469±431 pM.min^{-1}), concentrations compared with placebo (P<0.05, for all). Vildagliptin modestly increased resting energy expenditure (P:1821±54 vs. V:1896±65 kCal/day, P<0.05), with no effect on energy intake. Vildagliptin (i) modulates the effects of ID fat to enhance active GLP-1 and GIP, stimulate insulin and suppress glucagon, thereby reducing glycemia, and (ii) increases energy expenditure, without affecting energy intake. These observations suggest that the fat content of a meal, by enhancing GLP-1 and GIP secretion, may contribute to the response to DPP-IV inhibition.

2.2 Introduction

The incretin hormones, GLP-1 and GIP, are major determinants of postprandial glycemia (212), and GLP-1 also suppresses energy intake (213). In health, GLP-1 and GIP account for ~70% of the insulin response to enteral glucose (214). In type 2 diabetes, the incretin effect is impaired (215), reflecting a markedly diminished insulinotropic effect of GIP (71) and, possibly, reduced GLP-1 secretion (216). The enzyme DPP-IV rapidly degrades the incretins, and inhibitors of DPP-IV have been developed as a therapeutic strategy for type 2 diabetes (74). DPP-IV inhibitors enhance postprandial intact GLP-1 and GIP concentrations, and in type 2 diabetes their use is associated with reductions in pre- and postprandial blood glucose and glycated hemoglobin (HbA1c) (217). The glucose-lowering efficacy of DPP-IV inhibitors is primarily, but not exclusively, mediated by GLP-1 (218). However, in contrast to GLP-1 receptor agonists, which promote weight loss (likely via suppression of appetite), DPP-IV-based therapy tends to be weight neutral. Given this, it is surprising that the effects of DPP-IV inhibition on energy intake and expenditure have received little attention.

The efficacy of DPP-IV inhibition is likely to be potentiated by strategies to enhance food-induced GLP-1 secretion. For example, we demonstrated in type 2 patients that a D-xylose preload given before a carbohydrate meal attenuated the postprandial glycemic response, an effect that was enhanced by DPP-IV inhibition (219). To date, the primary focus has been on carbohydrate-induced incretin secretion. However, the fat content of a meal is likely to be highly relevant to the incretin response, since enteral fatty acids may be the most potent stimulus for GLP-1 secretion (220). Fat ingestion also stimulates insulin and glucagon secretion (221) and slows gastric emptying (222). Since intravenous lipid has no effect on insulin secretion (223), effects of enteral fat may be dependent on

incretin release. Indeed, ingestion of a fat "preload" attenuates the glycemic response to a carbohydrate-containing meal in type 2 diabetes (224). Given the potent effect of fat on GLP-1 concentrations, it is possible that the combination of DPP-IV inhibition with enteral fat may markedly attenuate postprandial glycemia.

Clinical trials of incretin-based therapies have demonstrated potentially beneficial cardiovascular effects, including a reduction in plasma triglycerides (225). In animal studies, both GLP-1 and GIP decrease intestinal triglyceride absorption and apolipoprotein production (226, 227). Indeed, administration of vildagliptin with a high-fat meal markedly reduces postprandial triglyceride concentrations in type 2 diabetes (228). The effect of DPP-IV inhibition on postprandial triglycerides in healthy subjects has not, to our knowledge, been investigated. DPP-IV inhibitors have also been reported to augment postprandial lipid mobilization and fat oxidation (75), and this may explain why patients with type 2 diabetes treated with DPP-IV inhibitors do not gain weight (229). DPP-IV inhibition may promote fat oxidation (75), but the effects of DPP-IV inhibition on fat oxidation and energy expenditure require further investigation.

Exogenous administration of GLP-1 slows gastric emptying (44) and suppresses subsequent food intake (213), hence it may be expected that DPP-IV inhibition would also be associated with suppression of energy intake. Yet, despite a substantial increase in active GLP-1 concentrations following DPP-IV inhibition in type 2 diabetes, gastric emptying appears unchanged (230), or only modestly slower (218), and intake of a mixed nutrient meal appears unaffected (231). However, a major limitation of the latter study was that effects on energy intake were assessed by asking subjects to drink a liquid meal until maximum tolerance, which is unlikely to be representative of intake from a typical

meal. Additionally, fat is likely to stimulate a greater GLP-1 response than a mixed nutrient meal, so the combination of fat with DPP-IV inhibition (232) would be predicted to exert greater suppressive effects on food intake.

Therefore, the aims of this study were to determine whether DPP-IV inhibition during ID fat infusion in healthy lean volunteers would (i) increase plasma concentrations of active GLP-1 and GIP, (ii) modify the glycemic, insulinemic and triglyceride responses, (iii) increase energy expenditure and fat oxidation and (iv) potentiate the suppression of energy intake. The fat was administered intraduodenally in order to control for variations in the rate of gastric emptying that exist between individuals or as a result of potential drug effects. The use of DPP-IV inhibition allowed us to probe the physiological effects of prolonged elevation of active GLP-1 and GIP concentrations on responses to fat.

2.3 Materials and methods

2.3.1 Participants

Sixteen healthy males (age: 23.7±1.6 (18-45) years; BMI: 22.6±0.5 (19-25) kg/m^2) were studied. Power calculations based on effect size and variance from previous studies (233-236) indicated n=16 would allow detection of a ~360 kJ (SD 482 kJ) difference in energy intake ($\alpha<0.05$, $\beta\geq0.8$). All participants were unrestrained eaters (237), had no GI disease or symptoms, were non-diabetic, had normal iron levels, creatinine clearance and liver function tests, and were not taking medications. Consumption of a vegetarian diet, >20 g of alcohol/day, and smoking, also represented exclusion criteria. The study was approved by the Royal Adelaide Hospital Research Ethics Committee and conducted in accordance with the Declaration of Helsinki. All participants provided informed written consent.

2.3.2 Study design

In a double-blind, randomized, placebo-controlled cross-over design separated by at least 7 days, we evaluated the effects of a 120-min ID infusion of fat, following oral ingestion of 50 mg vildagliptin or a matched placebo tablet, on blood glucose, plasma insulin, glucagon, GLP-1 (total and active (GLP-1(7-36)), GIP (total and active (GIP(1-42)), PYY (total and PYY(3-36)), triglyceride and free fatty acid concentrations, energy expenditure and fat oxidation, appetite perceptions and energy intake. Vildagliptin and matched placebo were provided to the pharmacy by the sponsor (Novartis Pharmaceuticals Australia Pty. Ltd.).

2.3.3 Protocol

Participants were asked to maintain normal eating habits and to refrain from vigorous exercise and alcohol intake for 24 hours before visits. They were provided with a standardized beef lasagna (2,472 kJ, McCain Foods) for dinner at 7 pm the night before each visit, after which they fasted from all food and fluid (except water).

On each visit, participants arrived at the Discipline of Medicine at 0800 h, where a silicone catheter with antral and duodenal side-holes perfused with saline, and a terminal infusion port, was inserted through an anaesthetized nostril into the stomach, and allowed to pass into the duodenum (238). Catheter position was monitored by measurement of the transmucosal potential difference in the stomach and duodenum (238), using a saline filled subcutaneous cannula placed in the left forearm as a reference electrode (238). Once positioned, fasting resting energy expenditure (REE) and respiratory quotient (RQ) were measured over 30 minutes by indirect calorimetry, using a clear ventilated hood and the TrueOne® 2400 metabolic monitoring system (Parvo Medics, East Sandy, UT 84093, USA). After 30 minutes, the plastic hood was removed, and an intravenous cannula was inserted into an antecubital vein for blood sampling.

At t=-60 minutes, subjects inges 50 mg vildagliptin, or a matched placebo tablet, with 100 ml water (**Figure 2.1**). At t=0 minutes, an ID infusion of lipid (10% Intralipid at 1.8 ml/min (2 kcal/min)) was commenced and maintained for 120 minutes. Blood samples were collected and 100mm visual analog scale (VAS) questionnaires assessing appetite and GI sensations were completed, at intervals from t=-60 to 180 minutes. REE and RQ were assessed between t=-30 to 0 min, t=15-45 min and t=90-120 min. At t=120 minutes, the catheter and the ventilated hood were removed, and participants were offered a cold

buffet-style meal from which they were instructed to eat until comfortably full (t=120-150 min) (239). At t= 180 minutes, the intravenous cannula was removed, and the participant left the laboratory.

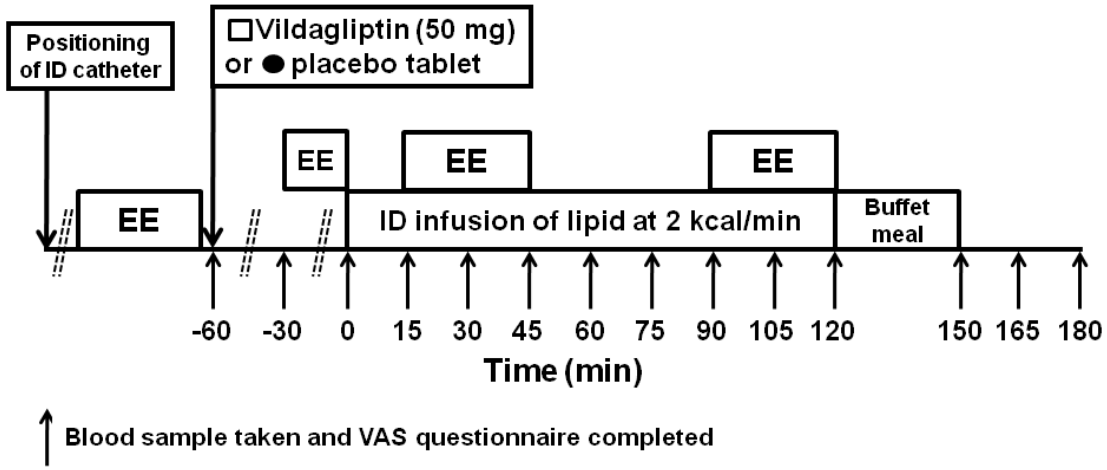

Figure 2.1.

Schematic representation of the study protocol. A catheter was positioned with an infusion port in the duodenum. Participants ingested 50 mg vildagliptin, or a matched placebo, tablet, with 100 ml water at t=-60 min. Resting energy expenditure (REE) and respiratory quotient (RQ) were assessed between t=-30 to 0 min, t=15-45 min and t=90-120 min using indirect calorimetry. Intraduodenal (ID) infusion of lipid (10% Intralipid 2 kcal/min, rate: 1.8 ml/min) was commenced at t=0 min, and maintained for 120 minutes. Blood samples were collected, and visual analogue scale questionnaires (VAS), assessing appetite and GI sensations, were completed at the time-points indicated. At t=120 minutes, the infusion was discontinued, and participants were offered a cold buffet-style meal (t=120-150 min), from which energy intake was quantified (239).

2.3.4 Measurements

2.3.4.1 Blood glucose and hormone concentrations

10 ml venous blood samples were collected in ice-chilled EDTA-treated tubes containing 100 µL DPP-IV inhibitor (DPP4-010, EMD Millipore Corporation, Billerica, MA, USA) for analysis of insulin, glucagon, GLP-1 (total and active), GIP (total and active), PYY (total and PYY(3-36)). 5 ml blood samples were collected into serum tubes and fluoride oxalate treated tubes for measurement of serum triglycerides, and plasma free fatty acid concentrations, respectively. Venous blood glucose concentrations (mmol/L) were determined by glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA). Plasma/serum was obtained by centrifugation (3200 rpm, 15 min, 4^{o}C), and stored at -80^{o}C.

Total GLP-1 concentrations were measured using a radioimmunoassay (antiserum:89390), specific for the amidated *C*-terminal end of the GLP-1 molecule that reacts equally with intact GLP-1 and the primary metabolite, while intact GLP-1 levels were measured using an in-house two-site (sandwich) assay (ELISA) (240). Total GIP was measured, using the *C*-terminally directed antiserum (code 80867:), which reacts fully with intact GIP and the *N*-terminally truncated metabolite and intact GIP was measured using an antiserum (no. 98171), which is specific for the intact *N*-terminus of GIP (Vilsbøll et al 2003). Total PYY (1-36+3-36) and PYY(3-36) were measured using commercially available radioimmunoassay kits from Linco (cat. no. PYY-66HK and PYY(3-36)-67HK, Millipore, St. Charles, MO, USA). Plasma insulin concentrations were measured using ElectroChemiLuminescense ImmunoAssay. Plasma glucagon was measured by radioimmunoassay using a C-terminally directed antiserum (no. 4305) (223) which recognizes fully processed pancreatic glucagon (240). Serum triglyceride and

plasma free fatty acid concentrations were assayed in commercial laboratories by SA Pathology (Adelaide, SA, Australia).

2.3.4.2 Appetite perceptions and energy intake

The use of 100mm VAS questionnaires to evaluate appetite and GI sensations has been described previously (241). Food consumption at the buffet-meal was determined by weighing meal items before and after presentation to the participant, and analyzed using commercially available software (Foodworks 3.01; Xyris Software, Highgate Hill, Queensland, Australia) (239).

2.3.4.3 Resting energy expenditure (REE), respiratory quotient (RQ) and thermic effect of feeding (TEF)

The first 10 minutes of data from each period (t=-90—60 (BL), 15-45, and 90-120 min) were discarded to ensure that participants had reached equilibrium, and the remaining values were averaged to provide REE and RQ at BL, and during the lipid infusion (average of values between t=15-45 and 90-120 min) (242). RQ was determined as the ratio of VCO_2/VO_2. A value of 0.7 is indicative of fat oxidation, while a value of 0.9 - 1.0 is indicative of carbohydrate oxidation (242). TEF was determined by subtracting baseline REE from the mean REE values during the ID fat infusion, and is expressed as % energy consumed during the ID fat infusion.

2.3.5 Data and statistical analysis

VAS scores and serum triglyceride concentrations are presented as changes from baseline (t=-60 min) values; all other data are presented as raw values. Data assessed over time were analyzed by repeated measures analysis of variance (ANOVA), with time and treatment as factors. Changes over time within a treatment were analyzed by repeated-measures ANOVA with time as a factor. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant effects. Energy intake (amount and energy consumed, and macronutrient distribution), REE, RQ and TEF, were analyzed by paired t-tests. Results are presented as means±SEM, and significance was accepted at $P<0.05$.

2.4 Results

The study was generally well tolerated. Some subjects experienced mild nausea (placebo: n=7, vildagliptin: n=6), mild abdominal cramps (placebo: n=3), and diarrhea (placebo: n=2) during the ID lipid infusion. Due to technical problems during indirect calorimetry, total data for REE, RQ and TEF were not available for 3 participants.

2.4.1 Blood glucose

There was a treatment*time interaction for blood glucose concentrations (P=0.000) (**Figure 2.2A)**, so that glucose was less between t=60-105 min following vildagliptin compared with placebo (P<0.05). During the lipid infusion, blood glucose concentrations decreased relative to baseline (t=0 min) between t=45-105 min following vildagliptin (P<0.05), while there was no change over time following placebo. After the buffet-meal, blood glucose concentrations increased on both days, but were markedly lower following vildagliptin, when compared with placebo (P<0.05).

2.4.2 Insulin

There was a treatment*time interaction for plasma insulin concentrations (P=0.04) (**Figure 2.2B**), so that concentrations were slightly greater between t=30-75 min following vildagliptin compared with placebo (P<0.05). During the lipid infusion, plasma insulin concentrations increased relative to baseline (t=0 min) between t=30-120 min following vildagliptin (P<0.05), while there was no change over time following placebo. After the buffet-meal, plasma insulin concentrations were markedly increased on both days, with no difference between treatments.

