Gastrointestinal mediation of glucose homeostasis and postprandial cardiovascular risk

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A thesis submitted by

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THESIS SUMMARY

In the majority of patients with type 2 diabetes, who have reasonably good glycemic control (HbA_1C ~7.5% or less), it is postprandial glycemia which predominates over fasting blood glucose in contributing to HbA_1C; postprandial glycemia may therefore represent an independent risk factor for diabetic complications and adverse cardiovascular events (Monnier et al., 2003). Also, at the same HbA_1C level, subjects with larger fluctuations in the blood glucose levels postprandially carry a higher cardiovascular risk (Del Prato, 2002b). The magnitude of postprandial glycemic excursions is largely determined by the rate of gastric emptying of nutrients to the small intestine which in turn regulates various metabolically important neurohumoral responses set off mainly by the release of incretin hormones from the enteroendocrine cells of the intestine. Conversely, acute glycemic excursions can regulate gastric emptying through feedback mechanisms. This thesis highlights the pivotal role of the upper gastrointestinal tract in the regulation of postprandial glycemia and attendant cardiovascular risks in health and type 2 diabetes, and the capacity for interventions aimed at modulation of upper gut function to be effective in the treatment of diabetes. Following is a brief outline of the experimental studies described in this thesis:

1. Changes in meal composition and duration affect postprandial endothelial function in healthy humans

Impaired endothelial function is now well-recognized as a forerunner of atherosclerosis (Juonala et al., 2004), and is predictive of long-term adverse cardiovascular outcomes (Vogel, 2001). The endothelial dysfunction after an oral glucose load is related to the degree of rise in blood glucose in both health and type 2 diabetes (Title et al., 2000, Kawano et al., 1999), with the duration of dysfunction being greater in the latter (Akbari et al., 1998). The rate of gastric emptying is a major determinant of postprandial glycemic increments (Chaikomin et al., 2006, Marathe et al., 2013) and modulating gastric emptying and/or nutrient absorption from the upper gut can be achieved non-pharmacologically by modifying the composition of a meal, for example by adding soluble fiber such as guar gum (Holt et al., 1979, Torsdottir et al., 1989), or by decreasing the rate of meal ingestion (Zhu et al., 2013). Though the effects of such dietary manipulations on glycemia have been described previously, their influence on postprandial endothelial function had not been investigated. We therefore evaluated the effects of dietary modifications designed to slow gastric emptying and/or small intestinal nutrient absorption on postprandial endothelial function in healthy humans.

2. The glucagon-like peptide-1 (GLP-1) receptor agonist, exenatide, inhibits small intestinal motility, flow, transit and absorption of glucose in health and type 2 diabetes: a randomised controlled trial.

The presence of glucose in the small intestine stimulates the release of several peptides, including glucagon-like peptide-1 (GLP-1) (Schirra et al., 1996); the latter has a critical role in determining postprandial insulin and glycemic responses, mainly via its ability to slow gastric emptying and regulate nutrient delivery to the small intestine, though other factors such as its insulinotropic property could also be involved. Lately, GLP-1 receptor agonists have been used widely in the treatment of patients with type 2 diabetes, (Holst and Gromada, 2004, Elahi et al., 1994). Exenatide belongs to this class of medications and is resistant to degradation by the plasma enzyme di-peptidyl peptidase-IV (DPP-IV) that rapidly degrades endogenous GLP-1. Previous studies in healthy humans have demonstrated that intravenous exogenous GLP-1 slows gastric emptying (Little et al., 2006b, Nauck et al., 1997b), but the effects of GLP-1, or agonists of its receptor, on small intestinal flow events and transit have not yet been evaluated in humans. Data from animal studies have shown that exogenous GLP-1 inhibits small intestinal motility and transit (Tolessa et al., 1998b, Tolessa et al., 1998a), which could represent an additional mechanism in the lowering of postprandial glycemia. This chapter describes the effects of the GLP-1 receptor agonist, exenatide on duodenal pressure waves, flow events, small intestinal transit time, and the rate of small intestinal glucose absorption in healthy humans as well as in those with type 2 diabetes.

3. Effects of glucagon-like peptide-1 (GLP-1) receptor agonist, exenatide, on blood pressure and heart rate in response to intraduodenal glucose infusion in type 2 diabetes: a randomised controlled trial.

It is well recognised that the magnitude of postprandial hypotension depends largely on the rate of gastric emptying (Trahair et al., 2012b); hence glucagon-like peptide-1 (GLP-1) receptor stimulation, which slows gastric emptying, could potentially be used as a treatment for postprandial hypotension. As exogenous GLP-1 has been found to increase blood pressure in some human studies, albeit not consistently (Edwards et al., 1998, Halbirk et al., 2010, Bharucha et al., 2008), and attenuate the hypotensive effect of both oral (Trahair et al., 2015) and intraduodenal (Trahair et al., 2014b) glucose loads, mechanisms other than just slowing of gastric emptying could potentially be involved. In this chapter, we present the effects of an intravenous infusion of a GLP-1 receptor agonist, exenatide, on systolic, diastolic and mean arterial blood pressure, and heart rate, measured in the course of Study 2 (above), during and after the intraduodenal glucose infusion.

4. Effects of hydroxycitrate (HCA) on intestinal glucose absorption and incretin release in healthy subjects and in type 2 diabetes.

It is now well-recognised that patients with greater fluctuations in blood glucose carry a greater cardiovascular risk than those with lesser excursions, even if HbA_1C levels are similar (Del Prato, 2002a). Therefore, there has been an emerging interest in exploring the therapeutic potential of inhibiting/delaying postprandial small intestinal carbohydrate absorption in order to dampen the glycemic spikes (Qualmann et al., 1995). Extracts from the fruit *Garcinia cambogia*, in which hydroxycitric acid (HCA) is the active ingredient, have been widely marketed for weight loss, but may also reduce postprandial

glycemia. The latter claim is based mainly on findings from rodent studies, where HCA was associated with a delay in glucose absorption, and stimulation of GLP-1 release from the distal gut. In this chapter, we describe the effects of small intestinal pre-treatment with HCA on glucose absorption and incretin release in healthy humans and in patients with diet-controlled type 2 diabetes.

5. Effects of 5 weeks resveratrol supplementation on GLP-1 secretion, gastric emptying, and postprandial glycemia in patients with type 2 diabetes.

Resveratrol, a phytoalexin, found in plant foods including red grapes, improves glycemic control in experimental animals with type 2 diabetes by uncertain mechanisms (Vang et al., 2011) and could be attractive as a potential therapy for type 2 diabetes in humans because it is safe and relatively inexpensive. In a high fat diet mouse model of diabetes, supplementation with resveratrol for 5 weeks was associated with a reduction in the glycemic excursion after an oral glucose tolerance test, together with an enhanced insulin response and substantially increased concentrations of GLP-1 in the portal vein (Dao et al., 2011), as well as increases in the proglucagon mRNA and GLP-1 content in the colon. However, effects of resveratrol on GLP-1 secretion have not previously been evaluated in humans with diabetes. This chapter describes the effects of 5 weeks of resveratrol supplementation on GLP-1 secretion, gastric emptying, and postprandial glycemia in patients with diet-controlled type 2 diabetes.

6. Comparative effect of intraduodenal and intrajejunal glucose infusion on the gut-incretin axis response in healthy males

Bariatric surgical procedures such as the Roux-en-Y gastric bypass are known to result in improvements in glycemic control in patients with type 2 diabetes, which is associated with an enhanced incretin response. In this chapter, we examined whether bypassing the duodenum would elicit a greater response of the gut-incretin axis to small intestinal glucose infusion, by comparing plasma GLP-1, GIP, insulin and glucagon, and blood glucose responses to a standardised glucose infusion into the proximal jejunum and duodenum in healthy humans.

7. Mechanism of increase in plasma intact GLP-1 by metformin in type 2 diabetes: stimulation of GLP-1 secretion or reduction in plasma DPP-4 activity?

The critical role of GLP-1 in glucose homeostasis is being increasingly recognised. In this chapter, we describe the effects of the widely used anti-diabetic medication, metformin, on total and intact GLP-1 concentrations, and on the activity of plasma dipeptidyl peptidase -4 (DPP-4), the enzyme responsible for the degradation of GLP-1, before and during an intraduodenal glucose infusion in patients with type 2 diabetes.

DECLARATION

Name: **Sony Sebastian Thazhath** ***Program:*** **Doctor of Philosophy**

I certify that this work contains no material which has been accepted for the award of any other degree or diploma 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 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 THE THESIS

1. Thazhath SS, Wu T, Young RL, Horowitz M, Rayner CK. Glucose absorption in small intestinal diseases. Expert Rev Gastroenterol Hepatol. 2014 Mar;8(3): 301-12.

2. Thazhath SS, Jones KL, Horowitz M, Rayner CK. Diabetic gastroparesis: recent insights into pathophysiology and implications for management. Expert Rev Gastroenterol Hepatol. 2013 Feb;7(2):127-39.

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Statements of Authorship

NOTE: Statements of authorship appear in the print copy of the thesis held in the University of Adelaide Library.

CHAPTER 1. GLUCOSE ABSORPTION IN SMALL INTESTINAL DISEASES

(Adapted from Expert Rev Gastroenterol Hepatol. 2014 Mar;8(3): 301-12.) (Thazhath et al., 2014b)

1.1 Abstract

Recent developments in the field of diabetes and obesity management have established the central role of the gut in glucose homeostasis; not only is the gut the primary absorptive site, but it also triggers neurohumoral feedback responses that regulate the pre- and post-absorptive phases of glucose metabolism. Structural and/or functional disorders of the intestine have the capacity to enhance (e.g.: diabetes) or inhibit (e.g.: short-gut syndrome, critical illness) glucose absorption, with potentially detrimental outcomes. In this review, we first describe the normal physiology of glucose absorption and outline the methods by which it can be quantified. Then we focus on the structural and functional changes in the small intestine associated with obesity, critical illness, short gut syndrome and other malabsorptive states, and particularly type 2 diabetes, which can impact upon carbohydrate absorption and overall glucose homeostasis.

1.2 Introduction

The growing burden of type 2 diabetes and obesity in modern societies has stimulated research into the complexities of glucose absorption, utilisation and

disposal in humans. It is now well recognised that the gut plays a pivotal role in glucose homeostasis, not only as the site of absorption, but also as a neuroendocrine modulator of pre- and post-absorptive glucose disposition. Recent insights into the roles of intestinal sweet taste receptors have improved our understanding of glucose-gut interactions. In this review, we will outline the physiology of glucose absorption and then discuss the structural and functional changes in the small intestine associated with various disease states, with particular reference to type 2 diabetes, which can influence carbohydrate absorption and blood glucose control.

1.3 Physiology of glucose absorption

The small intestinal absorption of glucose from a carbohydrate-containing meal is a complex process influenced by multiple factors, which include the rate of delivery of chyme into the small intestine, digestion of complex carbohydrates, glucose sensing by specialised intestinal cells, and transport of glucose across the intestinal mucosa to the portal circulation. These processes are described in the following sections.

1.3.1 Gastrointestinal motility

Differences in the rate of gastric emptying account for ~35% of the initial variance in the blood glucose response to an oral glucose load in both health (Horowitz et al., 1993) and type 2 diabetes (Jones et al., 1996). In health, the overall rate that nutrients empty from the stomach is maintained in the range of 1 to 4 kcal/minute by neurohumoral feedback originating from nutrient sensing in the small intestine, with resultant modulation of gastroduodenal motor function (Chang et al., 2011). The

intra-individual variability of this rate of gastric emptying on different days is relatively low when compared to the inter-individual variability (Lartigue et al., 1994). Pharmacological acceleration of gastric emptying induced by erythromycin augments the postprandial glycemic profile in type 2 diabetes, while deceleration with morphine dampens the glycemic response substantially (Gonlachanvit et al., 2003). This glycemic variability in relation to the rate of gastric emptying relates at least partly to the altered availability of glucose in the small intestine for absorption, particularly in the first hour after ingestion of a meal (Woerle et al., 2008, Gonlachanvit et al., 2003).

Gastrointestinal motor function is also responsive to changes in the glycemic state (Rayner and Horowitz, 2006). Reversible slowing of gastric emptying can be observed, not only with marked acute hyperglycemia (~16-20 mmol/L (290-360 mg/dL)), but also during more modest elevation of blood glucose within the physiological postprandial range (~8 mmol/L (140 mg/dL)) (Thazhath et al., 2013). Conversely, insulin-induced hypoglycemia increases the rate of gastric emptying, probably as a counter-regulatory process to enhance the availability of carbohydrates for absorption in the small intestine (Russo et al., 2005).

The pathogenesis of pathological slowing of gastric emptying (gastroparesis), which is a known complication of type 2 diabetes, and its implications in the management of diabetes is discussed in the next chapter.

Small bowel transit times vary significantly in the same individual during fasting as well as between fasting and postprandial states. In a scintigraphic study, two

separate observations in each healthy subject showed the small intestinal transit times to be 135 +/- 70 and 103 +/- 40 min respectively in fasting state for water, and 209 +/- 47 and 209 +/- 29 min respectively in postprandial state (Lartigue et al., 1991). Small intestinal motility and the flow of luminal contents, as assessed by manometry and impedance techniques in healthy humans, are major determinants of the rate of glucose absorption. After a carbohydrate-rich meal, duodenal absorptive capacity is rapidly overwhelmed so that glucose reaches the jejunum (Schirra et al., 1996), since small intestinal glucose absorption is limited to about ~0.5 grams per minute per 30 cm (Duchman et al., 1997). Increased small intestinal motility and flow events result in greater exposure of glucose over longer lengths of the intestine, leading to recruitment of more glucose transporters and consequent increased rates of absorption (Duchman et al., 1997). Moreover, an increase in luminal flow events results in thinning of the unstirred water layer over the mucosal surface, probably contributing to enhanced absorption (Lewis and Fordtran, 1975). Thus, pharmacological suppression of small intestinal motility and flow events by the muscarinic receptor antagonist, hyoscine, induces a delay in glucose absorption in healthy humans (Chaikomin et al., 2007) (Figure 1).

1.3.2 Digestion of carbohydrates

Complex carbohydrates reaching the small intestine must be hydrolyzed to monosaccharides such as glucose or galactose, in order to be transported across the mucosa. This occurs via disaccharidases located in the brush border membrane, including sucrase-isomaltase, lactase phlorizin hydrolase, maltase-glycoamylase and trehalase (Gudmand-Hoyer and Skovbjerg, 1996). In addition, carbohydrates derived

from dietary fruits and vegetables must initially be liberated from within the cellular plant structure and this process probably constitutes substantially to the lower glycemic index of carbohydrates from these sources (Thomas et al., 2007), as opposed to acellular forms of carbohydrate such as flour or sugars (Spreadbury, 2012).

Pseudo-carbohydrates such as acarbose, voglibose, and miglitol are competitive inhibitors of intestinal α-glucosidase enzymes and can induce carbohydrate malabsorption with consequent dampening of early postprandial glycemic excursions (Derosa and Maffioli, 2012). Carbohydrates that are absorbed incompletely from the proximal small intestine traverse to the distal gut where the digestive action of gut flora converts them into short chain fatty acids (SCFAs), which are readily absorbed by the colonic mucosa (Hammer et al., 1990). The relative slowing of colonic transit in the presence of these fatty acids further enhances absorptive capacity (Fritz et al., 2005).

1.3.3 Effects of sweet 'tasting' by the intestine

It is now recognised that glucose in the intestine is sensed or 'tasted' by specialised intestinal enteroendocrine cells as well as other specialised cells in the brush border membrane. This 'sweet tasting', is akin to sweet taste sensing by the tongue, evidenced by the presence of transcripts for G-protein coupled receptors, such as heterodimers of taste receptor type 1 member 2 (T1R2) and taste receptor type 1 member 3 (T1R3) which form a broadly-tuned heterodimeric receptor (T1R2/R3) for sweet taste at both sites (Young, 2011). These sweet taste receptors (STRs) trigger

intracellular signal transduction pathways, involving activation of the alpha subunit of gustducin, the subsequent release of intracellular Ca^{2+}, gating of the taste-specific cation channel TRPM5 (Chandrashekar et al., 2006) and changes in intracellular cAMP levels (Clapp et al., 2008), all of which culminate in membrane depolarization and the release of various neurohumoral mediators (Figure 2). The effects of STR stimulation on the expression of glucose transporters are described in the following section.

Mediators released from enteroendocrine cells as a result of STR signalling may exert endocrine, paracrine and neural actions, fundamental to glucose homeostasis. For example, STR stimulation has been linked to the release of gut peptides such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) from L-cells mainly located in the distal gut (ileum and colon), glucose-dependent insulinotropic polypeptide (GIP) from K-cells, and cholecystokinin (CCK) from I-cells of the more proximal small intestine (duodenum and jejunum) (Young, 2011). Of these, GLP-1 and GIP have been characterised as 'incretin' hormones, which stimulate insulin release from the pancreatic islet cells in a glucose dependent manner (Schirra et al., 1996). GLP-1 also slows gastric emptying (Deane et al., 2010b) and suppresses glucagon release from the alpha cells of the pancreas (Baggio and Drucker, 2007), thus contributing substantially towards the maintenance of blood glucose homeostasis. (Figure 3)

The release of GLP-1 is dependent not only on the magnitude of the glucose load reaching the small intestine (Trahair et al., 2012a), (Ma et al., 2012)but also on the length/region of the small intestine exposed to glucose (Little et al., 2006a). Although studies employing intragastric and intraduodenal artificial sweeteners to

stimulate STRs failed to induce GLP-1 secretion in healthy humans (Ma et al., 2010, Ma et al., 2009a), blockade of STRs by lactisole (an STR antagonist) attenuates glucose-stimulated GLP-1 secretion (Gerspach et al., 2011, Steinert et al., 2011), suggesting that activation of STRs is necessary, albeit insufficient, to stimulate the L-cells. CCK and PYY also have roles relevant to glucose homeostasis, including regulation of gastric and intestinal motility, and of pancreatic and biliary secretion, and signalling of satiation (Liddle et al., 1988, Rehfeld, 2004, Ballantyne, 2006)

STR signalling in the small intestine is a dynamic process responsive to acute metabolic fluctuations, including the presence of glucose in the gut lumen and also the prevailing blood glucose concentration, which will be discussed in more detail subsequently.

1.3.4 Glucose transport across the small intestinal mucosa

Even when the luminal glucose concentration in the small intestine is less than that in blood, glucose can be transported actively across the apical cell membrane via the Na^+-glucose co-transporter, SGLT1, which couples the 'up-hill' glucose transport to a 'down-hill' sodium gradient, maintained by removal of intracellular Na^+ by the Na^+/K^+ ATPase at the basolateral membrane (Kellett et al., 2008). Emerging evidence suggests a role for intestinal STRs in directing the absorptive function of SGLT1. Animal studies have revealed that small intestinal exposure to glucose, or indeed, artificial sweeteners such as saccharin, sucralose or acesulfame-K (Margolskee et al., 2007), results in rapid up-regulation of intestinal SGLT1 mRNA levels, with an increase in protein levels within 3 hours. The magnitude of this

SGLT1 response is reduced in the distal intestine - matching the distribution of STRs (Stearns et al., 2010). Moreover, the SGLT1 response in rodents is abolished by disrupting vagal pathways with capsaicin, and attenuated by pretreatment with the 5-HT_3 antagonist, ondansetron - implicating STR-triggered gut serotonin release and vagal signalling (Stearns et al., 2010). However, such a phenomenon remains to be confirmed in humans. Indeed, sucralose infused in advance of glucose in healthy lean humans did not increase small intestinal glucose absorption acutely (Ma et al., 2010), arguing against STR effects on acute SGLT1 responses in humans. The effects of a chronic high dietary intake of artificial sweeteners on intestinal SGLT1 function and glucose absorption have yet to be determined in humans.

Apart from being the primary intestinal glucose transporter, SGLT1 may have an indirect role in post absorptive glucose metabolism by acting as a luminal glucose sensor in the K and L-cells, to promote incretin release (Figure 2). Both in vitro and in vivo observations have confirmed the expression of SGLT1 in K and L cells (Wu et al., 2010), and the electrical activity associated with the sodium coupled glucose transport appears sufficient to depolarize their cell membranes, and prompt voltage-gated calcium entry and consequent incretin release (Gribble et al., 2003, Wu et al., 2012). Monosaccharide substrates of SGLT1, regardless of whether they are metabolised (e.g. glucose), or not (e.g. 3-O-methylglucose (3-OMG)), are capable of stimulating incretin secretion (Moriya et al., 2009, Wu et al., 2012). Conversely, SGLT1 inhibitors suppress glucose-dependent GLP-1 secretion, further supporting a role of these glucose transporters in incretin release (Parker et al., 2012). However, in in vivo studies in rodents, the dual SGLT1 and SGLT-2 blocker, LX4211, has been shown to reduce proximal intestinal glucose absorption, yet increase its availability

in the distal intestine where the L-cell density is greater, with consequent increments in GLP-1 release and dampening of the glycemic profile (Powell et al., 2013). This may potentially reflect an SGLT1-independent mechanism involving the colonic formation of SCFAs from unabsorbed glucose and subsequent activation of free fatty acid receptors (FFARs) on colonic L-cells (Kaji et al., 2011).

It has been shown in rodents that when the glucose concentration is higher in the intestinal lumen than in plasma, rapid trafficking of GLUT2 glucose transporters to the apical membrane occurs in enterocytes, which then accounts for the majority of glucose absorption by way of facilitated transport (Gouyon et al., 2003). Stimulation of intestinal STRs by sucralose also results in 'up-regulation' of apical GLUT2 and enhanced glucose absorption in rodent models (Mace et al., 2007). Insulin has been shown to reduce this 'up regulation' of GLUT2 transporters, thereby reducing the rate of glucose absorption and maintaining glucose homeostasis (Tobin et al., 2008).

The interaction between SGLT1 and GLUT2 is illustrated by studies of extreme starvation in rats, where GLUT2 levels become undetectable in the apical membrane, whereas SGLT1 levels are up-regulated (Habold et al., 2005). Refeeding restores baseline SGLT1 levels and triggers rapid (within 2 hours) apical trafficking of GLUT2. Further studies on SGLT1-deficient mice have confirmed that SGLT1 plays a key role in the up regulation of apical GLUT2 (Gorboulev et al., 2012).

Insertion of GLUT2 in the apical membrane has also been demonstrated in the human enterocytic colon carcinoma TC7 sub clone (Caco-2/TC7) model in vitro (Tobin et al., 2008). To date, direct in vivo evidence of apical GLUT2 in healthy

humans is lacking, and its relevance is questioned in humans by the apparent lack of GLUT2 mediated absorption to compensate for monosaccharide malabsorption in infants with rare mutations of SGLT1, resulting in glucose–galactose malabsorption (Turk et al., 1991). In fact, studies on gene expression profiles of intestinal transporters in animal models and humans indicate that there is substantially less duodenal GLUT2 expression in humans than in rats, but contrastingly higher SGLT1 expression in humans (Kim et al., 2007), indicative of a dominant role of SGLT1 in glucose absorption in healthy individuals.

Studies in animals have also indicated that the expression of GLUT2 and SGLT1, and as a result, the capacity of the gut to absorb glucose, may follow a circadian rhythm, which follows the usual eating patterns (Balakrishnan et al., 2008); however, this phenomenon has yet to be adequately evaluated in humans.

1.4 Quantification of glucose absorption

Efforts to quantify glucose absorption from the small intestine using intestinal perfusion and aspiration techniques have been documented since the 1960s (Holdsworth and Dawson, 1964). These techniques involved the use of multi-lumen tubes to perfuse the jejunum with a known concentration of glucose solution mixed with non-absorbable polyethylene glycol (PEG), at a predetermined rate. A separate lumen allowed simultaneous aspiration of intestinal fluid from a fixed distal point in the jejunum (Modigliani and Bernier, 1971), where an inflatable balloon prevented proximal reflux of the infused solution. Glucose absorption was calculated from the difference in the glucose concentrations of the infusate and the aspirate, taking into

account dilution from intestinal secretions, which was evaluated from the dilution of the co-infused PEG. Due to the inconvenience of insertion of an intra-jejunal tube and the high likelihood of errors, this technique is not presently used routinely.

It is widely recognised that the postprandial glycemic excursion reflects a combined effect of the rate of appearance of exogenous glucose absorbed from the meal ($R_{a\ meal}$), endogenous glucose production (EGP), and glucose disposal (R_d), all of which fluctuate considerably over the postprandial period. The current gold standard for measurement of the rate of absorption of exogenous glucose, distinct from the rate of EGP, is the triple tracer technique (Haidar et al., 2012, Thomaseth et al., 2008). This involves a primed continuous intravenous infusion of [3-^{3}H]-labeled, or d-[6,6-$^{2}H_2$]-labeled glucose in the fasting state with repeated blood glucose measurements, until a steady state is achieved. The rate of glucose infusion at this steady state indicates the rate of EGP. This is then followed by oral loading with a second tracer, such as [1-^{14}C]-glucose or ^{13}C-labeled glucose, along with an intravenous infusion of a third tracer, such as [U-(13)C; 1,2,3,4,5,6,6-(2)H(7)]glucose at a rate mimicking the expected $R_{a\ meal}$. Repeated blood sampling is performed for the analysis of isotopic enrichments of blood glucose using gas chromatography-mass spectrometry (Selz et al., 2003). To calculate the $R_{a\ meal}$, Steele's one-compartment model with fixed unknown glucose distribution volume, or more precisely, a two compartment model, (Mari et al., 1994) or possibly an even more accurate circulatory model, can be used (Mari et al., 2003). The expertise required and the costs involved in this sophisticated technique restrict its use to specialised centres.

3-OMG is a non-metabolisable glucose analogue that is absorbed via SGLT1 (Fordtran et al., 1962b) and GLUT2. Plasma concentrations of 3-OMG can be measured after a fixed dose is administered into the gut lumen, in order to demonstrate variations in intestinal absorption of exogenous glucose (Ma et al., 2010, Kuo et al., 2010a). This technique allows comparison of rates of 3-OMG absorption under different conditions, but does not provide absolute quantification of glucose absorption, because it does not take into account 3-OMG disposal.

Judicious use of these techniques is required to quantify glucose absorption in the appropriate setting.

1.5 Diseases affecting glucose availability in the small intestine

Structural or functional alterations in the gut, resulting from iatrogenic interventions or diseases, can affect the rate and magnitude of glucose absorption as detailed below.

1.5.1 Disorders of gastric motility

Abnormalities of gastric motor function occur in a variety of disease states, including diabetes and critical illness, and also as a result of the actions of various drugs, leading to changes in the rate of glucose absorption from a meal (Thazhath et al., 2013). The effects of gastric motility on small intestinal glucose delivery and glucose absorption are discussed in detail in the subsequent section on diabetes.

1.5.2 Bariatric surgery

Numerous therapeutic approaches to the management of obesity and its complications have aimed to control appetite via actions within the central nervous system, and have had limited efficacy and/or unacceptable adverse effects. The most effective and durable approach currently is bariatric surgery, which likely contributes to weight loss by restricting food intake and/or inducing malabsorption of nutrients. Of the various operations, Roux-en-Y gastric bypass (RYGB) has emerged as the procedure of choice in many countries (Hammer, 2012). It primarily involves restriction of food intake by creation of a small gastric pouch with a narrow outlet. Nutrient liquids empty very rapidly from this pouch into a 'Roux limb' of the jejunum, where they provoke greater release of 'satiety' hormones via a chemosensory mechanism (Picot et al., 2009). In contrast, the rate of emptying of solid nutrients from the pouch is variable (Horowitz et al., 1982). Lengthening the Roux limb further contributes to weight loss by inducing malabsorption, which acts synergistically with restricted food intake (Odstrcil et al., 2010).

RYGB markedly improves glucose tolerance in patients with type 2 diabetes, and alters the postprandial glycemic profile in glucose-tolerant individuals, resulting in an elevated glycemic peak after an oral glucose load, followed by a rapid fall to lower than basal levels. Rapid emptying of nutrient liquids into the distal gut brings about the glycemic peak, associated with exaggerated GLP-1 and insulin responses (Dirksen et al., 2012), leading to the subsequent fall in glycemia. Long-limb RYGB, at least partially, exerts its bariatric properties by inducing fat and protein malabsorption, however, overall carbohydrate absorption does not seem to be perturbed significantly, despite the rapid transit of meals through the anatomically

altered proximal gut. This is likely to reflect compensatory gut mechanisms, such as an increased expression of distal small intestinal glucose transporters, though this has yet to be clearly demonstrated.

