# The role of gastrointestinal function in the regulation of postprandial glycaemia and energy balance in health and type 2 diabetes

*A thesis by*

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# TABLE OF CONTENTS

# THESIS ABSTRACT

This thesis includes a series of clinical studies, focussing on the pivotal role of gastrointestinal (GI) function, particularly gastric emptying and GI hormones (e.g. glucagon-like peptide-1 (GLP-1)), in the regulation of postprandial glycaemia, energy expenditure and energy intake in both health and type 2 diabetes (T2D). The key themes relate to evaluation of: 1) gastric emptying of solid and liquid meals in healthy individuals and subjects with T2D, 2) the bidirectional relationship between gastric emptying and postprandial secretion of GLP-1, 3) the role of endogenous GLP-1 signalling in the regulation of postprandial glycaemia and energy expenditure in T2D, and 4) effects of intestinal bitter taste signalling on GI hormone secretion, gastric emptying, postprandial blood glucose and energy intake in health and T2D.

Gastric emptying is a major determinant of the blood glucose response to dietary carbohydrate in both health and diabetes. The interaction of luminal nutrients and bioactive compounds with the intestines gives rise to the secretion of numerous GI hormones. Of particular importance to glycaemic control in T2D is the so-called incretin hormone, GLP-1, which has the capacity to stimulate insulin, suppress glucagon secretion and energy intake and slow gastric emptying.

In T2D, gastric emptying is frequently abnormal, but may be either delayed, unchanged or accelerated. This discrepancy has reflected the substantial heterogeneity in subject characteristics (e.g. age, duration of diabetes, glycaemic status,

pharmacotherapy and presence or absence of diabetic complications) of cohorts studied and the test meals employed (e.g. emptying of solid and liquid test meals is frequently disconcordant). The study reported in **Chapter 4** evaluated gastric emptying of a semisolid high carbohydrate meal in a group of community-based individuals with relatively well-controlled T2D (HbA1c ≤ 7.9%), managed by diet or metformin monotherapy, in comparison with a cohort of age- and body mass index (BMI)-matched healthy subjects, and a group of healthy young subjects. The study described in **Chapter 5**, evaluated the gastric emptying of an oral glucose drink in two groups of community-based individuals with relatively well- (HbA1c ≤ 7.9%) and poorly- (HbA1c ≥ 9%) controlled T2D managed by diet or metformin alone, together with young and older subjects without diabetes.

There is a complex bidirectional relationship between gastric emptying and the secretion of GLP-1 after a meal. In a given individual, the magnitude of GLP-1 secretion is related to the rate of nutrient delivery into the small intestine (i.e. gastric emptying); conversely, GLP-1 signalling slows gastric emptying. Gastric emptying exhibits a relatively modest intra-individual, but substantial inter-individual, variation. It remains unknown whether the latter reflects the differences in the 'intestinal sensitivity' to nutrients and hence secretion of GLP-1. In **Chapter 6**, the relationship between gastric emptying and the postprandial GLP-1 response was evaluated in subjects with T2D, the inter- and intra-individual variations in plasma GLP-1 response to enteral nutrient infusions were evaluated in health and T2D, and the relationship

between gastric emptying of a glucose drink and the responsiveness of GLP-1 to intestinal glucose was further evaluated in subjects with and without T2D.

Subsequent to its secretion, GLP-1 is rapidly degraded by the enzyme, dipeptidyl peptidase 4 (DPP-4). DPP-4 inhibitors are therefore a logical treatment option to augment intact GLP-1 levels for glycaemic control in T2D. In healthy humans, a single dose of DPP-4 inhibitor was shown to lower the blood glucose response to fat and increase energy expenditure and the thermic effect of feeding; the latter would favour a reduction in body weight with sustained use of DPP-4 inhibitors. The fact that DPP-4 inhibitors are weight neutral in subjects with T2D suggests that the effect of DPP-4 inhibition on energy expenditure may be compromised in this disorder. The study reported in **Chapter 7**, therefore, evaluated the effect of DPP-4 inhibition on the glycaemic and energy expenditure responses to an intraduodenal fat in subjects with T2D, including the role of endogenous GLP-1, assessed using the GLP-1 receptor antagonist, exendin (9-39).

There is emerging evidence from preclinical studies suggesting that stimulation of GI bitter taste receptors (BTRs) has the potential to reduce postprandial glycaemia and suppress energy intake by modulating the secretion of GI hormones and slowing gastric emptying. The study reported in **Chapter 8** evaluates the effects of a non-nutritive bitter taste compound, denatonium benzoate (DB), encapsulated for oral administration, on gastric emptying, postprandial glycaemia and energy intake in subjects with T2D. In **Chapter 9,** the effects of DB and a bitter tasting bile acid,

taurocholic acid, administered via rectal perfusion, on GLP-1 and peptide YY secretion were evaluated in the presence or absence of a BTR antagonist, probenecid, in healthy humans.

# DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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# PUBLICATIONS ARISING FROM PHD

## Publications included in the thesis:

1. **Xie, C**, Wang, X, Young, R. L, Horowitz, M, Rayner, C. K, & Wu, T. Role of intestinal bitter sensing in enteroendocrine hormone secretion and metabolic control. *Frontiers in Endocrinology* 2018;9:576. doi: 10.3389/fendo.2018.00576.

2. Watson, L. E*, **Xie, C***, Wang, X, Li, Z, Phillips, L. K, Sun, Z, Jones, K. L, Horowitz, M, Rayner, C. K, & Wu, T. Gastric emptying in patients with well-controlled type 2 diabetes compared with young and older control subjects without diabetes. *Journal of Clinical Endocrinology and Metabolism* 2019;104(8):3311-3319. doi: 10.1210/jc.2018-02736. (* joint first authorship)

3. **Xie, C**, Jones, K. L, Rayner, C. K, & Wu, T. Enteroendocrine hormone secretion and metabolic control: importance of the region of the gut stimulation. *Pharmaceutics* 2020;12(9):1-23. doi: 10.3390/ph13110410.

4. **Xie, C**, Wang, X, Jones, K. L, Horowitz, M, Sun, Z, Little, T. J, Rayner, C. K, & Wu, T. Role of endogenous glucagon-like peptide-1 enhanced by vildagliptin in the glycaemic and energy expenditure responses to intraduodenal fat infusion in type 2 diabetes. *Diabetes, Obesity & Metabolism* 2020;22(3):383-392. doi: 10.1111/dom.13906.

5. **Xie, C**, Huang, W, Wang, X, Trahair, L. G, Pham, H. T, Marathe, C. S, Young, R. L, Jones, K. L, Horowitz, M, Rayner, C. K, & Wu, T. Gastric emptying in health and type 2 diabetes: An evaluation using a 75 g oral glucose drink. *Diabetes Research and Clinical Practice* 2021;171:108610. doi: 10.1016/j.diabres.2020.108610.

6. **Xie, C***, Huang, W*, Young, R. L, Jones, K. L, Horowitz, M, Rayner, C. K, & Wu, T. Role of bile acids in the regulation of food intake, and their dysregulation in metabolic disease. *Nutrients* 2021;13(4):1104. doi: 10.3390/nu13041104. (* joint first authorship)

7. **Xie, C**, Huang, W, Watson, L. E, Soenen, S, Young, R. L, Jones K. L, Horowitz M, Rayner C. K, & Wu T. Plasma GLP-1 response to oral and intraduodenal nutrients in health and type 2 diabetes – impact on gastric emptying. *Journal of Clinical Endocrinology and Metabolism* 2021 [in press].

8. **Xie, C**, Wang, X, Bound, M. J, Grivell, J, Young, R. L, Jones K. L, Horowitz M, Rayner C. K, & Wu T. Effects of the bitter taste receptor agonist, denatonium benzoate, on gastric emptying, postprandial glycaemia, and energy intake in type 2 diabetes. *To be submitted for publication*.

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flavouring, denatonium benzoate, and the bitter-tasting physiological bile acid, taurocholic acid, with and without the bitter taste receptor antagonist, probenecid, on glucagon-like peptide-1 and peptide YY secretion in healthy humans. *To be submitted for publication.*

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10. **Xie, C,** Wang, X, Jones, K. L, Horowitz, M, Sun, Z, Little, T. J, Rayner, C. K, & Wu, T. Comparative effects of intraduodenal glucose and fat infusion on blood pressure and heart rate in type 2 diabetes. *Frontiers in Nutrition* 2020;7:582314. doi: 10.3389/fnut.2020.582314.

11. Borg, M. J, Rayner, C. K, Jones, K. L, Horowitz, M, **Xie, C** & Wu, T. Gastrointestinal mechanisms underlying the cardiovascular effect of metformin. *Pharmaceuticals* 2020;13(11):410. doi: 10.3390/ph13110410.

12. Huang, W, **Xie, C**, Young, R. L, Zhao, J, Ebendorff-Heidepriem, H, Jones, K. L, Rayner, C. K, & Wu, T. Development of innovative tools for investigation of nutrient-gut interaction. *World Journal of Gastroenterology* 2020;26(25):3562–3576. doi:10.3748/wjg.v26.i25.3562.

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acids. *Diabetes, Obesity and Metabolism* 2020;22(2):141-148. doi:10.1111/dom.13869.

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15. Wu, T, Rayner, C. K, Jones, K. L, **Xie, C**, Marathe, C. H, & Horowitz, M. Role of intestinal glucose absorption in glucose tolerance. *Current Opinion in Pharmacology* 2020;55:116-124. doi: 10.1016/j.coph.2020.10.017.

16. Borg, M. J, **Xie, C**, Rayner, C. K, Horowitz, M, Jones, K. L, & Wu, T. Potential for gut peptide-based therapy in postprandial hypotension. *Nutrients* 2021;13:2826. doi: 10.3390/nu13082826.

17. Huang, W, Wu, T, Xie C, Rayner, C. K, Priest, C, Ebendorff-Heidepriem, H, & Zhao J. Sensing intra- and extra-cellular Ca2+ in the islet of langerhans. *Advanced Functional Materials* 2021 [in press].

# CONFERENCE PRESENTATIONS

**Xie C**, Wang X, Bound M. J, Grivell J, Young R. L, Jones, K. L, Horowitz, M, Rayner, C. K, Wu, T. Effects of the bitter taste receptor agonist, denatonium benzoate, on postprandial glycaemia, gastric emptying and energy intake in type 2 diabetes. Australian Society for Medical Research (ASMR) South Australian Scientific Meeting, Adelaide, Australia, June 2018 (**Poster presentation**).

**Xie C**, Wang X, Bound M. J, Grivell J, Young R. L, Jones, K. L, Horowitz, M, Rayner, C. K, Wu, T. Effects of the bitter taste receptor agonist, denatonium benzoate, on postprandial glycaemia, gastric emptying and energy intake in type 2 diabetes. Australian Diabetes Congress (ADC), Adelaide, Australia, August 2018 (**Poster presentation, Clinical Science Poster Award**).

**Xie C**, Wang X, Bound M. J, Grivell J, Young R. L, Jones, K. L, Horowitz, M, Rayner, C. K, Wu, T. Effects of the bitter taste receptor agonist, denatonium benzoate, on postprandial glycaemia, gastric emptying and energy intake in type 2 diabetes. 54$^{th}$ Annual Meeting of the European Association for the Study of Diabetes (EASD), Berlin, Germany, October 2018 (**Oral presentation**).

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# CHAPTER 1: ENTEROENDOCRINE HORMONE SECRETION AND METABOLIC CONTROL: IMPORTANCE OF THE REGION OF THE GUT STIMULATION

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## 1.1 Abstract

It is now widely appreciated that gastrointestinal function is central to the regulation of metabolic homeostasis. Following meal ingestion, the delivery of nutrients from the stomach into the small intestine (i.e. gastric emptying) is tightly controlled to optimise their subsequent digestion and absorption. The complex interaction of intraluminal nutrients (and other bioactive compounds, such as bile acids) with the small and large intestine induces the release of an array of gastrointestinal hormones from specialised enteroendocrine cells distributed in various regions of the gut, which in turn to regulate gastric emptying, appetite and postprandial glucose metabolism. Stimulation of gastrointestinal hormone secretion, therefore, represents a promising strategy for the management of metabolic disorders, particularly obesity and type 2 diabetes (T2D). That enteroendocrine cells are distributed distinctively between the proximal and distal gut suggests that the region of the gut exposed to intraluminal stimuli is of major relevance to the secretion profile of gastrointestinal hormones and associated metabolic responses. This review discusses the process of intestinal digestion and absorption and their impacts on the release of gastrointestinal hormones and the regulation of postprandial metabolism, with an emphasis on the differences between the proximal and distal gut, and implications for the management of obesity and T2D.

## 1.2 Introduction

As the key interface between ingested nutrients and the body, the gastrointestinal (GI) tract is now recognised to play a central role in regulating postprandial metabolism. During the fasting state, ghrelin is released from the gastric Gr-cells to drive food intake (Steinert et al., 2017). After meal ingestion, the stomach accommodates the nutrients, grinds digestible solids into small particles, and releases the resultant chyme into the small intestine in a regulated fashion to optimise intestinal digestion and absorption. It is now widely appreciated that distinctive enteroendocrine cells scattered along the GI tract, comprising up to 1% of the gut epithelium, constitute the largest endocrine organ in the body, accounting for the release of an array of peptides that orchestrate appetite, energy intake and the blood glucose responses to meals (Gribble & Reimann, 2019). Of particular importance are cholecystokinin (CCK) and glucose-dependent insulinotropic polypeptide (GIP) released from the upper small intestine, and glucagon-like pepetide-1 (GLP-1) and peptide YY (PYY) secreted mainly from the distal gut. These integrated hormonal responses convey important regulatory signals governing subsequent gastric emptying, insulin and glucagon secretion from the pancreas, energy intake, and postprandial glycaemic control. Stimulation of GI postprandial hormone secretion therefore represents an attractive strategy for the management of metabolic disorders, such as obesity and type 2 diabetes (T2D) (Gribble & Reimann, 2019). Given that the distribution of the respective types of enteroendocrine cells varies substantially along the GI tract, the region of the gut exposed to intraluminal stimuli is likely to be of major relevance to the secretion profile of GI hormones and associated metabolic responses. This review

discusses nutrient digestion and absorption along the GI tract and how these processes influence the secretion of GIP, CCK, GLP-1 and PYY, and highlights the importance of which region of the gut is stimulated to the secretory profiles of these GI hormones, the regulation of postprandial metabolism, and the implications for the management of obesity and T2D. Other hormones, such as ghrelin and leptin, are also important metabolic regulators, but are not specifically discussed as they are outside the scope of this review.

## 1.3 Nutrient transport, digestion, and absorption

Following meal ingestion, the stomach stores the ingested content and grinds digestible solids into small particles prior to delivering them into the small intestine. The latter occurs at a relatively constant caloric rate (in the range of 1-4 kcal/min in healthy individuals), driven by antral and duodenal contractions against tonic and phasic pyloric pressures (L. K. Phillips, Deane, Jones, Rayner, & Horowitz, 2015) , to optimise the subsequent digestion and absorption of nutrients in the small intestine. Due to the inactivation of salivary amylase in the gastric environment, there is limited digestion of carbohydrate in the stomach, whereas fat and protein are digested into lipid emulsions (Iqbal & Hussain, 2009) and oligopeptides (Erickson & Kim, 1990), respectively. Upon entering the duodenum, nutrients stimulate the release of a range of digestive enzymes from the exocrine pancreas and bile acids from the gallbladder, influenced by both the load and composition of the meal. Starch is broken down by pancreatic $\alpha$-amylase and disaccharidases into glucose and other monosaccharides (e.g., fructose and galactose) (Gray, 1975). Dietary fat (90-95% in the form of

triglycerides) is digested by pancreatic lipase, a process relying largely on emulsification by bile acids, to form monoacylglycerol, glycerol and free fatty acids (Iqbal & Hussain, 2009). Digestion of protein involves both pancreatic enzymes, including chymotrypsin and trypsin, and aminopeptidases secreted by the small intestine mucosa, and yields individual amino acids, dipeptides and tripeptides (Erickson & Kim, 1990).

The digestive products are transported by peristalsis and absorbed by passive diffusion and/or active transport via distinctive transporters at specific regions of the gut. For example, absorption of glucose involves both active transport from the lumen into the enterocytes via sodium-glucose cotransporter 1 (SGLT-1) and facilitated diffusion across the basolateral side of enterocytes through the glucose transporter 2 (GLUT2), taking place predominantly in the upper small intestine (Balen et al., 2008; Song, Onishi, Koepsell, & Vallon, 2016; Thazhath, Wu, Young, Horowitz, & Rayner, 2014; Wright, Hirayama, & Loo, 2007). Unlike glucose, fructose is absorbed mainly through GLUT5 (Gouyon et al., 2003), which is well expressed in both the human jejunum and ileum (Davidson et al., 1992). Dietary fat typically binds to bile acids to form mixed micelles, which are absorbed by fatty acid transport proteins (FATPs) (e.g., FATP4 and FAT/CD36) and Niemann-Pick C1 like-1 (NPC1L1) (Abumrad & Davidson, 2012; Paalvast, de Boer, & Groen, 2017). Although a small fraction of bile acids are absorbed passively in the jejunum, the majority of them (~90%) are actively absorbed in the ileum through the apical sodium dependent bile acid transporter (ASBT) (Carter, Howlett, Wiernsperger, & Bailey, 2003). The uptake of amino acids

depends on a variety of "amino acid transport systems" that preferentially transport amino acids of similar biophysical properties (Broer, 2008; Broer & Fairweather, 2018), whereas dipeptides and tripeptides are absorbed via the proton-dependent intestinal peptide transporter 1 (PEPT1) (Daniel & Zietek, 2015). The large intestine hosts a diversity of gut bacteria, which are involved in fermentation of products that escape digestion/absorption in the small intestine, such as dietary fibre, resistant starches and proteins, leading to the production of short-chain fatty acids (SCFAs) which can be absorbed through facilitated diffusion (Gill, Chater, Wilcox, Pearson, & Brownlee, 2018; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Topping & Clifton, 2001).

## 1.4 Secretion and actions of GI hormones

While intestinal enteroendocrine cells maintain a low secretory profile during fasting, the interaction between intraluminal contents and enteroendocrine cells during the digestive process represents the main driver of the secretion of GI hormones. The latter, including GLP-1, GIP, CCK and PYY released from distinctive enteroendocrine cells in various regions of the GI tract **(Figure 1.1)**, are now recognised as key regulators of energy intake and postprandial glucose metabolism **(Figure 1.2).**

Enteroendocrine cells are equipped with a variety of chemo-sensors linking the sensing of intraluminal stimuli to the secretion of GI hormones. For example, carbohydrates can be detected by both their sweet taste through sweet taste receptors

(STRs) (Jang et al., 2007; Kreuch et al., 2018; T. Wu, M. J. Bound, S. D. Standfield, M. Bellon, et al., 2013), and via the glucose transporters, SGLT-1 and GLUT2 (Gorboulev et al., 2012; Mace, Schindler, & Patel, 2012), although stimulation of STRs by artificial sweeteners alone does not seem to be sufficient to induce GLP-1 or GIP secretion in humans (T. Wu, M. J. Bound, S. D. Standfield, M. Bellon, et al., 2013; T. Wu et al., 2012). Enteroendocrine cell sensing of intraluminal fat is dependent on the degree of its digestion (S. Beglinger et al., 2010; Kuo et al., 2011; Perano et al., 2014), and involves a number of G-protein coupled receptors (GPCRs), such as GPR40, GPR119 and GPR120 (Edfalk, Steneberg, & Edlund, 2008; Lauffer, Iakoubov, & Brubaker, 2009; Sankoda et al., 2017), and intestinal fat transporters FATP4 and FAT/CD36 (Poreba et al., 2012; Sundaresan et al., 2013). Amino acids are detected by the calcium-sensing receptors (CasR) (Mace et al., 2012; Pais, Gribble, & Reimann, 2016) and amino acid transporters (Clemmensen, Jorgensen, Smajilovic, & Brauner-Osborne, 2017; Jiang et al., 2015). Non-nutritive compounds are also effectively sensed by enteroendocrine cells. In particular, bile acids are known to induce GLP-1 and PYY secretion via inhibition of nuclear farnesoid X receptor (FXR) (Trabelsi et al., 2015) and/or stimulation of Takeda G-protein coupled receptor 5 (TGR-5) (Kuhre et al., 2018; Thomas et al., 2009). Of note, TGR5 is expressed on the basolateral membrane of enteroendocrine cells (Tough, Schwartz, & Cox, 2020), such that intestinal bile acid absorption is necessary to achieve TGR5 activation (Kuhre et al., 2018; Tough et al., 2020). There is recent evidence that a group of specialised GPCRs responsible for the sensing of bitter taste (i.e. bitter taste receptors; BTRs) are also abundantly expressed on enteroendocrine cells. Activation of BTRs

on enteroendocrine cells by a variety of natural or chemosynthetic bitter taste compounds therefore has potential to trigger the secretion of GI hormones (Xie, Wang, Young, et al., 2018).

### *1.4.1 GLP-1*

GLP-1 is secreted from the enteroendocrine L-cells located mainly in the ileum and colon in response to each of the macronutrients, although fat, relative to glucose and protein, is generally more potent at stimulating GLP-1 secretion when administered into the duodenum in humans (Ryan et al., 2013; T. Wu, C. K. Rayner, et al., 2017). However, in subjects who have undergone Roux-en-Y gastric bypass, oral ingestion of glucose was shown to be more effective than fat or protein to stimulate GLP-1 secretion (Jensen et al., 2020). The discrepancy observed in the latter is likely to be attributable to the influence of gastric emptying and digestion of fat or protein. Other bioactive compounds released into the lumen following meal ingestion, such as bile acids, are also responsible for postprandial GLP-1 secretion (Katsuma, Hirasawa, & Tsujimoto, 2005; Kuhre et al., 2018). After its secretion, GLP-1 is rapidly inactivated by the enzyme dipeptidyl peptidase IV (DPP-4) with a half-life of 1-2 min, such that only 10-15% intact GLP-1 reaches the peripheral circulation (Andersen, Lund, Knop, & Vilsboll, 2018; Holst, Albrechtsen, Rosenkilde, & Deacon, 2019). While obesity is associated with attenuated GLP-1 secretion, accumulating evidence suggests that the latter is otherwise unaltered in subjects with T2D (M. A. Nauck & Meier, 2018; T. Wu, Rayner, & Horowitz, 2016a). Importantly, the action of GLP-1 is also relatively well preserved in T2D (M. A. Nauck & Meier, 2018).

GLP-1 binds to its receptor, expressed in a variety of metabolic tissues, to regulate glucose, lipid and energy metabolism. Within the pancreas, GLP-1 stimulates insulin secretion from the pancreatic β-cells and suppresses glucagon secretion from the α-cells in a glucose-dependent manner (Hare et al., 2010). For this reason, GLP-1-based glucose-lowering therapies in general entail a low risk of hypoglycaemia. Although there is preclinical evidence that GLP-1 receptor signalling is involved in β-cell survival and regeneration (Maida, Hansotia, Longuet, Seino, & Drucker, 2009), such effects have not been established in humans. Within the liver, GLP-1-signalling is linked to the control of endogenous glucose production, an effect that can be independent of changes in insulin or glucagon (Seghieri et al., 2013). Moreover, GLP-1 slows gastric emptying in both healthy individuals and those with T2D (Deane, Nguyen, et al., 2010; Jones et al., 2019; Little, Pilichiewicz, et al., 2006; M. A. Nauck et al., 1997). That the reduction in postprandial glycaemia induced by exogenous GLP-1 is associated with less, rather than more, postprandial insulin secretion suggests that the slowing of gastric emptying outweighs its insulinotropic effect in controlling postprandial glycaemia (Lorenz et al., 2013). In contrast to the GLP-1 receptor agonists, the DPP-4 inhibitors – which prolong the half-life of endogenous GLP-1 – have little, if any, effect on gastric emptying (DeFronzo et al., 2008; Stevens et al., 2020).

Effects of GLP-1 signalling on lipid metabolism have been noted in both preclinical and clinical studies. GLP-1 has been shown to inhibit the production of lipid proteins

(e.g. apolipoprotein B-48 (apob-48)) that are involved in the synthesis and transport of chylomicrons in the enterocytes, thereby improving lipid metabolism in rodents (Hsieh et al., 2010; Qin et al., 2005). Similarly, the GLP-1 receptor agonist, exenatide, and the DPP-4 inhibitor, sitagliptin, have been shown to reduce plasma apob-48 concentrations in humans (Xiao, Bandsma, Dash, Szeto, & Lewis, 2012; Xiao, Dash, Morgantini, Patterson, & Lewis, 2014), while in obesity, treatment with GLP-1 receptor agonists improves dyslipidaemia (Hjerpsted et al., 2018).

GLP-1 also has the capacity to regulate energy intake and expenditure. Both exogenous GLP-1 and the GLP-1 receptor agonists suppress energy intake (Holst, 2007; T. Wu, Rayner, et al., 2016a), and this effect has been shown to be mediated primarily via vagal afferents (Krieger et al., 2016; Plamboeck et al., 2013) and the activation of GLP-1 receptors in the central nervous system (ten Kulve et al., 2015; Turton et al., 1996). GLP-1 receptor agonists are therefore often associated with weight loss in both obesity and T2D (Andersen et al., 2018; R. Pratley et al., 2019). The role of GLP-1 in the regulation of energy expenditure is controversial. In mice, GLP-1 receptor agonists have been reported to induce browning of white adipose tissue and increase β-oxidation of fatty acids (Beiroa et al., 2014; Lynch et al., 2016; Xu et al., 2016), and administration of both GLP-1 receptor agonists and DPP-4 inhibitors increases energy expenditure and reduces body weight (Beiroa et al., 2014; Fukuda-Tsuru, Kakimoto, Utsumi, Kiuchi, & Ishii, 2014; Goldsmith et al., 2015; Tomas et al., 2015). However, a recent meta-analysis suggests that GLP-1 receptor agonists have little, if any, effect on energy expenditure in humans (Maciel et al.,

2018). Although inhibition of DPP-4 by vildagliptin was found to augment the energy expenditure response to an intraduodenal fat infusion in healthy humans (Heruc et al., 2014), this effect was not evident in subjects with T2D (Xie, Wang, et al., 2020). That antagonism of GLP-1 signalling by exendin (9-39) increased energy expenditure in the latter group during treatment with vildagliptin suggests that endogenous GLP-1 signalling may rather be associated with suppression of energy expenditure (Xie, Wang, et al., 2020).

### *1.4.2 GIP*

GIP is released from the enteroendocrine K-cells, distributed predominantly in the upper small intestine (M. A. Nauck & Meier, 2018). GIP is also co-expressed with GLP-1 in a subset of "K/L-cells" in the duodenum and proximal jejunum (Mortensen, Christensen, Holst, & Orskov, 2003; Svendsen et al., 2015). Similar to GLP-1, GIP is released in response to macronutrients – with fat being a more potent stimulus than glucose or protein (T. Wu, C. K. Rayner, et al., 2017) – and rapidly inactivated by DPP-4 after secretion (M. A. Nauck & Meier, 2018; T. Wu, Rayner, Jones, & Horowitz, 2010). However, the secretion of GIP does not seem to be affected by T2D or obesity (T. Wu, Rayner, et al., 2016a).

In health, GIP stimulates insulin secretion in a glucose-dependent manner by binding to the GIP receptor expressed on the pancreatic β-cells (Holst & Gromada, 2004), which contributes equally with GLP-1 to the augmented insulin response that is observed during enteral glucose administration when compared to an 'isoglycaemic'

intravenous glucose infusion, i.e. the so-called the 'incretin effect'. However, the insulinotropic effect of GIP is markedly diminished in subjects with T2D (Holst et al., 2019; Mentis et al., 2011). Unlike GLP-1, GIP stimulates glucagon secretion from the pancreatic α-cells, particularly in the face of hypoglycaemia (M. Christensen et al., 2015), and has little effect on appetite (Bergmann et al., 2019) or GI motility (Meier et al., 2004). Moreover, GIP exhibits numerous extra-glycaemic actions; blockade of GIP signalling in mice preferentially increases fat oxidation (Miyawaki et al., 2002; Naitoh et al., 2008; Zhou et al., 2005), reduces fat accumulation in adipocytes (Hansotia et al., 2007; Miyawaki et al., 2002; Zhou et al., 2005) and skeletal muscle (Naitoh et al., 2008; Zhou et al., 2005), decreases triglyceride deposition in the liver (Hansotia et al., 2007; Miyawaki et al., 2002), and prevents the development of obesity (Boylan, Glazebrook, Tatalovic, & Wolfe, 2015; Miyawaki et al., 2002; Naitoh et al., 2008) in the context of overfeeding. In line with these findings, antagonism of the GIP receptor is associated with reduced blood flow and triglyceride deposition in adipose tissue in healthy subjects (Asmar et al., 2017). Although compounds that display dual GIP and GLP-1 receptor agonism appear to be more effective for weight loss and glycaemic control than the GLP-1 receptor agonists, liraglutide and dulaglutide, in subjects with T2D (Frias et al., 2017; Frias et al., 2018), the relative contribution of GIP signalling to this superiority remains to be characterised in humans. Counterintuitively, acute administration of exogenous GIP failed to show any effect on energy intake or expenditure, but rather, augmented postprandial glycaemia in subjects with T2D receiving long-acting GLP-1 receptor agonists (Bergmann et al., 2020). The mechanism underlying this phenomenon is

unclear, but may be related to the stimulation of glucagon by intravenous GIP administration (Bergmann et al., 2020; Mentis et al., 2011).

### *1.4.3 CCK*

CCK is secreted from the enteroendocrine I-cells located in the duodenum and upper jejunum (Steinert et al., 2017). In mice, there is also evidence that a subset of enteroendocrine cells co-express CCK, GIP, GLP-1 and PYY in the proximal small intestine (Svendsen et al., 2015; Sykaras et al., 2014). However, this is unlikely to be the case in humans, since exposure to glucose in the proximal 60 cm segment of the small intestine, while inducing substantial GIP and CCK secretion, has no effect on GLP-1 secretion (Little, Doran, et al., 2006). CCK is secreted within 10-15 min in response to oral ingestion of macronutrients (fat > protein > carbohydrate) (Steinert et al., 2017). This is critical for subsequent digestion, since CCK stimulates the release of digestive enzymes and bile from the pancreas and the gallbladder, respectively.

Exogenous CCK is reported to attenuate the postprandial glycaemic excursion in humans. This effect is secondary to slowing of gastric emptying, rather than a direct effect on glucose metabolism; intravenous administration of CCK at physiological doses diminishes the glycaemic response to an oral, but not an intraduodenal, glucose load in healthy males (Liddle et al., 1988). Similarly, in subjects with well-controlled T2D, administration of CCK at a physiological dose (0.4 pmol/kg/min) was shown to slow gastric emptying and reduce postprandial blood glucose excursions (Ahren, Holst, & Efendic, 2000). However, for those with longstanding T2D, both the

secretion and action of CCK appear to be impaired, due probably to the development of autonomic neuropathy (M. Horowitz et al., 1989; L. K. Phillips et al., 2015). In addition to its effects on upper GI motor function, CCK has an established role in the regulation of appetite through both vagal and endocrine pathways. In rats, the effect of exogenous CCK to suppress food intake was abolished by small molecule CCK antagonists or after vagotomy (Reidelberger, Hernandez, Fritzsch, & Hulce, 2004). Similarly, intravenous infusion of CCK at both physiological (0.6-0.8 pmol/kg/min) and supraphysiological doses (1.8 and 2.6 pmol/kg/min) suppresses hunger and energy intake in healthy subjects (Brennan et al., 2005; Brennan et al., 2008; MacIntosh et al., 2001), effects abolished in the presence of a CCK antagonist (C. Beglinger, Degen, Matzinger, D'Amato, & Drewe, 2001). In a population-based study, genetic polymorphisms of the CCK receptor, e.g. increased CCK_H3 haplotype frequency, may be responsible for over-eating in obesity (de Krom et al., 2007). However, acute administration of exogenous CCK showed a comparable effect on suppressing appetite in non-diabetic obese and lean subjects (Lieverse, Jansen, Masclee, & Lamers, 1995).

### *1.4.4 PYY*

PYY is a 36-amino-acid peptide co-released with GLP-1 from L-cells (Habib, Richards, Rogers, Reimann, & Gribble, 2013). Like other GI hormones, postprandial secretion of PYY is dependent on the composition and load of macronutrients (Batterham et al., 2006; Brennan et al., 2012; Essah, Levy, Sistrun, Kelly, & Nestler, 2007; Helou, Obeid, Azar, & Hwalla, 2008). In contrast to GLP-1 and GIP, enzymatic

conversion of PYY1-36 to PYY3-36 by DPP-4 is necessary for the systemic effects of PYY, namely suppression of appetite and slowing of gastric emptying (Steinert et al., 2017).

PYY participates in the regulation of appetite and energy intake. PYY-null mice exhibit increased daily food intake and weight gain when compared with wild type mice, and this phenotype is reversed with PYY3-36 administration (Batterham et al., 2006). PYY binds to Neuropeptide Y receptor Y2 (NPY2R), which is highly expressed in the hypothalamic arcuate nucleus (Broberger, Landry, Wong, Walsh, & Hokfelt, 1997). That the effect of PYY on energy intake is abolished in NPY2R knockout mice and by the selective NPY2R antagonist BIIE0246, suggests a key role of NPY2R signalling in mediating the effect of PYY to suppress energy intake (Abbott et al., 2005; Batterham et al., 2002). In healthy humans, postprandial PYY levels are positively correlated with changes in satiety scores (Guo et al., 2006; Stoeckel, Weller, Giddings, & Cox, 2008), and intravenous PYY(3-36) infusion (up to 0.8 pmol/kg/min) reduces food intake in a dose-dependent manner (Degen et al., 2005; le Roux et al., 2008). Recently, the long-acting PYY3-36 analogue, mAb-cycPYY, was shown to reduce food intake and body weight over 7 days in rhesus macaques, effects further augmented when combined with the GLP-1 receptor agonist, liraglutide (Rangwala et al., 2019).

PYY may be also involved in the regulation of postprandial glycaemia, given its effect to slow gastric emptying in both rodents and humans (Chelikani, Haver, &

Reidelberger, 2004; Moran et al., 2005; Savage, Adrian, Carolan, Chatterjee, & Bloom, 1987; Witte et al., 2009). Furthermore, PYY may influence insulin secretin; PYY1-36, but not PYY3-36, was found to inhibit insulin secretion from the pancreatic $\beta$-cells ex vivo (Bottcher, Ahren, Lundquist, & Sundler, 1989; Lafferty, Gault, Flatt, & Irwin, 2019; Nieuwenhuizen, Karlsson, Fridolf, & Ahren, 1994), and isolated islets from PYY-knockout mice showed higher glucose-induced insulin levels (Boey, Heilbronn, et al., 2006). PYY-knockout mice exhibit relative hyperinsulinaemia during fasting and postprandially (Boey, Lin, et al., 2006). However, exogenous PYY infusion had little effect on glucose-stimulated insulin in healthy humans (Ahrén & Larsson, 1996).

## 1.5 Regional differences in nutrient absorption and GI hormone secretion, and associated impact on postprandial glycaemia and appetite

### *1.5.1 Nutrient absorption*

The upper small intestine (duodenum and proximal jejunum) represents a major site of nutrient absorption. Given that the delivery of nutrients into the small intestine is controlled by gastric emptying, it is not surprising that the rate of nutrient absorption (such as glucose) is related directly to the rate of gastric emptying (N. Q. Nguyen et al., 2018). In the case of glucose, the maximum capacity of absorption of the upper small intestine approximates ~0.5 g/min per 30 cm (Duchman et al., 1997). Of note, glucose transporters (SGLT-1, GLUT2 and GLUT5) are less abundant in the distal than proximal small intestine (Balen et al., 2008; Yoshikawa et al., 2011).

Accordingly, intra-ileal administration of glucose is associated with slower absorption than intraduodenal, in both healthy individuals and subjects with T2D (X. Zhang et al., 2019). Moreover, duodenal-jejunal bypass improves glucose tolerance, associated with a reduction in SGLT-1-mediated glucose absorption in both obese rats and T2D subjects (de Jonge et al., 2013; Jurowich et al., 2013; Koehestanie et al., 2014; Yan et al., 2013). Similarly, the expression of the majority of lipid transporters (e.g., FAT/CD36, FATP4 and NPC1L1) decreases from the duodenum and jejunum to the ileum in rodents (M. Chen, Yang, Braunstein, Georgeson, & Harmon, 2001; Lobo et al., 2001; Nassir, Wilson, Han, Gross, & Abumrad, 2007; D. V. Nguyen et al., 2009; Ockner & Manning, 1974), and fatty acid and cholesterol uptake is slower in the distal than proximal small intestine (D. V. Nguyen et al., 2009; A. L. Wu, Clark, & Holt, 1975). In mice, ablation of FAT/CD36 and FATP4 is associated impaired lipid absorption in the proximal (Nassir et al., 2007; Nauli et al., 2006), but not in the distal (Nassir et al., 2007; Shim et al., 2009) small intestine. However, fatty acid transporters have been shown to be abundantly expressed in both proximal and distal small intestine in humans (Masson et al., 2010). The expression of transporters of amino acids and peptides varies substantially along the GI tract (Broer, 2008). While the majority of digestive products of protein are absorbed in the proximal jejunum, a considerable proportion is also absorbed in other small intestinal segments (Broer & Fairweather, 2018). Amino acid transporters are abundant in the distal jejunum and ileum (Mutch et al., 2004), such that obese subjects who have undergone Roux-en-Y gastric bypass (RYGB) exhibit accelerated uptake of amino acids (Bojsen-Moller et al., 2015). The absorption of nutrients in the large intestine is minimal. Undigested

nutrients are fermented by microbiota localised in the large intestine to produce SCFAs which can be absorbed passively across colonic mucosa.

### *1.5.2 GI hormone secretion*

Enteroendocrine cells secreting GIP, CCK, GLP-1 and PYY exhibit high regional specificity of distribution along the GI tract. Their secretory profiles are, therefore, largely dependent on the region of the gut exposed to intraluminal stimuli. In response to meal ingestion, increments in plasma GIP and CCK usually occur earlier than those of GLP-1 and PYY (Dirksen et al., 2019), consistent with the proximal distribution of K- and I-cells, and distal predominance of L-cells. Studies employing intraduodenal infusion of nutrients at different rates that mimic the physiological range of gastric emptying have shown that the secretion of GIP and CCK increases in an approximately linear pattern with increasing rates of infusion in health, obesity and T2D (Hutchison et al., 2015; Little et al., 2005; Ma et al., 2012; Pilichiewicz, Chaikomin, et al., 2007; Pilichiewicz, Papadopoulos, et al., 2007; Trahair et al., 2012; T. Wu, Rayner, & Horowitz, 2016b; T. Wu, Zhang, et al., 2016). In obese and T2D subjects, both the GIP and CCK responses to oral meals are increased after Roux-en-Y gastric bypass (Dirksen et al., 2013; Laferrere et al., 2007), due probably to rapid gastric pouch emptying (N. Q. Nguyen et al., 2014). However, postprandial GIP secretion is decreased after biliopancreatic diversion, since this procedure bypasses the majority of K-cell rich regions of the small intestine (Mingrone et al., 2009; Salinari et al., 2009). By contrast, intraduodenal infusion of nutrients needs to exceed a threshold (e.g. ~2 kcal/min for glucose) in order for sufficient nutrient to escape

proximal small intestinal absorption and stimulate more distal L-cells; accordingly, the GLP-1 response is minimal to intraduodenal glucose infusion at rates between 1-2 kcal/min, but increases substantially in response to infusions of 3-4 kcal/min (Ma et al., 2012; Pilichiewicz, Chaikomin, et al., 2007; Trahair et al., 2012).

The extent to which nutrients are delivered to the more distal regions of the gut is dependent not only on their rate of entry to the small intestine, but also on their digestion and absorption in the upper gut. For example, ablation or inhibition of SGLT-1 that reduces proximal intestinal glucose absorption augments the GLP-1 and PYY responses to oral glucose in rodents (Oguma et al., 2015; Powell et al., 2013). In humans, intestinal SGLT-1 inhibition (e.g. by GSK-1614235 (Dobbins et al., 2015) or licogliflozin (He et al., 2019)), while reducing GIP secretion, is associated with overall increased, albeit relatively delayed, responses of GLP-1 and PYY to carbohydrate meals. Similarly, malabsorption of carbohydrate induced by an α-glucosidase inhibitor (e.g. acarbose) was shown to increase GLP-1 and PYY secretion in both health and T2D (Qualmann, Nauck, Holst, Orskov, & Creutzfeldt, 1995; M. Y. Zheng et al., 2013). Alternatively, poorly absorbed carbohydrates, such as tagatose (T. Wu et al., 2012), xylose (Vanis et al., 2011) and resistant starch (Crapo, Reaven, & Olefsky, 1976), also have the capacity to induce sustained secretion of GLP-1. Consistent with this principle, consumption of a small amount of tagatose or xylose as a 'preload' has been shown to slow gastric emptying and improve the glycaemic response to the subsequent main meal by stimulating GLP-1 secretion in both health and T2D (T. Wu, M. J. Bound, B. Y. R. Zhao, et al., 2013; T. Wu et al., 2012).

Treatment with the lipase inhibitor, orlistat, however, has been reported to either increase (2004) or decrease (Ellrichmann et al., 2008; Enc et al., 2009; D. O'Donovan et al., 2004) postprandial GLP-1 secretion. These discrepancies may have reflected differences in the test meals (including the forms of dietary fat) and associated impact of orlistat on their digestion between studies.

Compared with intraduodenal infusion, administration of nutrients directly into the jejunum or ileum is more effective at stimulating GLP-1 and PYY secretion (Chaikomin et al., 2008; Maljaars et al., 2008; Mangan et al., 2019; Poppitt et al., 2017; Rigda et al., 2016; van Avesaat, Troost, Ripken, Hendriks, & Masclee, 2015; T. Wu et al., 2015; X. Zhang et al., 2019). Interestingly, intra-ileal infusion of glucose is also associated with a considerable, albeit relatively lower, GIP response than intraduodenal infusion in both healthy subjects and subjects with T2D (Poppitt et al., 2017; X. Zhang et al., 2019), suggesting that a considerable number of enteroendocrine cells capable of secreting GIP are found even in the distal small intestine. The large intestine represents another major source of GLP-1 and PYY production. Microbial metabolites, including SCFAs and secondary bile acids, are known to stimulate GLP-1 and PYY secretion (Canfora et al., 2017; Chambers et al., 2015; Christiansen et al., 2018; Christiansen et al., 2019; Freeland & Wolever, 2010; Katsuma et al., 2005; Psichas et al., 2015; Tolhurst et al., 2012) and may also induce differentiation of stem cells towards L-cells (N. Petersen et al., 2014). Inhibition of ileal ASBT (by elobixibat), increasing the exposure of the large intestine to bile acids, is therefore associated with an increase in GLP-1 and PYY secretion in humans

(Rudling, Camilleri, Graffner, Holst, & Rikner, 2015). In both healthy individuals and subjects with T2D (T. E. Adrian et al., 2012; T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013), rectal administration of a primary bile acid, taurocholic acid (TCA), has also been shown to stimulate GLP-1 and PYY secretion in in a dose-dependent manner, although the PYY response seems to be more robust than that of GLP-1.

### *1.5.3 Regulation of postprandial glycaemia and appetite*

As discussed, the upper gut coordinates the delivery of nutrients for intestinal digestion and absorption, and is primarily responsible for the release of GIP and CCK after meals, whereas the interaction of intraluminal nutrients and bioactive compounds with the distal gut gives rise to the secretion of both GLP-1 and PYY. These variations in nutrient absorption and GI hormone secretion along the GI tract are of major relevance to the regulation of postprandial glycaemia and appetite.

It is now widely recognised that the rate of gastric emptying represents a major determinant of the glycaemic profile in response to carbohydrates in both health and diabetes (Marathe, Rayner, Jones, & Horowitz, 2013; L. E. Watson, Xie, et al., 2019). While obesity *per se* does not seem to have a major impact on gastric emptying (Seimon et al., 2013), recent evidence suggests that gastric emptying in subjects with 'early-stage' uncomplicated type 1 and 2 diabetes is more rapid than in non-diabetic controls (Perano et al., 2015; L. E. Watson, Xie, et al., 2019), which may predispose them to glucose intolerance. By contrast, in subjects with longstanding diabetes who

have poor glycaemic control and autonomic dysfunction, gastric emptying is often abnormally delayed (Jones et al., 1995). Nevertheless, nutritional and/or pharmacological strategies that slow gastric emptying have been shown to attenuate postprandial glycaemic excursions in both type 1 and 2 diabetes (Ghazi, Rink, Sherr, & Herold, 2014; Jones et al., 2019; L. Watson et al., 2018; T. Wu, Little, et al., 2016). However, it should be noted that the relationship between the rise in postprandial blood glucose and the gastric emptying rate is not necessarily linear. Intraduodenal glucose infusion at 2 kcal/min results in a much greater increase in blood glucose levels than 1 kcal/min in healthy humans, while minimal additional increase occurs in glycaemia at rates of 3 or 4 kcal/min (Ma et al., 2012; Pilichiewicz, Chaikomin, et al., 2007; Trahair et al., 2012), due probably to the increasing contribution of the distal gut to provide counter-regulation to glycaemic excursions.

In health, GIP contributes to approximately 50% of the incretin effect (Vilsbøll, Krarup, Madsbad, & Holst, 2003), and may serve to stabilise blood glucose by stimulating glucagon secretion during hypoglycaemia (M. Christensen et al., 2015). However, the loss of the insulinotropic effect of GIP in T2D, and the potential for GIP to increase fat deposition, have rendered it an unappealing target for the management of T2D. Recently, novel compounds with dual GIP and GLP-1 receptor agonism have been developed to treat T2D, with promising glucose-lowering efficacy (Frias et al., 2018). However, as mentioned earlier, the relative contribution of GIP receptor agonism to the overall metabolic benefits of these compounds remains unclear. The rapid release of CCK in response to meal ingestion is necessary for the digestion of

complex macronutrients, particularly fat, so it represents a determinant of subsequent gastric emptying and appetite responses.