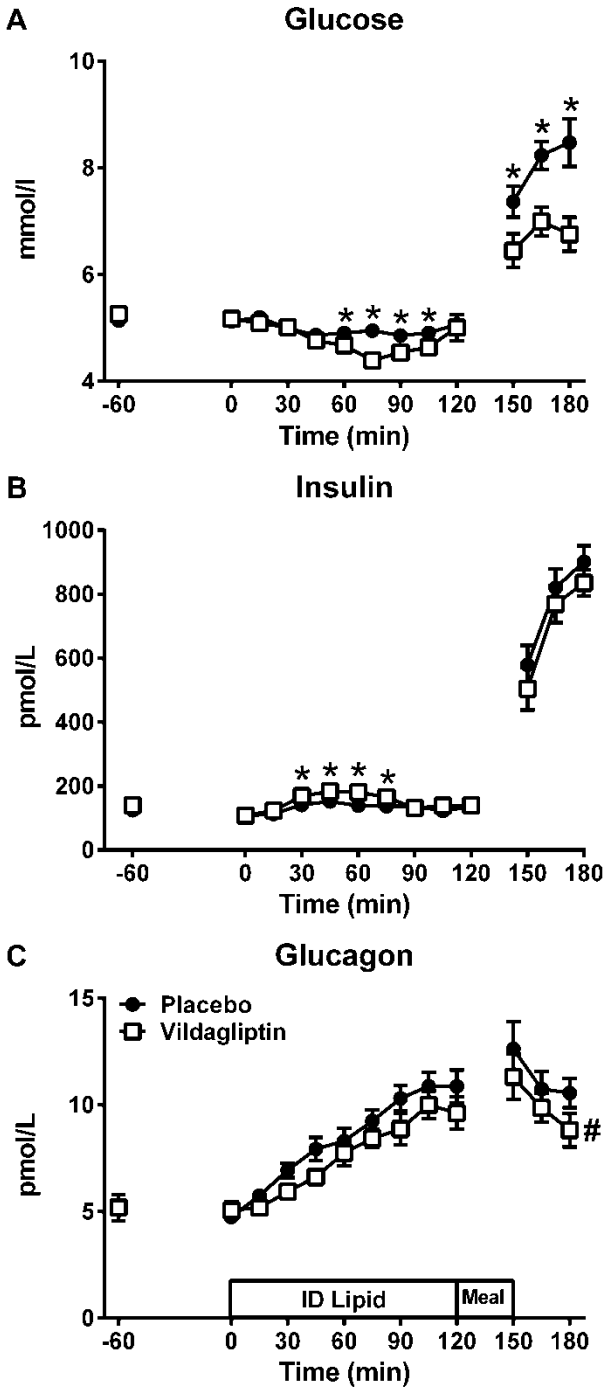

Figure 2.2.

Plasma glucose (A), insulin (B) and glucagon (C) responses to intraduodenal (ID) lipid infusion following administration of the DPP-IV inhibitor, vildagliptin, or matched placebo, in healthy lean males. Data are means±SEM, n=16. *placebo vs. vildagliptin, P< 0.05, #Treatment effect: placebo vs. vildagliptin, P<0.05.

2.4.3 Glucagon

There was an effect of treatment on plasma glucagon concentrations, with glucagon being lower after treatment with vildagliptin compared with placebo (P=0.000) (**Figure 2.2C**). During the lipid infusion, plasma glucagon concentrations increased relative to baseline (t=0 min) between t=45-120 min following vildagliptin (P<0.05), and between t=15-120 min following placebo. After the buffet-meal, plasma glucagon concentrations decreased on both days (P<0.05).

2.4.4 Total and active GLP-1

There was a treatment*time interaction for total plasma GLP-1 concentrations (P=0.000). Plasma total GLP-1 concentrations increased in response to the ID lipid infusion on both study days, but were lower at t=30, 45, 90 and 120 min, and after ingestion of the buffet-meal (between t=150-180 min), following vildagliptin compared with placebo (P<0.05) (**Figure 2.3A**).

There was a treatment*time interaction for plasma active GLP-1 concentrations (P=0.000). Plasma active GLP-1 concentrations increased in response to the ID lipid infusion on both study days, but were greater between t=30-180 min following vildagliptin compared with placebo (P<0.05) (**Figure 2.3B**).

2.4.5 Total and active GIP

There was a treatment effect for plasma total GIP concentrations (P=0.02). Plasma total GIP concentrations increased in response to the ID lipid infusion on both study days, but total GIP was lower following vildagliptin compared with placebo (**Figure 2.3C**).

There was a treatment*time interaction for plasma active GIP concentrations (P=0.0001). Plasma active GIP concentrations increased in response to the ID lipid infusion on both study days, but were greater at t=0 and between t=30-180 min following vildagliptin compared with placebo (P<0.05) (**Figure 2.3D**).

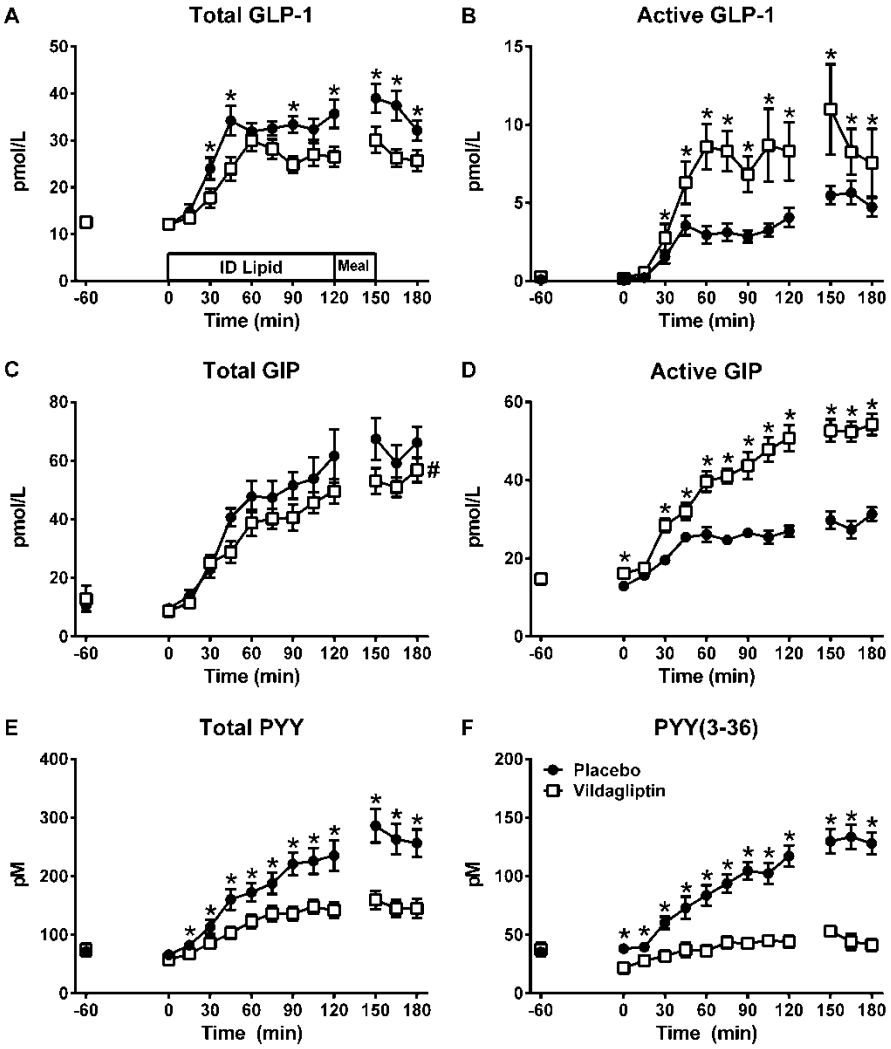

Figure 2.3.

Plasma total GLP-1 (A), active GLP-1 (B), total GIP (C), active GIP (D), total PYY (E), and PYY (3-36) (F) responses to intraduodenal (ID) lipid infusion following administration of the DPP-IV inhibitor, vildagliptin, or matched placebo, in healthy lean males. Data are means±SEM, n=16. *placebo vs. vildagliptin, P<0.05, #Treatment effect: placebo vs. vildagliptin, P<0.05.

2.4.6 Total PYY and PYY(3-36)

There was a treatment*time interaction for total plasma PYY concentrations (P=0.000). Plasma total PYY concentrations were lower between t=15-180 min following vildagliptin compared with placebo (P<0.05) (**Figure 2.3E**).

There was a treatment*time interaction for plasma PYY(3-36) concentrations (P = 0.000). Plasma PYY(3-36) was lower between t=0-180 min following vildagliptin compared with placebo (P<0.05) (**Figure 2.3F**).

2.4.7 Triglycerides and free fatty acids

There was a treatment*time interaction for serum triglyceride concentrations (P = 0.000). During the lipid infusion, serum triglyceride concentrations did not increase relative to baseline on either day. After the buffet-meal, serum triglyceride concentrations were markedly increased on both days. Serum triglyceride concentrations were lower after the buffet-meal at t=165-180 min following vildagliptin compared with placebo (P<0.05) (**Figure 2.4**).

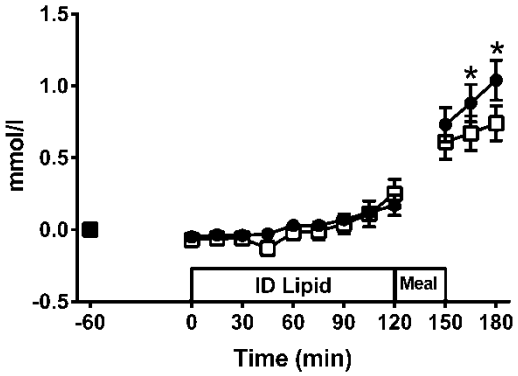

Figure 2.4.
Serum triglyceride response to intraduodenal (ID) lipid infusion following administration of the DPP-IV inhibitor, vildagliptin, or matched placebo, in healthy lean males. Data are means±SEM, n=16. *placebo vs. vildagliptin, P<0.05.

There was no effect of treatment on circulating concentrations of free fatty acids (P=0.998) (data not shown). During the lipid infusion and after the buffet-meal, there was no change in plasma free fatty acids relative to baseline on both days.

2.4.8 REE, RQ and TEF

There was no difference in baseline REE or RQ between study days. On both days REE increased during the ID lipid infusion compared with baseline (P<0.01), however during the lipid infusion, REE was greater following vildagliptin compared with placebo (P=0.01) (**Figure 2.5A**). On both days, RQ decreased during the ID lipid infusion compared with baseline, indicative of a shift toward lipid oxidation (P<0.01), however, there was no effect of vildagliptin on RQ (data not shown). There was an effect of treatment on the thermic effect of food, such that vildagliptin increased the thermic effect of food when compared with placebo (P=0.049) (**Figure 2.5B**).

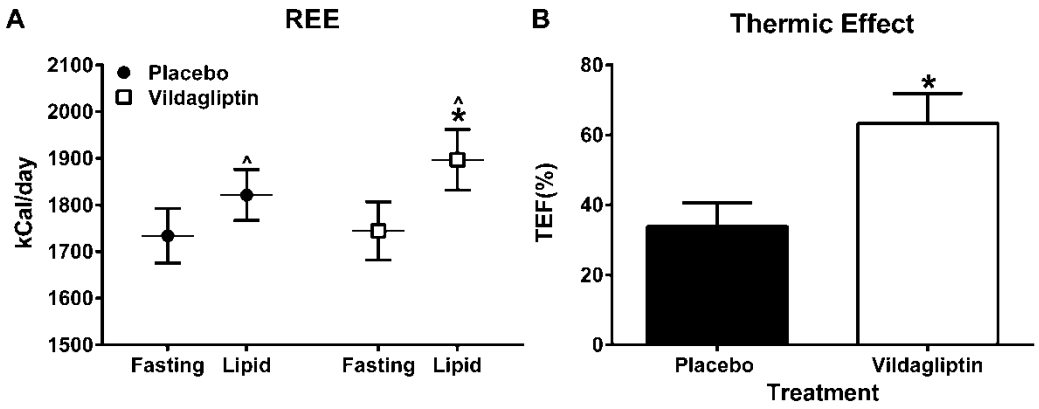

Figure 2.5.

Resting energy expenditure (REE, A) and thermic effect of lipid (TEF, B) following intraduodenal (ID) lipid infusion following administration of the DPP-IV inhibitor, vildagliptin, or matched placebo, in healthy lean males. Data are means±SEM, n=13. ^fasted vs. lipid, P<0.05, *placebo vs. vildagliptin, P< 0.05.

2.4.9 Appetite and GI symptom perceptions and energy intake at subsequent buffet meal

There was no effect of treatment on appetite perceptions or ratings of nausea or fullness (data not shown). There was no effect of treatment on the amount (g), energy (kJ) or macronutrient composition of the food consumed at the buffet-meal (**Table 2.1**).

Table 2.1.

Energy (kJ), amount (g), and macronutrient distribution (percentage of energy derived from fat, carbohydrate or protein) of food consumed at the buffet meal following intraduodenal (ID) lipid infusion paired with administration of the DPP-IV inhibitor, vildagliptin, or matched placebo, in healthy lean males.

Variable	Placebo	Vildagliptin	*P*-value
Energy intake (kJ)	4647±374	4497±374	0.4
Amount consumed (g)	1156±74	1077±80	0.2
Protein (%)	20±1	20±1	0.5
Carbohydrate (%)	52±2	51±2	0.4
Fat (%)	28±1	29±1	0.7

Data are presented as mean values ± SEM. *n*=16.

2.5 Discussion

This study shows that acute administration of the DPP-IV inhibitor, vildagliptin, during euglycemia, modulates the effects of an ID fat infusion in healthy males to enhance active GLP-1, active GIP, and insulin, and suppresses glucagon, glycemia and postprandial triglycerides. Vildagliptin also reduced total PYY, and PYY(3-36), and was associated with a modest increase in resting energy expenditure during ID fat infusion, but had no effect on *ad libitum* energy intake.

We have confirmed that fat is a potent stimulus for GIP and GLP-1 release in health, and that these effects are augmented by DPP-IV inhibition, with evidence of feedback inhibition on the L-cell, i.e. the observed reductions in total GLP-1 and GIP concentrations, as has been reported previously (231). This is consistent with the results of Ohlsson *et al.,* who observed potentiated responses to all 3 macronutrients, but particularly to fat, following administration of the DPP-IV inhbitor, sitagliptin (232). Increased active GIP and GLP-1 presumably account for the stimulation of insulin. In previous studies in health, stimulation of GIP and GLP-1 following oral fat increased both insulin and glucagon, but had no effect on glycemia (221, 223). Exogenous GIP has been reported to stimulate glucagon at euglycemia and hypoglycemia, but not with hyperglycemia (243). In the current study, during euglycemia, the augmented active GIP and GLP-1 response to ID fat was associated with increased insulin, but decreased glucagon. Thus, with DPP-IV inhibition, at euglycemia, the effect of GLP-1 to reduce glucagon appears to be dominant. These observations suggest that fat plays an important role in determining the glycemic response to DPP-IV inhibition, which may have implications for the therapeutic effects of DPP-IV inhibitors. For example, the reduction in HbA1C induced by DPP-IV inhibition may be greater in type 2 diabetes with a

habitually greater fat intake. Dietary macronutrient patterns may, thus, account for variability in the HbA1C lowering effects of DPP-IV inhibitors. This is an issue that warrants further investigation.