1.5.3 Dumping Syndrome

Rapid delivery of osmotically active chyme from the stomach to the small intestine, particularly after bariatric and other gastric surgery, can result in 'early dumping syndrome' characterised by abdominal and vasomotor symptoms such as nausea, cramping, bloating, vomiting, diarrhoea, dizziness, and fatigue, which may manifest during, or immediately after, a meal. In some patients, rapidly absorbed glucose may trigger a disproportionate surge in insulin secretion resulting in 'late dumping syndrome', usually 1 to 3 hours after a meal, characterised by hypoglycemic symptoms such as sweating, tiredness and dizziness. 'Late' dumping is relatively uncommon, but can co-exist with 'early' dumping (Hammer, 2012). It is unclear as to why only some patients experience 'late' dumping. Numerous gut peptides and vasoactive substances including neurotensin, vasoactive intestinal peptide (VIP), catecholamines, serotonin and substance P have been implicated in the pathogenesis of this syndrome (Geer et al., 1990). Diagnosis of dumping syndrome is based on history and symptoms of vasomotor disturbance and/or reactive hypoglycemia, while gastric emptying studies, preferably using scintigraphic methods, may be of assistance in confirming the diagnosis (Lipp et al., 1997). Usually, restriction of dietary intake of simple carbohydrates may be enough to improve symptoms. Alpha glucosidase inhibitors such as acarbose (Buscemi et al., 2013) and miglitol (Fujita et al., 2012) can be utilised to dampen the increase in postprandial glycemia. In severe

cases, pre-prandial subcutaneous doses of somatostatin analogues, such as octreotide (Geer et al., 1990), can be considered to reduce the rate of gastric emptying, increase intestinal transit time, and restrict the release of vasoactive peptides.

1.6 Disorders of carbohydrate digestion

Disaccharidase deficiencies may be associated with carbohydrate malabsorption. Lactase deficiency is widely prevalent throughout most of the world, whereas sucrose-isomaltase and trehalase deficiencies are relatively rare, except in Greenland (Gudmand-Hoyer and Skovbjerg, 1996). Nonspecific disaccharidase deficiencies may result from certain infections, (Solaymani-Mohammadi and Singer, 2011) coeliac disease, (Murray et al., 2001) other inflammatory insults to the small intestine and exocrine pancreatic insufficiency, all of which may interfere with the breakdown of complex sugars (Hammer et al., 1990) in the proximal gut. These act to reduce glucose availability and absorption in the small intestine and increase provision of substrate for anaerobic metabolism by the distal gut flora resulting in the formation of lactic acid and SCFAs including acetate, propionate, and butyrate, along with H_2, CO_2 and methane (Cummings and Macfarlane, 1997). If these products of bacterial metabolism overwhelm the colonic absorptive capacity, they can give rise to symptoms of bloating, flatulence, and cramping. Osmotic diarrhoea, often associated with the use of antibiotics, may be a consequence of suppression of metabolism of unabsorbed carbohydrates by the gut microbiome (Hammer and Hammer, 2012).

Symptomatic patients with suspected disaccharidase deficiencies can be evaluated by elimination diets, noninvasive hydrogen breath tests (Fordtran et al., 1962b), or by

direct disaccharidase activities estimated from duodenal biopsies; the latter may also allow diagnosis of associated gastrointestinal disorders, such as coeliac disease (Murray et al., 2001). Dietary restriction or elimination of the causative disaccharide, or appropriate enzyme supplementation (eg: lactase supplements for lactase deficiency or sacrosidase supplements for sucrase deficiency), or appropriate use of probiotics, may benefit many of these patients (Montalto et al., 2006, Robayo-Torres et al., 2009).

1.7 Disorders of glucose transporters in the intestinal mucosa

Autosomal recessive mutations in the SGLT1 gene may result in a structurally, or functionally, deficient SGLT1 protein, or to trafficking errors within enterocytes that prevent insertion into the plasma membrane. Such mutations lead to congenital glucose-galactose malabsorption, a rare disorder characterised by early onset malabsorption of glucose and galactose (Wright, 1998), which is typically diagnosed via H_2 breath tests. Fructose replacement formulas are used to mitigate life-threatening diarrhoea in affected infants.

In contrast, a congenital defect in the GLUT2 transporter (Fanconi–Bickel syndrome), does not result in significant intestinal glucose malabsorption (Santer et al., 2003). An alternative transport mechanism for glucose at the basolateral membrane is implicated in affected individuals, involving glucose phosphorylation, transport across the microsomal membrane via the glucose-6-phosphate transporter G6PT1, cleavage by glucose-6-phosphatase within the microsome to free glucose, and exit of the free glucose across the basolateral membrane through exocytosis to the portal circulation. Not surprisingly, such patients are unable to absorb 3-OMG, as it cannot

be metabolised to its phosphorylated form for microsomal transport. Though intestinal glucose transport is unaffected, patients with Fanconi–Bickel syndrome exhibit tubular nephropathy, fasting hypoglycemia, rickets, stunted growth and hepatomegaly secondary to glycogen accumulation due to the extra-intestinal distribution of the defective GLUT2 transporters.

1.8 Glucose absorption in critical illness

Critical illness is typically associated with a period of nutritional deprivation, resulting from deficient, or delayed enteral feeding, which has detrimental effects on small intestinal mucosal integrity and absorptive function (Stechmiller et al., 1997, Nguyen et al., 2012). Short-term nutrient deprivation (~ 4 days) has been shown to result in a decrease in villus height and crypt depth (Hernandez et al., 1999), which is associated with diminished nutrient absorption and prolongation of malnutrition. Furthermore, recent evidence in critically ill patients indicates that optimization of glycemia is associated with better clinical outcomes (Lanspa et al., 2013), which underscores the necessity to improve understanding of gut-glucose interactions in

Critical illness induced ('stress') hyperglycemia (CIIH), is observed in a substantial proportion of critically ill patients without a previous history or evidence (elevated glycated hemoglobin) of diabetes or disordered glucose metabolism (Umpierrez et al., 2002) (Finfer et al., 2009). Elevated levels of endogenous catecholamines, corticosteroids and glucagon, along with a relative decline in insulin secretion and the development of peripheral insulin resistance, are likely to contribute (Dahn and Lange, 1982). There is evidence that the deleterious effects of hyperglycemia may be greater in patients with CIIH than in those with pre-existing diabetes probably

because the former group is poorly conditioned to the effects of hyperglycemia (Egi et al., 2008).

Critical illness is also associated with a reduction in the rate of intestinal glucose absorption which cannot fully be accounted for by the concomitant reduction in the rate of gastric emptying that is observed frequently in these patients (Chapman et al., 2009). We have recently shown that transcript levels of intestinal glucose transporters SGLT1 and GLUT2, as well as glucose absorptive function, are profoundly reduced in critically ill patients. Furthermore, we revealed that intestinal levels of STR (T1R2) transcript are lower in critically ill patients when compared to healthy controls, and lower in a murine model of critical illness, supporting the hypothesis that suppression of intestinal STR signalling in critical illness may contribute to reduced availability of glucose transporters, and thereby, glucose malabsorption (Deane et al., 2013). The well described increase in superior mesenteric artery (SMA) blood flow in response to small intestinal glucose infusion is also markedly diminished in elderly critically ill patients, which may further compromise glucose absorption (Sim et al., 2013). Any attempts to correct impaired glucose absorption in critical illness, however, could potentially be detrimental to overall glycemic status if not accompanied by other glucose lowering factors, such as augmentation of the incretin response (Deane et al., 2011c).

Even though overall GLP-1 levels are maintained in critical illness, fasting levels are higher than in health, most likely due to the delivery of undigested carbohydrates to the distal gut, where they stimulate L-cells via non-SGLT1 mechanisms (Deane et al., 2011b). The conversion of glucose to SCFA by colonic bacteria, and subsequent

stimulation of L-cell FFARs is likely to be the dominant signalling pathway for GLP-1 release here (Kaji et al., 2011), aided by the high density of L-cells in this gut region, and is likely to compensate, in large part, for the reduced glucose-dependent GLP-1 release from proximal L-cells (Deane et al., 2013). Indeed, intrinsic responses to GLP-1 appear to be preserved in the critically ill, as evidenced by the fact that acute exogenous GLP-1 administration effectively lowers postprandial glycemia in these patients, at least in part by slowing gastric emptying when the latter is normal, but not when it is delayed (Deane et al., 2010a). Moreover, exogenous GLP-1 also effectively reduces the glycemic response to nutrients delivered directly to the small intestine in the critically ill, apparently via relative stimulation of insulin and suppression of glucagon (Deane et al., 2009).

1.9 Glucose absorption in short gut syndrome

Extensive small bowel resection for indications such as malignancies, radiation injury, ischemic gut, Crohn's disease, trauma or necrotizing enterocolitis, may result in 'short gut syndrome' – a term used when the remnant length is less than 200 cm. Such resections result in marked loss of absorptive mucosal surface area, potentially leading to nutrient malabsorption and intestinal failure requiring long-term parenteral nutrition (Messing et al., 1999). Over months to years after the surgery, the remnant gut undergoes structural adaptation to increase villus height and crypt depth, with proliferation of enterocytes, as well as functional adaptation involving reduced gut motility to enable more efficient nutrient absorption. The prognosis, and nutritional recommendations, for such patients are dependent not only on the remaining length of small intestine, but also on continuity with the terminal ileum and colon. Even after substantial jejunal resection, carbohydrate malabsorption is greatly mitigated by

salvage mechanisms in the more distal gut. Conversely, loss of the ileum and colon is associated with diminished secretion of enteric hormones including GLP-1, GLP-2 and PYY, which can disrupt the 'ileal brake mechanism', resulting in exacerbation of fluid and nutrient loss (Madsen et al., 2013).

Conventional management consists of parenteral nutrition and optimization of the remnant intestinal function using anti-diarrhoeal and antisecretory agents. Recent studies on short-gut patients showed a decrease in diarrhoea and faecal load with continuous infusions of the gut hormones GLP-1 and GLP-2, either individually or in combination. GLP-2 was more effective than GLP-1, and the combination demonstrated an additive effect (Madsen et al., 2013). GLP-2 has intestinotrophic actions and augments intestinal adaptation with resultant enhancement of nutrient absorption by the remnant intestine. Evidence derived from animal studies, and studies with the human enteroendocrine cell line NCI-H716, support a role of STR stimulation in the release of GLP-2 from L-cells (Sato et al., 2013), and GLP-2 induced augmentation of glucose transport via SGLT1 (Cheeseman, 1997) and, in rodents, GLUT2 trafficking to the brush-border (Au et al., 2002), indicating a potential link between intestinal STR stimulation and GLP-2-facilitated glucose absorption. Long-term treatment with a novel recombinant GLP-2 analogue, teduglutide, which is resistant to breakdown via endothelial dipeptidyl peptidase-IV, has been approved in the USA and European Union for the treatment of adults with short gut syndrome, who are dependent on parenteral nutrition (Burness and McCormack, 2013). Teduglutide increases villus height and crypt depth, and decreases gastric motility and intestinal secretory losses, thereby improving nutrient absorption (Vipperla and O'Keefe, 2013).

1.10 Glucose absorption in type 2 diabetes

The following section pertains to the structural and functional alterations of the gut in type 2 diabetes, which have an impact on the various stages of glucose absorption described previously.

1.10.1 Disordered gastric motility

Gastroparesis is a common complication of diabetes, and slows the rate of delivery of carbohydrates to the small intestine (Samsom et al., 1996). In patients not being treated with insulin, slower gastric emptying may well be of benefit for glycemic control, provided it is not associated with symptoms such as nausea and vomiting (Thazhath et al., 2013). However, in insulin-treated patients, the timing of nutrient delivery to the small intestine needs to be coordinated with the administration of exogenous insulin in order to obviate early postprandial hypoglycemia ('gastric hypoglycemia') and/or late postprandial hyperglycemia (Horowitz et al., 2006).

It appears logical to speculate that the relatively newer classes of anti-diabetic medications, short acting GLP-1 agonists (eg: exenatide bd and lixisenatide) and amylin analogues, which exert their glucose lowering action primarily by reducing the rate of glucose delivery to the small intestine, may be more effective in patients with relatively more rapid gastric emptying at baseline (Linnebjerg et al., 2008), suggesting that quantification of gastric emptying may be helpful in guiding the choice of antidiabetic therapy (Thazhath et al., 2013). The potential for GLP-1 agonists to impair small intestinal motility (Tolessa et al., 1998b), and thus glucose absorption, has yet to be adequately evaluated in humans.

1.10.2 Disordered carbohydrate digestion

Animal models of type 2 diabetes display intestinal mucosal hyperplasia and an increase in disaccharidase activity, with resultant enhancement of nutrient absorption (Adachi et al., 2003). Increased duodenal sucrase and lactase are also reported in human type 2 diabetes, along with specific increases in intestinal SGLT1, GLUT2 and GLUT5 mRNA expression and protein, and Na^+-dependent glucose transport, in comparison to healthy control subjects; such phenomena are likely to worsen glycemic control, especially when associated with consumption of a high glycemic index diet (Dyer et al., 2002). Indeed, competitive inhibitors of intestinal α-glucosidase enzymes, such as acarbose (Gentilcore et al., 2005), voglibose, and miglitol, reduce carbohydrate digestion and glucose availability in the proximal small intestine and are effective as antidiabetic medications (Derosa and Maffioli, 2012), by ameliorating postprandial hyperglycemia.

1.10.3 Disorders of intestinal 'sweet tasting'

We have shown that duodenal expression of STRs is comparable in fasted control subjects and type 2 patients, and unaltered by acute hyperglycemia in either group (Young et al., 2009, Young et al., 2013). However, duodenal T1R2 transcript levels are differentially regulated by luminal glucose according to glycemic status in healthy subjects, with expression being increased during euglycemia, and decreased during hyperglycemia. As STR activation up-regulates SGLT1 in animal models (Stearns et al., 2010), this phenomenon may represent a counter-regulatory

mechanism to limit the rate of glucose absorption in the face of hyperglycemia. In contrast, T1R2 levels in type 2 patients are increased by luminal glucose irrespective of prevailing glycemia – a defect associated with higher absorption of glucose (or its analogue, 3-OMG) during hyperglycemia (Young et al., 2013), providing strong support for T1R2-mediated regulation of glucose absorption via SGLT1 in humans. Furthermore, a positive association can be observed between glucose-induced changes in duodenal STR expression and the magnitude of GLP-1 and GIP secretion, supporting an additional role for duodenal STRs in incretin release. Consequently, interventions aimed at modulating STR function may represent a potential therapeutic approach to improve glycemic control in diabetes.

1.10.4 Disorders of glucose transporters

While apical expression of GLUT2 is absent in lean subjects and type 2 patients (Dyer et al., 2002), it has recently been demonstrated in morbidly obese humans using confocal and electron microscopy and Western blot, where it correlates with fasting glycemia and insulin resistance (Ait-Omar et al., 2011). Such permanent, apical insertion of GLUT2 would predispose to accelerated post-prandial glucose absorption. Indeed, the GLUT2 transporter has bidirectional capability and the direction of glucose transport is dependent on the gradient between the luminal and the blood glucose concentrations. For example, permanent apical GLUT2 in obese mice facilitates glucose transport from the enterocytes into the lumen as soon as the blood glucose concentration rises above luminal concentration. This glucose efflux might alter the composition of the gut microbiota to induce a pro-inflammatory state

(Turnbaugh et al., 2006). This phenomenon is, however, yet to be demonstrated in humans.

1.11 Expert Commentary

Recent studies in the field of diabetes and obesity surgery have established the central role of the gut in glucose homeostasis. Disorders of the gastrointestinal system associated with different diseases affect various aspects of glucose absorption as well as the post-absorptive phase, through nutrient-gut and gut-brain interactions, including alterations in the enteroendocrine response to nutrient exposure. The increasingly recognised role of the microbiome in salvaging unabsorbed carbohydrates in the distal gut may be beneficial in cases of malnutrition, while contributing to obesity in other individuals. Further research in the field of intestinal sweet-taste sensing mechanisms is required, and has the potential to lead to the development of novel therapeutic options for the management of type 2 diabetes and obesity.

1.12 Five year view

Further insights into the molecular mechanisms underlying intestinal glucose absorption are anticipated in the coming years. Future developments in the management of diabetes and obesity will further increase the focus on the gut as a primary target of treatment. Ongoing research in the field of the gut microbiome may lead to incorporation of novel strategies in the management of the metabolic syndrome. Intestinal sweet taste receptor modulation has the potential to be a therapeutic intervention in the areas of diabetes, obesity, critical illness, short-gut

syndrome and various malabsorptive states. All these developments may afford a tailored treatment approach for patients with specific metabolic derangements.

1.13 Key issues

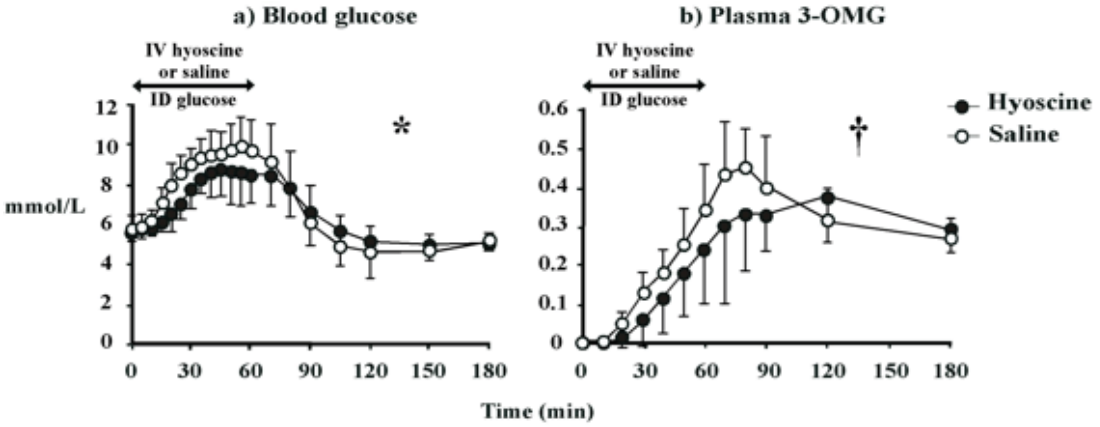
- The central role of gut in glucose homeostasis is being increasingly recognised with the ongoing developments in the areas of diabetes and obesity management; gut is not only the site of nutrient absorption, but also a regulator of pre- and post-absorptive phases of glucose metabolism via neurohumoral feedback responses.

- Small intestinal glucose absorption is influenced by the rate of gastric emptying, motility of the small intestine, flow of luminal contents, digestive capacity of the gut, and the efficacy of glucose transporters.

- Disorders of one or more of these interrelated factors could potentially stimulate, or attenuate, glucose absorption; interventions to modulate these elements could, therefore, be therapeutically important.

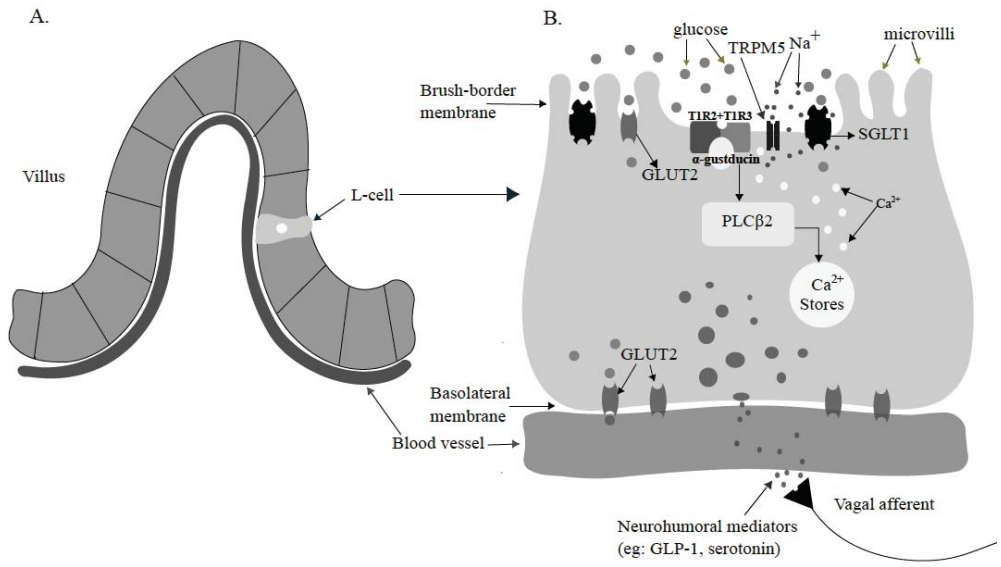
- Recent insights into the role of sweet taste receptor (STR) signalling in the regulation of intestinal glucose transporters and release of neurohumoral mediators, such as incretin and satiety hormones, have deepened our understanding of nutrient-gut and gut-brain interactions, especially in conditions such as diabetes, obesity and critical illness. However, further human studies are necessary before pharmacological modulation of STRs can be considered as a therapeutic option.

Figure 1



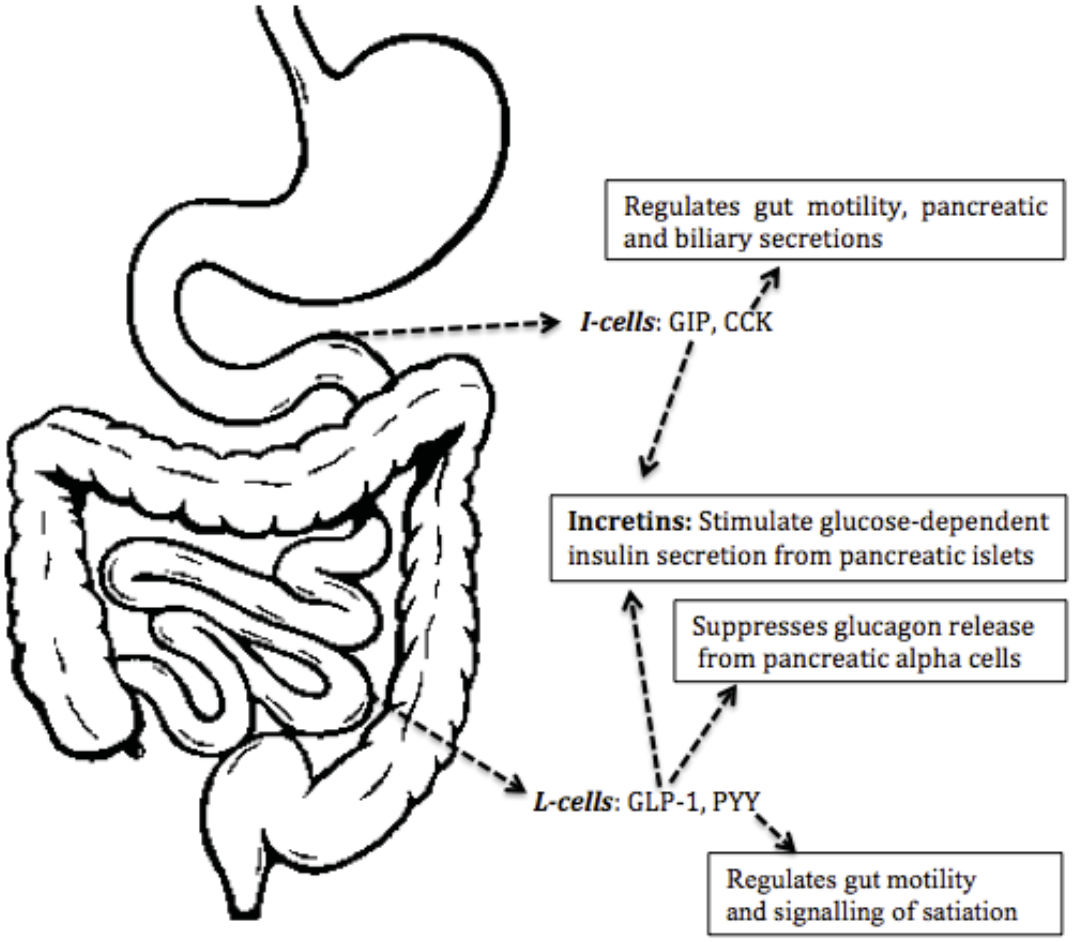
Glucose, together with the non-metabolized glucose analogue 3-O-methylglucose (3-OMG), was infused via an intra-duodenal catheter for 60 minutes, with concurrent intravenous (IV) administration of either hyoscine or placebo (saline). There was a reduction in (A) blood glucose concentrations and (B) plasma 3-OMG concentrations with hyoscine when compared to placebo (*P < 0.05 in the first 60 min; †P<0.05 for area under the curve over 180 min). Concurrent intraluminal impedance measurements showed that the reduction in glucose absorption was associated with a decrease in the number of small intestinal flow events. (Reproduced from Am J Physiol Gastrointest Liver Physiol. 2007; 292: G1099-104.)

Figure 2



Schematic representation of sweet taste signaling in the L-cell. A) L-cell located in the villus. B) Enlarged view of the L-cell. Glucose is sensed by the heterodimeric taste receptor type 1, members 2 and 3 (T1R2+T1R3) located on the brush-border membrane (BBM). This stimulates α-gustducin, which subsequently activates phospholipase C beta 2 (PLCβ2) leading to release of ionized calcium (Ca^{+}) from intracellular stores. This opens up the taste-specific transient receptor potential cation channel, subfamily M, member 5 (TRPM5) allowing influx of sodium ions (Na^{+}), membrane depolarization and release of neurohumoral mediators, which can produce endocrine and paracrine actions as well as trigger impulses via the mucosal vagal afferents. Sodium-glucose linked transporter-1 (SGLT1) is the main glucose transporter in the BBM and glucose transporter-2 (GLUT2) in the basolateral membrane. The electrical activity associated with sodium coupled glucose transport appears sufficient to depolarize the cell membrane, and prompt voltage-gated calcium entry (not shown here) and consequent mediator release.

Figure 3



Schematic representation of regional variations in the release of gut hormones such as GLP-1, PYY, GIP and CCK and a brief description of their functions.

CHAPTER 2. DIABETIC GASTROPARESIS – RECENT INSIGHTS INTO PATHOPHYSIOLOGY, AND IMPLICATIONS FOR MANAGEMENT.

(Adapted from Expert Rev Gastroenterol Hepatol. 2013 Feb;7(2):127-39.)(Thazhath et al., 2013)

2.1 Abstract

Delayed gastric emptying affects a substantial proportion of patients with long-standing diabetes, and when associated with symptoms and/or disordered glycemic control, affects quality of life adversely. Important clinicopathological insights have recently been gained by the systematic analysis of gastric biopsies from patients with severe diabetic gastroparesis, which may stimulate the development of new therapies in the coming decade. Experience with prokinetic therapies and treatments, such as pyloric botulinum toxin injection and gastric electrical stimulation, has established that relief of symptoms does not correlate closely with acceleration of delayed gastric emptying, and that well-designed controlled trials are essential to determine the efficacy of emerging therapies.

2.2 Introduction

Reports of gastric motor dysfunction in patients with diabetes have existed in the literature since at least 1925 (Boas, 1925), and in 1958 Kassander used the term “gastroparesis diabeticorum” to describe abnormally increased gastric retention in these patients (Kassander, 1958). Diabetic gastroparesis is increasingly recognized as an important clinical disorder, probably reflecting both greater awareness, and

advances in diagnostic techniques. Although there is no universally accepted definition for diabetic gastroparesis, we consider it to be present when gastric emptying is (i) known to be delayed for more than three months in patients with diabetes, (ii) not attributable to mechanical obstruction, transient effects of hyperglycemia (blood glucose>12mmol/L) or other potential etiologies (Khoo et al., 2009), and (iii) associated with upper gastrointestinal symptoms such as early satiation, fullness, abdominal pain or bloating, or postprandial hypoglycemia (in insulin-treated patients). The disorder can range from being mildly symptomatic to having severe symptoms leading to malnutrition, electrolyte imbalances and impaired glycemic control (Khoo et al., 2009). This review will discuss the pathophysiology and management of diabetic gastroparesis, focusing particularly on recent insights.

2.3 Epidemiology

Because the correlation between symptoms and the magnitude of the delay in gastric emptying is poor, it is difficult to make an accurate assessment of the incidence or prevalence of diabetic gastroparesis (Khoo et al., 2009). Existing estimates are highly dependent on the definition of gastroparesis chosen by the investigators, and whether the measurements were conducted in the euglycemic state, but delayed gastric emptying per se has a reported prevalence amongst patients with longstanding type 1 or type 2 diabetes ranging from 28% to as high as 65% (Samsom et al., 2003, Jones et al., 2001b). Although the prevalence of gastroparesis is thought to be greater among type 1 compared to type 2 diabetic patients, the absolute number is likely to be higher in the latter group, given their larger number overall (Camilleri et al., 2011). Moreover, a 158% rise in hospitalizations for gastroparesis was recorded in the

United States between 1995 and 2004(Wang et al., 2008). Female sex and symptoms of abdominal bloating and fullness have been shown to have some predictive value for the presence of delayed gastric emptying of both solids and liquids in type 1 and type 2 diabetes (Jones et al., 2001b).

2.4 Physiology of normal gastric motility

The gastrointestinal tract is innervated by extrinsic autonomic nerves providing a connection with the central nervous system, and by the enteric nervous system consisting of the myenteric (Auerbach's) plexus between the longitudinal and circular muscle layers of the muscularis externa, and the submucosal (Meissner's) plexus which is involved in the regulation of absorption, secretion and mucosal blood flow (Camilleri, 2006). Interstitial cells of Cajal (ICC), which are non-neural cells with pacemaker properties, function at the interface between the enteric nervous system and the smooth muscle.