However, when nutrients are administered intraduodenally, a threshold of caloric load is required to achieve suppression of appetite (Alleleyn, van Avesaat, Troost, & Masclee, 2016), indicative of a greater relevance of stimulating the distal gut to the control of appetite. Indeed, relative to the upper gut, the lower gut appears to be more effective at mediating postprandial glucose metabolism and suppressing appetite due to substantially augmented GLP-1 and PYY secretion. Recently, the comparative effects of the proximal and distal small intestine on postprandial glucose metabolism were evaluated using targeted intraluminal glucose infusion in both healthy individuals and subjects with T2D (X. Zhang et al., 2019). In both groups, intra-ileal administration of glucose (2 kcal/min over 60 min) was associated with substantially greater GLP-1 secretion, incretin effect and GI-mediated glucose disposal (GIGD), when compared with intraduodenal infusion (**Figure 1.3**). That the absorption of glucose occurs at a lower rate in the ileum, probably because of fewer glucose transporters in the distal gut, may not only attenuate the glycaemic response to glucose infusion, but also allow enteroendocrine cells to be stimulated over a longer duration than those in the proximal gut (X. Zhang et al., 2019). In a similar study setting, Poppitt and colleagues compared the effects of a small load of glucose (~0.65 kcal/min over 90 min), administered into either the ileum or the duodenum, on GI hormone secretion, appetite and food intake in healthy subjects. In this study, ileal infusion of glucose induced greater GLP-1 and PYY secretion and less food intake

than did intraduodenal infusion (Poppitt et al., 2017). Compared with oral ingestion or duodenal perfusion, delivering fat or protein into the ileum also induces a greater suppression of food intake in healthy humans (Maljaars et al., 2011; Maljaars et al., 2008; van Avesaat, Troost, Ripken, Hendriks, et al., 2015). Moreover, administration of a relatively small load of lauric acid (5 g; 20 kcal) in enterically coated pellets for release in the ileum and colon has been shown to stimulate sufficient GLP-1 secretion to improve the glycaemic response to a standardised breakfast and lunch in subjects with T2D (Ma et al., 2013). Similarly, ileo-colonic delivery of mixed bile acids (1 g/day) increases GLP-1 secretion and reduces postprandial blood glucose levels in subjects with obesity and T2D during a 4-week treatment (Calderon et al., 2020). Alternatively, enteroendocrine cell stimuli can be delivered through rectal administration; rectal perfusion with TCA has been shown to stimulate GLP-1 and PYY secretion, and suppress appetite scores in health (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013) and reduces energy intake and glycaemia in T2D (T. E. Adrian et al., 2012). Accordingly, enhancing the exposure of the distal gut to nutrients, and associated bioactive compounds such as bile acids, either by pharmacological inhibition of nutrient digestion and absorption in the upper gut (Qualmann et al., 1995; M. Y. Zheng et al., 2013), surgical reconstruction of the GI tract (such as Roux-en-Y gastric bypass) (Fruhbeck, 2015; Rubino, Schauer, Kaplan, & Cummings, 2010), or endoscopic implantation of a duodenal-jejunal bypass sleeve device (de Jonge et al., 2013; Koehestanie et al., 2014), has been shown to improve blood glucose control in T2D and reduce body weight in obesity. The causal links of these metabolic outcomes to GLP-1 and PYY signalling have been further validated

in T2D subjects undergoing Roux-en-Y gastric bypass, in whom glucose tolerance is attenuated by the GLP-1 receptor antagonist exendin9-39) (Jørgensen et al., 2013; Svane, Bojsen-Moller, et al., 2016), while energy intake is increased with either GLP-1 receptor antagonism, or inhibition of PYY activation using a DPP-4 inhibitor (Svane, Jorgensen, et al., 2016).

In recognition that the gut microbiota are an essential regulator of the host energy metabolism (Nicholson et al., 2012), and that insulin resistance, obesity and T2D are linked to dysbiosis (Seck et al., 2019), the role of the large intestinal bacteria in the regulation of glycaemia and food intake is now receiving increasing attention. While the mechanisms by which the gut microflora participate in the regulation of metabolic homeostasis remain incompletely understood, many of their metabolites are linked to GI hormone secretion (Canfora, Meex, Venema, & Blaak, 2019; Wahlstrom, Sayin, Marschall, & Backhed, 2016). For example, SCFAs, including acetate, butyrate and propionate, have been shown to stimulate GLP-1 and PYY from colonic L-cells in a dose-dependent manner (Canfora et al., 2017; Christiansen et al., 2018; Tolhurst et al., 2012) and to enhance insulin secretion either directly or indirectly in both rodents and humans (Canfora et al., 2017; Chambers et al., 2015; Christiansen et al., 2018; Freeland & Wolever, 2010; Psichas et al., 2015; Tolhurst et al., 2012). Oral supplementation with propionate and butyrate improves blood glucose control and promotes weight loss in rats (De Vadder et al., 2014). In obese individuals, acute administration of inulin-propionate ester (10 g), designed to be released in the colon, was shown to increase postprandial GLP-1 and PYY concentrations and decrease

energy intake, without affecting gastric emptying (Chambers et al., 2015). Moreover, administration of inulin-propionate ester (10 g/day) over 24 weeks showed a tendency to reduce body weight in obese subjects (Chambers et al., 2015). However, this phenomenon is complicated by evidence of a central effect contributing to suppression of energy intake after a single dose of colonic inulin-propionate ester, independent of changes in peripheral GLP-1 or PYY concentrations (Byrne et al., 2016).

## 1.6 Summary

The GI tract serves not only as the site of nutrient digestion and absorption, but also as an endocrine organ secreting a variety of GI hormones as a result of the complex interaction between ingested nutrients, bioactive compounds and enteroendocrine cells to regulate postprandial glucose metabolism and energy intake. Given the major difference in the distribution of enteroendocrine cells between the upper and lower gut, the load and delivery of nutrients, as well as the digestive processes in the GI tract, have major implications on how these enteroendocrine cells are stimulated. Exposure of the upper gut to nutrients is associated with predominantly GIP and CCK release, whereas increasing the delivery of nutrients to the distal small intestine and colon is associated with augmented secretion of GLP-1 and PPY. These distal gut hormones appear more potent in mediating postprandial glucose metabolism and suppressing energy intake than those secreted from the proximal gut. Accordingly, the distal gut is becoming an appealing target for the management of T2D and obesity, using nutritional, pharmacological or surgical approaches to increase its exposure to

nutrients and other bioactive compounds. Future development in this area is likely to yield novel therapies for T2D and obesity of high efficacy without the need of surgical procedures.

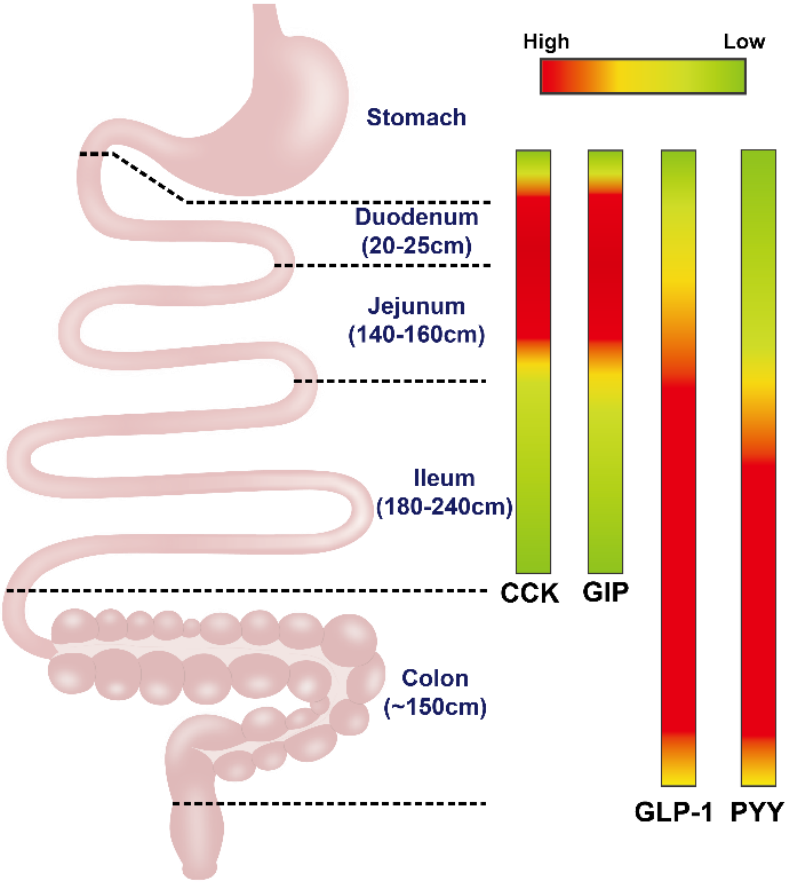


**Figure 1.1.** The density of enteroendocrine cells secreting cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) in the duodenum, jejunum, ileum and colon.

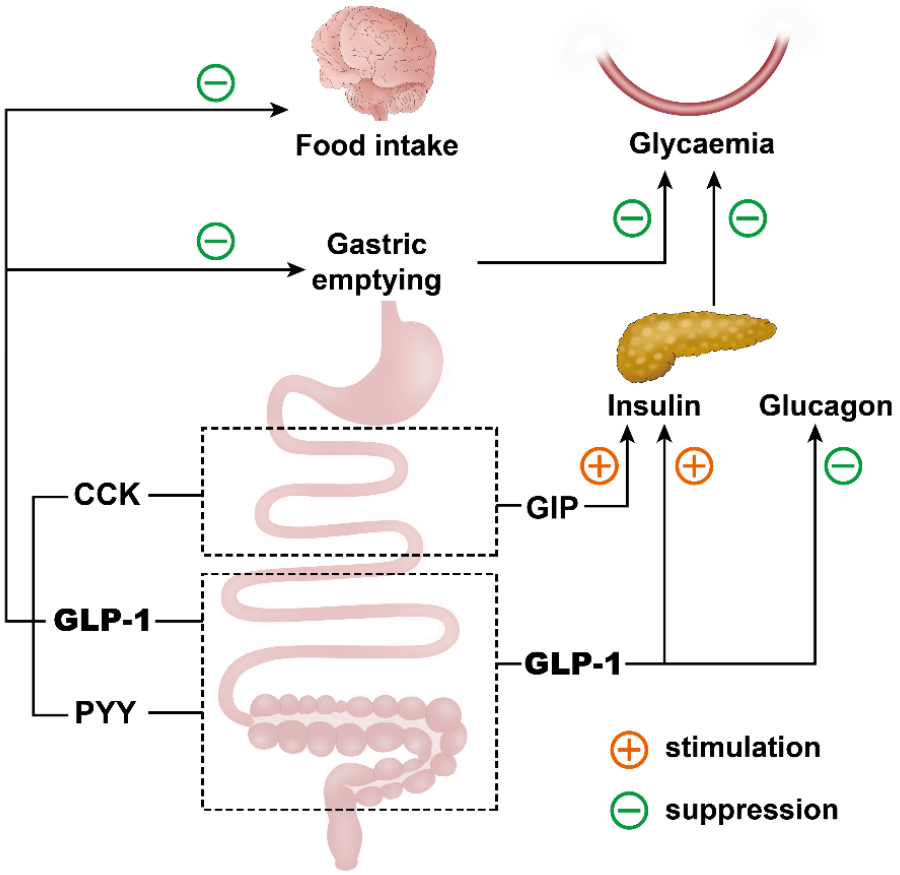


**Figure 1.2.** The roles of gastrointestinal hormones, including cholecystokinin (CCK), glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide 1 (GLP-1) and peptide YY (PYY), released in response to meal ingestion, in the regulation of gastric emptying, postprandial glycaemia and energy intake.

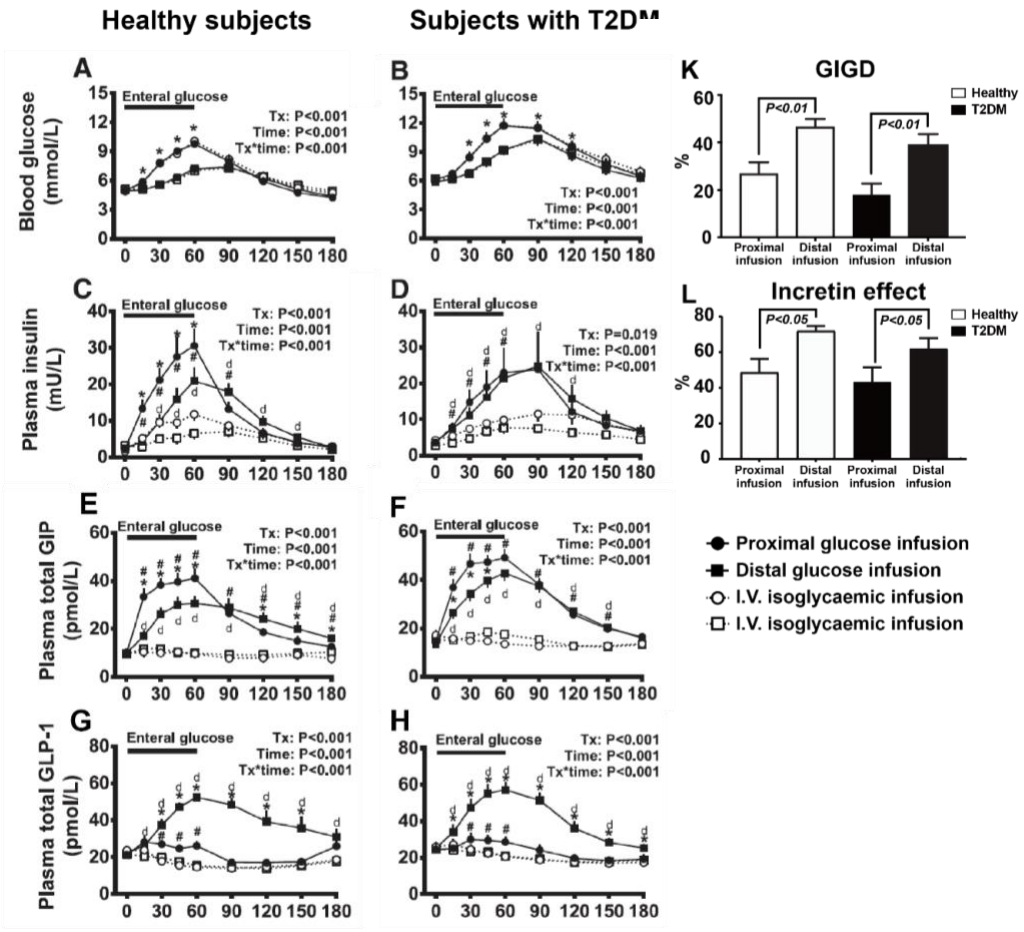


**Figure 1.3.** Comparative effects of proximal and distal small intestinal glucose exposure on glycaemia, incretin hormone secretion, and the incretin effect in both healthy individuals and subjects with type 2 diabetes (T2D) (n=10 each). A and B: Blood glucose levels, C and D: plasma insulin, E and F: plasma total glucose dependent insulinotropic polypeptide (GIP), G and H: plasma total glucagon- like peptide 1 (GLP-1), K: Gastrointestinal-mediated glucose disposal (GIGD), and L: Incretin effect. *P<0.05 for proximal vs. distal enteral glucose infusion; #P<0.05 for proximal enteral vs. corresponding i.v. glycaemic glucose infusion; dP<0.05 for distal enteral vs. corresponding i.v. glycaemic glucose infusion. Data are mean ± SEM. [Figures are adapted from reference (X. Zhang et al., 2019) , with permission from Diabetes Care].

# CHAPTER 2: ROLE OF INTESTINAL BITTER SENSING IN ENTEROENDOCRINE HORMONE SECRETION AND METABOLIC CONTROL

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

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## 2.1 Abstract

The gastrointestinal (GI) tract stores ingested nutrients in the stomach which are then delivered to the small intestine at a controlled rate to optimize their digestion and absorption. The interaction of nutrients with the small and large intestine generates feedback that slows gastric emptying, induces satiation, and reduces postprandial glycemic excursions. The mechanisms underlying these nutrient-gut interactions are complex; it has only recently been appreciated that the gut has the capacity to detect intraluminal contents in much the same way as the tongue, via activation of specific G-protein-coupled receptors, and that ensuing signalling mechanisms modulate the release of an array of gut hormones that influence GI motility, appetite and glycaemia. Interestingly, evidence from preclinical models supports a functional link between intestinal bitter taste receptor (BTRs) and GI hormone secretion, and the outcomes of recent studies indicate that stimulation of intestinal BTRs may be used to modulate GI function, to diminish energy intake and limit postprandial blood glucose excursions in humans. This review summarizes current evidence about the expression and function of intestinal BTRs in relation to enteroendocrine hormone release and discusses the clinical implications of this pathway for the management of obesity and type 2 diabetes.

## 2.2 Introduction

Recent decades have witnessed the conceptual evolution of the gastrointestinal (GI) tract from being solely a site of nutrient digestion and absorption to its recognition as the largest endocrine system in the body – more than 30 peptides are now known to be released from enteroendocrine cells within the GI mucosa. These gut-derived hormones communicate with tissues both within and outside the gut, and play a pivotal role in the regulation of metabolic homeostasis. Of particular importance are ghrelin, released from the enteroendocrine Gr-cells (within the stomach); cholecystokinin (CCK), from I-cells (mainly in the upper small intestine); glucose-dependent insulinotropic polypeptide (GIP), from K-cells (largely in the upper small intestine); and glucagon-like pepetide-1 (GLP-1) and peptide YY (PYY), from L-cells (predominantly in the distal small and large intestine) (**Figure 2.1**). Ghrelin is secreted predominantly during fasting and is suppressed after meals. It is regarded as a 'hunger' hormone that drives food intake and accelerates gastric emptying (Date et al., 2000; Steinert et al., 2017). In contrast, CCK, GIP, GLP-1 and PYY are predominately released postprandially and, in concert, mediate intestinal feedback to limit postprandial glycaemic excursions and suppress energy intake (Steinert et al., 2017; T. Wu, Rayner, Young, & Horowitz, 2013). In health, GIP and GLP-1 are responsible for the substantially greater insulin response to oral, or enteral, glucose administration when compared with 'isoglycaemic' intravenous glucose infusion – the so-called 'incretin' effect (M. A. Nauck et al., 1986). In type 2 diabetes (T2D), the insulinotropic effect of GLP-1 remains relatively intact, although that of GIP is markedly diminished, which may account for the diminished incretin effect in this

group (T. Wu, Rayner, et al., 2016a). GLP-1 also exerts a glucose-dependent glucagonostatic effect (T. Wu, Rayner, et al., 2016a) and, together with CCK and PYY, acts to slow gastric emptying and suppress energy intake (Steinert et al., 2017). Accordingly, modulation of gut hormone secretion has been actively pursued as a therapeutic option in the management of obesity and T2D (Amori, Lau, & Pittas, 2007; Jakubowicz et al., 2014; Ma et al., 2013; Owens, Monnier, & Hanefeld, 2017; T. Wu, M. J. Bound, S. D. Standfield, K. L. Jones, et al., 2013; T. Wu, Little, et al., 2016; T. Wu, Rayner, et al., 2016a; T. Wu et al., 2012). To this end, it has been suggested that a wide array of chemo-sensors expressed on different enteroendocrine cells is responsible for the detection of carbohydrate (e.g. ATP-sensitive K+ channel and sodium glucose co-transporter-1 (Kuhre, Frost, Svendsen, & Holst, 2015; H. E. Parker, Habib, Rogers, Gribble, & Reimann, 2009)), fat (e.g. G-protein-coupled receptors (GPCRs) 119 and 120 (Hirasawa et al., 2005; Lauffer et al., 2009)) and protein (e.g. oligopeptide transporter 1 and calcium sensing receptor (Daly et al., 2013; Liou et al., 2011)) and associated stimulation of gut hormone secretion. Emerging evidence also attests to the functional importance of 'taste' signals arising from intraluminal contents in modulating gut hormone release. For example, blockade of intestinal sweet taste receptors (STRs) by lactisole attenuates glucose-induced incretin hormone secretion substantially in healthy humans (Steinert et al., 2011), although stimulation of STRs (by low-calorie sweeteners) alone appears insufficient to stimulate GIP or GLP-1 secretion in humans (T. Wu, M. J. Bound, S. D. Standfield, M. Bellon, et al., 2013). Unlike STRs, activation of intestinal bitter taste receptors (BTRs), either by pharmacological BTR agonists or physiological bitter compounds,

has been shown to modulate gut hormone secretion in various preclinical and clinical experimental settings, leading to reductions in blood glucose and energy intake (Iven et al., 2019; Kim, Egan, & Jang, 2014). In this review, we summarize current evidence relating to the expression and function of intestinal BTRs in relation to enteroendocrine hormone release, as well as the clinical implications of this pathway for the management of obesity and T2D.

## 2.2 Intestinal bitter taste receptors

Taste stimuli are detected by a group of specialised G protein-coupled receptors, initially identified in the taste buds of the oral cavity (Calvo & Egan, 2015). Subtypes of taste 1 receptors heterodimerize to detect sweet (T1R2/T1R3) and umami (T1R1/T1R3) stimuli, while multiple type 2 receptors (T2Rs) are characterised as BTRs and detect bitter stimuli, and may trigger mechanisms which prevent the ingestion and absorption of potentially noxious bitter compounds. Binding of ligands to these taste receptors initiates a signalling cascade involving the dissociation of the G-protein gustducin into Gα and Gβγ subunits, activation of phospholipase C $\beta_2$, production of diacylglycerol and inositol 1,4,5-trisphophate (Kim et al., 2014; Yu et al., 2015; Yue, Liang, Gu, Du, & Chen, 2018), and opening of the transient receptor potential ion channel M5, leading to the release of intracellular $Ca^{2+}$ (Avau & Depoortere, 2016; Avau, Rotondo, et al., 2015; Barrea et al., 2017; Kim et al., 2014; Yu et al., 2015), $Na^{+}$ influx (Avau, Rotondo, et al., 2015; J. Li et al., 2017), cellular depolarization and the secretion of neurotransmitters (Avau & Depoortere, 2016). The increases in intracellular Gα subunit also activate phosphodiesterase to degrade cyclic

adenosine monophosphate (cAMP), whereas diacylglycerol and intracellular $Ca^{2+}$ activate the protein kinase C pathway (Avau, Rotondo, et al., 2015; Kim et al., 2014) (**Figure 2.2**). It has only recently been appreciated that taste receptors and their downstream signalling molecules are also found in extra-oral locations, including the airway, kidney, brain, immune system and the GI tract (Depoortere, 2014; P. Lu, Zhang, Lifshitz, & ZhuGe, 2017). For example, in rodents, inhalation of BTR agonists decreases airway resistance (Deshpande et al., 2010), while intravenous administration of the BTR agonist, denatonium benzoate (DB), causes a transient fall in blood pressure (Lund et al., 2013). The focus of this review, however, is the biology of intestinal BTRs, and in particular their relevance to the secretion of GI hormones from enteroendocrine cells.

In a seminal study reported in 2002, Wu *et al.* demonstrated gene expression of several BTRs in both the stomach and duodenum of mice and rats using reverse transcriptase-PCR (S. V. Wu et al., 2002). In addition, BTRs were also found to be expressed on the secretin tumour cell line (STC-1), an enteroendocrine cell model derived from murine enteroendocrine tumours (S. V. Wu et al., 2002). That the exposure of STC-1 to different bitter compounds resulted in a rapid increase in intracellular $Ca^{2+}$ indicated that a functional BTR-sensing system may be present on the enteroendocrine cells (S. V. Wu et al., 2002). These observations were further validated in subsequent studies employing reverse transcriptase- and quantitative-PCR assays on small and large intestinal tissues and enteroendocrine cells of both rodents and humans (**Table 2.1**) (M. C. Chen, Wu, Reeve, & Rozengurt, 2006; Jeon,

Zhu, Larson, & Osborne, 2008; Yue et al., 2018). Consistent with PCR observation, studies using double-labelling immunofluorescence have also shown co-localization of chromogranin A (a cellular marker of enteroendocrine cells) with BTRs in the mouse small and large intestine (Jeon et al., 2008; Vegezzi et al., 2014). More specifically, co-expression of GLP-1 with various BTRs in human enteroendocrine L cell lines (i.e. HuTu-80 and NCI-h716) and in small and large intestinal tissues has been observed (Kim et al., 2014; Park et al., 2015; Pham et al., 2016; Rozengurt et al., 2006). However, the co-expression of BTRs with enteroendocrine cells containing other hormones is not well characterised in rodents or humans. Moreover, the expression of intestinal BTRs in metabolic disorders has not been consistently reported. In the study reported by Chao *et al.* (Herrera Moro Chao et al., 2016), the expression of both STR and BTR subtypes were shown to be less in the hypothalamus, brainstem and duodenum in ob/ob mice than C57Bl/6 controls. By contrast, the expression of the BTR, T2R38, in the colonic mucosa was shown to be related directly to BMI in humans, such that the abundance of T2R38 tended to be higher in those who were overweight/obese, when compared to lean subjects (Latorre et al., 2016). In both healthy individuals and subjects with T2D, the expression of STRs in duodenal biopsy samples did not correlate with BMI or HbA1c, although the dynamic response of STR expression to intraduodenal glucose infusion was found to be impaired in T2D (Young et al., 2013). Of note, the downstream signalling molecules of taste receptors have also been identified in non-endocrine cells of the gut. For example, α-gustducin and transient receptor potential ion channel M5 are expressed abundantly in subsets of brush cells in mouse and rat gut (Bezencon et al., 2008; Hofer, Puschel, &

Drenckhahn, 1996; Sutherland, Young, Cooper, Horowitz, & Blackshaw, 2007). In murine gastric tissue, α-gustducin-expressing brush cells have been found adjacent to ghrelin-releasing Gr-cells (Hass, Schwarzenbacher, & Breer, 2010; Janssen et al., 2011). Given that the latter are not in direct contact with the intraluminal contents, i.e. "closed-type", it is possible that brush cells may act as a sensor for intraluminal contents to regulate ghrelin secretion (Iwatsuki & Uneyama, 2012).

## 2.3 Effects of BTR signalling on gut hormone secretion

An increasing number of studies in both preclinical and clinical models have evaluated the effects of BTR agonists on ghrelin, CCK, GLP-1 and PYY secretion, although the specificity of bitter compounds for different BTRs is poorly defined and the function of intestinal BTR sensing in either obesity or T2D has not been thoroughly investigated. In contrast, information regarding GIP secretion in response to BTR stimulation is limited (**Table 2.2**).

### *2.3.1 Ghrelin*

The potential role of BTR signalling in the regulation of ghrelin secretion has evaluated in mice and humans, albeit with strikingly different outcomes. In mice, intragastric administration of a mixture of BTR agonists (including DB, phenylthiocarbamide (PTC), quinine and D-[-]salicin) was shown to increase plasma total ghrelin and octanoyl ghrelin levels without affecting ghrelin mRNA expression (Janssen et al., 2011). BTR agonist-induced ghrelin secretion was markedly attenuated in α-gustducin-/- mice. This was consistent with a functional involvement

of taste signalling in ghrelin release (Janssen et al., 2011), although α-gustducin is a non-specific downstream signalling molecule and, as discussed, an indirect interaction between brush cells and Gr cells is an alternative possibility. Paradoxically, intragastric gavage of BTR agonists in mice was associated with only a transient increase in food intake during the first 30 min, followed by a sustained suppression of intake over the subsequent 4 hours (Janssen et al., 2011). In contrast to the stimulation of ghrelin observed in mice, intragastric administration of another bitter tastant, quinine-hydrochloride (HCl quinine, 10umol/kg), reduced fasting plasma ghrelin and motilin levels in healthy women (Deloose, Corsetti, Van Oudenhove, Depoortere, & Tack, 2018) (Iven et al., 2019), associated with increased activity in hedonic and homeostatic brain regions on functional magnetic resonance imaging, and suppressed antral motility and energy intake (Iven et al., 2019). These observations suggest a role of BTR signalling in communications between the gut and brain in the control of energy intake. However, in another study, intragastric DB at a dose of 1umol/kg, which suppressed motilin secretion, appetite scores and energy intake, failed to affect either plasma ghrelin or the rate of gastric emptying in healthy women (Deloose et al., 2017). Accordingly, further studies are required to determine the secretory pattern of ghrelin in response to different types and doses of BTR agonists and the associated metabolic effects in humans, including those with obesity and T2D.

### *2.3.2 CCK*

Initial evidence to support the potential for BTR-evoked CCK secretion was reported in STC-1 cells, where both DB and PTC increased intracellular $Ca^{2+}$ and stimulated CCK secretion in a dose-dependent manner (M. C. Chen et al., 2006; Jeon, Seo, & Osborne, 2011). Subsequently, steroid glycoside H.g.-12, extracted from the plant *Hoodia gordonii* (which tastes bitter, and has potent appetite-suppressant effects in both animals and humans (van Heerden, 2008)) was found to induce CCK secretion both *ex vivo* from rat intestine, and from HuTu-80 cells (Le Neve, Foltz, Daniel, & Gouka, 2010). That the effect of H.g.-12 on CCK secretion was abolished by a BTR inhibitor, compound 03A3, supports a functional role of BTR signalling in H.g.-12-induced CCK release (Le Neve et al., 2010). While co-expression of BTRs with CCK-secreting I-cells has not been assessed in humans, oral administration of encapsulated HCl quinine (18 mg) was recently reported to increase plasma CCK concentrations and reduce energy intake at an *ad libitum* meal in healthy young individuals (Andreozzi et al., 2015). Moreover, in this study the magnitude of suppression of energy intake in response to HCl quinine was related directly to the subjects' sensitivity to the bitter taste of PTC (Andreozzi et al., 2015). These observations warrant further investigation on the potential of targeting the intestinal BTR signalling pathway to stimulate CCK secretion and reduce energy intake in obesity.

### *2.3.3 GLP-1 and PYY*

Underpinned by the successful clinical application of GLP-1 receptor agonists and dipeptidyl peptisase-4 inhibitors to the management of T2D (Amori et al., 2007;

Owens et al., 2017; T. Wu, Rayner, et al., 2016a), there has been great interest in the potential for BTR agonists to augment L-cell secretion, and thereby increase concentrations of endogenous GLP-1.

At the cellular level, numerous bitter compounds have been reported to induce GLP-1 secretion from enteroendocrine cells via BTR pathways. For example, in both NCI-716 and STC-1 cells, berberine, a natural bitter plant alkaloid commonly used as an antibiotic, was shown to dose-dependently stimulate GLP-1 secretion via T2R38 (Yu et al., 2015; Yue et al., 2018). Similarly, a specific T2R38 agonist, phenylthiourea, induced GLP-1 secretion from HuTu-80 cells, an effect markedly inhibited by silencing of T2R38 with small interfering RNA(Pham et al., 2016), In contrast, 1,10-phenanthroline stimulates GLP-1 via T2R5 (Park et al., 2015), and DB appears to induce GLP-1 secretion via a broad range of BTRs (including T2R4, T2R43 and T2R46 at least), in NCI-h716 cells (Kim et al., 2014). Furthermore, blockade of BTRs (e.g. by probenecid), or the downstream pathways relating to BTR signalling, including inositol 1,4,5-trisphophate, phospholipase C $\beta_2$, protein kinase C and/or phosphodiesterase, attenuates GLP-1 secretion induced by bitter tastants (Huang, Lu, Pai, Chin, & Huang, 2013; Kim et al., 2014; Suh et al., 2015).

In rodents, exposure of the gut to BTR agonists has also been shown to augment plasma GLP-1 levels (Huang et al., 2013; Kim et al., 2014; Pham et al., 2016; Suh et al., 2015). In acute settings, an intragastric preload of DB prior to enteral glucose administration increased plasma GLP-1 and insulin concentrations (Kim et al., 2014),

slowed gastric emptying (Avau, Rotondo, et al., 2015; Glendinning, Yiin, Ackroff, & Sclafani, 2008) and reduced blood glucose (Kim et al., 2014). Consistent with the role of BTR signalling in GLP-1 secretion, the effect of DB to slow gastric emptying was abolished by co-administration of probenecid (Avau, Rotondo, et al., 2015). Similarly, intragastric administration of PTC has been reported to augment plasma GLP-1 concentrations (Pham et al., 2016) and slow gastric emptying (Avau, Rotondo, et al., 2015) in mice. The latter effect was, however, not inhibited by probenecid (Avau, Rotondo, et al., 2015). This discrepancy necessitates further investigation to determine whether probenecid sufficiently blocks the BTRs activated by PTC, and whether mechanisms other than BTR-gut hormone pathways account for the slowing of gastric emptying by PTC in mice. In support of the latter, the slowing of gastric emptying induced by a mixture of bitter substances (including PTC) was not affected by concurrent administration of GLP-1 and CCK antagonists in mice (Janssen et al., 2011). In the longer-term (i.e. 4 weeks), intragastric administration of DB remained effective at increasing meal-induced GLP-1 secretion, associated with a reduction in body weight in obese mice, whereas another bitter tastant, quinine, had minimal effect on GLP-1 or ghrelin, despite reducing body weight (Avau, Bauters, et al., 2015).

While BTRs (e.g. T2R5 and T2R38) have been reported to localize on L-cells in the small and/or large intestine, effects of BTR agonists on GLP-1 secretion are not well characterised in humans. Recently, Mennella *et al.* evaluated the effect of a single low dose of *Gentiana lutea* root extract encapsulated for release in the small intestine in healthy subjects (Mennella et al., 2016), and observed a tendency for a higher GLP-1

response to a standardised breakfast, and a reduction in post-lunch energy intake compared to placebo (Mennella et al., 2016). Accordingly, additional human studies are needed to evaluate the potential for targeting intestinal BTRs to stimulate GLP-1 secretion.

In contrast to GLP-1, information relating to the effect of BTR agonists on PYY secretion (also released from L-cells) is limited. Although DB stimulates PYY secretion from NCI-H716 cells in a similar manner to GLP-1 (Kim et al., 2014), this effect has hitherto not been assessed *in vivo*.

## 2.4 Clinical implications of targeting intestinal BTRs

That BTR signalling is functionally linked to the secretion of hormones integral to the regulation of energy intake and glycaemia, as well as the control of gastric emptying, has stimulated substantial interest in targeting this pathway for the management of obesity and T2D (publications from clinical studies are summarised in **Table 2.3**). The relative absence of calories in bitter compounds represents a substantial asset of this approach.

### *2.4.1 Effects on energy intake*

The impact of BTR sensing in the control of energy intake has been evaluated in both preclinical and clinical studies. Despite variable effects of different BTR agonists on each GI hormone, the majority of studies in rodents have reported energy intake to be suppressed following exposure to acute doses of BTR agonists (Kratz, Levitsky, &

Lustick, 1978; Leng, Lu, & Xu, 2004; van Heerden et al., 2007), although one study reported a transient increase, followed by a sustained suppression of food intake after intragastric administration of a mixture of DB, PTC and salicin (Janssen et al., 2011). Arguably, of greater interest is evidence that intragastric gavage of DB (60 μmol/kg) or quinine (160 μmol/kg) once daily for four weeks in high fat-fed obese mice reduced weight gain substantially, and in an α-gustducin-dependent manner (Avau, Bauters, et al., 2015). In healthy women, a single dose of HCl quinine (10umol/kg), administrated intragastrically 60 min before an *ad libitum* liquid meal (chocolate milk shake), reduced food intake (346 ± 37 g for HCl quinine vs. 414 ± 46 g for water control), in association with reduced ghrelin levels and increased neural activity in the hypothalamus, hedonic regions, and parts of the medulla associated with appetite homeostasis (Iven et al., 2018). Consistent with these observations, oral administration of encapsulated HCl quinine (18 mg) also modestly suppressed energy intake at a subsequent *ad libitum* buffet meal (514 ± 248 kcal for HCl quinine vs. 596 ± 286 kcal for placebo) in healthy young subjects (12 females and 8 males) without inducing nausea (Andreozzi et al., 2015). Likewise, administration of encapsulated bitter compounds derived from *Gentiana lutea* root with a standardised breakfast reduced total daily energy intake by ~20% in healthy individuals (Mennella et al., 2016), while oral insensitivity to the bitter taste of 6-n-propylthrouracil was associated with increased energy intake in female subjects (Shafaie, Koelliker, Hoffman, & Tepper, 2013). It remains to be determined whether stimulation of intestinal BTRs has the capacity to reduce energy intake and, hence, body weight in obese individuals.

### *2.4.2 Effects on blood glucose*

The rate of emptying of carbohydrates from the stomach for absorption in the small intestine is a major determinant of the glycaemic response to meals (M. Horowitz, Edelbroek, Wishart, & Straathof, 1993). In the majority of T2D subjects with modestly elevated glycated haemoglobin (HbA1c less than ~8% or 64 mmol/mol), postprandial glycaemia makes the dominant contribution to overall glycaemic control (Monnier, Lapinski, & Colette, 2003; T. Wu, Rayner, et al., 2016b). In addition, postprandial glycaemia is an independent cardiovascular risk factor and predicts all-cause mortality (Cavalot et al., 2011), and accordingly, represents a specific target for the treatment of T2D. Preclinical models indicate that stimulating intestinal BTRs has the potential to improve blood glucose control. In wild type mice, intragastric administration of DB, PTC or a mixture of bitter compounds slowed gastric emptying substantially (Avau, Rotondo, et al., 2015; Janssen et al., 2011), while oral administration of DB (1 mg/kg) (Kim et al., 2014) or *Gentia scabra* root extract (300mg/kg; containing several bitter compounds such as loganic acid, gentiopicrin and rindoside) (Kim et al., 2014; Suh et al., 2015) in db/db mice was associated with higher GLP-1 and lower blood glucose responses following glucose gavage when compared with saline. In mice fed a high fat diet, oral administration of bitter gourd extract prior to an oral or intraperitoneal glucose load also resulted in higher GLP-1 and insulin levels and lower blood glucose responses (Huang et al., 2013). That the magnitude of reduction in glycaemia was attenuated substantially by concurrent administration of the GLP-1 receptor antagonist, exendin(9-39), attests to the

importance of GLP-1 to glucose-lowering induced by bitter substances (Huang et al., 2013).

Hitherto, there is limited information about the effect of BTR agonists on blood glucose in humans. Studies to date have reported inconsistent effects on gastric emptying. In healthy women, sham-feeding with quinine sulphate (10 mg) was reported to slow the emptying of subsequently ingested 'electrolyte soup', when compared to sham-feeding with a 'pleasant' strawberry flavouring or control (no sham-feeding) (Wicks, Wright, Rayment, & Spiller, 2005). Little *et al.* compared the rate of gastric emptying of three 'test meals' in healthy subjects, consisting of 500 mL water (control) and two bitter-tasting solutions containing either a small dose of quinine (1 mM) or naringin (0.198 mM), delivered via intragastric infusion. Although these doses of quinine and naringin yielded a medium intensity of bitterness during an oral perception test, gastric emptying did not differ between the bitter solutions and water alone (Little, Gupta, Case, Thompson, & McLaughlin, 2009). More recently, intragastric administration of DB at a dose of 1umol/kg suppressed appetite sensations, but failed to affect gastric emptying in healthy women (Little et al., 2009). However, it remains unclear whether the disparity in findings between studies in mice and humans reflect species differences, or whether the relatively low doses of BTR agonists employed in the human studies were insufficient to interact with L-cells located predominantly in the distal small and large intestine. In the case of GLP-1, infusion of glucose into the duodenum at 2 kcal/min (where glucose is absorbed in the

upper gut) elicits minimal GLP-1 secretion, while ileal infusion of glucose at the same rate induces substantial GLP-1 release (X. Zhang et al., 2019).

The genetic phenotype of GPCRs is now known to be an important determinant of physiological function, may predispose to human diseases (Thompson et al., 2014). There is evidence that polymorphisms of BTR genes that impair the sensitivity to bitterness may be associated with changes in food intake and dysregulation of blood glucose. For example, women with gestational diabetes mellitus exhibited a lower T2R9 gene (rs3741845) frequency, and consumed more meat, dairy and sweet beverages compared to pregnant women without gestational diabetes mellitus (Bartakova, Kuricova, Zlamal, Belobradkova, & Kankova, 2018). Similarly, dysfunction of T2R9 due to a single nucleotide polymorphism is associated with higher blood glucose and insulin responses to an oral glucose tolerance test in Amish individuals with and without T2D (Dotson et al., 2008). In German individuals without T2D, variations in the T2R38 gene (rs713598, rs1726866 and rs10246939) are also reported to have significant associations with body composition in women, and the glycaemic response to oral glucose in men (Keller et al., 2013).

## 2.5 Conclusions and prospective views

In recognition of the pleiotropic actions of GI hormones in the regulation of metabolic homeostasis, exogenous peptides or mimetics (e.g. GLP-1 receptor agonists and GLP-1/GIP dual receptor agonists) are under rapid development within the pharmaceutical industry to better manage both T2D and obesity. This approach, however, is often

limited by cost, side effects (predominantly GI symptoms), and suboptimal efficacy (particularly for obesity). Dietary strategies to modulate endogenous GI hormone secretion represent an alternative that shows substantial promise. For example, consuming a nutrient 'preload' prior to the main meal has been shown to reduce postprandial blood glucose in both health and T2D by stimulating GLP-1 secretion in advance of the meal, and by slowing gastric emptying (Ma et al., 2009; T. Wu, M. J. Bound, B. Y. R. Zhao, et al., 2013; T. Wu, Little, et al., 2016). However, this approach entails additional energy intake associated with the preload. Modulation of GI hormone secretion by low- or non-caloric compounds, such as bitter tastants, would therefore be advantageous compared with nutrient preloads.

There is a large body of preclinical studies that provide compelling evidence of a functional BTR signalling system in enteroendocrine cells, the effects of non-nutritive BTR agonists on enteroendocrine hormone secretion, and the potential for stimulating intestinal BTRs to suppress energy intake and reduce postprandial glycaemic excursions (Avau, Bauters, et al., 2015; Huang et al., 2013) . However, there are only a handful of clinical studies in healthy subjects (mostly females) that have evaluated the effects of BTR signalling on gut hormone secretion and associated metabolic effects, and no studies in subjects with obesity and/or T2D. Moreover, the doses of BTR agonists administered in human subjects have been low, probably because bitter tastants are considered to be potentially toxic and aversive (Avau & Depoortere, 2016). Bitter taste perception in the mouth is unpleasant, and naturally serves as an aversive signal for the termination of eating. However, stimulation of intestinal BTRs by

administration of different BTR agonists directly into the stomach or duodenum, thereby bypassing oral perception, has not been reported to cause any adverse effects in preclinical models and healthy subjects. Nevertheless, the tolerability of BTR agonists at higher doses remains to be established.

Relative to STRs (T1R2/T1R3) and umami taste receptors (T1R1/T1R3), the biology of BTRs appears to be more complex due to their diversity. Moreover, expression of BTRs varies substantially along the GI tract. For example, T2R2 and T2R6 showed higher expression in gastric than duodenal mucosa in rats (S. V. Wu et al., 2002), whereas in mice, T2R118 and T2R131 are expressed abundantly in the colon, but minimally in the duodenum and jejunum (Prandi et al., 2013). As summarised in **Table 2.1**, multiple BTRs are often co-expressed on the same enteroendocrine cell. However, the relative importance of each has not been characterised. Accordingly, it remains to be determined whether the expression of BTRs also exhibits regional specificity, in a similar pattern to enteroendocrine cells and, therefore, whether more targeted delivery of BTR agonists is needed for effective stimulation of enteroendocrine hormone secretion. Notably, physiological bitter substances, including bile acids and products of digestion (e.g. amino acids), are abundantly present in the gut after a meal; it is also important, therefore, to understand the physiological role of intestinal bitter taste sensing in the regulation of GI hormone secretion, appetite and postprandial glycaemia.