The postprandial triglyceride response was modestly decreased following vildagliptin, while free fatty acids did not change. It is unlikely that the former reflected differences in intake at the buffet-meal given that participants consumed the same macronutrient composition on both days. These observations are consistent with reports that vildagliptin lowers postprandial triglyceride concentrations following a high-fat meal, after 4 weeks of treatment in patients with type 2 diabetes (228). While the mechanisms underlying this effect are poorly defined, the increases in active GIP and GLP-1 are likely to be involved. In animal studies, GLP-1 reduces intestinal triglyceride absorption and apolipoprotein production (226, 244), and the GLP-1 receptor has been demonstrated to be essential for intestinal lipoprotein synthesis and secretion (244). Furthermore, GIP reduces postprandial triglyceride levels, which may be mediated by effects on both intestinal triglyceride absorption and peripheral tissue uptake (227).

DPP-IV inhibition also modestly increased resting energy expenditure during the intralipid infusion, which may be attributable to the increase in the thermic effect of ID lipid. If maintained during prolonged treatment, this could contribute to weight neutrality, and perhaps even weight loss, depending on the macronutrient composition of the diet, so this issue clearly warrants further evaluation. There was a decrease in respiratory quotient in response to ID lipid, indicative of increased fat oxidation. In contrast to previous reports (75), this was not augmented by vildagliptin, but this may reflect the fact that only a single dose was administered. This metabolic response to DPP-IV inhibition

may be mediated through GLP-1 receptor-mediated activation of the sympathetic nervous system (75), as evidenced by an increase in plasma norepinephrine (75). Increased insulin secretion may contribute to this effect through effects on lipid metabolism. It is also possible that DPP-IV inhibition exerts its effects through modulation of other hormones that we did not measure. For example, DPP-IV inhibition augments the antilipolytic effect of neuropeptide Y in human adipose tissue (245).

Administration of exogenous GLP-1(7-36) (213) or PYY(3-36) (94) potently suppresses food intake in humans. In the current study, vildagliptin had no effect on ratings of hunger and fullness, nor energy intake at the buffet-meal. This may be due to any elevation of active GLP-1 being counterbalanced by a concomitant reduction in total PYY and inhibition of conversion of PYY(1-36) (which has orexigenic effects) to PYY (3-36) (which has anorexigenic effects), and is consistent with the weight neutral effect of DPP-IV inhibitors in type 2 diabetes (246), as opposed to the weight loss observed with GLP-1 receptor agonists (247). Furthermore, the latter are associated with much higher levels of receptor stimulation when compared with endogenous stimulation with DPP-IV inhibition.

The study had a number of limitations. First, only a single dose of vildagliptin was administered; effects may differ with prolonged therapy. Fat was administered intraduodenally to exclude the confounding effects of variations in gastric emptying between individuals, which in themselves would also be likely to impact on the efficacy of DPP-IV inhibition on glycemic control (248). While fat may enhance the response to DPP-IV inhibition, direct comparison with other nutrients, and examination of the effects of mixed-nutrient meals consumed orally on glycemic profiles are warranted. Finally,

this study was limited to healthy subjects, in whom GIP has substantial insulinotropic effects; it will be important to determine whether DPP-IV inhibitors have comparable effects following fat ingestion in patients with type 2 diabetes.

In conclusion, in healthy males, acute administration of the DPP-IV inhibitor, vildagliptin, has significant effects on the glycemic, triglyceride and energy expenditure responses to ID fat. These observations may have implications for the development of dietary strategies to enhance the efficacy of DPP-IV inhibition.

Chapter 3: Effects of starvation and short-term refeeding on gastric emptying and postprandial blood glucose regulation in adolescent females with anorexia nervosa

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Contribution to the Paper	Designed the research, conducted the research, analysed the data and performed statistical analysis, contributed to interpretation of the results, wrote the manuscript		
Overall percentage (%)	50%		
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Signature		Date	05/10/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate to include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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3.1 Abstract

Postprandial glucose is reduced in malnourished patients with AN, but the mechanisms and duration for this remain unclear. We examined blood glucose, gastric emptying and glucoregulatory hormone changes in malnourished patients with AN, and during two weeks of acute refeeding, compared with HCs. 22 female adolescents with AN and 17 age-matched female HCs were assessed after a 4-hour fast. Patients were commenced on a refeeding protocol of 2400 kcal/day. Gastric emptying (^{13}C-octanoate breath-test), glucose absorption (3-O-methylglucose), blood glucose, GLP-1, GIP, insulin, C-peptide and glucagon responses to a mixed-nutrient test-meal were measured on admission and one and two weeks post-refeeding. HCs were assessed once. On admission, patients had slower gastric emptying, lower postprandial glucose and insulin, and higher glucagon and GLP-1, than HCs ($P<0.05$). In AN, the rise in glucose (0-30 min) correlated with gastric emptying ($P<0.05$). With refeeding, postprandial glucose and 3-O-methylglucose were higher, gastric emptying faster, and baseline insulin and C-peptide less ($P<0.05$), compared with admission. After two weeks of refeeding, postprandial glucose remained lower, and glucagon and GLP-1 higher, in patients compared with HCs ($P<0.05$), without differences in gastric emptying, baseline glucagon or postprandial insulin. Delayed gastric emptying may underlie reduced postprandial glucose in starved patients with AN, however, postprandial glucose and glucoregulatory hormone changes persist after two weeks of refeeding, despite improved gastric emptying. Future research should explore whether reduced postprandial glucose in AN is related to medical risk by examining associated symptoms alongside continuous glucose-monitoring during acute refeeding in AN.

3.2 Introduction

AN is characterised by a marked restriction of energy intake leading to malnutrition, serious medical complications (28) and high mortality (249). Hypoglycemia, defined as episodes of abnormally low plasma glucose concentrations exposing individuals to potential harm (250), occurs in up to 30% of hospitalised patients (5), may lead to coma, cardiogenic shock (251) or cardiac arrest (4), and is associated with an increased risk of death (252). Moreover, disturbances in glycemic regulation may be responsible for clinical symptoms (e.g. fatigue, dizziness, collapsing, etc.) that are commonly attributed to vital sign instability in AN. Studies in underweight AN patients have demonstrated blunted glucose responses to mixed-nutrient meals (172, 180), however, the determinants of postprandial hypoglycemia in AN remain uncertain.

In health, postprandial glycemia is determined by a number of factors, including fasting glucose, meal composition, gastric emptying, glucose absorption rate, incretin hormones (GLP-1 and GIP), insulin secretion, and hepatic and peripheral glucose metabolism and clearance (16). Gastric emptying accounts for ~35% of the variance in the initial (0-30 min) rise in postprandial glucose in health and diabetes (42, 253), and is affected by acute changes in dietary intake. For example, a four-day fast slows gastric emptying and is associated with a slower rise in postprandial glucose (17), while overfeeding (e.g. two weeks of a hypercaloric fat- or glucose-supplemented diet) accelerates gastric emptying of fat or glucose (148, 150). Although chronic caloric restriction in AN is associated with delayed gastric emptying (6, 155, 160, 254), the impact of gastric emptying on glycemia remains uncharacterised. Furthermore, it is unknown whether higher-calorie refeeding, within a two-week time-frame, improves glucose regulation by reversing delayed gastric emptying.

This study aimed to evaluate whether in malnourished adolescent females with AN (i) gastric emptying, blood glucose, plasma insulin, glucagon and incretin responses to a mixed-nutrient meal were disturbed compared with HCs, and (ii) whether two weeks of refeeding would normalise these responses.

3.3 Materials and methods

3.3.1 Participants

Participants were 22 female, adolescent inpatients with AN (restricting sub-type), as defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM5) (1) (age: 15.9±0.4 (range 13-19) years), and 17 healthy, age-matched female controls (age: 16.5±0.7 (range 12-19) years). Of the 34 eligible patients admitted with medical instability (255) to the Children's Hospital at Westmead and Westmead Hospital in Sydney, Australia, between August 2013 and June 2014, 12 declined to participate due to the requirement of cannulation, the test meal or non-specific anxiety. One patient took olanzapine throughout the study and another patient during week 1 only. Exclusion criteria included taking any other medication known to affect GI function, vomiting, smoking, consuming >20g alcohol/day or a history of GI disease (including gastroparesis), or GI surgery. No participant had diabetes. HCs were recruited through advertisements (e.g. community noticeboards, online fora, etc.). HCs were unrestrained eaters (scoring <12 for eating restraint (Factor-1) in the Three-Factor Eating Questionnaire (237)) and not following any special diet. All participants had reached Tanner stage-3. All patients with AN had primary or secondary amenorrhoea with no oral contraception usage. HCs had regular menstruation over the previous three months and were studied during the follicular phase of the menstrual cycle to minimise potential confounding effects of the menstrual cycle on GI function (256) and growth hormone (257). Five HCs were taking an oral contraceptive.

Participant characteristics are outlined in **Table 3.1**. Patients with AN scored significantly higher on EDE-Q global and subscale scores than HCs ($P<0.05$). Of the 22 patients, 15 had not previously undergone treatment and reported 36±4 weeks of recent dietary

restriction (weight loss: 12±1 kg; total illness duration: 11±3 months). The 7 previously treated patients reported 10±2 weeks of recent dietary restriction (weight loss: 6±1 kg; total illness duration: 27±6 months).

Table 3.1.
Characteristics of healthy controls (HCs) and patients with anorexia nervosa (AN) on admission (Wk0), and after one (Wk1) and two (Wk2) weeks of refeeding[1]

	HC	AN		
		Wk0	**Wk1**	**Wk2**
Weight (kg)	55.7±1.4	43.3±1.4*	46.1±1.4†	46.9±1.5*†‡
Age (years)	16.5±0.7	15.9±0.4		
BMI (kg/m^2)	21.1±0.5	16.1±0.4*	17.2±0.4†	17.5±0.4*†‡
% EBW	102.8±2.2	78.7±1.8*	83.8±1.8	85.2±1.9*
RMR (kJ/day)	6499±175	5105±148*	5850±181†	5605±189*
RQ	0.78±0.0	0.87±0.0*	0.94±0.0†	0.88±0.0*‡
EDE-Q – Global score	0.5±0.1	3.1±0.3*		
- EDE-Q Restraint score	0.2±0.1	2.8±0.3*		
- EDE-Q Eating Concern score	0.2±0.1	2.5±0.3*		
- EDE-Q Weight Concern score	0.9±0.3	4.3±0.5*		
- EDE-Q Shape Concern score	0.6±0.2	2.7±0.3*		

[1]Data are means ± SEM; n=22 anorexia nervosa (AN) patients and n=17 healthy controls (HCs). In AN patients, main treatment effects were determined using one-factor repeated-measures ANOVA with treatment as a within-participant factor. Comparisons between AN patients at both Wk0 and Wk2 and with HCs were conducted using independent-samples t tests. *Significantly different from HCs; †significantly different from Wk0; ‡significantly different from Wk1 (all $P<0.05$). BMI = body mass index; %EBW = percentage of expected body weight; RMR = resting metabolic rate; RQ = respiratory quotient; EDE-Q = Eating Disorders Examination Questionnaire (258).

The study was approved by the Human Research Ethics Committees at the Sydney Children's Hospital Network, the Royal Adelaide Hospital and the University of Adelaide, and performed in accordance with the Declaration of Helsinki. All participants, and parents of those <18 years old, provided informed, written consent prior to their enrolment.

3.3.2 Study design

Upon admission, all patients were commenced on oral phosphate supplementation and a standardized refeeding protocol commencing at 2400 kcal/day. Calories were provided through a combination of supported meals and, in the presence of medical instability (bradycardia, hypotension, or hypothermia), nocturnal nasogastric feeds (100 mL/hr; Jevity, Abbott Nutrition (1 kcal/mL)). Total caloric intake was adjusted to achieve a weight gain of approximately 1 kg per week and ranged from 2400-3200 kcal/day, and nasogastric feeds were ceased once patients were medically stable. This protocol has been previously described in detail (259).

To investigate the 'early' effects of refeeding on blood glucose and GI mechanisms regulating blood glucose, patients were studied on three occasions: once medically stable within the first five days (2.2±0.2 days) of admission ('Wk0'), then one ('Wk1') and two ('Wk2') weeks post-Wk0. HCs were studied once. On each study day, we evaluated gastric emptying, blood glucose and plasma GLP-1, GIP, insulin, C-peptide (co-secreted with insulin from the pancreas and a measure of insulin secretion) and glucagon responses to an oral mixed-nutrient test-meal.

A self-report questionnaire assessing eating disorder-specific psychopathology (Eating Disorders Examination-Questionnaire (258)) was completed on the first study day in AN patients and HCs (**Table 3.1**). Patients were also asked how much weight they had lost during their most recent episode of dietary restriction and over what duration this occurred.

3.3.3 Protocol

Patients were studied during admission and, if discharged prior to Wk2 (n=6), re-attended hospital. Both discharged patients and HCs were asked to refrain from vigorous exercise and alcohol intake for 24 hours before each study, and, on the study day, attended the hospital at 0800 hours after fasting from 2000 hours the night before.

On each study day, participants were weighed in the morning. To allow for regular blood sampling, an intravenous cannula was inserted into an antecubital vein at 0800 hours. At 0830 hours, patients on an oral diet (n=17 at Wk0, n=22 at Wk1 and Wk2) and HCs were provided with a standardised breakfast (30 g cereal-wheat biscuits (Weetbix, Sanitarium, Australia), 400 mL full-cream milk and an apple; 479 kcal, 65 g carbohydrate, 19 g protein, 15 g fat, 7 g fiber), and allowed up to 30 min for complete consumption under supervision. Patients still receiving continuous nasogastric feeds at Wk0 (n=5), but medically stable and, thus, could participate in the study, had feeds ceased at 0900 hours in lieu of the standardised breakfast. No further food or fluid (except water) was consumed prior to the test-meal.

At 1200 hours, respiratory quotient (RQ) and resting metabolic rate (RMR) were determined by indirect calorimetry using a clear plastic ventilated hood and metabolic

monitor (DeltatracTM II, MBM-200 Datex Ohmeda, under standardised conditions) (242, 260), while participants rested in the supine position for 30 min. Energy expenditure was quantified using the amount of O_2 consumed and CO_2 produced in breath.

At 1300 hours, immediately before consumption of the test-meal (t=0 min), baseline blood (15 mL) and end-expiratory breath (baseline sample prior to measuring gastric emptying) samples were collected. Following this, a mixed-nutrient semi-solid test-meal identical to that given at breakfast, and with 150 ml of the milk labelled with 100 µL ^{13}C-octanoate (Novachem, Australia) (261) (for assessment of gastric emptying by ^{13}C-breath-test), was consumed under supervision within 15 min to ensure a standardised ingestion rate. Further blood samples were collected at t=30, 60 and 120 min, with timing commencing as soon as test-meal ingestion was complete. End-expiratory breath samples were collected in foil bags every 5 min for the first hour, and every 15 min for a further hour, to quantify ^{13}C-enrichment of breath samples, as a measure of gastric emptying. In a sub-sample of n=7 patients with AN, 5 g 3-O-methylglucose (3-OMG) was mixed into the meal to assess glucose absorption (262).