The gastric pacemaker is located in the greater curvature of the mid to upper corpus (O'Grady et al., 2010). ICCs generate electrical slow waves from this region, at a rate of three per minute (O'Grady et al., 2010), which are propagated slowly longitudinally, but rapidly in the circumferential direction, and set the maximum possible rate of contraction. In the fasting state, the muscles of the stomach undergo different phases of contraction called the "migrating motor complex" (Wingate, 1981, Chang et al., 2011): phase 1 consists of motor quiescence lasting approximately 40 minutes, while phase 2 consists of irregular contractions for about 50 minutes, and phase 3 is characterized by regular high amplitude contractions at a rate of 3 per minute for about 5 to 10 minutes (Chang et al., 2011). It is primarily during phase 3

that large, indigestible solid residues are emptied into the duodenum; loss of phase 3 is associated with the formation of gastric “bezoars”.

The contraction patterns change postprandially: the gastric fundus relaxes, reflecting vagally mediated nitrergic inhibition of tone, to accommodate the ingesta; the antrum triturates solids into small particles; and the pylorus acts as a brake to gastric emptying by its tonic and phasic contractile activity. Normal gastric emptying, therefore, requires a combination of contraction (mediated by the excitatory neurotransmitters acetylcholine and substance P) and relaxation (mediated by inhibitory transmitters - nitric oxide (NO) and vasoactive intestinal peptide) - together with coordination of activity between regions (Kashyap and Farrugia, 2010).

When evaluated on a second-by-second basis, gastric emptying is predominantly pulsatile rather than continuous, so that most chyme enters the small intestine as a series of gushes (Jones et al., 1995a). There are, however, substantial differences in the determinants of overall gastric emptying patterns of solids and liquids. Solid emptying is largely dependent on the volume, consistency and fat content of the meal. There is an initial lag phase before emptying commences, followed by an emptying phase that approximates a linear pattern for the majority of emptying (Chang et al., 2011). Emptying of non-nutrient liquids is exponential whereas emptying slows to a more linear fashion with increasing calorie content (Chang et al., 2011).

The overall rate of emptying of nutrients from the stomach is closely regulated in the range of 1 to 4 kcal/minute, predominantly by feedback arising from the presence of nutrients in the small intestine to modulate gastroduodenal motor function (Chang et

al., 2011). Mediators of this feedback include the vagus nerve and gut-derived peptides including glucagon-like peptide-1 (GLP-1), cholecystokinin, peptide YY, and human islet amyloid polypeptide (which is co-secreted from the pancreas along with insulin) (Khoo et al., 2009).

2.5 Motor abnormalities in diabetic gastroparesis

Impairment of proximal gastric relaxation in response to meals (Samsom et al., 1995, Samsom et al., 1998) and hypomotility of the antrum, both during fasting (loss of phase 3 activity of the MMC) and postprandially (Samsom et al., 1996), are well described in patients with markedly delayed emptying associated with diabetic gastroparesis. In one series, pylorospasm was reported to make a frequent contribution towards gastroparesis (Mearin et al., 1986). Abnormalities of motor function are frequently associated with a disordered gastric electrical rhythm, such as abnormally low (bradygastria) or high (tachygastria) slow wave frequencies (Owyang and Hasler, 2002). Acute hyperglycemia itself can contribute to reversible slowing of gastric emptying during both marked elevation of blood glucose (~16-20 mmol/L) (Fraser et al., 1990) and during more modest elevation within the physiological postprandial range (~8 mmol/L) (Schvarcz et al., 1997), associated with reduced fundic tone (Hebbard et al., 1996), antral hypomotility(Fraser et al., 1991), and increased phasic and tonic pressures at the pylorus(Fraser et al., 1991). Acute hyperglycemia can also contribute to increased perception of symptoms such as fullness(Jones et al., 1997). Furthermore, gastric dysrhythmias can be induced by acute hyperglycemia. Conversely, insulin-induced hypoglycemia accelerates gastric emptying, even when the latter is delayed (Russo et al., 2005).

2.6 Etiology and natural history of symptoms in diabetic gastroparesis

Given the poor correlation between symptoms and the degree of delayed emptying (Horowitz et al., 1986), it is apparent that abnormalities of gastric emptying and intragastric meal distribution do not always fully account for the presence of symptoms in gastroparesis. Other potential contributing factors in the etiology of symptoms include altered gastric myoelectrical activity (Koch et al., 1989), disordered esophageal and intestinal motility (Horowitz et al., 1991, Wegener et al., 1990), an increased prevalence of psychiatric disorders, and increased sensitivity of the stomach or small intestine resulting in heightened feedback to distension or luminal nutrients (Chang et al., 2011). This multifactorial etiology needs to be considered when planning therapeutic interventions.

2.7 Pathophysiology of diabetic gastroparesis

Given the similarities in symptoms experienced by surgically vagotomised patients and those with long-standing diabetes, it was long assumed that diabetic gastroparesis was invariably the result of irreversible vagal damage. In support of this view, rodent models of diabetes have shown reductions in both the number of myelinated axons of the vagosympathetic trunk and in neurons in the dorsal root ganglia (Khoo et al., 2009). However, human studies have not consistently shown extrinsic nerve defects. Moreover, the outcome of standardized tests of cardiovascular autonomic function, which are used widely as a surrogate marker for the function of the abdominal vagus, does not correlate closely with the presence or absence of disordered gastric emptying in patients with diabetes (Khoo et al., 2009).

In recent years, novel insights into the pathogenesis of diabetic gastroparesis have been achieved through the analysis of full-thickness gastric biopsy specimens, which has been facilitated in particular by the establishment of the Gastroparesis Clinical Research Consortium in 2006, with the primary aim of enabling prospective clinical and pathological studies involving patients with gastroparesis. In the majority, if not all cases, the biopsies have been obtained from patients with severe gastroparesis unresponsive to conventional management. Reported abnormalities in gastric biopsies are heterogeneous and include smooth muscle fibrosis (Pasricha et al., 2008) and abnormal immune infiltrates, and there has been a particular focus on a reduction in inhibitory neurotransmission, mediated by NO, and on loss and/or dysfunction of the ICCs (Grover et al., 2011, Grover et al., 2012) .

2.7.1 Role of nitric oxide as an inhibitory neurotransmitter in diabetic gastroparesis

Nitric oxide (NO) is an inhibitory neurotransmitter that has a major role in regulating gastric emptying, particularly by facilitating pyloric relaxation. Rodent models of diabetes have revealed a defect in the activation of gastrointestinal nNOS resulting in impaired NO production. Interestingly, this appears to affect females of the species predominantly (Gangula et al., 2007), an observation that may potentially account for the female preponderance of diabetic gastroparesis. Reduced NO production due to experimental inhibition of nNOS in animals is associated with suppression of proximal gastric relaxation (Desai et al., 1991), and stimulation of pyloric contractions (Orihata and Sarna, 1996) with resultant slowing of gastric emptying. However, the NO donor, nitroglycerine, slows rather than accelerates gastric

emptying in healthy humans, reflecting increased meal retention in the proximal stomach and inhibition of propagated antral contractions (Sun et al., 1998). Furthermore, the phosphodiesterase type-5 inhibitor, sildenafil, which enhances NO-mediated responses, is ineffective at accelerating gastric emptying in patients with diabetic gastroparesis(Dishy et al., 2004). It is unclear, therefore, to what degree deficient NO transmission in animal models translates to humans with gastroparesis.

2.7.2 Role of ICC loss or dysfunction in diabetic gastroparesis

Since ICCs are fundamental to the generation and propagation of gastric electrical slow waves, it is not surprising that their loss or dysfunction might be associated with disordered gastric emptying (Sanders et al., 2006). Focal or diffuse depletion of ICCs has now been reported in animal models of diabetes (Ordog et al., 2000, Horvath et al., 2006) and in humans with diabetic gastroparesis (Iwasaki et al., 2006). Histopathological analysis of full thickness gastric biopsies demonstrated ICC depletion in 10 of 20 patients. The number of ICCs tended to be lower in older patients and was related to the rate of gastric emptying and to the loss of enteric nerves (Grover et al., 2012), while no differences were evident in terms of gender, type of diabetes, or glycemic control as determined by glycated hemoglobin (HbA1C) (Grover et al., 2012). The reduction in the number of ICCs appears to be relatively selective for the antrum, rather than the corpus of the stomach (Forster et al., 2005), and patients with more severe ICC depletion may have a greater tendency to tachygastria as well as higher symptom scores (Forster et al., 2005). Recent electron microscopic studies have compared ultrastructural changes in diabetic and idiopathic gastroparesis with healthy controls; all patients with gastroparesis exhibited

abnormalities of ICCs and enteric nerves on electron microscopy, whereas a few had apparently normal biopsies on light microscopy. In diabetic patients, the basal lamina around smooth muscle cells and nerves was thickened, whereas patients with idiopathic gastroparesis had perineural fibrosis (Faussone-Pellegrini et al., 2011), suggesting more severe irreversible pathology in the idiopathic than the diabetic group.

Potential causes of the ICC loss or damage in diabetes include autoimmune phenomena, oxidative damage induced by the formation of reactive oxygen species (ROS) as a result of chronic hyperglycemia, and/or dystrophic changes due to impairments in insulin and growth factor signaling (Ordog, 2008).

The concept of autoimmune depletion of ICCs is supported by the demonstration that the number of CD45 positive leukocytes in the myenteric plexus in full thickness gastric biopsies from patients with diabetic gastroparesis is increased by 25% when compared to healthy controls (Grover et al., 2012). However, the CD45 count does not apparently correlate with either the degree of ICC loss or the magnitude of delay in gastric emptying (Grover et al., 2012).

Acute hyperglycemia is known to increase the production of ROS in the endothelium, and this “oxidative stress” facilitates activation of protein kinase C, formation of advanced glycation end products (AGE), accumulation of sorbitol, and activation of NFkappaB, all of which may contribute to the development of the chronic complications of diabetes (Nishikawa et al., 2000). However, direct evidence for a causative association between hyperglycemia and loss of ICCs is currently lacking.

As well as being an important inhibitory neurotransmitter, NO may exert neuroprotective effects against ROS as a result of its potent antioxidant properties (Chiueh, 1999). Murine models of diabetic gastropathy have shown insulin-sensitive reversible loss of nNOS (Watkins et al., 2000), resulting in reduced NO production and increased likelihood of oxidative neuronal damage. This insulin-sensitivity in the early stages of diabetes appears to reflect a defect in the axonal transport of nNOS rather than an overall reduction; with progression of the disease, it is hypothesized that nNOS accumulates in the neuronal cell bodies and interacts with accumulating AGEs, resulting in irreversible neuronal apoptosis (Cellek, 2004). The implication is that efforts to replenish nNOS may represent an early therapeutic target to prevent irreversible damage, although it should be recognized that this concept is yet to be translated to humans with diabetes.

Heme-oxygenase-1 (HO-1), an enzyme involved in the metabolism of heme into biliverdin, iron and carbon monoxide (CO) in gastric macrophages, also has the potential to protect against oxidative stress-induced neuronal damage in diabetic gastroparesis(Choi et al., 2008). In a rodent model, induction of HO-1 by hemin was associated with a decrease in ROS, an increase in nNOS expression, restoration of the expression of Kit (a marker of ICC), and reversal of delayed gastric emptying(Choi et al., 2008). CO appears to mediate the protective effects of HO-1, and in a mouse model of diabetic gastroparesis, inhalation of low dose CO is accompanied by reduced oxidative stress, increased Kit expression, and normalization of gastric emptying(Kashyap et al., 2010). Facilitating the actions of nNOS and/or HO-1 therefore holds promise as a strategy for future therapies for

diabetic gastroparesis. A study in healthy humans recently confirmed that intravenous administration of hemin could indeed induce HO-1 activity(Bharucha et al., 2010).

In a murine model of diabetic gastroparesis, gastric smooth muscle atrophy has been implicated in the loss of ICCs, via a reduction in insulin or insulin-like growth factor-1 (IGF-1) signaling. Insulin and IGF-1 stimulate receptors on gastric smooth muscle, resulting in the production of stem cell factor (SCF), which is essential for the survival of gastric ICC, and is deficient in the setting of smooth muscle atrophy (Horvath et al., 2006).

It is clear from the discussion above that a number of factors are involved in the pathogenesis of diabetic gastroparesis, presenting both opportunities and challenges for formulating effective therapeutic interventions.

2.8 Diagnosis

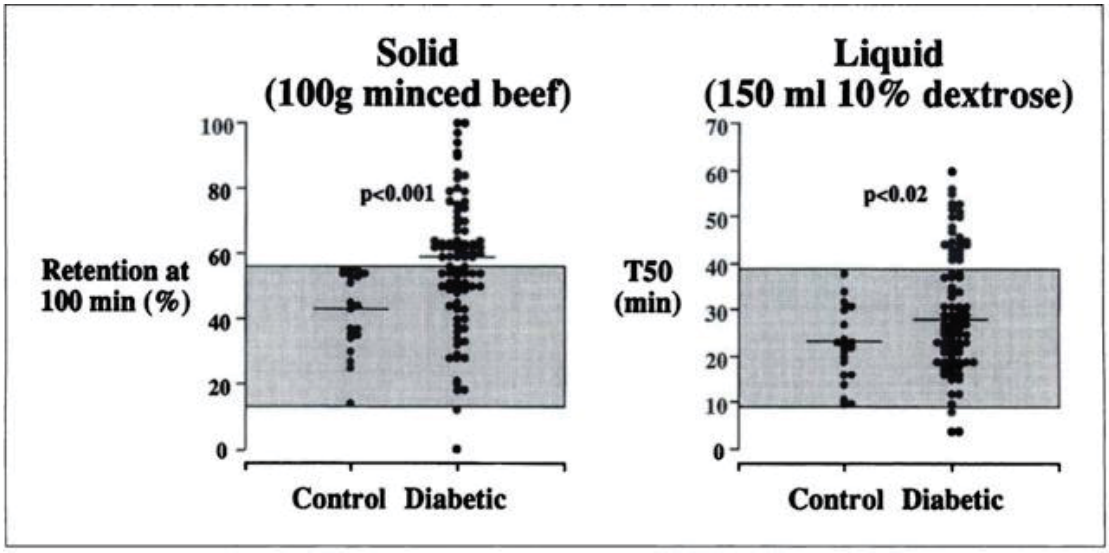
Diabetic gastroparesis has to be distinguished from "physiological" delays in gastric emptying occurring as a result of acute elevations in blood glucose, as previously mentioned. Measurements of gastric emptying should ideally be performed during euglycemia, or at a minimum when the blood glucose is > 4 mmol/L and ≤ 10mmol/L (Fraser et al., 1990). Any cause of mechanical gastric outlet obstruction should be ruled out radiologically and/or endoscopically (Camilleri et al., 2011). A delay in gastric emptying more than at least 1.5 standard deviations from the normal mean is one measure that has been used to diagnose gastroparesis (Stacher et al.,

1999). A variety of diagnostic techniques, as outlined below, have been employed to evaluate gastric emptying.

2.8.1 Scintigraphy

Scintigraphy remains the diagnostic method of choice to measure gastric emptying (Abell et al., 2008). A dual isotope study with distinct labels for solid and nutrient liquid components of the meal provides the most comprehensive information about gastric emptying (Horowitz et al., 1991)·(Sachdeva et al., 2011) (Figure 1). However, many centers assess only solid emptying, since delayed liquid emptying alone is a relatively infrequent finding (Sachdeva et al., 2011). A consensus statement relating to standardization of the test meal from the American Neurogastroenterology and Motility Society and the Society of Nuclear Medicine recommends a low fat, egg white meal labeled with ^{99m}Tc-sulfur colloid, taken with jam and toast as a sandwich, and consumed with a glass of water after an overnight fast. Scintigraphic imaging is undertaken at baseline and at 1, 2 and 4 hours, and retention of ≥ 10% of the meal at 4 hours is regarded as abnormal (Abell et al., 2008).

Figure 1



Gastric emptying of solid (% retention at 100 minutes) and nutrient liquid (half-emptying time (T50)) in 20 healthy controls and 86 patients with longstanding diabetes mellitus (66 type 1, 20 type 2). Horizontal lines represent median values. Solid emptying was abnormally slow in 57% of patients, and liquid emptying in 28%. (Reproduced with permission from that originally published in: Jones KL et al. J Nucl Med. 1995; 36(12): 2220-8. © Society of Nuclear Medicine, Inc.)

2.8.2 $^{13}CO_2$ Breath test

A convenient test for measuring gastric emptying is the $^{13}CO_2$ breath test involving a standard meal usually labeled with ^{13}C-octanoate or ^{13}C-acetate. It is less expensive than scintigraphy, does not need to be undertaken in a specialized center, and does not involve exposure to ionizing radiation. The underlying principle is that the ^{13}C containing substrate is absorbed in the small intestine and metabolized in the liver to enter the body's bicarbonate pool, before being excreted by the lungs as $^{13}CO_2$. The rate-limiting step in determining the amount of exhaled $^{13}CO_2$ over time is the rate of gastric emptying. This test has shown good reproducibility with a reasonable sensitivity and specificity of 86% and 80% respectively (Chew et al., 2003, Sanaka et al., 2006). However, it has not been adequately validated in patients with severe gastroparesis.

2.8.3 Wireless Motility Capsule (WMC or "Smart Pill")

The Wireless Motility Capsule is an orally ingestible device, capable of measuring and transmitting data on ambient pressure, temperature and pH during its transit through the gastrointestinal tract. It can provide information about motility and transit in the stomach, and also other regions of the gut (Saad and Hasler, 2011). This makes it especially useful in patients with diabetic gastroparesis in whom motor dysfunction in multiple regions of the gastrointestinal tract occurs frequently.

The WMC is administered after an after overnight fast with a standardized 260 kcal meal ("Smart bar") and 50 mL of water. Egg substitute with jam and toast can be used as an alternative (Kuo et al., 2008). The gastric emptying time that is derived

correlates relatively well with scintigraphy (Kuo et al., 2008) (correlation coefficient: 0.73 for a 4-hour scintigraphic study) giving a sensitivity and specificity of 87% and 92% respectively for detecting delayed gastric emptying. It should be recognized that while scintigraphic studies measure the emptying of digestible solids, the WMC measures emptying of a non-digestible solid object.

The use of the "Smart Pill" is contraindicated in patients with a history of gastric bezoar, swallowing disorders, suspected intestinal strictures or fistulae (e.g. Crohn's disease or diverticulitis), gastrointestinal obstruction or pseudo-obstruction, gastrointestinal surgery within the previous three months, or an implanted electromechanical medical device (e.g. a cardiac pacemaker) (Saad and Hasler, 2011). The single pressure sensor also creates a limitation of not being able to characterize the organization of small bowel pressure patterns (Saad and Hasler, 2011). The cost and the expertise required to analyze the data also impose restrictions to its use. Despite these limitations, the WMC could represent a useful tool for assessing multiple aspects of gut pathophysiology simultaneously.

2.8.4 Ultrasonography

Two-dimensional trans-abdominal ultrasonography (USG) can be used to quantify postprandial changes in antral cross-sectional area to provide an index of gastric emptying (Haruma et al., 2008), particularly that of liquids. More recently, three-dimensional ultrasound has been shown to provide information about the entire gastric volume, gastric contractility, and intragastric meal distribution; both two- and three-dimensional ultrasound have been validated against scintigraphy (Gilja et al.,

1999, Gentilcore et al., 2006). However, these techniques require considerable expertise, and can be challenging in cases of obesity or excessive intra-abdominal gas. Their main use at present is in research settings (Rao et al., 2011).

2.8.5 Magnetic Resonance Imaging (MRI)

MRI can be used to measure gastric volume, wall motion, and intragastric distribution of a meal, and has the advantage of being able to quantify gastric secretions independently of meal content (Parkman et al., 2010). The technique is largely restricted to liquid meals, has limitations in the obese or those with metal implants, and the requirement for sophisticated equipment and data processing essentially restricts its use to research settings (Fidler et al., 2009, Parkman et al., 2010).

2.8.6 Electrogastrography

Electrogastrography (EGG) uses surface electrodes attached to the skin of the epigastrium to record gastric electrical activity. Although used widely, the clinical relevance of this technique remains to be established. Recently, a model of the human stomach has been devised to investigate the potential for high-resolution gastric electrical mapping, to better understand the electrophysiological basis of abnormalities of the EGG (Du et al., 2010), which may correlate with ICC dysfunction/depletion (Lin et al., 2010).

2.9 Assessment of severity of gastroparesis

The intensity of gastrointestinal symptoms, the magnitude of the delay in gastric emptying, and the impact of delayed emptying on quality of life, nutritional status and/or glycemic control, should all be considered in defining the severity of gastroparesis. The recommendations for standardization of scintigraphy referred to previously, proposed a grading of severity based on gastric retention at 1, 2 or 4 hours (Abell et al., 2008), but this takes no account of symptoms, which, as discussed, are known to correlate poorly with the rate of gastric emptying (Jones et al., 1995b).

The Gastroparesis Cardinal Symptom Index (GCSI) was introduced primarily as a tool to grade the severity of upper gastrointestinal symptoms for use in clinical trials. The index incorporates three sub-scales: postprandial fullness/early satiety, nausea/vomiting, and bloating, with a total of 9 symptoms, assessed over a 2 week recall period (Revicki et al., 2003). Such an index can be used to monitor gastroparesis and to assess the response to treatment. The GCSI has been shown to correlate with both physician assessments of severity and with health-related quality of life as assessed by the SF-36 scale (Revicki et al., 2003). Recently, it has been modified and validated for use as a daily diary (GCSI-DD), in an attempt to determine responses to treatment on a more precise time-scale (Revicki et al., 2012).

2.10 Management

The goal of management of diabetic gastroparesis should not simply be to accelerate gastric emptying. Rather, a comprehensive and targeted approach is required aimed at restoring hydration, electrolyte balance and nutritional status (preferably enteral); optimization of glycemic control; and symptom relief. The latter includes relief of

nausea/vomiting, and of pain, which occurs frequently in gastroparesis, preferably without the use of narcotics. Particular care must be taken to avoid drug interactions (Camilleri, 2007), and the potential for delayed absorption of orally administered medications needs to be recognized (Hebbard et al., 1995). Evaluation of new therapies needs to be undertaken in a randomized, controlled manner where possible, given the potential for substantial placebo responses. The following discussion provides examples of medical and surgical interventions that have shown substantial promise in initial "open-label" studies, but much less, if any, efficacy in adequately controlled trials.

2.10.1 Glycemic control and gastroparesis

Since both acute and chronic hyperglycemia appear to be important in the pathogenesis of diabetic gastroparesis and its symptoms, overall glycemic control is pivotal to the management of this disorder. Conversely, it is now recognized that the stomach and small intestine play a major role in determining postprandial glycemic control, and that the latter predominates over fasting blood glucose in determining the glycated hemoglobin in patients who have relatively good glycemic control (Monnier et al., 2003, Standl et al., 2011). At least a third of the variance in the glycemic response to oral glucose is attributable to differences in the rate of gastric emptying in healthy subjects (Horowitz et al., 1993), as well as patients with type 1 (Horowitz et al., 1986) and type 2 diabetes (Jones et al., 1996), which is not surprising given the substantial inter-individual variation in health, which is greater in diabetes (Nowak et al., 1995). As would be predicted, pharmacological acceleration of gastric emptying with erythromycin in patients with type 2 diabetes

increases the postprandial blood glucose excursion (both peak and area under the curve), while slowing of gastric emptying with morphine reduces the glycemic response substantially (Gonlachanvit et al., 2003). Therefore, in patients not being treated with insulin, slower gastric emptying may well be advantageous for glycemic control, provided that it is not associated with symptoms.

The other major contribution of the gut to postprandial glycemic control is the secretion of the so-called "incretin" hormones, GLP-1 and glucose-dependent insulinotropic polypeptide. The incretin effect accounts for ~70% of the plasma insulin response to enteral glucose (Nauck et al., 1986). In addition to this insulinotropic effect, both endogenous and exogenous GLP-1 slows gastric emptying (Little et al., 2006b) (Deane et al., 2010b), and at least acutely, this effect is primarily responsible for the consequent reduction in postprandial glycemia (Nauck et al., 1997b). GLP-1 agonists have now entered the mainstream of therapy for type 2 diabetes, with widespread use as "add-on" therapy to metformin (Zander et al., 2001), and clear potential for use in combination with basal insulin (Baruah and Kalra, 2012), based on the rationale that both pre- and postprandial hyperglycemia will be addressed, potentially with a lower risk of weight gain and hypoglycemia than is the case with insulin alone. There is evidence that the effect of "longer-acting" agonists such as liraglutide (Jelsing et al., 2012) and exenatide-LAR (Drucker et al., 2008) on gastric emptying may diminish with time, although this has not been studied using sensitive techniques. However, it appears that the dominant effect of "short-acting" preparations, such as the twice daily formulation of exenatide, to diminish postprandial glycemic excursions is related to slowing of gastric emptying, which accounts for the observed reduction in postprandial insulinemia (Nauck et al., 1997b).

The magnitude of slowing of gastric emptying and the consequent reduction in glycemia are most marked when baseline gastric emptying is relatively more rapid (Linnebjerg et al., 2008), and it is intuitively likely, albeit not established, that these medications may be more effective in type 2 diabetic patients with more rapid gastric emptying, providing an indication for routine measurement of the latter.

Another antidiabetic medication, which exerts at least part of its therapeutic action by slowing gastric emptying, is pramlintide, an amylin analog (Vella et al., 2002) (Samsom et al., 2000). It has not been reported whether the baseline rate of gastric emptying affects the efficacy of pramlintide, but it might be anticipated that this would be the case.

While patients with diabetic gastroparesis, especially those with impaired gastric phase 3 activity, may experience a delay in the onset of therapeutic effects of oral hypoglycemic agents (OHA) due to altered pharmacokinetics, which could potentially result in difficult glycemic control (Hebbard et al., 1995), the majority of OHAs have relatively long half-lives.

In insulin-treated type 1 or type 2 patients, it is critical that nutrient absorption is coordinated with the action of exogenous insulin in order to avoid early postprandial hypoglycemia and/or late postprandial hyperglycemia. The initial postprandial insulin requirement of type 1 diabetic patients with gastroparesis is predictably less than in those without gastroparesis (Ishii et al., 1994). In patients with recurrent episodes of hypoglycemia, particularly soon after meals, gastroparesis should be considered as a potential cause (“gastric hypoglycemia”), even in the absence of

gastrointestinal symptoms, and gastric emptying should be measured (Horowitz et al., 2006). Continuous subcutaneous insulin infusion can be a useful therapy in patients with diabetic gastroparesis, and can achieve improvements in glycemic control and glycemic variability, and reduce the number of days in hospital (Sharma et al., 2011). Anecdotal evidence also suggests that patients who have received combined kidney-pancreas transplants experience improvements in autonomic function, awareness of hypoglycemia, and delayed gastric emptying (Navarro et al., 1997, Hathaway et al., 1994, Gaber et al., 1991). There is a paucity of information as to whether gastroparesis responds to islet cell transplantation.

2.10.2 Dietary modification in diabetic gastroparesis

Several recommendations have been put forward for dietary modifications in patients with symptomatic diabetic gastroparesis based on physiological principles, but none has been rigorously evaluated by a prospective randomized controlled trial. As fat is known to be potent at slowing gastric emptying, a low fat diet is advisable (Olausson et al., 2008). It is also logical, albeit unproven, to minimize intake of fiber-rich food, particularly large indigestible solids that carry a risk of bezoar formation. Small frequent meals (4 to 6 per day) with an increased proportion of energy in liquid form are generally recommended, and patients are advised to chew food thoroughly; a vitamised diet in which solid particles are of a small size empties more rapidly than foods with a large particle size (Olausson et al., 2008). Walking or assuming an upright (Lipp et al., 2000), semi supine or right lateral position (Appadu et al., 1995) postprandially may potentially facilitate gastric emptying. The glycemic profile may

require close monitoring for alterations resulting from interventions to facilitate gastric emptying, especially in patients treated with insulin.

The nutritional status of patients with diabetic gastroparesis should be carefully monitored. Unintentional loss of body weight of 5% in 3 months or 10% in 6 months is indicative of severe malnutrition (Parkman et al., 2010). If supplementation with oral nutrient drinks is insufficient or poorly tolerated, then direct enteral feeding into the small intestine should be considered (Colemont and Camilleri, 1989). Naso-jejunal feeding may be tried initially before considering endoscopic or surgical jejunostomy. Venting gastrostomy may reduce hospitalizations for some patients (Colemont and Camilleri, 1989). However, there is dearth of formal studies to assess the effects of gastroparesis symptoms on nutritional outcomes.

2.10.3 Prokinetics

Prokinetic agents represent the mainstay of treatment in established cases of gastroparesis, but it should be recognized that acceleration of gastric emptying correlates poorly with improvements in symptoms (Sturm et al., 1999). Metoclopramide exerts its prokinetic effect by activation of 5-HT_4, while its dopamine receptor antagonism in the central nervous system (CNS) can cause somnolence and diminished mental acuity, which may limit its efficacy (Tack et al., 2012). Moreover, long-term use of metoclopramide can occasionally lead to permanent neurological side effects including tardive dyskinesia. Domperidone is equally effective, but with a lower prevalence of CNS adverse effects (Khoo et al., 2009). Although cisapride, and to a lesser extent tegaserod, were useful therapies for

gastroparesis, they were withdrawn from the market following reports of life-threatening cardiac side effects (Tack et al., 2012). Newer drugs with 5-HT_4 agonist properties, including clebopride, prucalopride, mosapride, renzapride, TD-5108 (velusetrag) and ATI-7505 (naronapride) appear to have an acceptable cardiac safety profile (Tack et al., 2012), but their efficacy in diabetic gastroparesis remains to be determined.