**Table 2.1. Summary of published reports on the presence of different BTRs in enteroendocrine cells and gastrointestinal tissues in rodents and humans.**

| Species | Models | T2Rs expressed | Reference |
|---|---|---|---|
| **Human** | HuTu-80 cell | T2R4, T2R5, T2R13, T2R14, T2R16, T2R38, T2R39, T2R40, T2R44, T2R46, T2R47, T2R49, T2R50, T2R60 | Rozengurt, et al. 2006; Pham et al. 2016, Le Neve et al. 2010 |
| | NCI-H716 cell | T2R1, T2R3, T2R4, T2R5, T2R7, T2R8, T2R9, T2R10, T2R13, T2R14, T2R19, T2R20, T2R30, T2R38, T2R39, T2R40, T2R41, T2R45, T2R46, T2R50, T2R60 | Kim et al. 2014, Dotson et al. 2008, Park et al. 2015, Yu et al. 2015 |
| | small intestine | T2R5 T2R14 T2R38 | Le Neve et al. 2010, Park et al. 2015, Pham et al. 2016; |
| | large intestine | T2R1, T2R3, T2R4, T2R5, T2R10, T2R13, T2R38, T2R39, T2R40, T2R42, T2R43, T2R44, T2R45, T2R46, T2R47, T2R49, T2R50, T2R60 | Rozengurt, et al. 2006, Laorre et al. 2016, Kaji et al. 2009, Dotson et al. 2008, Pham et al. 2016 |
| **Mouse** | STC-1 cells | mT2R102, mT2R104, mT2R105, mT2R106, mT2R107, mT2R108, mT2R109, mT2R110, mT2R113, mT2R114, mT2R116, mT2R117, mT2R118, mT2R119, mT2R121, mT2R122, mT2R123, mT2R124, mT2R125, mT2R126, mT2R129, mT2R130, mT2R131, mT2R134, mT2R135, mT2R136, mT2R137, mT2R138, mT2R139, mT2R140, mT2R143, mT2R144 | Jeon et al. 2008, Chen et al. 2006, Yue et al. 2018 |
| | small intestine | mT2R102, mT2R104, mT2R105, mT2R106, mT2R107, mT2R108, mT2R110, mT2R113, mT2R114, mT2R116, mT2R117, mT2R119, mT2R121, mT2R122, mT2R123, mT2R124, mT2R126, mT2R129, mT2R130, mT2R134, mT2R135, mT2R136, mT2R137, mT2R138, mT2R139, mT2R140, mT2R143, mT2R144 | Vegezzi et al. 2014, Avau et al. 2015, Gu et al. 2015, Prandi et al. 2013 Prandi et al. 2017 |
| | large intestine | mT2R108, mT2R113, mT2R117, mT2R118, mT2R119, mT2R125, mT2R126, mT2R131, mT2R135, mT2R136 mT2R137, mT2R138, mT2R140, mT2R143 | Avau et al. 2015, Prandi et al. 2017, Wu et al. 2005, Prandi et al. 2013 |

Table 2.1 continues

| **Species** | **Models** | **T2Rs expressed** | **Reference** |
|---|---|---|---|
| **Rat** | small intestine | rT2R1, rT2R2, rT2R3, rT2R4, rT2R5, rT2R6, rT2R7, rT2R8, rT2R9, rT2R10, rT2R12, rT2R16, rT2R34, rT2R38 | Wu et al. 2002, Wu et al. 2005 |
| | large intestine | rT2R, rT2R16, rT2R26 | Kaji et al. 2009 |

**Table 2.2. Effects of bitter tastants on gut hormone secretion in preclinical and clinical models.**

| Hormone | Preclinical/Clinical | Vitro/Vivo | Model | Bitter tastants | Reference |
|---|---|---|---|---|---|
| **Ghrelin** | Preclinical | vivo | mice | mixture of DB, quinine, PTC, D-salicin | Janssen et al. 2011 |
| | | | human | HCl quinine 10umol/kg | Deloose et al. 2017 |
| | Clinical | | | HCl quinine 10umol/kg | Iven et al. 2018 |
| **GLP-1** | Preclinical | vitro | HuTu-80 cells | Phenylthiourea | Pham et al. 2016 |
| | | | NCI-716 cells | Berberine | Yu et al. 2015 |
| | | | | 1,10-phenanthroline | Park et al. 2015 |
| | | | | Gentiana scabra | Suh et al. 2015 |
| | | | | DB | Kim et al. 2014 |
| | | | STC-1 cells | extract from wild bitter gourd | Huang et al. 2013 |
| | | | | Berberine | Yue et al. 2018 |
| | | vivo | mice | extract from wild bitter gourd | Huang et al. 2013 |
| | | | | DB | Kim et al. 2014 |
| | | | | Qing-Hua Granule | Li et al. 2017 |
| | | | | Gentiana scabra | Suh et al. 2015 |
| | Clinical | | healthy volunteer | Gentiana lutea root | Mennella et al. 2016 |

Table 2.2 continues

| Hormone | Preclinical/Clinical | Vitro/Vivo | Model | Bitter tastants | Reference |
|---|---|---|---|---|---|
| **CCK** | Preclinical | vitro | STC-1 cells | DB and PTC | Chen et al. 2006 |
| | | | HuTu-80 cells | H.g. -12 (extract of the plant Hoodia gordonii) | Le Neve et al. 2010 |
| | | | Caco-2 cells | PTC | Jeon et al. 2011 |
| | | vivo | mice | mixture of DB, quinine, PTC, D-salicin | Jeon et al. 2011 |
| | Clinical | | healthy volunteer | HCl quinine 10mg | Andreozzi et al. 2015 |
| **PYY** | Preclinical | vitro | NCI-716 cells | DB | Kim et al. 2014 |

**Table 2.3. Effects of bitter tastants in clinical studies**

| Authors | Subjects | Bitter tastants and doses | Main method | Key observation |
|---|---|---|---|---|
| Wicks et al. Eu J Gastroenterol. Hepatol, 2005 | healthy women (n=16) | 10 mg quinine sulphate | Sham feeding | Slowed gastric emptying substantially. |
| Little et al. Am J Physiol Regul Integr Comp Physiol, 2009 | healthy subjects (n=12) | 0.198mM 500 mL quinine (3.24mg) | Intragastric administration | Had no effect on gastric emptying. |
| Andreozzi et al. J Neurogastroenterol Motil, 2015 | healthy subjects (n=20) | 18 mg HCl quinine | encapsulated | Suppressed energy intake; increased CCK secretion; had no effect on gastric emptying. |
| Mennella et al. Br J Nutr, 2016 | healthy subjects (n=20) | 100mg extracts (from Gentiana lutea root) | encapsulated | Increased GLP-1; suppressed energy intake; had no effect on blood glucose. |
| Deloose et al. Am J Clin Nutr, 2017 | healthy women (n=39) | 1μmol/kg DB | Intragastric administration | Had no effect on gastric emptying; reduced hungry rating and increased satiety ratings. |
| Deloose et al.Neurogastroenterol & Motil, 2017 | healthy women (n=10) | 10 μmol/kg HCl quinine | Intragastric administration | Reduced plasma motilin and ghrelin levels; inhibited the antral motility. |
| Deloose et al. Nutr Neurosci, 2018 | healthy women (n=16) | 10 μmol/kg HCl quinine | Intragastric administration | Suppressed energy intake; reduced plasma motilin and ghrelin levels; reduced hungry ratings and increased satiety ratings. |

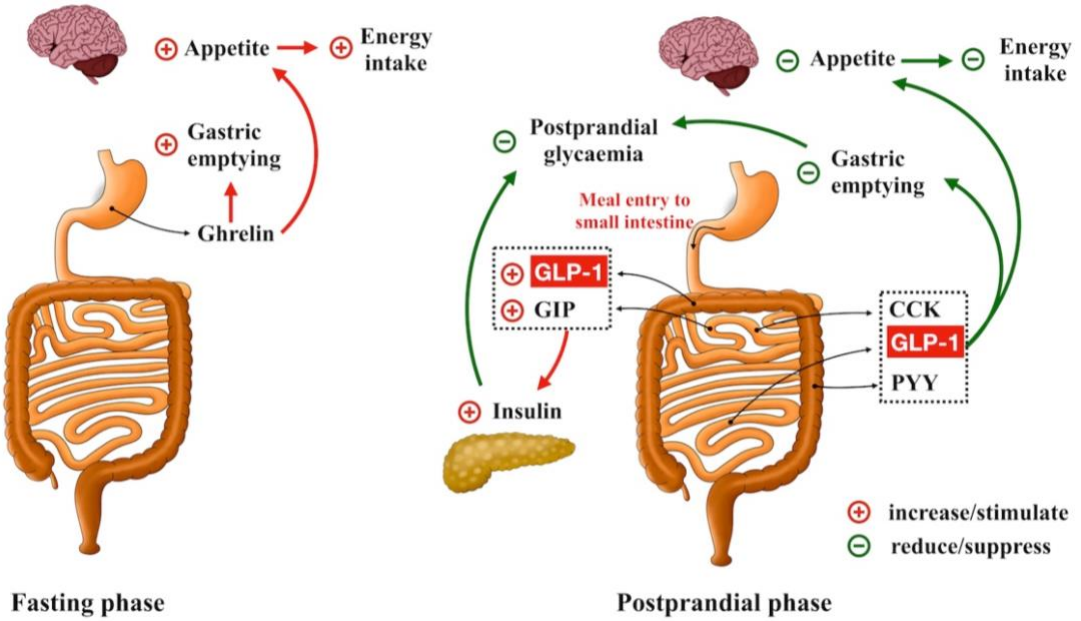


**Figure 2.1: Role of gastrointestinal hormones in the regulation of gastric emptying, postprandial glycaemia and energy intake.** Ghrelin is secreted during fasting and acts to accelerate gastric emptying, promote appetite and drive energy intake. Glucagon-like pepetide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), cholecystokinin (CCK) and peptide YY (PYY) are released in the postprandial phase. GLP-1 and GIP are the 'incretin' hormones, stimulating insulin secretion in a glucose-dependent manner. GLP-1, CCK and PYY also form intestinal feedback to slow gastric emptying and suppress energy intake.

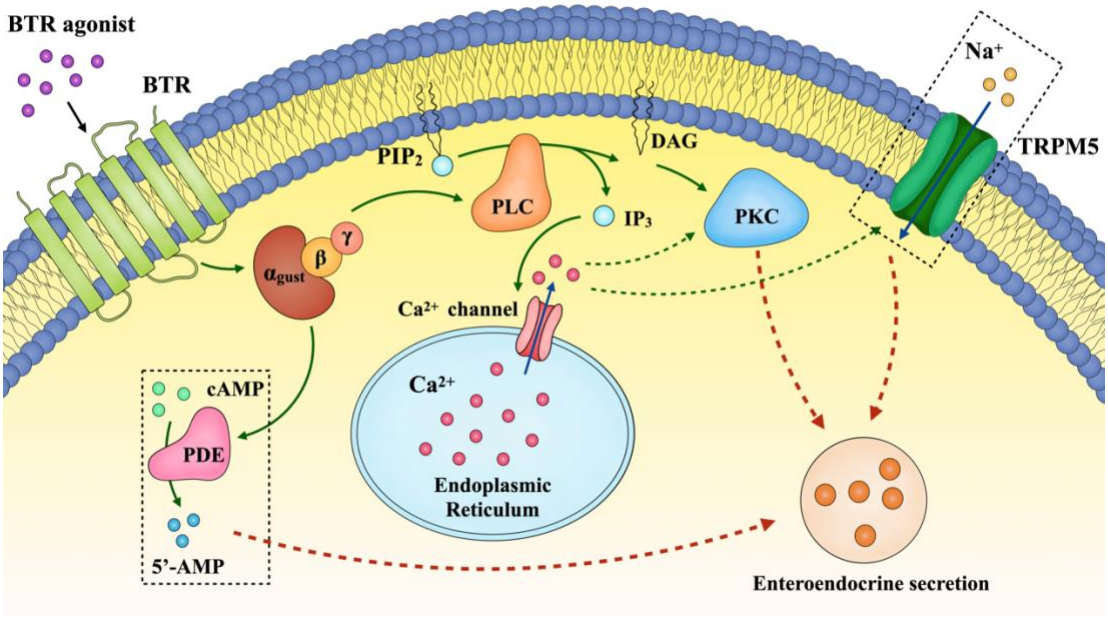


**Figure 2.2. Proposed mechanisms underlying enteroendocrine secretion in response to BTR agonists.** Binding of ligands to bitter taste receptors (BTRs) triggers a signalling cascade involving the dissociation of the G-protein gustducin into Gα and Gβγ subunits, activation of phospholipase C $\beta_2$ (PLC$\beta_2$), production of diacylglycerol (DAG) and inositol 1,4,5-trisphophate ($IP_3$), and opening of the transient receptor potential ion channel M5 (TRPM5), thereby leading to the release of intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$), $Na^+$ influx, cellular depolarization and the secretion of neurotransmitters. DAG and $[Ca^{2+}]_i$ also activate the protein kinase C (PKC) pathway. In addition, increases in intracellular Gα subunit activate phosphodiesterase.

# CHAPTER 3: ROLE OF BILE ACIDS IN THE REGULATION OF FOOD INTAKE, AND THEIR DYSREGULATION IN METABOLIC DISEASE

## STATEMENT OF AUTHORSHIP

| | |
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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 | September 2021 |

## 3.1 Abstract

Bile acids are cholesterol-derived metabolites with a well-established role in the digestion and absorption of dietary fat. More recently, the discovery of bile acids as natural ligands for the nuclear farnesoid X receptor (FXR) and membrane Takeda G-protein coupled receptor 5 (TGR5), and the recognition of effects of FXR and TGR5 signalling, have led to a paradigm shift in knowledge regarding bile acid physiology and metabolic health. Bile acids are now recognised as signalling molecules that orchestrate blood glucose, lipid and energy metabolism. Changes in FXR and/or TGR5 signalling modulates the secretion of gastrointestinal (GI) hormones, including glucagon-like peptide-1 and peptide YY, hepatic gluconeogenesis, glycogen synthesis, energy expenditure, and the composition of the gut microbiome. These effects may contribute to the metabolic benefits of bile acid sequestrants, metformin and bariatric surgery. This review focuses on the role of bile acids in energy intake and body weight, particularly their effects on GI hormone secretion, the changes in obesity and T2D, and their potential relevance to the management of metabolic disorders.

## 3.2 Introduction

Bile acids are synthesised in the liver, where cholesterol is converted via 7α-hydroxylase (CYP7A1) and, to a lesser extent, 27α-hydroxylase (CYP27A1) and 24-hydroxylase (CYP46A1), to the primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) in humans (CA and muricholic acid in rodents). These are then conjugated to glycine or taurine, prior to their secretion into bile (Russell, 2003). Following meal ingestion, bile acids are released into the gut upon gallbladder emptying, and about 95% of intestinal bile acids is absorbed in the ileum via the apical sodium bile acid co-transporter (ASBT), returning to the liver for re-secretion – a highly efficient process known as "enterohepatic circulation". A small fraction of bile acids reach the large intestine, where they are modified (through de-conjugation and dihydroxylation) by gut bacteria to secondary bile acids, such as deoxycholic acid (DCA), lithocholic acid (LCA) and ursodeoxycholic acid (UDCA, a secondary bile acid in humans, but a primary bile acid in rodents), and absorbed passively into the circulation or excreted in the faces (Thomas, Pellicciari, Pruzanski, Auwerx, & Schoonjans, 2008) (**Figure 3.1**). Bile acids lost to the large intestine are replenished by *de novo* hepatic synthesis, which is regulated by fibroblast growth factor-19 (FGF19) signalling in the small intestine in humans (or FGF15 in rodents). Thus, bile acids are found in high concentrations in the liver (Setchell et al., 1997), bile (Shiffman, Sugerman, Kellum, & Moore, 1992) and small intestine (Northfield & McColl, 1973).

For more than a century, bile acids have been regarded solely as "intestinal detergents" which emulsify dietary fat for digestion and transport. The recognition that bile acids are also pivotal signalling molecules orchestrating glucose, lipid and energy metabolism is recent. Bile acids also bind to numerous nuclear and cytoplasmic receptors, such as the vitamin D receptor (Makishima et al., 2002), pregnane X receptor (Ihunnah, Jiang, & Xie, 2011) and constitutive androstane receptor (Wagner et al., 2005). However, it was the identification of the bile acid-specific nuclear farnesoid X receptor (FXR) in 1999 and membrane Takeda G-protein-coupled receptor 5 (TGR5) in 2002 that provided a mechanistic framework for a role of BA signalling in the context of metabolism (Makishima et al., 1999; Maruyama et al., 2002). FXR and TGR5 are present in numerous tissues, including the central and peripheral nervous systems; bile acid signalling in the latter has been shown to regulate energy intake (Mertens, Kalsbeek, Soeters, & Eggink, 2017), as supported by the observation that suppression of energy intake induced by intravenous injection of DCA is attenuated when TGR5 was silenced in the vagal nodose ganglia in rats (X. Wu et al., 2020). However, the clinical relevance of this concept is unclear, particularly given that plasma bile acid concentrations are low and that in obese individuals relative elevation in plasma bile acid levels are not associated with reduced energy intake. In line with the high turnover of bile acids in the enterohepatic circulation, both FXR and TGR5 are expressed abundantly in the liver and the intestine. Signalling through both receptors has been linked to the secretion of gastrointestinal (GI) hormones, known to be integral to maintenance of metabolic homeostasis (**Figure 3.1**). For example, the release of ghrelin from gastric G-cells

during fasting appears pivotal to sensations of hunger, and stimulation of energy intake. After meals, the secretion of cholecystokinin (CCK) from enteroendocrine I-cells located in the upper gut, and glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) from L-cells located most abundantly in the distal gut, form an integrated signalling system that slows GI motility and transit, drives the secretion of insulin to regulate postprandial glucose metabolism (via GLP-1) and suppresses appetite and energy intake (Xie, Jones, Rayner, & Wu, 2020). The role of bile acids in the control of blood glucose and lipid metabolism has been reviewed in detail (T. R. Ahmad & Haeusler, 2019; Fiorucci, Mencarelli, Palladino, & Cipriani, 2009; Kuipers, Bloks, & Groen, 2014; Lefebvre, Cariou, Lien, Kuipers, & Staels, 2009), but their potential to impact on the regulation of energy intake has received less attention, despite the recognition, since 1968, that oral administration of CDCA and DCA stimulated PYY secretion and suppressed appetite in obese individuals (Bray & Gallagher, 1968). The current review addresses the effects of bile acids on GI hormone secretion, energy intake and body weight, as well as the relevance of bile acid dysregulation in obesity and type 2 diabetes (T2D).

## 3.3 Effects of bile acids on GI hormone secretion

The last two decades have witnessed a substantial effort to increase the understanding of the effects of bile acids on GI hormone secretion and the consequent impact on metabolism. In healthy individuals, postprandial plasma bile acid concentrations have been reported to correlate negatively with ghrelin, and positively with GLP-1 and PYY (Roberts et al., 2011). Similar relationships have also been observed in obese

subjects following bariatric surgery (Nakatani et al., 2009). However, bile acids per se do not appear to affect ghrelin secretion in rats; intestinal infusion of a mixture of physiological bile acids did not affect portal ghrelin levels (Kuhre et al., 2018). In contrast, small intestinal sensing of bile acids has been reported to inhibit CCK secretion in both rodents and humans (Gomez et al., 1988; Koop et al., 1996), supporting the existence of a negative feedback loop between the two. In contrast, the effects on GLP-1 and PYY release from L-cells have been studied extensively in preclinical and clinical models (Guida & Ramracheya, 2020; Sonne, Hansen, & Knop, 2014; T. Wu, M. J. Bound, S. D. Standfield, K. L. Jones, et al., 2013), stimulating the potential development of bile acid-based interventions for metabolic disorders. While bile acid-induced release of GLP-1 and PYY has been linked to signalling via FXR and TGR5, the data are inconsistent, which may relate to differences in the binding affinity of individual bile acids at FXR and TGR5 (**Table 3.1**) and/or complex interactions between the two signalling pathways.

### *3.3.1 FXR*

FXR is expressed abundantly in the liver and the intestine, and the binding affinity of individual bile acids is variable (CDCA > DCA > LCA > CA > UDCA, **Table 3.1**). FXR was initially identified as a regulator of bile acid metabolism (Lefebvre et al., 2009), and subsequently as a modulator of L-cell secretion. Indeed, FXR is expressed by the murine L-cell line, GLUTag. However, the FXR agonist GW4064 and CDCA (which preferentially binds FXR) were shown to suppress glucose-induced proglucagon expression and GLP-1 secretion in this cell line by decreasing glycolysis,

whereas silencing FXR abolished these effects (Trabelsi et al., 2015). These observations have been replicated in studies with different L-cell lines (i.e. NCI-H736 (X. Zheng et al., 2021) and STC-1 (P. Li et al., 2019)). In a similar manner, GW4064 blunted the GLP-1 response to short-chain fatty acids (SCFA) in both GLUTag and NCI-H716 cell lines (Ducastel et al., 2020). Consistent with these observations, FXR-deficient mice exhibit increased GLP-1 secretion in response to both dietary fibre, which increases colonic SCFA (Ducastel et al., 2020), and oral glucose (Xie et al., 2017). Oral intake of GW4064 (10mg/kg, 2 doses over 12 hours) also decreased active GLP-1 levels in the plasma of mini-pigs (X. Zheng et al., 2021). However, in an isolated perfusion model of rat intestine, both luminal and vascular perfusion of GW4064 failed to affect the GLP-1 response to a physiological mixture of bile acids in rats (Kuhre et al., 2018). In mice, diversion of bile acids from the gallbladder to the ileum was shown to modestly increase GLP-1 secretion, improve glucose tolerance and induce weight loss (Albaugh et al., 2019). The reductions in postprandial blood glucose and body weight induced by this procedure were abolished in intestinal FXR-knockout mice, suggesting that intestinal FXR-signalling can potentially promote GLP-1 secretion. Unfortunately, the study failed to determine whether the rise in GLP-1 was specifically induced by FXR-activation (Albaugh et al., 2019). Of note, oral administration of the intestine-restricted FXR agonist, fexaramine, in mice was reported to increase the abundance of LCA-producing gut bacteria to activate TGR5-signalling indirectly, leading to enhanced GLP-1 secretion and improvement in insulin sensitivity and lipid profile as well as the promotion of adipose tissue browning (Pathak et al., 2018). Accordingly, outcomes derived from ex vivo and in vivo

experiments are, by and large, inconsistent, although the intestine-restricted FXR signalling appears to have an overall favourable effect on metabolic health.

### *3.3.2 TGR5*

TGR5, also known as GPBAR1, is a G-protein coupled receptor that is expressed widely in the GI tract, pancreas, liver, gallbladder and adipose tissue. Like FXR, its binding affinity for individual bile acids varies substantially (LCA > DCA > CDCA > CA > UDCA, **Table 3.1**) (Kawamata et al., 2003). TGR5 activation has been reported to suppress hepatic macrophages, induce gallbladder relaxation and refilling, and promote intestinal motility (Lefebvre et al., 2009). TGR5 is also expressed on L-cells. Unlike FXR, stimulation of TGR5 by LCA and DCA was shown to potently stimulate GLP-1 secretion from STC-1 cells in a dose-dependent manner, an effect suppressed by downregulation of TGR5 expression (Katsuma et al., 2005). The stimulatory effect of TGR5 on GLP-1 secretion required the closure of ATP-sensitive potassium (KATP) channels and elevated intracellular concentrations of cAMP and Ca2+ (H. E. Parker et al., 2012; Thomas et al., 2009). A major observation in relation to TGR5 signalling was the demonstration of its basolateral location on L-cells. Thus, to activate TGR5, it is necessary for bile acids or other TGR5 ligands to be transported through the epithelial layer (Brighton et al., 2015). However, the readily absorbed TGR5 agonist SB-756050 failed to stimulate GLP-1 secretion significantly, or improve glycaemic control at various doses compared with placebo in acute studies involving subjects with T2D (Hodge et al., 2013). It is noteworthy that L-cells are distributed most densely in the distal gut regions (Xie, Jones, et al., 2020). It would

therefore be of interest to investigate whether delivery of TGR5 agonists should be targeted at the distal gut.

PYY is co-released with GLP-1 from L-cells, and it was initially noted that perfusion of DCA (1-25 mM) into the isolated rabbit colon increased PYY secretion substantially in a dose-dependent manner (Bray & Gallagher, 1968). Intracolonic administration of DCA or TCA in humans has also been shown to induce a rapid and substantial rise in plasma PYY (T. Adrian et al., 1993; T. E. Adrian et al., 2012; T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013). Similar to TGR5-mediated GLP-1 secretion, the outcomes of studies using isolated rat colon indicate that bile acid-induced PYY secretion is dependent on bile acid translocation from the luminal to basolateral side (Tough et al., 2020). That PYY secretion is less evident in response to bile acids with poor affinity to TGR5, and attenuated in TGR5-knockout models, attests to the fundamental relevance of TGR5-signalling to bile acid-induced PYY secretion (Christiansen et al., 2019).

In summary, there is compelling evidence for a role of bile acids in the modulation of GLP-1 and PYY secretion in both animals and humans. Stimulation of TGR5 on L-cells induces the secretion of both hormones, while effects of FXR signalling remain controversial. The interactions between FXR and TGR5 signalling remain poorly characterised and an improved understanding may be of relevance to the development of novel strategies for the management of metabolic disorders.

## 3.4 Effects of bile acid signalling on energy intake and body weight

In view of the effects of bile acids on appetite regulation, particularly via the secretion of GI hormones, it is intuitively likely that modulating bile acid signalling affects energy balance. Genetic ablation of the bile acid synthesis enzyme CYP8B1 leading to a deficiency of 12α-hydroxylated bile acids (e.g. CA) has been shown to be associated with reduced energy intake and subsequent weight gain in mice fed a fat enriched diet (Bertaggia et al., 2017; Higuchi et al., 2020). However, these effects appeared to be secondary to impaired fat hydrolysis and the increased exposure of unabsorbed fat to the distal gut, as in these mice there was an increase in energy intake when fed a fat-free diet (Higuchi et al., 2020). Nevertheless, this study supports the fundamental role of endogenous bile acids in fat digestion and absorption, which may influence energy intake and body weight indirectly.

The outcomes of preclinical and clinical studies, involving administration of various bile acids, have been equivocal in relation to effects on energy intake and body weight (**Table 3.2**). For example, supplementation with CA or UDCA prevented weight gain in mice fed a high-fat diet (Watanabe et al., 2006; Wei et al., 2020; Zietak & Kozak, 2016), possibly reflecting a TGR5-related increase in energy expenditure (Watanabe et al., 2006). Moreover, a number of other bile acid species with high affinity for TGR5, including hyocholic acid (HCA), hyodeoxycholic acid (HDCA), DCA and TCA, failed to affect energy intake or body weight in rodents with or without diabetes (Cheng, Liu, Zhang, Bi, & Hu, 2018; Zaborska, Lee, Garribay, Cha, & Cummings, 2018; X. Zheng et al., 2021). Information relating to the effects of bile acids on

appetite and energy intake in humans are limited. In healthy individuals, rectal administration of TCA substantially stimulated GLP-1 and PYY secretion and suppressed hunger in a dose-dependent manner (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013). Similarly, in obese individuals with T2D, rectally administered TCA significantly suppressed energy intake dose-dependently (T. E. Adrian et al., 2012). However, these observations could be confounded by the concurrent urge for defecation induced by rectal TCA perfusion (**Figure 3.2**) (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013). More recently, a double-blind, randomised, placebo-controlled 4-week trial that delivered a mixture of encapsulated bile acids (1000mg/day) designed for release in the ileum and colon (to provide dual agonism of FXR and TGR5) showed little effect on body weight in subjects with T2D, despite increases in plasma GLP-1 and serum and intestinal bile acids (Calderon et al., 2020).

As discussed, physiological bile acids often activate both FXR and TGR5, but with preferential affinity depending on their molecular structure. Selective FXR- and TGR5-knockout mice, or specific FXR and TGR agonists, have been pivotal to delineation of the respective signalling pathways to the metabolic effects of bile acids. However, outcomes remain inconclusive. Administration of the intestinal FXR agonist, fexaramine, for 5 weeks to mice fed a high-fat-diet was reported to prevent weight gain. However, this may have reflected an increase in metabolic rate, rather than a reduction in energy intake (Fang et al., 2015). In contrast, GW4064 had no effect on either energy intake or body weight in diabetic or obese mice (Watanabe et

al., 2006; Y. Zhang et al., 2006). Notably, mice with FXR deficiency (either whole body or intestine-specific knockout) fed a high-fat diet also exhibited reductions in energy intake and body weight compared with wild-type mice (K. Li et al., 2020; Xie et al., 2017). Similarly, TGR5 agonism (e.g. by INT-777) was associated with reduced weight gain, apparently by augmenting energy expenditure, without affecting energy intake (Thomas et al., 2009), whereas knockout of TGR5 had no significant effect on body weight or energy intake in mice fed a high-fat diet (McGavigan et al., 2017; Thomas et al., 2009). Clinical outcomes relating to TGR5 or FXR agonism have been disappointing. As discussed, the TGR5 agonist, SB-756050, failed to stimulate GLP-1 secretion or improve glycaemic control in individuals with T2D (Hodge et al., 2013). The effects of TGR5 agonists on energy intake and body weight in humans have not been reported. Treatment with the semi-synthetic FXR agonist, obeticholic acid, over 72 weeks only achieved a modest reduction in body weight (~2 kg) in subjects with non-alcoholic fatty liver disease (NAFLD), with or without, T2D (Neuschwander-Tetri et al., 2015). In another 24-week double-blind, randomised, placebo-controlled trial, the non-bile acid FXR agonist, cilofexor, had no effect on body weight in subjects with non-alcoholic steatohepatitis (Patel et al., 2020). Accordingly, the concept of supplementing bile acids or targeting BA signalling pathways to reduce energy intake and body weight is currently not supported by current clinical evidence.

## 3.5 Bile acid dysregulation in obesity and T2D

The emerging link between bile acid signalling and the regulation of metabolic homeostasis has stimulated substantial interest in potential phenotypical changes in bile acid profiles in metabolic disorders, particularly obesity and T2D. Although bile acids are present at high concentrations in the liver, bile and small intestine, bile acid profiles have hitherto been compared in peripheral blood and faecal samples predominantly due to their easy accessibility. Accordingly, processes in relation to small intestinal bile acid transport and absorption are poorly characterised, although gallbladder emptying can be readily assessed using ultrasound.

There is a substantial variation in circulating bile acid levels both between and within individuals (Steiner et al., 2011). In the context of obesity, most studies have reported that fasting serum/plasma bile acid levels are increased as a result of augmented bile acid synthesis (reflected by an increase in 7α-hydroxy-4-cholesten-3-one (C4)) (Cariou et al., 2011; Prinz et al., 2015; Straniero et al., 2017). There is evidence that the expression of both hepatic Na+-taurocholate co-transporting polypeptide (NTCP) (Haeusler et al., 2016) (responsible for the uptake of bile acids from the portal vein to the liver) and intestinal ASBT is lower in obese individuals (Renner et al., 2014), and that intestinal FGF-19 secretion is also decreased (Gomez-Ambrosi et al., 2017; Renner et al., 2014). It is, therefore, conceivable that the augmented hepatic bile acid secretion observed during fasting represents a compensatory response to deficiencies in the enterohepatic circulation. In support of this concept, the postprandial increase in circulating bile acids is significantly blunted in obesity (N. N. Ahmad, Pfalzer, &

Kaplan, 2013; Glicksman et al., 2010; Haeusler et al., 2016) and restored after Roux-en-Y gastric bypass (N. N. Ahmad et al., 2013). In addition, the production and faecal excretion of secondary bile acids (e.g. DCA) are increased in obese individuals (Aleman et al., 2018; Kudchodkar, Sodhi, Mason, & Borhani, 1977), which may be secondary, or contribute to, alterations in gut microbiota ("dysbiosis") (Gomes, Hoffmann, & Mota, 2018), leading to impaired energy metabolism in the host (Fiorucci & Distrutti, 2015). Obesity-related increases in fasting bile acid levels primarily reflect increases in 12α-hydroxylated bile acids (e.g. CA) (Cariou et al., 2011; Haeusler et al., 2016), which are more effective in emulsifying dietary fat than non-12α-hydroxylated bile acids (Higuchi et al., 2020). The shift in the bile acid composition in obesity may, therefore, favour improved fat digestion. Although fasting plasma unconjugated primary bile acids (CA and CDCA) and numerous conjugated primary and secondary bile acids (TCA, GCA, GCDCA, TDCA and GLCA) are related positively with insulin resistance in obesity (De Vuono et al., 2019; Legry et al., 2017), it remains to be determined whether changes in plasma bile acids represent a manifestation, or the drivers, of obesity.

T2D individuals, with or without obesity, exhibit higher fasting bile acid concentrations in the peripheral circulation compared with non-diabetic controls, mainly due to increased unconjugated and glycine-conjugated DCA and UDCA (Brufau et al., 2010; Cariou et al., 2011; Haeusler, Astiarraga, Camastra, Accili, & Ferrannini, 2013; Sonne et al., 2016; Wewalka, Patti, Barbato, Houten, & Goldfine, 2014). This rise in plasma secondary bile acids may reflect increased bile acid delivery

and a relative abundance of bile acid de-conjugating bacteria in the large intestine (Bennion & Grundy, 1977; H. Wu et al., 2020). Interestingly, the expression of ASBT has been reported to be increased in diabetic rats (Annaba et al., 2010), which would favour enhanced ileal bile acid resorption. However, this does not necessarily lead to increased FGF-19 secretion (Brufau et al., 2010; Sonne et al., 2016; Wewalka et al., 2014), or suppression of bile acid synthesis in T2D. Hepatic bile acid synthesis, particularly CA, is, in fact, known to be increased in subjects with T2D (Brufau et al., 2010). In a small group of individuals with T2D (n=15), the plasma bile acid responses to oral glucose or fat-containing mixed nutrients were reported to be modestly elevated (Sonne et al., 2016). Gallbladder emptying in this group of subjects was similar to healthy controls (Sonne, Rehfeld, Holst, Vilsboll, & Knop, 2014). However, in this study T2D subjects had relatively poor glycaemic control (mean HbA1c = 7.5%) and a long duration of diabetes (6-20 years), with the majority receiving medication (e.g. metformin (Sansome et al., 2020)) known to affect bile acid metabolism.

The magnitude of the increase in fasting bile acids in plasma or serum has been shown to correlate positively with fasting and 2h-postprandial glucose levels and HbA1c in T2D, and with the degree of insulin resistance in individuals, regardless of the presence of diabetes (Lee, Lee, Choi, Cho, & Kim, 2019; Wewalka et al., 2014). In a recently reported longitudinal study, 23 bile acid species were analysed to evaluate their baseline association with incident T2D during a median 3-year follow-up in a large cohort of individuals with normal glucose tolerance (J. Lu et al., 2021). Serum

fasting unconjugated primary and secondary bile acids (CA, CDCA and DCA) were reported to be negatively associated with the risk of T2D, while conjugated primary and secondary bile acids (GCA, TCA, GCDCA, TCDCA and TUDCA) were positively associated. Moreover, the ratios of conjugated to unconjugated bile acids (TCA/CA, GCA/CA, TCDCA/CDCA and GCDCA/CA) were positively associated with the development of T2D. These observations support the concept that impaired catalysis of conjugated bile acids by the hepatic bile acid-CoA:amino acid N-acyltransferase (BAAT) (Chiang, 2013) and/or intestinal resorption of unconjugated bile acids contribute to the development of T2D. The relevance of postprandial bile acid levels, particularly in the small intestine and liver, to the risk of T2D, however, remains unknown. Further studies are, therefore, required to clarify how bile acid metabolism changes with progression of glucose dysregulation.

## 3.6 Relevance of bile acids to therapies for metabolic disorders

As discussed, it remains to be clarified whether alterations in bile acids underpin the pathogenesis, or represent a consequence, of metabolic derangement. However, there is increasing persuasive evidence to support a role for bile acids in mediating the metabolic benefits of therapies used to treat metabolic disorders, including bile acid sequestrants, ASBT inhibitors, metformin and bariatric surgery.

### *3.6.1 Bile acid sequestrants*

Bile acid sequestrants are resins which bind to intestinal bile acids to disrupt their enterohepatic circulation and increase hepatic bile acid synthesis from cholesterol to

reduce intestinal secretion of FGF19 (or FGF15 in rodents) (Fuchs et al., 2018; Watanabe et al., 2012), elevate plasma C4 levels (Schadt et al., 2018) and augment expression of hepatic CYP7A1 (Fuchs et al., 2018; Herrema et al., 2010; Schadt et al., 2018). The increase in de novo bile acid synthesis is sufficient to maintain the size of the total bile acid, but often changes its composition (Beysen et al., 2012; Brufau et al., 2010). For example, in T2D subjects, treatment with colesevelam (3.75 g/day) over 8 weeks increased CA, but decreased CDCA and DCA (Brufau et al., 2010), shifting the bile acid pool towards a more hydrophilic phenotype. Because of their effects on the enterohepatic circulation, bile acid sequestrants were initially developed to treat hypercholesterolemia. Surprisingly, they were also shown to be associated with a substantial improvement in glycaemic control in subjects with T2D, leading to potential re-purposing for the management of T2D (Karhus, Bronden, Sonne, Vilsboll, & Knop, 2017), although the mechanism of their glucose-lowering action remains elusive. Several preclinical and clinical studies have reported a significant increase in GLP-1 secretion, associated with the use of bile acid sequestrants (Beysen et al., 2012 ; Fuchs et al., 2018; Shang, Saumoy, Holst, Salen, & Xu, 2010), although some studies have reported minimal (Hansen et al., 2016; Smushkin et al., 2013), or the opposite effect (Bronden et al., 2018; Hansen et al., 2016). Similarly, evidence for effects of bile acid sequestrants on energy intake and energy expenditure is also inconsistent. In high-fat fed C57BL/6J mice, the bile acid sequestrant, colestimide, was reported to increase energy expenditure in brown adipose tissue and prevent diet-induced obesity, without affecting energy intake or lipid absorption (Watanabe et al., 2012). In a similar study of hyperlipidaemic transgenic mice, colestilan was reported

to reduce body weight, accompanied by an increase in energy intake, a reduction in total energy expenditure, and enhanced carbohydrate catabolism (Sugimoto-Kawabata et al., 2013). In clinical trials of healthy individuals and subjects with obesity and/or T2D, bile acid sequestrants have been found to be weight-neutral (Bays, Goldberg, Truitt, & Jones, 2008; Beysen et al., 2012; Fonseca, Rosenstock, Wang, Truitt, & Jones, 2008; Goldberg, Fonseca, Truitt, & Jones, 2008). While further studies are required to clarify the glucose-lowering mechanisms of bile acid sequestrants, the latter do not appear to be an effective treatment for obesity.

### *3.6.2 ASBT inhibitors*

Similar to bile acid sequestrants, ASBT inhibitors impair intestinal bile acid resorption, leading to increased delivery of bile acids to the large intestine and decreased bile acid concentrations in the circulation (Graffner, Gillberg, Rikner, & Marschall, 2016; Kitayama et al., 2006; Rao et al., 2016; Y. Wu et al., 2013). These agents were first developed to treat hypercholesterolemia, but were subsequently applied to the management of functional constipation and non-alcoholic steatohepatitis (Al-Dury & Marschall, 2018). While inhibition of ASBT remarkedly increases GLP-1 secretion in both rodents (L. Chen et al., 2012) and humans (Rudling et al., 2015), ASBT inhibitors have not affected energy intake or body weight in animals (Kitayama et al., 2006; Rao et al., 2016). Their effect on energy intake in humans has not been reported.

### *3.6.3 Metformin*

Metformin remains the first-line therapy for glucose-lowering in T2D (Sansome et al., 2020), but also suppresses appetite and reduces body weight modestly (Adeyemo et al., 2015; Coll et al., 2020; Day et al., 2019; H. Wu et al., 2017). The potential for metformin to increase plasma GLP-1 and PYY levels has been widely recognised in both preclinical and clinical studies (Bahne et al., 2018; Borg, Bound, et al., 2019; E. W. Sun et al., 2019; T. Wu, Thazhath, et al., 2014). There is evidence that the latter may be attributable, at least in part, to inhibition of intestinal bile acid resorption by metformin. Indeed, metformin substantially decreases serum FGF-19, and increases faecal bile acid excretion and serum C4 levels in T2D (L. Sun et al., 2018). In high-fat-fed mice, metformin was also shown to prevent weight gain, apparently by increasing energy expenditure through upregulation of the thermogenic gene (Ucp1) in white adipose tissue, without affecting energy intake (L. Sun et al., 2018). That the effect on body weight was abolished in mice with intestinal-specific FXR knockout supports an important role for intestinal FXR signalling in metformin-induced weight loss in mice (L. Sun et al., 2018). Moreover, metformin modifies the gut microbiota (Forslund et al., 2015); metformin therapy (1700 mg/day) over 4 months results in major shifts in over 50 bacterial strains, which may account for glucose-lowering in T2D (H. Wu et al., 2017). In mice weight loss induced by metformin may be attributable to a reduction in intestinal Bacteroides fragilis and resultant increases in GUDCA; the latter antagonizes FXR signalling to improve glucose metabolism and reduce body weight (L. Sun et al., 2018). In this context, delayed-released metformin

(of minimal intestinal absorption) may be desirable to maximize the interaction between metformin and the gut microbiota for the management of T2D.

### *3.6.4 Bariatric surgery*

Despite emerging pharmaceutical treatments, bariatric surgery remains the most effective intervention for obesity and T2D. Relative to adjustable gastric banding and sleeve gastrectomy, procedures that bypass segments of the small intestine (e.g. Roux-en-Y gastric bypass, duodenal-jejunal bypass and biliopancreatic diversion) are in general more effective (Fruhbeck, 2015; Rubino et al., 2010). While the underlying mechanisms remain incompletely understood, emerging evidence suggests that the expedited flow of bile acids to the distal gut may be important. Indeed, bile acid diversion from the duodenum to distal ileum increases GLP-1 (X. Zhang et al., 2016), decreases blood glucose (Albaugh et al., 2019; Flynn et al., 2015; X. Zhang et al., 2016) and reduces body weight substantially (Albaugh et al., 2019; Flynn et al., 2015) in rodents with diet-induced obesity. While the expression of bile acid receptors (i.e. TGR5 and FXR) in the distal gut is not affected by bariatric surgery (Flynn et al., 2015), increased delivery of bile acids into the large intestine may alter the composition of the gut microbiome after bariatric surgery (or vice versa) (Albaugh et al., 2019; Flynn et al., 2015; W. Wang et al., 2019), thereby influencing host energy metabolism (Nicholson et al., 2012). That FXR-knockout abolishes (Albaugh et al., 2019; K. Li et al., 2020), while TGR5 knock-out preserves (Albaugh et al., 2019), the weight loss effect of Roux-en-Y gastric bypass or ileal biliary diversion in high-fat-fed mice, suggests that FXR, but not TGR5, signalling is indispensable for weight loss

induced by the diversion of bile acids to the distal small intestine. However, the significance of FXR signalling in humans is questionable, since the administration of the FXR agonist, obeticholic acid, over 72 weeks, showed little effect on body weight in subjects with NAFLD (Neuschwander-Tetri et al., 2015).

## 3.7 Concluding comments

The recognition of bile acids as important signalling molecules that orchestrate metabolic homeostasis through specialised receptors (FXR and TGR5) has stimulated active research to determine their relevance to the pathogenesis of, and therapeutic potential for the management of, metabolic disorders. Recent studies, focusing on the enterohepatic circulation and bile acid sensing, are indicative of major shifts in plasma and faecal bile acid profiles in obesity and T2D, and of the potent effects of bile acids on GLP-1 and PYY secretion from enteroendocrine L-cells. Accordingly, assessment of the bile acid profile may be of relevance to predict the risk of obesity and T2D, while targeting bile acid signalling pathways may represent an attractive strategy for the prevention and management of these metabolic disorders. The efficacy of bile acids to stimulate gut hormone secretion is related to their affinity for TGR5 and FXR; activation of TGR5 (expressed on the basolateral side of the L-cells) mediates bile acid-induced GLP-1 and PYY secretion, whereas FXR signalling have been shown to suppress these actions, or modify TGR5 signalling indirectly, while studies of physiological bile acids or agonists of TGR5 and FXR have yielded inconsistent outcomes on blood glucose, energy intake and body weight changes in both animal and human studies. However, several interventions with proven benefits on metabolic

health are clearly associated with disrupted, or potentially accelerated, enterohepatic circulation. Studies are now warranted to determine whether there are causal links between the bile acid profile and metabolic outcomes and, if so, the underlying mechanisms. Finally, it would also be of interest to explore whether bile acids have additive or synergistic effects with other (dietary or pharmaceutical) interventions to promote weight loss and glycaemic control.

**Table 3.1.** Binding affinities of bile acids to human TGR5 and FXR

| Bile acid | TGR5 | | | FXR | | |
|---|---|---|---|---|---|---|
| | **Subjects** | **Indicator** | **$EC_{50}$** | **Subjects** | **Indicator** | **$EC_{50}$** |
| **Primary bile acids** | | | | | | |
| **CA** | CHO cells/ HEK293 | Intracellular cAMP | 7.72 μM (Kawamata et al., 2003)/ >10 μM (Maruyama et al., 2002) | CV-1 cells | Reporter gene activation | No effect (H. Wang, Chen, Hollister, Sowers, & Forman, 1999) |
| **CDCA** | CHO cells/ HEK293 | Intracellular cAMP | 4.43 μM (Kawamata et al., 2003)/ 4 μM (Maruyama et al., 2002) | HepG2 cells /CV-1 cells | Reporter gene activation | 10 μM (Makishima et al., 1999)/ 50 μM (H. Wang et al., 1999) |
| | CHO cells | Reporter gene activation | 6.71 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | 4.5 μM (Parks et al., 1999) |
| **Conjugated primary bile acids** | | | | | | |
| **TCA/ GCA** | CHO cells | Reporter gene activation | 4.95 μM/ 13.6 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | No effect (Parks et al., 1999) |
| **TCDCA/ GCDCA** | CHO cells | Reporter gene activation | 1.92 μM/ 3.88 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | 10 μM (Parks et al., 1999) |
| **HCA** | | | | Cell-free | TR-FRET FXR coactivator assay | 70.06 μM ($IC_{50}$) (X. Zheng et al., 2021) |

Table 3.1 Continues.

| Bile acid | TGR-5 | | | FXR | | |
|---|---|---|---|---|---|---|
| | **Subjects** | **Indicator** | **$EC_{50}$** | **Subjects** | **Indicator** | **$EC_{50}$** |
| **Secondary bile acids** | | | | | | |
| **DCA** | CHO cells | Intracellular cAMP | 1.01 µM (Kawamata et al., 2003) | HepG2 cells | Reporter gene activation | 100 µM (Makishima et al., 1999) |
| | HEK293 | Intracellular cAMP | 575 nM (Maruyama et al., 2002) | CV-1 cells | Reporter gene activation | 50 µM (H. Wang et al., 1999) |
| **LCA** | CHO cells | Intracellular cAMP | 0.53 µM (Kawamata et al., 2003) | CV-1 cells | Reporter gene activation | 50 µM (H. Wang et al., 1999) |
| | HEK293 | Intracellular cAMP | 35 nM (Maruyama et al., 2002) | Cell-free | Ligand-sensing assay | 25 µM (Makishima et al., 2002) |
| **UDCA** | CHO cells | Reporter gene activation/ Intracellular cAMP | 36.4 µM (Sato et al., 2008)/ No effect (Kawamata et al., 2003) | CV-1 cells | Reporter gene activation | No effect (H. Wang et al., 1999) |
| **HDCA** | CHO cells | Reporter gene activation | 31.6 µM (Sato et al., 2008) | Cell-free | TR-FRET FXR coactivator assay | 62.43 µM (X. Zheng et al., 2021) ($IC_{50}$) |

Table 3.1 Continues.

| Bile acid | TGR-5 | | | FXR | | |
|---|---|---|---|---|---|---|
| | **Subjects** | **Indicator** | **$EC_{50}$** | **Subjects** | **Indicator** | **$EC_{50}$** |
| **Conjugated secondary bile acids** | | | | | | |
| **TDCA/ GDCA TLCA/ GLCA** | CHO cells | Reporter gene activation | 0.79 μM/1.18 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | 500 μM (Parks et al., 1999) ($IC_{50}$) |
| | CHO cells | Reporter gene activation | 0.29 μM/0.54 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | 3.8 μM /4.7 μM (Parks et al., 1999) ($IC_{50}$) |
| **TUDCA/ GUDCA THDCA/ GHDCA** | CHO cells | Reporter gene activation | 30.0 μM/33.9 μM (Sato et al., 2008) | Cell-free | Ligand-sensing assay | No effect (Parks et al., 1999) |
| | CHO cells | Reporter gene activation | 24.2 μM /36.7 μM (Sato et al., 2008) | | | |

Note: EC50: the concentration for a half maximal effect; IC50: the concentration for a half maximal inhibitory effect; CHO: Chinese hamster ovary cells; HepG2 cells: Human hepatoma cell line; CV-1 cells: Monkey kidney fibroblast cells (CV-1 line); HEK293: human embryonic kidney cell line 293; TR-FRET FXR coactivator assay: commercial assay kit for screening ligand for FXR.

**Table 3.2.** Reported effects of bile acids on energy intake and body weight in preclinical and clinical models

| Bile acid | | Model | Dose | Method | Effect | Reference |
|---|---|---|---|---|---|---|
| **Conjugated bile acid** | | | | | | |
| **Primary** | **TCA** | HFD Sprague-Dawley rat + streptozotocin | 0.05% or 0.3% | High-fat diet fed for 12 weeks | Body weight −<br>Energy intake − | (Cheng et al., 2018) |
| | | T2D subjects | 0.66, 2, 6.66 or 20 mmol | Rectal administration | Energy intake ↓ (~47% at 20mmol) | (T. E. Adrian et al., 2012) |
| | **HCA** | db/db mice; HFD C57BL/6J mice +streptozotocin;C57BL/6J mice | 100 mg/kg/day | Oral gavage for 28 days | Body weight −<br>Energy intake − | (X. Zheng et al., 2021) |
| | **TUDCA** | db/db mice; HFD C57BL/6J mice +streptozotocin; C57BL/6J mice | 100 mg/kg/day | Oral gavage for 28 days | Body weight −<br>Energy intake − | (X. Zheng et al., 2021) |
| **Secondary** | **HDCA** | db/db mice; HFD C57BL/6J mice + streptozotocin; C57BL/6J mice | 100 mg/kg/day | Oral gavage for 28 days | Body weight −<br>Energy intake − | (X. Zheng et al., 2021) |
| **Unconjugated bile acid** | | | | | | |
| **Primary** | **CA** | C57BL/6J mice | 0.5% | High-fat diet fed for 47 days | Body weight ↓ (24%); Energy intake −<br>Energy expenditure ↑ (~50%) | (Watanabe et al., 2006) |
| | | | 0.5% | High-fat diet fed for 9 weeks | Body weight ↓ (6g, ~18%);<br>Energy intake ↑ (20%);<br>Energy expenditure ↑(29%) | (Zietak & Kozak, 2016) |
| | **UDCA** | | 0.5% | High-fat diet fed for 8 weeks | Body weight ↓ (15%) | (Wei et al., 2020) |
| **Secondary** | **DCA** | C57BL/6J mice | 0.1% | High-fat diet fed for 3 weeks | Body weight −; Energy intake − | (Zaborska et al., 2018) |

Note: Both UDCA & TUDCA are primary bile acids in rodents, but secondary bile acids in humans. Given the effects of TUDCA and UDCA on energy intake and body weight were shown in rodents, they are grouped into the primary bile acids in the table.