3.3.4 Measurements

3.3.4.1 Gastric emptying

Gastric emptying of the liquid phase of the test-meal was estimated using the ^{13}C-octanoate breath-test (261), which has been validated against scintigraphy (263). The concentration of CO_2 and percentage of $^{13}CO_2$ were measured in each breath sample using mass spectroscopy (FANci2 Infrared Spectroscopy ^{13}C Breath Test Analyser, Fischer Analysen Instrumente GmbH, Germany). Gastric emptying is reported as the percentage of $^{13}CO_2$ recovery per hour in breath.

3.3.4.2 Blood glucose, serum 3-OMG and plasma hormone analysis

Blood glucose concentrations (mmol/L) were measured immediately upon sampling using a glucometer (Optium Xceed; Abbott Laboratories). Blood samples were collected in ice-chilled EDTA-treated tubes, containing 20 uL/mL blood of the serine protease inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Pefabloc, Roche, Australia), and in serum tubes containing zinc. Samples were separated by centrifugation (3200 rpm, 15 min, 4°C) within 15 min of collection, and stored at -80°C until assayed. Serum 3-OMG was measured by liquid chromatography and mass spectrometry by CPR Pharma Services (264). Serum insulin (mU/L) was measured by ELISA immunoassay (10-1113, Mercodia, Uppsala, Sweden). The minimum detectable limit was 1.0 mU/L, and the intra- and inter-assay coefficients of variation (CVs) were 2.8% and 6.7%, respectively. Plasma C-peptide (pg/ml), total GLP-1 (pg/ml) and total GIP (pg/ml) concentrations were measured via multiplex assay (Milliplex® MAP Human Metabolic Hormone Magnetic Bead Panel, HMHEMAG-34K) and analysed on a Bio-plex® MAGPIXTM Multiplex Reader (Luminex®, Millipore Corporation) using xPONENT® software (Luminex®, Millipore Corporation, version 4.2) according to manufacturer's instructions. There was negligible antibody cross-reactivity. The minimum detectable limits were 9.5 pg/ml for C-peptide, 2.5 pg/ml for GLP-1 and 0.6 pg/ml for GIP. Intra- and inter-assay coefficient of variations (CVs) were <10% and <15%, respectively, for all analytes. Plasma glucagon was measured by radioimmunoassay (GL-32K, Millipore, Billerica, MA). The minimum detectable limit was 20 pg/mL, and the intra- and inter-assay CVs were 3.8% and 8.1%, respectively.

3.3.5 Data and statistical analysis

All analyses were performed in collaboration with a professional biostatistician. Due to a lack of available data in similarly malnourished and refed patients with AN undertaking a similar study design, power calculations for the primary study outcome (postprandial blood glucose concentrations) were based on the results from the first 12 patients. We calculated that a clinically relevant within-participant mean difference of 0.6±0.9 mmol/L in AUC/min of blood glucose would be detectable with a sample size of n=22 patients with $P<0.05$ and statistical power (β) 80%.

Body weight was expressed as %EBW (% of expected body weight, determined using the 50th percentile for body mass index (BMI; kg/m^2) for age and sex from Centers for Disease Control and Prevention (CDC) growth charts (265)). Due to baseline differences in some variables, we calculated (using the trapezoidal rule) both total area under the curves (AUCs; to represent the overall response), and incremental AUCs (iAUCs; accounting for differences in pre-meal concentrations, thus, representing the magnitude of response to the test-meal) for all variables (except for 3-OMG and gastric emptying where baseline values were zero). AUC and iAUC values were divided by the time of last measurement to obtain a final weighted average to account for samples that could not be collected in n=3 participants due to cannula failure at t=120 min. The number of patients with a blood glucose concentration of <3.9 mmol/L at any postprandial time-point was also recorded (5, 266).

In patients, repeated-measures ANOVAs were used to analyse the data over the refeeding period, with post-hoc paired comparisons, adjusted for multiple comparisons by Bonferroni's correction, performed when ANOVAs revealed significant effects. Baseline

concentrations for all variables, AUCs and iAUCs for glucose and hormones, and AUCs for gastric emptying and 3-OMG, were analysed using independent samples t-tests to compare patients (at Wk0 and Wk2) with HCs. Pearson correlation was used to analyse the relationships between percentage of $^{13}CO_2$ AUC (0-30 min) with change in glucose from 0-30 min, as well as postprandial glucose and insulin with each other and glucagon, C-peptide, GIP and GLP-1, in patients at Wk0 and Wk2, and in HCs. Data were analysed using SPSS v23 (IBM, 2015). Statistical significance was accepted at $P<0.05$. All data are presented as mean±SE.

3.4 Results

At Wk0, %EBW was less in patients with AN than in HCs (P<0.001). In patients, %EBW was higher at Wk1 and Wk2 compared with Wk0 (both P<0.001), and higher at Wk2 compared with Wk1 (P=0.03). At Wk2, %EBW remained lower in patients than in HCs (P<0.001) (**Table 3.1**). At Wk0, RMR was lower, and RQ higher, in patients than in HCs (both P<0.001) (**Table 3.1**). In patients, RMR (P=0.007) and RQ (P=0.002) were higher at Wk1 than Wk0, though RQ was lower at Wk2 than Wk1 (P=0.003). There were no differences in RMR (P=0.12) or RQ (P=1.00) between Wk2 and Wk0, or for RMR between Wk2 and Wk1 (P=0.14). At Wk2, RMR remained lower (P=0.002), and RQ higher (P<0.001), in patients than in HCs.

3.4.1 Blood glucose

There were no differences in baseline blood glucose concentrations between AN and HCs at Wk0 (P=0.51) or Wk2 (P=0.77), or in patients with AN between Wk0, Wk1 and Wk2 (P=0.52) (**Table 3.2, Figure 3.1A**).

At Wk0, blood glucose AUC and iAUC were lower in patients than in HCs (both P<0.001) (**Table 3.3**). In patients, blood glucose AUC and iAUC were higher at Wk1 and Wk2 than Wk0 (both P<0.001), with no difference between Wk1 and Wk2 (P=1.00). At Wk2, blood glucose AUC and iAUC were lower in patients than in HCs (both P<0.01).

At Wk0, 18% (n=4) of patients, and at Wk2, 9% (n=3) of patients, had a blood glucose of <3.9 mmol/L at ≥1 time-point following the test-meal (**Table 3.3**). While the low n did not allow formal statistical comparisons, %EBW appeared lower in patients with

blood glucose <3.9 mmol/L (W0: 81.9±2.4%, W2: 86.9±2.1%) than those with blood glucose >3.9 mmol/L (W0: 90.0±2.6%, W2: 93.4±2.2%).

Table 3.2.
Baseline (pre-meal) blood glucose and plasma glucose-regulatory hormone concentrations in healthy controls (HC), and in patients with anorexia nervosa (AN) on admission (Wk0), and after one (Wk1) and two (Wk2) weeks of refeeding[1]

	HC	AN		
		Wk0	Wk1	Wk2
Glucose (mmol/L)	5.5±0.1	5.6±0.2	5.6±0.1	5.4±0.1
Insulin (mU/L)	5.0±1.5	3.9±0.6	2.4±0.4†	1.7±0.2*†
C-peptide (pg/mL)	1003±156	1273±106	839±74†	674±71*†‡
Glucagon (pg/mL)	68.8±3.8	79.4±3.4*	73.6±3.6	69.0±4.8
GIP (pg/mL)	96±14	140±20	112±20	87±16
GLP-1 (pg/mL)	57±6	86±14	81±14	71±14

[1]Data are means ± SEMs; n=22 anorexia nervosa (AN) patients and n=17 healthy controls (HCs). In AN patients, main treatment effects were determined using one-factor repeated-measures ANOVA with treatment as a within-participants factor. Comparisons between AN patients at both Wk0 and Wk2 and with HCs were conducted using independent-samples t tests. GIP = glucose-dependent insulinotropic peptide; GLP-1 = glucagon-like peptide-1. *Significantly different from HCs; †significantly different from Wk0; ‡significantly different from Wk1 (all P<0.05).

3.4.2 Gastric emptying

At Wk0, $\%^{13}CO_2$ recovery AUC was lower in patients than in HCs (P<0.001) (**Table 3.3**, **Figure 3.1B**), indicating slower gastric emptying. In patients, $\%^{13}CO_2$ recovery AUC was higher at Wk2 (P=0.02), but not at Wk1 (P=0.41), compared with Wk0, with no difference between Wk1 and Wk2 (P=0.36). At Wk2, there was no difference in $\%^{13}CO_2$ recovery AUC between patients and HCs (P=0.13), although mean values remained lower in patients.

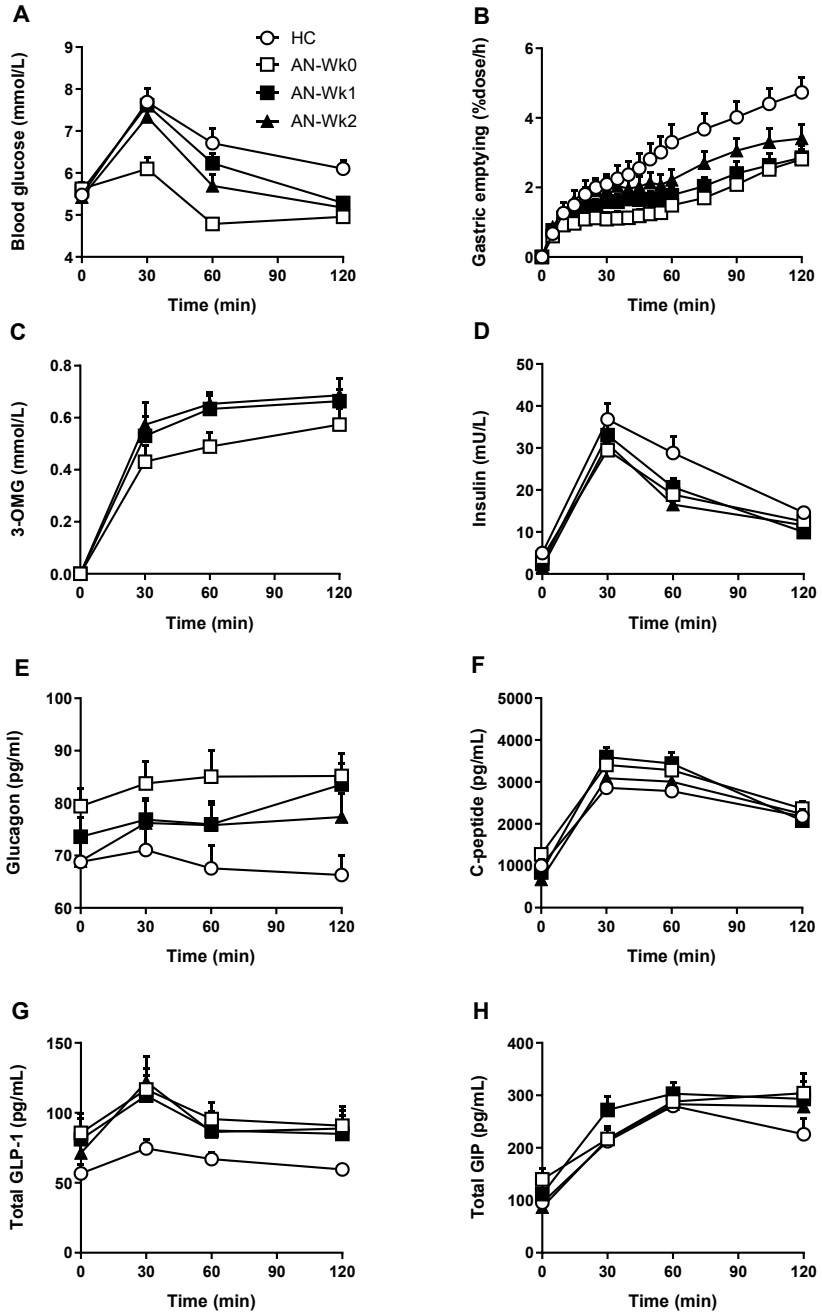

Figure 3.1.

Blood glucose (A), gastric emptying (B), serum 3-orthomethylglucose (3-OMG) (C), insulin (D), glucagon (E), C-peptide (F), and total glucagon-like peptide-1 (GLP-1) (G) and total glucose-dependent insulinotropic polypeptide (GIP) (H) responses to a mixed nutrient, semi-solid meal in 22 adolescent females with AN on admission (AN-Wk0), and after one (AN-Wk1) and two weeks (AN-Wk2) of refeeding, and in 17 age-matched healthy controls (HCs). Serum 3-OMG data are from n=7 AN patients. Data are means±SEM.

Table 3.3.

Area under the curves (AUCs) for blood glucose, gastric emptying, serum 3-OMG and plasma hormone concentrations, incremental AUCs per minute (iAUCs) for blood glucose and plasma hormone concentrations, and proportion of AN patients with blood glucose ≤3.9 mmol/L1.

	HC	AN		
		Wk0	Wk1	Wk2
Blood glucose				
AUC (mmol/L)	6.6±0.2	5.3±0.1*	6.3±0.1†	6.0±0.2*†
iAUC (mmol/L)	1.2±0.9	0.2±0.1*	0.8±0.1†	0.7±0.1*†
≤3.9 mmol/L (n)	0	4/22	0	2/22
Gastric emptying				
AUC (%dose/hr)	3.0±0.3	1.6±0.2*	1.9±0.2	2.4±0.3†
3-OMG				
AUC (mmol/L)	-	0.4±0.0	0.5±0.0†	0.6±0.0†
Insulin				
AUC (mU/L)	24.3±9.3	18.1±1.0*	18.8±1.0	17.0±1.5*
iAUC (mU/L)	19.4±2.2	14.2±1.2*	16.4±0.9	15.4±1.3
C-peptide				
AUC (pg/mL)	2399±171	2848±160	2813±165	2543±181
iAUC (pg/mL)	1445±155	1579±179	1977±166	1869±155
Glucagon				
AUC (pg/mL)	68.3±3.8	84.1±4.0*	77.8±3.6	75.4±3.9
iAUC (pg/mL)	2.7±0.7	7.2±2.1	7.5±1.5	8.0±1.6*
GIP				
AUC (pg/mL)	224.8±22	253.7±22	268.9±20	240.9±21
iAUC (pg/mL)	137±19	124±22	160±16	156±18
GLP-1				
AUC (pg/mL)	65.6±4	98.8±13*	92.2±12	94.1±14
iAUC (pg/mL)	13.8±2.8	16.9±3.4	15.0±3.0	24.2±4.2*

[1]Data are means ± SEMs; n=22 anorexia nervosa (AN) patients and n=17 healthy controls (HCs), and n=7 AN patients only for 3-OMG). In the AN group, main treatment effects were determined with the use of one-factor repeated-measures ANOVA with treatment as a within-participants factor. Comparisons between AN patients at both Wk0 and Wk2 and with HCs were conducted with the use of independent-samples t tests. GIP = glucose-dependent insulinotropic peptide; GLP-1 = glucagon-like peptide-1. *Significantly different from HC; †significantly different from Wk0 (all $P<0.05$).

3.4.3 Serum 3-OMG concentrations

In patients with AN, 3-OMG AUC was higher at Wk1 (P=0.01) and Wk2 (P=0.004) compared with Wk0, indicating greater glucose absorption, with no difference between Wk1 and Wk2 (P=1.0) (**Table 3.3, Figure 3.1C**).