The macrolide antibiotic, erythromycin, which stimulates motilin receptors on the cholinergic neurons and gastric smooth muscle, is one of the most potent prokinetic drugs when given intravenously in the acute setting (Richards et al., 1993). However, the effects of orally administered erythromycin tend to wane over around four weeks due to tachyphylaxis (Richards et al., 1993). The prokinetic effect is also attenuated during acute hyperglycemia (Jones et al., 1999a, Jones et al., 1999b); whether this phenomenon also applies to other prokinetic drugs has not been established, but is likely to be the case (Horowitz et al., 2002). Erythromycin is also known to cause drug interactions due to cytochrome P450 inhibition (Okudaira et al., 2007). Furthermore, it can prolong the QT interval (Wisialowski et al., 2006) accounting for its pro-arrhythmic potential. Recently, concerns have been raised regarding the cardiovascular safety profile of another macrolide, azithromycin (Ray et al., 2012).

Mitemcinal is a promising new macrolide motilin receptor agonist without antibiotic properties, which has the capacity to accelerate gastric emptying and improve upper gastrointestinal symptoms (McCallum and Cynshi, 2007) in patients with gastroparesis, although the relationship between these two endpoints is predictably weak and further studies are required (McCallum and Cynshi, 2007). Other motilin

receptor agonists – GSK962040 and RQ-00201894 – are in development (Sanger et al., 2009, Takahashi N, 2010).

In diabetic gastroparesis, the regulation of ghrelin secretion from the stomach is impaired (Suzuki et al., 2011), possibly as a result of autonomic impairment, and both ghrelin (GRLN) and ghrelin receptor (GRLN-R) agonists accelerate gastric emptying (Murray et al., 2005). However, there are conflicting reports about the effects of exogenous ghrelin on symptoms (Murray et al., 2005, Tack et al., 2005), with the potential for some patients to experience a worsening of upper gut symptoms because of a reduction in gastric accommodation (Ang et al., 2009). The orexigenic and antioxidant properties (Suzuki et al., 2011) of ghrelin represent a possible advantage, but excessive secretion of growth hormone (GH) and inhibition of insulin release (Greenwood-Van Meerveld et al., 2011) represent potential concerns for long term use. Synthetic non-peptide GRLN-R agonists, such as TZP-101 (ulimorelin) (Fraser et al., 2008) and EX-1314/5 (Charoenthongtrakul et al., 2009) have been developed. In preliminary human trials on patients with severe gastroparesis, TZP-101 appears to be safe and effective in accelerating emptying and improving symptoms (Ejskjaer et al., 2010, Ejskjaer et al., 2009).

Acotiamide (Z-338) is a novel muscarinic M1/M2 receptor antagonist, which increases acetylcholine release, resulting in enhanced gastric accommodation in a rodent model(Seto et al., 2008). Its place in the management of human diabetic gastroparesis remains to be determined.

2.10.4 Antiemetics

Antiemetics such as diethylperazine (a dopamine antagonist), aprepitant (a neurokinin NK_1 antagonist), promethazine (an H_1 antagonist), ondansetron (a 5-HT_3 antagonist) and dronabinol (a cannabinoid) are all used to treat nausea and vomiting associated with gastroparesis (Hasler, 2011), while tricyclic antidepressants (Sawhney et al., 2007) and mirtazapine (Kim et al., 2006), despite their anticholinergic properties, may also have beneficial effects. However, none of these agents have been evaluated in well-controlled clinical trials in the setting of gastroparesis, nor is there any evidence that more recently developed and more expensive therapies are more effective than older ones such as prochlorperazine. An advantage of metoclopramide over other antiemetics is its prokinetic property.

2.10.5 Pain management

As discussed, abdominal pain is a frequent symptom in diabetic gastroparesis, which affects quality of life (Hoogerwerf et al., 1999). Though its pathogenesis is poorly understood, neuropathy is thought to contribute (Lahr et al., 2010); as with other symptoms, its severity correlates poorly with the degree of impairment in gastric emptying (Cherian et al., 2010). Patients with gastroparesis are usually advised to avoid opiates and analgesics with anti-cholinergic properties, because of their potential to impair gut motility, although tricyclic antidepressants (amitriptyline or nortriptyline) may nonetheless be helpful as pain-modifying agents. Tramadol, a weak opiate agonist may be useful, while asimadoline, a new kappa opioid agonist, appears to have future potential (Camilleri, 2008). Selective serotonin reuptake inhibitors (paroxetine or citalopram), serotonin-epinephrine reuptake inhibitors

(duloxetine or venlafaxine), and some antiepileptics (gabapentin or pregabalin), may be of benefit (Chang et al., 2011). However, it should be recognized that, as is the case with antiemetics, none of these drugs have been rigorously evaluated for the management of pain associated with gastroparesis. Other options with minimal supporting evidence include ketamine, acupuncture, dorsal cord stimulators, and repetitive transcranial magnetic stimulation (Chiarioni and Whitehead, 2008).

2.10.6 Intrapyloric botulinum injection

Botulinum toxin causes irreversible inhibition of acetylcholine release from nerve fibers, leading to sustained muscle paralysis for at least several months before new axonal sprouting occurs (Thomas et al., 2012), necessitating repeated injections. Several uncontrolled series indicated that intrapyloric injection of botulinum toxin was associated with improvement in both gastric emptying and gastrointestinal symptoms (Ezzeddine et al., 2002, Gupta and Rao, 2002, Lacy et al., 2004, Thomas et al., 2012, Ben-Youssef et al., 2006). However, two randomized controlled trials (one with only two of 23 patients having diabetes, but the other with 18 out of 32) failed to demonstrate any difference between toxin and saline injections in the improvements in either symptoms or gastric emptying (Arts et al., 2007, Friedenberg et al., 2008), indicating that it should not be used clinically.

2.10.7 Gastric electric stimulation (GES)

As discussed above, ICC loss or dysfunction in diabetes can result in impaired gastric myoelectrical activity, which may contribute to the pathogenesis of

gastroparesis. This suggests the potential for therapies employing gastric electrical stimulation to treat this disorder. Two modes of electrical stimulation of the stomach have been evaluated (Rayner and Horowitz, 2005); long-pulse/high-energy stimulation at physiological frequencies (3 cycles per minute) can entrain gastric myoelectrical activity, but is currently impractical due to the prohibitively high energy requirements for an implantable device, while short-pulse/low energy stimulation at a much higher frequency than the gastric slow wave has been incorporated into the implantable device known as Enterra, which was approved by the FDA under an Humanitarian Device Exemption in 2000. Enterra delivers stimulation at a frequency of 12 per minute, which does not entrain the gastric slow wave or for the most part accelerate gastric emptying, and is thought to act by stimulation of vagal afferent pathways which influence central mechanisms for nausea and vomiting, and/or possibly to enhance postprandial gastric accommodation (McCallum et al., 2010a).

Enterra therapy has been associated with marked symptomatic improvement in several uncontrolled case series (O'Grady et al., 2009). In such studies, patients with severe ICC depletion (Lin et al., 2010) and those dependent on narcotic analgesia were reported to respond less well, and patients with diabetic gastroparesis had a more favorable outcome than those with idiopathic gastroparesis (Maranki et al., 2008). In the only prospective controlled study published to date, patients with diabetic gastroparesis experienced marked reductions in weekly vomiting frequency during the initial uncontrolled run-in period of 6 weeks (McCallum et al., 2010b). However in the subsequent phase, where patients were studied in randomized crossover fashion during 3 months with the stimulator on and 3 months off, there was

no difference in symptoms between the “on” and “off” periods. It is accordingly clear that better controlled trial evidence is essential before GES can be considered a mainstream therapy for diabetic gastroparesis. Meanwhile, experimentation continues with alternative modes and sequencing of gastric electrical stimulation in canine models (Chen et al., 2005, Song et al., 2008).

2.10.8 Surgical treatment

The efficacy of surgical interventions, such as sub-total or complete gastrectomy (Watkins et al., 2003) with or without Roux-limb construction or Nissen fundoplication (Van Sickle et al., 2007) and pyloroplasty for the treatment of diabetic gastroparesis remains uncertain, due to a lack of evidence other than small case series and isolated reports. In a retrospective observation, Hibbard et al. reported symptomatic improvement after laparoscopic pyloroplasty in 28 patients with gastroparesis, seven of whom had diabetes (Hibbard et al., 2011), but these data were again uncontrolled and follow-up was limited to one month. Better studies are required to determine the place of surgery in the management of diabetic gastroparesis, and surgery should ideally be carried out in specialized referral centers where the full range of options for treating gastroparesis can be considered.

2.10.9 Stem Cells

If deficiency of neuronal NOS is indeed one of the key abnormalities in gastroparesis, as suggested by mouse models (Gangula et al., 2007), then intrapyloric transplantation of neural stem cells (NSCs) may be a therapeutic option. In support

of this concept, NSCs transplanted into nNOS-/- mice had differentiated within one week into neurons expressing NOS, associated with acceleration of gastric emptying (Micci et al., 2005). The durability and safety of such an approach clearly needs to be established before it can be translated to the management of gastroparesis in humans.

2.10.10 Psychological disorders and gastroparesis

The presence and severity of anxiety and depression are related to the severity of symptoms, and also correlate with the use of antiemetic and prokinetic drugs in patients with gastroparesis, regardless of whether the etiology is diabetic or idiopathic (Hasler et al., 2010). Nevertheless, psychological disorders remain under-recognized and under-treated in gastroparesis (Hasler et al., 2010), potentially contributing substantially to morbidity, and are associated with suboptimal glycemic control in patients with diabetes (Lustman et al., 2000). Given the high prevalence of psychological/psychiatric illness in diabetes including eating disorders in type 1 patients (d'Emden et al., 2012), consideration should be given to involving a clinical psychologist/psychiatrist in the multidisciplinary management of diabetic gastroparesis.

2.10.11 Alternative therapies

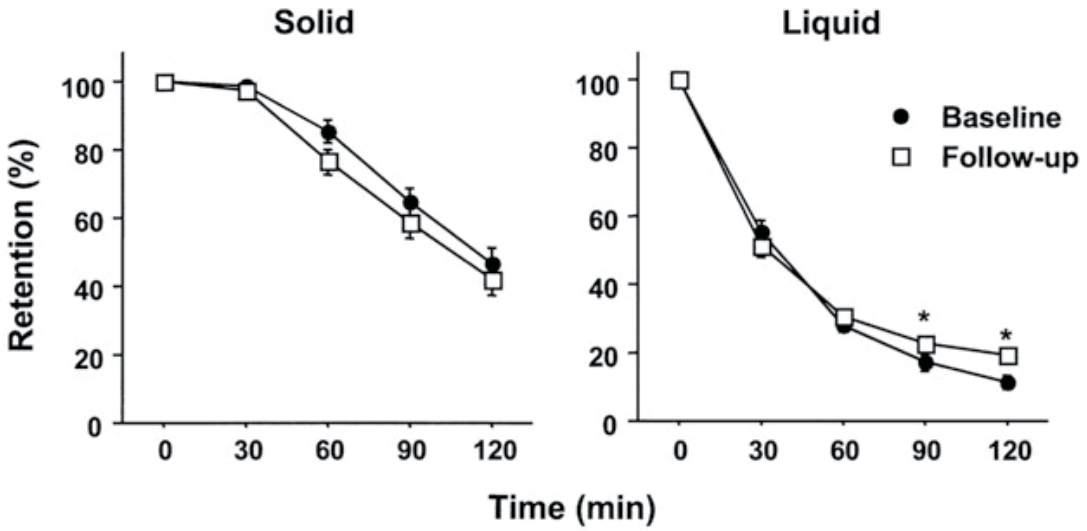
Acupuncture, electro-acupuncture, and acupressure stimulation of specific pressure points are some of the alternative therapies that have been employed for symptom relief in diabetic gastroparesis (Ouyang et al., 2002), and potentially act through vagal stimulation, though a substantial contribution from a placebo effect has not yet

been ruled out. Behavioral treatment involving autonomic training with verbally directed imagery ("biofeedback") has been reported to be useful in some patients (Rashed et al., 2002) although further controlled studies are essential to establish this as a therapeutic option.

2.11 Prognosis of diabetic gastroparesis

Gastroparesis reduces the quality of life in diabetes, and a recent report indicated that in those with severe symptoms, there was an increase in morbidity as measured by hospitalization and visits to physicians and emergency departments (Hyett et al., 2009). Nevertheless, gastroparesis has hitherto not been shown to increase mortality per se (Kong et al., 1999), and the prognosis of delayed emptying itself is not necessarily poor. In one study, 20 patients with long-standing diabetes were followed over a mean 12 year period and there was no significant change in the rate of gastric emptying (Jones et al., 2002), perhaps at least in part because glycemic control had improved over this interval [Figure 2]. Just as symptoms correlate poorly with the rate of gastric emptying, so too is the degree of delay in gastric emptying a poor predictor of response to therapy (Hejazi et al., 2011).

Figure 2



Gastric emptying (mean±SEM) of solid and liquid meal components measured at baseline and follow-up (12.3± 3.1 years later) in 20 patients with diabetes mellitus (16 type 1, four type 2). Both solid and liquid emptying rates were remarkably stable over several years. * $p<0.05$ for baseline versus follow-up by analysis of variance. (Reproduced from: Jones KL et al Am J Med. 2002; 113:449-55, with permission from Elsevier)

2.12 Expert commentary

Increased awareness and advances in diagnostic techniques have resulted in the recognition of diabetic gastroparesis as a significant disorder, which can potentially affect quality of life. The weak correlation between the degree of delay in gastric emptying and symptoms, along with the multifactorial nature of its pathogenesis, makes it difficult to formulate effective therapeutic strategies. However, loss or dysfunction of ICCs has emerged as a key underlying feature. Variations in the rate of gastric emptying, which could be a cause or effect of glycemic variations, have a significant impact on the management of diabetes and co-ordination of anti-diabetic medications. Indeed, delayed gastric emptying can be beneficial for glycemic control in patients with diabetes, as long as it does not result in symptoms, or an unpredictable mismatch between absorption of nutrients and the action of exogenous insulin. Pharmacological modulation of the rate of gastric emptying represents an important mode of action of some of the newer anti-diabetic agents, such as GLP-1 agonists.

2.13 Five-year view

Further insights into the pathogenesis of diabetic gastroparesis are likely to be achieved through clinicopathological correlation using gastric biopsies from patients with severely symptomatic disease. Though several nutritional, pharmacological and interventional strategies currently appear promising, more robust clinical trials are essential to determine their position in the management of gastroparesis. Better understanding of the pathophysiology and the evolution of gastroparesis and its symptoms could make it possible to tailor management strategies to suit individual patients, using a multidisciplinary approach. An understanding of the variation in

rates of gastric emptying between patients with diabetes will become more important in the rational use of new therapies, such as incretin-based drugs.

CHAPTER 3. METHODOLOGY

3.1 Introduction

In this chapter, the methodologies and techniques utilized in my experimental studies are detailed. These include evaluation of flow-mediated dilatation of the brachial artery, assessments of glucose absorption, glycemia, gastric emptying, duodenal motility and flow events, small intestinal transit, appetite perception and energy intake, and gut hormone concentrations in response to either oral or intraduodenal stimuli. Appropriate use of these techniques allowed a thorough assessment of the inter-relations between upper gut function and glycemic control in health and type 2 diabetes.

3.2 Approval by the ethics committee

All my study protocols were reviewed and accepted by the Royal Adelaide Hospital Ethics Committee. Those involving experimental drugs were assessed and approved by the Investigational Drugs Sub-Committee. Prior to the commencement of such studies, 'Clinical Trial Notification (CTN) Scheme' forms were lodged with the Australian Government Department of Health and Aging, Therapeutic Goods Administration, and the clinical studies were registered with the Australia New Zealand Clinical Trials Registry (ANZCTR). The principles of the Declaration of Helsinki as revised in 2000 were meticulously observed in all studies.

3.3 Recruitment of study subjects

Patients with type 2 diabetes and healthy volunteers were recruited through advertisements on notice boards in the Royal Adelaide Hospital and local universities as well as in local diabetes magazines. Appropriate subjects were selected using the inclusion and exclusion criteria of each study. Each subject provided informed, written consent at a screening visit, prior to being included in any study.

3.4 Gastrointestinal symptom questionnaires

Gastrointestinal symptoms – including those which might constitute a criterion for exclusion – were evaluated at the screening visit using a 'gastrointestinal symptom questionnaire' evaluating 9 symptoms: 1)'poor appetite for food', 2) 'nausea, or a feeling of sickness', 3) 'feeling full after eating only a little food', 4) 'discomfort or distension in the upper abdomen', 5) 'vomiting', 6) 'pain in the abdomen', 7) 'difficulty in swallowing', 8) 'heart burn', and 9) 'acid regurgitation'. The severity of each symptom in the preceding three months was scored from '0' to '3': 0 = none; 1 = mild (the symptom can be ignored); 2 = moderate (the symptom cannot be ignored, but does not influence daily activities); 3 = severe (the symptom affects daily activities) (Horowitz et al., 1991, Jones et al., 2001a).

3.5 Autonomic nerve function

Standardized cardiovascular reflex tests were employed to assess the autonomic nerve function in patients with type 2 diabetes (Ewing and Clarke, 1982, Vinik and

Mehrabyan, 2003). Parasympathetic function was assessed from the variations in R-R interval in response to deep breathing and change in posture from supine to standing (30:15 ratio). Sympathetic function was assessed from the fall in systolic blood pressure in response to standing from the supine position. The findings were scored in accordance with age-adjusted, predefined criteria (Ewing and Clarke, 1982), as 0 = normal, 1 = borderline, and 2 = abnormal. The maximum total score possible was 6 and a score of 3 or more was taken to suggest the presence of autonomic dysfunction (Ewing and Clarke, 1982).

3.6 Flow mediated dilatation

Measuring the "flow-mediated dilatation" (FMD) of the brachial artery using high resolution vascular ultrasound as an index of endothelial function is a well established non-invasive technique (Charakida et al., 2010) (Celermajer et al., 1992). In my studies, the assessment of FMD involved inflation of a sphygmomanometer cuff on the forearm to 200 mmHg for 5 minutes to induce ischemia, followed by deflation. The diameter of the brachial artery was measured 5 cm above the olecranon process using ultrasound (Logiq e, GE Medical systems, Australia), with a 12 MHz transducer placed in a longitudinal plane over the artery. 3-second scans were recorded at baseline (before inflation of the cuff) and every 15 seconds for 2 minutes, after cuff release. A concomitant electrocardiogram (ECG) recording was superimposed on the ultrasonographic images. The transducer position was marked on the skin, and the arm was held immobile with the aid of a foam support and stereotactic clamp throughout the study duration. A single trained operator (the author of this thesis) made all the observations. Images corresponding to end-diastole,

coincident with the beginning of the R wave on the ECG, were compared to determine differences in arterial diameter. FMD responses were expressed as percentage change in diameter of the artery from baseline at each time point (Stoner and Sabatier, 2012). Heart rate (HR) was calculated from the R-R interval of the ECG.

3.7 Gastric emptying

Although the scintigraphic technique is regarded as the gold standard for measurement of gastric emptying, it has the limitations of exposure to ionising radiation, and the requirement for access to a gamma camera. Therefore, the technique of stable isotope breath tests was employed to evaluate gastric emptying in the studies reported in this thesis.

Evaluation of gastric emptying of solid (Perri et al., 2005), liquid (Kurita et al., 2011) and semi-solid (Cardoso-Junior et al., 2007) meals in humans using stable isotope breath tests is well established (Ghoos et al., 1993) and validated (Chew et al., 2003). ^{13}C-octanoic acid was mixed with the semi-solid meals in my studies. After oral ingestion, the ^{13}C-octanoic acid label is absorbed from the small intestine, metabolised in the liver, and exhaled as $^{13}CO_2$ in the breath. Emptying of the labelled meal from the stomach into the duodenum is the rate-limiting step. In my studies, breath samples were stored in air-tight tubes for analysis using an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas chromatographic purification system. The gastric half-emptying time and the gastric emptying coefficient (GEC) were calculated from the $^{13}CO_2$ excretion curves, i.e. the

percentage of dose per hour and cumulative dose per hour, using established formulas (Ghoos et al., 1993).

3.8 Intraduodenal infusion

As gut hormone secretion is highly dependent on the rate of gastric emptying (Ryan et al., 2012, Ma et al., 2012, Pilichiewicz et al., 2007), which varies widely between healthy individuals in the range 1-4 kcal/min (Brener et al., 1983, Macdonald, 1996), and even more so in patients with diabetes, we elected to employ intraduodenal infusion of nutrients at a controlled rate, to bypass gastric emptying and allow more precise comparison of gut hormone responses. In my studies, an intraduodenal catheter (diameter 3.5 mm; Dentsleeve, Ontario, Canada) was inserted through an anaesthetized nostril and allowed to pass through the stomach and into the duodenum by peristalsis. The assembly contained an infusion channel, located about 12 cm distally from the pylorus, used to infuse nutrients and experimental drugs into the duodenum. The correct positioning of the catheter was maintained by continuous measurement of the transmucosal potential difference (TMPD) from the manometry channels in the antrum and duodenum (antral potential < -20mV, duodenal potential > -15mV, difference > 15mV) (Heddle et al., 1988).

3.9 Measurements of duodenal motility and flow events

Well-established techniques using a combined manometry and impedance assembly were employed in my study to assess duodenal motility and flow events, as described in chapter 5. The assembly incorporated a multilumen silicone manometry catheter

(external diameter 4 mm, containing 20 side holes (each 1.5cm apart) + 1 extra side hole for infusion) to which an impedance catheter with 7 electrode pairs (external diameter 2 mm, each electrode 2cm apart) was bound, so that the electrodes spanned the same region as the duodenal manometry channels. The assembly was introduced into the stomach through an anaesthetized nostril, and allowed to pass into the duodenum by peristalsis. Channels 1-12 were positioned within the duodenum, channels 13-15 across the pylorus, and channels 16-20 in the antrum. The manometry catheter was perfused with degassed water or saline, and its intraduodenal position was monitored continuously during the study by measurement of the transmucosal potential difference between the most distal antral (channel 16) and the most proximal duodenal (channel 12) side holes, using established criteria (antral potential < -20mV, duodenal potential > -15mV, difference > 15mV) (Heddle et al., 1988). A 20G saline-filled cannula was inserted subcutaneously in the forearm as a reference (Heddle et al., 1988). Occlusion of the side-holes by lumen-occlusive contractions generated changes in pressure that were registered in each channel by an external transducer, and digitally recorded using appropriate software (Flexisoft, Oakfield Instruments of Oxford, UK).

Analyses of the recorded pressures was carried out using custom-designed software (Prof AJ Smout, Academic Medical Center, Amsterdam, The Netherlands) to yield the number and amplitude of duodenal pressure waves over successive 15 min periods and the motility index was calculated as MI=ln(number of pressure waves * sum amplitude)+1 (Abell et al., 1991). The frequency of propagated sequences of waves was analyzed, and sequences classified by the length and direction of propagation (Rayner et al., 2002). An investigator, who was blinded to the

intervention, analyzed the impedance recordings for the occurrence of flow events. A flow event was defined as a transient reduction in the impedance of at least 12% from baseline in at least three sequential electrode pairs (i.e. at least 6 cm) (Chaikomin et al., 2007). Each flow event was classified as either “antegrade” or “retrograde”.

3.10 Small intestinal transit

Small intestinal transit was measured using scintigraphy. The intraduodenally infused glucose solution in chapter 5 was labelled with 20 MBq of 99mTc-sulfur colloid in order to assess the transit time of chyme in the small intestine from the duodenum to the cecum. The study was conducted in the Department of Nuclear Medicine, where the subjects remained supine under a gamma camera throughout the duration of the study. A cobalt marker placed over the right superior iliac spine was used as reference point to correct for subject movement and to determine the approximate position of the cecum (Deane et al., 2012). Scintigraphic images (anterior) were acquired every 15 minutes (5 minute static images) in the first hour of infusion of labelled intraduodenal glucose and every 30 min thereafter, until the completion of the study (T = 240 min). Data were corrected appropriately for radionuclide decay and subject motion (Deane et al., 2012). The acquired images were stored digitally and analyzed later using GE Entegra software (v2.5202) by a trained investigator, who was blinded to the experimental interventions. Regions of interest were drawn around the cecum and the stomach (based on the position of the cobalt marker), and a percentage retention/time curve for the cecum was derived (Deane et al., 2012). The cecal arrival time was calculated by identifying the initial

appearance of isotope in the region of interest in association with an increase in the retention/time curve (Deane et al., 2012).

3.11 Appetite perception and energy intake

3.11.1 Visual analog scales (VAS)

Questionnaires incorporating visual analog scales (VAS) are a commonly used tool to assess appetite perception and gastrointestinal sensations (Parker et al., 2004). Each VAS evaluated different sensations on 100 mm horizontal lines, where 0 mm represented "sensation not felt at all" and 100 mm represented "sensation felt greatest". Subjects were asked to place a vertical mark at the point along the line corresponding to the intensity of each sensation. "Hunger", "fullness", "desire to eat" and "prospective consumption" were all evaluated at each time point throughout the studies.

3.11.2 Energy intake

In chapter 8, the daily food consumption was assessed by a 3-day food diary kept by the study subjects during the latter half of the week, immediately prior to each study visit, and analysed using FoodWorks software (FoodWorks 3.01; Xyris Software, Highgate Hill, QLD, Australia) to calculate energy and macronutrient intake.

3.12 Assessment of intestinal glucose absorption

3.12.1 3-O-methylglucose (3-OMG)

The transport of glucose from the lumen into the enterocyte is effected mainly through sodium glucose co-transporter 1 (SGLT1) (Shirazi-Beechey et al., 2011), with a minor role for other transporters (eg. glucose transporter 2 or GLUT-2) (Shirazi-Beechey et al., 2011, Ait-Omar et al., 2011). SGLT-1 actively transports both glucose and 3-OMG, in the presence of sodium (Uhing and Kimura, 1995). However, ~30% of the glucose absorbed from the gut undergoes hepatic metabolism (Cherrington, 1999), whilst 3-OMG is not metabolised. Therefore, measurement of serum 3-OMG concentrations represents an acceptable method of comparing the magnitude of glucose absorption from the small intestine between different study days in a cross-over study design (Fordtran et al., 1962a). In my studies, serum 3-OMG concentrations were measured by liquid chromatography/mass spectrometry (Ma et al., 2010).

3.13 Biochemical measurements

3.13.1 Blood glucose

Blood glucose concentrations in my studies were measured using a portable bedside glucometer (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA), which uses the glucose oxidase technique. The mean of two successive readings on the same blood sample, within a range of 0.6 mmol/L, was used.

3.13.2 Glucagon like-peptide 1 (GLP-1)

Plasma total GLP-1 concentrations were measured by radioimmunoassay (GLPIT-36HK; Linco Research, St. Charles, MO, USA). The sensitivity was 3 pmol/L, and intra- and inter-assay CVs were 6.8% and 8.5% respectively.

3.13.3 Glucose-dependent insulinotropic peptide (GIP)

Plasma total GIP concentrations were measured by radioimmunoassay (Wishart et al., 1992a), with a sensitivity of 2 pmol/L and intra- and inter-assay CVs of 10.2% and 11.2% respectively.

3.13.4 Insulin

Serum insulin concentrations were measured by ELISA immunoassay (10-1113; Mercodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L, and intra- and inter-assay CVs of 2.1% and 6.6% respectively.

3.13.5 C-peptide

Plasma C-peptide concentrations were measured by ELISA immunoassay (10-1136-01, Mercodia, Uppsala, Sweden). The sensitivity of the assay was 15 pmol/L and the CV was 3.6% within assays and 3.3% between assays.

3.13.6 Glucagon

Plasma glucagon concentrations were measured by radioimmunoassay (GL-32K, Millipore, Billerica, MA) with sensitivity 20 pg/ml, and intra- and inter-assay CVs of 15 % and 10.5 %.

3.14 Statistical analysis

All analyses were performed using SPSS software (version 20.0 or version 21.0), in consultation with a biomedical statistician (Ms Kylie Lange, Centre of Research Excellence in Translating Nutritional Science to Good Health, Discipline of Medicine, University of Adelaide). Normally distributed data were presented as mean values ± standard error; $P < 0.05$ was considered statistically significant. Data which were not normally distributed (eg: nausea in Chapter 5) were analysed using Wilcoxon matched-pairs signed rank test.

3.15 Conclusions

The methods and techniques employed in this thesis have all been validated and are well tolerated by subjects. They are considered as either the best available, or the most practical techniques to address the hypotheses of each study.