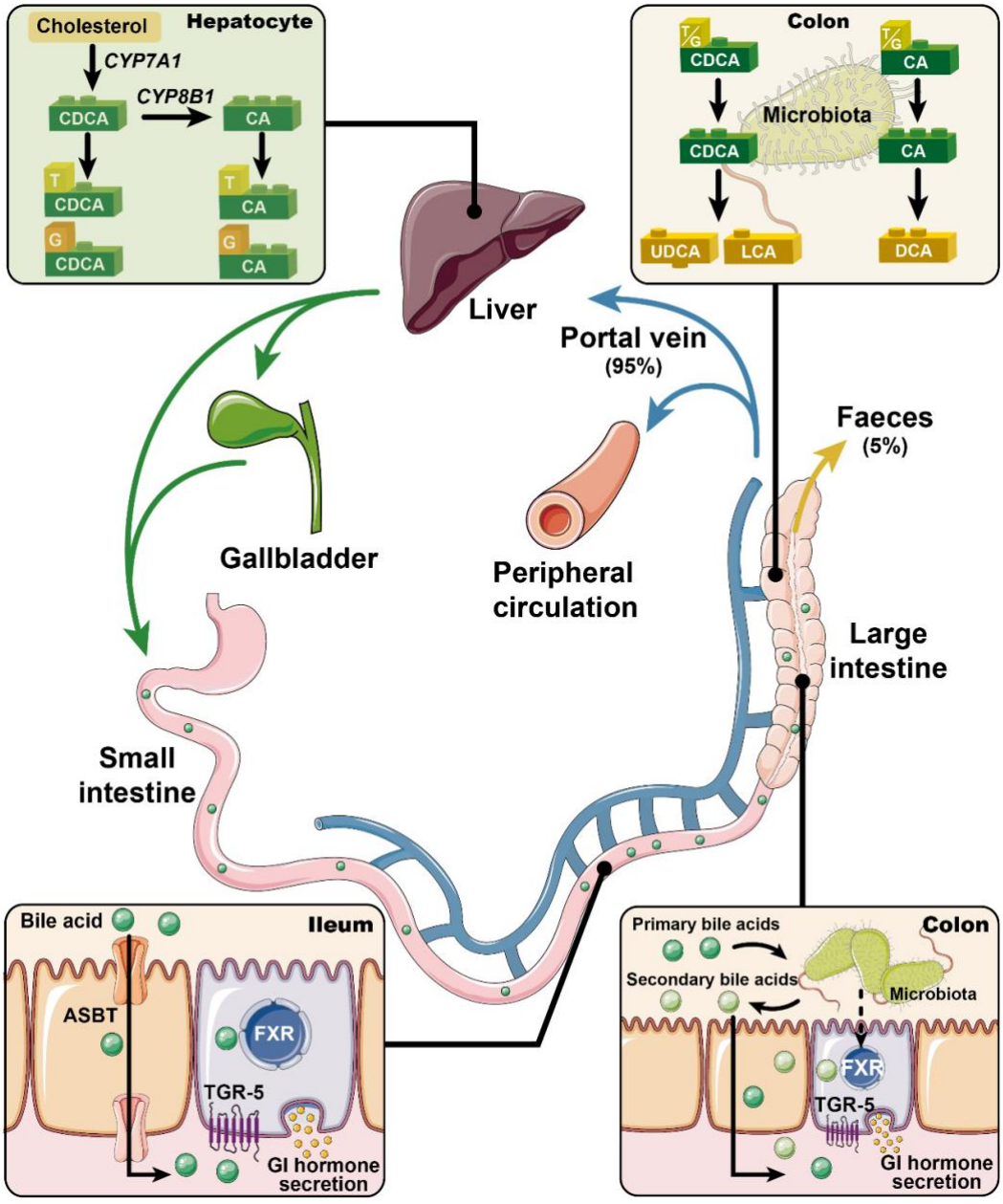


**Figure 3.1.** Primary bile acids (i.e. CDCA and CA) are synthesised from cholesterol in the liver, and conjugated to glycine and taurine prior to their secretion into bile. In response to meals, bile acids are discharged into the intestine. ~95% of the intestinal bile acids are absorbed in the ileum via apical sodium bile acid co-transporter (ASBT)

and return to the liver for re-secretion (i.e. the enterohepatic circulation). Only ~5% of bile acids escape into the large intestine and are modified by gut microbiota into secondary bile acids (e.g. DCA, LCA and UDCA). Bile acids are now recognised as pivotal signalling molecules that participate in the regulation of metabolic homeostasis through regulating the secretion of gastrointestinal hormones. This complex process has been linked to activation of the nuclear farnesoid X receptor (FXR) and/or the membrane Takeda G-protein-coupled receptor 5 (TGR5). Accordingly, modulation of FXR and/or TGR5 signalling has been actively pursued for the management of metabolic disorders.

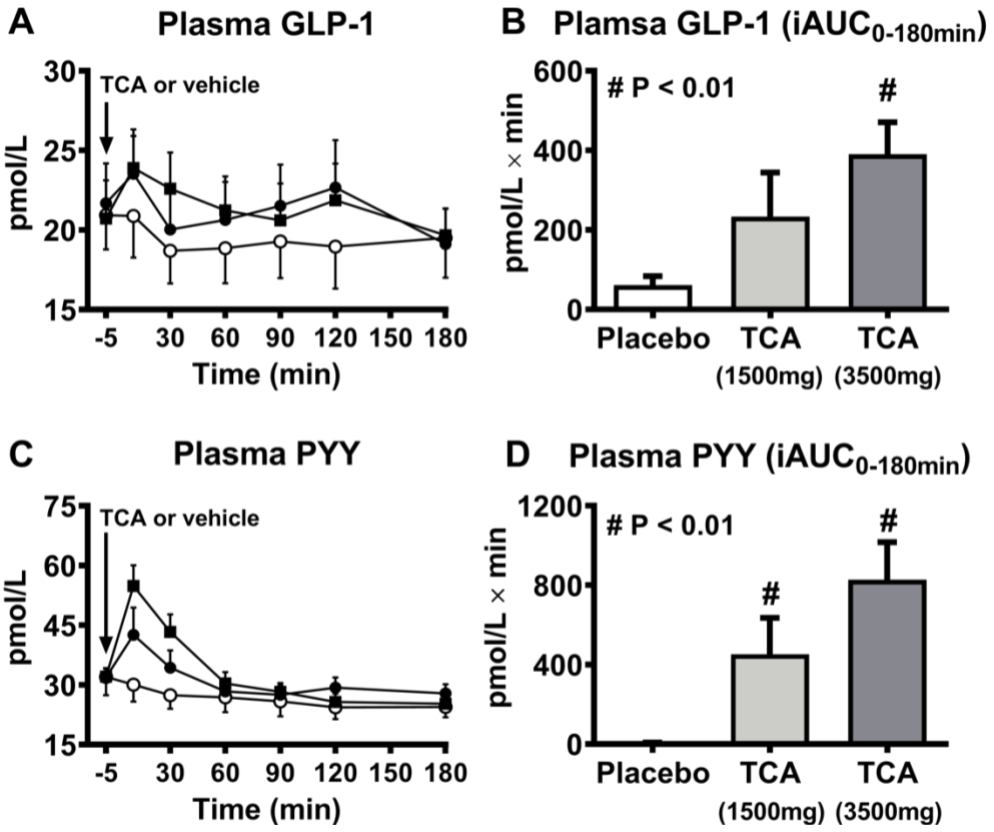


**Figure 3.2.** Plasma glucagon-like peptide-1 (GLP-1) (**A-B**), and peptide YY (PYY) (**C-D**) (mean values ± SEM.) after rectal taurocholic acid (TCA) enema in 10 healthy humans. (B) p = 0.019 for incremental area under the curves (iAUC); r = 0.48, p = 0.004 for dose-dependent effect; (D) p = 0.0005 for iAUC; r = 0.56, p = 0.001 for dose-dependent effect. Reproduced with permission from (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013) © (2013).

# KEY RESEARCH THEMES

As discussed in **Chapters 1-3**, there is increasing recognition that the gastrointestinal (GI) tract plays a central role in the regulation of metabolic homeostasis. Gastric emptying is a major determinant of the blood glucose response to dietary carbohydrate in both health and diabetes. The presence of nutrients and bioactive compounds in the small and large intestine can induce the secretion of GI hormones, including glucagon-like peptide-1 (GLP-1), which, in turn, slow gastric emptying, stimulate insulin, suppress glucagon, and modulate energy intake and metabolism. Modulation of endogenous GI hormone secretion may therefore represent a novel approach for the management of obesity and type 2 diabetes (T2D).

**Chapters 4 – 9** were set to address whether:

1. Gastric emptying of a semi-solid meal is slowed with aging but more rapid in community-based subjects with relatively well-controlled and uncomplicated T2D managed by diet or metformin alone;
2. Gastric emptying of an oral glucose drink is faster in both community-based T2D subjects with relatively well- (HbA1c $\leq$ 7.9%) or poorly- (HbA1c $\geq$ 9%) controlled T2D than non-diabetic controls;
3. Gastric emptying predicts the postprandial GLP-1 secretion in a given individual, and is related to the GLP-1 response to small intestinal nutrient infusion;
4. Augmentation of endogenous active GLP-1 by DPP-4 inhibition (vildagliptin) lowers plasma glucose and decreases energy expenditure during intraduodenal fat

infusion in subject with T2D, effects abolished by the GLP-1 receptor antagonist, exendin(9-39);

5. Stimulation of intestinal bitter taste receptors (BTRs) by administrating encapsulated denatonium benzoate (DB) slows gastric emptying, reduces postprandial glycaemia and supresses energy intake in subjects with T2D, in association with modulation of GI hormone secretion;
6. Rectal perfusion of the BTR agonist, DB, and physiological bitter bile acid, taurocholic acid, stimulates GLP-1 and peptide-YY secretion in healthy subjects and, if so, whether this effect is attenuated by the BTR antagonist, probenecid.

# CHAPTER 4: GASTRIC EMPTYING IN SUBJECTS WITH WELL-CONTROLLED TYPE 2 DIABETES COMPARED WITH YOUNG AND OLDER CONTROL SUBJECTS WITHOUT DIABETES

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| 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. LEW and I shared first authorship of this manuscript. | | |
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## 4.1 Abstract

**Background and aim:** Gastric emptying is a major determinant of postprandial glycaemia, and is often delayed in longstanding, complicated type 2 diabetes (T2D). There is, however, little information about gastric emptying in well-controlled T2D. Here, we evaluated the rate of gastric emptying in community-based subjects with relatively well-controlled T2D, compared to non-diabetic young and older controls.

**Methods:** 111 subjects with T2D managed by diet (n = 52) or metformin monotherapy (n = 59) (HbA1c 6.6 ± 0.1% / 49.0 ± 0.9 mmol/mol), 18 age- and BMI-matched older non-diabetic subjects, and 15 young healthy subjects consumed a standardised mashed potato meal (368.5 kcal) containing 100 μL $^{13}$C-octanoic acid. Gastric emptying (by breath test) and blood glucose were evaluated over 240min.

**Results:** Gastric emptying was slower in the non-diabetic older than young subjects (2.3 ± 0.1 vs. 3.0 ± 0.1 kcal/min, P = 0.0008). However, relative to the age- / BMI-matched non-diabetic subjects, gastric emptying (2.8 ± 0.1 kcal/min) was faster in T2D subjects (P = 0.0005). Furthermore, gastric emptying was faster in the metformin-treated (3.0 ± 0.1 kcal/min) than diet-controlled (2.7 ± 0.1 kcal/min) T2D subjects (P = 0.011), although there were no differences in age, BMI, HbA1c or the duration of known diabetes. The increments in blood glucose (at t = 30 and 60 min and the incremental area under the curve during t = 0-120 min) after the meal were related directly to the rate of gastric emptying in the T2D subjects, regardless of treatment with or without metformin (P < 0.05 each).

**Conclusions:** Gastric emptying is slowed with aging, but otherwise is relatively more rapid in subjects with well-controlled T2D. This provides a strong rationale for slowing gastric emptying to improve postprandial glycaemic control in these subjects.

## 4.2 Introduction

It is now appreciated that the rate of gastric emptying, which exhibits substantial inter-individual, but low intra-individual, variation in both health (Camilleri et al., 2012; Cremonini, Mullan, Camilleri, Burton, & Rank, 2002) and diabetes (M. Horowitz et al., 1989), is a major determinant of the glycaemic response to a glucose drink (M. Horowitz et al., 1989; Jones et al., 1995) or a solid meal containing carbohydrate (Linnebjerg et al., 2008). Interventions that slow gastric emptying, including "short-acting" glucagon-like peptide-1 (GLP-1) receptor agonists, pramlintide, acarbose, and nutrient preloads (Ma et al., 2015), have the capacity to attenuate postprandial glycaemic excursions in type 2 diabetes (T2D) (L. K. Phillips et al., 2015). Moreover, the reduction in postprandial glycaemia with short-acting GLP-1 receptor agonists is related to the magnitude of the slowing of gastric emptying, such that when baseline gastric emptying is relatively more rapid, the reduction of postprandial glycaemia is greater (Deane, Chapman, et al., 2010; Little, Pilichiewicz, et al., 2006). Accordingly, the baseline rate of gastric emptying is likely to be an important determinant of the response to therapy.

After meal ingestion, the stomach usually empties, after an initial lag phase for solid, at a relatively constant caloric rate of 1-4 kcal/min in health (L. K. Phillips et al., 2015). In people with T2D, gastric emptying is often disordered, although there have been marked differences in outcomes between studies which may relate, at least in part, to the characteristics of the cohort selected. Studies from tertiary referral centres involving subjects with longstanding (typically 8-12 years) T2D, poor glycaemic

control (HbA1c >8.5%), and a high prevalence of microvascular complications, indicate that 30-50% have abnormally delayed emptying of solids and/or nutrient liquids, whether studied by scintigraphy (Bharucha, Kudva, et al., 2015; M. Horowitz et al., 1989) or a stable isotope breath test (Matsumoto et al., 2007), and ~5% have rapid emptying. Conversely, subjects with "early" T2D (< 2 years) (W. T. Phillips, Schwartz, & McMahan, 1992; Schwartz, Green, Guan, McMahan, & Phillips, 1996) and/or an absence of autonomic neuropathy (Bertin et al., 2001; Frank et al., 1995) have been reported to have abnormally rapid emptying of solids and/or liquids, although this has not been observed in all series (Jones et al., 1995). A recent study of a small group of heterogeneous subjects with longstanding T2D, but relatively good glycaemic control (HbA1c ~7%), indicated that the rate of gastric emptying (measured by $^{13}$C octanoic acid breath test) did not differ from healthy controls (Boronikolos et al., 2015). In interpreting these observations, it is well established that acute hyperglycaemia – even at physiological postprandial levels – is associated with slowing of gastric emptying when compared with euglycaemia (Schvarcz et al., 1997), although the influence of chronic glycaemic control, as assessed by HbA1c, is less clear (Bharucha, Kudva, et al., 2015; Boronikolos et al., 2015). Other factors that potentially influence the rate of gastric emptying include obesity, which may be associated with accelerated emptying (Cardoso-Júnior et al., 2007; Gryback, Naslund, Hellstrom, Jacobsson, & Backman, 1996), advancing age, in which emptying is modestly slowed (M. Horowitz et al., 1984; Moore, Tweedy, Christian, & Datz, 1983), and anti-diabetic treatments, such as metformin, which was recently shown to slow

gastric emptying acutely in drug-native subjects with T2D, probably in part due to its stimulation of GLP-1 secretion (Borg, Bound, et al., 2019).

Accordingly, to assess the effects of T2D on gastric emptying, we sought to evaluate a cohort of community-based subjects with good glycaemic control (HbA1c ≤ 7.9%), managed by diet or metformin monotherapy, to reduce heterogeneity, and also in view of the fact that this group is most likely to benefit from interventions that specifically target postprandial glycaemia (Monnier et al., 2003). We compared subjects (with or without metformin) to both non-diabetic young and older subjects, with the latter selected to match T2D subjects in both age and BMI, to determine the impact of each of these variables on gastric emptying. While the gold standard technique to quantify gastric emptying is scintigraphy, this involves exposure to ionising radiation and requires specialised equipment and personnel to undertake the assessment. We, therefore, evaluated gastric emptying with a stable isotope breath test, which can be used in an office-based setting, and has been validated against scintigraphy in both health (Ghoos et al., 1993) and T2D (Ziegler et al., 1996). We derived the gastric half-emptying time (T50), corrected by the Wagner-Nelson method, which has been shown to be of comparable accuracy to scintigraphy in assessing gastric emptying of both liquid and solid meals (Sanaka, Nakada, Nosaka, & Kuyama, 2007; Sanaka, Yamamoto, Ishii, & Kuyama, 2004) and allows for calculation of the caloric rate of gastric emptying. Our hypotheses were that gastric emptying would be slowed with aging, but otherwise more rapid in community-based subjects with relatively well-controlled T2D.

## 4.3 Methods

### *4.3.1 Subjects*

Subjects with T2D were recruited from the community by advertisement for studies evaluating nutritional and/or pharmacological therapies for diabetes in our centre (L. Watson et al., 2018; T. Wu, M. J. Bound, B. Y. R. Zhao, et al., 2013; T. Wu, Little, et al., 2016). The T2D subjects had been diagnosed by American Diabetes Association (ADA) criteria, were managed by diet and/or metformin monotherapy (500-3000 mg/day, stable for >3 months), and had HbA1c $\geq$ 6.0% and $\leq$ 7.9% at the time of screening. Non-diabetic controls, including young volunteers of normal BMI and older volunteers with age and BMI in the range of the T2D subject group, were also recruited from the community by advertisement, and were excluded if they had evidence of T2D by fasting blood glucose or HbA1c. Both non-diabetic controls and T2D subjects were excluded if they reported significant gastrointestinal (GI) symptoms, a history of GI disease including known gastroparesis, bariatric surgery, or a requirement for medication known to affect GI function or appetite, and they were screened to exclude those with kidney or liver disease. Based on our previous work (O'Donovan et al., 2005), a sample size of 15 in each of the control group was calculated to have at least 80% power (at $\alpha = 0.05$) to detect ~ 18% difference in the gastric-half emptying time (T50) between the groups. The Royal Adelaide Hospital Human Research Ethics Committee approved the study, and all subjects provided written informed consent.

### *4.3.2 Protocol*

Gastric emptying of a standardised meal and postprandial glycaemia were evaluated on a single study visit. All subjects were asked to refrain from strenuous physical activity for 24 h before the study, and were provided with a standardised evening meal consisting of beef lasagne (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia) to be consumed with bread, a non-alcoholic beverage, and one piece of fruit at 1900h. Subjects were then instructed to abstain from all food and nutrient beverages, but were allowed to drink water until midnight, before attending the laboratory at 0800h. Metformin-treated T2D subjects were instructed to withhold any scheduled dose the evening before each visit, and to defer their morning dose until the end of the study. On arrival, an intravenous cannula was inserted into a forearm vein for repeated blood sampling. The semi-solid test meal, consisting of 65 g powdered potato (Deb; Unilever Australia) and 20g glucose, reconstituted with 200 mL water and one egg yolk containing 100uL $^{13}$C-octanoic acid (368.5 kcal: 61.4 g carbohydrate, 7.4 g protein and 8.9 g fat), was then consumed over five minutes (t = 0 – 5 min). Breath samples were collected immediately before, and every 5 minutes after meal ingestion in the first hour, and every 15 minutes for a further 3 hours. Venous blood was sampled immediately before the meal (at t = 0) and at t = 15, 30, 60, 90, 120, 180 and 240 min.

### *4.3.3 Measurement of gastric emptying*

$^{13}CO_2$ in each breath sample was measured by a non-dispersive infrared spectrometer (FANci2, Fischer ANalysen Instrumente, Germany). The gastric half-emptying time

(T50) was calculated using the Wagner-Nelson method, as described previously (Sanaka et al., 2007) , from which the rate of gastric emptying was derived and expressed in kcal/min.

### *4.3.4 Measurement of blood glucose concentrations and cardiovascular autonomic function*

Blood glucose concentrations were assessed using a glucometer (Optium Xceed, Abbott Laboratories, USA), and are reported as the mean of duplicate measurements at each time point. After completion of the gastric emptying measurement, autonomic nerve function was evaluated using standardised cardiovascular reflex tests (variation in heart rate during deep breathing, heart rate response to standing, and fall in systolic blood pressure in response to standing). Each test result was scored as 0 = normal, 1 = borderline, 2 = abnormal. A score $\geq$ 3 was considered to indicate autonomic dysfunction (Piha, 1991).

### *4.3.5 Assessment of upper GI symptoms*

A subset of T2D subjects (n = 71) and non-diabetic older subjects (n = 18) also completed a standardised questionnaire to assess upper GI symptoms (Quan et al., 2003). “Gastric” (anorexia, nausea, early satiation, abdominal bloating/fullness, vomiting, abdominal pain) and “oesophageal” symptoms (dysphagia, heartburn, acid regurgitation) were graded as 0 (none), 1 (mild; the symptom could be ignored), 2 (moderate; the symptom could not be ignored, but did not influence daily activities),

or 3 (severe; the symptom influenced daily activities) for a total possible score of 27 (Michael Horowitz et al., 1991).

### *4.3.6 Statistical analysis*

Demographic data, as well as the rate of gastric emptying, between the non-diabetic young and older subjects and between the non-diabetic older subjects and T2D subjects were compared using unpaired Student's t-tests after confirming their normality of distribution, except that the proportions of each gender and those with autonomic neuropathy in each group were compared using Fisher's exact test. Changes in blood glucose after the standardised test meal were evaluated by 2-way repeated measures analysis of variance (ANOVA) using group and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant interactions. Relationships between change in blood glucose from baseline, the incremental area under the curve (iAUC) for blood glucose, HbA1c and gastric emptying rate in the T2D group were evaluated using the Pearson correlation analysis. Analyses were performed using Prism 7.0 (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant. Data are expressed as mean values ± SEM.

## 4.4 Results

111 subjects with T2D managed by diet or metformin monotherapy, 15 young healthy subjects, and 18 older, non-diabetic subjects were studied (**Table 4.1**). In the non-diabetic older group, 14 subjects were found to have impaired glucose tolerance. All

tolerated the protocol well. The non-diabetic older controls matched the T2D subjects well for gender, age and BMI, but were older ($P < 0.01$) and had a higher BMI ($P < 0.05$) compared with the young controls. 6 of 111 subjects with T2D and 1 of 18 non-diabetic older subjects had mild autonomic dysfunction.

### *4.4.1 Comparisons between the non-diabetic young and older subjects*

Fasting blood glucose concentrations were slightly higher in the non-diabetic older than young subjects ($P < 0.001$) (**Table 4.1**). Following meal ingestion, blood glucose concentrations increased promptly, and peaked at ~30 min in the young, and at ~60 min in the older, group before returning to baseline. There were both a group effect ($P < 0.001$) and a group by time interaction on blood glucose concentrations ($P < 0.0001$), such that blood glucose concentrations were higher between t = 60-180 min in the non-diabetic older than young subjects ($P < 0.05$ each) (**Figure 4.1A**).

The rate of gastric emptying ranged from 2.2 – 3.9 kcal/min in the young subjects, and 1.6 – 3.3 kcal/min in the older subjects. The mean emptying rate was slower in the non-diabetic older (2.3 ± 0.1 kcal/min) than healthy young (3.0 ± 0.1 kcal/min) subjects ($P = 0.0008$) (**Figure 4.1B**).

### *4.4.2 Comparisons between T2D and age- and BMI-matched non-diabetic older subjects*

Both fasting and postprandial blood glucose concentrations were higher in subjects with T2D than age- and BMI-matched non-diabetic older subjects (two-way repeated

measures ANOVA: group effect $P < 0.0001$; group by time interaction $P < 0.0001$), with significant differences at all time points ($t$ = 0 – 240min, $P < 0.05$ for each) (**Figure 4.1A**).

The rate of gastric emptying ranged from 1.6 – 4.7 kcal/min in the whole cohort of T2D subjects, and the mean emptying rate (2.8 ± 0.1 kcal/min) was significantly faster than the non-diabetic older subjects ($P = 0.0005$) (**Figure 4.1B**).

The prevalence of upper GI symptoms was comparable in the subset of T2D subjects who completed the questionnaire (n = 71) and the non-diabetic older controls; 47 of 71 subjects in the T2D group reported upper GI symptoms (66%), and 12 of 18 in the control group (67%). The median total score for upper GI symptoms in T2D was 1 (0-8) and the main symptoms were acid regurgitation (32%), heartburn (27%) and fullness (27%). The median total score in the age-matched non-diabetic controls was also 1 (0-4).

### *4.4.3 Subgroup comparisons between T2D subjects with and without metformin*

There were no differences in gender, age, BMI, HbA1c or duration of known diabetes between T2D subjects treated with and without metformin. However, both fasting and postprandial blood glucose concentrations were lower in the subgroup of T2D subjects managed by diet alone (2-way repeated measures ANOVA: group effect $P = 0.007$; group by time interaction $P = 0.01$, with significant differences at t = 0, 15, 30, 120, 180 and 240 min ($P < 0.05$ for each)) (**Table 4.1; Figure 4.2A**).

The rate of gastric emptying ranged from 1.6 -3.9 kcal/min in the subgroup of T2D subjects without metformin therapy and from 2.0 – 4.7 kcal/min in the those treated with metformin monotherapy, with the mean rate of emptying being faster in the latter (3.0 ± 0.1 kcal/min for subjects on metformin vs. 2.7 ± 0.1 kcal/min for subjects managed by diet alone, P = 0.011). Gastric emptying in the diet-controlled T2D subjects remained faster than in the non-diabetic older controls (P = 0.009) (**Figure 4.2B**).

### *4.4.4 Relationships between gastric emptying, glycaemic markers and demographic data*

There was no significant relationship of the rate of gastric emptying with BMI across the three groups, or with HbA1c, in subjects with T2D.

In the two control groups, there was a tendency for a direct relationship between the increment in blood glucose at 30 min after the meal and the rate of gastric emptying (r = 0.31, P = 0.07) (data not shown). In T2D subjects, the increments in blood glucose at t = 30min (r = 0.49, P < 0.01) and 60min (r = 0.51, P < 0.01), as well as the iAUC for blood glucose between 0-120min (r = 0.34, P < 0.01), were directly related to the rate of gastric emptying (**Figure 4.3A-C**). These direct relationships between gastric emptying and the increments in postprandial glycaemia remained evident in the subgroup of T2D subjects treated with metformin (**Figure 4.3D-F**), and in subjects managed by diet alone (**Figure 4.3G-I**).

## 4.5 Discussion

In this cross-sectional study, we observed that gastric emptying was slower in older than young individuals without T2D (mean age about 65 vs. 23 years), but more rapid in relatively well controlled subjects with T2D when compared with age- and BMI-matched non-diabetic controls. In keeping with the concept that gastric emptying is a critical determinant of postprandial glycaemia, the increments in blood glucose after a standardised test meal were closely related to the rate of gastric emptying in this group of T2D subjects. These observations, therefore, add to the rationale for slowing of gastric emptying as a therapeutic strategy in the management of T2D, particularly in subjects who are relatively well controlled. However, in subjects with more advanced T2D treated by prandial insulin administration, gastric emptying and insulin dosing should probably be coordinated to improve postprandial glycaemia and minimise the risk of hypoglycaemia (L. K. Phillips et al., 2015).

The observed range of the rate of gastric emptying derived from the isotope breath test using the Wagner-Nelson method in the control groups was in line with the literature (Sanaka et al., 2004). Our observation that gastric emptying was slower in the non-diabetic older than young subjects is also consistent with previous studies using a scintigraphic technique (M. Horowitz et al., 1984; Moore et al., 1983), although the older subjects also had a higher BMI, which has been associated with somewhat accelerated gastric emptying in some (Cardoso-Júnior et al., 2007; Gryback et al., 1996), but not all (Seimon et al., 2013) studies. On the other hand, the majority of the non-diabetic older subjects had impaired fasting glucose, with significantly

higher fasting and postprandial blood glucose concentrations than the young subjects, which may at least in part contribute to the slowing of gastric emptying in this group (Schvarcz et al., 1997). The elevated blood glucose levels are likely to reflect greater insulin resistance relating to aging and the higher BMI in the non-diabetic older group (K. F. Petersen et al., 2003). In this context, the relationship between gastric emptying and postprandial glycaemia in the non-diabetic controls was weaker.

In contrast to some studies of subjects with T2D which indicate a high prevalence of delayed gastric emptying (Bharucha, Kudva, et al., 2015; M. Horowitz et al., 1989; Matsumoto et al., 2007), we observed that gastric emptying was more rapid in T2D subjects who were relatively well controlled by diet and/or metformin when compared with age- and BMI-matched non-diabetic controls. However, our subjects differed from those studied previously, in that they were well controlled (mean HbA1c ~6.6%), had a relatively short duration of known diabetes, and were managed on diet and/or metformin only. Although blood glucose concentrations were predictably higher in the T2D subjects than non-diabetic controls, this might be expected to contribute towards slowing of gastric emptying (Marathe et al., 2013). Our data are consistent with reports that gastric emptying of liquid (W. T. Phillips et al., 1992) or solid (Schwartz et al., 1996) high-carbohydrate meals is accelerated in “early” T2D, when studied using a scintigraphic technique. The consistency between our observations and those reported previously raises the question as to whether accelerated gastric emptying may, in fact, precede the development of T2D (W. T. Phillips et al., 1992; Schwartz et al., 1996). The lack of any subjects with abnormally slow gastric

emptying in our cohort is in agreement with the study of Boronikolos et al (Boronikolos et al., 2015), where a $^{13}$C-octanoic acid breath test was also used, and the subjects were also well-controlled (HbA1c ~7%), albeit with a longer duration of diabetes (10 years) and heterogenous profile of therapies for T2D. Finally, it should be noted that the T2D subjects studied in our study were essentially uncomplicated; few had evidence of autonomic neuropathy, and those with significant GI symptoms were excluded.

There is recent evidence that acute administration of metformin slows gastric emptying in subjects with T2D (Borg, Jones, et al., 2019). In the present study, gastric emptying was counter-intuitively faster in the subgroup of subjects treated with metformin monotherapy than those managed by diet alone, despite comparable gender, age, BMI, HbA1c and duration of T2D at the time of assessment in the two subgroups. However, it would be expected that the "baseline" characteristics between the two subgroups prior to any diabetes intervention, would differ substantially; both fasting and postprandial blood glucose concentrations were modestly higher in the subgroup of subjects receiving metformin. Because of the lack of baseline emptying data, it is not possible to draw conclusions from the present study about the chronic effects of metformin on gastric emptying in T2D, which warrant further investigation. Nevertheless, our observation that metformin-treated subjects often have more rapid gastric emptying suggests that selecting an agent that slows gastric emptying may be ideal if add-on therapy is needed.

Gastric emptying has a major impact on postprandial blood glucose concentrations, particularly the "early" glycaemic response (M. Horowitz et al., 1993; Jones et al., 1996). In the current study, we observed close relationships between the increments in postprandial glycaemia and gastric emptying in subjects with T2D. It has been established that, in T2D subjects who are not treated with insulin, postprandial blood glucose excursions are increased by interventions that accelerate gastric emptying, and diminished by those that delay it (Gonlachanvit et al., 2003), even when the slowing of emptying is quite modest (D. G. O'Donovan et al., 2004; Pilichiewicz, Chaikomin, et al., 2007). It is also evident that therapies that slow gastric emptying are effective in reducing postprandial glycaemic excursions in subjects who have normal or rapid emptying at baseline, but have minimal impact in those in whom emptying is already delayed (Linnebjerg et al., 2008). Our findings are, therefore, of high clinical relevance, because the subset of subjects with relatively good glycaemic control (HbA1c < 7.9%) is most likely to benefit from interventions that target postprandial glycaemia (Monnier et al., 2003; Riddle, Umpierrez, DiGenio, Zhou, & Rosenstock, 2011). Evaluation of gastric emptying at baseline, such as using a point-of-care $^{13}$C-octaonoic breath test, may potentially individualise therapy in T2D, although considering our data, it could also be argued that this is not essential in the well-controlled subgroup.

There is a poor correlation between upper GI symptoms and gastric emptying in diabetes (Jones et al., 1995), which was confirmed in our study, as there was no difference in gastric emptying between those with or without upper GI symptoms in

the T2D group. The prevalence of symptoms was, moreover, similar between the T2D and age- and BMI-matched non-diabetic controls. It should, however, be appreciated that subjects who had significant symptoms were excluded.

A strength of our study is that we recruited a quite homogenous group of essentially uncomplicated T2D subjects who were relatively well controlled by diet and/or metformin monotherapy, and evaluated appropriate control groups. While the lack of inclusion of subjects with poorly controlled diabetes, taking other anti-diabetic medications, or with high prevalence of complications might be perceived as a limitation, it was not our intention to study subjects in these categories, given that they are intuitively less likely to benefit from interventions that slow gastric emptying. Several other limitations should also be noted. First, the sample size of the control groups was relatively small. However, the subjects in each group were carefully controlled for potential confounding factors, and the sample size has provided adequate power to detect a difference in the rate of gastric emptying (i.e. primary endpoint). In this context, our observations were clear-cut, and increasing the sample size would be unlikely to alter our study conclusions substantially. Second, the majority of the age- and BMI-matched non-diabetic controls had impaired fasting glucose, such that it would be of interest to have included an additional older group without any glycaemic dysregulation. Third, blood glucose levels were determined using a portable glucometer, yet the correlations observed in the present study are robust. Furthermore, a high carbohydrate meal was used in the present study, which may not be typical for subjects with T2D, but is ideal to investigate the relationship

between gastric emptying and postprandial glycaemia. Finally, previous dietary habits, which may affect gastric emptying, were not evaluated in the current study.

In conclusion, gastric emptying is delayed modestly with aging, but otherwise more rapid in T2D subjects of relatively short duration and with good glycaemic control. These observations support the potential utility of interventions that slow gastric emptying to improve postprandial glycaemia and overall glycaemic control in this group.

**Table 4.1. Subject characteristics (mean values ± SEM).**

| | Subjects with T2D | | | Non-diabetic subjects | |
|---|---|---|---|---|---|
| | **Whole group** | **Metformin subgroup** | **Diet alone subgroup** | **Older** | **Young** |
| **Gender (male)** | 71/111 | 42/59 | 29/52 | 11/18 | 9/15 |
| **Age (years)** | 64.8 ± 0.6 | 63.8 ± 0.8 | 65.9 ± 0.8 | 65.4 ± 1.8 | 21.3 ± 0.7 $^{\#\#}$ |
| **BMI (kg/m$^2$)** | 29.7 ± 0.5 | 30.4 ± 0.6 | 29.0 ± 0.7 | 27.9 ± 1.1 | 23.7 ± 1.1 $^{\#}$ |
| **HbA1c (%)** | 6.6 ± 0.1 | 6.6 ± 0.1 | 6.6 ± 0.1 | 5.5 ± 0.1 $^{**}$ | - |
| **HbA1c (mmol/mol)** | 49.0 ± 0.6 | 49.0 ± 0.9 | 49.0 ± 0.9 | 36.1 ± 0.9 | - |
| **Duration of T2D (years)** | 5.5 ± 0.5 | 6.4 ± 0.8 | 4.9 ± 0.6 | - | - |
| **Fasting glucose** | 8.1 ± 0.1 | 8.4 ± 0.2 | 7.8 ± 0.2 $^{\phi}$ | 5.9 ± 0.1 $^{**}$ | 5.4 ± 0.1 $^{\#\#}$ |
| **Prevalence of autonomic neuropathy** | 6/111 | 3/59 | 3/52 | 1/18 | 0 |

Compared with overall T2D subjects, * $P < 0.05$; **, $P < 0.01$.

Compared with non-diabetic older subjects, $^{\#}$ $P < 0.05$; $^{\#\#}$, $P < 0.01$.

Compared with T2D subjects treated with metformin, $^{\phi}$ $P < 0.05$.

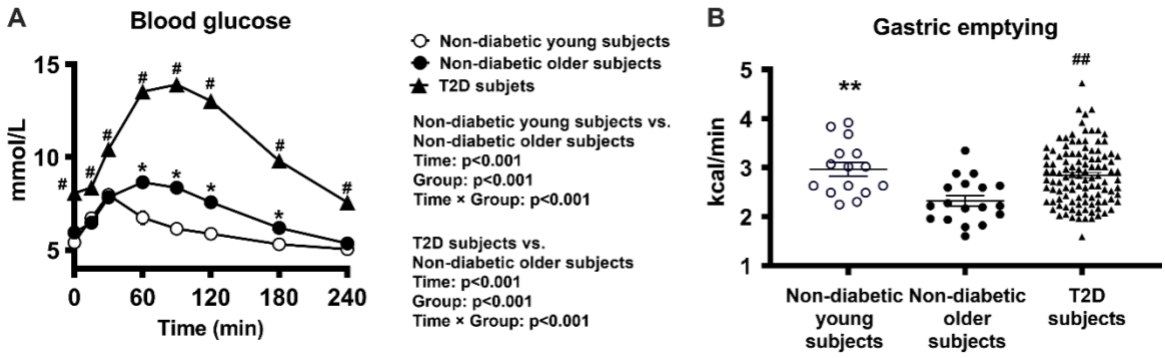


**Figure 4.1.** A: Blood glucose concentrations in response to a standardised carbohydrate meal (given at t = 0) in T2D subjects and non-diabetic young and older controls. Repeated-measures ANOVA was used to determine statistical difference. Results of ANOVA are reported as P values for differences by group, differences over time, and differences due to group by time interaction. Post hoc comparisons were adjusted by Bonferroni's correction. B: Gastric emptying rate in T2D subjects and non-diabetic young and older controls assessed by $^{13}$C-octanoic acid breath test. Unpaired t test was used to determine statistical difference. Data are mean ± SEM. * P<0.05, ** P<0.01 for non-diabetic young vs. older subjects; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, for T2D subjects vs. non-diabetic older subjects.

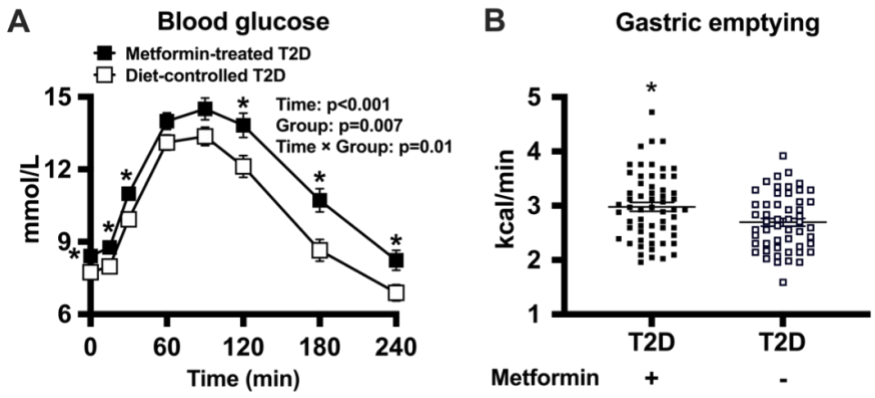


**Figure 4.2.** A: Blood glucose concentrations in response to a standardised carbohydrate meal (given at t = 0) in T2D subjects managed with or without metformin. Results of ANOVA are reported as P values for differences by group, differences over time, and differences due to group by time interaction. Post hoc comparisons were adjusted by Bonferroni's correction. B: Gastric emptying rate in T2D subjects and healthy older subjects as assessed by $^{13}$C-octanoic acid breath test. Data are mean ± SEM. * P<0.05 for T2D subjects with metformin vs. T2D subjects without metformin.

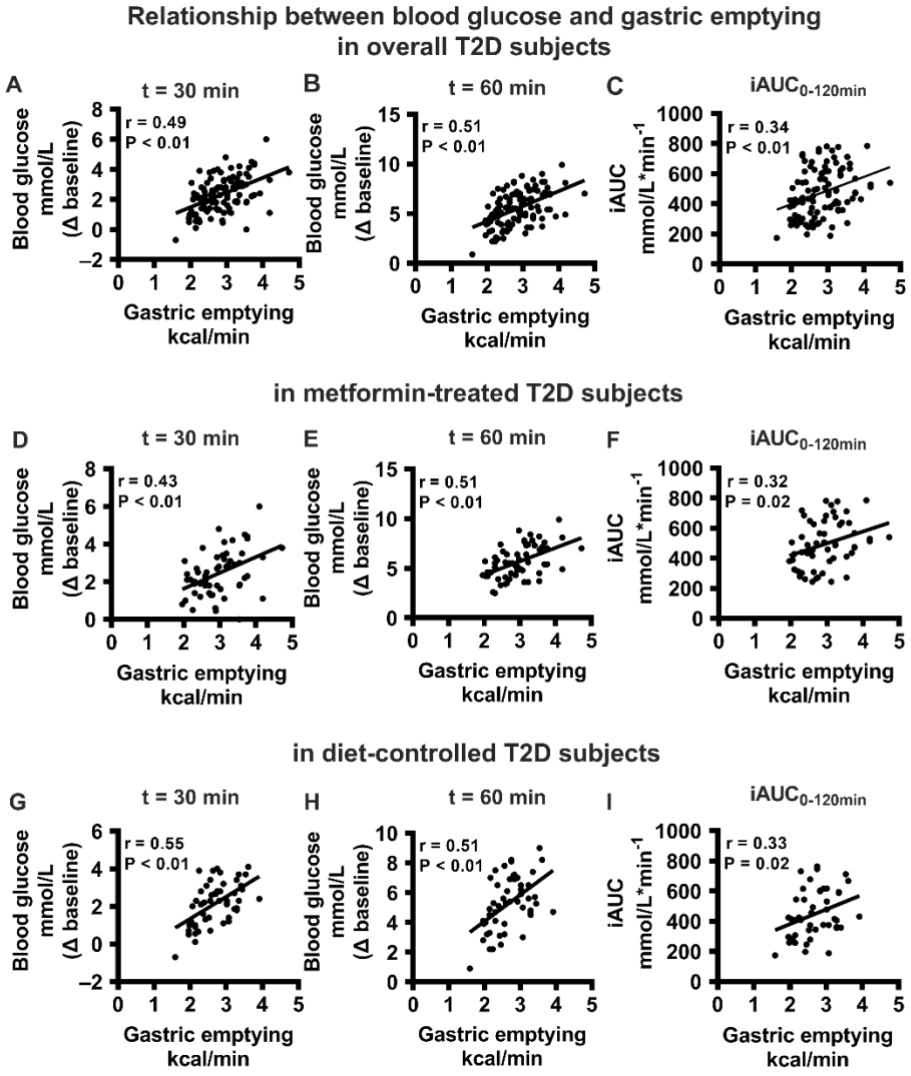


**Figure 4.3.** A-C: Relationship between the blood glucose increment (at t = 30 min and 60 min after the meal and the incremental area under the curve (iAUC) for blood glucose during 0-120min) and gastric emptying rate in overall subjects with T2D. D-F: Relationship between the blood glucose increment (at t = 30 min and 60 min after the meal and the iAUC for blood glucose during 0-120min) and gastric emptying rate in the T2D subjects taking metformin. **G-I:** Relationship between the blood glucose increment (at t = 30 min and 60 min after the meal and the iAUC for blood glucose during 0-120min) and gastric emptying rate in the T2D subjects without metformin.

# CHAPTER 5: GASTRIC EMPTYING IN HEALTH AND TYPE 2 DIABETES: AN EVALUATION USING A 75 G ORAL GLUCOSE DRINK

# STATEMENT OF AUTHORSHIP

| | |
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| Title of the paper | Gastric emptying in health and type 2 diabetes: an evaluation using a 75 g oral glucose drink. |
| Publication status | Published |
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| 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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Co-Author Contributions

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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## 5.1 Abstract

**Aims:** Gastric emptying is a major determinant of the glycaemic response to carbohydrate and is frequently abnormal in type 2 diabetes (T2D). There is little information about how chronic glycaemic control affects gastric emptying in T2D. We evaluated gastric emptying of a 75 g glucose drink in community-based subjects with T2D of short duration with good or poor glycaemic control, and compared this to young and older controls.

**Methods:** T2D subjects managed by diet and/or metformin, either well-controlled or poorly-controlled, together with young and age-matched older controls without diabetes, consumed a 75 g oral glucose drink containing 150 mg $^{13}$C-acetate for evaluation of gastric emptying (breath test) and blood glucose over 180 min.

**Results:** The gastric half-emptying time (T50) was longer in the older than the young non-diabetic subjects ($P = 0.041$), but shorter in well-controlled T2D subjects than age-matched older controls ($P = 0.043$). The T50 in poorly-controlled T2D subjects was shorter than in older controls ($P = 0.006$), but similar to young non-diabetic subjects.

**Conclusions:** Gastric emptying of a glucose drink is delayed with ageing, but more rapid in subjects with T2D of relatively short duration, regardless of their glycaemic status. These observations support interventions that slow gastric emptying to improve postprandial glycaemia in these subjects with T2D.

## 5.2 Introduction

Gastric emptying is a major determinant of the blood glucose response to dietary carbohydrate in both health and diabetes (Jones et al., 1995; Marathe et al., 2015; L. E. Watson, Xie, et al., 2019), accounting for more than a third of the variation in the initial glycaemic response (Rayner, Samsom, Jones, & Horowitz, 2001). Both pharmacological and nutritional interventions that modulate gastric emptying have a profound impact on the postprandial blood glucose profiles, and even relatively small changes in the rate of gastric emptying may have a substantial impact on the postprandial glycaemic profile (L. K. Phillips et al., 2015). For example, acceleration of gastric emptying by erythromycin increases (Janssens et al., 1990), while slowing of gastric emptying by morphine attenuates, postprandial glycaemic excursions in type 2 diabetes (T2D) (Gonlachanvit et al., 2003). Moreover, glucagon-like peptide-1 (GLP-1) receptor agonists (Jones et al., 2020; Jones et al., 2019) (particularly those that are short-acting) and administration of nutrient 'preloads' before a meal (L. Watson et al., 2018; T. Wu, Little, et al., 2016; T. Wu et al., 2012) slow gastric emptying to diminish postprandial glycaemic excursions in T2D. Conversely, gastric emptying influences the response to antidiabetic therapies. For example, the reduction in postprandial glucose by GLP-1 receptor agonists (Lorenz et al., 2013) and dipeptidyl peptidase-4 inhibitors (Stevens et al., 2020; T. Wu, Zhang, et al., 2016) is greater when baseline gastric emptying is relatively more rapid. Accordingly, gastric emptying has major implications for the management of postprandial hyperglycaemia in T2D.