3.4.4 Baseline insulin, C-peptide, glucagon, GLP-1 and GIP concentrations

There were no differences in baseline plasma insulin (P=0.44), C-peptide (P=0.14), GLP-1 (P=0.10) or GIP (P=0.10) concentrations between patients with AN and HCs at Wk0, although mean GLP-1 values were lower in HCs (**Table 3.2, Figure 3.1D-H**). At Wk0, baseline plasma glucagon was higher in patients than in HCs (P=0.04). In patients, there were no differences in baseline GLP-1 (P=0.08) or GIP (P=0.07) between Wk0, Wk1 and Wk2. Baseline insulin and C-peptide were lower at Wk1 (insulin: P=0.04; C-peptide: P=0.006) and Wk2 (insulin: P=0.003; C-peptide: P<0.001) compared with Wk0, and although there was no difference in insulin between Wk1 and Wk2 (P=0.08), C-peptide was lower at Wk2 than Wk1 (P=0.002). For baseline glucagon, there was an overall treatment effect in patients (P=0.04), and although post-hoc comparisons revealed no difference between Wk0, Wk1 and Wk2, concentrations tended to be lower at Wk2 compared with Wk0 (P=0.08). At Wk2, there were no differences in baseline glucagon (P=0.97), GLP-1 (P=0.39) or GIP (P=0.70) between patients and HCs, however, baseline insulin (P=0.015) and C-peptide (P=0.04) were lower in patients than in HCs.

3.4.5 Postprandial insulin, C-peptide, glucagon, GLP-1 and GIP concentrations

At Wk0, insulin AUC (P=0.015) and iAUC (P=0.02) were lower, while there was a trend for C-peptide AUC (P=0.07), but not C-peptide iAUC (P=0.58), to be higher in patients with AN than HCs (**Table 3.3**). Glucagon AUC was higher (P=0.009), and there was a

trend for glucagon iAUC to be higher (P=0.05), in patients than in HCs. Although GLP-1 AUC was higher in patients than in HCs (P=0.03), there was no difference in GLP-1 iAUC (P=0.49), nor GIP AUC (P=0.37) or iAUC (P=0.66) between patients and HCs.

In patients with AN, insulin, C-peptide, glucagon and GIP AUCs (insulin: P=0.31; C-peptide: P=0.11; glucagon: P=0.05; GIP: P=0.25) and iAUCs (insulin: P=0.18; C-peptide: P=0.06; glucagon: P=0.94; GIP: P=0.15), as well as GLP-1 AUC (P=0.59), did not differ between Wk0, Wk1 and Wk2. Although there was no difference between Wk1 (P=1.00) or Wk2 (P=0.25) and Wk0, there was a trend for GLP-1 iAUC to be higher at Wk2 than Wk1 (P=0.05).

At Wk2, insulin AUC (P=0.008), but not insulin iAUC (P=0.10), was lower in patients with AN than HCs. There was no difference in C-peptide (P=0.06), glucagon (P=0.21) or GIP AUC, nor GIP iAUC, between patients and HCs. Glucagon (P=0.21) and GLP-1 (P=0.09) AUCs did not differ between patients and HCs, but there was a trend for C-peptide iAUC to be higher (P=0.06). Glucagon iAUC was higher (P=0.005), and there was a trend for GLP-1 iAUC (P=0.06) to be higher, in patients than in HCs.

3.4.6 Relationships between blood glucose, hormones, gastric emptying and body weight

In patients with AN at Wk0, but not at Wk2 or in HCs, the increase in blood glucose from 0-30 min correlated directly with $^{13}CO_2$ recovery AUC 0-30 min at Wk0 (r=0.53, P=0.01). In patients with AN, there was a direct correlation between blood glucose iAUC with C-peptide iAUC at Wk2 (r=0.49, P=0.02). In HCs, blood glucose iAUC correlated directly with insulin iAUC (r=0.52, P=0.03) and C-peptide (r=0.50, P=0.04). In patients with AN,

there were significant correlations between insulin iAUC and GIP iAUC at Wk0 (r=0.72, P=0.04) and Wk2 (r=0.48, P=0.02). In HCs, insulin iAUC was directly correlated with GIP (r=0.55, P=0.02). There were also direct correlations between insulin iAUC and C-peptide iAUC in patients at Wk0 (r=0.63, P=0.002) and Wk2 (r=0.60, P=0.003), and in HCs (r=0.84, P<0.001). In patients with AN, the increase in weight from Wk0 to Wk2 was directly correlated with the increase in blood glucose iAUC from Wk0 to Wk2 (r= 0.57, P=0.006). No other correlations were observed.

3.5 Discussion

Our study demonstrated that on admission, compared with HCs, malnourished AN patients (i) had 'flattened' postprandial glucose curves, with the reduced initial rise associated with slower gastric emptying, (ii) plasma insulin was lower, while C-peptide tended to be higher, suggesting increased insulin clearance, (iii) fasting glucagon was elevated and not suppressed post-prandially, and (iv) GLP-1 appeared higher, probably due to higher baseline concentrations. After two weeks of refeeding, patients had (v) a greater postprandial rise in glucose, faster gastric emptying, increased 3-OMG and lower baseline insulin and C-peptide than in Wk0, and (vi) while overall glucose in patients remained lower, there were no significant differences in gastric emptying, baseline glucagon or postprandial insulin compared with HCs. The changes in postprandial glucose regulation in the patients are likely due to chronic caloric deficiency, and while two weeks of nutritional rehabilitation led to improvements (e.g. gastric emptying, postprandial insulin), postprandial glucose regulation remained markedly different from HCs.

Fasting glucose in AN at Wk0 did not differ from HCs, in line with some (169, 175), but not all (179, 180), previous studies. However, due to the short 4-hour fast, necessitated by the refeeding protocol, values may not have been true fasting levels. There was a marked difference in postprandial glucose, with lower peak glucose, and glucose falling below baseline ~60 min post-meal. The observed hypoglycemia in a few patients is consistent with other studies reporting a "flattened" postprandial glucose curve and postprandial hypoglycemia in starvation (5, 180, 267). Previous observations in AN have, however, been mixed, including lower (180), comparable (268), delayed (181) and higher (179) postprandial glucose relative to controls, discrepancies which probably reflect

experimental factors, including patient malnutrition, duration and level of pre-study nutritional intake, inclusion of purging vs. restricting AN sub-types, and test-meal composition (e.g. pure-glucose vs. mixed-nutrient meals) (179-181, 268).

Delayed gastric emptying in patients with AN is consistent with previous work (154, 155). Although gastric emptying is an important determinant of postprandial glucose, accounting for ~35% of the variance in health (42), this relationship has not previously been examined in AN. In AN at Wk0, the rate of gastric emptying correlated with the rise in glucose at 30 min, suggesting that slower gastric emptying may be driving the reduced postprandial glucose response. This is supported by the lower 3-OMG concentrations at Wk0 compared with Wk2 in a subset of patients, suggesting that in the starved state, small intestinal glucose absorption is reduced, and improved with refeeding. While this provides further evidence that small intestinal glucose absorption is reduced with short-term starvation (1.5-11 days) and improved with refeeding (269), further studies in a larger sample and over a longer period are required.

Since at Wk0 postprandial glucose barely rose above pre-meal levels in AN, the rise in insulin and C-peptide ~30 min after the meal, similar to HCs, was unexpected and may indicate dysregulated insulin secretion in AN. The rise in insulin was unlikely driven by glucose given that glucose levels were lower, but may reflect increased insulin sensitivity, as previously suggested (174, 178, 270). Moreover, there is evidence of a potential genetic contribution to increased insulin sensitivity in AN (271). It is also possible that amino acids in the test-meal may have augmented insulin secretion (272), and intestinal amino acid absorption may also increase with malnutrition (273), although this remains unstudied in AN. In any case, our study confirms previous findings of lower overall

insulin responses in AN following oral (179) or intravenous (173) glucose tolerance tests, or meals (180). Due to controlled timing of meal intake, we observed a shorter time to peak and no shift in profile described by others (180). In contrast, C-peptide responses were similar to those in HCs, in line with previous reports that patients may clear insulin more rapidly (175, 178). In addition, both baseline and postprandial glucagon were elevated at Wk0, confirming previous findings (179) (186), possibly due to the relative increase in insulin. Moreover, the meal failed to suppress glucagon, potentially acting to offset the low glucose and insulin rises. Although elevated GLP-1 was observed in patients, this contrasts previous research in a smaller sample finding a decrease in GLP-1 (169). GLP-1 only displays its insulinotropic effect once blood glucose reaches ~8 mmol/L, suggesting that GLP-1 is unlikely to have stimulated insulin in this setting. Furthermore, GIP, whose insulinotropic effect is also glucose-dependent, did not differ between patients and HCs. However, GIP is known to enhance postprandial glucagon in health, and 'sensitivity' to GIP may be modified in AN. Examination of amino acid absorption as well as the role of glucagon in glycemic control in chronic starvation may provide further insight into the postprandial insulin rise.

After two weeks of refeeding, postprandial glucose improved, likely reflecting faster gastric emptying (160, 163, 165), and postprandial insulin increased, possibly due to improved insulin sensitivity with nutrient exposure. Despite these improvements, postprandial glucose responses remained below HCs. We also found a trend for overall GLP-1 to remain higher, perhaps related to enhanced nutrient sensitivity, or small intestinal transit, since GLP-1 is mainly released in the distal small intestine (33). Taken together, since two weeks of refeeding appears inadequate to return gastric emptying and

postprandial glucose profiles to those observed in health, continued clinical monitoring is indicated.

Several study limitations should be noted. Initial refeeding, required to achieve medical stability, may have improved GI and glycemic responses before studies at the 'baseline visit' (Wk0), thus, early improvements may have been missed. Moreover, to minimise medical risk, the treatment protocol only allowed for patients with AN to be fasted for four hours prior to the Wk0 test-meal, hence, the previous meal, which was standardized in all but n=5 still on nasogastric feeds at Wk0, may have impacted the response to the test-meal. Although caloric intake was individualized for each patient's refeeding requirements, the standardised refeeding protocol ensured comparable nutrient intakes between patients. While the ^{13}C breath-test has only been validated in healthy children (274) and those with dyspeptic or respiratory complaints (275), it was the safest and least invasive method to measure gastric emptying in our patients. Blood glucose was assessed by glucometer, potentially affecting the accuracy of absolute glucose values, particularly in relation to hypoglycemia (276). Two patients took olanzapine, which rarely increases blood glucose, hence, a major effect on this outcome is unlikely. Finally, since 68% of the patients were first admits, the results may not be generalizable to more chronically ill patients with AN.

In conclusion, postprandial glucose is markedly reduced in starvation, leading to hypoglycemic levels in some patients with AN. Delayed gastric emptying appears to be a major driver for this, and the relationship with glucose absorption warrants further investigation. The acceleration of gastric emptying towards normal after two weeks of refeeding, associated with improvements in glycemic regulation, implicates chronic

nutritional deprivation in the disturbances observed in starvation. However, further research is needed to determine the extent of refeeding necessary for complete restoration, or whether some changes persist beyond weight restoration. This work suggests that GI nutrient-sensing mechanisms may be altered in AN and highlights the importance of close medical monitoring during refeeding while glucoregulatory disturbances persist. Future studies should examine whether a range of symptoms that patients with AN often present with (e.g. dizziness, fainting, tiredness, poor attention, amongst others), which are often attributed to low blood pressure or heart rate, are in fact due to reduced postprandial glucose or hypoglycemic events, by examining symptoms alongside continuous glucose monitoring during starvation and with acute refeeding in AN.

Chapter 4: Appetite perceptions, gastrointestinal symptoms, ghrelin, peptide YY and state anxiety are disturbed in adolescent females with anorexia nervosa and only partially restored with short-term refeeding

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4.1 Abstract

Factors underlying disturbed appetite perception in anorexia nervosa (AN) are poorly characterized. We examined in patients with AN whether fasting and postprandial appetite perceptions, GI hormones, GI symptoms and state anxiety (i) differed from healthy controls (HCs) and (ii) were modified by two weeks of refeeding. 22 female adolescent inpatients with restricting AN, studied on hospital admission, once medically stable ('Wk0'), and after one ('Wk1') and two ('Wk2') weeks of high-calorie refeeding, were compared with 17 age-matched HCs. After a 4-hour fast, appetite perceptions, GI symptoms, state anxiety, and plasma acyl-ghrelin, CCK, PYY and pancreatic polypeptide (PP) concentrations were assessed at baseline and in response to a mixed-nutrient test-meal (479 kcal). Compared with HCs, in patients with AN at Wk0, baseline ghrelin, PYY, fullness, bloating and anxiety were higher, and hunger less, and in response to the meal, ghrelin, bloating and anxiety were greater, and hunger less (all $P < 0.05$). After two weeks of refeeding, there was no change in baseline or postprandial ghrelin or bloating, or postprandial anxiety, but baseline PYY, fullness and anxiety decreased, and baseline and postprandial hunger increased ($P < 0.05$). We conclude that in AN, refeeding for 2 weeks was associated with improvements in PYY, appetite and baseline anxiety, while increased ghrelin and bloating, as well as postprandial anxiety persisted.

4.2 Introduction

AN is characterised by severe dietary restriction, weight loss and high levels of anxiety (2, 277). GI symptoms (e.g. bloating and nausea) are commonly reported (160) and may contribute to a reduction in caloric intake. Moreover, despite significant malnutrition (278, 279), patients frequently report reduced hunger and increased fullness (6, 7). It is, however, unclear whether these self-reported symptoms are related to alterations in the GI mechanisms underlying appetite regulation or to heightened anxiety.

In healthy individuals, the GI tract plays a pivotal role in appetite regulation. Slowed gastric emptying, and in particular, increased content of the distal stomach (antral distension), are related directly to greater fullness (280). Moreover, as nutrients reach the small intestine, GI hormones (e.g. CCK (281), PYY (91), pancreatic polypeptide (PP) (282)) are released, and ghrelin is suppressed (118), providing feedback to further slow gastric emptying and reduce food intake. Both gastric emptying and GI hormone secretion are sensitive to changes in diet (17, 18). For example, a 4-day fast slows gastric emptying in healthy individuals (17), and 30% dietary restriction for 12 weeks modifies postprandial GI hormone release in obesity (18). Thus, in AN, prolonged energy restriction has the potential to induce changes in the GI mechanisms involved in appetite regulation. Previous research has reported that both fasting and postprandial total ghrelin and PYY(3-36) concentrations are higher in patients with AN than healthy individuals, with levels decreasing towards those in healthy individuals after three months of refeeding (177). Observations in relation to CCK and PP in untreated patients with AN have been inconsistent, with plasma concentrations increased (180, 197), or not different (155, 200), from HCs. Gastric emptying is also severely delayed in AN (155, 254, 283), and we have reported recently, in the current cohort, that two weeks of standardized high-

calorie refeeding improves gastric emptying (283). However, it remains unclear whether changes in GI hormones contribute to the disturbed appetite perceptions reported by patients with AN and to what extent these factors may improve with short-term refeeding.

Anxiety, a primary feature of AN, can potently influence appetite in healthy individuals, to potentially increase or decrease hunger and food intake (26, 27). Psychological stress also appears to influence GI motility (284, 285) and GI hormone secretion (286). Moreover, GI symptoms (e.g. nausea, bloating and epigastric discomfort) are frequent complaints in patients with AN (8). In other conditions, such as functional dyspepsia, which is also associated with high levels of anxiety (287), there is a positive association between anxiety levels with postprandial symptom intensity (288). Interestingly, functional dyspepsia is also associated with disturbances in GI motility (289) and circulating ghrelin (290), CCK and PYY (291) concentrations. It is, therefore, conceivable that anxiety may contribute to the appetite and GI disturbances, and GI symptoms, in AN. In AN, greater pre-meal anxiety is associated with reduced food intake (292), and pre- and post-meal anxiety is at least partially reduced after nutritional rehabilitation (25).