CHAPTER 4. CHANGES IN MEAL COMPOSITION AND DURATION AFFECT POSTPRANDIAL ENDOTHELIAL FUNCTION IN HEALTHY HUMANS

(Adapted from Am J Physiol Gastrointest Liver Physiol. 2014 Dec 15;307(12):G1191-7.)(Thazhath et al., 2014a)

4.1 Abstract

Endothelial function, measured by flow-mediated dilatation (FMD), predicts cardiovascular events and is impaired postprandially. The objective of this study was to evaluate the effects of changes in composition or duration of ingestion of a meal, which slows gastric emptying and/or small intestinal nutrient exposure, on postprandial endothelial function. Twelve healthy subjects (6 male, 6 female; 33 ± 6 yr) were each studied on three occasions, in a randomized crossover design. After an overnight fast, subjects consumed a [(13)C]octanoic acid-labeled mashed potato meal ("meal 1"), or meal 1 mixed with 9 g guar ("meal 2") within 10 min, or meal 1 divided into 12 equal portions over 60 min ("meal 3"). Brachial artery FMD was measured every 30 min for 120 min. Blood glucose, serum insulin, and gastric emptying (breath test) were evaluated for 240 min. Data are means ± SE. Compared with meal 1, meal 2 was associated with slower gastric emptying (half-emptying time 285 ± 27 vs. 208 ± 15 min, $P < 0.05$), lower postprandial blood glucose and insulin ($P < 0.001$ for both), and a delayed, but more sustained, suppression of FMD ($P < 0.001$). After meal 3, both glycemic increment and reduction in FMD were less than after meal 2 ($P < 0.05$ for both). The decrement in FMD was directly related to the

increment in blood glucose (r = 0.46, P = 0.02). We conclude that, in health, postprandial FMD is influenced by perturbation of gastric emptying and the duration of meal consumption, which also impact on glycemia.

4.2 Introduction

In arteries with intact endothelial function, increased blood flow following an ischaemic stimulus results in dilatation (Rubanyi et al., 1986, Laurent et al., 1990), via the release of the endothelium-derived relaxing factor (Pohl et al., 1986), nitric oxide (NO). Impaired endothelial function is now well-recognized as a forerunner of atherosclerosis (Juonala et al., 2004) and is predictive of long-term adverse cardiovascular outcomes (Vogel, 2001). Factors known to affect endothelial function acutely include diet (Ceriello et al., 2002), exercise (Currie et al., 2012), ambient temperature (Zanobetti et al., 2014), and time of the day (Ringqvist et al., 2000). As gastric emptying of a meal usually approximates a rate of 1 to 4 kcal/min, people who generally consume three meals daily spend most of the day in the postprandial state, with only a few hours of true fasting before breakfast (Monnier et al., 2003). Therefore, endothelial function in the postprandial state is arguably more likely to contribute to overall cardiovascular risk than fasting endothelial function. In the case of majority of patients with type 2 diabetes, who have reasonably good glycemic control (HbA_1C ~7.5% or less), it is now well-recognised that postprandial glycemia predominates over fasting blood glucose in contributing to HbA_1C, and may be an independent risk factor for cardiovascular events (Monnier et al., 2003).

The mechanisms of postprandial endothelial dysfunction (Shige et al., 1999, Kawano et al., 1999) are poorly understood, although several variables appear to be of

relevance, including the glycemic index (Lavi et al., 2009) and salt content (Dickinson et al., 2011) of the meal, as well as the postprandial elevation of triglycerides (Ceriello et al., 2002). After an oral glucose load, endothelial dysfunction is related to the degree of rise in blood glucose in both health and type 2 diabetes (Title et al., 2000, Kawano et al., 1999), with the duration of dysfunction being greater in the latter (Akbari et al., 1998).

The rate of gastric emptying is a major determinant of postprandial glycemic increments (Chaikomin et al., 2006, Marathe et al., 2013). Modulating gastric emptying and/or nutrient absorption from the upper gut can be achieved non-pharmacologically by modifying the composition of a meal, for example by adding soluble fiber such as guar gum (Holt et al., 1979, Torsdottir et al., 1989), or by decreasing the rate of meal ingestion (Zhu et al., 2013). The glycemic reduction achieved by incorporating guar into a meal is most pronounced at around 30 minutes postprandially, when gastric emptying plays a major role in determining the glycemic profile (Torsdottir et al., 1989). Though the effects of such dietary manipulations on glycemia have been described previously, their influence on postprandial endothelial function has not yet been investigated.

We therefore evaluated the effects of dietary modifications designed to slow gastric emptying and/or small intestinal nutrient absorption on postprandial endothelial function in healthy humans, with the hypothesis that, in addition to lowering postprandial blood glucose, they would attenuate postprandial endothelial dysfunction, resulting in improved vasodilation in response to increased blood flow induced by an ischaemic stimulus.

4.3 Methods

4.3.1 Subjects

12 healthy subjects (6 male and 6 female; mean age (± standard error) 33 ± 5.6 years; mean BMI 23.3 ± 0.8 kg/m^2) were studied after providing written, informed consent. None was a smoker, or was taking medication known to affect gastrointestinal and/or endothelial function. The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

4.3.2 Protocol

Each subject was studied on three separate occasions, at least five days apart, in a randomised, crossover design. On each study day, the subject arrived in the laboratory at ~0800 h after a 12 hour overnight fast, and rested in a temperature-controlled room for 20 minutes. Baseline endothelial function was evaluated by measuring the flow-mediated dilatation (FMD) of the right brachial artery (Stoner and Sabatier, 2012), as described subsequently. An intravenous cannula was then inserted into an antecubital vein of the left arm for blood sampling. Thereafter, each subject ate a semisolid mashed potato meal, consisting of 65 g powdered potato (Deb Instant Mashed Potato, Continental, Epping, NSW, Australia) and 20 g glucose reconstituted with 250 mL boiling water and an egg yolk containing 100μL ^{13}C-octanoic acid (total volume 300 mL; energy 368.5 kcal (with 'meal 2', there was an additional 36 kcals from guar gum); carbohydrate 61.4 g; protein 7.4 g; fat 8.9 g). On each of the three study days, the meal was given in a different way, in randomised order: 1) 'meal 1': consumption of the meal within 10 minutes, 2) 'meal 2': addition

of 9 g guar gum (Russo et al., 2003) (Lotus Gums & Chemicals, Jodhpur, India) to the meal, and ingestion within 10 minutes, and 3) 'meal 3': division of 'meal 1' into 12 equal portions, each of 25 mL, ingested over 5 minutes (^{13}C-octanoic acid in the first portion only), with a total meal duration of 60 min (T = 0 to 60 min).

With 'meal 1' and 'meal 2', T = 0 min was defined as the time of finishing the meal, whereas with 'meal 3', T = 0 min was defined as the beginning of the meal. Venous blood was sampled at baseline (ie. immediately prior to meal ingestion) and then at T = 15, 30, 60, 90, 120, 180 and 240 min. Blood samples were collected into ice-chilled serum tubes, and stored on ice before centrifugation at 3200 rpm for 15 min at 4°C within 15 min of collection. Serum was separated and stored at –80°C for subsequent analysis. Breath samples were collected into airtight tubes at baseline and every 5 minutes for the first hour, and every 15 minutes for a further 3 hours, for measurement of gastric emptying.

FMD was measured at baseline and then at T = 30, 60, 90 and 120 min. At the time of each FMD measurement, heart rate (HR) was also recorded. At T = 140 min, 400 mcg glyceryl trinitrate (GTN) spray was administered sublingually and the vascular response recorded, to demonstrate the capacity for vasodilation independent of endothelial function (Faulx et al., 2003).

4.4 Measurements

4.4.1 Flow mediated dilatation and heart rate

Measuring the "flow-mediated dilatation" (FMD) of the brachial artery as an index of endothelial function is a well established non-invasive technique (Charakida et al., 2010), using high resolution vascular ultrasound, with FMD expressed as the percentage change in arterial diameter relative to baseline (Celermajer et al., 1992). In our study, the assessment of FMD involved inflation of the sphygmomanometer cuff on the forearm to 200 mmHg for 5 minutes to induce ischemia, followed by deflation. The diameter of the brachial artery was measured 5 cm above the olecranon process using ultrasound (Logiq e, GE Medical systems, Australia), with a 12 MHz transducer placed in a longitudinal plane over the artery. 3-second scans were recorded at baseline (before inflation of the cuff) and every 15 seconds for 2 minutes, after cuff release. A concomitant electrocardiogram (ECG) recording was superimposed on the ultrasonographic images. The transducer position was marked on the skin, and the arm was held immobile with the aid of a foam support and stereotactic clamp until T = 120 min. A single trained operator blinded between 'meal 1' and 'meal 2', but not with 'meal 3', made all the observations. Images corresponding to end-diastole, coincident with the beginning of the R wave on the ECG, were compared to determine differences in arterial diameter. FMD responses were expressed as percentage change in diameter of the artery from baseline at each time point (Stoner and Sabatier, 2012). Heart rate (HR) was calculated from the R-R interval of the ECG.

4.4.2 Blood glucose and serum insulin concentrations

Blood glucose was measured by glucometer, using the glucose oxidase technique (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). Serum insulin was measured by ELISA (10-1113; Mercodia, Uppsala, Sweden), with sensitivity of 1.0 mU/L and coefficient of variation of 2.1% within assays and 6.6% between assays.

4.4.3 Gastric emptying

Assessment of gastric emptying by the measurement of $^{13}CO_2$ concentrations in breath samples has previously been validated against scintigraphy (Chew et al., 2003). $^{13}CO_2$ concentrations in the breath samples from our study were measured by an isotope ratio mass spectrometer (ABCA 2020; Europa Scientific, Crewe, UK) with an on-line gas chromatographic purification system. The cumulative $^{13}CO_2$:$^{12}CO_2$ ratio was reported as a measure of meal delivery into the small intestine. The half-emptying time (T50) of 'meal 1' and 'meal 2' was calculated, using the formula described by Ghoos et al (Ghoos et al., 1993). Breath test data from 'meal 3' were not included in the analysis, since they would not have provided a comparable measure of gastric emptying to 'meal 1' and 'meal 2'.

4.5 Statistical analysis

Based on a previous study (Dickinson et al., 2011), we determined that in a crossover design, 12 subjects were required to detect a mean difference in FMD of 2.0% (α = 0.05; β = 0.2). The incremental area under the curves (iAUC) from T = 0 to 240 min

for HR, blood glucose and serum insulin, and the decremental area above the curve (dAAC) for FMD, were calculated using the trapezoidal rule. The fasting values, peaks and $iAUCs_{(0\text{-}240\ min)}$ of postprandial blood glucose, serum insulin and HR, as well as the nadirs and dAACs of FMD on the three study days, were compared using one-factor repeated measures ANOVA. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant effects. Repeated-measures ANOVA, with treatment and time as factors, was used to compare blood glucose, serum insulin, FMD and HR responses only between 'meal 1' and 'meal 2' because of the different definition of T = 0 min for 'meal 3'. Pearson's correlation analysis was used to assess the relationships of basal FMD with age and BMI. Within-subject correlation analysis was used to assess the relationships of the dAAC for FMD with the iAUC for blood glucose and serum insulin (Bland and Altman, 1995). All analyses were performed using SPSS 21 (IBM Corporation, Armonk, NY, USA). Results are expressed as mean ± SEM; $P < 0.05$ was considered statistically significant.

4.6 Results

Of the 14 subjects recruited, 12 completed the study. The others were unable to ingest the test meal within 10 min and subsequently withdrew from the study. All subjects reported that 'meal 2' was less palatable than 'meal 1'.

4.6.1 Gastric emptying

Addition of guar to the meal ('meal 2' vs 'meal 1') was associated with prolongation of gastric half-emptying time and a reduction of cumulative $^{13}CO_2$: $^{12}CO_2$ ratio (treatment effect: $P = 0.002$; treatment × time interaction: $P < 0.001$; AUC: $P < 0.001$). The latter was manifest in the first half hour postprandially (AUC: $P < 0.001$) (Table 1 and Figure 1A).

4.6.2 Blood glucose concentrations

Fasting blood glucose did not differ between the three study days. After all three meals, blood glucose concentrations increased and subsequently returned to baseline ($P < 0.001$ for each), with the peak occurring ~30 min after 'meal 1', and ~60 min after 'meal 2'. Although the peak blood glucose concentrations did not differ between the three study days ($P = 0.49$), there was a treatment effect on iAUC ($P = 0.03$), such that the rise in blood glucose was less after 'meal 3' than 'meal 2' ($P = 0.03$), without a significant difference between 'meal 1' and 'meal 3', or between 'meal 1' and 'meal 2'. However, when compared to 'meal 1', the addition of guar to the meal ('meal 2') was associated with an initially delayed, but more sustained, rise in blood glucose (treatment × time interaction: $P < 0.001$), so that blood glucose was lower at $T = 30$ min and higher at $T = 240$ min ($P < 0.05$ for each) (Table 1 and Figure 1B).

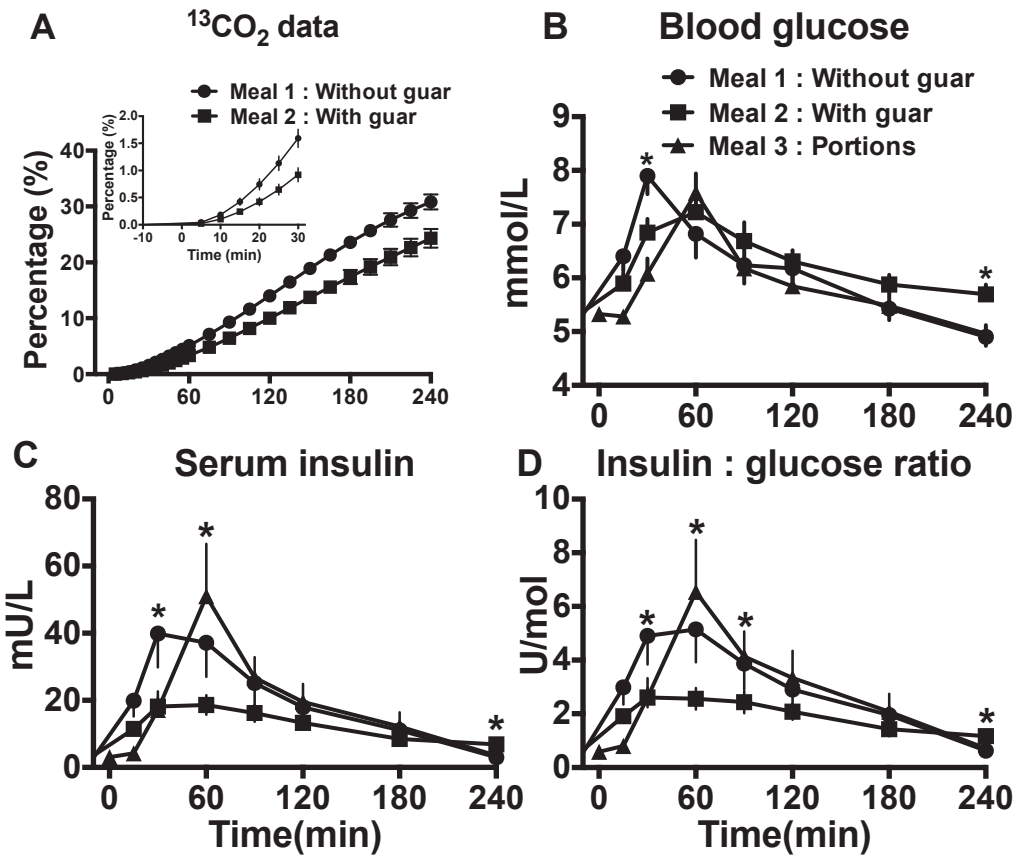


Figure 1

Cumulative $^{13}CO_2$:$^{12}CO_2$ ratios ('meal 1' vs 'meal 2'(A), blood glucose concentrations (B), serum insulin concentrations (C), and insulin: glucose (I/G) ratio (D) in response to 'meal 1', 'meal 2' and 'meal 3'. Data are mean ± SEM. * $P < 0.05$ for 'meal 1' vs 'meal 2'.

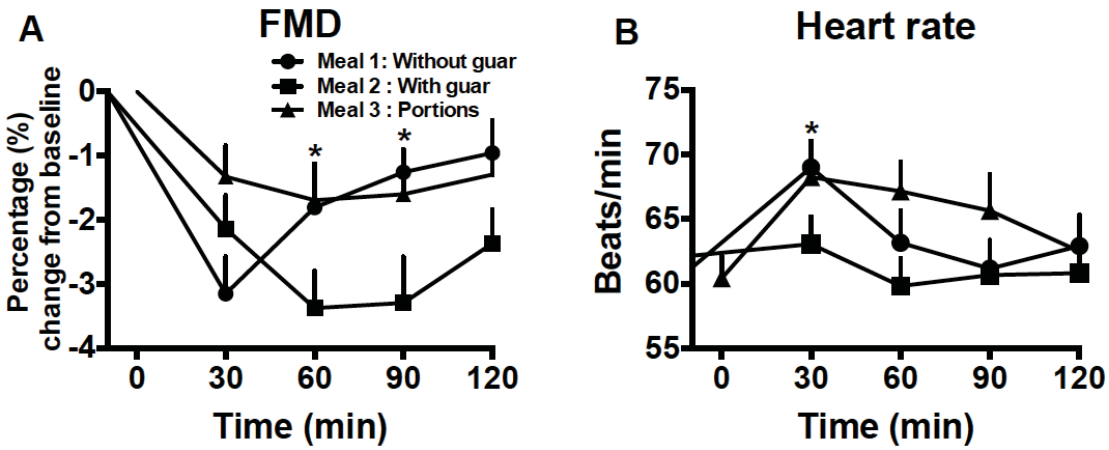


Figure 2

Flow mediated dilatation (FMD) (A) and heart rate (B) in response to 'meal 1', 'meal 2' and 'meal 3'. Data are mean ± SEM. * $P < 0.05$ 'meal 1' vs 'meal 2'.

Table 1:

Basal and peak values and the incremental area under the curves (iAUCs) for blood glucose, serum insulin, heart rate (HR), area under the curve for insulin : glucose (I/G) ratio and basal and nadir values and the decremental area above the curve (dAAC) for flow-mediated dilatation (FMD) after a high carbohydrate meal consumed within 10 min ('meal 1'), a high carbohydrate meal mixed with 9 g guar consumed within 10 min ('meal 2'), and the same high carbohydrate meal consumed as 12 equal portions over 60 min ('meal 3') (n = 12). Data are mean ± SEM. †P < 0.05 'meal 2' vs 'meal 3'.

	Meal 1	Meal 2	Meal 3	P
Gastric half-emptying time (min)	208 ± 15	285± 27	-	0.02
Fasting blood glucose (mmol/L)	5.3 ± 0.1	5.4 ± 0.1	5.3 ± 0.1	0.95
Peak blood glucose (mmol/L)	7.9 ± 0.4	7.5 ± 0.3	7.6 ± 0.4	0.49
Blood glucose iAUC ($mmolL^{-1}min$)	182.9 ± 44.3	221.6 ± 43.3†	131.3 ± 56.8†	0.03
Fasting serum insulin (mU/L)	2.9 ± 0.4	3.6 ± 0.5	3.1 ± 0.3	0.19
Peak serum insulin (mU/L)	45.3 ± 9.9	21.4 ± 2.7	51.2 ± 16.2	0.02
Insulin: glucose ratio AUC (U/mol)	714.3 ± 174.0	469.4 ± 70.5	699.2 ± 184.9	0.05
Serum insulin iAUC ($UL^{-1}min$)	4.8 ± 1.3	3.0 ± 4.7	4.5 ± 1.2	0.05
Basal HR (beats per min)	61 ± 2	62 ± 2	60 ± 2	0.64
Peak HR (beats per min)	72 ± 2	66 ± 2	72 ± 3	0.02
HR iAUC (beats)	351.3 ± 210.3	-102.9 ± 163.3	626.3 ± 238.1	0.00
Basal FMD (%)	5.4 ± 0.58	6.1 ± 0.93	4.8 ± 0.56	0.22
Max. reduction of FMD (%)	-3.2 ± 0.6	-4.2 ± 0.7	-2.6 ± 0.60	0.20
FMD dAAC (%min)	-216.3 ± 42.3	-310.1 ± 48.1†	-157.9 ± 50.1†	0.08

4.6.3 Serum insulin concentrations

Fasting serum insulin did not differ between the three study days. After all three meals, serum insulin increased before gradually returning to baseline ($P < 0.001$ for each), with the peak insulin occurring ~30 min after 'meal 1' and 'meal 2', and ~60 min with 'meal 3'. There was a treatment effect on both the peak and iAUC for serum insulin between the three study days ($P = 0.02$ for each), such that peak insulin was lower after 'meal 2' than 'meal 1' ($P = 0.02$), and tended to be lower after 'meal 2' than 'meal 3' ($P = 0.15$), with no significant difference between 'meal 1' and 'meal 3'. The iAUC for serum insulin tended to be less after 'meal 2' than 'meal 1' ($P = 0.09$), without a significant difference between 'meal 1' and 'meal 3', or between 'meal 2' and 'meal 3'. The addition of guar to the meal ('meal 2' vs. 'meal 1') was associated with changes in serum insulin concentrations (treatment effect: $P = 0.04$; treatment × time interaction: $P < 0.001$), with serum insulin being lower at T = 30 and 60 min, and higher at T = 240 min ($P < 0.05$ for each) (Table 1 and Figure 1C).

4.6.4 Serum insulin to blood glucose ratio (I/G ratio)

The addition of guar to the meal ('meal 2' vs. 'meal 1') influenced the insulin response to the prevailing blood glucose concentration (I/G ratio) (treatment effect: $P = 0.03$; treatment × time interaction: $P < 0.001$; AUC : $P = 0.04$), with the I/G ratio being lower at T = 30, 60 and 90 min, and higher at T = 240 min ($P < 0.05$ for each). The I/G ratio (iAUC) with 'meal 2' tended to be lower than with 'meal 3', ($P = 0.06$) (Table 1 and Figure 1D).

4.6.5 FMD

Fasting FMD did not differ between the three study days. The basal arterial diameter did not change during the postprandial period ($P = 0.22$), but FMD decreased postprandially on all three days ($P < 0.001$ for each), with distinct patterns: after 'meal 1', there was a rapid reduction in FMD reaching a nadir at 30 min, followed by partial recovery by 60 min; after 'meal 2' there was a delayed, but more sustained, FMD reduction and with 'meal 3', the reduction in FMD was minimal. The overall reduction in FMD (dAAC) did not quite show a significant treatment effect ($P = 0.08$). However, addition of guar to the meal ('meal 2' vs 'meal 1') was associated with a relatively delayed, but more sustained, suppression of postprandial FMD (treatment × time interaction: $P = 0.002$), with greater reductions at T = 60 and 90 min ($P < 0.05$ for each). Furthermore, when compared to 'meal 2', prolonging the duration of meal consumption ('meal 3') was associated with attenuation of the postprandial reduction of FMD (dAAC: $P < 0.05$).

Endothelium independent vasodilation of the brachial artery, assessed by sublingual GTN, did not differ between the three study days (13.5 ± 1.4 %; 12.8 ± 1.1% and 12.0 ± 0.8%; $P = 0.59$) (Table 1 and Figure 2A).

4.6.6 Heart rate

Basal HR did not differ between the three study days, but increased after 'meal 1' and 'meal 3', peaking at ~ 30 min before gradually returning to baseline ($P = 0.002$ and 0.003, respectively). HR was unchanged after 'meal 2'. Accordingly, both the peak and iAUC for HR were greater after 'meal 1' and 'meal 3' than 'meal 2' ($P < 0.05$ for

both), without significant difference between 'meal 1' and 'meal 3'. When compared with 'meal 1', the addition of guar to the meal ('meal 2') was associated with attenuation of the postprandial increase in HR (treatment × time interaction: P = 0.05), with HR being lower at T = 30 min (P = 0.02) (Table 1 and Figure 2B).

4.6.7 Relationships between variables

Basal FMD was related inversely to age (r = -0.62; P = 0.03), but was not related to BMI. The magnitude of the increment in blood glucose (iAUC) in the early postprandial period after 'meal 1' and 'meal 2' (T = 0 - 30 min), was related directly to gastric emptying, indicated by the cumulative $^{13}CO_2$:$^{12}CO_2$ ratio (r = 0.86; P < 0.001). The magnitude of reduction in FMD within each subject at T = 30 min in response to the three meals was related directly to the $iAUC_{0\text{-}30}$ for blood glucose (r = 0.43, P = 0.03). In addition, the dAAC for FMD within each subject in response to the three meals, was related directly to the $iAUC_{(0\text{-}30)}$ for blood glucose (r = 0.46, P = 0.02), and inversely to the iAUC for HR (r = -0.55, P = 0.005).

4.7 Discussion

Our study shows that, in healthy humans, (i) the inclusion of guar gum in a meal, leading to slowing of gastric emptying, results in a relatively delayed but more sustained suppression of postprandial FMD, which is associated with attenuation of the early rise in postprandial blood glucose concentration, as well as the overall rise in serum insulin and HR; and (ii) when compared to adding guar gum, consuming a meal more slowly attenuates the overall increase in postprandial glycemia as well as

the postprandial decline in FMD. It is unlikely that the changes in FMD demonstrated in this study were an artefact related to postprandial variations in the basal arterial diameter (Rudolph et al., 2007), since the latter did not change during our study. We also noted an inverse relation between FMD and age, in keeping with existing reports (Taddei et al., 1995).

Previous studies on postprandial endothelial function have focussed primarily on the effects of nutritional content (Ceriello et al., 2002) and glycemic index (Lavi et al., 2009) of the test-meal. Here, we gave the same meal on three days, modifying only its composition (by the addition of guar) or duration of consumption (by dividing it into small portions) with the aim of altering gastric emptying and/or small intestinal nutrient absorption. The addition of 9g guar in our study, a dose previously known to slow gastric emptying (Leclere et al., 1994), resulted in a reduction in the rate of small intestinal nutrient absorption, reflected by the reduced $^{13}CO_2$: $^{12}CO_2$ ratio. However, the degree of glycemic variation was modest, as would be anticipated in subjects with normal glucose tolerance (Wu et al., 2012). Regardless, postprandial FMD was substantially altered, suggesting that manipulation of gastrointestinal function by dietary means can modulate postprandial endothelial function.

The “glycemic index” (GI) is a measure of the ability of dietary carbohydrates to increase blood-glucose concentrations postprandially (Wolever et al., 2008); whether it predicts what foods are healthy has recently been a subject of debate (Wolever, 2013). In the present study, addition of guar gum ('meal 2') reduced the effective GI of the standardised high carbohydrate meal but was associated with a sustained suppression of endothelial function, which suggests that the GI value of foods, which

is based on measurements of postprandial glycemic responses for only up to 2 hours, may not be the ideal predictor of the relatively longer lasting postprandial cardiovascular risks.

We demonstrated a direct relationship between the reduction in FMD and the increase in blood glucose, consistent with existing literature (Suzuki et al., 2012). However, in our study, after 'meal 2', FMD remained persistently low even after the first 30 min, despite improvements in glycemia, and with 'meal 3', the glycemic spike at 60 min was not accompanied by a marked reduction in FMD. These discrepancies may be accounted for, at least in part, by the differences in postprandial insulin secretion, since insulin is known to have vasodilatory and anti-inflammatory properties (Dandona et al., 2009), and would be expected to counteract the postprandial reduction in FMD. Therefore, we cannot rule out the possibility that the markedly elevated insulin levels at T = 60 min after 'meal 3' might have counterbalanced any tendency for postprandial suppression of FMD due to hyperglycemia, while the attenuated insulin response associated with 'meal 2', could potentially have contributed to the sustained suppression of postprandial FMD.

Even though a higher serum insulin concentration appears to have a favourable effect on FMD, we could not demonstrate a relationship between these outcome measures in this study, probably due to interfering factors, as described subsequently. Furthermore, the rise in serum insulin did not correlate with the magnitude of the postprandial increment in glycemia. Though this is difficult to explain, it seems plausible that the high variability of insulin concentrations could have contributed.

Other dynamic factors such as gastric distension associated with meal ingestion might be of importance in the regulation of postprandial FMD, especially in the early postprandial phase. Gastric distension is known to induce sympathetic stimulation in proportion to the distending pressure in healthy young adults (van Orshoven et al., 2004), and sympathetic activation has been reported to suppress FMD (Hijmering et al., 2002). In the present study, it is likely that slowing of gastric emptying by guar was associated with prolonged gastric distension, while consumption of the meal in portions over one hour ('meal 3') resulted in the least gastric distension; this could explain the sustained suppression of postprandial FMD after 'meal 2', and a lesser reduction in FMD after 'meal 3'. That we observed a slight delay in the maximal suppression of FMD with 'meal 2' when compared to 'meal 1', despite similar gastric volumes in the early postprandial phase, suggests that the difference in glycemia at this point (T = 30 min) may potentially have suppressed FMD in the early part of the study more for 'meal 1' than 'meal 2'.

Addition of guar to the meal was also associated with attenuation of the rise in HR, an effect probably attributable to slowing of gastric emptying (Russo et al., 2003) and/or inhibition of nutrient absorption in the small intestine (O'Donovan et al., 2005). We observed an inverse relationship between the dAAC for FMD and iAUC for HR, and accordingly cannot exclude potential enhancement of the local shear stimulus on the endothelium as a result of the rise in postprandial HR, which would tend to enhance FMD.