In T2D, gastric emptying has been reported to be delayed (Michael Horowitz et al., 1991), accelerated (W. T. Phillips, Schwartz, & McMahan, 1991), or unchanged (Boronikolos et al., 2015), which probably reflects the substantial heterogeneity in subject characteristics (e.g. age, duration of diabetes, glycaemic status, pharmacotherapy, and presence or absence of diabetic complications) of the cohorts studied and the test meals employed (e.g. emptying of solid and liquid test meals is frequently inconsistent (Jones et al., 1995; L. E. Watson, Phillips, et al., 2019)). In subjects with longstanding (typically >10 years), poorly-controlled T2D (HbA1c >8.5%), associated with a high prevalence of microvascular complications, the emptying of solid meals has been reported to be delayed in 30-50% of subjects (Bharucha, Kudva, et al., 2015; M. Horowitz et al., 1989; Jones et al., 1995; Matsumoto et al., 2007). Conversely, well-controlled T2D subjects with few complications have gastric emptying of solids that is similar to controls without diabetes (Boronikolos et al., 2015). More recently, we reported that in T2D subjects, well controlled by diet or metformin monotherapy (HbA1c ~6.6%) and without autonomic dysfunction, gastric emptying of a high carbohydrate semi-solid was more rapid than in age-matched controls (L. E. Watson, Xie, et al., 2019), supporting the concept that rapid gastric emptying may predispose to glucose intolerance in "early" T2D (Schwartz et al., 1996) (Bertin et al., 2001). As for solids, previous studies have shown that gastric emptying of a nutrient-containing liquid meal (e.g. oral glucose) is also delayed in longstanding poorly-controlled T2D subjects with a high rate of complications (M. Horowitz et al., 1989; Jones et al., 1995), but may be more rapid in T2D subjects without autonomic dysfunction (Frank et al., 1995; W. T. Phillips et

al., 1992). However, the latter studies suffered from small sample sizes (n = 8-10) and lack of information on antidiabetic medications and glycaemic control, which may confound the interpretation of the outcomes substantially.

The 75 g oral glucose tolerance test (OGTT) is a widely performed diagnostic test for diabetes. The impact of gastric emptying on the interpretation of OGTT has hitherto not been given adequate consideration. Recently, the glycaemic response to oral glucose at one hour has also been shown to be a strong independent predictor of T2D in adults (Abdul-Ghani, Abdul-Ghani, Ali, & Defronzo, 2008). It is intuitively likely that a higher glucose level at one hour after OGTT may reflect more rapid gastric emptying in a given individual (M. Horowitz et al., 1993; Jones et al., 1996; Marathe et al., 2015). Accordingly, evaluation of gastric emptying of an oral glucose drink is likely to yield important insights into its clinical application.

There is evidence that acute hyperglycaemia, even within the physiological range, slows gastric emptying in both health and type 1 diabetes (Fraser et al., 1990; Schvarcz et al., 1997). In the latter, delayed gastric emptying is predictive of both early and long-term hyperglycaemia (Bharucha, Batey-Schaefer, et al., 2015), whereas normalisation of hyperglycaemia accelerates gastric emptying (Samsom et al., 1997). However, the influence of chronic glycaemic control, as measured by HbA1c, on gastric emptying remains controversial in T2D (Halland & Bharucha, 2016; Laway, Malik, Khan, & Rather, 2013). Although a handful of small studies reported that obesity is associated with an acceleration of gastric emptying (Cardoso-

Júnior et al., 2007; Gryback et al., 1996), more recent data suggest it has minimal influence (Seimon et al., 2013). By contrast, ageing is associated with a modest delay in gastric emptying (M. Horowitz et al., 1984; Pham et al., 2020).

Accordingly, the present study evaluated the gastric emptying of an oral glucose drink in two cohorts of community-based subjects with relatively well- (HbA1c ≤ 7.9%) or poorly- (HbA1c ≥ 9%) controlled T2D managed by diet or metformin alone, together with young and older subjects without diabetes.

## 5.3. Methods

### *5.3.1 Subjects*

Four separate cohorts were included in the study. Subjects with T2D (consistent with American Diabetes Association criteria) were recruited from the community by advertisement for studies evaluating nutritional and/or pharmacological therapies for diabetes in our centre (Trahair, Horowitz, & Jones, 2015; Young et al., 2009). They were managed by diet and/or metformin monotherapy (500-2000 mg/day, stable for > 3 months), and were classified as having either 'reasonable' (HbA1c ≤ 7.9% (63 mmol/mol)) or 'poor' (HbA1c ≥ 9% (75 mmol/mol)) glycaemic control at screening. Young subjects with normal BMI and older subjects with an age in the range of the well-controlled T2D subjects group, both without diabetes, were also recruited from the community by advertisement (Trahair et al., 2015; T. Wu, M. J. Bound, S. D. Standfield, M. Bellon, et al., 2013; Young et al., 2009).

Both controls and T2D subjects were excluded if they reported significant gastrointestinal (GI) symptoms, a history of GI disease including known gastroparesis, bariatric surgery, or a requirement for medication known to affect GI function or appetite. They were screened to exclude renal dysfunction (based on the estimated glomerular filtration rate) or liver disease (based on liver enzymes). The studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee, and all subjects provided written informed consent.

### *5.3.2 Protocol*

Gastric emptying of, and the glycaemic response to, a 75 g glucose drink was evaluated on a single study visit. All subjects were asked to refrain from strenuous physical activity for 24 h before the study, and were provided with a standardised dinner, consisting of beef lasagne (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia), bread, a non-alcoholic beverage and one piece of fruit, to be consumed at 1900h on the evening before the study. Subjects were then instructed to refrain from all food and nutrient beverages, but were allowed to drink water until 2200h, before attending the laboratory at 0800h. Metformin-treated T2D subjects were instructed to withhold any scheduled dose the evening before each visit, and to defer their morning dose until the end of the study. On arrival, an intravenous cannula was inserted into a forearm vein for repeated blood sampling. The drink, consisting of 75 g anhydrous glucose and 150 mg $^{13}$C-acetate dissolved in water to a final volume of 300 mL, was then consumed within 5 min (t = 0-5 min). Breath samples were collected immediately before, and every 5 minutes after the drink during the first hour, and every 15 minutes

for a further two hours. Venous blood was sampled immediately before (at t = 0), and then at t = 30, 60, 120 and 180 min after the drink. After completion of the gastric emptying measurement, autonomic nerve function (ANF) was evaluated in healthy older subjects and subjects with T2D using standardised cardiovascular reflex tests (Piha, 1991).

### *5.3.3 Measurement of gastric emptying*

$^{13}CO_2$ in each breath sample was measured by an isotope ratio mass spectrometer (ABCA 20/20; Europa Scientific, Crewe, UK) with an online gas chromatographic purification system. The gastric half-emptying time (T50) and intragastric retention were calculated using the Wagner-Nelson method, as described (Sanaka et al., 2004). This method has been shown to be of comparable accuracy to scintigraphy in the measurement of gastric emptying of both sloid and liquid meals (Sanaka et al., 2007; Sanaka et al., 2004).

### *5.3.4 Measurements of blood glucose concentrations and autonomic function*

Blood glucose concentrations were assessed using a glucometer (Optium Xceed, Abbott Laboratories, USA), and reported as the mean of duplicate measurements at each time point. ANF was evaluated according to the variation in heart rate during deep breathing, heart rate response to standing, and fall in systolic blood pressure in response to standing. Each test result was scored as 0 = normal, 1 = borderline, 2 = abnormal. A score $\geq 3$ was considered to indicate autonomic dysfunction (Piha, 1991).

### *5.3.5 Statistical analysis*

Demographic data between designated groups were compared using unpaired Student's t-tests after confirming their normality of distribution, except that the proportions of each gender and those with autonomic neuropathy in each group were compared using Fisher's exact test. T50 in designated groups were compared using unpaired Student's t-tests with and without adjustment for differences in age and/or BMI. Changes in blood glucose after the glucose drink and gastric retention were evaluated by two-way repeated measures analysis of variance (ANOVA) using group and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant interactions. Relationships between the glycaemic outcomes, including the changes in blood glucose from baseline at different time points, the incremental area under the curve (iAUC) for blood glucose between t = 0-60 min and HbA1c, and the T50 in each group, were evaluated using the Pearson correlation analysis. Based on our previous work (L. E. Watson, Xie, et al., 2019), a sample size of 18 would provide at least 80% power (at $\alpha = 0.05$) to detect ~20% difference in the T50 between groups. Accordingly, the primary outcomes of the present study were the differences in the T50 between groups (i.e. young vs. older non-diabetic subjects, well-controlled T2D subjects vs. age-matched non-diabetic older subjects, and poorly-controlled T2D subjects vs. non-diabetic young and older subjects). Analyses were performed using SPSS Statistics (Version 26, IBM, NY, USA). $P < 0.05$ was considered statistically significant. Data are expressed as mean values ± SEM.

## 5.4 Results

The study included 20 young subjects without diabetes, 31 older subjects without diabetes, 20 well-controlled and 13 poorly-controlled T2D subjects (**Table 5.1**). All subjects tolerated the protocol well. Older controls without diabetes were ~42 years older than young controls without diabetes, without any difference in BMI between them. When compared with older controls, the well-controlled T2D subjects had a similar age, but a slightly higher BMI ($P < 0.05$), while poorly-controlled T2D subjects were ~12 years younger and had a higher BMI ($P < 0.05$ for both). Five of 20 subjects with well-controlled T2D and 3 of 13 subjects with poorly-controlled T2D had mild autonomic dysfunction as assessed by cardiovascular reflex tests.

### *5.4.1 Comparisons between the young and older subjects without diabetes*

Fasting blood glucose concentrations did not differ between the young and older subjects without diabetes (**Table 5.1**). After the glucose drink, blood glucose concentrations increased promptly and peaked at ~30 minutes (~9.0 mmol/L) before returning to baseline without any difference between the two groups (**Figure 5.1**).

Gastric emptying was slower in the older than young subjects (T50: 85.6 ± 5.7 min vs. 68.9 ± 4.9, $P = 0.041$, **Figure 5.2A**). Similarly, two-way ANOVA showed a significant group difference on the intragastric retention time profiles with gastric retention being greater ($P = 0.039$) in the older group (**Figure 5.2B**).

### *5.4.2 Comparisons between well-controlled T2D and young and older (age-matched) subjects without diabetes*

Both fasting ($P < 0.01$) and post-drink (group effect: $P < 0.001$; group by time interaction, $P < 0.001$) blood glucose concentrations were higher in subjects with well controlled T2D than young and age-matched older subjects without diabetes, with significant differences between t = 0 -180 min, $P < 0.05$ for each, **Figure 5.1**).

Gastric emptying was faster in subjects with well controlled T2D (T50: 67.6 ± 4.4 min) than age-matched controls ($P = 0.037$ before, and $P = 0.043$ after adjustment for differences in BMI, **Figure 5.2A**), but did not differ from young controls ($P = 0.98$ before, and $P = 0.27$ after, adjustment for differences in age and BMI, **Figure 5.2A**). Two-way ANOVA analysis also showed a significant group difference ($P = 0.026$) and a group by time interaction ($P = 0.034$) on the intragastric retention time profiles between the well-controlled T2D subjects and age-matched controls, with gastric retention being greater in the latter between t = 30-180 min ($P < 0.05$ for each). There was no difference in intragastric retention profile over time between the well-controlled T2D subjects and young controls (**Figure 5.2B**).

### *5.4.3 Comparisons between poorly-controlled T2D subjects and young and older subjects without diabetes*

Fasting and post-drink blood glucose concentrations were markedly higher in poorly-controlled T2D subjects than both young and older subjects without diabetes ($P < 0.05$ for each, **Figure 5.1**).

Gastric emptying was faster in the poorly-controlled T2D subjects (T50: 58.8 ± 5.5 min) than older controls (P = 0.002 before, and P = 0.006 after, adjustment for differences in age and BMI, **Figure 5.2A**). Likewise, there was a significant group difference in the gastric retention curves (P = 0.002), and a group by time interaction (P = 0.001) on the intragastric retention time profiles between older controls and poorly-controlled T2D subjects, with gastric retention being greater in older controls between t = 30-180 min (P < 0.05 for each, **Figure 5.2B**). Although T50 in the poorly-controlled T2D subjects did not differ from that in the young subjects without diabetes (P = 0.10 before, and P = 0.16 after, adjustment for differences in age and BMI), there was a significant group by time interaction (P = 0.015) for the intragastric retention curves between the two groups.

### *5.4.4 Comparisons between well-controlled and poorly-controlled T2D subjects*

Both fasting (P<0.01) and post-drink blood glucose concentrations (group effect: P<0.001, group by time interaction, P<0.001) were markedly higher in subjects with poorly-controlled, than well-controlled T2D, with significant differences between t = 0-180 min (P<0.05 for each) (**Figure 5.1**). The T50 did not differ from well-controlled T2D subjects (P = 0.20 before, and P = 0.74 after, adjustment for differences in age and BMI, **Figure 5.2A**). Similarly, there was no difference in intragastric retention profile over time between the two groups (**Figure 5.2B**).

### *5.4.5 Relationships between glycaemic measures and gastric emptying*

The T50 did not correlate with BMI or baseline blood glucose levels across the four groups, or with HbA1c, duration of diabetes, or ANF scores in subjects with T2D. There was no significant correlation between the T50 and the increment in blood glucose at t = 30, 60, 90 or 120 min in either (young and older) control groups without diabetes or poorly-controlled T2D subjects. However, there was a direct relationship between the increment of blood glucose at t = 180 min after the glucose drink and the T50 ($r = 0.50$, $P = 0.024$ and $r = 0.70$, $P < 0.001$, respectively) in the young and older subjects without diabetes, (**Figure 5.3A-B**). In subjects with well-controlled T2D, the increment in blood glucose at t = 30 min ($r = -0.66$, $P = 0.002$) and t = 60 min ($r = -0.47$, $P = 0.037$), and the iAUC for blood glucose levels between t = 0-60 min ($r = -0.63$, $P = 0.003$), but not the increment in blood glucose at t = 120 min, were related inversely to the T50 (**Figure 5.3C-E**).

## 5.5 Discussion

We evaluated gastric emptying of a 75 g oral glucose drink (a 'test meal' for diagnosis of diabetes) in both well- (HbA1c ~6.9%) and poorly- (HbA1c ~10.5%) controlled T2D subjects with a relatively short duration of diabetes (~3 and 6 years, respectively), minimal pharmacological treatment (diet or metformin alone), and an absence of severe complications, and compared this with young and older subjects without diabetes. The major observations were that gastric emptying of glucose was slower in older than young subjects without diabetes, but faster in both well- and poorly-controlled T2D subjects than age-adjusted individuals without diabetes. These

findings suggest that accelerated gastric emptying, particularly of nutrient liquids which are highly consumed in modern populations, occurs frequently in T2D regardless of glycaemic status.

Ageing is known to be associated with slowing of gastric emptying (M. Horowitz et al., 1984; Pham et al., 2020). In keeping with previous findings with both solid (L. E. Watson, Xie, et al., 2019) and liquid (M. Horowitz et al., 1984) meals, the gastric emptying of glucose was slightly slower in older than BMI-matched young subjects without diabetes. The lack of difference in blood glucose response between the two groups may reflect increased insulin resistance (K. F. Petersen et al., 2003) and/or an attenuated response of the incretin hormone glucagon-like peptide-1 (Pham et al., 2019) related to ageing. Similarly, the observation that the gastric emptying of oral glucose, either with or without adjustment for BMI, was more rapid in well-controlled T2D subjects than age-matched controls without diabetes is in support of the concept that gastric emptying in "early" T2D is, as a group, accelerated (Bertin et al., 2001; W. T. Phillips et al., 1991, 1992; Schwartz et al., 1996; L. E. Watson, Xie, et al., 2019). In a previous study in a cohort of T2D subjects with relatively good glycaemic control (HbA1c ~7%), but a long duration of known diabetes (~10 years), gastric emptying of a solid meal was shown to be similar to that of age-matched subjects without diabetes (Boronikolos et al., 2015), although the interpretation of these observations was complicated by heterogeneity in pharmacotherapy. The blood glucose response to oral glucose was predictably greater in well-controlled T2D subjects than in subjects without diabetes, and was associated with the T50, suggesting that slowing

of gastric emptying, for example by the GLP-1 receptor agonists, would be beneficial for lowering postprandial glycaemia in this group of subjects.

To our surprise, gastric emptying in subjects with poorly-controlled T2D was also faster when compared with the controls without diabetes, and did not differ from that of subjects with well-controlled T2D, in the context of substantially higher blood glucose levels and differences in age. In particular, hyperglycaemia, even within the physiological range, is known to slow gastric emptying (L. K. Phillips et al., 2015). Relative to the young controls without diabetes, the poorly-controlled T2D subjects were ~30 years older, yet the gastric retention-time profile suggested that gastric emptying was modestly faster in the latter. This finding contrasts with previous studies, in which poorly-controlled T2D subjects typically had a long duration of diabetes (~15 years) and high prevalence of autonomic disfunction, and required more intensive therapeutic regimens (Jones et al., 1995). Nine out of 13 poorly-controlled T2D subjects were treated with metformin, which would, if anything, potentially slow gastric emptying (Borg, Bound, et al., 2019). Accordingly, in poorly-controlled T2D of relatively short duration and in the absence of severe complications, gastric emptying is often modestly accelerated. However, it is noteworthy that an improvement of HbA1c from ~10.5% to 5.8% with intensive lifestyle and pharmacological interventions was shown to accelerate gastric emptying in a group of T2D subjects who had delayed gastric emptying (Laway et al., 2013). In this regard, intensification of glucose-lowering therapy in poorly-controlled T2D may further accelerate gastric emptying and (hence) adversely influence postprandial glycaemia.

The GLP-1 receptor agonists are often used as an add-on therapy in T2D subjects who are inadequately managed by metformin. Their potential to slow gastric emptying is therefore likely to be of major relevance to the glucose-lowering efficacy in subjects with both well- and poor-controlled T2D.

In line with our previous report (Trahair et al., 2014), the relationship between gastric emptying and the increment in glycaemia appeared to be bidirectional – being positive in the early phase, but negative later in the postprandial period – and subject to the glycaemic status. In the present study, we observed that the T50 correlated negatively with the "early" glycaemic response in relatively well-controlled T2D, but positively with the rise in blood glucose at 3 hours in subjects without diabetes. Such a phenomenon might be of relevance to the measurement of blood glucose at 1 hour after OGTT, which is a strong predictor of the onset of T2D (Abdul-Ghani et al., 2008). Of note, we did not observe a significant relationship between the T50 and blood glucose concentrations at t = 120 min in either group, arguing against a major impact of gastric emptying on the diagnosis of dysglycaemia with the standard OGTT. It is also noteworthy that marked elevation of blood glucose levels following oral glucose in the poorly-controlled subjects would intuitively increase urinary glucose excretion. While this is unlikely to be relevant to the regulation of gastric emptying, it may, to some extent, have accounted for the lack of significant correlation between T50 and the rises in postprandial glycaemia in this group.

Several limitations need to be noted. First, the sample size of each group was modest; nevertheless, apart from subjects with poorly-controlled T2D, sample sizes were estimated to have adequate power to compare gastric emptying between the groups (L. E. Watson, Xie, et al., 2019), and proved sufficient to reveal significant differences. Increasing the sample size is therefore unlikely to modify the conclusions. Second, blood glucose concentrations were measured by a portable glucometer, yet the differences in blood glucose profiles between the groups are clear-cut. Third, we did not measure plasma gluco-regulatory hormones (e.g. insulin/C-peptide and GLP-1), given that the primary outcome of the current study was the differences in gastric emptying between groups. Fourth, relative to other groups, the well-controlled T2D group had a relatively higher proportion of female subjects. There is evidence that gastric emptying of liquids is slower in women than men (Datz, Christian, & Moore, 1987; Hutson, Roehrkasse, & Wald, 1989). Despite this, gastric emptying in well-controlled T2D subjects was found to be more rapid than the age-matched older controls. Finally, given that the relationship between gastric emptying of solids and nutrient-containing liquids is weak, we should be cautious in extrapolating our findings for liquids to the gastric emptying of solids (or vice versa).

In conclusion, gastric emptying of a liquid meal is delayed with ageing, but is more rapid in subjects with T2D of relatively short duration without severe complications, regardless of their glycaemic status. These observations are in support of interventions that slow gastric emptying for the management of postprandial hyperglycaemia in these subjects with T2D.

**Table 5.1. Subject characteristics (mean values ± SEM).**

| | Subjects without diabetes | | Subjects with T2D | |
|---|---|---|---|---|
| | **Young** | **Older** | **Well controlled** | **Poorly controlled** |
| **Gender (male)** | 15/20 | 19/31 | 7/20 | 11/13 # |
| **Age (years)** | 27.5 ± 2.0 ** | 69.8 ± 0.7 | 70.7 ± 1.6 | 57.1 ± 2.7 ## ** |
| **BMI (kg/m$^2$)** | 24.3 ± 0.8 | 25.7 ± 0.5 | 27.7 ± 0.8 * | 31.6 ± 1.6 # ** |
| **HbA1c (%)** | - | - | 6.9 ± 0.2 | 10.5 ± 0.5 ## ** |
| **HbA1c (mmol/mol)** | - | - | 52 ± 2 | 91 ± 6 ## ** |
| **Duration of known T2D (years)** | - | - | 2.9 ± 0.8 | 5.8 ± 1.6 # |
| **Fasting glucose (mmol/L)** | 5.6 ± 0.1 | 5.6 ± 0.1 | 7.1 ± 0.3 ** | 12.8 ± 1.1 ## ** |
| **Prevalence of autonomic neuropathy (n)** | - | 0/31 | 5/20 * | 3/13 * |
| **Use of metformin (n)** | - | - | - | 9/13 |

Compared with older subjects without diabetes, * P < 0.05; **, P < 0.01.

Compared with well controlled T2D subjects, # P < 0.05; ##, P < 0.01.

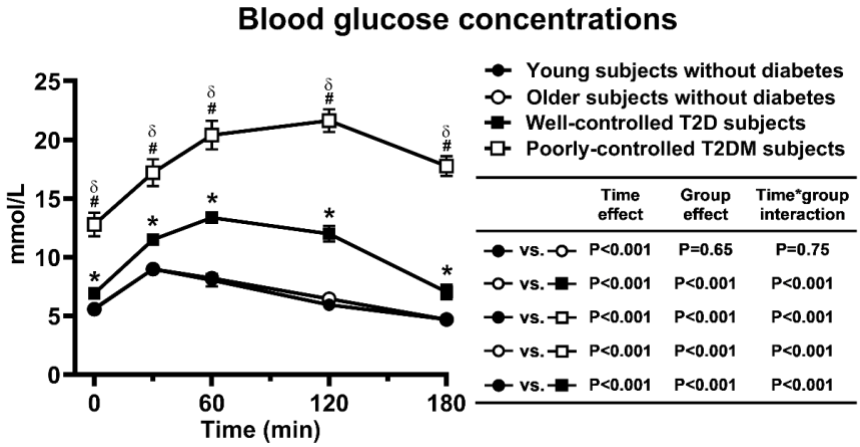


**Figure 5.1.** Blood glucose concentrations in response to a 75 g glucose drink (given at t = 0) in subjects with well- and poorly controlled T2D and young and older control subjects without diabetes. Repeated-measures ANOVA was used to determine statistical significance, with results reported as P values for differences over time and by group, and for group by time interactions. Post hoc comparisons were adjusted by Bonferroni correction. Data are mean ± SEM. * $P < 0.05$ for older subjects without diabetes vs. well-controlled T2D subjects. # $P < 0.05$ for young subjects without diabetes vs. poorly-controlled T2D subjects. δ $P < 0.05$ for older subjects without diabetes vs. poorly-controlled T2D subjects.

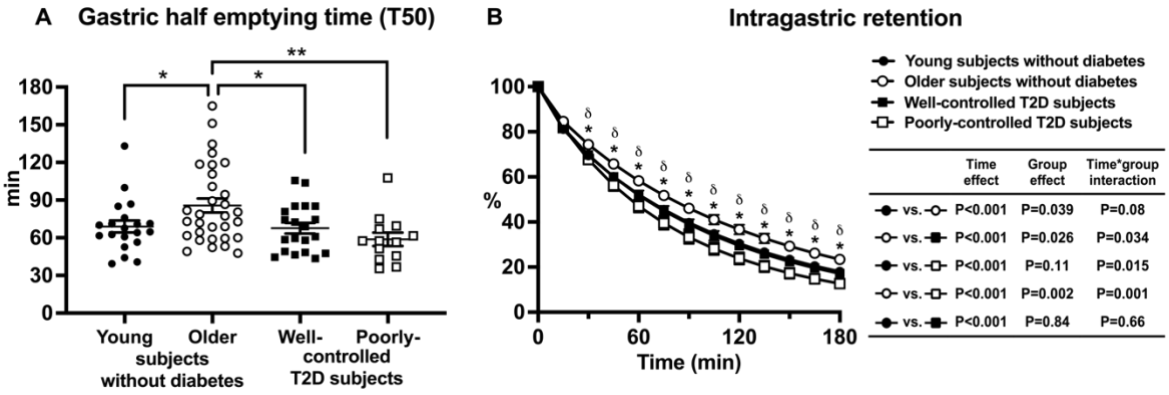


**Figure 5.2.** (A) Gastric half emptying time (T50) of a 75 g glucose drink in subjects with well- and poorly-controlled T2D, and young and older control subjects without diabetes, assessed by $^{13}$C-acetate breath test. Unpaired Student's t tests were used to determine statistical significance after adjusting for age- and/or BMI. * $P < 0.05$, ** $P < 0.01$. (B) Intragastric retention of the 75 g glucose drink (given at t = 0) over time in each group. Repeated-measures ANOVA was used to determine statistical significance, and ANOVA results are reported as P values for differences over time and by group, and for group by time interactions. Post hoc comparisons were adjusted by Bonferroni correction. Data are mean ± SEM. * $P < 0.05$ for older subjects without diabetes vs. well-controlled T2D subjects. $\delta$ $P < 0.05$ for older subjects without diabetes vs. poorly-controlled T2D subjects.

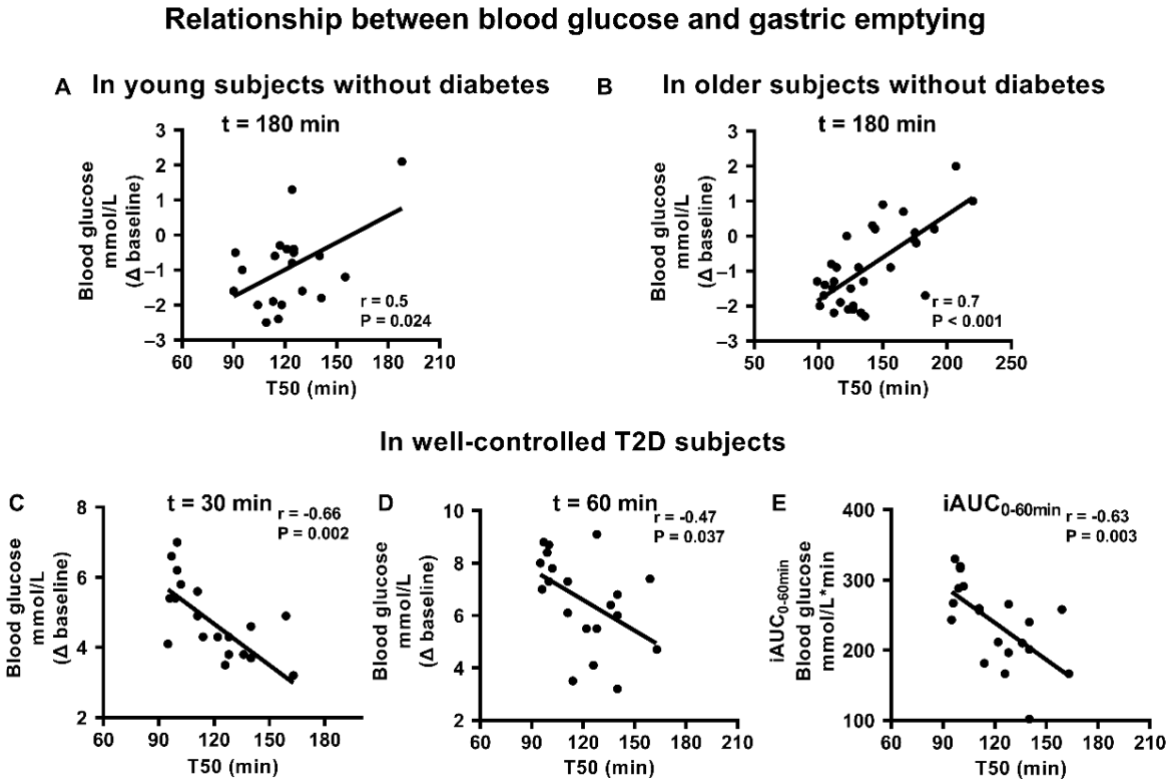


**Figure 5.3.** (A-B) Relationships between the blood glucose increment (at t = 180 min after the glucose drink) and Gastric half emptying time (T50) in young and older subjects without diabetes. (C-E) Relationships between the blood glucose increment (at t = 30 and 60 min after the glucose drink and the incremental area under the curve (iAUC) for blood glucose during 0 to 60 min) and T50 in well-controlled T2D subjects.

# CHAPTER 6: PLASMA GLP-1 RESPONSE TO ORAL AND INTRADUODENAL NUTRIENTS IN HEALTH AND TYPE 2 DIABETES – IMPACT ON GASTRIC EMPTYING

# STATEMENT OF AUTHORSHIP

| | |
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| Overall percentage | 60% | | |
| 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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Co-Author Contributions

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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## 6.1 Abstract

**Aims:** Both gastric emptying and the secretion of glucagon-like peptide-1 (GLP-1) are major determinants of postprandial glycaemia in health and type 2 diabetes (T2D). GLP-1 secretion after a meal is dependent on the entry of nutrients into the small intestine, which, in turn, slows gastric emptying. This study aimed to define the relationship between gastric emptying and the GLP-1 response to both oral and small intestinal nutrients in healthy subjects and those T2D.

**Methods:** We evaluated: (i) the relationship between gastric emptying (measured by the $^{13}$C-isotope breath test) and postprandial GLP-1 levels after a mashed potato meal in 73 T2D subjects; (ii) inter-individual variations in GLP-1 response to (a) intraduodenal glucose (4 kcal/min) during euglycaemia and hyperglycaemia in 11 healthy, and 12 T2D, subjects, (b) intraduodenal fat (2 kcal/min) in 15 T2D subjects, and (c) intraduodenal protein (3 kcal/min) in 10 healthy subjects; and (iii) the relationship between gastric emptying of oral glucose and the GLP-1 response to intraduodenal glucose (4 kcal/min) in 21 subjects (9 healthy, 12 T2D).

**Results:** The gastric half-emptying time (T50) was not related to the integrated area under the curve (iAUC) for plasma GLP-1 after the mashed potato meal. The GLP-1 responses to intraduodenal glucose, fat and protein varied substantially between individuals, but intra-individual variation to glucose was modest. The T50 of oral glucose was related directly with the GLP-1 response to intraduodenal glucose ($r = 0.65$, $P = 0.002$).

**Conclusion**: In a given individual, gastric emptying is not a determinant of the postprandial GLP-1 response. However, the intrinsic gastric emptying rate is determined in part by the magnitude of the GLP-1 response to intestinal nutrients.

## 6.2 Introduction

Gastrointestinal function is pivotal to the regulation of postprandial glycemia. In particular, gastric emptying, which controls the delivery of ingested nutrients into the small intestine, accounts for a substantial proportion of the variation in the blood glucose response to carbohydrate in both health and diabetes (Marathe et al., 2015; L. E. Watson, Xie, et al., 2019; Xie, Huang, Wang, et al., 2021). In patients with type 2 diabetes (T2D), interventions that accelerate gastric emptying (e.g. intravenous administration of erythromycin) increase the postprandial glycemic excursion (Gonlachanvit et al., 2003; Janssens et al., 1990), while those that slow gastric emptying (e.g. morphine (Gonlachanvit et al., 2003) and nutrient 'preloads' consumed before the main meal (Ma et al., 2009; L. Watson et al., 2018; T. Wu, Little, et al., 2016)) attenuate it. The entry of nutrients into the small intestine induces the secretion of a variety of gut hormones(Xie, Jones, et al., 2020), including the incretin hormone glucagon-like peptide-1 (GLP-1), which stimulates insulin, and suppresses glucagon secretion in a glucose-dependent manner (Hare et al., 2010), inhibits energy intake (Edwards et al., 2001) and, in concert with cholecystokinin and peptide YY, slows gastric emptying (Deane, Nguyen, et al., 2010; Little, Pilichiewicz, et al., 2006). Accordingly, there is a complex, bidirectional, relationship between gastric emptying and the secretion of postprandial GLP-1. Understanding this relationship is critical to the development of gut-based interventions for the management of postprandial hyperglycemia in T2D.

In healthy individuals, gastric emptying usually occurs at a relatively constant overall caloric rate in the range of 1-4 kcal/min (L. E. Watson, Xie, et al., 2019). Given that gastric emptying is often accelerated in 'early' uncomplicated T2D subjects (L. E. Watson, Xie, et al., 2019; Xie, Huang, Wang, et al., 2021) and frequently delayed in longstanding complicated T2D (Michael Horowitz et al., 1991), the variation in the rate of gastric emptying is even wider in T2D. The impact of gastric emptying, or the rate of the entry of nutrients into the small intestine, on the secretion of GLP-1 has been examined in experimental models involving intraduodenal delivery of standardized macronutrients via a nasoduodenal catheter. In these studies, glucose-induced GLP-1 secretion was shown to be dependent on the rate of glucose delivery, exhibiting a minimal response when glucose was infused at rates of 1-2 kcal/min, but a substantially greater response at rates of 3-4 kcal/min in both health and T2D (Ma et al., 2012; Trahair et al., 2012). The latter is likely to reflect an increased exposure of the distal gut – where the GLP-1-releasing L-cells are most densely located – to glucose that has exceeded the absorptive capacity of the proximal gut (Schirra et al., 1996). It might therefore be anticipated that in individuals with more rapid gastric emptying, the GLP-1 response to an orally ingested meal would be greater. However, in a small set of healthy young subjects (n = 12), the GLP-1 response to a 75 g oral glucose drink over 2-3 hours did not correlate with the rate of gastric emptying (J. M. Wishart, Horowitz, Morris, Jones, & Nauck, 1998). Similarly, in a larger group of healthy 'older' subjects (n = 87), plasma GLP-1 levels over 240 min after oral glucose were unrelated to gastric emptying (Trahair et al., 2014). While GLP-1 secretion in response to intraduodenal glucose did not seem to differ between health and T2D (Ma

et al., 2012; Trahair et al., 2012), the GLP-1 response to oral glucose was reported to be reduced in individuals with 'early' T2D (Faerch et al., 2015), despite that gastric emptying may be faster in these groups than healthy controls (W. T. Phillips et al., 1992; L. E. Watson, Xie, et al., 2019). The relationship between gastric emptying and the GLP-1 response to a physiological meal in T2D remains poorly characterized.

Despite increasing recognition of the substantial variation in the rate of gastric emptying between individuals, the underlying mechanisms is still unknown. Gastric emptying is dependent on coordinated antral contractions to pump gastric content against pyloric and duodenal resistance, and is inhibited by neurohormonal feedback arising from the small intestine (T. Wu, C. K. Rayner, et al., 2013). In both health and T2D, intravenous administration of physiological doses of exogenous GLP-1 slows gastric emptying substantially (Little, Pilichiewicz, et al., 2006), while antagonism of endogenous GLP-1 signaling by the GLP-1 receptor antagonist, exendin(9-39), accelerates gastric emptying (Deane, Nguyen, et al., 2010). These observations provide strong support for the involvement of intestinal nutrient-evoked GLP-1 release in the regulation of gastric emptying. The reciprocal actions of gastric emptying and GLP-1, on the one hand, complicate the evaluation of the relationship between them, but on the other hand, suggests that the intrinsic rate of gastric emptying in a given individual may be tuned by the intestinal GLP-1 response to nutrient stimulation.

In a series of experimental settings involving subjects with and without T2D, we examined whether: (i) the rate of gastric emptying predicts GLP-1 secretion after a high carbohydrate meal, (ii) the GLP-1 response to standardized intestinal nutrient stimulation within the physiological range of gastric emptying exhibits a wide inter-individual, but modest intra-individual variation, and (iii) the intrinsic rate of gastric emptying is related to the GLP-1 response to small intestinal nutrient infusion.

## 6.3 Methods

### *6.3.1. Subjects*

Both healthy and T2D subjects were recruited from the community by advertisement for studies evaluating nutritional and/or pharmacological therapies for T2D (Giezenaar et al., 2018; L. Watson et al., 2018; Xie, Wang, et al., 2020; Young et al., 2013). T2D subjects were managed by diet or metformin monotherapy (the latter was ceased 48 hours prior to the study visit). Subjects were excluded if they reported significant gastrointestinal symptoms, a history of gastrointestinal surgery, or a requirement for medication known to affect gastrointestinal function or appetite. They were screened to exclude renal dysfunction (based on the estimated glomerular filtration rate), liver disease (based on serum liver enzymes), or autonomic dysfunction (assessed by the standardized cardiovascular reflex tests (Piha, 1991)). The studies were approved by the Royal Adelaide Hospital Human Research Ethics Committee, and all participants provided written informed consent.

### *6.3.2. Protocol*

All subjects were asked to refrain from strenuous physical activity for 24 h before each study visit and were provided with a standardized evening meal consisting of beef lasagne (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia) to be consumed with bread, a non-alcoholic beverage, and one piece of fruit at 1900 h. Subjects were instructed to then abstain from food and nutrient beverages, but were allowed to drink water until midnight, before attending our clinical research laboratory at The University of Adelaide at ~0800 h. On arrival, an intravenous cannula was inserted into a forearm vein for repeated blood sampling. Specific procedures on each study day are detailed below. At the end of each study visit, subjects were given a meal and their blood glucose levels were checked to exclude hypoglycemia (blood glucose < 3.9 mmol/L) before they left the research facility.

***Part 1: Evaluation of the relationship between postprandial GLP-1 secretion and gastric emptying***

Seventy-three T2D subjects (**Table 6.1**) were evaluated on a single study visit, when they consumed a semi-solid test meal within 5 minutes (t = 0 - 5 min). The meal consisted of 65 g powdered potato (Deb; Unilever, Australia) and 20 g glucose, reconstituted with 200 mL boiling water and one egg yolk containing 100 µL $^{13}$C-octanoic acid (368.5 kcal; 61.4 g carbohydrate, 7.4 g protein and 8.9 g fat). Breath samples were collected immediately before and every 5 min for the first hour after the meal, and then every 15 min in the next 3 hours, for the measurement of gastric emptying. “Arterialised” venous blood was sampled at t = 0, 15, 30, 60, 90, 120, 180

and 240 min for the measurement of blood glucose and plasma total GLP-1 concentrations.

***Part 2: Evaluation of the plasma GLP-1 response to intraduodenal nutrients***

*2.1 Intra- and inter-subject variations in the GLP-1 response to intraduodenal glucose*

Eleven healthy and 12 T2D subjects were studied on two days, separated by at least seven days, in a single-blinded, randomised, crossover fashion (**Table 6.2**). On each study day, an insulin/glucose clamp was established to maintain either euglycaemia (~5 mmol/L) or hyperglycaemia (~12 mmol/L) (t = -30 to 60 min), as described (Young et al., 2013). Once blood glucose concentrations were stable for 30 min, a small diameter video endoscope (GIF-XP160; Olympus, Tokyo, Japan) was inserted through an anesthetised nostril into the second part of the duodenum (Young et al., 2013) to infuse glucose at a rate of 4 kcal/min (5 mL/min) between t = 0-30 min. "Arterialised" venous blood samples were taken at t = 0, 10, 20, 30, 40, 50, and 60 min for the measurement of plasma total GLP-1 concentrations.

2.2 *Inter-individual variations in the GLP-1 response to intraduodenal protein*

Ten healthy subjects (**Table 6.3**) were evaluated on a single study day, when a multi-lumen silicone catheter (Dentsleeve International, Ontario, Canada) was inserted into a nostril and passed into the duodenum by peristalsis, with the infusion port positioned ~12 cm distal to the pylorus. The correct positioning of the catheter was monitored by continuous measurement of transmucosal potential difference in the antrum and

duodenum (Turner, Powell, Carney, Orlando, & Bozymski, 1978; T. Wu, Ma, et al., 2014). After correct positioning of the catheter, a whey protein solution (Fonterra Co-Operative Group Ltd., Palmerston North, New Zealand) was infused at rate of 3 kcal/min (4 mL/min) between t = 0-60 min. "Arterialised" venous blood samples were collected at t = 0, 15, 30, 45 and 60 min for the measurement of plasma total GLP-1 concentrations.

*2.3 Inter-individual variations in the GLP-1 response to intraduodenal fat*

Fifteen T2D subjects (**Table 6.3**) were evaluated on a single study day, when a multi-lumen silicone catheter was inserted (as detailed in 2.2) for intraduodenal infusion of a fat emulsion (20% Intralipid; Fresenius Kabi, Hornsby, NSW, Australia) at a rate of 2 kcal/min (1 mL/min) between t = 0-120 min. "Arterialised" venous blood samples were collected at t = 0, 15, 30, 45, 60, 90, and 120 min for the measurement of plasma total GLP-1 concentrations.

***Part 3: Evaluation of the relationship between gastric emptying of oral glucose and the GLP-1 response to intraduodenal glucose***

Twenty-one male subjects (mean age 47.0 ± 4.1 years, BMI 28.1 ± 1.1 kg/m$^2$), including 9 healthy and 12 T2D subjects (HbA1c 9.7 ± 0.9%, 6 subjects were treated with metformin), were studied on two occasions. On the first study day, subjects received an intraduodenal glucose infusion (4 kcal/min between t = 0-30 min) under euglycaemic clamp conditions (~5 mmol/L), as per the protocol detailed in Part 1. "Arterialised" venous blood samples were taken at t = 0, 10, 20, 30, 40, 50, and 60

min for the measurement of plasma GLP-1 concentrations. On the second study day, subjects consumed a glucose solution, containing 75 g glucose and 150 mg $^{13}$C-acetate dissolved in water to a final volume of 300 mL, within 5 min (t = 0-5 min). Breath samples were collected immediately before and every 5 min in the first hour after consumption, and then every 15 min in the next 2 hours, for the measurement of gastric emptying.

### *6.3.3. Measurement of blood glucose and plasma total GLP-1*

Blood glucose concentrations were assessed using a glucometer (Optium Xceed, Abbott Laboratories, USA), and reported as the mean of duplicate measurements at each time point. Plasma total GLP-1 concentrations were measured by radioimmunoassay (GLPIT-36HK; Millipore, Billerica, MA, USA) with a sensitivity of 3 pmol/L and intra- and inter-assay coefficients of variation (CVs) of 4.2% and 10.5%, respectively.

### *6.3.4. Measurement of gastric emptying*

$^{13}CO_2$ in each breath sample was measured by a non-dispersive infrared spectrometer (FANci2, Fischer ANalysen Instrumente, Germany) which has been validated against the gold standard of scintigraphy, in both health (Ghoos et al., 1993) and T2D (Ziegler et al., 1996). The gastric half-emptying time (T50) was calculated using the Wagner-Nelson method, as described (Sanaka et al., 2004), and has comparable accuracy to scintigraphy for the measurement of gastric emptying of both solid and liquid meals (Sanaka et al., 2007; Sanaka et al., 2004).

### *6.3.5. Statistical analysis*

The integrated area under the curve from baseline (iAUC) for plasma total GLP-1 was calculated using the trapezoidal rule. In Part 1, the relationships of gastric emptying (T50) with the iAUC for plasma total GLP-1 between t = 0-120 min and 0-240 min were evaluated using univariate linear regression analysis. Subjects were also divided into tertiles based on their T50. Demographic data, baseline plasma GLP-1 levels and the iAUC for GLP-1 between the T1 and T3 subgroups were compared using unpaired Student's t-tests after confirming normality of distribution, while the proportions of gender and usage of metformin were compared using Fisher's exact test. Blood glucose and changes in plasma GLP-1 levels were evaluated by two-way repeated measures analysis of variance (ANOVA) using group and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant interactions.

In Part 2, the iAUC for plasma total GLP-1 was calculated between 0-60 min in Studies 2.1 and 2.2 and between 0-120 min in Study 2.3. The inter-subject variability of the GLP-1 response was assessed by the coefficient of variation ($CV_{inter}$) of the GLP-1 iAUC on each study day. The intra-subject variation in plasma GLP-1 response to intraduodenal glucose was assessed by both the coefficient of variation ($CV_{intra}$) and the intraclass correlation coefficient (ICC) (two-way mixed model effect). CV was calculated as: CV(%) = (Standard deviation/Mean)*100%. ICC results were

interpreted as follows: > 0.75 suggested excellent reproducibility, and < 0.35 indicated poor reproducibility (Cicchetti, 1994).

In Part 3, the relationships of T50 with age, BMI, HbA1c, blood glucose levels and the iAUC for plasma GLP-1, as well as the relationships of the iAUC for plasma GLP-1 with age, BMI, HbA1c and blood glucose levels, were evaluated using univariate linear regression analysis.

Analyses were performed using GraphPad Prism 9 software (GraphPad, La Jolla, California) and SPSS Statistics Version 26 (IBM, New York). $P < 0.05$ was considered statistically significant. Data are expressed as means ± SEM.

## 6.4 Results

### *6.4.1. Gastric emptying does not predict the postprandial GLP-1 response in T2D*

Amongst a total of 73 T2D subjects, there was no significant relationship between the T50 and the $iAUC_{0\text{-}120min}$ or $iAUC_{0\text{-}240min}$ for plasma GLP-1. When subjects were divided into tertiles, based on their T50 (i.e. 87 ± 2 min for the T1 subgroup, 70 ± 1 min for the T2 subgroup, and 56 ± 1 min for the T3 subgroup, **Figure 6.1A**), gender, age, BMI and use of metformin did not differ between T1 and T3 subgroups ($P > 0.05$ each), while the duration of known T2D was ~ 3 years greater ($P = 0.01$) and HbA1c tended to be higher ($P = 0.07$) in the T3 than T1 subgroup (**Table 6.1**). Both fasting ($P = 0.005$) and postprandial (group effect: $P = 0.001$; group by time interaction, $P <$

0.001) blood glucose concentrations were higher in T3 than T1, with significant differences between t = 30 - 90 min, P < 0.05 for each (**Figure 6.1B**). Fasting plasma total GLP-1 did not differ between the T1 and T3 subgroups. After the meal, plasma total GLP-1 increased promptly and peaked at ~60 min before returning to baseline (time effect: P < 0.001 for each) in both the T1 and T3 subgroups, without any difference between them (ANOVA: P = 0.9 for the group effect, P = 0.1 for the group by time interaction; unpaired Student's t-test: P = 0.6 for the $iAUC_{0\text{-}240min}$, P = 0.9 for the $iAUC_{0\text{-}120min}$) (**Figure 6.1C and D**).