We, therefore, investigated in malnourished patients with AN whether appetite perceptions, GI hormones, GI symptoms and state anxiety at baseline, and in response to a mixed-nutrient semi-solid test-meal (i) differed from HCs and (ii) were modified by short-term refeeding.

4.3 Materials and methods

4.3.1 Participants

Twenty-two female adolescent inpatients with AN (restricting sub-type), as defined by the Diagnostic and Statistical Manual of Mental Disorders (5th edition, DSM-5 (1)) criteria, and 17 healthy, age-matched female control participants, were included in the study. Fifteen of the 22 patients had not received previous treatment and reported 36±4 weeks of recent dietary restriction (weight loss: 12±1 kg; total illness duration: 11±3 months) (283). The 7 patients who had been previously treated reported 10±2 weeks of recent dietary restriction (weight loss: 6±1 kg; total illness duration: 27±6 months). Based on previous studies (172, 195, 293), sample sizes of n = 22 patients with AN and n = 17 HCs were powered to detect a difference between groups in fasting PYY of 42.5 pg/ml, and a difference in postprandial PYY at 120 min of 31.6 pg/ml, with $P < 0.05$ and statistical power (1-β) 80%. These numbers also allowed detection of a mean difference of 24 mm on a visual analogue scale (VAS) for hunger and fullness, based on post-hoc power calculations of previous research (7).

Participant recruitment and characteristics have been described previously (283). Patients were excluded if they had current diarrhoea, constipation, GI disease or previous surgery, or a history of other medical conditions unrelated to complications of AN-related malnutrition, vomiting, smoking or consumption of >20 g alcohol per day. One patient took olanzapine throughout the study and another patient during week 1 only, but there was no use of any other medication.

The study protocol was approved by the Human Research Ethics Committees at the Sydney Children's Hospital Network (reference no. 11CHW88, approval: May 23, 2011),

and ratified by the Royal Adelaide Hospital and the University of Adelaide, and all studies were carried out in accordance with the Declaration of Helsinki. All participants, and parents for those <18 years old, provided informed, written consent prior to their enrolment. The study was registered as a clinical trial with the Australia and New Zealand Clinical Trial Registry (http://www.anzctr.org.au; Trial ID: 12616000134426).

4.3.2 Study design

Upon admission, all patients with AN commenced a standardized rapid high-calorie refeeding protocol commencing on 2400 kcal/day (100 mL/hr; Jevity, Abbott Nutrition (1 kcal/mL)), and then transitioned to an energy delivery of 2400-3200 kcal/day (259). Patients were studied on three occasions: once medically stable within the first five days (2.2 ± 0.2 days) of admission ('Wk0'), then one ('Wk1') and two ('Wk2') weeks post-Wk0 while undergoing refeeding treatment. HCs were studied on one occasion only. On each study day, appetite perceptions, plasma acyl-ghrelin, CCK, PYY and PP concentrations, as well as GI symptoms and state anxiety were evaluated at baseline and in response to an oral mixed-nutrient test-meal.

4.3.3 Protocol

Patients were studied during their inpatient admission and, if discharged prior to Wk2 (n = 6), re-attended hospital. Both discharged patients and HCs were asked to refrain from vigorous exercise and alcohol intake for 24 h before the study, and, on the study day, attended the hospital at 0800 hours after fasting from 2000 h the night before.

On each study day, participants were weighed in the morning. To allow for regular blood sampling, an intravenous cannula was inserted into an antecubital vein at 0800 hours. At

0830 hours, patients with AN on an oral diet (n = 17 at Wk0, n = 22 at Wk1 and Wk2) and all HCs were provided with a standardised breakfast (30 g cereal wheat biscuits (Weetbix, Sanitarium, Australia), 400 mL full-cream milk and an apple; 479 kcal, 65 g carbohydrate, 19 g protein, 15 g fat, 7 g fibre), and allowed up to 30 min for consumption. Medically stable patients still receiving continuous nasogastric feeds (100 mL/hr; Jevity, Abbott Nutrition (1 kcal/mL)) at Wk0 (n = 5), had feeds ceased at 0900 hours in lieu of the standardised breakfast. No further food or fluid (except water) was consumed prior to the test-meal.

At 1300 hours, before consumption of the test-meal (t = 0 min), a baseline blood sample (15 mL) was collected, and, using VAS questionnaires, participants rated appetite perceptions, GI symptoms (nausea and bloating) and state anxiety (baseline values). Following this, a mixed-nutrient semi-solid test-meal identical to that given at breakfast was consumed over 15 min. Further blood samples and VAS ratings were collected at t = 30, 60 and 120 min, with timing commencing as soon as test-meal ingestion was complete. At the end of the study day, participants were provided with afternoon tea before returning to the ward or home.

4.3.4 Measurements

4.3.4.1 Appetite perceptions, GI symptoms and state anxiety

Appetite (hunger, desire to eat, prospective consumption and fullness), nausea, bloating and state anxiety were assessed with a previously described 100-mm VAS questionnaire, anchored by ‘not at all’ and ‘very much’ (241). Assessment of state anxiety by VAS has been validated in adolescents against the State-Trait Anxiety Inventory (STAI) (294). More detailed GI symptoms (including nausea, sickness, vomiting, bloating, abdominal

cramps, early satiety, acidic eructation/heartburn, loss of appetite, retrosternal discomfort and epigastric pain/upper abdominal pain) were assessed using the GI Symptom Score (GIS) questionnaire (295) once on each study day at baseline.

4.3.4.2 Plasma hormone analysis

Blood samples were collected in ice-chilled EDTA-treated tubes, containing 20 uL/mL blood of the serine protease inhibitor, 4-(2-aminoethyl) benzene sulfonyl fluoride hydrochloride (Pefabloc, Roche, Australia) (296). Samples were separated by centrifugation (3200 rpm, 15 min, 4 °C) within 15 min of collection, and plasma stored at −80 °C until assayed, for later analysis of plasma concentrations of plasma acyl-ghrelin, CCK-8, PYY and PP. Plasma acyl-ghrelin (pg/mL), total PYY (pg/mL) and PP (pg/mL) concentrations were measured using a multiplex assay (Milliplex® MAP Human Metabolic Hormone Magnetic Bead Panel, HMHEMAG-34K, Millipore Corporation, Temecula, CA, USA) and analysed on a Bio-plex® MAGPIXTM Multiplex Reader (Luminex®, Millipore Corporation, Temecula, CA, USA) using xPONENT® software (Luminex®, Millipore Corporation, Temecula, CA, USA, version 4.2) according to manufacturer's instructions. There was negligible antibody cross-reactivity. The minimum detectable limits were 13 pg/mL for ghrelin, 28 pg/mL for PYY and 2 pg/mL for PP. Intra- and inter-assay coefficients of variation (CVs) were <10% and <15%, respectively, for all analytes. Plasma CCK-8 (pmol/L) was measured by radioimmunoassay using an adaption of the method of Santangelo et al. (297). Samples were extracted in 66% ethanol, extracts were dried down and resuspended in assay buffer (50 mM phosphate, 10 mM EDTA, 2 g/L gelatin, pH 7.4). Standards were prepared using synthetic sulphated CCK-8 (Sigma Chemical, St Louis, MO, USA), antibody (C2581, Lot 105H4852, Sigma Chemical, St Louis, MO, USA) was added at a working dilution

of 1/17,500 and sulphated CCK-8 125I-labeled with Bolton and Hunter reagent (Perkin Elmer, Boston, MA, USA) was used as tracer. Incubation was for 7 days at 4˚C. The antibody bound fraction was separated by the addition of dextran-coated charcoal containing gelatin (0.015 g gelatin, 0.09 g dextran, 0.15 g charcoal in 30 ml assay buffer) and the radioactivity determined in the supernatants following centrifugation. Intra- and inter-assay CVs were 6.2% and 13.4%, respectively. The minimal detectable limit was 1 pmol/L.

4.3.5 Data and statistical analyses

The analyses were performed in collaboration with a professional biostatistician. To compare patients with AN with HCs, age, EDE-Q and RCADS were analysed using independent samples t-tests. Body weight was expressed as %EBW (% of expected body weight, determined using the 50th percentile for body mass index (BMI; kg/m2) for age and sex from CDC growth charts (37)).

4.3.5.1 Baseline and responses to the test meal

Incremental areas under the curves (iAUCs) were calculated using the trapezoidal rule (correcting for differences in baseline (pre-meal) concentrations to represent the magnitude of the response to the test meal) for all variables. Where data decreased from baseline (i.e. ghrelin, hunger, desire to eat and prospective consumption), inverse iAUCs were calculated. For gut hormones, iAUC values were divided by the time of last measurement to obtain a final weighted average to account for plasma samples that could not be collected in n = 3 participants (on one occasion each) due to cannula failure at t = 120 min. For consistency, iAUCs for all other variables were also divided by 120 to

obtain a final weighted average. Appetite, bloating, nausea and anxiety VAS scores were skewed and analysed using the Friedman test.

4.3.5.2 Longitudinal comparisons

GI hormone data were normally distributed and analysed using repeated-measures ANOVAs with visit (Wk0, Wk1, Wk2) as the factor. Post-hoc paired comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed when ANOVAs revealed significant effects. To compare patients with AN (at both Wk0 and Wk2) with HCs, baseline values and iAUCs were analysed using independent samples t-tests. Non-parametric Mann-Whitney tests were used to compare baseline values and iAUCs for GI symptoms and anxiety scores in patients with AN (at Wk0 and Wk2) with HCs.

Data were analysed using SPSS version 23 (IBM, 2015). Statistical significance was accepted at $P < 0.05$. Parametric data are presented as means ± standard errors (SEs), and non-parametric data as medians (25^{th}-75^{th} quartiles).

4.4 Results

The experimental conditions were well tolerated by all study participants. At Wk0, %EBW was less in patients with AN than in HCs (78.7% ± 1.8 vs 102.8% ± 2.2, $P < 0.001$). In patients, %EBW was higher at Wk1 (83.8% ± 1.8) and Wk2 (85.2% ± 1.9) compared with Wk0 (both $P < 0.001$), and higher at Wk2 compared with Wk1 ($P = 0.03$) (283). At Wk2, %EBW remained lower in patients than in HCs ($P < 0.001$).

Patients with AN had significantly higher scores on both the Eating Disorder Examination Questionnaire (EDE-Q) global score (258) (3.1 ± 0.3 vs 0.5 ± 0.1, $P <0.05$) and Revised Children's Anxiety and Depression Scale (RCADS) Total Anxiety score (298) (55.9 ± 2.6 vs 39.6 ± 1.5, $P < 0.05$). There was no significant difference in age between the patients with AN (15.9 ± 0.4 years, range 13-19 years) and HCs (16.5 ± 0.7 years, range 12-19 years, $P > 0.05$).

4.4.1 Appetite perceptions

Baseline ratings (t = 0 min)

At Wk0, baseline hunger, desire-to-eat and prospective consumption were less, and fullness greater, in patients than HCs ($P < 0.05$) **(Table 4.1, Figure 4.1A-D)**. In patients, baseline hunger, desire-to-eat, prospective consumption and fullness did not differ between Wk0, Wk1 and Wk2, although median values for hunger, desire-to-eat and prospective consumption increased, and for fullness decreased. At Wk2, there were no longer any differences in baseline hunger, desire-to-eat or prospective consumption between patients and HCs, but there was a trend for baseline fullness to remain higher in patients than in HCs ($P = 0.085$).

Table 4.1.
Appetite perceptions, GI symptoms and state anxiety at baseline[1].

	HCs	AN patients		
		Wk0	**Wk1**	**Wk2**
Hunger (mm)	56(36–68)	14(5–24)*	35(3–51)	48(2–66)
Desire to eat (mm)	58(43–74)	20(6–59)*	29(2–52)	47(12–65)
Prospective consumption (mm)	60(48–67)	32(21–49)*	39(25–56)	47(31–62)
Fullness (mm)	18(0–48)	65(36–85)*	48(12–73)	39(13–62)
Gastrointestinal symptom score (points)	0(0–2)	7(5–13)*	3(2–8)$^{\#}$	3(0–8)*$^{\#}$
Nausea (mm)	3(0–14)	9(3–44)	11(0–35)	2(0–49)
Bloating (mm)	1(0–6)	32(11–73)*	41(11–64)	24(3–49)*
State anxiety (mm)	4(2–13)	27(12–57)*	21(3–65)	9(3–30)

[1]Data are medians (25^{th}–75^{th} quartiles); n = 22 patients with anorexia nervosa (AN) and n = 17 healthy controls (HCs). In the patients with AN, main treatment effects were determined using Friedman tests. Comparisons between patients with AN and HCs at both Wk0 and Wk2 were conducted using Mann-Whitney tests. GI, gastrointestinal. *significantly different from HCs, $P < 0.05$; $^{\#}$significantly different from Wk0, $P < 0.05$.

Responses to the test meal

At Wk0, hunger inverse iAUC was less in patients than HCs ($P < 0.05$) **(Table 4.2, Figure 4.1A)**. However, there was no difference in fullness iAUC, or desire-to-eat and prospective consumption inverse iAUCs, between patients and HCs **(Table 4.2, Figure 4.1B-D)**. In patients, fullness iAUC and hunger, desire-to-eat and prospective consumption inverse iAUCs did not differ between Wk0, Wk1 and Wk2. At Wk 2, there were no differences in fullness iAUC, or hunger, desire-to-eat or prospective consumption inverse iAUCs, between patients and HCs.