Consumption of dietary fiber, especially soluble fiber such as guar gum, is generally recommended (Rideout et al., 2008), and is reported to reduce the risk of

cardiovascular disease (Threapleton et al., 2013), via metabolic effects, including production of short chain fatty acids through colonic bacterial fermentation, slowing of gastric emptying, promotion of satiety, improvement in glycemic and lipid profiles, and loss of body weight (James et al., 2003). While the long term benefits of fiber rich food remain undisputed, our study points to the possibility of an acute detrimental effect of adding guar gum (a soluble fiber) to a high carbohydrate meal, on endothelial function postprandially, which could potentially be of clinical significance in high-risk patients with pre-existing cardiovascular disease. In hyperinsulinemic patients with obesity and type 2 diabetes, the cardiovascular effect of suppression of postprandial insulin release by guar gum or by other dietary fiber needs further evaluation.

Slowing of gastric emptying by adding guar, by reducing the rate of carbohydrate digestion and absorption in the proximal small intestine, could also potentially allow greater exposure of nutrient to the L-cells, located more densely in the distal gut, which might enhance secretion of endogenous GLP-1 (Thazhath et al., 2014b), although we did not measure this hormone. It is uncertain whether the addition of guar to a meal, in conjunction with the use of a GLP-1 receptor agonist in patients with type-2 diabetes, would have an additive effect on the deceleration of gastric emptying leading to further improvements in postprandial glycemia and endothelial function. There is now persuasive evidence that with long term use, GLP-1 agonists improve vascular function directly by stimulating endothelial GLP-1 receptors, indirectly by triggering insulin release, and also by reducing weight gain (Ceriello et al., 2014), but there have been few attempts to assess their acute postprandial effects, and those showed either a direct protective effect on the endothelium (Ceriello et al.,

2011) or an indirect effect via improvements in the lipid profile (Schwartz et al., 2010). If our current observations in healthy lean adults are applicable to type 2 patients, slowing of gastric emptying induced by GLP-1 agonists (Nauck et al., 1997b, Angeli and Shannon, 2014) may result in more prolonged gastric distension (Delgado-Aros et al., 2002). Therefore their potential for an acute detrimental impact on postprandial endothelial function should now be evaluated, as it could be relevant in patients with significant cardiovascular disease.

This study was conducted as 'proof of principle' and has several limitations. The sample size was relatively small and some comparisons had the potential to be affected by type 2 error; however, our observations appeared clear-cut. The regulation of postprandial FMD is clearly multifactorial, and our study was not designed to address potential mechanisms in isolation. Further studies incorporating non-nutrient gastric distension, such as with an intragastric balloon, and measurement of splanchnic blood flow, may enable us to dissect out the individual roles of gastric distension, sympathetic activation, glycemia and hormonal profiles on postprandial FMD. The use of a breath test, rather than scintigraphy, to assess gastric emptying of a meal containing guar does not discriminate between slowing of gastric emptying or of nutrient absorption. Measurements of GLP-1 concentrations and markers of oxidative stress such as isoprostanes, which induce vasoconstriction, would have been of interest; however, these are unlikely to alter our main conclusions.

In summary, we have shown in healthy humans that postprandial endothelial function is affected less when a meal is consumed in small portions over a longer

period of time, than when consumed more rapidly with guar gum, which slows gastric emptying. Further studies are indicated, in order to develop therapeutic strategies to reduce postprandial endothelial dysfunction in high-risk populations such as those with type 2 diabetes.

CHAPTER 5: THE GLUCAGON-LIKE PEPTIDE-1 (GLP-1) RECEPTOR AGONIST, EXENATIDE, INHIBITS SMALL INTESTINAL MOTILITY, FLOW, TRANSIT AND ABSORPTION OF GLUCOSE IN HEALTH AND TYPE 2 DIABETES: A RANDOMISED CONTROLLED TRIAL.

(Adapted from Diabetes. 2016 Jan; 65(1): 269-75.) (Thazhath et al., 2016a)

5.1 Abstract

The short-acting glucagon-like peptide 1 receptor agonist exenatide reduces postprandial glycemia, partly by slowing gastric emptying, although its impact on small intestinal function is unknown. In this study, 10 healthy subjects and 10 patients with type 2 diabetes received intravenous exenatide (7.5 μg) or saline (-30 to 240 min) in a double-blind randomized crossover design. Glucose (45 g), together with 5 g 3-O-methylglucose (3-OMG) and 20 MBq (99m)Tc-sulfur colloid (total volume 200 mL), was given intraduodenally (t = 0-60 min; 3 kcal/min). Duodenal motility and flow were measured using a combined manometry-impedance catheter and small intestinal transit using scintigraphy. In both groups, duodenal pressure waves and antegrade flow events were fewer, and transit was slower with exenatide, as were the areas under the curves for serum 3-OMG and blood glucose concentrations. Insulin concentrations were initially lower with exenatide than with saline and subsequently higher. Nausea was greater in both groups with exenatide, but suppression of small intestinal motility and flow was observed even in subjects

with little or no nausea. The inhibition of small intestinal motor function represents a novel mechanism by which exenatide can attenuate postprandial glycemia.

5.2 Introduction

Glycemic control, as assessed by glycated hemoglobin, is a major determinant of the development and progression of long term microvascular and, to a lesser extent, macrovascular complications in patients with type 1 (DCCT, 1993) and type 2 (UKPDS, 1998) diabetes. In type 2 patients, when HbA1c is ≤ 7.5%, postprandial glycemia usually predominates over preprandial blood glucose in contributing to overall glycemic control, and may be an independent risk factor for cardiovascular events (Monnier et al., 2003). Accordingly, therapies that target postprandial glycemia play an important role in the management of type 2 diabetes (Ceriello, 2010).

Key determinants of postprandial glycemia include: (i) the rate of postprandial glucose absorption from the gut, which itself is dependent upon the rate of gastric emptying, the frequency of flow events of chyme in the small intestine, digestion of complex carbohydrates, sensing of glucose by specialised enteroendocrine cells and glucose transport across the small intestinal mucosa (Thazhath et al., 2014b), and (ii) postprandial glucose metabolism, influenced amongst other factors by the release of the incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), from intestinal K- and L-cells respectively, which stimulate insulin release in a glucose-dependent manner in health (Vilsboll et al.,

2003, Nauck, 1998). In type 2 diabetes, the insulinotropic effect of GIP is substantially diminished, while that of GLP-1 is largely retained (Nauck et al., 1993). This has stimulated the therapeutic development of GLP-1 receptor agonists, which are resistant to degradation in the circulation, and are now widely used in the management of type 2 diabetes (Ryden et al., 2014).

Aside from their insulinotropic effects, the ability of short-acting GLP-1 receptor agonists specifically to target postprandial glycemia appears to be mediated via their ability to slow gastric emptying (Nauck et al., 1997b), as a result of excitatory and inhibitory motor activity in different gastric regions, including relaxation of the gastric fundus (Schirra et al., 2002), inhibition of antral motility (Brennan et al., 2005, Schirra et al., 2000), and stimulation of pyloric contractions (Schirra et al., 2000). However, on repeated dosing with GLP-1, the suppression of gastric emptying is subject to tachyphylaxis (Umapathysivam et al., 2014). Conversely, antagonism of GLP-1 receptors using exendin (9-39) amide leads to stimulation of antral contractility, as well as a reduction in pyloric motor activity (Schirra et al., 2006), associated with modest acceleration of gastric emptying (Deane et al., 2010b).

While the rate of gastric emptying has been established as a key determinant of postprandial glycemic excursions in health (Horowitz et al., 1993), and type 1 (Merio et al., 1997) and type 2 (Jones et al., 1996) diabetes, there is little information about the contribution of motility of the small intestine and the flow of chyme within its lumen. We previously reported, using an impedance technique (described subsequently) in healthy humans, that the anti-cholinergic agent, hyoscine butyl-

bromide, decreased the number of duodenal flow events and this was associated with a delay in absorption of glucose infused directly into the small intestine (Chaikomin et al., 2007), indicating that modulation of small intestinal motor function can impact substantially on postprandial glycemia. However, to our knowledge, a comprehensive evaluation of the relationship between glucose absorption and small intestinal function in patients with type 2 diabetes has never been reported.

Data from rodents have shown that exogenous GLP-1 inhibits small intestinal motility and transit (Tolessa et al., 1998b, Tolessa et al., 1998a), which might therefore represent a mechanism – in addition to slowing of gastric emptying – by which GLP-1 can lower postprandial glycemia. In healthy humans, exogenous GLP-1 reduces the frequency of migrating motor complexes and the motility index in the small intestine during fasting (Hellstrom et al., 2008), and inhibits postprandial duodenal motility (Schirra et al., 1997), although no study has evaluated the impact on the flow of chyme or its transit along the small intestine. The GLP-1 analog, ROSE-010, has been reported to inhibit gastric emptying substantially, and accelerate colonic transit, in women with constipation-predominant irritable bowel syndrome, but there was no effect on transit through the small intestine (Camilleri et al., 2012). Despite the relatively high prevalence of adverse effects, such as diarrhea – which could be referable to small intestinal motility – observed with short acting GLP-1 receptor agonists (Sun et al., 2014), the influence of these agents on small intestinal function has essentially been ignored, in making the assumption that slowing of gastric emptying is pivotal to their capacity to lower postprandial blood glucose.

In the present study, we examined the effects of intravenous exenatide (7.5 mcg) on the frequency of duodenal pressure waves and flow events, small intestinal transit, the rate of absorption of the glucose analog, 3-O-methylglucose (3-OMG), from the small intestine (Fordtran et al., 1962b), and the resultant glycemic excursion, in response to intraduodenal (ID) glucose, infused at a controlled physiological rate of 3 kcal/min in both healthy subjects and patients with well-controlled type 2 diabetes. Our hypothesis was that exenatide would reduce the frequency of small intestinal pressure waves and flow events and increase the small intestinal transit time, with a consequent decrease in the rate of glucose absorption and reduction in glycemia in both health and type 2 diabetes.

5.3 Methods

5.3.1 Subjects

We studied 10 healthy subjects (8 male, 2 female; mean age (±SE) 37±5 years; body mass index (BMI) 27.4±1.7 kg/m^2) and 10 patients with type 2 diabetes (7 male, 3 female; mean age 60.4 ± 2.3 years; BMI 29.1 ± 1.5 kg/m^2; mean known duration of diabetes 5 ± 1 years and HbA1c 6.1 ± 0.2 % (43.4 ± 2.6 mmol/mol)) (Figure 1). None was a smoker or consumed more than 20g alcohol per day, had any known diabetic complications or any other significant medical co-morbidity, or had been taking any medication known to affect gastrointestinal motility. Nine subjects with type 2 diabetes were controlled by diet alone, while the remaining subject, who was treated with metformin in addition to diet, was instructed to withhold this drug for four days before entering the study.

5.3.2 Protocol

Each subject underwent two study visits separated by at least five days, in a double blind, randomized fashion. Computerised randomization was undertaken by the Royal Adelaide Hospital Pharmacy, which supplied a 50 mL solution on each study day containing either saline alone (0.9% sodium chloride solution) as a control, or exenatide (40 mcL Byetta® solution (AstraZeneca Pharmaceuticals, North Ryde, Australia) containing metacresol as an antimicrobial preservative, mannitol as a tonicity-adjusting agent, and glacial acetic acid and sodium acetate tri hydrate as buffers (pH 4.5), diluted in 0.5 mL of 20% human serum albumin to prevent adhesion to tubing and 49.5 mL of 0.9% sodium chloride solution, to a final concentration of 10 mcg/50 mL). Each solution was stored at 2–8 degrees Celsius in a masked bag, and both the subject and the investigators were blinded to the contents.

After an overnight fast from solids and liquids for 12 hours, each subject attended the Royal Adelaide Hospital Department of Nuclear Medicine, PET and Bone Densitometry at approximately 0830, when a combined manometry and impedance assembly, consisting of a multilumen silicone catheter (Dentsleeve International, Mississauga, Ontario, Canada) closely bound to an impedance catheter (Sandhill Scientific, Highlands Ranch, CO) was introduced into the stomach through an anesthetized nostril, and allowed to pass into the duodenum by peristalsis, with continuous monitoring of its position by measurement of the antral and duodenal transmucosal potential difference (TMPD) (Chaikomin et al., 2007). The assembly

was positioned with 6 manometry side-holes (spaced at 3 cm intervals), 7 impedance electrode pairs (2 cm between each electrode), and an infusion port in the duodenum. The manometry channels were perfused with degassed water, while additional proximal duodenal and distal antral sideholes were perfused with 0.9% saline for TMPD measurements.

After positioning the assembly, an intravenous cannula was inserted into a forearm vein in each arm, one for infusion of exenatide or control, and the other for blood sampling. Subjects then remained supine with a gamma camera (Model: GE Millennium MPR; GE healthcare, Little Chalfont, UK), placed anteriorly (Bonapace et al., 2000, Gryback et al., 2002). At T = -30 min, a baseline blood sample was collected and an intravenous infusion of exenatide or control was commenced and continued for 270 min. For the initial 30 min (T = -30 to 0 min), exenatide was administered at a rate of 50 ng/min, and then maintained at 25 ng/min for 240 min (T = 0 – 240 min); corresponding volumes of saline were infused on the control days. Between T = 0 – 60 min, an ID glucose infusion (45 g glucose, mixed with 5 g 3-OMG (Carbosynth Limited, Berkshire, United Kingdom) and 20 MBq of ^{99m}Tc-sulfur colloid in water to a total volume of 200 mL) was administered at a rate of 3.3 mL/min (3 kcal/min). Anterior scintigraphic images were acquired, in dynamic mode, every 3 minutes from T = 0 – 240 min. A cobalt marker was placed over the right superior iliac spine as a reference point (Deane et al., 2011b).

Venous blood was sampled frequently between T = -30 and T = 240 min for measurements of blood glucose, serum 3-OMG and insulin, and plasma C-peptide concentrations. Blood samples were collected into ice-chilled serum and EDTA

tubes, which were immediately centrifuged at 3200 rpm for 15 min at 4°C. Serum and plasma were separated and stored at -80°C for later analyses. At the same intervals used for blood sampling, gastrointestinal sensations including nausea were assessed using 100 mm visual analog questionnaires (Parker et al., 2004). After T = 240 min, the ID assembly and both intravenous cannulae were removed and each subject was given a meal prior to leaving the laboratory.

5.3.3 Measurements

Both the manometric and impedance data were recorded digitally (Insight stationary system, Sandhill Scientific, Oxford, UK) and the manometric data analyzed using custom-designed software (Professor A.J. Smout, Academic Medical Center, Amsterdam, The Netherlands) (Chaikomin et al., 2007) to determine the number and amplitude of duodenal waves and the frequency of propagated duodenal wave sequences over successive 15 min periods (Chaikomin et al., 2007). An investigator (CKR), who was blinded to the intervention, analyzed the impedance recordings for the occurrence of flow events, defined as a transient reduction in impedance of at least 12% from baseline in at least three sequential electrode pairs (i.e. at least 6 cm) (Chaikomin et al., 2007). Each flow event was classified as either “antegrade” or “retrograde”.

Small intestinal transit time was determined from the scintigraphic data, after correction for radionuclide decay and subject movement; the latter was determined by the position of the cobalt marker (Deane et al., 2011b). Images were reviewed, using purpose built software (IDL v6.2, RSI, Boulder, Colorado, USA) by an

experienced nuclear medicine technologist (KLJ) who was blinded to the intervention. A region-of-interest was drawn around the colon using a composite image. The frame in which activity was first observed in the colon was then identified to calculate the cecal arrival time (Deane et al., 2011b). Where no colonic activity appeared to be present during the imaging period (T = 0 – 240min), the cecal arrival time was recorded as >240 min.

Blood glucose concentrations were measured by the glucose oxidase method using a glucometer (MediSense Optium, Bedford, MA, USA). Serum 3-OMG concentrations were measured by liquid chromatography and mass spectrometry, with a sensitivity of 10 pmol/L (Deane et al., 2011a). Serum insulin was measured by ELISA immunoassay (10-1113, Mercodia, Uppsala, Sweden) with a sensitivity of 1.0 mU/L and intra- and inter-assay coefficient of variations (CVs) of 2.8% and 8.1% respectively. Plasma C-peptide was also measured by ELISA immunoassay (10-1136-01, Mercodia, Uppsala, Sweden), with a sensitivity of 15 pmol/L and intra- and inter-assay CVs of 2.4% and 8.4%, respectively.

5.3.4 Statistical analysis

Based on our previous study (Chaikomin et al., 2007), we determined that with a crossover design, 10 subjects would provide 80% power to detect a 50% reduction in duodenal pressure waves, and 90% power to detect a 50% reduction in duodenal flow events, with exenatide compared to control (α = 0.05). The area under the curves (AUCs) for blood glucose, serum 3-OMG and insulin, and plasma C-peptide concentrations were calculated using the trapezoidal rule. These parameters, as well

as the frequency of duodenal pressure waves, propagated sequences and antegrade flow events, were analyzed using Student's paired *t*-test for intragroup comparisons, and Student's unpaired *t*-test for intergroup comparisons. Two-factor repeated measures ANOVA, with treatment and time as factors, were also used to compare these variables within each group. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant effects. The number and amplitude of duodenal waves were used to calculate motility indices (MI) using the following equation: MI = ln [(sum of amplitudes × number of duodenal waves) + 1] (Camilleri and Malagelada, 1984). Scores for nausea between exenatide and control were compared using Wilcoxon matched-pairs signed rank test. Within-subject correlation analysis was used to assess the relationships between the variations in variables after exenatide compared to control (Bland and Altman, 1995), for which data from both healthy and type 2 subjects were pooled. All analyses were performed using SPSS 21 (IBM Corporation, Armonk, NY, USA). Results are expressed as mean ± SEM; $P < 0.05$ was considered statistically significant.

5.3.5 Study approval

The study protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000. All subjects provided written informed consent prior to their inclusion in the study.

5.4 Results

Ten healthy subjects and 10 patients with well-controlled type 2 diabetes were studied on two occasions each, with exenatide and saline control (Figure 1).

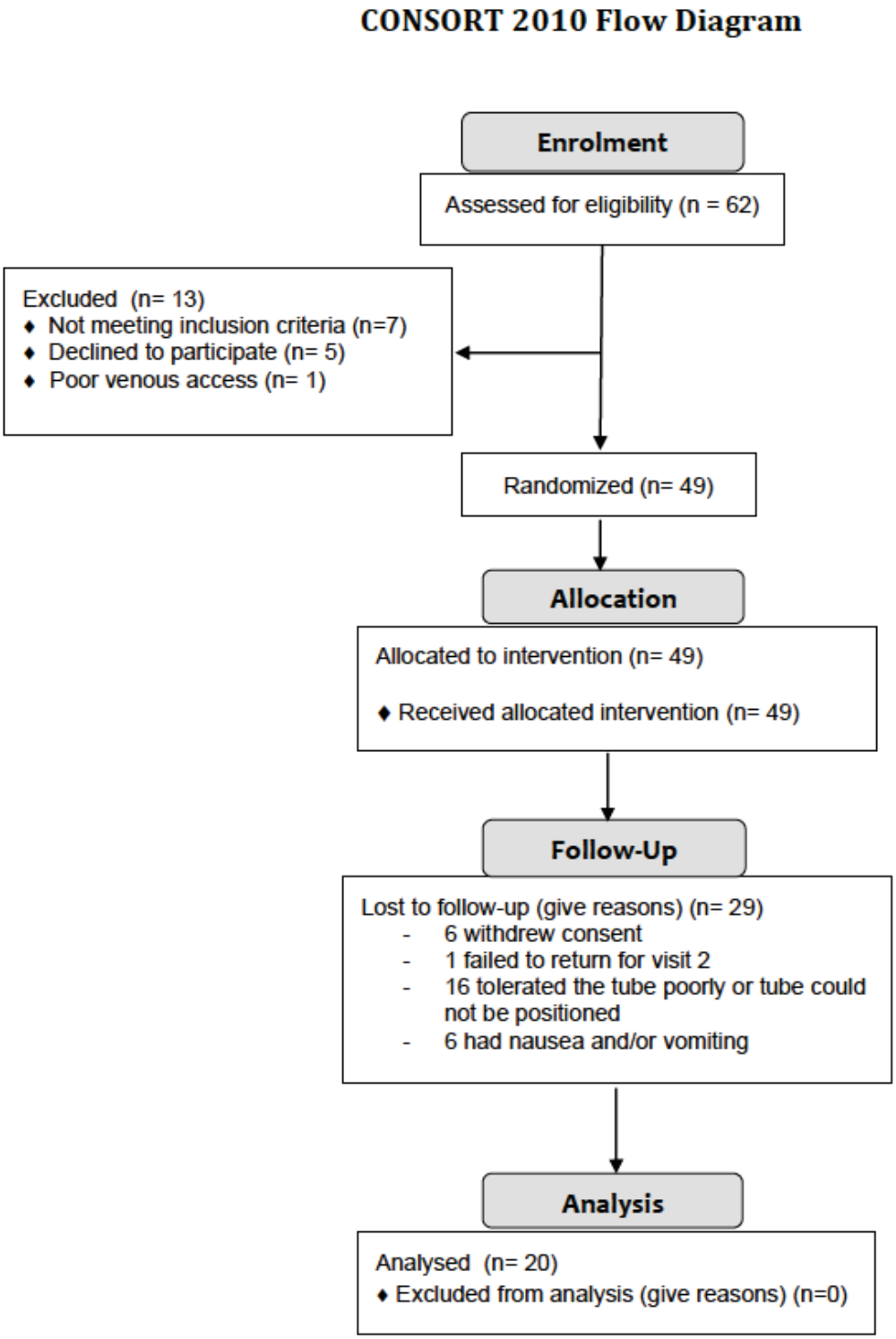


Figure 1

CONSORT diagram outlining the number of subjects involved in enrolment, intervention allocation, follow-up, and data analysis.

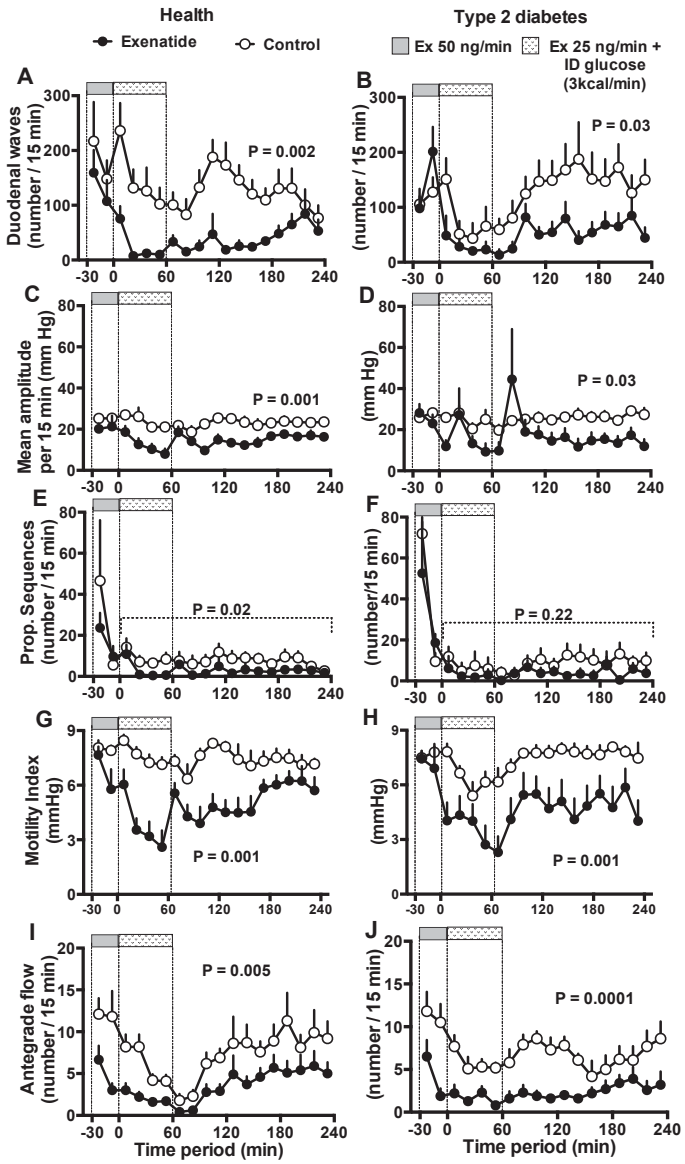


Figure 2

Effects of intravenous exenatide compared to saline control, in health (n=10) and in type 2 diabetes (n=10), on duodenal pressure waves (A, B), mean amplitude of pressure waves (C, D), propagated pressure waves sequences (E, F), duodenal antegrade flow events (G, H) and motility indices (I, J) during fasting and in response to intraduodenal (ID) glucose infusion. Two-factor repeated measures ANOVA, with treatment and time as factors, adjusted by Bonferroni correction, was used to compare these variables.

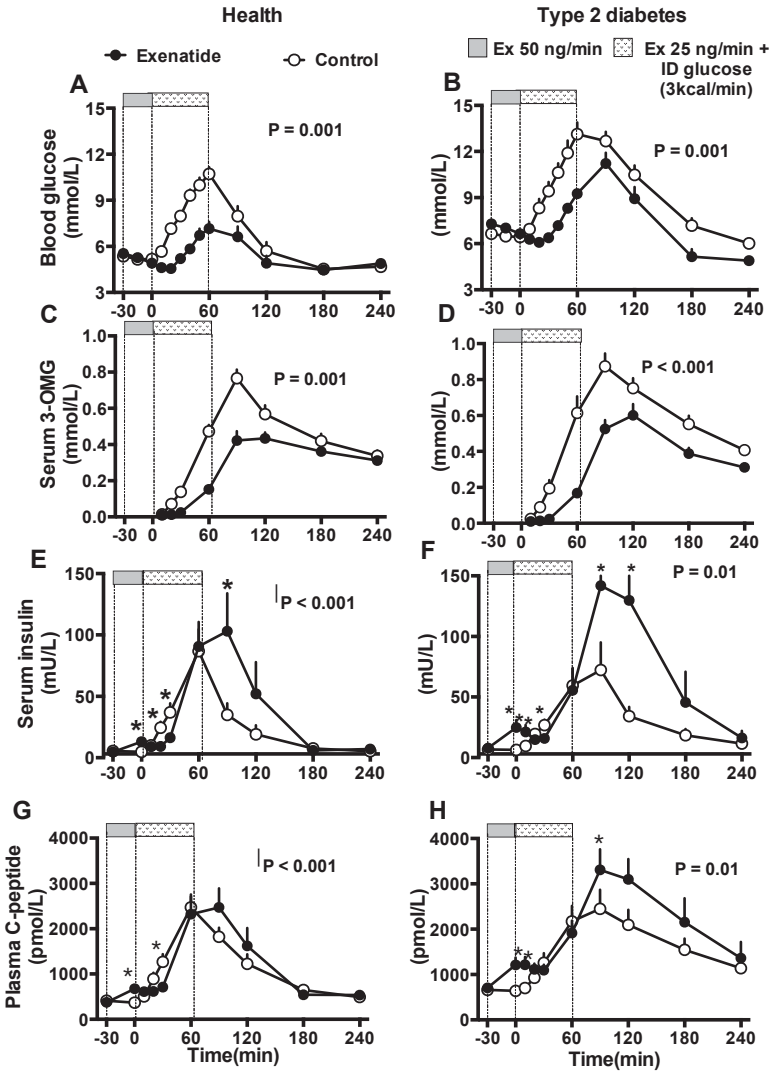


Figure 3

Effects of intravenous exenatide compared to saline control, in health (n=10) and in type 2 diabetes (n=10), on blood glucose (A, B), serum 3-OMG (C, D), serum insulin (E, F) and plasma C-peptide concentrations (G, H) during fasting and in response to intraduodenal (ID) glucose infusion. Two-factor repeated measures ANOVA, with treatment and time as factors, adjusted by Bonferroni correction, was used to compare these variables. ^ treatment x time interaction. *P < 0.05 for post hoc comparison of specified time points.