### *6.4.2. The GLP-1 response to intraduodenal nutrients exhibits a substantial inter-, but modest intra-individual, variation in subjects with and without T2D*

In response to intraduodenal infusion of glucose (4 kcal/min), fat (2 kcal/min) and protein (3 kcal/min), plasma GLP-1 levels increased substantially, with a substantial difference between subjects in each study. The $CV_{inter}$ for the GLP-1 $iAUC_{0\text{-}60min}$ was 144.4% on the euglycemic day and 115.0% on the hyperglycemic day in healthy subjects, and 59.1% on the euglycemic day and 84.4% on the hyperglycemic day in the T2D subjects, respectively (**Table 6.2**). Similarly, the $CV_{inter}$ for the GLP-1 $iAUC_{0\text{-}60min}$ in response to intraduodenal protein was 65.5% in healthy subjects, and the $CV_{inter}$ for the GLP-1 $iAUC_{0\text{-}120min}$ in response to intraduodenal fat was 48.4% in T2D subjects (**Table 6.2**). By contrast, the intra-subject variability for the GLP-1 $iAUC_{0\text{-}60min}$ in response to intraduodenal glucose between the two study days was relatively modest (with a $CV_{intra}$ of 31.5% and 24.5% in the healthy and T2D subjects, respectively). Moreover, the ICC was 0.965 in the healthy subjects, and 0.832 in the

T2D subjects, indicative of a high reproducibility of intestinal glucose-evoked GLP-1 secretion within subjects (**Table 6.2**).

### *6.4.3. Gastric emptying of oral glucose is related to GLP-1 response to intraduodenal glucose*

Both the T50 of oral glucose and the GLP-1 $iAUC_{0\text{-}60min}$ in response to intraduodenal glucose infusion (4 kcal/min over 30 min) exhibited wide inter-individual variation, with the T50 of oral glucose ranging from 39 to 164 min, and the GLP-1 $iAUC_{0\text{-}60min}$ in response to intraduodenal glucose ranging from -64 to 1125 pmol/L*min. There was a direct relationship between the T50 of oral glucose and the GLP-1 $iAUC_{0\text{-}60min}$ in response to intraduodenal glucose ($r = 0.65$, $P = 0.002$, **Figure 6.2**). There was no relationship between the T50 and age, BMI, fasting or postprandial glucose levels, or HbA1c. Likewise, the GLP-1 response to intraduodenal glucose did not correlate with age, BMI or HbA1c.

## 6.5 Discussion

Characterization of the relationship between gastric emptying and GLP-1 secretion is of relevance to the development of 'gut-based' approaches to reduce postprandial hyperglycemia in T2D. The current study showed that: (i) the GLP-1 response to a high carbohydrate meal was unrelated to the rate of gastric emptying in T2D, i.e. postprandial GLP-1 levels did not differ between subjects with faster or slower gastric emptying, (ii) the GLP-1 response to intraduodenal nutrients (glucose, protein and fat)

exhibited substantial inter-individual variation in healthy and T2D subjects, but intra-individual variation to glucose was modest, and (iii) the GLP-1 response to intestinal glucose was related inversely to the intrinsic gastric emptying rate (or directly to the T50) of an oral glucose drink. These observations indicate that, in a given individual, gastric emptying does not determine postprandial GLP-1 secretion, rather that the intrinsic rate of gastric emptying is tuned by the GLP-1 response to small intestinal nutrients. Accordingly, dietary and/or pharmacological strategies that enhance the GLP-1 response to 'intestinal nutrients' may slow gastric emptying to reduce postprandial glycemic excursions.

As anticipated (Part 1), in T2D subjects, relatively more rapid gastric emptying was associated with a greater glycemic response to the mashed potato meal, consistent with the concept that rapid gastric emptying predisposes to postprandial hyperglycemia in T2D. However, the T50 was not related with postprandial GLP-1 secretion after a high carbohydrate meal in T2D subjects. The latter observation apparently contrasts findings derived in experimental models employing intraduodenal perfusion (Ma et al., 2012; Trahair et al., 2012), where standardized intraduodenal macronutrient loads induced dose-dependent GLP-1 secretion, but the potential for small intestinal feedback to modulate gastric emptying was circumvented. In the subgroup analysis of Part 1, despite marked differences in the rate of gastric emptying and, hence, delivery of intraduodenal nutrients between T1 and T3 subgroups, postprandial GLP-1 responses between the two subgroups were comparable. This may suggest that the capacity of intestinal nutrients to induce GLP-

1 release is attenuated in subjects with more rapid gastric emptying – a phenomenon unrelated to age, gender, BMI or glycemic status. Macronutrients exhibit substantial differences in stimulating GLP-1 secretion in humans; fat is generally more potent at inducing GLP-1 secretion relative to glucose and protein, when administered intraduodenally (Ryan et al., 2013; T. Wu, C. K. Rayner, et al., 2017). In Part 2, by standardizing the small intestinal exposure to glucose (4 kcal/min), fat (2 kcal/min) and protein (3 kcal/min) in subjects with and without T2D, via intraduodenal infusion, we affirmed the concept of a substantial inter-individual variation in GLP-1 response to intestinal nutrients is independent of gastric emptying, which may have accounted for the absence of a relationship between gastric emptying and postprandial GLP-1 levels in Part 1.

The mechanisms underlying the differences in GLP-1 response to intraduodenal administered nutrients remain unclear, but could involve differences in small intestinal digestion, transit, and L-cell nutrient sensing. That subjects were free from gastrointestinal disease and that glucose does not require intraluminal digestion excludes these as potential confounders. Since GLP-1-releasing L-cells are most densely located in the distal gut regions, absorption and transit of nutrients in the upper gut may influence the stimulation of distal L-cells. For example, acarbose-induced malabsorption of carbohydrate is associated with stimulation of GLP-1 secretion in both health and T2D (Qualmann et al., 1995; M. Y. Zheng et al., 2013). Similarly, poorly absorbed carbohydrates (e.g., tagatose (T. Wu et al., 2012) and xylose (Vanis et al., 2011)) induce sustained GLP-1 secretion. It is unclear whether the density of

L-cells in either the upper or lower gut, or their capacity to sense intraluminal nutrients, differs substantially between individuals. More recently, evidence has been presented in rodents that somatostatin released from the intestinal D-cells (Jepsen et al., 2019) and the nature of the gut microbiome may influence nutrient-evoked GLP-1 secretion (Wichmann et al., 2013). The respective contributions of these factors to the secretion of GLP-1 induced by intestinal nutrients warrant further investigation.

It is noteworthy that the intra-individual variation in GLP-1 response to intraduodenal glucose infusion was small in both healthy and T2D subjects, indicating that in a given individual, intestinal nutrient-evoked GLP-1 release is tuned at a specific set point, as seen with gastric emptying (Collins, Horowitz, Cook, Harding, & Shearman, 1983). Accordingly, we hypothesized that the variation in GLP-1 response to intestinal nutrients might be related to difference in gastric emptying between individuals. In Part 3, we evaluated both the intrinsic gastric emptying rate of oral glucose and the GLP-1 response to intraduodenally administered glucose in a cohort of subjects with and without T2D, and observed a direct relationship between the T50 and the GLP-1 response to intraduodenal glucose. It, therefore, appears that individuals with lower GLP-1 response to intestinal nutrient stimulation (probably reflecting that the intestine is less responsive to nutrients) would naturally exhibit more rapid gastric emptying of an oral meal, while those with greater GLP-1 response have slower gastric emptying; or vice versa.

Several limitations in the current study should be noted. First, blood glucose concentrations were clamped at different levels while evaluating the intra-subject variations in the GLP-1 response to intraduodenal glucose. However, variations in peripheral glucose concentrations (due to intravenous glucose infusion) do not affect GLP-1 secretion in either healthy or T2D subjects (X. Zhang et al., 2019). Although hyperglycemia may potentially affect GI motility (Kuo et al., 2010; Lingenfelser, Sun, Hebbard, Dent, & Horowitz, 1999), the GLP-1 response to intraduodenal glucose infusion did not differ between the two study days in either group. Second, we did not delineate the mechanisms accounting for the inter-individual variation in intestinal nutrient-induced GLP-1 secretion. Third, in Part 2, the intraduodenal glucose, protein and fat was administrated at different caloric rates and duration. The varied rates of infusion, however, reflected the potency of each at stimulating GLP-1 secretion. Fourth, the subjects included in each part of our analysis had specific clinical traits. For example, in Part 2, the GLP-1 response to intraduodenal glucose, fat and protein were evaluated in different groups. However, these datasets complemented each other to show consistently a high level of inter-individual variation in intestinal nutrient-induced GLP-1 secretion. In Part 3, we included a small sample size of male subjects with mixed glycemic status, and did not assessed plasma GLP-1 levels during the oral glucose tolerance test. Accordingly, caution should be exercised in generalizing our findings to a broader population. Fifth, the current findings may not be applicable in setting where gastric emptying differs substantial from the 'normal' range. For example, gastric emptying is markedly accelerated in patients who have undergone bariatric surgery, in whom the mechanisms involved in the regulation of gastric

emptying would most likely be disrupted. Finally, we note that the secretion of glucose - dependent insulinotropic polypeptide (GIP), another incretin hormone released predominantly from the proximal small intestine, is expected to show a linear relationship with the rate of gastric emptying (or the rate of nutrient entry into the small intestine). However, since GIP does not slow gastric emptying (Meier et al., 2004), we did not measure GIP in the current study.

In conclusion, while gastric emptying does not predict postprandial GLP-1 secretion, the intrinsic rate of gastric emptying in a given individual is related to GLP-1 response to a standardized intestinal nutrient stimulus. In T2D subjects with more rapid gastric emptying, therapies that improve the GLP-1 response may represent a novel approach to slow gastric emptying and reduce postprandial hyperglycemia.

**Table 6.1. Table 1. Comparison of three groups of type 2 diabetes (T2D) subjects with slower, intermediate, and faster gastric emptying rate of a high carbohydrate meal (mean values ± SEM).**

| | **Slower (T1) (n = 24)** | **Intermediate (T2) (n = 25)** | **Faster (T3) (n = 24)** | **T1 vs. T3 P value** |
|---|---|---|---|---|
| **Gender (male/female)** | 12/12 | 12/13 | 11/15 | 0.8 |
| **Age (years)** | 65.0 ± 1.4 | 64.5 ± 1.1 | 64.8 ± 1.6 | 0.8 |
| **BMI (kg/m$^2$)** | 30.2 ± 0.9 | 29.9 ± 1.2 | 29.8 ± 1.0 | 0.8 |
| **Duration of T2D (years)** | 3.6 ± 0.8 | 6.0 ± 1.1 | 6.3 ± 0.9 | 0.01 |
| **HbA1c (%)** | 6.5 ± 0.1 | 6.7 ± 0.1 | 6.8 ± 0.1 | 0.07 |
| **HbA1c (mmol/mol)** | 47.8 ± 0.8 | 50.1 ± 1.3 | 50.4 ± 1.1 | 0.07 |
| **Usage of metformin (n)** | 11 | 11 | 14 | 0.6 |
| **Baseline blood glucose (mmol/L)** | 7.4 ± 0.2 | 7.9 ± 0.2 | 8.5 ± 0.3 | 0.005 |
| **Baseline plasma GLP-1 (pmol/L)** | 20.2 ± 1.4 | 22.8 ± 1.9 | 23.1 ± 1.8 | 0.2 |
| **Gastric half-emptying time (T50, min)** | 87 ± 2 | 70 ± 1 | 56 ± 1 | <0.001 |

**Table 6.2. Characteristics of healthy and T2D subjects receiving intraduodenal glucose (4 kcal/min, for 30 min), protein (3 kcal/min for 60 min) and fat (2 kcal/min for 120 min) infusion. Data are means ± SEM.**

| | | Intraduodenal glucose (4 kcal/min) | | | Intraduodenal protein (3 kcal/ min) | Intraduodenal fat (2 kcal/min) |
|---|---|---|---|---|---|---|
| | | **Healthy subjects (n =11)** | **T2D subjects (n = 12)** | | **Healthy subjects (n = 10)** | **T2D subjects (n = 15)** |
| **Gender (male/female)** | | 9/2 | 4/8 | | 10/0 | 10/5 |
| **Age (years)** | | 31.0 ± 3.2 | 61.0 ± 5.4 | | 74.4 ± 1.5 | 68.8 ± 2.2 |
| **BMI (kg/m$^2$)** | | 25.1 ± 1.6 | 27.6 ± 1.3 | | 26.2 ± 0.6 | 30.2 ± 1.3 |
| **HbA1c (%)** | | - | 6.3 ± 0.3 | | - | 6.7 ± 0.2 |
| **HbA1c (mmol/mol)** | | - | 45.1 ± 2.7 | | - | 49.3 ± 2.1 |
| **Duration of T2D (years)** | | - | 5.3 ± 0.9 | | - | 6.6 ± 1.5 |
| **Usage of metformin (n)** | | - | 0 | | - | 8 |
| **GLP-1 response (pmol/L*min$^{-1}$)** | **iAUC$_{0\text{-}60min}$ (euglycemia)** | 434.2 ± 198.3 | 1037.5 ± 184.9 | **iAUC$_{0\text{-}60min}$** | 361.9 ± 79.0 | - |
| | **iAUC$_{0\text{-}60min}$ (hyperglycemia)** | 648.2 ± 236.0 | 1131.46 ± 287.8 | **iAUC$_{0\text{-}120min}$** | - | 2644.5 ± 330.2 |
| **CV$_{inter}$** | **Euglycemia** | 144.4% | 59.1% | | 65.5% | 48.4% |
| | **Hyperglycemia** | 115.1% | 84.4% | | | |
| **CV$_{intra}$** | | 31.5% | 24.5% | | | |
| **ICC (euglycemia vs. hyperglycemia)** | | 0.965 | 0.832 | | - | - |

The inter-subject variability of the GLP-1 response, i.e. the coefficient of variation ($CV_{inter}$) of the GLP-1 iAUC on each study day. The intra-subject variation in plasma GLP-1 response to intraduodenal glucose was represented by coefficient of variation ($CV_{intra}$) and the intraclass correlation coefficient (ICC) (two-way mixed model effect).

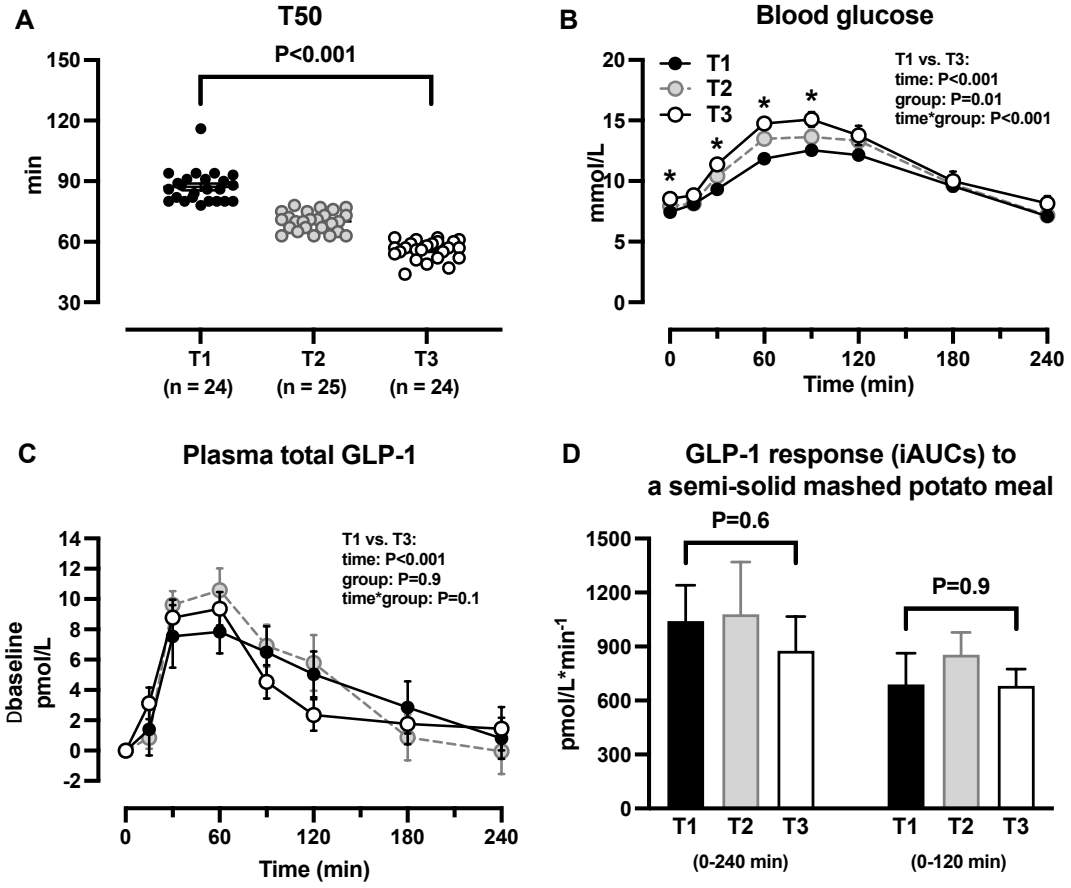


**Figure 6.1.** Based on their gastric emptying of a mashed potato meal, subjects with T2D were categorised into tertiles (T1, T2 and T3). (A) Gastric half emptying time (T50) in the three groups. Unpaired Student's t tests were used to determine statistical significance between T1 and T3. Blood glucose (B) and changes in plasma total GLP-1 levels (C) after the mashed potato meal in the three groups. Repeated-measures ANOVA was used to determine statistical significance between T1 and T3, and ANOVA results are reported as P values for differences over time and by group, and for group by time interactions. Post hoc comparisons were adjusted by Bonferroni correction. (C) Plasma total GLP-1 levels ($iAUC_{0\text{-}240min}$ and $iAUC_{0\text{-}120min}$) in the three groups. Unpaired Student's t tests were used to determine statistical significance between T1 and T3. Data are mean values ± SEM.

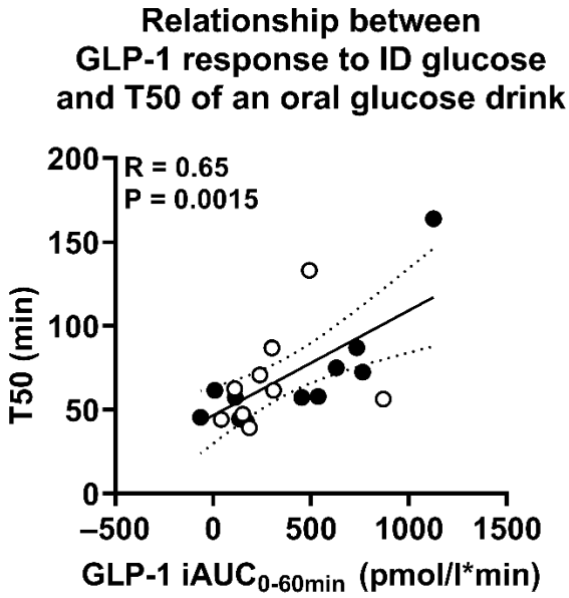


**Figure 6.3.** Relationship between GLP-1 responses to intraduodenal glucose infusion (integrated area under the curve from baseline for plasma GLP-1 over 60min (GLP-1 $iAUC_{0\text{-}60min}$)) and the gastric half-emptying time (T50) of 75 g glucose drink in male subjects with (n = 12) and without (n = 9) type 2 diabetes (total n = 21). White circle: healthy subjects; black circle: subjects with type 2 diabetes.

# CHAPTER 7: ROLE OF ENDOGENOUS GLUCAGON-LIKE PEPTIDE-1 IN THE GLYCAEMIC AND ENERGY EXPENDITURE RESPONSES TO VILDAGLIPTIN DURING INTRADUODENAL FAT INFUSION IN TYPE 2 DIABETES

# STATEMENT OF AUTHORSHIP

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| Overall percentage | 60% | | |
| 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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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 | September 2021 |

## 7.1 Abstract

**Aims:** Dipeptidyl peptidase-4 (DPP-4) inhibitors prolong the action of endogenous glucagon-like peptide-1 (GLP-1), the secretion of which is influenced by dietary macronutrients, particularly fat. In health, the DPP-4 inhibitor, vildagliptin, has been shown to lower blood glucose and glucagon and increase energy expenditure during an intraduodenal fat infusion. We evaluated the effects of vildagliptin on these responses, and the contribution of endogenous GLP-1-signalling in type 2 diabetes (T2D).

**Methods:** 15 T2D subjects, managed by diet and/or metformin (HbA1c 6.7 ± 0.2%), were studied on 3 occasions (two with vildagliptin and one placebo) in a double-blind, randomised, crossover fashion. On each day, vildagliptin (50 mg) or placebo was given orally, followed by intravenous exendin(9-39) (600 pmol/kg/min, on 1 of the 2 vildagliptin treatment days) or 0.9% saline over 180 min. Between 0-120 min, a fat emulsion was infused intraduodenally at 2 kcal/min. Energy expenditure, plasma glucose and glucose-regulatory hormones were evaluated.

**Results:** Intraduodenal fat increased plasma GLP-1 and GIP, insulin and glucagon, and energy expenditure, and decreased plasma glucose (all $P < 0.05$). On the two intravenous saline days, plasma glucose and glucagon were lower, plasma intact GLP-1 was higher (all $P < 0.05$), and energy expenditure tended to be less after vildagliptin ($P = 0.08$) than placebo. On the two vildagliptin days, plasma glucose, glucagon and GLP-1 (both total and intact), and energy expenditure were higher during intravenous exendin(9-39) than saline (all $P < 0.05$).

**Conclusion:** In well controlled T2DM during intraduodenal fat infusion, vildagliptin lowered plasma glucose and glucagon, and tended to decrease energy expenditure, effects that were mediated by endogenous GLP-1.

## 7.2 Introduction

The incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), are secreted from the gut upon nutrient exposure and, while degraded rapidly by dipeptidyl peptidase 4 (DPP-4), account for the substantially greater insulin response to enteral, compared to intravenous (IV), glucose in health (Marathe et al., 2014; M. A. Nauck et al., 1986; X. Zhang et al., 2019). In type 2 diabetes (T2D), the insulinotropic effect of GIP is markedly diminished, whereas that of GLP-1 remains relatively intact (M. A Nauck et al., 1993). Although the insulinotropic response to 'exogenous' GIP was shown to be partially restored in T2D with good/normalised glycaemic control (Aaboe et al., 2015; Højberg et al., 2008), its contribution to glucose metabolism appeared , if any, relatively modest (T. Wu, Ma, et al., 2014). That activation of GLP-1 receptors is also linked to slowing of gastric emptying (Deane, Nguyen, et al., 2010) and suppression of glucagon (Hare et al., 2010) and energy intake (Steinert et al., 2017) has stimulated the development and current widespread use of GLP-1-based therapies for T2D.

Both DPP-4-resistant GLP-1 receptor agonists (GLP-1RAs) and DPP-4 inhibitors are mainstream options for glycaemic control in T2D, which lower blood glucose with minimal, if any, risk of hypoglycaemia when used as monotherapy (T. Wu, Rayner, et al., 2016a). In contrast to GLP-1 RAs, which promote weight loss, DPP-4 inhibitors are weight neutral (R. E. Pratley et al., 2010; Rosenstock et al., 2019), and their glucose-lowering efficacy in T2D appears to be largely (although not exclusively) dependent on the secretion of endogenous GLP-1 (Aulinger et al., 2014; M. A. Nauck

et al., 2016), and is influenced by dietary intake and the rate of nutrient entry into the small intestine (i.e. gastric emptying) (T. Wu et al., 2010). For example, administration of either a poorly absorbed carbohydrate (xylose) or whey protein before a carbohydrate meal increases GLP-1 secretion, slows gastric emptying and augments the lowering of postprandial blood glucose by DPP-4 inhibitors (T. Wu, M. J. Bound, B. Y. R. Zhao, et al., 2013; T. Wu, Little, et al., 2016). Furthermore, in T2D, the reduction in blood glucose by DPP-4 inhibitors is greater when the rate of intraduodenal glucose infusion is higher, an effect attributable to increased GLP-1 secretion (T. Wu, Zhang, et al., 2016). While it is appreciated that fat is more potent than glucose in stimulating GLP-1 (T. Wu, C. K. Rayner, et al., 2017), the relevance of the fat content of a meal to the effects of DPP-4 inhibition is poorly defined.

Although DPP-4 inhibitors do not affect appetite or energy intake, emerging preclinical and clinical evidence has increasingly linked DPP-4 inhibition to changes in energy expenditure. In a high-fat-fed mouse model of T2D, DPP-4 inhibitors (e.g. teneligliptin and sitagliptin) have been reported to increase energy expenditure and reduce adiposity and body weight (Fukuda-Tsuru et al., 2014; Goldsmith et al., 2015), effects absent in GLP-1 receptor knockout mice (Goldsmith et al., 2015). These observations suggest a key role of GLP-1-signalling in the regulation of energy homeostasis in T2D. Indeed, GLP-1RAs have been reported to induce browning of white adipose tissue and increase β-oxidation of fatty acids and energy expenditure in rodents (Beiroa et al., 2014; Lynch et al., 2016; Xu et al., 2016). By contrast, a recent meta-analysis suggests that administration of GLP-1RAs has, if any, little effect on

energy expenditure in humans (Maciel et al., 2018). Moreover, intravenous infusion of GLP-1, either alone or in combination with peptide YY (an appetite-regulating hormone co-released with GLP-1 (Steinert et al., 2017)), does not affect energy expenditure in healthy humans (Schmidt et al., 2014). However, the effects of endogenous GLP-1 signalling on energy expenditure has not been well characterised. We recently observed in healthy humans that during an intraduodenal fat infusion (2 kcal/min, a rate within the physiological range of gastric emptying (L. K. Phillips et al., 2015)), a single dose of the DPP-4 inhibitor, vildagliptin (50 mg), increased plasma intact GLP-1 and GIP, lowered plasma glucose and increased energy expenditure (75 kcal/day) and the thermic effect of feeding (TEF) (~60%) (Heruc et al., 2014); the latter would favour a reduction in body weight with sustained use of DPP-4 inhibitors. The fact that DPP-4 inhibitors are weight-neutral in subjects with T2D therefore suggests that the effect of DPP-4 inhibition on energy expenditure may be compromised in this disorder.

The current study was designed to clarify the effect of DPP-4 inhibition on the glycaemic and energy expenditure responses to fat in T2D, including the role of endogenous GLP-1, assessed using the GLP-1 receptor antagonist, exendin(9-39). As in our previous study (Heruc et al., 2014), fat was administered at 2kcal/min via intraduodenal infusion to circumvent the potential influence of variations in gastric emptying. We hypothesised that vildagliptin would lower plasma glucose and increase energy expenditure during intraduodenal fat infusion, and that these effects would be abolished by exendin(9-39).

## 7.3 Methods

### *7.3.1 Subjects*

20 subjects with T2D, managed by diet and/or metformin monotherapy (at a stable dose of 500-2000 mg/day for ≥ 3 months), were recruited after providing written informed consent. Two subjects withdrew prior to the first study visit, and 3 withdrew before completing the study, due to intolerance of the nasoduodenal catheter (2 subjects) or the intraduodenal fat infusion (1 subject, on the placebo/saline day). Accordingly, 15 subjects (10 male and 5 female, mean age 68.8 ± 2.2 years, BMI 30.2 ± 1.3 kg/m$^2$, HbA1c 6.7 ± 0.2% (49.3 ± 2.1 mmol/mol), and duration of known diabetes 6.6 ± 1.5years) completed the study and were included in the final analysis. None had impaired liver or renal function, diabetic microvascular complications, was a smoker, or was taking medication known to affect gastrointestinal function. The protocol (**Figure 7.1**) was approved by the Royal Adelaide Hospital Human Research Ethics Committee and prospectively registered on the Australian New Zealand Clinical Trials Registry (ACTRN12614001117606).

### *7.3.2 Protocol*

Subjects were each studied on 3 days, separated by at least 7 days, in a double-blind, randomised, placebo-controlled, crossover fashion, facilitated by the Royal Adelaide Hospital Pharmacy. Subjects were asked to maintain their usual diet between study days and to refrain from vigorous exercise and alcohol for 24 hours prior to each

study visit. Subjects on metformin were instructed to cease this for 48 hours before each study visit. On the evening before each study day (~1900h), subjects consumed a standardised beef lasagne meal (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia), and then fasted from solids and liquids (with the exception of water which was allowed until 2200h) until the following morning, when they attended the Clinical Research Facility of the Adelaide Medical School at the University of Adelaide.

On each study day, a multi-lumen silicone catheter (Dentsleeve International, Ontario, Canada) was inserted transnasally and allowed to pass into the duodenum by peristalsis, with its position monitored continuously by measurement of the antral and duodenal transmucosal potential difference, as previously (Heruc et al., 2014; T. Wu, Ma, et al., 2014; T. Wu, Zhang, et al., 2016). An infusion port was positioned 12 cm distal to the pylorus. A cannula was inserted into a forearm vein to allow for IV infusion of exendin(9-39) or 0.9% saline. An IV cannula was placed on the other arm for repeated blood sampling and the arm kept warm with a heat pad for sampling of "arterialised" blood. Subjects then rested in a supine position for 30min. Subsequently, fasting energy expenditure and respiratory quotient (RQ) were measured over 30 min (t = -90 to -60min) by indirect calorimetry, using a clear ventilated hood and the TrueOne®2400 metabolic monitoring system (Parvo Medics, East Sandy, UT 84093, USA). The hood was then removed, and a baseline blood sample collected. At t = -60 min, vildagliptin 50 mg or a matching placebo tablet was taken orally with 50 mL water, followed immediately by an IV infusion of exendin(9-39) (Bachem AG, Bubendorf, Switzerland) at 600pmol/kg/min (shown to

achieve >95% inhibition of GLP-1 receptor activity (Schirra & Goke, 2014)) on one of the two vildagliptin treatment days, or 0.9% saline on the other 2 days, over 180 min (i.e. between t = -60-120 min). Accordingly, the three treatments were: (i) oral placebo + IV 0.9% saline (PLBO/NS), (ii) oral vildagliptin + IV 0.9% saline (VILD/NS), and (iii) oral vildagliptin + IV exendin(9-39) (VILD/EX9-39), respectively. Between t=0-120min, a fat emulsion (20% Intralipid; Fresenius Kabi, Hornsby, NSW, Australia) was infused intraduodenally at 2 kcal/min (1 mL/min). Measurements of energy expenditure and RQ were repeated between t = 15-45 and t = 90-120 min. Blood samples were collected at t = -60, 0, 15, 30, 45, 60, 90, 120 min for measurements of plasma glucose, insulin, glucagon, total GIP, and total and intact GLP-1. Samples were stored on ice and in tubes containing EDTA and DPP-4 inhibitor (DPP4-010; Linco Research Inc., St. Charles, MO, USA) before centrifugation at 3200 rpm for 15 min at 4 ℃. Plasma was separated and stored at -80 ℃ for subsequent analysis. Gastrointestinal symptoms and appetite sensations were evaluated at the same time points as blood sampling using 100 mm visual analogue scales (B. A. Parker et al., 2004). At t = 120 min, the ventilated hood and intraduodenal catheter were removed and the participant provided with a mixed nutrient meal before leaving the laboratory.

### *7.3.3 Measurements of plasma glucose and hormone concentrations*

Plasma glucose was measured using the glucose oxidase technique (2300 STAT Plus; YSI, Yellow Springs, OH, USA). Plasma insulin was measured by ELISA immunoassay (10-1113, Mercodia, Uppsala, Sweden). Plasma glucagon and (total

and intact) GLP-1 were measured by radioimmunoassay (GL-32K, GLPIT-36HK and GLPIA-35HK, respectively, Millipore, Billerica, MA, USA). Plasma total GIP were measured by radioimmunoassay using some modifications of a previously published method (J. Wishart, Morris, & Horowitz, 1992). The standard curve was prepared in buffer rather than extracted charcoal stripped serum and the radio-iodinated label was supplied by Perkin Elmer (Boston, MA, USA).

### *7.3.4 Assessments of energy expenditure, RQ and TEF*

To ensure that subjects had reached equilibrium, the first 10min of data from each period were discarded, and the remaining values were averaged to provide energy expenditure and RQ at baseline (t = -90 to -60 min), and during intraduodenal fat infusion (mean of values between t = 15-45 min and 90-120 min) as previously (Heruc et al., 2014; Luscombe, Clifton, Noakes, Parker, & Wittert, 2002). RQ was determined as the ratio of $VCO_2/VO_2$. A value of 0.7 is indicative of fat oxidation, whereas a value of 0.8-1.0 is indicative of mixed oxidation of nutrients (e.g., protein and carbohydrate) (Heruc et al., 2014; Luscombe et al., 2002). The TEF was assessed by subtracting baseline energy expenditure from the mean energy expenditure values during intraduodenal fat infusion, expressed as percent energy consumed during intraduodenal fat infusion.

### *7.3.5 Statistical Analysis*

Fasting plasma glucose and hormone levels at t = -60 and 0 min were compared using one-factor repeated-measures ANOVA. The differences between t = -60 and 0 min on

each study day were compared using paired Student's t-test. Plasma glucose, hormones and visual analogue scale scores between PLBO/NS and VILD/NS and between VILD/NS and VILD/EX9-39 were analysed by two-factor repeated measures ANOVA, using treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs showed significant interaction between treatment and time. The baseline energy expenditure and RQ were compared using one-factor repeated measures ANOVA. Differences in energy expenditure, RQ and TEF between baseline and post-infusion and between two specified treatments (i.e. PLBO/NS vs. VILD/NS, and VILD/NS vs. VILD/EX9-39) were compared using paired Student's t test after confirming their normality of distribution. The relationship between energy expenditure and the incremental areas under the curves (iAUCs) for plasma glucagon and GLP-1 was evaluated using univariate linear regression analysis. Based on our previous work (Heruc et al., 2014), a sample size of 15 subjects was calculated to have at least 80% power at $\alpha = 0.05$ to detect a difference of 75 kcal/day (with an SD of 76 kcal/day) in the effect of intraduodenal fat on energy expenditure between treatments. All analyses were performed using Prism 8.1 software (GraphPad, La Jolla, CA, USA). Data are presented as mean values ± SEM; $P < 0.05$ was considered statistically significant.

## 7.4 Results

The 15 subjects who completed the protocol all tolerated the study well. Total data for energy expenditure, RQ and TEF were available for only 14 subjects, because of technical issues during indirect calorimetry in one subject.

### *7.4.1 Plasma glucose*

Fasting plasma glucose at t = -60 min did not differ between the 3 study days (**Table 7.1**). During the first hour of IV infusion (t = -60-0 min), fasting plasma glucose decreased by ~0.3-0.4 mmol/L on the PLBO/NS and VILD/NS days, but increased by ~0.5 mmol/L on the VILD/EX9-39 day (all $P < 0.05$). During intraduodenal fat infusion (t = 0-120 min), there was a further modest reduction in glucose concentrations on the PLBO/NS and VILD/NS days (time effect: $P < 0.05$ for each), while they remained relatively stable on the VILD/EX9-39 days, such that plasma glucose concentrations were lower with VILD/NS vs. PLBO/NS (treatment effect: $P = 0.04$, treatment by time interaction: $P < 0.001$, with a significant difference at t = 60-120 min), and higher with VILD/EX9-39 vs. VILD/NS (treatment effect: $P < 0.001$; treatment by time interaction: $P < 0.001$, with significance differences between t = 0-120 min) (**Figure 7.2A**).

### *7.4.2 Plasma insulin*

Fasting plasma insulin at t = -60 min did not differ (**Table 7.1**), and remained unchanged during the first hour of IV infusion (t = -60-0 min) on all 3 study days. During intraduodenal fat infusion (t = 0-120 min), plasma insulin increased modestly on all 3 study days (time effect: $P < 0.05$ each), without any difference between PLBO/NS and VILD/NS or between VILD/NS and VILD/EX9-39 (**Figure 7.2B**).

### *7.4.3 Plasma glucagon*

Fasting plasma glucagon at t = - 60 min did not differ between the 3 study days (**Table 7.1**). During the first hour of IV infusion (t = -60-0 min), fasting glucagon remained unchanged on the PLBO/NS and VILD/NS days, but increased on the VILD/EX9-39 days ($P < 0.01$). During intraduodenal fat infusion (t = 0-120 min), plasma glucagon increased markedly and progressively on all 3 study days (time effect: $P < 0.05$ each), but plasma glucagon concentrations were lower after VILD/NS vs. PLBO/NS (treatment effect: $P < 0.05$), and higher with VILD/EX9-39 vs. VILD/NS (treatment effect: $P < 0.001$; treatment by time interaction: $P < 0.001$, with significant differences between t = 0-120 min) (**Figure 7.2C**).

### *7.4.4 Plasma total and intact GLP-1*

Fasting plasma total and intact GLP-1 did not differ between the 3 study days (**Table 7.1**). During the first hour of IV infusion (t = -60-0 min), plasma total GLP-1 remained unchanged on the PLBO/NS and VILD/NS days, but increased on the VILD/EX9-39 days ($P < 0.01$). However, intact GLP-1 levels were unchanged on all 3 study days. During intraduodenal fat infusion (t = 0-120 min), plasma total GLP-1 increased minimally within the first 15 minutes, but substantially thereafter on all 3 study days (time effect: $P < 0.001$ each), such that plasma total GLP-1 concentrations tended to be lower with VILD/NS vs. PLBO/NS (treatment effect: $P = 0.099$), and were higher with VILD/EX9-39 vs. VILD/NS (treatment effect: $P < 0.001$; treatment by time interaction: $P < 0.001$, with significant differences between t = 0-120 min) (**Figure 7.2D**). Plasma intact GLP-1 also increased on all 3 study days (time effect: $P < 0.001$

each), with concentrations being higher with VILD/NS vs. PLBO/NS (treatment effect: $P < 0.001$; treatment by time interaction: $P<0.001$, with significant differences between t = 45-120 min), and higher with VILD/EX9-39 vs. VILD/NS (treatment effect: $P < 0.001$; treatment by time interaction: $P = 0.02$, with significant differences between t = 30-60 min) (**Figure 7.2E**).

### *7.4.5 Plasma total GIP*

Fasting plasma total GIP at t = -60 min did not differ between the 3 study days (**Table 7.1**) and remained unchanged during the first hour of IV infusion (t = -60-0 min). During intraduodenal fat infusion (t = 0-120 min), plasma GIP levels increased promptly on all 3 study days (time effect: $P < 0.001$ each), but plasma GIP concentrations were lower with VILD/NS vs. PLBO/NS (treatment effect: $P = 0.02$; treatment by time interaction: $P = 0.01$, with a significant difference at t = 60 min) and higher with VILD/EX9-39 vs. VILD/NS (treatment by time interaction: $P = 0.02$, with a significant difference at t = 45 min) (**Figure 7.2F**).

### *7.4.6 Energy expenditure, RQ, and TEF*

Neither fasting energy expenditure nor RQ differed between the 3 study days (**Table 7.1**). During intraduodenal fat infusion (t = 0-120 min), there was an increase in energy expenditure on all 3 study days ($P < 0.05$ each), such that energy expenditure was greater after VILD/EX9-39 than VILD/NS (1745 ± 78 vs. 1671 ± 68 kcal/day, $P < 0.05$), and tended to be less after VILD/NS than PLBO/NS (1671 ± 68 vs. 1712 ± 72 kcal/day, $P = 0.08$) (**Figure 7.3A**). By contrast, RQ decreased comparably in

response to intraduodenal fat infusion on all 3 study days ($P < 0.01$ each), indicative of increased fat oxidation (**Figure 7.3B**). Similar to energy expenditure, TEF was greater after VILD/EX9-39 than VILD/NS (13.0 ± 2.4 vs. 4.9 ± 1.8%, $P < 0.05$), and tended to be less after VILD/NS than PLBO/NS (9.4 ± 1.3 vs. 4.9 ± 1.8%, $P = 0.09$) (**Figure 7.3C**).

### *7.4.7 GI symptoms and appetite sensations*

Intraduodenal fat infusion was associated with modest increases in nausea and bloating (time effect: $P < 0.05$ each), without any difference between the treatments. Appetite sensations (including hunger, fullness, desire to eat and prospective consumption) were minimally altered during intraduodenal fat infusion on all 3 study days, without any difference between the treatments (data not shown).

### *7.4.8 Relationships between variables*

By pooling the data from the 3 study days, the increment in energy expenditure induced by intraduodenal fat infusion was related directly to the $iAUC_{(-60\text{-}120min)}$ for glucagon ($r = 0.43$, $P = 0.006$) (**Figure 7.4**), but not related to the changes in total or intact GLP-1.

## 7.5 Discussion

In the current study of subjects with T2D, we evaluated the effects of endogenous GLP-1-signalling on the glycaemic and energy expenditure responses to DPP-4 inhibition during an intraduodenal fat infusion that induced marked secretion of both

GLP-1 and GIP. Under this unique experimental setup, we observed that (i) acute dosing with the DPP-4 inhibitor vildagliptin increased plasma intact GLP-1, lowered plasma glucose and glucagon, and tended to decrease energy expenditure and TEF, and (ii) exendin(9-39) abolished the lowering of plasma glucose by vildagliptin, and augmented plasma glucagon, energy expenditure and TEF. These observations therefore support a major role of endogenous GLP-1 in the glycaemic and energy expenditure responses to dietary fat in T2D, and provide a rationale for combining interventions that increase energy expenditure with GLP-1-based therapies for the management of obesity and T2D.

Consistent with our previous study in healthy subjects (Heruc et al., 2014; T. Wu, C. K. Rayner, et al., 2017), intraduodenal infusion of fat at 2 kcal/min induced substantial GIP and GLP-1 secretion in subjects with T2D. Furthermore, as anticipated, vildagliptin markedly increased plasma intact GLP-1 and reduced plasma total GLP-1 concentrations modestly, consistent with the concept of negative feedback on the regulation of GLP-1 secretion (Bock et al., 2009; M. A. Nauck et al., 2016; T. Wu, Ma, et al., 2014). Indeed, blockade of GLP-1-signalling with exendin(9-39) increased both fasting and intraduodenal fat-induced GLP-1 secretion substantially, a phenomenon also evident when IV exendin(9-39) was administered with oral glucose (Aulinger et al., 2014) or a mixed meal (M. A. Nauck et al., 2016). Likewise, plasma total GIP levels were also reduced by vildagliptin (intact GIP was not measured). Surprisingly, exendin(9-39) was associated with an early, albeit modest, increase in plasma total GIP. Since GLP-1 accounts for the slowing of gastric emptying and small

intestinal transit, i.e. the so-called "ileal brake", antagonism of GLP-1 may have resulted in an increase in the transit of fat within the proximal small intestine and therefore stimulated additional GIP secretion– our recent study in both healthy subjects and those with T2D has shown that the distal small intestine is also capable of secreting GIP (T. Wu et al., 2015; X. Zhang et al., 2019).

Intraduodenal fat was associated with a modest reduction in plasma glucose and increases in plasma insulin and glucagon; the latter are likely to reflect direct stimulation of the pancreatic α- and β-cells by fatty acids (Hong et al., 2005). As expected, vildagliptin resulted in a further slight reduction in the plasma glucose response to intraduodenal fat infusion, associated with a decrease in plasma glucagon. The observations that antagonism of GLP-1 by exendin(9-39) led to a marked increase in plasma glucagon and abolished the lowering of plasma glucose by vildagliptin, and that neither vildagliptin nor exendin(9-39) altered plasma insulin levels (since plasma glucose concentrations did not exceed a threshold of ~8 mmol/L for the introduction of the incretin effect (M. B. Christensen, Calanna, Holst, Vilsboll, & Knop, 2014)), are indicative of a key role of the GLP-1/glucagon axis in the regulation of plasma glucose in the current experimental setting. Notably, IV exendin(9-39) also increased fasting plasma glucose and glucagon, suggesting that GLP-1, even at low basal levels, has a physiological role in suppressing glucagon secretion and maintaining glucose homeostasis. The contribution of endogenous GIP to the glucose lowering effect of vildagliptin may be further clarified by administering a GIP receptor antagonist. However, blockade of GLP-1 signalling by exendin(9-39) abolished the lowering of

glucose by vildagliptin completely, suggesting that GIP is, if any, a minor player of glucose metabolism in this experimental setup. This is in line with our previous studies in which subjects with relatively well controlled T2D received an intraduodenal infusion of glucose at 2 kcal/min and exhibited marked GIP, but minimal GLP-1 secretion (ie. GIP being the predominant incretin hormone in the peripheral circulation) and the DPP-4 inhibitor sitagliptin, failed to lower blood glucose, an effect however evident in healthy lean and obese subjects (T. Wu, Ma, et al., 2014). Moreover, addition of sitagliptin (that augments endogenous GIP substantially) to the GLP-1RA liraglutide failed to show any meaningful effect on blood glucose in subjects with T2D (M. A. Nauck, Kahle, Baranov, Deacon, & Holst, 2017).

As was reported in health (Heruc et al., 2014), intraduodenal infusion of fat increased energy expenditure and TEF as a result of increased fat oxidation (reflected by a reduction in RQ) in the present study. In contrast to findings in healthy humans(Heruc et al., 2014) and high-fat-fed obese mouse models of T2D (Fukuda-Tsuru et al., 2014; Goldsmith et al., 2015), we observed a tendency for vildagliptin to decrease, rather than increase, energy expenditure and TEF during intraduodenal fat. Moreover, exendin(9-39) increased energy expenditure and TEF substantially. That the effects of vildagliptin and exendin(9-39) on energy expenditure and TEF paralleled the changes in plasma glucagon, suggests that endogenous GLP-1-signalling may exhibit an inhibitory effect on energy expenditure through suppressing glucagon secretion in T2D. Indeed, we observed a positive relationship between the increase in energy

expenditure and plasma glucagon levels. This is in line with an increasingly recognised role of glucagon-signalling in the regulation of energy expenditure (T. Wu, Rayner, Marathe, Jones, & Horowitz, 2018), and the observations that exogenous GLP-1 or GLP-1RAs do not affect fasting energy expenditure, but may decrease diet-induced thermogenesis (Maciel et al., 2018). Accordingly, dual agonism of GLP-1 and glucagon receptors is likely to be useful in the management of obesity and T2D (T. Wu et al., 2018). Emerging clinical trials have shown profound effects of novel GLP-1/glucagon receptor dual agonists on body weight and glycaemic control in overweight/obese subjects with T2D (Ambery et al., 2018; Tillner et al., 2019). On the other hand, antagonism of the GLP-1 receptor in subjects with T2D, while increasing blood glucose, is associated with a favourable increase in energy expenditure; the latter may be protective from insulin resistance, as was seen in GLP-1 receptor knockout mice (Ayala et al., 2010).