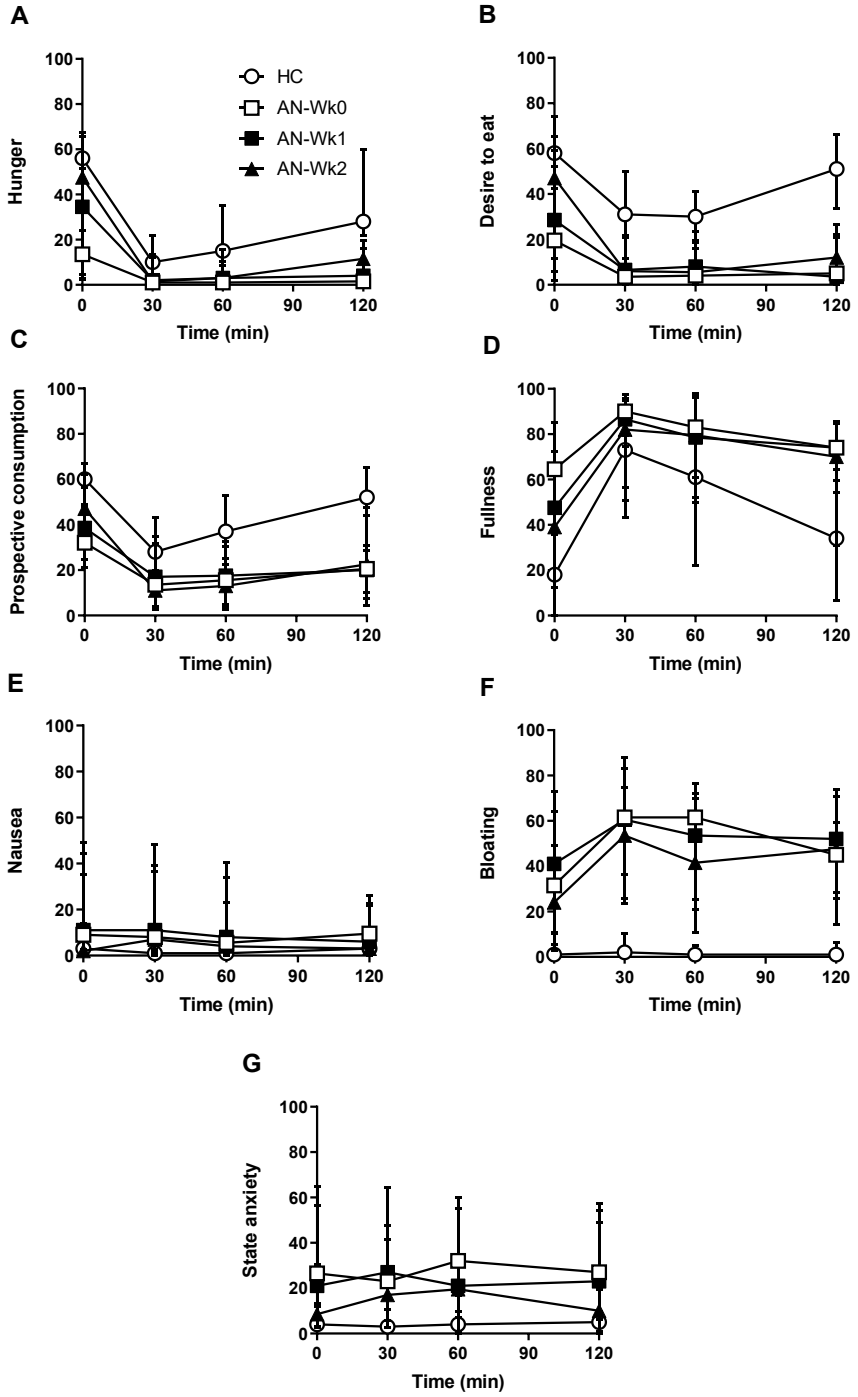

Figure 4.1.

Hunger (A), desire to eat (B), prospective consumption (C), fullness (D) nausea (E), bloating (F) and state anxiety (G) scores before and after a mixed-nutrient, solid-liquid meal in 22 adolescent females with anorexia nervosa on admission (AN-Wk0), and after one (AN-Wk1) and two weeks (AN-Wk2) of refeeding, as well as in 17 age-matched healthy controls (HC). Data are medians (25th-75th quartiles).

Table 4.2.

Inverse incremental area under the curves (iAUCs) per minute for hunger, desire to eat and prospective consumption perceptions, and iAUCs per minute for fullness perceptions, GI symptoms and state anxiety[1].

	HCs	AN patients		
		Wk0	Wk1	Wk2
Hunger (iAUC, mm)	23(9–40)	10(2–20)*	22(1–39)	26(1–43)
Desire to eat (iAUC, mm)	16(3–32)	10(3–28)	19(1–27)	29(8–35)
Prospective consumption (iAUC, mm)	12(8–21)	16(6–25)	17(3–33)	21(13–38)
Fullness (iAUC, mm)	24(10–42)	16(1–30)	22(10–31)	25(12–40)
Nausea (iAUC, mm)	0(0–2)	0(0–5)	1(0–2)	1(0–4)
Bloating (iAUC, mm)	0(0–2)	6(0–21)*	7(5–19)	12(2–21)*
State anxiety (iAUC, mm)	0(0–0)	2(0–23)*	1(0–7)	3(0–6)*

[1]Data are medians (25^{th}-75^{th} quartiles); n = 22 anorexia nervosa (AN) patients and n = 17 healthy controls (HCs). In patients with AN, main treatment effects were determined using Friedman tests. Comparisons between patients with AN at both Wk0 and Wk2 and with HCs were conducted using Mann-Whitney tests. GI, gastrointestinal. *Significantly different from HC, $P < 0.05$.

4.4.2 Gastrointestinal symptoms

Baseline ratings

At Wk0, the total GIS score was greater in patients than HCs ($P < 0.05$) **(Table 4.1)**. In patients, the GIS was lower at Wk1 and Wk2 than Wk0 ($P < 0.05$), but there was no difference between Wk1 and Wk2. At Wk2, the GIS remained greater in patients than in HCs ($P < 0.05$). Examining VAS ratings at Wk0, there was no difference in nausea between patients and HCs, but bloating was greater in patients ($P < 0.05$) **(Table 4.1, Figure 4.1E-F)**. In patients, nausea and bloating did not differ between Wk0, Wk1 and Wk2. At Wk2, bloating, but not nausea, was greater in patients than HCs ($P < 0.05$).

Responses to the test meal

At Wk0, bloating, but not nausea, iAUC was greater in patients than HCs ($P < 0.05$) (**Table 4.2, Figure 4.1E-F**). In patients, nausea and bloating iAUC did not differ between Wk0, Wk1 and Wk2. At Wk2, bloating, but not nausea, iAUC was greater in patients than in HCs ($P < 0.05$).

4.4.3 State anxiety

Baseline ratings

At Wk0, baseline anxiety was higher in patients than HCs ($P < 0.05$) **(Table 4.1, Figure 4.1G)**. In patients, there was a trend for a treatment effect for baseline anxiety (P = 0.06), with mean values declining over time, but there was no difference between Wk0, Wk1 and Wk2. At Wk2, there was also no difference in baseline anxiety between patients and HCs.

Responses to the test meal

At Wk0, anxiety iAUC was greater in patients with AN than in HCs ($P < 0.05$) **(Table 4.2, Figure 4.1G)**. In patients, anxiety iAUC did not differ between Wk0, Wk1 and Wk2. At Wk2, anxiety iAUC was greater in patients than in HCs ($P < 0.05$).

4.4.4 Gut hormones

4.4.4.1 Plasma acyl-ghrelin

Baseline concentrations

At Wk0, baseline ghrelin was higher in patients than HCs ($P < 0.05$) **(Table 4.3, Figure 4.2A)**. In patients, baseline ghrelin did not differ between Wk0, Wk1 and Wk2, and, at Wk2, baseline ghrelin remained higher than in HCs ($P < 0.05$).

Table 4.3.

Plasma hormone concentrations at baseline[1].

	HCs	AN patients		
		Wk0	**Wk1**	**Wk2**
Acyl ghrelin (pg/ml)	110 ± 11	237 ± 37*	270 ± 36	261 ± 26*
CCK[2] (pmol/L)	1.2 ± 0.2	1.6 ± 0.2	1.4 ± 0.1	1.8 ± 0.2
Total PYY (pg/ml)	64 ± 6.7	91 ± 8.7*	84 ± 6.2	80 ± 5.8
PP (pg/ml)	73 ± 42	111 ± 28	106 ± 28	113 ± 28

[1]Data are means ± SEMs; n = 22 patients with anorexia nervosa (AN) and n = 17 healthy controls (HCs). In the patients with AN, main treatment effects were determined using one-factor repeated-measures ANOVA with treatment as a within-subjects factor. Comparisons between HCs and patients with AN at both Wk0 and Wk2 were conducted using independent-samples t-tests. CCK, cholecystokinin; PYY, peptide tyrosine tyrosine; PP, pancreatic polypeptide. *significantly different from HC, $P < 0.05$. [2]n = 16 HCs and n = 19 patients with AN due to missing data.

Table 4.4.

Inverse incremental area under the curves (iAUCs) per minute for acyl ghrelin, and iAUCs per minute for CCK, total PYY and PP[1].

	HCs	AN patients		
		Wk0	**Wk1**	**Wk2**
Acyl ghrelin (inverse iAUC, pg/ml)	39 ± 6	101 ± 17*	119 ± 19	112 ± 15*
CCK[2] (iAUC, pmol/L)	1.3 ± 0.2	1.0 ± 0.2	1.5 ± 0.2	1.1 ± 0.2
Total PYY (iAUC, pg/ml)	19 ± 4	17 ± 3	16 ± 3	21 ± 5
PP (iAUC, pg/ml)	51 ± 10	58 ± 13	55 ± 8	54 ± 8

[1]Data are means ± SEMs; n = 22 patients with anorexia nervosa (AN) and n = 17 healthy controls (HCs). In the AN group, main treatment effects were determined using 1-factor repeated-measures ANOVA with treatment as a within-subjects factor. Comparisons between patients with AN and HCs at both Wk0 and Wk2 were conducted using independent-samples t tests. CCK, cholecystokinin; PYY, peptide tyrosine tyrosine; PP, pancreatic polypeptide. *significantly different from HC, $P < 0.05$. [2]n = 16 HCs and n = 19 patients with AN due to missing data.

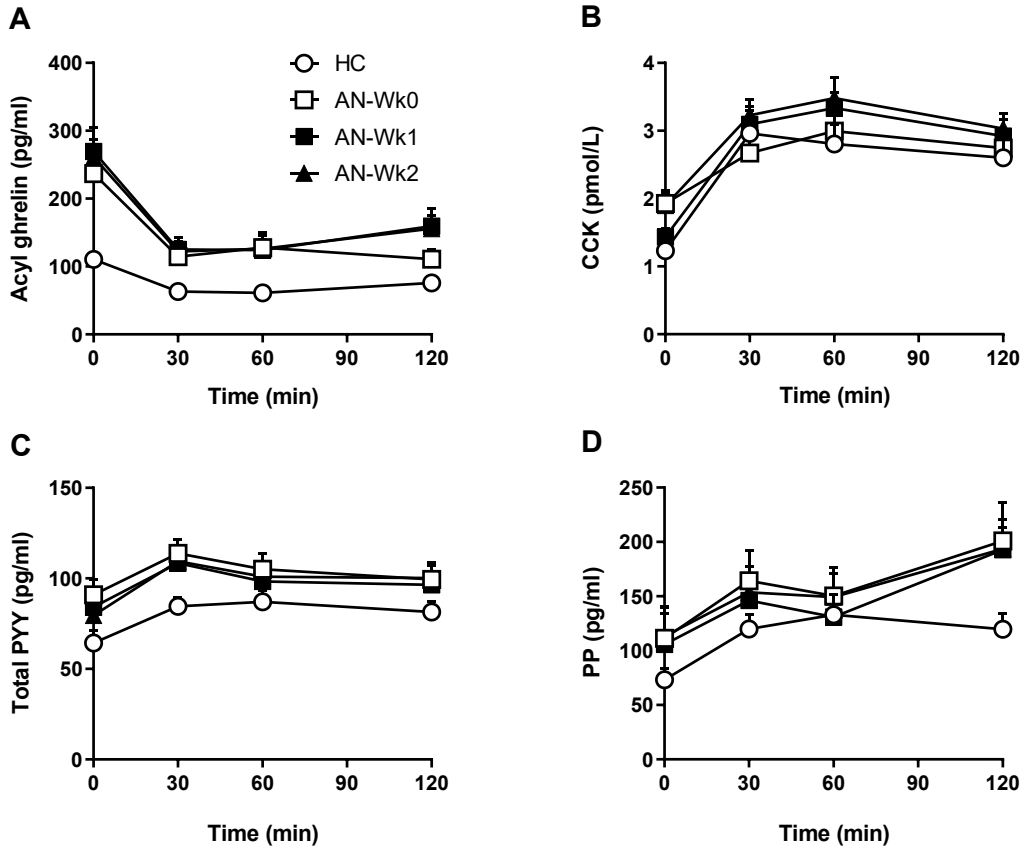

Figure 4.2.

Acyl ghrelin (A), CCK (B) total PYY (C), and PP (D) concentrations before and after a mixed-nutrient, solid-liquid meal in 22 adolescent females with anorexia nervosa on admission (AN-Wk0), and after one (AN-Wk1) and two weeks (AN-Wk2) of refeeding, as well as in 17 age-matched healthy controls (HC). Data are means ± SEM. For CCK: n = 16 HCs and n = 19 patients with AN due to missing data.

Responses to the test meal

At Wk0, ghrelin inverse iAUC was greater in patients with AN than in HCs ($P < 0.05$) **(Table 4.4, Figure 4.2A)**. In patients, ghrelin inverse iAUC did not differ between Wk0, Wk1 and Wk2, and, at Wk2, ghrelin inverse iAUC remained greater than in HCs ($P < 0.05$).

4.4.4.2 Plasma CCK

Baseline concentrations

There was no difference in baseline CCK between patients and HCs at Wk0 or Wk2, nor in patients between Wk0, Wk1 and Wk2 **(Table 4.3, Figure 4.2B)**.

Responses to the test meal

There were no differences in CCK iAUC between patients with AN and HCs at Wk0 or Wk2, nor in patients across Wk0, Wk1 and Wk2 **(Table 4.4, Figure 4.2B)**.

4.4.4.3 Plasma total PYY

Baseline concentrations

At Wk0, baseline PYY concentrations were higher in patients than HCs ($P < 0.05$) **(Table 4.3, Figure 4.2C)**. In patients, baseline PYY did not differ between Wk0, Wk1 and Wk2. At Wk2, there was no difference in baseline PYY between patients and HCs.

Responses to the test meal

There were no differences in PYY iAUC between patients with AN and HCs at Wk0 or Wk2, nor in patients across Wk0, Wk1 and Wk2 **(Table 4.4, Figure 4.2C)**.

4.4.4.4 Plasma PP

Baseline concentrations

There was no difference in baseline PP concentrations between patients and HCs at Wk0 or Wk2, nor in patients between Wk0, Wk1 and Wk2 **(Table 4.3, Figure 4.2D)**.

Responses to the test meal

There were no differences in PP iAUC between patients with AN and HCs at Wk0 or Wk2, nor in patients across Wk0, Wk1 and Wk2 **(Table 4.4, Figure 4.2D)**.

4.5 Discussion

The current study established that malnourished patients with AN display greater fullness, bloating and overall GI symptoms, and less hunger, desire to eat and prospective consumption when compared with HCs, and that two weeks of high-calorie refeeding was associated with changes in ratings of hunger, prospective consumption, desire to eat and fullness towards normal, while bloating and GIS remained higher. The patients also exhibited disturbances in GI hormones. At Wk0, baseline concentrations of acyl-ghrelin and total PYY were higher, while postprandial suppression of ghrelin was greater than in HCs; at Wk2, the disturbances in ghrelin remained, but PYY no longer differed from HCs. At Wk0, in the patients baseline and postprandial anxiety were also greater than in HCs, and while baseline anxiety improved with refeeding, postprandial anxiety remained higher in patients than in HCs at Wk2.

Patients with AN exhibited substantially lower fasting and postprandial hunger and greater fullness than HCs, despite chronically restricted energy intake prior to Wk0. These observations are consistent with previous studies (6, 7). It has been suggested that patients with AN may be unaware of, or not respond to, internal and external eating-related cues in a manner comparable to healthy people (299). Refeeding was associated with increases in baseline hunger, desire to eat and prospective consumption, as well as the suppression of hunger in response to the test-meal, suggesting that refeeding partially restores perceptions of appetite. Reduced hunger and increased fullness evident in the patients may reflect pathophysiologically enhanced sensitivity to the appetite-suppressant effect of nutrients, which appears to be, at least in part, reversed by re-exposure to feeding. Indeed, sensitivity to nutrients is altered in other conditions, including obesity (300), functional dyspepsia (301) and anorexia of ageing (233). Moreover, in obesity,

the altered sensitivity can be modified by changes in dietary intake (18, 149). For example, while ID lipid infusion failed to suppress hunger in obese individuals at baseline, following 4 days of 70% dietary energy restriction lipid potently suppressed hunger, and this was associated with a reduction in *ad libitum* energy intake (149). Of note, these changes in appetite and food intake were associated with greater lipid-induced suppression of ghrelin and stimulation of PYY (149), demonstrating that intestinal nutrient sensing mechanisms underlie, at least in part, the adaptation of appetite regulation following changes in nutritional status.