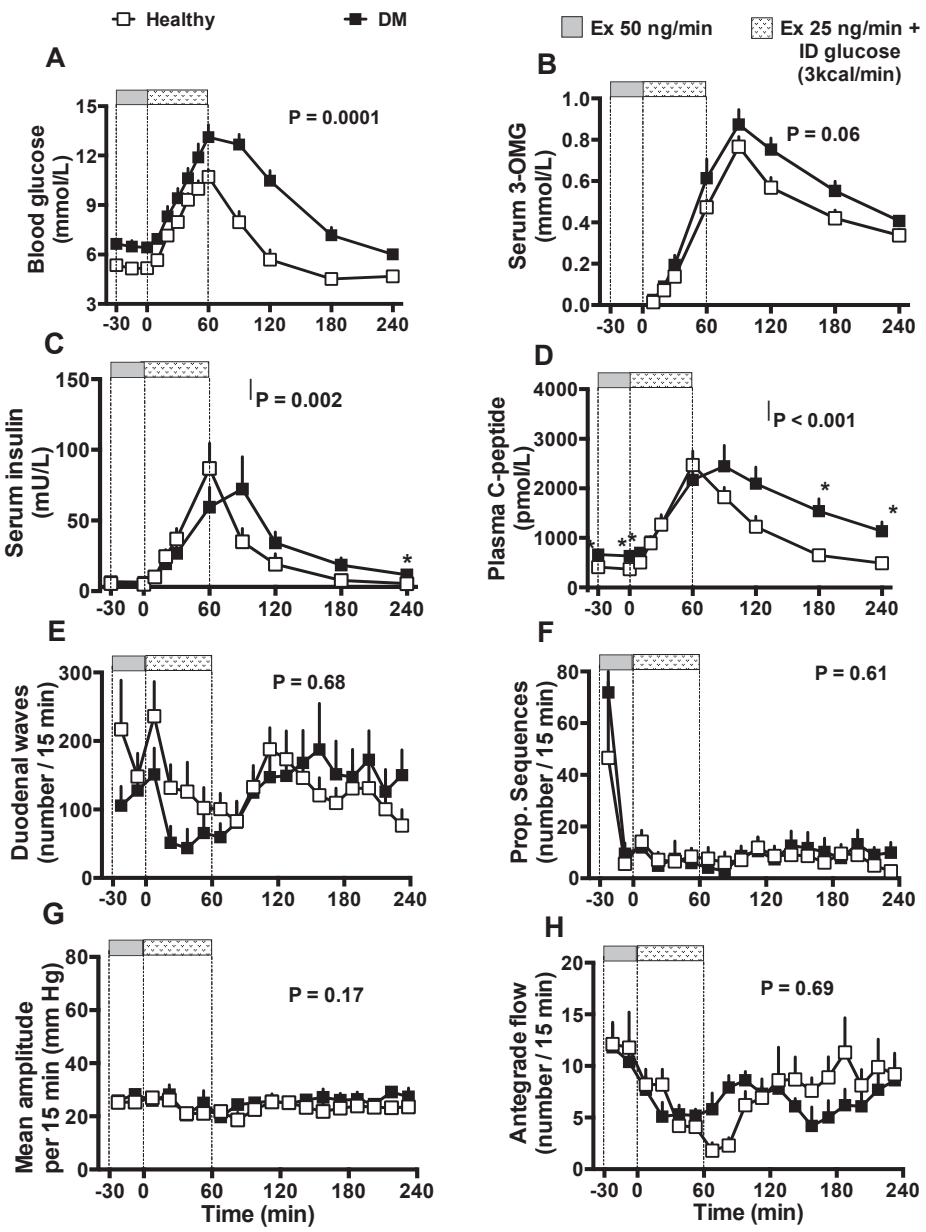


Figure 4

Blood glucose (A), serum 3-OMG (B), serum insulin (C), plasma C-peptide concentrations (D), duodenal pressure waves (E), propagated pressure waves sequences (F), mean amplitude of pressure waves (G) and duodenal antegrade flow events (H), during fasting and in response to intraduodenal (ID) glucose infusion, in patients with type 2 diabetes (DM) (n=10) compared to healthy subjects (n=10), on the control day. Two-factor repeated measures ANOVA, with treatment and time as factors, adjusted by Bonferroni correction, was used to compare these variables. ^ treatment x time interaction. *P < 0.05 for post hoc comparison of specified time points.

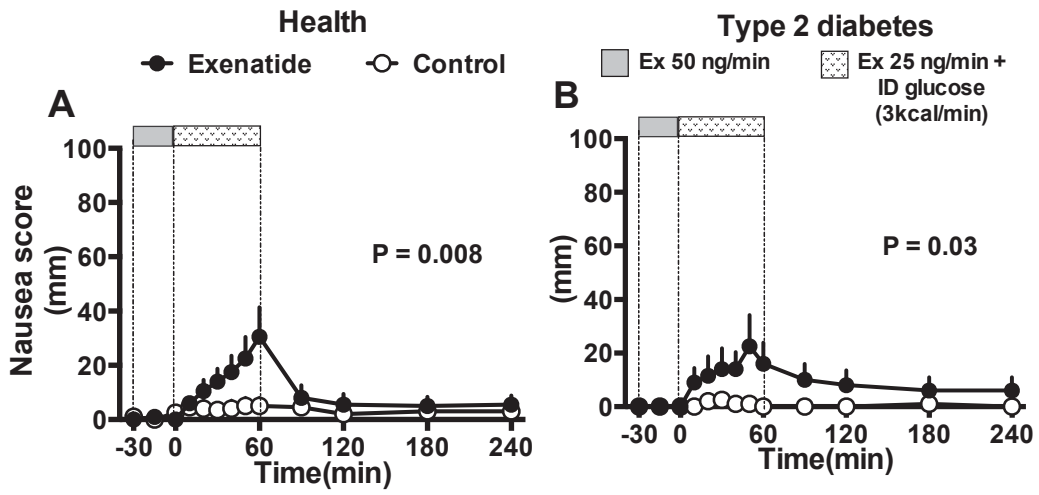


Figure 5

Mean nausea scores associated with intravenous exenatide compared to intravenous saline control in healthy subjects (n=10) (A) and patients with type 2 diabetes (DM) (n=10) (B). Wilcoxon matched-pairs signed rank test was used for comparison.

Table 1

Demographics of the study subjects, number of duodenal pressure waves (DW), mean duodenal pressure wave amplitude (DWA), motility index, number of propagated pressure wave sequences (PPWS) and number of anterior flow events (AFE) in the duodenum during fasting (T = -30 to 0 min), and also in response to intraduodenal glucose infusion (3kcal/min; T = 0 to 240 min), with intravenous (IV) exenatide or saline control, in healthy subjects (n = 10) and patients with type 2 diabetes (n = 10).

	Healthy (H)		**Type 2 diabetes (DM)**		**Healthy (Ex vs C) P**	**DM (Ex vs C) P**	**Control (H vs DM) P§**
	Exenatide (Ex)	**Control (C)**	**Exenatide (Ex)**	**Control (C)**			
Age (yrs)	36.8 ± 4.5		60.5 ± 2.3		–	–	0.001
Gender (male : female)	8 : 2		7 : 3		–	–	–
BMI (kg. m^{-2})	27.4 ± 1.7		29.1 ± 1.5		–	–	0.40
Duration of diabetes (yrs)	–		60.5 ± 2.3		–	–	–
HbA1c % (mmol.mol^{-1})	–		6.1 ± 0.2 (43.4 ± 2.6)		–	–	–
Number of DW (-30 to 0 min)	267 ± 67	365 ± 90	299 ± 58	233 ± 57	0.426	0262	0.209
Number of DW (0 to 240 min)	577 ± 98	2088 ± 282	781±149	1976 ± 446	<0.001	0.016	0.825
DWA (mm Hg) (-30 to 0 min)	21.8 ± 2.6	25.3 ± 2.0	25.6 ± 3.6	27.1 ± 1.9	0.281	0.661	0.427
DWA (mm Hg) (0 to 240 min)	14.4 ± 1.3	23.2 ± 1.3	16.8 ± 2.7	25.4 ± 1.6	<0.001	0.021	0.164
MI (mm Hg) (-30 to 0 min)	6.7 ± 0.6	8.0 ± 0.3	7.2 ± 0.8	7.6 ± 0.4	0.094	0.350	0.487
MI (mm Hg) (0 to 240 min)	4.8 ± 0.4	7.5 ± 0.3	4.5± 0.7	7.3 ± 0.3	0.001	0.001	0.716
Number of PPWS (-30 to 0 min)	33 ± 11	52 ± 32	71 ± 27	81 ± 38	0.600	0.751	0.541
Number of PPWS (0 to 240 min)	46 ± 17	127 ± 15	59 ± 23	138 ± 60	0.015	0.222	0.857
Number of AFE (-30 to 0 min)	10 ± 2	24 ± 4	8 ± 2	22 ± 4	<0.001	0.015	0.907
Number of AFE (0 to 240 min)	55 ± 12	114 ± 15	36 ± 8	105 ± 10	<0.05	<0.001	0.587

BMI: Body Mass Index; DW: Duodenal pressure Waves; DWA: Duodenal pressure wave amplitude; MI: Motility index; PPWS: Propagated Pressure Wave Sequences; AFE: Antegrade Flow Events; § Unpaired student *t* test

Table 2

Basal values, fasting values after intravenous (IV) exenatide or saline control (T = -30 to 0 min), and area under the curves (AUC) in response to intraduodenal glucose infusion (3kcal/min) with IV exenatide or saline control (T = 0 to 240 min), for blood glucose, serum insulin and plasma C-peptide, and serum 3-O-methyl glucose (3-OMG) concentrations in healthy subjects (n = 10) and patients with type 2 diabetes (n = 10).

	Healthy (H)		**Type 2 diabetes (DM)**		**Healthy (Ex vs C) P**	**DM (Ex vs C) P**	**Control (H vs DM) P§**
	Exenatide (Ex)	**Control (C)**	**Exenatide (Ex)**	**Control (C)**			
Basal glucose (mmol. L^{-1})	5.5 ± 0.2†	5.4 ± 0.2*	7.3 ± 0.4‡	6.6 ± 0.4	0.196	0.003	0.006
Glucose at 0 min (mmol. L^{-1})	4.9 ± 0.2†	5.2 ± 0.2*	6.6 ± 0.3‡	6.4 ± 0.4	0.057	0.345	0.005
Peak glucose (mmol.L^{-1}.min)	7.9 ± 0.4	10.7 ± 0.5	11.5 ± 0.7	13.4 ± 0.7	<0.001	0.023	0.004
Glucose AUC (0-240 min) (mmol.L^{-1}.min)	1272 ± 65	1548 ± 75	1754 ± 81	2229 ± 128	<0.001	<0.001	<0.001
Peak 3-OMG (mmol. L^{-1})	0.5 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.001	0.005	0.149
3-OMG AUC (0-240 min) (mmol.L^{-1}.min)	68.3 ± 3.4	101.5 ± 6.7	81.0 ± 6.1	128.7 ± 10.3	0.001	<0.001	0.031
Basal insulin (mU. L^{-1})	4.7 ± 1.4#	5.5 ± 1.9	7.8 ± 1.5	6.7 ± 1.2	0.235	0.100	0.559
Insulin at 0 min (mU. L^{-1})	13.1± 4.6#	4.8 ± 1.6	24.9 ± 6.6	6.3 ± 0.9	0.019	0.011	0.404
Peak insulin (mU.L^{-1})	140.5 ± 29.0	86.8 ± 18.9	155.4 ± 35.3	77.8 ± 22.7	0.060	0.013	0.751
Insulin AUC (0-240 min) (mU.L^{-1}.min)	9300 ± 2444	6227 ± 1260	15787 ± 4312	7803 ±1850	0.078	0.013	0.467
Basal C-peptide (pmol. L^{-1})	374 ± 64.0^	410 ± 80∫	703 ± 87$	662 ± 80	0.132	0.279	0.031
C-peptide at 0 min (pmol. L^{-1})	675 ± 112^	369 ± 77∫	1212 ± 174$	638 ± 71	0.001	0.002	0.014
Peak C-peptide (pmol. L^{-1})	3137 ± 362	2500 ± 295	3661 ± 448	2490 ± 437	0.105	0.007	0.984
C-peptide AUC (0-240 min) (pmol.L^{-1}.min)	295234 ± 40832	278725 ± 28433	517356 ± 77199	404219 ± 64075	0.536	0.023	0.075

Data are mean ± SEM. †, *, ‡, #, ^, ∫, $ P < 0.05 (T = -30 min vs 0 min) by student paired *t* test. § Unpaired student *t* test

5.4.1 Duodenal pressure waves and flow events

Prior to ID glucose infusion (T= -30 to 0 min), the frequency, amplitude and motility index (MI) of duodenal pressure waves did not differ between exenatide and control in either healthy subjects or type 2 diabetes patients. During and after ID glucose infusion (T = 0 to 240 min), there were marked reductions in the frequency (health: $P < 0.001$; type 2 diabetes: $P = 0.016$), amplitude ($P < 0.001$ and 0.021), and MI ($P = 0.001$ for both) of duodenal pressure waves with exenatide compared to control in both groups. (Figure 2 and Table 1)

Prior to ID glucose infusion (T= -30 to 0 min), the frequency of propagated duodenal pressure wave sequences did not differ between exenatide and control in either group ($P = 0.60$ and 0.75 respectively). In response to ID glucose infusion (T = 0 to 240 min), propagated pressure wave sequences were fewer with exenatide compared to control in health ($P = 0.02$), but not in type 2 diabetes ($P = 0.22$).

Duodenal flow events were almost exclusively antegrade on both study days, and in both groups (health: 96 ± 1% with exenatide and 99 ± 0% with control; type 2 diabetes: 98 ± 1% with exenatide and 99 ± 1% with control). There were fewer antegrade flow events with exenatide compared to control, both before ID glucose (T = -30 to 0 min) and in response to ID glucose infusion (T = 0 to 240 min) in both groups (health: $P < 0.001$ and 0.01; type 2 diabetes: $P = 0.02$ and 0.001, respectively). (Figure 2 and Table 1)

On the control days, there were no significant differences in the frequency, amplitude, or MI of duodenal pressure waves, or in the frequency of propagated pressure wave sequences or antegrade flow events, between healthy subjects and patients with type 2 diabetes (Figure 4).

5.4.2 Small intestinal transit

With saline control, the radioactive label reached the cecum in all subjects within 240 min, other than one patient with diabetes. Excluding this subject, the mean small intestinal transit time was 121 (95% CI: 98 to 144) min in healthy subjects (n = 10) and 120 (95% CI: 85 to 155) min in type 2 patients (n = 9), without a difference between the groups. With exenatide, there was a marked delay in small intestinal transit such that the radiolabel did not reach the cecum within 240 min in any healthy or type 2 subject.

5.4.3 Blood glucose concentrations (Figure 3A and B and Table 2)

Fasting blood glucose concentrations did not differ between the two study days in either group. Prior to ID glucose infusion (T= -30 to 0 min), blood glucose concentrations in health decreased slightly on both days (exenatide: $P = 0.001$ and control: $P = 0.05$), with a tendency to be lower after exenatide than control at T = 0 min ($P = 0.06$). In type 2 diabetes, blood glucose concentrations (T = -30 to 0 min) remained unchanged with control, and decreased slightly (mean ~0.7 mmol/L) with exenatide ($P < 0.001$). In response to ID glucose infusion (T = 0 to 240 min), blood glucose increased on both study days before returning to baseline. Both the peak

blood glucose (health: $P < 0.001$; type 2 diabetes: $P = 0.02$) and area under the curve (AUC) ($P < 0.001$ for both groups) were lower with exenatide than control. With exenatide, blood glucose decreased slightly during the initial 30 min of ID glucose infusion in both groups, in contrast to control. (Figure 3A and B and Table 2)

On the control days, fasting blood glucose concentrations at T = -30 min and T = 0 min ($P = 0.006$ for both), as well as the AUC for blood glucose (T = -30 to 240 min), were greater ($P < 0.001$) in type 2 diabetes than in health (Figure 4A and Table 2).

5.4.4 Serum 3-OMG concentration

Serum 3-OMG increased from T = 0 min in both groups with the peak concentrations and AUCs being markedly lower with exenatide than control ($P < 0.005$ for each, in both groups); 3-OMG concentrations also peaked later with exenatide than control ($P < 0.001$), by about 30 min. (Figure 3C and D and Table 2)

On the control days, the AUC for serum 3-OMG, but not the peak concentration, was greater in type 2 diabetes than in health ($P = 0.031$) (Figure 4B).

5.4.5 Serum insulin concentrations

Fasting insulin concentrations did not differ between the two study days in either group. Prior to ID glucose infusion (T= -30 to 0 min), serum insulin concentrations remained unchanged during control, and were modestly increased with exenatide ($P = 0.03$ in health, $P = 0.01$ in type 2 diabetes). In response to ID glucose infusion (T =

0 to 240 min), there was a significant treatment × time interaction ($P < 0.001$ for each group), such that serum insulin concentrations were lower between T = 20 and 60 min with exenatide compared to control, and higher after 60 min ($P < 0.05$ for each). Overall, the peak and AUC for serum insulin tended to be higher with exenatide than control in health ($P = 0.06$ and 0.07 respectively), and were higher with exenatide in type 2 diabetes ($P = 0.01$ for both). (Figure 3E and F and Table 2)

On the control days, fasting insulin concentrations (T = -30 and 0 min) did not differ between the two groups. The peak serum insulin concentration in response to ID glucose occurred later in type 2 diabetes than health ($P =0.002$), but the AUC did not differ (Figure 4C).

5.4.6 Plasma C-peptide concentrations

Fasting plasma C-peptide concentrations did not differ between the two study days in either group. Prior to ID glucose infusion (T= -30 to 0 min), plasma C-peptide remained unchanged with control, but increased with exenatide (health: $P < 0.001$; type 2 diabetes: $P = 0.002$). In response to ID glucose infusion, plasma C-peptide increased progressively with control, and in both groups, this rise was delayed with exenatide. There was a significant treatment × time interaction (health: $P < 0.001$; type 2 diabetes: $P = 0.005$), with plasma C-peptide concentrations being higher with exenatide than control at T = 0 and lower at T = 30 min in health, and higher with exenatide at T = 10, 20 and 90 min in type 2 diabetes ($P < 0.05$ for each). Neither the peak nor the AUC for plasma C-peptide differed between the two study days in

health, whereas each was higher after exenatide in type 2 diabetes (P = 0.01 and 0.02 respectively). (Figure 3G and H and Table 2).

On the control days, fasting C-peptide concentrations (T = -30 and 0 min) were higher in type 2 diabetes than in health (P = 0.017 and 0.036, respectively). Peak plasma C-peptide concentrations in response to ID glucose infusion occurred later in type 2 diabetes than in health (P < 0.001), and the AUC for plasma C-peptide tended to be greater in type 2 diabetes (P = 0.075) (Figure 4D and Table 2).

5.4.7 Gastrointestinal sensations

Prior to ID glucose infusion (T = -30 to 0 min), nausea scores were low and did not increase with either control or exenatide. During ID glucose infusion (T = 0 to 60 min), nausea increased with exenatide, but not with control in each group, and returned to baseline after the end of ID glucose infusion. The mean and peak nausea scores were higher with exenatide than control (health: P = 0.008 and 0.039; type 2 diabetes: P = 0.03 and 0.047).

In both health and type 2 diabetes, there was substantial inter-subject variability in nausea scores. When restricting the analysis of small intestinal motor function to those with a peak nausea score below the median of 27.5 mm (n = 10; 4 healthy and 6 type 2 subjects), the observations of suppression of duodenal pressure waves (678 ± 137 vs 1963 ± 467; P < 0.01) and antegrade flow events (58 ± 9 vs 106 ± 6; P < 0.001) with exenatide, associated with a decrease in the mean blood glucose and serum 3-OMG concentrations (P < 0.001 for each), persisted. (Figure 5).

Other sensations including hunger, fullness, abdominal rumbling, anxiety, sleepiness and dizziness did not differ between exenatide and control in health and type 2 diabetes. On the control days, there were no differences in these sensations between health and type 2 diabetes.

5.4.8 Relationships between variables

On pooling the data from healthy and type 2 subjects, the reduction in AUC for blood glucose concentrations observed with exenatide compared to placebo, in response to ID glucose infusion, was related directly to the reduction in AUC for serum 3-OMG concentrations ($r = 0.83$, $P < 0.001$). On pooling the data from both groups on both study days, the AUCs for serum 3-OMG and blood glucose concentrations were related directly to the frequency of duodenal pressure waves ($r = 0.66$, $P = 0.001$ and $r = 0.58$, $P = 0.006$ respectively), duodenal MI ($r = 0.84$, $P < 0.001$ and $r = 0.67$, $P = 0.001$ respectively), and antegrade flow events ($r = 0.75$, $P < 0.001$ and $r = 0.59$, $P = 0.005$ respectively), but were not related to small intestinal transit time on control days.

5.5 Discussion

We evaluated the acute effects of intravenous exenatide on small intestinal motility, flow events, transit time and glucose absorption in healthy subjects and patients with relatively well-controlled type 2 diabetes. The main observations, in both health and

type 2 diabetes, were that exenatide (i) markedly suppressed duodenal motility and flow events, (ii) slowed small intestinal transit, (iii) decreased the absorption of the glucose analog, 3-OMG, and (iv) delayed and suppressed glycemic increments in response to an ID infusion of glucose. The glucose absorption and glycemic response showed a positive correlation with duodenal motility and the frequency of flow events.

It is well established that GLP-1 receptor agonists can improve glycemia by a number of mechanisms, including augmentation of insulin secretion (Nauck et al., 1997a), and suppression of glucagon release and endogenous glucose production (Seghieri et al., 2013). While the long-acting GLP-1 receptor agonists mainly target preprandial hyperglycemia, the short-acting formulations, such as exenatide, at doses similar to the one used in our study (McCormack, 2014), are known to be effective in treating postprandial hyperglycemia (Pinelli and Hurren, 2011). The capacity of the short-acting agonists to slow gastric emptying has been taken to be the major mechanism of action by which the latter is achieved, even though their effects on small intestinal function have not hitherto been evaluated. In the present study, the intraduodenal administration of glucose – as opposed to the oral route – bypassed any effects of gastric emptying, to enable the effects of exenatide on the small intestine be examined in isolation. The available evidence suggests that, in contrast to the stomach, there is no difference in the way nutrients derived from ingested solids and liquids progress through the small intestine (Malagelada et al., 1984); therefore, the infusion of glucose into the duodenum at a controlled rate, approximating the physiological rate of gastric emptying, allows a valid assessment of chyme transport in the small intestine, and highlights a novel mechanism by which

GLP-1 receptor agonists can specifically address postprandial glycemia. It is likely, on the basis of the actions of GLP-1 (Tolessa et al., 1998b, Tolessa et al., 2001, Hellstrom et al., 2008), that exenatide exerts its inhibitory effects on small intestinal motility via neurocrine, rather than endocrine pathways, which involve nitric oxide (NO)-dependent vagal parasympathetic outflow.

Manometric data from previous human studies have demonstrated a relationship between the frequencies of small intestinal pressure waves and propagated pressure sequences, and the absorption of 3-OMG (Schwartz et al., 2002, Rayner et al., 2002). In the current study, in addition to the manometric measurements, we also evaluated small intestinal flow events in response to exenatide or control, by recording intraluminal impedance between pairs of electrodes positioned along a catheter (Nguyen et al., 1999). This sophisticated technique is well established and validated to detect liquid and semisolid flow in the esophagus (Nguyen et al., 1997a), to characterise antropyloroduodenal flow patterns in health (Nguyen et al., 1995, Savoye et al., 2003, Savoye-Collet et al., 2003), and to evaluate abnormalities of duodenal chyme transport in patients with diabetic gastroparesis (Nguyen et al., 1997b). When compared against videofluoroscopy as the "gold-standard", impedance monitoring was found to be more sensitive in detecting intraluminal flow than manometry (Imam et al., 2004). We observed a direct relationship between glucose absorption and the frequency of both duodenal pressure waves and antegrade flow events, supporting the concept that small intestinal motor function is a major determinant of glucose absorption. Previously, when manometric and impedance data were collected simultaneously during ID glucose infusion, with concurrent administration of intravenous hyoscine or placebo, flow events were found to be

more important than the frequency of pressure waves in determining the rate of glucose absorption (Chaikomin et al., 2007). The main factors that affect intestinal glucose absorption are the length of the intestinal mucosa exposed to glucose (Little et al., 2006a), the thickness of the unstirred water layer at the interface of the chyme and intestinal mucosa (Lewis and Fordtran, 1975), the sensing of glucose by intestinal cells resulting in regulation of glucose transporters and release of neurohumoral mediators fundamental to glucose homeostasis, transport across the intestinal mucosa to the portal circulation by glucose transporters (Thazhath et al., 2014b), and the blood flow in the mesenteric circulation (Sim et al., 2013). In the present study, the inhibition of duodenal motility and flow events, together with the slowing of small intestinal transit that we observed with exenatide, could have reduced the effective length of small intestinal mucosa exposed to glucose, and diminished the thinning of the unstirred water layer which occurs with flow of chyme, resulting in a reduction in glucose absorption in spite of a possible increase in mesenteric blood flow associated with GLP-1 receptor stimulation, as has been shown previously (Trahair et al., 2014a).

Our finding of slowing of small intestinal transit with exenatide differs from the previous observation with the GLP-1 receptor agonist, ROSE-010, where no effect was reported (Camilleri et al., 2012). This disparity could be due to a different patient population (ROSE-010 was studied only in women with constipation-predominant irritable bowel syndrome), different technique (oral meal and 6 hour colonic filling measurement for ROSE-010), and differences in drug administration (subcutaneous injection for ROSE-010).

After the initiation of ID glucose infusion, there was a relative delay before blood glucose began to rise with exenatide, when compared to control, in both healthy and diabetic subjects, consistent with decreased absorption of glucose from the lumen. Serum insulin and plasma C-peptide concentrations were also lower at T = 20 and 30 min with exenatide than control, indicating that initially, inhibition of small intestinal motility and glucose absorption outweighed the insulinotropic effects of exenatide. Subsequently, the release of insulin was greater with exenatide than control, once elevated blood glucose concentrations allowed the insulinotropic effects to be manifest. A similar phenomenon has been demonstrated previously, when gastric emptying of a liquid meal was slowed by intravenous GLP-1 infusion in healthy subjects (Nauck et al., 1997b).

We observed on the control days that neither the frequency of duodenal pressure waves or flow events, nor the small intestinal transit time, differed between healthy subjects and patients with type 2 diabetes. This contrasts with previous studies, where type 2 patients had reduced frequency of duodenal pressure waves during ID glucose infusion compared to healthy controls (Chang et al., 2013), although a wide variation is reported in small intestinal transit (Wegener et al., 1990), which may sometimes be accelerated (Triantafyllou et al., 2007).These disparities might relate to better glycemic control in our patients, or to a relatively shorter duration of ID glucose infusion, resulting in less marked hyperglycemia.

We observed that serum 3-OMG concentrations were higher in type 2 diabetes than in health on the control days, indicating enhanced small intestinal glucose absorption in the former group. Animal data (Adachi et al., 2003), and those from obese humans

with evidence of chronic hyperglycemia (HbA1c > 6.0%) (Verdam et al., 2011), suggest that small intestinal mucosal hyperplasia and hypertrophy occur in type 2 diabetes, which can facilitate glucose absorption. Furthermore, there is emerging evidence that dysregulation of sweet taste receptor expression in type 2 diabetes could result in inappropriate up-regulation of sodium-glucose linked transporter-1 (SGLT-1), the major transporter of glucose into the enterocyte, even under hyperglycemic conditions, with a consequent increase in glucose absorption (Young et al., 2013).

Nausea is one of the most common adverse effects associated with the use of GLP-1 receptor agonists, including exenatide (Kendall et al., 2005, DeFronzo et al., 2005). In a previous study using an identical intravenous dosing schedule to ours, patients with type 2 diabetes did not experience significant nausea (Fehse et al., 2005). Indeed, it has recently been suggested that exenatide could be better tolerated intravenously than subcutaneously (Nauck et al., 2013). In our study, nausea tended not to occur with exenatide until the added stimulus of intraduodenal glucose was provided. It is unlikely that the suppression of small intestinal function that we observed with exenatide could be accounted for by nausea, because the inhibition of gut motility, flow events and glucose absorption were evident even in subjects with little or no nausea.

Our study has several limitations. First, the sample size was relatively small; however our findings showed clear-cut and statistically significant differences between the treatments, which are unlikely to be altered by increasing the sample size in this technically challenging study. Second, as our patients with type 2 diabetes

had no diabetic complications and had relatively good glycemic control, it is uncertain whether our findings can be extended to patients with poorly controlled diabetes. Third, our study demonstrates only the acute effects of intravenous exenatide; further studies will be required to evaluate the potential for tachyphylaxis of the suppression of small intestinal motor function after repeated dosing. It should also be examined whether the reduction of glucose absorption due to inhibition of small intestinal motility, flow events and transit with exenatide could be relatively less pronounced in subjects who already have relative suppression of these parameters at baseline, as is the case with gastric emptying (Deane et al., 2010a). In our study, only one subject with diabetes had substantially slow small intestinal transit at baseline. Finally, intergroup comparisons between patients with type 2 diabetes and healthy subjects in our study could potentially have been affected by the age difference between the groups.

In summary, our study shows that the inhibition of small intestinal motility, flow, and transit of chyme induced by exenatide is associated with reductions in the absorption of glucose from the gut lumen, and provides evidence for a novel mechanism for lowering of postprandial glycemia by this agent in type 2 diabetes.

CHAPTER 6: ACUTE EFFECTS OF GLUCAGON-LIKE PEPTIDE-1 (GLP-1) RECEPTOR AGONIST, EXENATIDE, ON BLOOD PRESSURE AND HEART RATE RESPONSES TO INTRADUODENAL GLUCOSE INFUSION IN TYPE 2 DIABETES: A RANDOMISED CONTROLLED TRIAL.

6.1 Abstract

Our aim was to evaluate the effects of the glucagon-like peptide-1 receptor agonist, exenatide, on blood pressure and heart rate (HR) during an intraduodenal glucose infusion in type 2 diabetes. Nine subjects with type 2 diabetes were randomised to receive intravenous exenatide or saline control in a crossover design. Glucose (3kcal/min) was infused via an intraduodenal manometry catheter for 60 min. Blood pressure, HR, and the frequency and amplitude of duodenal pressure waves were measured at regular intervals. Gastrointestinal symptoms were monitored using 100mm visual analog scales. During intraduodenal glucose infusion (0–60min), diastolic (DBP) ($P_{(0–60)}$ = 0.03) and mean arterial (MAP) ($P_{(0–60)}$= 0.03) blood pressures and HR ($P_{(0–60)}$= 0.06; $P_{(0–120)}$= 0.03)) were higher with exenatide compared to placebo. The increase in the AUCs for DBP and MAP were related directly to the suppression of the duodenal motility index with exenatide compared to control (P = 0.007 and 0.04, respectively).So we concluded that in type 2 diabetes, intravenous

exenatide increases MAP and HR during an intraduodenal glucose infusion supporting its potential use in the management of postprandial hypotension.