The discrepancy in the effects of vildagliptin (or endogenous GLP-1) on energy expenditure and TEF between healthy subjects and those with T2D may also be attributable, at least in part, to the differential effects of GLP-1 on glucagon secretion in the context of different plasma glucose concentrations. In euglycemic healthy subjects, the glucagon-suppressive effect of vildagliptin during intraduodenal fat infusion was marginal, so that the effect of vildagliptin on energy expenditure was minimally confounded by glucagon. By contrast, the suppression of plasma glucose by vildagliptin was predictably more marked in T2D, which may have outweighed any other effects on energy expenditure during DPP-4 inhibition.

Appetite ratings were minimally affected in response to intraduodenal fat infusion, with and without vildagliptin or exendin(9-39), despite differences in GLP-1-signalling. Previous studies have shown that DPP-4 inhibition and antagonism of GLP-1 receptors are also associated with changes in plasma levels of PYY that tend to confound the effect of GLP-1 on appetite and energy intake (Steinert et al., 2014; Ten Kulve et al., 2017). Moreover, the T2D subjects in the current study had a mean age of ~69 years, so that their appetite sensations are expected to be decreased (Rolls, 1992).

Several limitations should be noted in interpreting our findings. First, the number of subjects was relatively small, such that the lack of statistical significance in energy expenditure between vildagliptin and placebo might have reflected a type 2 error. However, this finding contrasted sharply with that made in healthy subjects (Heruc et al., 2014). Moreover, the effects of exendin(9-39) observed were clear-cut. Accordingly, increasing the sample size would be unlikely to alter our conclusions. Second, the delivery of fat into the small intestine was standardised by infusing through a nasoduodenal catheter, which is, by definition, a non-physiological model, but circumvented the influence of variations in gastric emptying on the secretion of the incretin hormones. Third, the subjects involved in the current study had relatively well controlled, uncomplicated T2D, so that the effects of DPP-4 inhibition (and the role of endogenous GLP-1) in subjects with suboptimal glycaemic control and/or complications remain to be determined. Fourth, we infused fat rather than mixed

nutrients in the interest of delineating the relevance of the fat content of a meal to the gut-incretin axis in the regulation of postprandial metabolism. Further studies involving a physiological meal would be of interest. Finally, our study could not differentiate the effects of different fat on the response of the gut-incretin axis since the fat emulsion contains a balanced supply of saturated and unsaturated dietary fat. Given the potential difference in the incretin response to saturated and unsaturated fat (Thomsen, Storm, Holst, & Hermansen, 2003), and that dietary intake of saturated fat may be associated with deteriorated glycaemic control by DPP-4 inhibitors in T2D (Kuwata et al., 2018), studies to evaluate the influence of the type of fat on the metabolic outcomes are warranted.

In summary, in relatively well controlled T2D, acute administration of the DPP-4 inhibitor vildagliptin lowered plasma glucose and glucagon, increased intact GLP-1, and tended to decrease energy expenditure and thermic responses to intraduodenal fat, and these effects were mediated by endogenous GLP-1. These observations highlight the relevance of the fat content of a meal to the gut-GLP-1 axis in the regulation of postprandial plasma glucose and energy expenditure in T2D, particularly when treated with DPP-4 inhibitors.

**Table 7.1. Fasting plasma glucose, insulin, glucagon, total GLP-1, intact GLP-1 and total GIP levels, energy expenditure and respiratory quotient (RQ) on the three study days in subjects with type 2 diabetes. One-factor repeated-measures ANOVA was used to compare the differences at t = -60 and 0 min, respectively. Difference between time points (t = -60 and 0 min) in each study day were compared using Paired Student's t-test was used to compare the differences between t = -60 and 0 min on respective study days; * P < 0.05 and ** P < 0.01 (mean values ± SEM).**

| | t = -60 min | | | | t = 0 min | | | |
|---|---|---|---|---|---|---|---|---|
| | PLBO/NS | VILD/NS | VILD /EX9-39 | ANOVA P value | PLBO/NS | VILD/NS | VILD /EX9-39 | ANOVA P value |
| Glucose (mmol/L) | 6.8 ± 0.5 | 6.7 ± 0.4 | 6.9 ± 0.5 | 0.37 | 6.6 ± 0.4** | 6.3 ± 0.3** | 7.3 ± 0.5** | <0.01 |
| Insulin (mU/L) | 5.2 ± 0.6 | 5.0 ± 0.8 | 5.4 ± 0.8 | 0.68 | 5.2 ± 0.6 | 5.1 ± 0.7 | 5.2 ± 0.9 | 0.96 |
| Glucagon (pg/mL) | 47.8 ± 4.5 | 47.1 ± 4.6 | 48.1 ± 4.6 | 0.94 | 46.9 ± 3.9 | 46.6 ± 4.3 | 58.6 ± 5.6** | <0.01 |
| GLP-1 $_{total}$ (pmol/L) | 19.6 ± 1.6 | 20.3 ± 1.6 | 20.0 ± 1.9 | 0.80 | 18.6 ± 1.4 | 19.6 ± 1.7 | 32.6 ± 2.7** | <0.01 |
| GLP-1 $_{intact}$ (pmol/L) | 10.6 ± 1.2 | 11.0 ± 1.2 | 11.2 ± 1.4 | 0.79 | 10.8 ± 0.9 | 11.1 ± 1.0 | 12.3 ± 0.9 | 0.07 |
| GIP $_{total}$ (pmol/L) | 16.9 ± 1.5 | 16.5 ± 1.1 | 16.3 ± 0.8 | 0.83 | 17.2 ± 1.1 | 16.9 ± 1.1 | 17.7 ± 1.8 | 0.88 |
| Energy expenditure (kcal/day) | 1567 ± 65.7 | 1592 ± 53.0 | 1553 ± 74.1 | 0.63 | - | - | - | - |
| RQ | 0.81 ± 0.02 | 0.83 ± 0.02 | 0.83 ±0.02 | 0.65 | - | - | - | - |

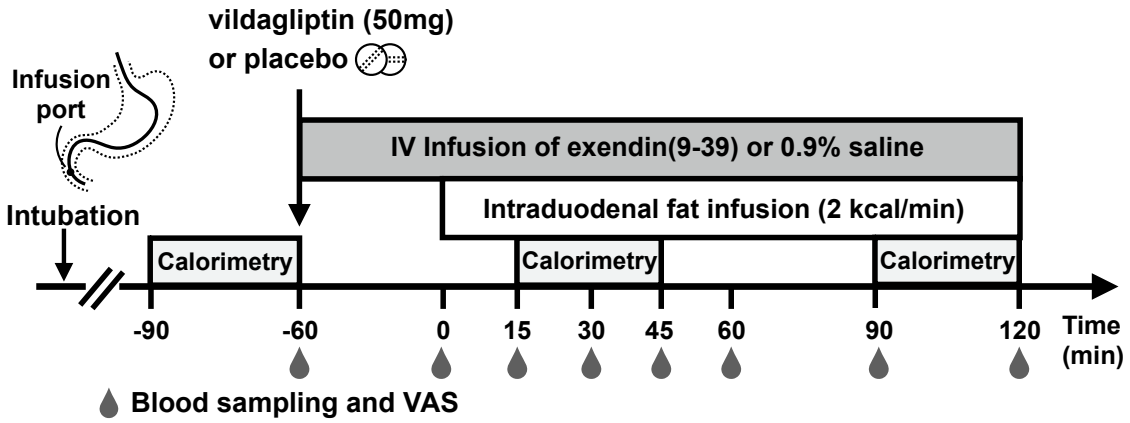


**Figure 7.1.** Schematic representation of the study protocol. VAS, visual analogue scale.

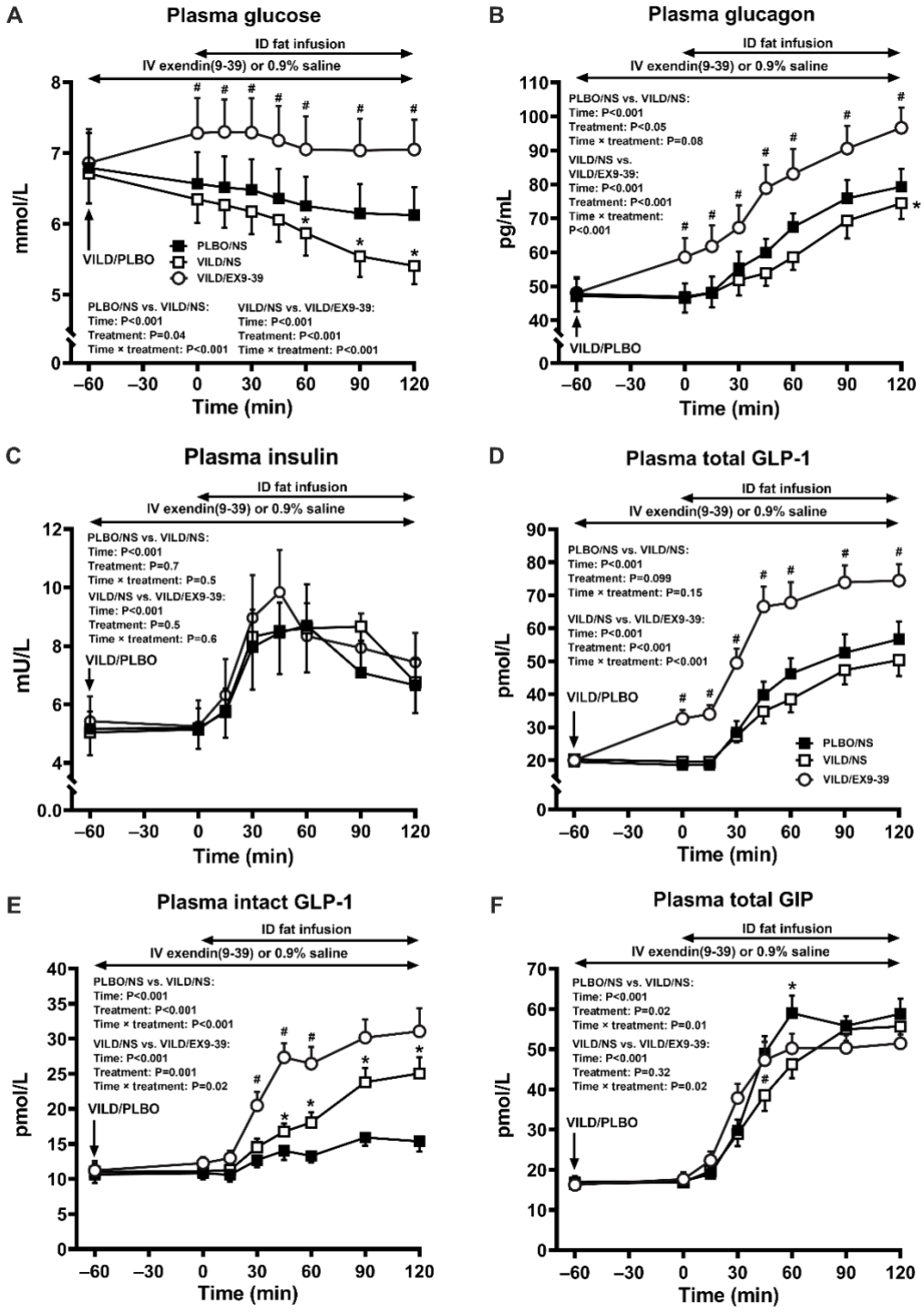


**Figure 7.2.** Plasma glucose (A), insulin (B), glucagon (C), total (D) and intact (E) GLP-1 and total GIP (F) before (t = -60-0 min) and after (t = 0-120 min) intraduodenal (ID) fat infusion (2 kcal/min) in subjects with type 2 diabetes (n = 15). The three

treatments were: (i) oral placebo + IV 0.9% saline (PLBO/NS), (ii) oral vildagliptin (50 mg) + IV 0.9% saline (VILD/NS), and (iii) oral vildagliptin (50 mg) + IV exendin(9-39) (600 pmol/kg/min) (VILD/EX9-39). Two-factor repeated measures ANOVA, with treatment and time as factors, was used to compare the differences between VILD/NS and PLBO/NS, and between VILD/EX-9-39 and VILD/NS; * $P < 0.05$ for PLBO/NS vs. VILD/NS; # $P < 0.05$ for VILD/NS vs. VILD/EX9-39. Data are mean values ± SEM.

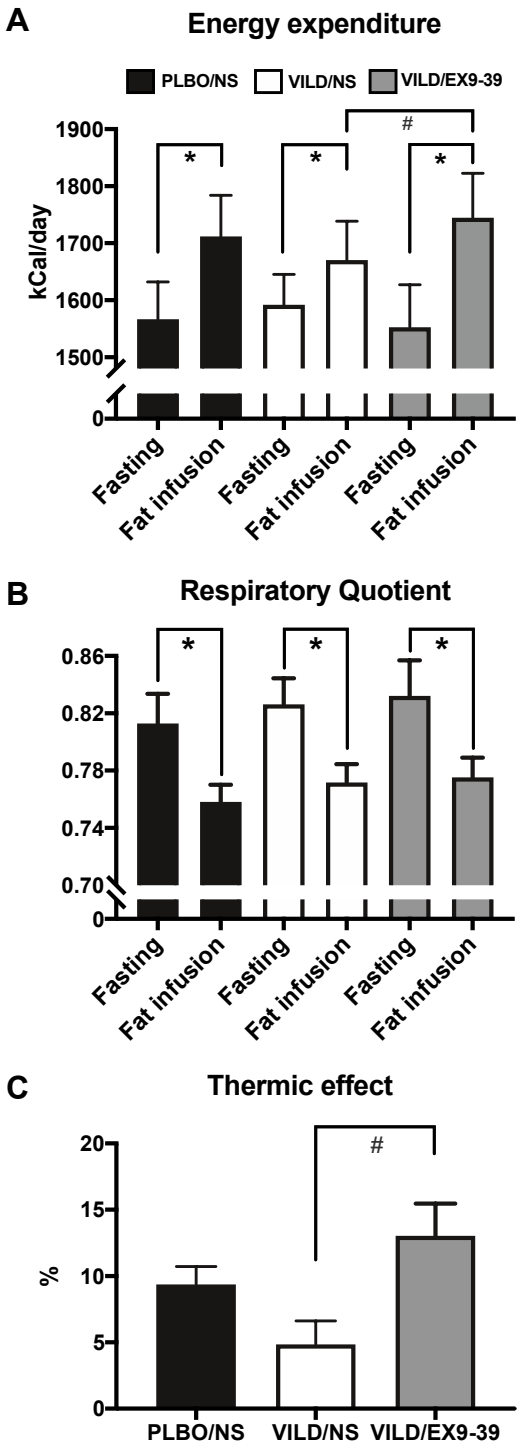


**Figure 7.3**. Energy expenditure (A), respiratory quotient (RQ) (B), and the thermic effect (C) in response to intraduodenal (ID) fat infusion (2 kcal/min) in subjects with type 2 diabetes (n = 14). The three treatments were: (i) oral placebo + IV 0.9% saline (PLBO/NS), (ii) oral vildagliptin (50 mg) + IV 0.9% saline (VILD/NS), and (iii) oral vildagliptin (50 mg) + IV exendin(9-39) (600 pmol/kg/min) (VILD/EX9-39). Paired Student's t-test was used to compare the differences before and after ID fat infusion, and the differences between VILD/NS and PLBO/NS, and between VILD/EX-9-39 and VILD/NS; * $P < 0.05$ for fasting vs. fat infusion; # $P < 0.05$ for VILD/NS vs. VILD/EX9-39. Data are mean values ± SEM.

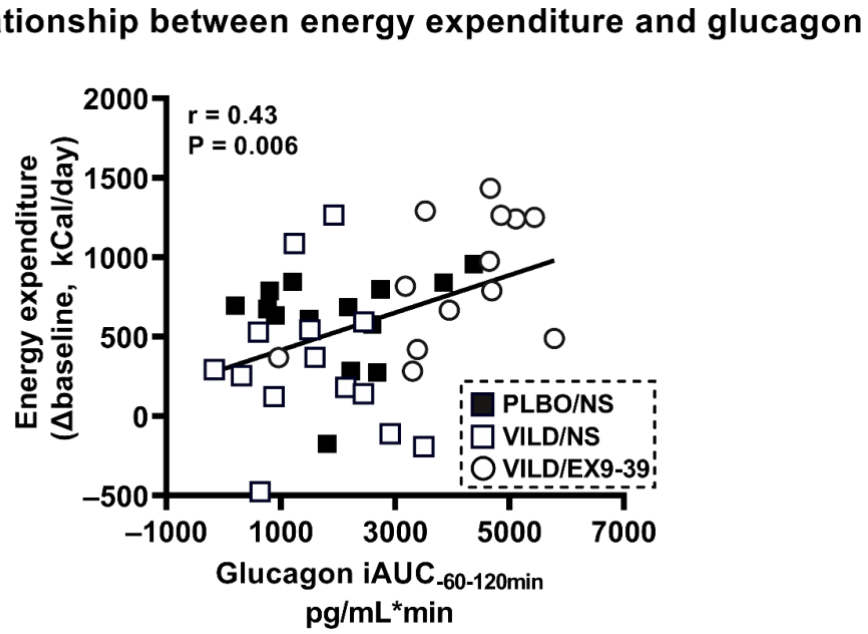


**Figure 7.4.** Relationship between energy expenditure and the plasma glucagon increment (iAUC for plasma glucagon during -60-120 min) in subjects with type 2 diabetes (n = 14).

# CHAPTER 8: EFFECTS OF THE BITTER TASTE RECEPTOR AGONIST, DENATONIUM BENZOATE, ON GASTRIC EMPTYING, POSTPRANDIAL GLYCAEMIA, AND ENERGY INTAKE IN TYPE 2 DIABETES

# STATEMENT OF AUTHORSHIP

| | |
|---|---|
| Title of the paper | Effects of the bitter taste receptor agonist, denatonium benzoate, on gastric emptying, postprandial glycaemia, and energy intake in type 2 diabetes |
| Publication status | To be submitted for publication |
| Publication details | - |

Principal Author

| | | | |
|---|---|---|---|
| Candidate | Cong Xie | | |
| Contribution | Data collection and interpretation, statistical analysis and drafting of the manuscript. | | |
| Overall percentage | 60% | | |
| 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. | | |
| Signature | | Date | September 2021 |

Co-Author Contributions

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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## 8.1 Abstract

**Aims:** There is emerging evidence that the gastrointestinal (GI) tract can sense luminal contents through activation of taste specific G-protein coupled receptors, and that activation of GI bitter taste receptors (BTRs) may modulate ghrelin, cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) secretion, thereby slowing gastric emptying, reducing postprandial glycaemia and suppressing energy intake. We evaluated the effects of the BTR agonist, denatonium benzoate (DB), on gastric emptying, postprandial glycaemia, and energy intake in type 2 diabetes (T2D).

**Methods:** 16 T2D subjects managed by diet and/or metformin were studied on 4 occasions (Parts A and B) in double-blind, randomised, crossover fashion. In Part A, subjects consumed encapsulated DB (30 mg) or sodium chloride (placebo), followed 30 min later by a $^{13}$C-octanoic acid-labelled mashed potato meal to evaluate gastric half-emptying time (T50) and postprandial glycaemia. In Part B, subjects consumed the capsule containing DB or control and were offered an *ad libitum* buffet meal after 30 min, to evaluate energy intake. In Part B, plasma ghrelin, total GLP-1 and CCK were also evaluated before and 30 min after the treatments.

**Results:** In Part A, DB had no effect on T50 (DB: 71.2 ± 4.0 vs. control 70.3 ± 3.7 min, P = 0.66) or plasma glucose (P > 0.05) after the mashed potato meal. In Part B, DB suppressed plasma ghrelin levels and reduced both the weight (DB 935 ± 78 vs. control 1034 ± 93 g, P = 0.03) and energy (DB 814 ± 101 vs. control 924 ± 125 kcal, P = 0.03) of food consumed, without affecting GLP-1 or CCK. The reduction in energy intake after DB was related to the suppression of ghrelin (r = 0.58, P = 0.038).

**Conclusion:** In T2D, a single dose of orally administered DB (30 mg) had no effect on gastric emptying or postprandial glycaemia, but suppressed ghrelin secretion and energy intake. Stimulation of intestinal BTRs may represent a novel approach to the management of obesity in T2D.

## 8.2 Introduction

The gastrointestinal (GI) tract is increasingly recognised as a novel target for the management of type 2 diabetes (T2D). The interaction of intraluminal contents with the GI tract modulates the secretion of an array of GI hormones that are fundamental in determining subsequent gastric emptying, postprandial glycaemia and appetite (Xie, Jones, et al., 2020). For example, ghrelin, released from the Gr-cells in the stomach during fasting and suppressed after meal ingestion, is a hunger hormone that drives food intake (Date et al., 2000; Steinert et al., 2017). The entry of nutrients into the intestine induces the secretion of postprandial GI hormones, including cholecystokinin (CCK) from the I-cells, and glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) from the L-cells. These stimulate insulin and suppress glucagon secretion, slow gastric emptying and inhibit energy intake (Xie, Jones, et al., 2020). While administration of exogenous gut peptides and their analogues has been used to treat obesity and T2D (e.g. the GLP-1 receptor agonists) (Meier, 2012), an alternative strategy is to stimulate the secretion of endogenous GI hormones.

A wide array of chemo-sensors are reported to account for the detection of carbohydrate (e.g., ATP-sensitive $K^+$ channel and sodium glucose co-transporter-1 (Kuhre et al., 2015; H. E. Parker et al., 2009)), fat (e.g., G-protein coupled receptor (GPCR) 119 and 120 (Hirasawa et al., 2005; Lauffer et al., 2009)) and protein (e.g., oligopeptide transporter 1 and calcium sensing receptor (Daly et al., 2013; Liou et al., 2011)) by the enteroendocrine cells to modulate GI hormone secretion (Xie, Jones, et al., 2020). More recently, specialised GPCRs that mediate taste signals (e.g. sweet,

bitter and umami) are found to be localised abundantly in the intestine, beyond the taste buds in the oral cavity (Avau, Rotondo, et al., 2015; S. V. Wu et al., 2002). A family of taste 2 receptors (T2Rs) detect intraluminal bitter taste stimuli and are recognised as bitter taste receptors (BTRs). That BTRs are co-expressed with enteroendocrine cells in both rodents and humans (Kok et al., 2018; Latorre et al., 2016; Masuho, Tateyama, & Saitoh, 2005), and that non-nutritive bitter taste agonists (e.g. denatonium benzoate (DB) and propylthiouracil) have been shown to trigger GLP-1 and PYY secretion from enteroendocrine L-cells in both a dose- and BTR-dependent manner (Kim et al., 2014; Pham et al., 2016), has stimulated substantial interest in targeting intestinal BTRs to modulate glucose metabolism and energy intake.

In mice, oral administration of bitter compounds, such as DB (Avau, Rotondo, et al., 2015; Kim et al., 2014) and extracts from hops (Kok et al., 2018), *Gentia scabra* root (Suh et al., 2015) and bitter gourd (Huang et al., 2013), has been shown to stimulate GLP-1 and PYY secretion, slow gastric emptying and attenuate the glycaemic response to a subsequent oral glucose. That the glucose lowering effect of bitter gourd extract was abolished by the GLP-1 receptor antagonist, exendin(9-39) (Huang et al., 2013), provides strong support for the role of GLP-1 in glucose-lowering induced by bitter taste compounds. In healthy males, the bitter tasting compound, quinine (600 mg), given into either the stomach or duodenum, attenuated the glycaemic response to a subsequent mixed-nutrient drink, in association with augmented GLP-1 and insulin and delayed gastric emptying (Rose et al., 2021). The effects of bitter taste

compounds on gastric emptying and postprandial glycaemia, however, have not been evaluated in subjects with T2D.

Oral administration of DB (60 μmol/kg) or quinine (160 μmol/kg) once daily for 4 weeks significantly reduced energy intake and body weight in high-fat-diet-induced obese mice (Avau, Bauters, et al., 2015), while in healthy humans, intragastric perfusion of DB (1 μmol/kg) impaired gastric accommodation of a subsequent meal, and increased satiation (Deloose et al., 2017). Moreover, oral administration of encapsulated quinine (18 mg) was also shown to increase postprandial CCK levels and suppress energy intake (Andreozzi et al., 2015), although intraduodenal perfusion of quinine at doses of 37.5-225 mg failed to show any effect on CCK secretion or energy intake (Bitarafan et al., 2019). The effect of bitter compounds on energy intake in subjects with T2D is not known.

In the present study, we evaluated whether stimulation of extra-oral BTRs (by administering encapsulated DB) would (i) slow gastric emptying and reduce postprandial glycaemia after a standardised test meal, and (ii) suppress energy intake at an *ad libitum* buffet meal, in association with modulation of GI hormone secretion.

## 8.3 Methods

### *8.3.1 Subjects*

Sixteen T2D subjects (7 male and 9 female; mean age 66.6 ± 1.0 years; BMI 31.3 ± 1.1 kg/m2; HbA1c 6.6 ± 0.1 %; duration of known diabetes 6.4 ± 1.2 years) managed

by diet (n = 9) or metformin alone (n = 7) were studied after they provided written, informed consent. Subjects were excluded if they were smokers, consumed > 20 g alcohol on a daily basis, took medications affecting GI function, or had a history of GI surgery. They were screened to exclude renal dysfunction (based on the estimated glomerular filtration rate), liver disease (based on liver enzymes), or autonomic dysfunction (assessed by standardised cardiovascular reflex tests (Piha, 1991)). Females who were pre-menopausal were also excluded to minimise the potential influence of the menstrual cycle on gastric emptying (Brennan et al., 2009). The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital and registered on Australian New Zealand Clinical Trials Registry (ACTRN12618001764224).

### *8.3.2 Protocol*

Each participant was studied on four occasions (including 2 experimental settings: Parts A and B, 2 conditions each), separated by at least 7 days, in a double-blind, randomised, crossover fashion. Participants managed by metformin were instructed to withhold this medication for 48 hours before each study visit (T. Wu, Ma, et al., 2014). On the evening preceding the study day (~1900h), participants were given a standardised evening meal consisting of beef lasagne (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia), then refrained from all food and nutrient beverages (except water) until the following morning, when they attended the Clinical Research Facility of The University of Adelaide at 0800 h. On each study day, a cannula was inserted in an antecubital vein for regular blood sampling. Participants

then consumed a gelatin capsule containing either 30 mg DB (Sigma Aldrich, St Louis, Missouri, USA) or 30 mg sodium chloride (placebo) with 150 mL water (at t = -30 min). The dose of DB was based on a previous study (Deloose et al., 2017) and the unpublished work by Dr. Xuyi Wang of our research group, showing that it increased satiation and suppressed energy intake and was relatively well tolerated in healthy humans. Thirty minutes after the capsule, participants consumed a standardised test meal (between t = 0 - 5 min) consisting of 65 g powdered potato (Deb, North Rocks, Australia) and 20 g glucose reconstituted with 200 mL water and one egg yolk containing 100 μL $^{13}$C-octanoic acid (368.5 kcal: 61.4 g carbohydrate, 7.4 g protein, and 8.9 g fat) for evaluation of gastric emptying and postprandial glycaemia (Part A), or a standardised *ad libitum* buffet meal (between t = 0 - 30 min) including a variety of food and drink items for evaluation of energy intake (Part B). In Part A, blood samples were collected every 30 min between t = -30 - 240 min for measurement of plasma glucose levels, and breath samples were collected immediately before, and every 5 min in the first hour after the meal, and every 15 min in the subsequent 3 hours. In Part B, blood samples were collected at t = -30 and 0 min for measurements of plasma ghrelin, total GLP-1 and CCK. Appetite sensations were evaluated every 30 min using 100 mm visual analogue scales (B. A. Parker et al., 2004). Blood samples were collected into ice-chilled serum and EDTA tubes and centrifuged immediately at 3200 rpm for 15 min at 4°C. Plasma were separated and stored at -80°C until analysed.

### *8.3.3 Measurement of gastric emptying*

Gastric emptying of the mashed potato meal, labelled with 100 µL $^{13}$C-octanioc acid, was assessed by excretion of $^{13}CO_2$ in breath samples, measured by a non-dispersive infrared spectrometer (FANci2, Fischer ANalysen Instrumente, Germany). The gastric half-emptying time (T50) was calculated using the Wagner-Nelson method, as described previously (Sanaka et al., 2007; Sanaka et al., 2004), from which the T50 was derived.

### *8.3.4 Assessment of energy intake*

A standardised buffet meal was used to quantify *ad libitum* energy intake (Nair et al., 2009). The buffet meal had a total energy of ~2400 kcal, consisting of 4 slices (~120 g) of whole-meal bread, 4 slices (~120 g) of white bread, 100 g sliced ham, 100 g sliced chicken, 85 g sliced cheddar cheese, 100 g sliced tomato, 100 g sliced cucumber, 100 g lettuce, 22 g mayonnaise, 20 g margarine, 1 apple (~170 g), 1 banana (~190 g), 175 g strawberry yogurt, 100 g chocolate custard, 120 g fruit salad, 375 mL iced coffee, 300 mL orange juice, and 600 mL water. The weight of food consumed (g) and energy intake (kcal) were quantified by weighting the buffet meal before and after consumption and calculated using commercially available software (Foodworks Professional Edition, version 8, Xyris Software, Queensland, Australia).

### *8.3.5 Measurements of plasma glucose, ghrelin, total GLP-1 and CCK concentrations*

Plasma glucose was measured using the glucose oxidase technique (2300 STAT Plus YSI, Yellow Springs, OH). Plasma ghrelin was measured by using an adaptation of the method of Parker et al (B. A. Parker, Doran, Wishart, Horowitz, & Chapman, 2005). The radiolabel was supplied by Perkin Elmer, Boston, MA (NEX388). The standard and samples were incubated with the antibody and radiolabel for 3-4 days at 4 ˚C. The minimum detectable level was 40 pg/mL, and the intra- and inter- assay CVs were 10.6% and 8.2%, respectively. Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK, Millipore, Billerica, MA). The minimum detectable limit was 3 pmol/L, the intra- and inter-assay CVs were 8.3% and 8.7%, respectively.

Plasma CCK-8 was measured by radioimmunoassay using an adaption of the method of Santangelo et al (Santangelo, Peracchi, Conte, Fraquelli, & Porrini, 1998). Samples were extracted in 66% ethanol, and 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. Sulphated CCK-8 $^{125}$I-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. The minimum detectable limit was 1 pmol/L, and the intra- and inter-assay CVs were 8.0% and 20.1%, respectively.

### *8.3.6 Statistical analysis*

Plasma glucose concentrations and appetite sensations were evaluated by 2-way repeated measures analysis of variance (ANOVA) using treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant interactions. T50, energy intake and weight of food consumed were analysed by paired Student's t-test after confirming their normality of distribution. The integrated area under the curve from baseline (iAUC) for plasma glucose was calculated using the trapezoidal rule. Relationships between change in plasma glucose from baseline and the T50, and between the change in energy intake and plasma ghrelin levels after DB, were evaluated using univariate linear regression analysis.

Based on a previous study involving the same radiolabelled meal in T2D subjects (Ma et al., 2009) (for Part A) and our unpublished work by Dr. Xuyi Wang of our research group, in healthy participants (for part B), a sample size of 12 subjects was calculated to provide >80% power (at $\alpha$=0.05) to detect (i) a 30% reduction in the iAUC between $iAUC_{0\text{-}240min}$ for plasma glucose after the standardised test meal (Part A) after 30 mg DB versus control, and (ii) a 25% reduction in energy intake at the buffet meal after 30 mg DB vs. control (Part B). Analyses were performed using Prism 8.0 (La Jolla,

CA, USA). $P < 0.05$ was considered statistically significant. Data are expressed as mean values ± SEM.

## 8.4 Results

The study protocol was well tolerated, and no adverse effects were reported on the study days or during follow up. Data on energy intake were available for only 14 participants due to significant interruptions of personal situations over the period of Part B in 2 subjects.

### *8.4.1 Part A: Gastric emptying and plasma glucose*

The T50 of the standardised mashed potato meal ranged from 48 – 90 min on the DB day, and 49 – 96 min on the placebo day. The mean T50 did not differ between the two study days (Placebo 70.3 ± 3.7 vs. DB: 71.2 ± 4.0 min, $P = 0.66$, **Figure 8.1A**). Fasting plasma glucose also did not differ between the two study days, and remained unchanged after DB or control (t = -30-0 min). After the test meal, plasma glucose increased on both study days (time effect: $P < 0.001$ each) before returning to baseline, without any difference between the two days (treatment effect: $P = 0.87$, **Figure 8.1B**). On both two study days, the increments in blood glucose at t = 30 min (DB: $r = 0.81$, $P < 0.001$; Placebo: $r = 0.74$, $P < 0.001$), 60 min (DB: $r = 0.73$, $P < 0.001$; Placebo: $r = 0.84$, $P < 0.001$) and 90 min (DB: $r = 0.71$, $P = 0.002$; Placebo: $r = 0.70$, $P = 0.002$), were directly related to the T50 (**Figure 8.2A-F**).

### *8.4.2 Part B: Plasma ghrelin, total GLP-1 and CCK levels, and energy intake*

Plasma ghrelin, GLP-1 and CCK at baseline did not differ between the two study days ($P > 0.05$, **Table 8.1**). Plasma total GLP-1 and CCK remained unchanged after both DB and placebo ($P > 0.05$ each), without any difference between them. However, plasma ghrelin increased from baseline on the placebo day, but remained relatively stable on the DB day, with a significant difference in the change in plasma ghrelin between the two treatments (Placebo: 64.7 ± 23.8; DB -3.5 ± 30.3 pg/mL, $P = 0.04$). Energy intake was less after DB than placebo (814 ± 101 vs. 924 ± 125 kcal, $P = 0.03$, **Figure 8.3A**), and the weight of food consumed was also less after DB than placebo (935 ± 78 g vs 1034 ± 95 g, $P = 0.03$, **Figure 8.3B**). The reduction in energy intake after DB compared to placebo was related directly to the difference in plasma ghrelin between these treatments ($r = 0.58$, $P = 0.038$, **Figure 8.3C**).

### *8.4.3 Part B: Appetite sensations*

Hunger, desire to eat and amount of food subjects thought they could eat increased slightly, but fullness was unchanged before the test meal (during t = -30-0 min). After the buffet meal, hunger, desire to eat and amount of food subjects thought they could eat decreased, and fullness increased, before returning to baseline (time effect: $P < 0.001$ each), without any difference between the two study days (treatment effect: $P = 0.55$ for hunger, $P = 0.34$ for desire to eat, $P = 0.51$ for amount of food subjects thought they could eat, and $P = 0.45$ for fullness, **Fig 8.4A-D**).

## 8.5 Discussion

In the current study of obese subjects with relatively well-controlled T2D, we observed that a single dose of encapsulated DB (30 mg), administered orally, had no effect on the gastric emptying of, and the glycaemic response to, a high carbohydrate meal. DB did, however, suppress fasting ghrelin levels and energy intake, without affecting GLP-1 or CCK secretion. To our knowledge, this represents the first clinical evaluation of the effects of a non-nutritive bitter compound on postprandial glycaemia and energy intake in T2D. That a small amount of encapsulated DB suppressed energy intake in this group of subjects suggests that BTR agonists have therapeutic potential for the management of obesity in people with T2D. Indeed, bitter substances, such as bitter gourd and coffee, have been reported to reduce the risk of T2D and cardiometabolic disorders (Carlstrom & Larsson, 2018; Fuangchan et al., 2011; Shi et al., 2020).

In the present study, DB was administered orally in a gelatin capsule, thereby bypassing any oral perception of the intense bitterness of this compound. Gelatin capsules break down in the presence of gastric acid (Narayani & Rao, 1995), and it can therefore be expected that DB was released promptly within the stomach following oral ingestion. Accordingly, the concurrent ingestion of 150 mL water would yield a DB solution of ~0.5 mmol/L, which we had established exhibited a high intensity of bitterness in an oral perception test (data not shown) and was previously found to be effective for stimulating CCK secretion from STC-1 cells *in vitro* (M. C. Chen et al., 2006). In a study involving healthy young subjects, we observed that

intraduodenal infusion of DB (30 mg) given together with glucose (2 kcal/min over 120 min) augmented the GLP-1 response and suppressed the subsequent energy intake in an *ad libitum* buffet meal compared to control (unpublished work by Dr. Xuyi Wang of our research group). However, intraduodenal DB did not affect plasma glucose levels, which may, at least in part, be due to the fact that blood glucose was minimally elevated during intraduodenal glucose infusion in this group of healthy subjects.

In rodents, bitter tasting compounds have been shown both to slow gastric emptying (Avau, Rotondo, et al., 2015; Janssen et al., 2011) and to reduce postprandial glycaemia (Huang et al., 2013; Kim et al., 2014). In healthy young women, intragastric DB (1 μmol/kg, a comparable dose to the current study) impaired gastric accommodation to a subsequent meal, but did not affect gastric emptying (assessed by breath test) (Deloose et al., 2017). However, the number of subjects studied (n = 6) was small, and the outcomes were not controlled for the effect of different phases of the menstrual cycle, which has a substantial impact on gastric emptying in female subjects (Brennan et al., 2009). In Part A of the current study, while the rate of gastric emptying was related to the rise in blood glucose (at t = 30, 60 and 90 min) after the mashed potato meal as anticipated (L. E. Watson, Xie, et al., 2019; Xie, Huang, Wang, et al., 2021), DB had no effect on GLP-1 and CCK secretion, or on the rate of gastric emptying. Not surprisingly, postprandial plasma glucose levels did not differ between DB and placebo. In contrast to our previous study in healthy subjects (discussed above), the effects of DB on GLP-1 and CCK secretion were assessed over 30 min

only in the fasted state in the current study. Given that plasma glucose did not differ between the two study days, we did not measure postprandial GLP-1 and PYY levels. Recent studies on another bitter taste compound, quinine-HCl, suggested that the timing of delivery of bitter substances may influence their effects substantially. For example, a bolus infusion of 600 mg quinine-HCl into the stomach 30 min before a mixed nutrient drink, although increasing plasma GLP-1 and insulin concentrations and reducing the postprandial blood glucose response, had no effect on gastric emptying. By contrast, an intragastric bolus 600 mg quinine-HCl given 60 min before the same test meal was associated with slowing of gastric emptying subsequently, together with an augmented plasma GLP-1 response in healthy males (Rose et al., 2021). The disparity between these two studies may reflect a difference in how much quinine-HCl had emptied from the stomach and the extent of its interaction with the small intestine. In keeping with this concept, bolus infusion of 600 mg quinine-HCl directly into the duodenum 30 min prior to the test meal was effective to slow gastric emptying (Rose et al., 2021). Relative to quinine-HCI, the dose of DB employed in the current study was relatively small. Moreover, intragastric DB might, at least partly, have been absorbed in the stomach prior to entering the small intestine. Accordingly, future studies to evaluate the effects of DB at different doses, and delivered into specific gut regions, are warranted.

Of note, DB reduced the total energy consumed at the buffet meal in Part B of the current study, in association with a suppression in plasma ghrelin, even though appetite scores before and after the buffet meal were did not differ from placebo. In

both lean and overweight humans, plasma ghrelin levels at the start of a meal are known to predict subsequent energy intake (Gibbons et al., 2013). The effect of DB on ghrelin secretion is likely due to its interaction with the Gr-cells in the stomach, since BTRs co-localise on these cells (Janssen et al., 2011; Widmayer et al., 2020) and since neither CCK nor GLP-1 were altered after DB. However, intragastric bolus administration of DB (1 µmol/kg), despite suppressing plasma ghrelin levels (Verbeure et al., 2021) and increasing satiety scores after a standardised test meal (Deloose et al., 2017), failed to suppress energy intake of an *ad libitum* milkshake (Verbeure et al., 2021) or buffet meal (Deloose et al., 2017) in healthy women in previous studies. Notably, the young women in these studies consumed relatively little energy (~370 kcal in *ad libitum* milkshake and ~700 kcal in buffet meal), such that any suppression of energy intake by DB may have been more difficult to demonstrate. Similar to the findings in our current study, oral administration of 18 mg quinine-HCl in a capsule suppressed energy intake in young healthy men and women (Andreozzi et al., 2015). An intragastric bolus of quinine-HCl (10 µmol/kg, ~215 mg for a 60 kg subject) suppressed plasma ghrelin concentrations and increased neural activity in hedonic and homeostatic brain regions with a reduction in energy intake at an *ad libitum* liquid meal in healthy women (Iven et al., 2019). However, administration of quinine-HCl via intragastric or intraduodenal bolus infusions (215-600 mg) (Bitarafan et al., 2020; Verbeure et al., 2021) or via a continuous intraduodenal infusion (37.5-225 mg) (Bitarafan et al., 2019; van Avesaat, Troost, Ripken, Peters, et al., 2015) failed to affect energy intake in healthy participants. The reasons for these inconsistencies regarding the effect of quinine-HCl on energy intake remain to be

determined. Genetic variations encoding BTRs between individuals may contribute to differences in functional responses to bitter taste compounds (Xie, Wang, Young, et al., 2018). For example, polymorphism of T2R38 influences the recognition of the bitterness of 6-n-propylthiouracil (PROP) (Bufe et al., 2005), and women who are more sensitive to PROP were found to consume less energy at an *ad libitum* meal than those who are insensitive (Shafaie et al., 2013). It remains unclear as to what extent the suppression of energy intake by DB is mediated via the GI tract, or through systemic exposure following absorption.

Several potential limitations exist in the present study. First, the number of subjects was relatively small; however, the findings appeared clear-cut, so that a larger sample size would be unlikely to alter the outcomes. Second, a single dose of DB was administrated in the present study. The use of the gelatin capsule for oral administration would have yielded a rapid release of DB in the stomach. Accordingly, future studies involving delivery of DB at different doses, into specific gut regions, and over a longer term, are warranted. Third, we did not assess if participants had restrained eating habit which would cofound assessment of energy intake by the *ad libitum* buffet meal. Finally, genetic variation in BTRs was not taken into account when interpreting the effects of BTR agonists.

In summary, in obese subjects with relatively well controlled T2D, a single dose of orally administered DB (30 mg) had no effect on gastric emptying or postprandial glycaemia, but suppressed ghrelin secretion and energy intake. These observations

suggest that stimulation of intestinal BTRs may represent a novel approach to the management of obesity in T2D.

**Table 8.1. Plasma concentrations of glucose, ghrelin, CCK and total GLP-1 at baseline and following ingestion of encapsulated DB or placebo (given at t =-30 min) in 16 subjects with type 2 diabetes (mean values ± SEM).**

| | Placebo | DB | P value |
|---|---|---|---|
| Baseline (t = -30 min) | | | |
| Plasma ghrelin (pg/mL) | 820 ± 82 | 847 ± 82 | 0.4 |
| Plasma CCK (pmol/L) | 3.2 ± 0.4 | 3.0 ± 0.5 | 0.6 |
| Plasma total GLP-1 (pmol/L) | 21.3 ± 1.5 | 24.3 ± 2.2 | 0.2 |
| Change from baseline (t = -30 to 0 min) | | | |
| Plasma ghrelin (pg/mL) | 64.7 ± 23.8 | -3.5 ± 30.3 | 0.04 |
| Plasma CCK (pmol/L) | 0.6 ± 0.4 | -0.01 ± 0.19 | 0.2 |
| Plasma total GLP-1 (pmol/L) | -1.8 ± 1.4 | 2.7 ± 0.9 | 0.6 |

DB, denatonium benzoate; CCK, cholecystokinin; GLP-1, glucagon-like peptide-1.

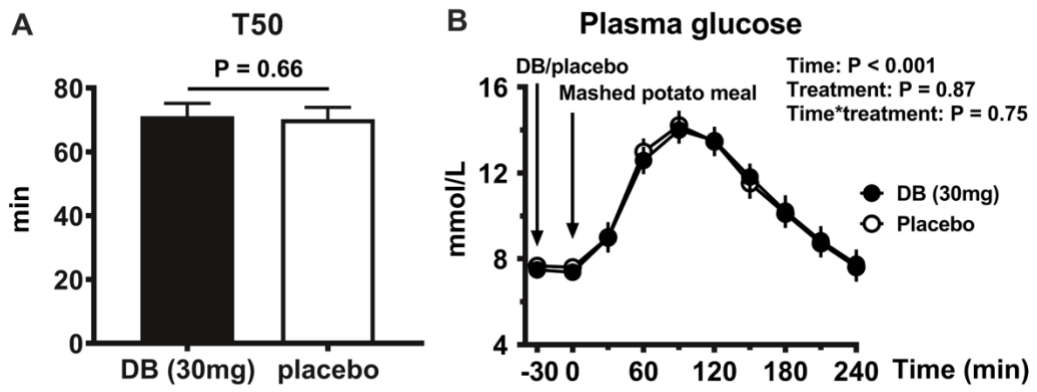


**Figure 8.1.** (A) Gastric half-emptying time (T50) of a standardised carbohydrate meal (given at t = 0 min) in 16 subjects with type 2 diabetes (T2D) as assessed by $^{13}$C-octanoic acid breath test after encapsulated 30 mg DB or placebo (given at t = -30 min). Statistical significance was assessed by paired t tests after confirming a normal distribution. (B) Plasma glucose concentrations in the same subjects. Differences by treatments and time, and related to treatment by time interactions, were analysed by 2-way repeated measures analysis of variance (ANOVA). Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed where ANOVAs revealed significant effects. DB, denatonium benzoate. Data are mean values ± SEM.