We also found disturbances in fasting and postprandial concentrations of gut hormones in AN. Ghrelin stimulates hunger and food intake in healthy individuals (302, 303). Patients had elevated acyl-ghrelin at baseline, and a greater suppression of ghrelin following the test meal when compared with HCs, and these responses did not change following two weeks of refeeding, consistent with previous studies (205, 209, 268, 304, 305). It has been suggested that elevated acyl-ghrelin is an adaptive response to increase hunger and energy intake following chronic nutrient restriction in AN. Despite this, at Wk0, baseline and postprandial hunger were markedly less, suggesting a disconnect between acyl-ghrelin and hunger in AN. Moreover, after two weeks of refeeding, patients reported greater hunger, while ghrelin remained unchanged, suggesting that with increased nutrient exposure, patients with AN became more sensitive to the orexigenic actions of acyl-ghrelin. Previous studies have also reported that circulating levels of desacyl-ghrelin, a degradation product of acyl-ghrelin, which has been shown in animal studies to suppress food intake (306) and to inhibit the orexigenic effect of acyl-ghrelin (307), are substantially higher in patients with AN than in controls (208, 209). Moreover, in a small study of patients with AN, desacyl-ghrelin concentrations were shown to

decrease after just one week of refeeding (208), thus, this may be another potential mechanism by which hunger perceptions are restored following acute refeeding. We did not measure desacyl-ghrelin concentrations.

At Wk0, baseline, but not postprandial, PYY concentrations were elevated in patients with AN, consistent with previous observations (177, 190, 195), while after two weeks of refeeding, baseline PYY no longer differed between patients and HCs, consistent with findings of reduced PYY(3-36) after ~3 months of nutritional rehabilitation (177) and reduced total PYY after weight restoration (190). PYY is an anorexigenic gut peptide (94), and elevated baseline levels of PYY could mediate the reduction in hunger ratings and increased fullness reported by patients at Wk0, while the decrease in baseline PYY at Wk2 may contribute to the improvements in appetite ratings. In healthy individuals, exogenously administered PYY(3-36) suppresses ghrelin (as well as hunger and food intake) (94, 95), suggesting a disruption of this interaction in AN. While absolute levels of PYY were higher across the postprandial time-points in patients compared with HCs, consistent with previous reports (177), we found no difference in the magnitude of the PYY response to the meal, suggesting that this was largely accounted for by the elevated baseline levels. This is in contrast to a previous study reporting increased postprandial PYY responses (308), however the inclusion of purging patients in that study may account for these discrepancies, with recent research finding elevated postprandial PYY in purging disorder (309). It is possible that elevated PYY was due to changes in the activity of dipeptidyl peptidase-IV, an enzyme that metabolises PYY, since its serum activity has been reported to be reduced in patients with AN (194), although results are inconsistent (310, 311).

In line with previous studies (155, 200), we found no difference in baseline or postprandial CCK concentrations during starvation or with refeeding. We also found no difference in baseline or postprandial PP at baseline or with refeeding. Two studies reported elevated baseline and postprandial PP in AN, but had much smaller sample sizes (180, 197) and studied medically stable outpatients (197). Moreover, meal responses were difficult to interpret due to the long duration (up to 50 min) of meal ingestion and the potential cephalic effect on PP release (180). Our data indicate that CCK and PP are either not involved in mediating disturbances in appetite regulation in AN, or, given that circulating levels are unchanged, patients with AN may be hypersensitive to the anorectic actions of CCK and PP, an effect reduced following refeeding. In support, hypersensitivity to CCK has been reported in patients with functional dyspepsia and in healthy older people with anorexia of ageing (312, 313).

Patients with AN exhibited greater GI symptoms, and higher baseline and postprandial bloating compared with HCs at Wk0, which remained unchanged following two weeks of refeeding. In contrast, the perception of fullness was reduced at Wk2. These observations suggest that in AN starvation compromises the patient's ability to discriminate between feelings of fullness and bloating, and that this capacity is, at least partially, restored by refeeding. We have reported that these patients have markedly slower gastric emptying compared with controls at Wk0 (283), thus, gastric distension was likely exaggerated. In healthy individuals, postprandial fullness is related directly to distension of the distal stomach (280), and patients with AN have greater antral distention than controls, which improves over time during nutritional rehabilitation (160). We have, however, reported that gastric emptying, assessed using a breath test, was no longer significantly different from controls after two weeks of refeeding (27). Other studies

found no correlation between the improvement in bloating and other GI symptoms with gastric emptying (as measured by antral area) with long-term rehabilitation (160). Thus, persistent bloating and GI symptoms may contribute to the ongoing difficulties many patients have with eating after weight restoration, potentially contributing to their risk of relapse.

In line with previous research (25), patients with AN had substantially higher baseline and postprandial state anxiety than HCs. Following short-term refeeding, baseline anxiety was reduced, while, in contrast, postprandial anxiety was not. Potentially the latter was due to the continued experience of postprandial bloating; in clinical practice the perception of bloating frequently triggers fears of weight gain. The improvement in baseline state anxiety was consistent with previous reports that refeeding and the restoration of body weight results in significant improvement in eating psychopathology, mood and anxiety symptoms (314). Pre-meal anxiety has been associated with lower caloric intake in patients with AN (292), while improvements in pre-meal anxiety following an exposure and response prevention treatment have been linked to greater food intake (315), thus, targeting pre-meal anxiety may have implications for feeding behaviour. In addition, persistent postprandial anxiety may compromise the recovery process, and potentially present a target for management. In this context, treatment specifically targeting a reduction in postprandial anxiety using relaxation techniques has been reported to diminish postprandial bloating/fullness (316), which may assist with longer-term recovery.

Some study limitations should be recognised. The immediate refeeding required in medically unstable patients with AN may have changed GI hormone responses before

studies could be conducted. Patients were only fasted for four hours prior to the study to minimise medical risk, thus, a potential effect of the breakfast consumed on baseline hormone concentrations cannot be excluded. Although calorically identical intakes for each patient over the refeeding period could not be guaranteed, standardised refeeding rates ensured maximal nutrient intake similarity between patients. Follow-up after weight restoration (e.g. up to 12 months) would be important to determine whether GI hormones, appetite, GI symptoms and anxiety return to healthy levels in the longer-term, or whether some changes, possibly resulting from malnutrition, persist beyond weight restoration.

Taken together our results indicate that two weeks of refeeding in patients with AN improves their ability to sense hunger and fullness in a manner more similar to HCs, and this may be mediated by changes in GI nutrient sensitivity, while GI symptoms, particularly bloating, and postprandial anxiety persisted. Further studies are required to examine the long-term effects of refeeding, and the impact of these changes in appetite and GI function on treatment success. Furthermore, future studies may examine whether treatment success could be improved by combining refeeding with more targeted treatments aimed at reducing GI symptoms and postprandial anxiety.

Chapter 5: Discussion

The studies outlined in this thesis have generated important insights into the physiological processes underlying the regulation of appetite, GI function and blood glucose concentrations in patients with AN during starvation and following short-term nutritional rehabilitation, as well as in healthy volunteers. The study described in Chapter 2 demonstrated that DPP-IV inhibition enhanced active GLP-1 and GIP concentrations in response to ID fat infusion, resulting in lowered postprandial blood glucose and triglycerides, and increased energy expenditure in healthy volunteers. The studies presented in Chapters 3 and 4 demonstrated that chronic starvation had significant effects on GI function in AN when compared with HCs, slowing gastric emptying and altering GI hormone concentrations, with both contributing to disturbances in appetite perceptions and postprandial glycemia. Partial improvements in GI function, appetite and glycemia occurred early in refeeding, suggesting a role for malnutrition in the development of these disturbances in AN, however, further research is required to determine whether these factors can be further improved in the longer term.

While GLP-1 and GIP are known to stimulate insulin and regulate glycemia, and GLP-1 also suppresses energy intake, their efficacy is limited by their rapid degradation by the enzyme, DPP-IV. The study presented in **Chapter 2** compared the effects of ID fat infusion with and without the DPP-IV inhibitor, vildagliptin. DPP-IV inhibition enhanced endogenous active GLP-1, active GIP and insulin, while suppressing glucagon, blood glucose and triglycerides. Furthermore, it reduced anorexigenic PYY(3-36) and increased resting energy expenditure without affecting food intake. While we had hypothesised that enhanced active GLP-1 would result in a greater suppression of food intake, its actions may have been counterbalanced by the effects of vildagliptin to concurrently decrease PYY(3-36). The observed changes in energy expenditure may underlie the weight neutral

effect observed in clinical studies of DPP-IV inhibition (246), however, further studies are required to determine if this effect is maintained with longer term treatment. Furthermore, it remains unclear whether DPP-IV inhibition may have the same effects on energy expenditure in patients with type 2 diabetes who have less metabolic flexibility (317), and future studies in this area are warranted. Our observations also strongly suggest that the therapeutic efficacy of DPP-IV inhibitors may be improved by manipulating dietary fat intake. GLP-1 targeted therapies may also have beneficial effects on reducing triglycerides and cardiovascular risk in Type 2 diabetes (318-320). **Chapter 2** thus provides evidence that DPP-IV inhibition had substantial effects on the glycemic, triglyceride and energy expenditure responses to ID fat.

The study reported in **Chapter 3** demonstrated reduced postprandial blood glucose levels in starved patients with AN compared with HCs. The blunted early rise in glucose observed in the first 30 minutes post meal ingestion was associated with slowed gastric emptying in patients with AN. In addition, patients with AN had markedly increased insulin and C-peptide responses when compared with HCs, despite having a lower postprandial glucose response, and insulin appeared to drive the postprandial hypoglycemia observed 60 minutes after the meal in a number of the patients. Elevated glucagon and GLP-1 were also observed. After two weeks of refeeding, while gastric emptying improved towards the rate observed in HCs, postprandial glucose and glucoregulatory hormone changes persisted, suggesting a disconnect between gastric emptying and postprandial glycemia in AN. It remains unclear whether additional improvements may be possible with further nutritional rehabilitation and weight restoration. Previous research supports our observations that gastric emptying rapidly improves following refeeding and increased nutrient exposure (160), however changes in

insulin, glucagon and GLP-1 persisted even after two weeks of refeeding in our study. Future studies warrant follow-up of glucoregulatory systems in patients under controlled testing conditions after weight restoration and longer-term recovery, clarifying whether glycemic regulation is fully restored to healthy levels, or the potential impact of longer-term impairment. More importantly, our study highlights that clinical assessment and management of hypoglycemia in AN is paramount. Prior to treatment, patients with AN often experience physical symptoms, such as dizziness, tiredness and poor attention, that are often attributed to hypotension or bradycardia. However, it is possible that undetected hypoglycemia also contributes, since it is associated with similar medical complications (e.g. dizziness, tiredness (321)), as well as coma and death (31). Close clinical monitoring of blood glucose during early refeeding is warranted, and further studies are required to determine whether new management strategies, such as continuous nasogastric feeding for a continuous glucose supply or a lower carbohydrate ratio in the overall macronutrient intake, can minimise hypoglycemic risk.

Using the same study protocol, **Chapter 4** explored potential factors associated with the appetite disturbances observed in AN. On admission to hospital, patients with AN had increased fasting fullness, bloating, anxiety, acyl-ghrelin and PYY, and decreased hunger. In response to the test-meal, patients also had increased bloating, anxiety and acyl-ghrelin, and decreased hunger. Overall, in starved patients with AN, there appeared to be a disconnect between acyl-ghrelin and hunger, with the elevated acyl ghrelin not increasing hunger as it does in health. However, after two weeks of refeeding, patients appeared more responsive to the persistently elevated acyl ghrelin as the sensation of hunger returned and baseline PYY, fullness and anxiety decreased. The improvements found in appetite regulation implicate malnutrition in the disturbances seen in AN.

Nevertheless, disturbances in baseline and postprandial acyl-ghrelin and bloating, as well as postprandial anxiety persisted, and further research is needed to clarify if these improve with longer-term refeeding or are predictors of treatment success. Future studies are required to evaluate the impact of desacyl ghrelin on appetite in AN (203, 208) and guide potential pharmaceutical interventions (322, 323). In addition, clarifying whether bloating or anxiety contribute to the increased fullness patients experience might direct the focus of future treatment and relapse prevention strategies.

It remains unknown whether the delayed gastric emptying, blood glucose and appetite dysregulation, upper GI symptoms and meal-related anxiety would partially or completely resolve with further nutritional rehabilitation and weight restoration, or whether further disturbances may result from the increased nutritional intake and nutrient exposure with high calorie refeeding. Following treatment for AN, 31-48% of patients develop binge eating (13, 15), with the potential for weight gain and associated impaired glucose tolerance. It is possible that persistent or new GI disturbances due to rapid high caloric intake with refeeding, and a chronic overexposure to nutrients in the gut, may contribute to further glycemic and appetite dysregulation in those who develop binge eating. Initial research suggests that people who regularly binge have greater gastric capacity and other changes in GI function (324), with a recent study finding greater overall ghrelin and less fullness (325). Those who are obese and binge eat may also have disturbances in glucose regulation (e.g. higher fasting insulin and higher insulin resistance (326)), however, this is yet to be examined in binge eating, independent from obesity. Larger studies are needed in both those with binge eating disorder and those who develop binge eating post-AN investigating potential changes in the GI factors examined in this thesis. GI symptoms can also persist in patients with AN even after nutritional

rehabilitation (9) and weight restoration (8), and may be a longer-term negative consequence of the malnutrition in AN or reflect a persistent increased sensitivity to meal-related stimuli. Additional research is needed to characterise whether GI symptoms or other GI factors may interfere with future treatment and/or contribute to relapse in certain patients. In this context, future research could examine whether different nutrient compositions, meal states (liquid vs solid) or other meal-related stimuli (e.g. dietary fibre, or other dietary components) in refeeding regimes may differentially influence GI function and symptoms and assist in the effectiveness of treatments. Furthermore, since studies have shown that prokinetic therapy can improve gastric emptying and self-reported meal-related discomfort (163, 327), it may be beneficial to investigate whether prokinetics might improve treatment compliance and weight gain, particularly in adult outpatient settings, and also whether they might reduce hypoglycemic risk in early refeeding.

In conclusion, the studies presented in this thesis demonstrate how the GI tract responds to nutrients in health, and in patients with AN in starvation and with refeeding, providing greater understanding of the physiological processes underlying glycemic and appetite regulation. In health, enhancing active GLP-1 and GIP concentrations by blocking the action of DPP-IV, resulted in changes in glycemic regulation and metabolism. In AN, the medical complications of hypoglycemia, frequent GI symptoms and appetite disturbance appear to be associated with alterations in GI function, including slowed gastric emptying, dysregulated insulin secretion and elevated glucagon, GLP-1, acyl-ghrelin and PYY. Longer term studies are needed to examine whether these GI changes improve with weight restoration or persist and contribute to relapse. Targeting gastric emptying may be beneficial for both reducing hypoglycemic risk and alleviating GI symptoms to

improve treatment outcomes, and closer monitoring of blood glucose in the outpatient setting and early stages of refeeding is also warranted. Future research should also specifically target bloating and meal-related anxiety to improve treatment success and reduce the risk of relapse.

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