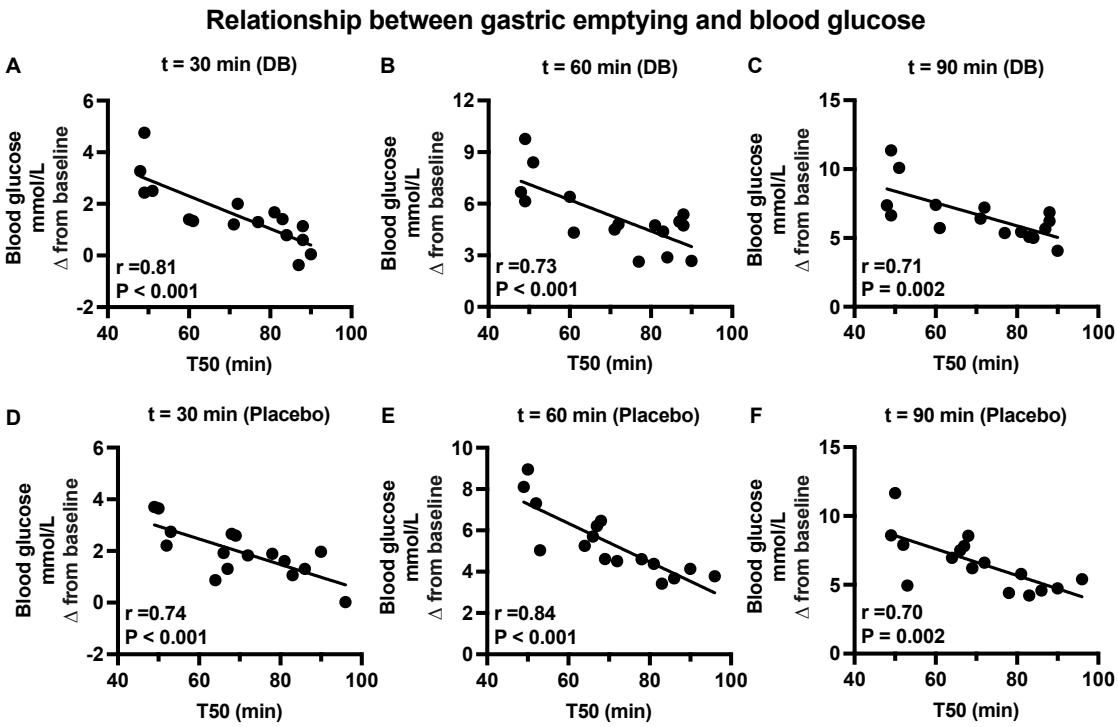


**Figure 8.2.** (A-F) Relationship between the blood glucose increment at t = 30, 60 and 90 min after the mashed potato meal and the gastric half emptying time (T50) in 16 subjects with type 2 diabetes on DB and placebo days. DB, denatonium benzoate.

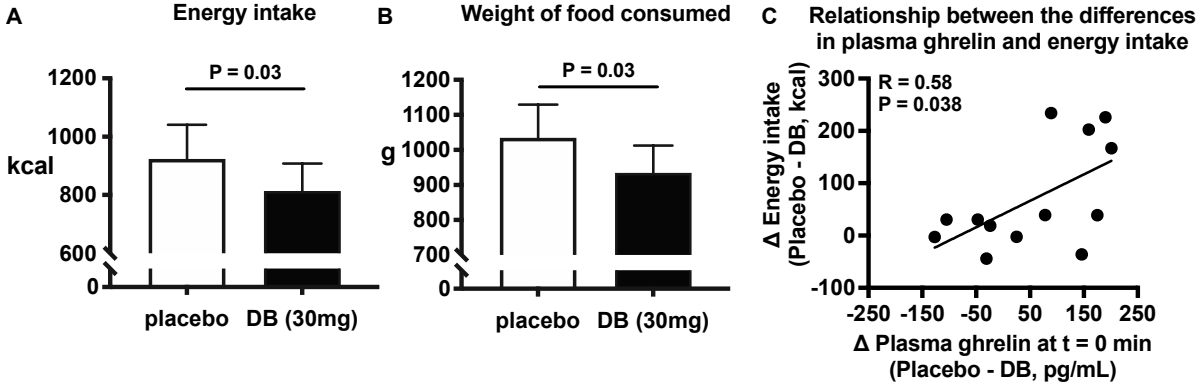


**Figure 8.3.** (A) Energy intake (kcal) and (B) weight of food consumed (g) at a standardised *ad libitum* buffet meal (t = 0 -30 min) after encapsulated 30 mg DB or placebo (given at t = -30 min) in 14 subjects with type 2 diabetes (T2D). Statistical significance was assessed by paired Student's t-test after confirming a normal distribution. (B) Relationship between the differences in plasma ghrelin at t = 0 min and energy intake between the two treatments in 13 subjects with T2D. Data are mean values ± SEM.

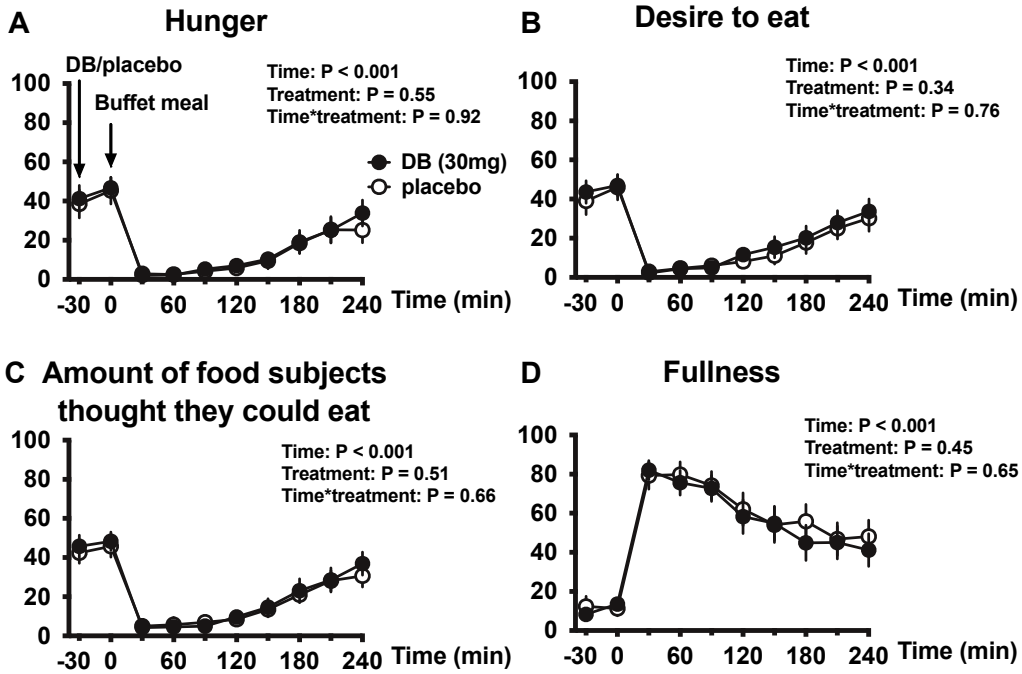


**Figure 8.4.** Scores for (A) Hunger, (B) desire to eat, (C) amount of food subjects thought they could eat and (D) fullness, in response to encapsulated DB (30 mg) or placebo (given at t = -30 min) and a standardised *ad libitum* buffet meal (t = 0 -30 min) in 16 subjects with type 2 diabetes (T2D), as assessed by 100 mm visual analogue scales. ANOVA results are reported as P values for differences by treatment and time, and due to treatment by time interactions. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed where ANOVAs revealed significant effects. Data are mean ± SEM.

# CHAPTER 9: EFFECTS OF RECTAL ADMINISTRATION OF THE NON-NUTRITIVE BITTER FLAVOURING, DENATONIUM BENZOATE, AND THE BITTER-TASTING PHYSIOLOGICAL BILE ACID, TAUROCHOLIC ACID, WITH AND WITHOUT THE BITTER TASTE RECEPTOR ANTAGONIST, PROBENECID, ON GLUCAGON-LIKE PEPTIDE-1 AND PEPTIDE YY SECRETION IN HEALTHY HUMANS.

# STATEMENT OF AUTHORSHIP

| | |
|---|---|
| Title of the paper | Effects of rectal administration of the non-nutritive bitter flavouring, denatonium benzoate, and the bitter-tasting physiological bile acid, taurocholic acid, with and without the bitter taste receptor antagonist, probenecid, on glucagon-like peptide-1 and peptide YY secretion in healthy humans. |
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| Contribution | Data collection and interpretation, statistical analysis and drafting of the manuscript. | | |
| Overall percentage | 60% | | |
| 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. | | |
| Signature | | Date | September 2021 |

Co-Author Contributions

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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## 9.1 Abstract

**Aims:** The gastrointestinal (GI) tract, like the tongue, can detect taste signals through activation of taste-specific G-protein coupled receptors. There is emerging evidence that stimulation of bitter taste receptors (BTRs) may trigger the release of GI hormones. We evaluated the effects of a non-nutritive bitter flavouring, denatonium benzoate (DB), and a bitter-tasting physiological bile acid, taurocholic acid (TCA), with and without the BTR antagonist, probenecid, on glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) secretion in healthy humans.

**Methods:** 16 healthy subjects were studied on 5 occasions each, in a double-blind, randomised, crossover fashion. On each study day, 20 mL of an aqueous gel (1% carboxymethyl cellulose) containing DB (30 mg), DB (30 mg) + probenecid (456 mg), TCA (3500 mg), TCA (3500 mg) + probenecid (456 mg), or vehicle only (placebo) was infused into the rectum via a soft catheter over 2 min. Subjects were offered an *ad libitum* buffet meal after 120 min to evaluate energy intake. Plasma GLP-1 and PYY concentrations and GI sensations were evaluated.

**Results:** TCA stimulated secretion of both PYY ($P < 0.001$) and GLP-1 ($P = 0.007$), and these effects were attenuated by probenecid. Neither DB, nor DB with probenecid, had any effect on plasma PYY or GLP-1. There was no difference in energy intake between any of the treatments.

**Conclusion:** In healthy humans, rectal administration of TCA stimulates PYY and GLP-1 secretion, mediated at least in part by bitter taste signalling. However, rectal administration of a non-nutritive bitter flavouring alone is not sufficient to induce GLP-1 or PYY secretion.

## 9.2 Introduction

The gastrointestinal (GI) tract is a key interface between the ingesta and the human body. The interaction of nutrients with the small and large intestine stimulates the secretion of an array of gut-derived hormones, including cholecystokinin (CCK) from the enteroendocrine I-cells and glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) from L-cells, which provide integrated feedback that slows gastric emptying, promotes satiation and limits postprandial glycaemia (Xie, Jones, et al., 2020).

While the mechanisms by which enteroendocrine cells detect, and respond to, intraluminal nutrients or bioactive compounds remain incompletely understood, there is recent evidence that the GI tract can detect taste signals (e.g. sweet, bitter and umami tastes) of luminal stimuli in the same way as the tongue, via activation of specific G-protein-coupled receptors (GPCRs) (Depoortere, 2014; Xie, Wang, Young, et al., 2018). Several lines of research are indicative of an important role of subtypes of the taste 2 receptor family (T2Rs), also known as bitter taste receptors (BTRs), in mediating the secretion of the aforementioned GI hormones and a potential for targeting these receptors to improve metabolic health (Xie, Wang, Young, et al., 2018). For example, a range of BTRs are found to be expressed on the enteroendocrine L-cells in both rodents (Avau, Rotondo, et al., 2015; Kim et al., 2014; S. V. Wu et al., 2002) and humans (Latorre et al., 2016; Le Neve et al., 2010; Pham et al., 2016). Moreover, the BTR agonists, denatonium benzoate (DB), 1,10-phenanthroline, and propylthiouracil, are known to induce GLP-1, CCK and PYY secretion from human (NCI-H716 (Kim et al., 2014; Park et al., 2015) and HuTu-80 (Pham et al., 2016)) and

mouse (STC-1 (M. C. Chen et al., 2006)) enteroendocrine cell lines in a dose- and BTR-dependent manner. In mice, an intragastric 'preload' of DB slowed gastric emptying and stimulated GLP-1 secretion, thereby increasing insulin secretion and attenuating the postprandial glycaemic excursion in response to glucose gavage (Kim et al., 2014). While we and others have shown that the pharmacological BTR agonists (e.g. DB and quinine) potently suppress energy intake in healthy individuals (Andreozzi et al., 2015) and subjects with type 2 diabetes (T2D) (Xie, Wang, Bound, et al., 2018), the capacity of BTR agonists to stimulate the secretion of GI hormones from L-cells (i.e. GLP-1 and PYY) has not been well established in humans.

Bitter substances, including bile acids, are present abundantly in the gut after a meal, yet the physiological role of intestinal bitter taste sensing is poorly understood. Over the last two decades, intestinal bile acids have been recognised as important signalling molecules that modulate metabolic homeostasis, at least in part, through the stimulation of GI hormone secretion (Xie, Huang, Young, et al., 2021). For example, administration of the physiological bile acid, taurocholic acid (TCA), into either the small (T. Wu, M. J. Bound, S. D. Standfield, K. L. Jones, et al., 2013; T. Wu, Horowitz, Jones, & Rayner, 2017) or the large intestine (T. E. Adrian et al., 2012; T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013), was shown to stimulate GLP-1 secretion, in association with a reduction in the blood glucose response to small intestinal glucose infusion in both health and T2D. Hitherto, the actions of bile acids have been attributed predominantly to the activation of the nuclear farnesoid X receptor (FXR) (Trabelsi et al., 2015) and/or Takeda G-protein coupled receptor 5

(TGR-5) at the basolateral membrane (Brighton et al., 2015; Kuhre et al., 2018). The potential involvement of the intestinal BTRs in relation to bile acid signalling has not been considered previously.

Probenecid, which is well recognised as an inhibitor of the organic anion transporters (OATs) and is used clinically to inhibit renal resorption of uric acid or reduce the renal excretion of penicillin, has recently been shown to block BTRs (e.g. T2R16, T2R38 and T2R43) both *in vitro* and *in vivo* (Avau, Rotondo, et al., 2015; Greene et al., 2011; Masamoto, Mitoh, Kobashi, Shigemura, & Yoshida, 2020; Wolfle et al., 2016). In rodents, a dose of 50 mg/kg completely abolishes the slowing of gastric emptying induced by DB (Avau, Rotondo, et al., 2015). Importantly, oral administration of probenecid (10 mM) effectively blocks bitter taste perception of salicin in humans (Greene et al., 2011). Furthermore, probenecid blocks bitter taste signalling without affecting non-BTR GPCRs, and its inhibitory effect on BTRs is rapid and unrelated to OATs (Greene et al., 2011), so that its effect to block intestinal BTRs is specific. Accordingly, probenecid appears to be a useful tool to elucidate the functional importance of bitter taste signalling in humans.

Given that the GLP-1- and PYY-releasing L-cells are located predominantly in the distal small intestine, colon and rectum (Jorsal et al., 2018), and that BTRs are abundantly expressed on the L-cells of human colonic mucosa (Latorre et al., 2016; Rozengurt et al., 2006), it is logical to investigate whether rectal perfusion of bitter taste compounds stimulates GLP-1 and PYY secretion in humans and, if so, whether

this effect can be antagonised by the BTR antagonist, probenecid. To establish 'proof-of-concept', the present study evaluated the effects of rectal infusion of the BTR agonist, DB, and a bitter-tasting physiological bile acid, TCA, on GLP-1 and PYY secretion, with and without probenecid.

## 9.3 Methods

### *9.3.1 Subjects*

Sixteen healthy subjects were studied after providing written informed consent (7 male and 9 female, age 23.5 ± 1.0 years, BMI 23.1 ± 0.8 kg/m$^2$, HbA1c 5.0 ± 0.1% (31 ± 0.9 mmol/mol)). Subjects were excluded if they were smokers, consumed > 20 g alcohol on a daily basis, took medications affecting GI function, or had a history of GI surgery, dietary bias, or impaired renal or liver function. Female subjects were excluded if they were pregnant (assessed by the urine pregnant test on each study day) or lactating. The protocol (**Figure 9.1**) was approved by the Royal Adelaide Hospital Human Research Ethics Committee and prospectively registered on the Australian New Zealand Clinical Trials Registry (ACTRN12618000093280). All subjects provided written informed consent.

### *9.3.2 Protocol*

Each subject was studied on five occasions, separated by 7 days, in a double-blind, randomised, placebo-controlled, crossover fashion, facilitated by the Royal Adelaide Hospital Pharmacy. All subjects were asked to maintain their usual diet between study days and to refrain from vigorous exercise and alcohol intake for 24 hours

before each study day. On the evening before each study day (~1900h), subjects consumed a standardised beef lasagne meal (591 kcal; McCain Foods Proprietary Ltd, Victoria, Australia). Following this meal, subjects were asked to fast from solids and liquids (other than water) until the following morning. On each study visit, subjects attended the Clinical Research Facility of the University of Adelaide at 0800 h. An intravenous cannula was inserted into a forearm vein for repeated blood sampling. Then the subject was positioned in the left lateral decubitus position, and 20 mL aqueous gel (1% carboxymethyl cellulose) containing DB (30 mg), DB (30 mg) + probenecid (456 mg, ~40 mM), TCA (3500 mg; this dose was shown to be effective at stimulating GLP-1 and PYY and relatively well tolerated in humans (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013)), TCA (3500 mg) + probenecid (456 mg), or vehicle only, was infused into the rectum via a soft catheter during t = -2 - 0 min. The pH of each solution was adjusted to 6 - 7 by 1 N sodium hydroxide (NaOH) to minimise the potential effect of acidic pH on GLP-1 and PYY secretion (Modvig et al., 2020). 'Arterialised' venous blood was collected at t = -5, 15, 30, 45, 60, 90 and 120 min for measurements of plasma glucose, GLP-1 and PYY concentrations. Blood samples were collected into ice-chilled serum and EDTA tubes and centrifuged immediately at 3200 rpm for 15 min at 4°C. Plasma were separated and stored at -80°C until analysed. At the same intervals used for blood sampling, appetite sensations and GI symptoms, including hunger, amount of food subjects thought they could eat and desire to defaecate, were assessed using 100 mm visual analogue scales (B. A. Parker et al., 2004). At t = 120 min, an *ad libitum* buffet meal was provided from which subjects were free to eat as much as they wished for 30 min (t = 120 to 150 min), and

from which energy intake was quantified (Foodworks Professional Edition, version 8, Xyris Software, Australia).

### *9.3.3 Measurements of plasma glucose, total GLP-1 and PYY levels*

Plasma glucose was measured using the glucose oxidase technique (2300 STAT Plus YSI, Yellow Springs, OH). Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK, Millipore, Billerica, MA) with a sensitivity of 3 pmol/L and intra- and inter-assay CVs of 8.3% and 8.7%, respectively. Plasma PYY was measured by radioimmunoassay using antiserum donated by Dr. B. Otto (University of Munich, Germany) raised in rabbits against human PYY (1-36); The minimum detectable limit was 1.5 pmol/L and the intra- and inter-assay CVs were 15.2% and 18%, respectively.

### *9.3.4 Statistical Analysis*

Based on data derived from our previous study (T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013), 16 healthy subjects would provide 90% power to detect a difference of 220 pmol/L*min$^{-1}$ in the 2-hour incremental area under the curve (iAUC) for plasma GLP-1 between treatments. Plasma glucose, total GLP-1 and PYY concentrations, appetite sensations and GI symptoms were evaluated by 2-way repeated measures analysis of variance (ANOVA), using treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant interactions. The iAUCs were calculated using the trapezoidal rule for plasma glucose, total GLP-1 and PYY concentrations. The baseline plasma glucose, total GLP-1 and PYY concentrations

were compared using one-factor repeated-measures ANOVA. Differences in plasma glucose, total GLP-1 and PYY iAUCs and energy intake between two specific treatments (i.e. DB vs. placebo, TCA vs. placebo, DB vs. DB + probenecid, and TCA vs. TCA + probenecid) were compared using paired Student's t-test after confirming a normal distribution. Data are presented as mean values ± SEM; $P < 0.05$ was considered statistically significant.

## 9.4 Results

The study protocol was generally well tolerated.

### *9.4.1 Plasma glucose concentrations*

Plasma glucose concentrations at baseline did not differ between the five study days ($P = 0.7$), and remained unchanged in response to either treatment, such that there were no differences in plasma glucose concentrations between the five study days (time effect: $P > 0.05$, treatment effect: $P > 0.05$, **Figure 9.2A**).

### *9.4.2 Plasma total GLP-1*

Plasma total GLP-1 concentrations at baseline did not differ between 5 study days ($P = 0.9$, **Table 9.1**). After the rectal perfusion, plasma total GLP-1 increased on the TCA day, peaking at t = 15 min before returning to baseline after t = 60 min (time effect: $P < 0.001$ for TCA, $iAUC_{0\text{-}60min}$ TCA vs. placebo: $P = 0.007$, **Table 9.1**). There were no significant changes in plasma GLP-1 levels after placebo, DB, DB + probenecid, or TCA + probenecid (time effect: $P > 0.05$ each, $iAUC_{0\text{-}60min}$ DB vs. placebo: $P = 0.87$,

**Table 9.1**). However, the $iAUC_{0\text{-}60min}$ for plasma GLP-1 was numerically, but not significantly, less after TCA + probenecid than TCA (P = 0.32, **Table 9.1**, **Figure 9.2B**).

### *9.4.3 Plasma PYY*

Plasma PYY concentrations at baseline did not differ between the five study days (P = 0.2, **Table 9.1**). After the rectal perfusion, plasma PYY increased promptly after TCA, peaking at t = 15 min before slowly returning to baseline at t = 60 min (time effect: P < 0.001 for TCA, $iAUC_{0\text{-}60min}$ TCA vs. placebo: P < 0.001). In the presence of probenecid, TCA-induced PYY secretion was attenuated substantially ($iAUC_{0\text{-}60min}$ for TCA vs. TCA + probenecid: P = 0.018, **Table 9.1**, **Figure 9.2C**). There was no significant change in plasma PYY after DB or DB + probenecid.

### *9.4.4 Energy intake*

Neither energy intake nor the weight of food consumed at the *ad libitum* buffet meal differed between the five study days (P > 0.05 each, **Figure 9.3A-B**).

### *9.4.5 Appetite sensations and GI symptoms*

Compared with placebo, hunger (**Figure 9.4A**) and amount of food subjects thought they could eat (**Figure 9.4B**) were not affected after rectal administration of DB (P > 0.05), but decreased immediately after TCA (P = 0.025 for hunger; P = 0.049 for amount of food subjects thought they could eat). Changes in hunger and amount of food subjects thought they could eat scores did not differ between TCA and TCA +

probenecid (treatment effect: P = 0.24 for hunger, P = 0.15 for amount of food subjects thought they could eat).

The desire to defaecate remained unchanged after rectal administration of placebo, DB or DB + probenecid, and no subject defaecated on these study days. The desire to defaecate increased promptly after rectal administration of both TCA and TCA + probenecid (time effect: $p < 0.001$), which quickly returned to baseline after defaecation (n = 16 on both days; mean time to defaecate: 13.4 ± 2.8 min for TCA and 11.0 ± 1.0 min for TCA + probenecid, P = 0.9), without any difference between the two study days (**Figure 9.4C**).

## 9.5 Discussion

In the present study, we observed in young healthy human subjects that a single dose of rectally administered TCA (3500 mg) stimulated both GLP-1 and PYY secretion, and these effects were markedly attenuated by the BTR antagonist, probenecid. However, the BTR agonist DB alone had no effect on GLP-1 or PYY secretion. These observations suggest that intestinal bitter taste signalling is critical, but may not be sufficient, to mediate the secretion of GI hormones in humans.

In consideration of the rich distribution of L-cells (Jorsal et al., 2018) and abundant expression of BTRs (Latorre et al., 2016; Rozengurt et al., 2006) in the colon, and particularly the rectum, our experimental model involving rectal perfusion of DB and TCA (with or without probenecid) should have been ideal to assess their effects on L-

cell section. In this model, we and others have previously shown that rectal administration of TCA stimulates both GLP-1 and PYY secretion in a dose-dependent manner in health and T2D (T. E. Adrian et al., 2012; T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013). As in our previous report, rectal TCA at the dose of 3500 mg induced both GLP-1 and PYY secretion in the current study, and the magnitude of the PYY response was demonstrably greater than GLP-1, probably because colonic L-cells express more PYY than GLP-1 (Martins et al., 2017). Bile acids are natural ligands of FXR and TGR5, both of which have been reported to account for bile acid-evoked GLP-1 and PYY secretion in preclinical and clinical studies (Castellanos-Jankiewicz et al., 2021; Kuhre et al., 2018; T. Wu, M. J. Bound, S. D. Standfield, B. Gedulin, et al., 2013; T. Wu, M. J. Bound, S. D. Standfield, K. L. Jones, et al., 2013). As a major primary bile acid in humans, TCA has a high affinity to TGR-5 (Sato et al., 2008) and has been shown to stimulate GLP-1 secretion in a TGR-5 dependent manner from mouse small and large intestinal tissue (Brighton et al., 2015). However, neither FXR nor TGR5 agonists have been shown to stimulate GLP-1 secretion potently in humans. Accordingly, it is plausible that additional mechanisms may be responsible for the sensing of intestinal bile acids.

To the best of our knowledge, the current study is the first to evaluate the relevance of bitter taste pathways to GI hormone secretion induced by bile acids in humans. We employed probenecid, which has been shown to block T2R16, T2R38 and T2R43 *in* both *in vitro* and *in vivo* studies (Avau, Rotondo, et al., 2015; Greene et al., 2011; Masamoto et al., 2020; Wolfle et al., 2016), to antagonise BTR signalling. The dose

of probenecid (~40 mM) exceeded the concentration (10 mM) that blocked bitter taste perception of salicin in humans (Greene et al., 2011). It should be noted that a wide range of BTRs have been reported in humans, and that probenecid does not block all BTR subtypes. Nevertheless, there is evidence that at least T2R38 and T2R43 are expressed in the human colon (Rozengurt et al., 2006), and that T2R38 is co-located with GLP-1 and PYY in this location (Latorre et al., 2016). In the current study, probenecid attenuated TCA-induced GLP-1 and PYY secretion, thus supporting a functional role of BTRs in mediating TCA-evoked GI hormone section. The relative contribution of TGR5 and FXR to this action, however, remains unclear. Future studies are warranted to delineate the respective role of bile acid sensing mechanisms in the regulation of GI hormone secretion.

Unlike TCA, the non-nutritive bitter taste flavouring, DB, did not affect GLP-1 or PYY secretion, despite its strong bitter taste at the given dose (30 mg; ~3.36 mmol/L), and that DB at this concentration was shown to induce GLP-1 secretion from an enteroendocrine L-cell line, NCI-H716, *in vitro* (Kim et al., 2014). Although a handful of recent studies have also reported that the non-nutritive BTR agonists DB (1 µmol/kg, a similar dose to the current study) (Verbeure et al., 2021) and quinine-hydrochloride (quinine-HCl, 37.5 - 225 mg) (Bitarafan et al., 2019; Verbeure et al., 2021), administered via either intragastric or intraduodenal perfusion, had no effect on plasma GLP-1 secretion in healthy human subjects, it cannot be ruled that the absorption of these compounds in the upper gut might have limited their interaction with L-cells located in the distal gut regions. The absence of GLP-1 and PYY

stimulation following rectal perfusion of DB in the current study therefore provides strong support for the concept that activation of BTRs alone may not be sufficient to stimulate L-cell secretion in humans. Interestingly, two recent studies in healthy subjects observed that an intragastric or intraduodenal 'preload' of quinine-HCl (600 mg) (Bitarafan et al., 2020; Rose et al., 2021) was associated with an augmented GLP-1 response to a subsequent mixed nutrient drink, with slower gastric emptying and a lower postprandial glycaemic response. Similarly, a low dose of encapsulated bitter extract from *Gentiana lutea* root also showed a tendency to augment plasma GLP-1 levels in response to a high carbohydrate breakfast in healthy subjects (Mennella et al., 2016). These findings are therefore indicative of a potential for intestinal BTR signalling to potentiate nutrient-gut interactions.

The duration of rectal exposure to TCA was relatively brief due to stimulation of defaecation (Sadik, Abrahamsson, Ung, & Stotzer, 2004). Since we did not observe any difference in defecation between the TCA and TCA + probenecid days, and since DB had no effect on the desire to defaecate, TCA-induced defecation does not appear to be BTR-dependent. In line with the changes in plasma GLP-1 and PYY, both hunger and amount of food subjects thought they could eat were decreased by TCA, although this may be associated with the effect of TCA to promote defaecation. However, energy intake did not differ between the treatments, consistent with GLP-1 and PYY concentrations already having returned to baseline at the time of the buffet meal. Plasma glucose levels remained stable after rectal gel administration during all study visits, since healthy subjects remained fasted throughout the study and the

insulinotropic and glucagonostatic effects of GLP-1 are glucose-dependent (De Heer & Holst, 2007).

Several limitations should be noted in the present study. First, a single dose of DB was examined, so it is unclear whether higher or lower doses of DB, or a longer-period of exposure, would be more efficacious at stimulating GLP-1 and PYY secretion. Second, there are currently 25 BTRs identified in humans. Blockade of selective BTRs (T2R16, T2R38 and T2R43) by probenecid is unlikely to reveal the full effects of BTR signalling. Future identification of BTR antagonists that both block either specific BTRs, or a wider range of BTRs, is critical to uncovering the physiological role of BTR signalling. Third, we did not assess if participants had restrained eating habit which would cofound assessment of energy intake by the *ad libitum* buffet meal. Finally, our sample size was relatively small, but the findings are clear-cut, so that increasing the number of the subjects would be unlikely to alter the results.

In conclusion, rectal administration of TCA in healthy humans stimulates PYY and GLP-1 secretion, mediated at least in part by bitter taste signalling. However, rectal administration of a non-nutritive bitter flavouring alone is not sufficient to induce GLP-1 or PYY secretion.

**Table 9.1. Baseline and the iAUCs for plasma total GLP-1 and PYY in response to rectal administration of DB, DB + probenecid, TCA, TCA + probenecid or placebo in healthy subjects (mean values ± SEM).**

| | Placebo | DB | DB + probenecid | TCA | TCA + probenecid |
|---|---|---|---|---|---|
| Baseline plasma total GLP-1 (pmol/L) | 10.0 ± 1.0 | 9.4 ± 0.7 | 10.1 ±1.1 | 9.5 ± 0.8 | 9.6 ± 0.7 |
| Baseline plasma PYY (pmol/L) | 16.7 ± 1.8 | 15.7 ± 2.0 | 13.0 ± 0.9 | 14.0 ± 1.7 | 16.2 ± 1.6 |
| GLP-1 $iAUC_{0\text{-}60min}$ (pmol/ L*min) | 17.7 ± 5.8 | 25.3 ± 15.5 | 39.5 ± 12.9 | 87.7 ± 21.4 * | 72.2 ± 28.4 |
| GLP-1 $iAUC_{0\text{-}120min}$ (pmol/ L*min) | 57.1 ± 17.7 | 62.0 ± 35.4 | 63.2 ± 21.0 | 145.6 ± 34.7 * | 121.3 ± 46.9 |
| PYY $iAUC_{0\text{-}60min}$ (pmol/ L*min) | 17.0 ± 7.1 | 13.7 ± 6.6 | 20.3 ± 7.0 | 670.7 ± 107.3 ** # | 396.6 ± 107.1 |
| PYY $iAUC_{0\text{-}120min}$ (pmol/ L*min) | 31.4 ± 17.2 | 28.1 ± 15.9 | 44.3 ± 17.6 | 729.2 ± 117.7 ** # | 446.0 ± 139.5 |

DB, denatonium benzoate; TCA, taurocholic acid; GLP-1, glucagon-like peptide-1; PYY, peptide YY; iAUC, incremental area under the curve. Data are as mean ± SEM, n = 16. * $P < 0.05$ and ** $P < 0.01$ for TCA vs. placebo; # $P < 0.05$ for TCA vs. TCA + probenecid.

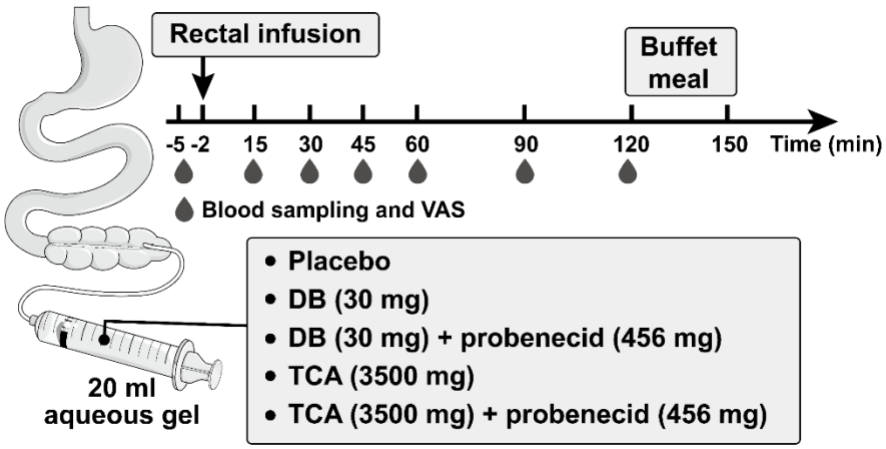


**Figure 9.1.** Schematic representation of the study protocol. VAS, visual analogue scale.

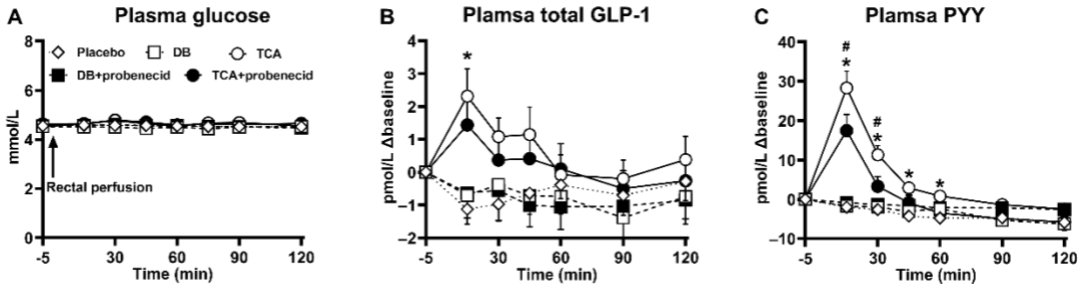


**Figure 9.2.** (A) Plasma glucose, (B) total GLP-1, and (C) PYY concentrations in response to rectal administration of DB, DB + probenecid, TCA, TCA + probenecid and placebo (given at t = -2 - 0 min) in healthy subjects. Data were analysed by repeated-measures two-way ANOVA with time and treatment as factors. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed where ANOVAs revealed significant effects Data are mean values ± SEM, n = 16. * $P < 0.05$ for TCA vs. placebo; # $P< 0.05$ for TCA vs. TCA + probenecid. GLP-1, glucagon-like peptide 1; PYY, peptide YY; DB, denatonium benzoate; TCA, taurocholic acid.

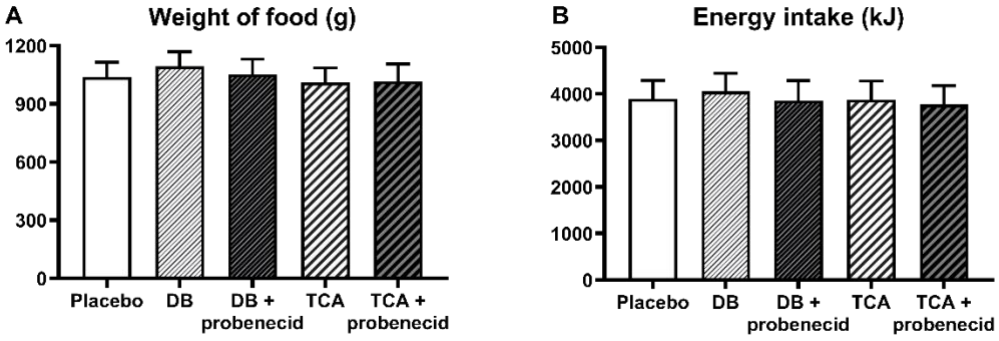


**Figure 9.3.** (A) Weight of food ingested and (B) energy intake at a standardised ad libitum buffet meal (t = 120 -150 min) after rectal administration of DB, DB + probenecid, TCA, TCA + probenecid and placebo (given at t = -2 - 0 min) in healthy subjects. Paired t tests were used to determine statistical difference between two specific treatments (i.e. DB vs. placebo, TCA vs. placebo, DB vs. DB + probenecid, and TCA vs. TCA + probenecid). Data are mean values ± SEM, n = 16. DB, denatonium benzoate; TCA, taurocholic acid.

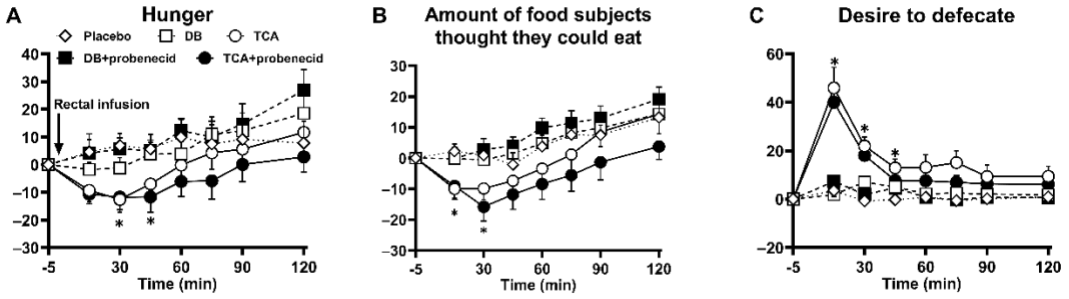


**Figure 9.4.** Scores for (A) Hunger, (B) amount of food subjects thought they could eat, and (C) desire to defaecate as measured from 100mm visual analogue scales, in response to rectal administration of DB, DB + probenecid, TCA, TCA + probenecid and placebo (given at t = -2 - 0 min) in healthy subjects. Data were analysed by repeated-measures two-way ANOVA with time and treatment as factors. Post-hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed where ANOVAs revealed significant effects. Data are mean values ± SEM, n = 16. * $P < 0.05$ for TCA vs. placebo. DB, denatonium benzoate; TCA, taurocholic acid.

# CHAPTER 10: CONCLUSION

The studies included in this thesis have yielded novel insights into the role of gastrointestinal (GI) function, particularly gastric emptying and GI hormones (e.g. glucagon-like peptide-1 (GLP-1), in the regulation of postprandial glycaemia, energy expenditure and energy intake in both health and type 2 diabetes (T2D).

Gastric emptying is a major determinant of the glycaemic response to a glucose drink or a solid high carbohydrate meal (Jones et al., 1995; L. E. Watson, Xie, et al., 2019; Xie, Huang, Wang, et al., 2021). Interventions that slow gastric emptying attenuate postprandial glycaemic excursions in T2D (Jones et al., 2019; Rayner et al., 2020). Importantly, the reduction in postprandial glycaemia is related to the magnitude of the slowing of gastric emptying, and when baseline gastric emptying is relatively more rapid, the reduction of postprandial glycaemia is greater (Little, Pilichiewicz, et al., 2006). Accordingly, the baseline gastric emptying is likely to be an important determinant of the response to therapy. In T2D, gastric emptying has been reported to be either delayed (Michael Horowitz et al., 1991), unchanged (Boronikolos et al., 2015) or accelerated (W. T. Phillips et al., 1991, 1992). This inter-individual variability may be, in part, related to the substantial heterogeneity in subject characteristics (e.g. age, duration of diabetes, glycaemic status, pharmacotherapy and presence or absence of diabetic complications) of cohorts studied and the test meals employed (e.g. emptying of solid and liquid test meals is frequently discordant). In the study reported in **Chapter 4** (L. E. Watson, Xie, et al., 2019), we found that gastric emptying of a semisolid meal (mashed potato) was slower in older than young individuals without T2D, but more rapid in relatively well controlled subjects with

T2D (HbA1c ~6.6%) when compared with age- and BMI-matched non-diabetic controls. In keeping with the concept that gastric emptying is a critical determinant of postprandial glycaemia, the increments in blood glucose after a standardised test meal were closely related to the rate of gastric emptying in this group of T2D subjects. In the study reported in **Chapter 5** (Xie, Huang, Wang, et al., 2021), we found that gastric emptying of an oral glucose drink was also slower in older than young subjects without diabetes, but faster in both well- (HbA1c ~6.9%) and poorly-controlled (HbA1c ~10.5%) uncomplicated subjects with T2D of short duration, when compared to age-adjusted individuals without diabetes, suggesting that accelerated gastric emptying occurs frequently in T2D regardless of glycaemic status. These observations therefore support the utility of interventions that slow gastric emptying for the management of postprandial hyperglycaemia in these groups of patients with T2D. It would also be intriguing to examine whether individuals with more rapid gastric emptying would be predisposed to glucose intolerance and type 2 diabetes.

There is a complex bidirectional relationship between gastric emptying and postprandial GLP-1 secretion (Xie, Jones, et al., 2020). In a given individual, the magnitude of GLP-1 secretion is related to the rate of nutrient delivery into the small intestine (i.e. gastric emptying) (Ma et al., 2012; Trahair et al., 2012); conversely, GLP-1, together with cholecystokinin and peptide YY (PYY), slows gastric emptying (Little, Pilichiewicz, et al., 2006). Gastric emptying exhibits a relatively modest intra-individual, but substantial inter-individual, variation (Camilleri et al., 2012; Cremonini et al., 2002). However, it remains unclear whether the latter is related to

the GLP-1 response to intestinal nutrient stimulation. Characterisation of the relationship between gastric emptying and GLP-1 secretion is of relevance to the development of "gut-based" approaches to reduce postprandial hyperglycaemia in T2D. The study reported in **Chapter 6** showed that (i) the GLP-1 response to a high carbohydrate meal was not related to the rate of GE in T2D subjects, such that postprandial GLP-1 levels did not differ between subjects with fast and slow gastric emptying, (ii) the GLP-1 response to intraduodenal nutrients, similar to GE, exhibited a substantial inter-individual, but a modest intra-individual variation in both healthy and T2D subjects, and (iii) the variation in the GLP-1 response to an intestinal glucose infusion was related directly to the intrinsic gastric emptying rate of an oral glucose drink in subjects with and without T2D. These observations indicate that, in a given individual, gastric emptying does not necessarily predict postprandial GLP-1 secretion, but that the intrinsic rate of gastric emptying is likely to be tuned, at least in part, by the responsiveness of GLP-1 to the presence of nutrients in the intestinal lumen. Further investigations are needed to understand the mechanisms underlying the variations in the GLP-1 response to the standardised load of intestinal nutrients, in order to develop effective dietary and/or pharmacological strategies that can enhance the GLP-1 response to "intestinal nutrients", slow gastric emptying and reduce postprandial glycaemic excursions substantially.

As well as slowing gastric emptying, GLP-1 has the capacity to stimulate insulin and inhibit glucagon secretion in a glucose-dependent manner, and to suppress energy intake. Upon secretion from the enteroendocrine L-cells, GLP-1 is rapidly degraded

by dipeptidyl peptidase 4 (DPP-4). Inhibition of DDP-4 is therefore a logical strategy to increase active GLP-1 levels for glycaemic control in T2D (Deacon, 2020). Previous observations in healthy humans showed that a single dose of the DPP-4 inhibitor, vildagliptin, lowered the blood glucose response to fat, and increased energy expenditure and the thermic effect of feeding (Heruc et al., 2014); the later would favour a reduction in body weight with sustained use of DPP-4 inhibitors. However, DPP-4 inhibitors are known to be weight neutral in subjects with T2D. Accordingly, the effect of DPP-4 inhibition on energy expenditure may be compromised in this disorder. In the study reported in **Chapter 7** (Xie, Wang, et al., 2020), we evaluated the effects of DPP-4 inhibition on the glycaemic and energy expenditure responses during intraduodenal fat infusion and the role of endogenous GLP-1 signalling (using the GLP-1 receptor antagonist exendin(9-39)) in the regulation of these effects in subjects with T2D. The key findings were that: 1) acute dosing with vildagliptin increased plasma intact GLP-1, lowered plasma glucose and glucagon, and tended to decrease energy expenditure and the thermic effect of feeding, and 2) exendin(9-39) abolished the lowering of plasma glucose by vildagliptin, and augmented plasma glucagon concentrations, energy expenditure, and the thermic effect of feeding. These observations therefore support a major role of endogenous GLP-1 in the glycaemic and energy expenditure responses to dietary fat in T2D, and provide a rationale for combining interventions that increase energy expenditure with GLP-1-based therapies for the management of obesity and T2D. The use of intraduodenal fat infusion, rather than a physiological meal, represents a limitation for generalisation of the findings made in this study. Further work in more physiological settings is required to validate

these observations. In addition, the subjects included in the current study had well-controlled T2D, the effects of DPP-4 inhibition (and the role of endogenous GLP-1) in subjects with suboptimal glycaemic control and/or complications remain to be determined.

In light of an essential role of GI hormones (e.g. GLP-1) in the regulation of metabolic homeostasis (Xie, Jones, et al., 2020), strategies that modulate endogenous GI hormone secretion have been actively pursued as a therapeutic option in the management of T2D and obesity. To this end, it has been suggested that a wide array of chemo-sensors expressed on different enteroendocrine cells account for the detection of nutrients and associated stimulation of GI hormone secretion (Daly et al., 2013; Kuhre et al., 2015; Lauffer et al., 2009; Liou et al., 2011; H. E. Parker et al., 2009). Emerging evidence has shown that stimulation of GI bitter taste receptors (BTRs) may have the potential to modulate GI hormone secretion and slow gastric emptying, thereby reducing postprandial glycaemic excursions and suppressing energy intake. The study reported in **Chapter 8** has shown that oral administration of the BTR agonist, denatonium benzoate (DB, 30 mg), while failing to affect gastric emptying or the glycaemic response to a high carbohydrate meal, suppressed ghrelin secretion and energy intake at an *ad libitum* buffet meal in subjects with T2D. These findings suggest that stimulation of intestinal BTRs may represent a novel approach to the management of obesity in T2D. Given that GLP-1- and PYY-releasing L-cells are located predominantly in the distal small intestine, colon and rectum (Jorsal et al., 2018), and that BTRs are abundantly expressed on the L-cells of human colonic

mucosa (Latorre et al., 2016; Rozengurt et al., 2006), it is logical to investigate the effects of rectal perfusion of bitter taste compounds on GLP-1 and PYY secretion in humans for 'proof-of-concept'. In **Chapter 9**, we observed that in young healthy humans, rectal administration of a physiological bitter bile acid (taurocholic acid, TCA), but not DB, acutely stimulated both GLP-1 and PYY secretion, and this effect was attenuated by the BTR antagonist, probenecid. However, neither TCA nor DB affected fasting plasma glucose concentrations, or energy intake at the *ad libitum* buffet meal 2 hours after rectal perfusion. Therefore, activation of BTRs alone appears to be insufficient to stimulate L-cell secretion to a degree that can be detected in the peripheral circulation, while TCA-induced gastrointestinal hormone secretion is, at least in part, mediated through BTR signalling. Further studies are required to explore whether intestinal BTRs operate in concert with other receptors to mediate GI hormone secretion.

In summary, the studies outlined in this thesis have revealed that gastric emptying in patients with 'uncomplicated' T2D, regardless of their glycaemic control, is abnormally accelerated, accounting for the exaggerated blood glucose response to meals. Interventions that slow gastric emptying are therefore a logical strategy to lower postprandial hyperglycaemia in these patients. The observation that variations in gastric emptying in subjects with and without T2D are related to the GLP-1 response to intestinal nutrients suggests that strategies capable of augmenting intestinal nutrient-induced GLP-1 secretion are desirable for slowing of gastric emptying. However, with the use of the GLP-1 receptor antagonist, exendin(9-39), a

potential for endogenous GLP-1 to suppress energy expenditure, beyond its glucose-lowering effect, is indicated, which may compromise the longer-term metabolic outcomes with GLP-1 agonism alone. While intestinal BTR-signalling is functionally linked to the secretion of endogenous GI hormones, further work is required to determine how this pathway should be exploited for the management of T2D.

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