6.2 Introduction

Postprandial hypotension (PPH) ie. a fall in systolic blood pressure (SBP) >20mmHg after a meal, triggered by pooling of blood in the splanchnic vasculature, occurs frequently and may result in syncope and/or falls, especially in older people and patients with type 2 diabetes and/or autonomic dysfunction (Trahair et al., 2014c). The hypotensive response to oral glucose is dependent on the rate of gastric emptying (Trahair et al., 2012b); accordingly, pharmacological interventions that slow gastric emptying could ameliorate PPH (Trahair et al., 2015). In this regard, glucagon-like peptide-1 (GLP-1) receptor stimulation has gained attention as a potential treatment for PPH; exogenous GLP-1 has been reported to increase blood pressure (BP) in some human studies, albeit not consistently (Edwards et al., 1998, Halbirk et al., 2010, Bharucha et al., 2008), and attenuate the hypotensive effect of both oral (Trahair et al., 2015), and intraduodenal (Trahair et al., 2014b) glucose loads, suggesting that the effect on BP may be via mechanisms other than slowing of gastric emptying.

We recently reported that in patients with type 2 diabetes, intravenous infusion of the GLP-1 receptor agonist, exenatide, suppressed small intestinal motility and flow of chyme and slowed the small intestinal transit of a glucose load, associated with an acute reduction in glucose absorption (Thazhath et al., 2016a). We now report the

effects of intravenous exenatide on SBP, diastolic blood pressure (DBP), mean arterial blood pressure (MAP), and heart rate (HR) in that study.

6.3 Patients and Methods

6.3.1 Subjects

Blood pressure and heart rate were evaluated in 9 patients with type 2 diabetes, managed by diet alone (6 male, 3 female; mean age 60.7±2.4 years; BMI 29.8±1.4kg/m^2; known duration of diabetes 5±1 years and HbA1c 6.2±0.2% (44.7±2.5mmol/mol)) (Thazhath et al., 2016a). In one other patient, these data were unavailable as a result of technical errors in recording BP. The inclusion and exclusion criteria have been reported (Thazhath et al., 2016a), and the protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000. All subjects provided written informed consent prior to their inclusion in the study.

6.3.2 Protocol

Each patient visited the laboratory on two days, at least five days apart, when a combined manometry and impedance assembly, which included a multilumen silicone catheter (Dentsleeve International, Mississauga, Ontario, Canada), was advanced into the stomach through an anesthetized nostril, and allowed to pass into the duodenum by peristalsis (Thazhath et al., 2016a). The position of six manometry

side-holes (perfused with degassed water), and an infusion port within the duodenum was monitored continuously by measuring the antral and duodenal transmucosal potential difference via a proximal duodenal and a distal antral side hole perfused with 0.9% saline, and the numbers and amplitudes of the duodenal pressure waves were recorded digitally for subsequent analysis (Chaikomin et al., 2007, Thazhath et al., 2016a). Patients were randomised in double-blind fashion to receive either intravenous exenatide (7.5mcg administered between -30 and 120min) or volume-matched saline control, while glucose (3kcal.min^{-1}) was given intraduodenally from 0–60min. Venous blood was sampled at regular intervals using a cannula inserted in a cubital fossa vein, for measurement of blood glucose and serum insulin concentrations (Thazhath et al., 2016a). SBP, DBP, MAP and HR were measured every 3 min from -30–120min, using an automatic sphygmomanometer (DINAMAP ProCare 100, Milwaukee, WI, USA).

6.3.3 Calculations and statistical analysis

Duodenal motility indices were calculated as ln[(sum of amplitudes × number of duodenal waves)+1] (Camilleri and Malagelada, 1984, Thazhath et al., 2016a) and MAP was calculated as [SBP+(2×DBP)]/3 (Moran et al., 1995). Areas under the curves (AUC) were measured using the trapezoidal rule. Mean values and AUCs were compared using Student's paired *t*-test. Repeated measures ANOVA (adjusted by Bonferroni's correction) was used to analyse the outcome measures, with treatment and time as factors. The AUCs for nausea scores did not conform to normality and were compared using Wilcoxon signed rank test. Pearson's correlation coefficient was used to assess correlations. All analyses were performed using SPSS

21 (IBM Corporation, Chicago, IL, USA). Data are mean values ± standard error; *P*<0.05 was considered statistically significant.

6.4 Results

6.4.1 Systolic, diastolic and mean arterial blood pressure and heart rate

Prior to (-30–0min), and after, the intraduodenal glucose infusion (60–120min) the SBP, DBP and MAP, as well as the HR, did not differ between exenatide and saline control, but during the intraduodenal glucose infusion (0–60min), SBP, DBP and MAP increased with exenatide (P<0.01 for each), but fell with saline control (P≤0.01 for each) before returning towards baseline. The $AUC_{(0\text{-}60)}$ for DBP (4479±195 vs 4157±177mmHg.min; P=0.03), and MAP (5692±217 vs 5309±186mmHg.min; P=0.03), but not SBP (8118±330 vs 7615±271mmHg.min; P=0.06), were higher with exenatide than control. (Figure 1B, 1C).

During and after intraduodenal glucose infusion (0–120min), HR increased progressively with both exenatide and control (P<0.001 for each), before declining. The $AUC_{(0\text{-}60)}$ for mean HR tended to be higher with exenatide than control (4546±287 vs 4307±315; P=0.06) and the $AUC_{(0\text{-}120)}$ was significantly higher with exenatide than control (9305±562 vs 8634±654 beats; P=0.03) (Figure 1D).

6.4.2 Blood glucose and serum insulin

As reported (Thazhath et al., 2016a), blood glucose and serum insulin concentrations increased in response to intraduodenal glucose infusion (0–60min), before returning to baseline. The $AUC_{(0\text{-}120)}$ for blood glucose was lower (P=0.002), and the $AUC_{(0\text{-}120)}$ for serum insulin higher (P=0.03), with exenatide than control (Figure 1E and 1F).

6.4.3 Nausea

During and after the intraduodenal glucose infusion (0–120min), nausea scores increased with exenatide, but not with control, and the difference between them (AUC) tended to be significant (P<0.06) (Figure 1G).

6.4.4 Duodenal motility index

During and after intraduodenal glucose infusion (0–120min), the duodenal motility index was suppressed markedly more with exenatide than control (P<0.001) (Figure 1H).

6.4.5 Relationships between outcome measures

During intraduodenal glucose infusion (0–60min), the increments in the AUC for DBP and MAP with exenatide compared to control were related significantly to the reduction in duodenal motility index (r=0.82, P=0.01 and r=0.67, P=0.05, respectively), but not to the changes in AUC for blood glucose or serum insulin.

Neither SBP, DBP, MAP nor HR were related to nausea scores during or after the intraduodenal glucose infusion (0–120min).

6.5 Discussion

In patients with type 2 diabetes receiving an intraduodenal glucose infusion at a rate within the physiological range for gastric emptying (3kcal.min^{-1}), we observed that both MAP and HR increased during intravenous administration of exenatide, and that these changes were in proportion to the suppression of duodenal motility by exenatide. The observed effects of exenatide on BP support its potential use in the management of PPH.

In contrast to previous observations where exogenous GLP-1 infusions attenuated, but did not abolish, the hypotensive response to an intraduodenal glucose load (Trahair et al., 2014b, Trahair et al., 2015), we found that the intravenous administration of the GLP-1 receptor agonist, exenatide, was associated with an increase in SBP, DBP, MAP and HR compared to baseline, though the increase in the SBP did not quite achieve statistical significance, possibly as a result of a type 2 error. This pressor response is likely to be due, at least in part, to an increase in cardiac output, as exogenous GLP-1 is known to increase stroke volume and HR (Asmar et al., 2015), where stimulation of atrial GLP-1 receptors (Wu et al., 2015a) may have contributed to the increase in HR, which persisted even after the completion of the intraduodenal glucose infusion. In the fasting state (-30–0min), exenatide was not associated with any chronotropic, or pressor response. Whether the decrease in gut motility induced by exenatide could have triggered some undefined

neurohumoral mechanism in the proximal gut ('gut-heart axis'), which favoured sympathetic stimulation is open to speculation.

Nausea scores tended to be higher with exenatide than placebo during and after the intraduodenal glucose infusion, but did not show any relationship with BP or HR. Therefore, it appears most unlikely that nausea accounts for the cardiovascular effects.

Studies involving chronic, subcutaneous dosing of exenatide have reported a decrease in SBP, although this has not been specifically measured in the postprandial phase, and was apparent mainly in those who had relatively higher BP at baseline (Seufert and Gallwitz, 2014). Although the cardiovascular effects of exenatide in type 2 diabetes are likely to be positive overall, perhaps particularly in those with postprandial hypotension (Seufert and Gallwitz, 2014), our observations with exenatide could suggest potential deleterious effects in a subgroup of patients with high cardiovascular risk, particularly as the observed increase in MAP (~16mmHg) was substantial.

Our study has several limitations. It was not primarily designed to assess cardiovascular outcomes, and the sample size was small; nonetheless, the differences in cardiovascular endpoints between treatments were quite marked. Secondly, as our subjects did not have PPH and had reasonably good glycemic control, it remains to be determined whether our findings apply to patients with PPH and/or less well controlled type 2 diabetes. Thirdly, this study has assessed only the acute effects of intravenous exenatide; in view of our observations, further studies are indicated to

evaluate the effects of subcutaneous exenatide, including the potential for tachyphylaxis of cardiovascular effects after repeated dosing, as is evident for the slowing effects of gastric emptying by intravenous GLP-1(Umapathysivam et al., 2014).

In summary, intravenous infusion of exenatide is associated with increases in the MAP and HR during an intraduodenal glucose infusion, in patients with well-controlled type 2 diabetes.

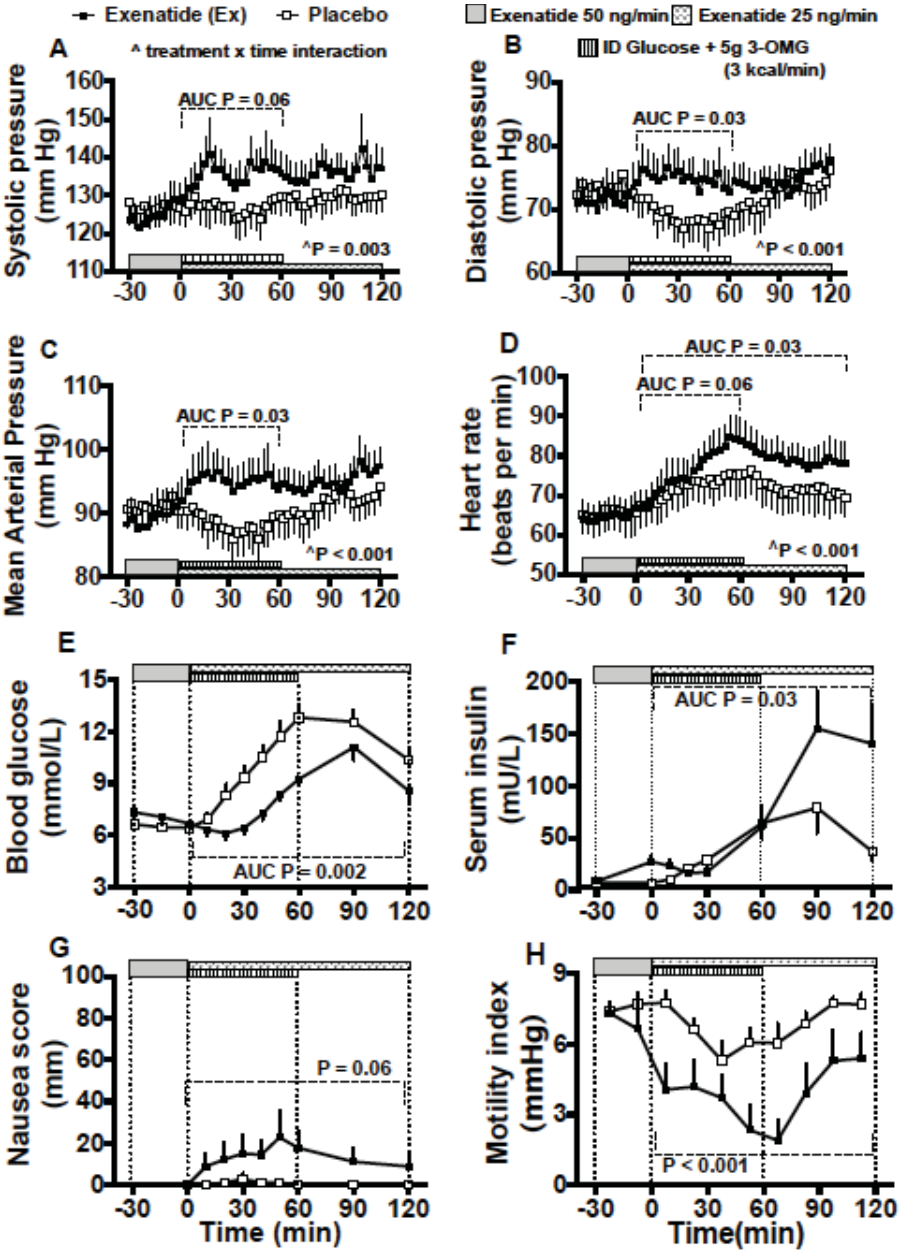


Figure legend

Effects of intravenous exenatide compared to saline control on systolic blood pressure (A), diastolic blood pressure (B), mean arterial pressure (C), heart rate (D) as well as blood glucose (E) and serum insulin concentrations (F), nausea scores (G) and motility index (H) during fasting and in response to intraduodenal glucose infusion, in patients with type 2 diabetes (n = 9). Two-factor repeated measures ANOVA, with treatment and time as factors, adjusted by Bonferroni correction, were used to compare these variables except nausea scores, the AUCs of which were compared using the Wilcoxon signed rank test. Areas under the curves were compared using Student's *t*-test.

CHAPTER 7: EFFECTS OF HYDROXYCITRATE (HCA) ON INTESTINAL GLUCOSE ABSORPTION AND INCRETIN RELEASE IN HEALTHY SUBJECTS AND IN TYPE 2 DIABETES.

(Adapted from Nutrition. 2016 May;32(5):553-9.)(Thazhath et al., 2016c)

7.1 Abstract

Hydroxycitric acid (HCA), derived from the fruit Garcinia cambogia, reduces the rate of glucose absorption and lowers postprandial glycemia in rodents, but its effect in humans is unknown. The aim of this study was to investigate the effects of small intestinal perfusion with HCA on glucose absorption, as well as the incretin and glycemic responses to a subsequent intraduodenal glucose infusion, in both healthy individuals and patients with type 2 diabetes. Twelve healthy participants and 8 patients with type 2 diabetes received an intraduodenal infusion of HCA (2800 mg in water) or control (water) over 60 min, followed by an intraduodenal infusion of 60 g glucose over 120 min, in a double-blind, randomized crossover design. In healthy individuals, 5 g 3-O-methylglucose (3-OMG) was co-infused with glucose as a marker of glucose absorption. Blood was sampled frequently. We found that in healthy individuals, blood glucose was lower with HCA than control, both before and during the intraduodenal glucose infusion ($P < 0.05$ for each). Plasma glucose-dependent insulinotropic polypeptide (GIP; $P = 0.01$) and glucagon ($P = 0.06$) were higher with HCA, but there were no differences in plasma glucagon-like peptide

(GLP)-1, insulin, or serum 3-OMG concentrations. In patients with type 2 diabetes, blood glucose, and plasma GIP, GLP-1, and insulin did not differ between HCA and control either before or after intraduodenal glucose, but during glucose infusion, plasma glucagon was higher with HCA ($P = 0.04$). We concluded that **i**n healthy individuals, small intestinal exposure to HCA resulted in a modest reduction in glycemia and stimulation of plasma GIP and glucagon, but no effect on plasma GLP-1 or insulin, or on glucose absorption. HCA had no effect on glycemia in patients with type 2 diabetes.

7.2 Introduction

In type 2 diabetes, poor glycemic control, indicated by glycated hemoglobin (HbA1c), predicts the development and progression of micro-
(Stratton et al., 2000) and to a lesser extent macro-vascular (Nathan et al., 2005) complications. In type 2 patients who have an HbA1c $\leq$ 7.5% (58 mmol/mol), postprandial hyperglycemia predominates over fasting blood glucose in determining HbA1c (Monnier et al., 2003). Even when HbA1c levels are comparable, patients with larger glycemic fluctuations may be at greater risk (Del Prato, 2002a). Therefore, there is considerable interest in exploring interventions that have the potential to minimise postprandial hyperglycemia.

The dried rind of the fruit of *Garcinia cambogia* has been used traditionally in India and Southeast Asia as a culinary ingredient to render meals more filling. Extracts from this fruit, which contain hydroxycitric acid (HCA) as the active ingredient, have gained popularity as a dietary supplement to reduce appetite and facilitate weight

loss (Downs et al., 2005), though with limited support for efficacy from short-term clinical trials (Onakpoya et al., 2011). Proposed mechanisms of weight loss, based on rodent studies, include a reduction in lipogenesis through inhibition of ATP-citrate-lyase (Sullivan et al., 1972), suppression of appetite by elevating brain serotonin levels (Ohia et al., 2001), reductions in plasma insulin levels (Hayamizu et al., 2003), and inhibition of enteral absorption of glucose (Wielinga et al., 2005). HCA has been shown to delay glucose absorption in rodents, with consequent reductions in peak blood glucose and plasma insulin concentrations in response to intragastric or intraduodenal glucose administration, although the overall area under the curve (AUC) for blood glucose was not affected (Wielinga et al., 2005).

A delay in glucose absorption could potentially alter the secretion of the "incretin" hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which are known to augment insulin secretion in healthy humans when glucose is given by the enteral route compared to an isoglycemic intravenous infusion (ie. the "incretin effect") (Elrick et al., 1964). In type 2 diabetes, the insulinotropic effect of GIP is diminished, while that of GLP-1 remains relatively intact (Nauck et al., 1993). The alpha-glucosidase inhibitor, acarbose, which delays the post-meal glucose release in the gut, has been shown to attenuate GIP release from the proximal small intestinal K-cells, and augment the release of GLP-1 from the L-cells, located more densely in the distal gut (Qualmann et al., 1995).

Here, we report the effects of intraduodenal administration of HCA on blood glucose concentrations, glucose absorption, and incretin hormone release in response to a subsequent intraduodenal glucose infusion, in both health and type 2 diabetes. Our

hypothesis was that pre-treatment with HCA would reduce the glycemic excursion in response to intraduodenal glucose, via inhibition of glucose absorption and augmentation of the release of the distal gut hormone, GLP-1, at the expense of the proximal intestinal hormone, GIP.

7.3 Experimental methods

7.3.1 Subjects

12 healthy subjects (7 male and 5 female; age (mean ± SEM) 33.4 ± 4.6 years; BMI 23 ± 0.8 kg/m^2 and 8 older and overweight patients with type 2 diabetes (4 male and 4 female; age 65.8 ± 2.2 years; BMI 31 ± 1.4 kg/m^2; known duration of diabetes 85 ± 24 months; HbA1c 6.7 ± 0.3% (49.7 ± 3.3 mmol/mol)) were studied after they provided written informed consent. Diabetes was managed by diet alone (4 subjects), or metformin (500 to 1000 mg/day) in addition to diet (4 subjects); metformin was stopped 5 days before each study visit. No patient had evidence of micro- or macro-vascular complications or any other significant co-morbidity, was a smoker, or was taking medication known to affect gastrointestinal function.

The protocol was approved by the Human Research Ethics Committee of the Royal Adelaide Hospital, and was conducted in accordance with the principles of the Declaration of Helsinki as revised in 2000.

7.3.2. Protocol

Each subject was studied at the Royal Adelaide Hospital on two different occasions, separated by at least 5 days, in a randomised, double-blind, crossover design. Randomisation was undertaken with the aid of established software. On the evening preceding each study day (~ 1900), subjects consumed a standardised evening meal consisting of frozen beef lasagne (McCain Foods Pty Ltd; 2472 kJ) with bread, a non-alcoholic beverage and one piece of fruit. Following the meal, subjects fasted until the following morning; water was allowed until 2200.

On each study day, an intraduodenal catheter (Dentsleeve International Ltd.) was inserted at about 0830 through an anesthetised nostril and allowed to pass into the duodenum by peristalsis. The catheter incorporated an infusion channel opening about 12 cm distal to the pylorus and its position was monitored by continuous measurement of transmucosal potential difference, monitored from sideholes in the antrum (~ -40 mV) and duodenum (~ 0 mV), as previously described (Heddle et al., 1988).

At -60 min, 4667 mg of HCA (SuperCitriMax® InterHealth Nutraceuticals Inc.), equivalent to 2800 mg of active HCA, dissolved in 420 mL water, or control (water only) was administered intraduodenally over 60 minutes (-60 to 0 min). This dose of HCA has been employed in several studies to induce weight loss, without any significant adverse effects (Chuah et al., 2012). This was immediately followed by an intraduodenal glucose infusion (60 g glucose dissolved in water to a volume of 240 mL) from 0 to 120 min; ie. 2 kcal/min. This rate of intraduodenal glucose infusion is within the range of the physiological rate of gastric emptying (Thazhath et al.,

2014b), and represents a submaximal stimulus for incretin hormone secretion (Marathe et al., 2014). In healthy subjects, 5 g 3-O-methylglucose (3-OMG (a non-metabolised glucose analog)), was also added to the glucose solution in order to evaluate glucose absorption (Fordtran et al., 1962b).

Venous blood sampled at frequent intervals via a cannula inserted into a forearm vein was stored on ice until centrifugation at 3200 rpm for 15 min at 4°C within 15 min of collection. Serum and plasma were separated and stored at -70 °C for subsequent analyses. Gastrointestinal sensations including nausea, hunger and fullness were assessed using 100 mm visual analog questionnaires at the same intervals used for blood sampling (Parker et al., 2004). Observations were continued until 240 min; data for healthy subjects are reported only up to 150 min, since several subjects developed asymptomatic hypoglycemia (blood glucose < 3 mmol/L) thereafter; in these instances the study was terminated, and a meal provided to avoid potential complications. At the end of the study, all subjects were given a meal and monitored to ensure stable blood glucose concentrations prior to leaving the laboratory.

7.3.3 Measurements

Blood glucose was measured by the glucose oxidase method using a glucometer (MediSense Optium; MediSense Inc.); serum 3-OMG by liquid chromatography and mass spectrometry with a sensitivity of 10 pmol/L (Deane et al., 2011a); plasma insulin by ELISA (10-1113, Mercodia) with a sensitivity of 1.0 mU/L and with intra- and inter-assay coefficient of variations (CVs) of 2.8% and 8.1% respectively; plasma glucagon by radioimmunoassay (GL-32K, Millipore) with a sensitivity of 20

pg/ml, and intra- and inter-assay CVs of 15 % and 10.5 % respectively; plasma total GIP by radioimmunoassay modified from a previously published method (Wishart et al., 1992b) with a sensitivity of 2 pmol/L and intra- and inter-assay CVs of 5.1% and 8.8% respectively; and plasma total GLP-1 by radioimmunoassay (GLPIT-36HK, Millipore) with a sensitivity of 3 pmol/L and with intra- and inter-assay CVs of 7.9% and 6.9% respectively.

7.3.4 Calculations and statistical analysis

Based on a previous study (Wu et al., 2014a), we estimated that a sample size of 12 healthy subjects would provide 80% power (at $\alpha = 0.05$) to detect a 10% difference in AUC for blood glucose concentrations and the sample size of 8 patients with type 2 diabetes would provide 80% power (at $\alpha = 0.05$) to detect a 15% difference in AUC for blood glucose concentrations. The homeostatic model assessment 2 (HOMA2) model was used to calculate beta cell function, insulin sensitivity and insulin resistance, prior to the glucose infusion (Caumo et al., 2006). During the intraduodenal glucose infusion, insulin sensitivity was assessed using the Matsuda index (Matsuda and DeFronzo, 1999). The AUCs for blood glucose, serum 3-OMG and plasma GLP-1, GIP and insulin concentrations were calculated using the trapezoidal rule and compared using Student's paired *t*-tests. These parameters were also analyzed using two-factor repeated measures ANOVA with treatment and time as factors, as were the gastrointestinal sensations. Post hoc comparisons, adjusted by Bonferroni's correction, were performed if ANOVAs revealed significant effects. All analyses were performed using SPSS 21 (IBM Corporation). Data are expressed as mean ± SEM; $P < 0.05$ was considered statistically significant.

7.4 Results

None of the subjects who completed the study reported any adverse effects from the use of HCA (Figure 1).

7.4.1 Blood glucose

In healthy subjects, fasting blood glucose concentrations (-60 min) did not differ between the two study days. After HCA infusion (0 min), blood glucose concentrations were slightly lower than after control infusion (P= 0.002). In response to intraduodenal glucose infusion, blood glucose concentrations increased on both study days (P< 0.001 for each), but were lower after HCA than control (P= 0.02), with a significant treatment x time interaction, such that concentrations were lower at 120 and 150 min after HCA (P= 0.02 for each). However, the peak blood glucose concentration did not differ significantly between the two days (P= 0.12) (Table 1 and Figure 2).

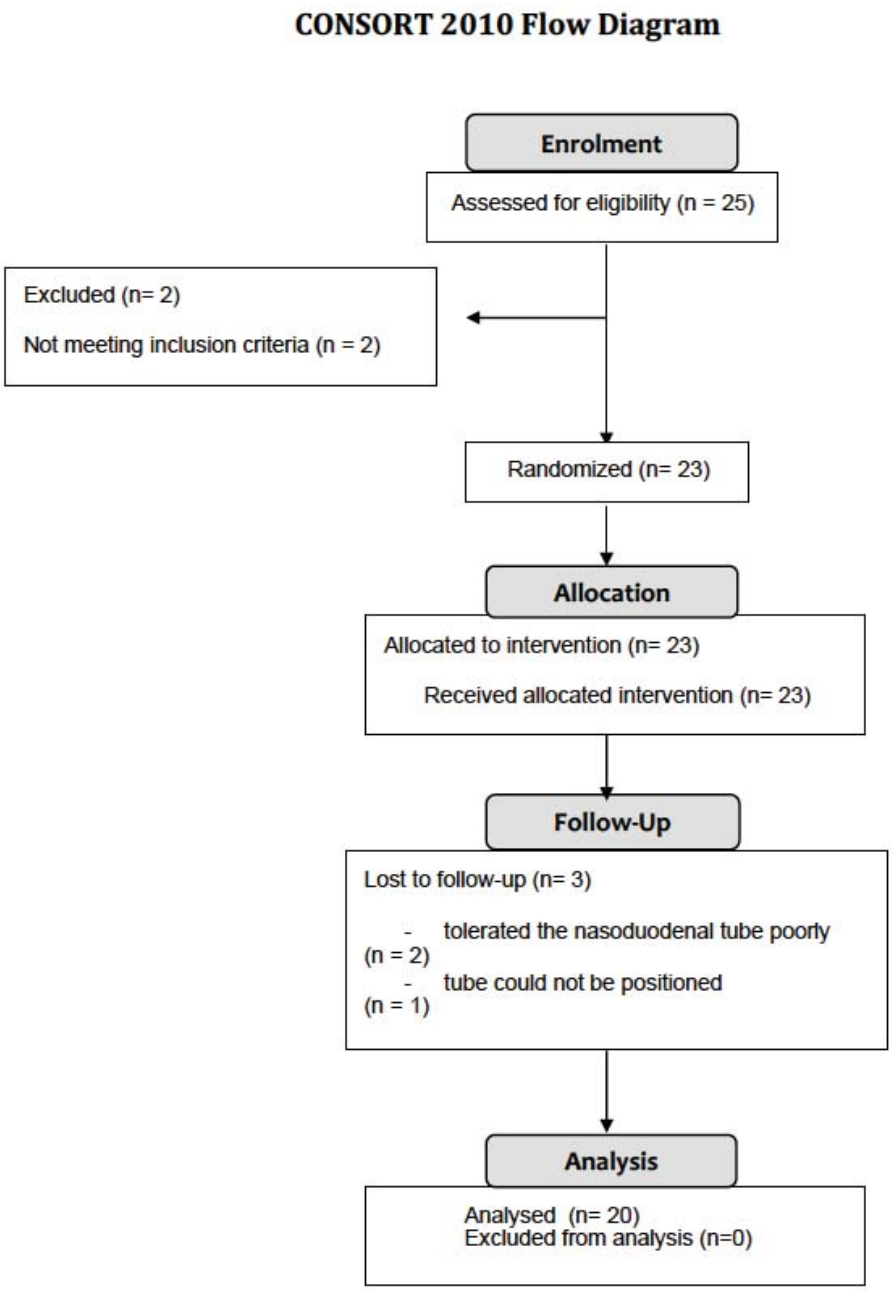


Figure 1:

CONSORT diagram outlining the number of subjects involved in enrolment, intervention allocation, follow-up, and data analysis.

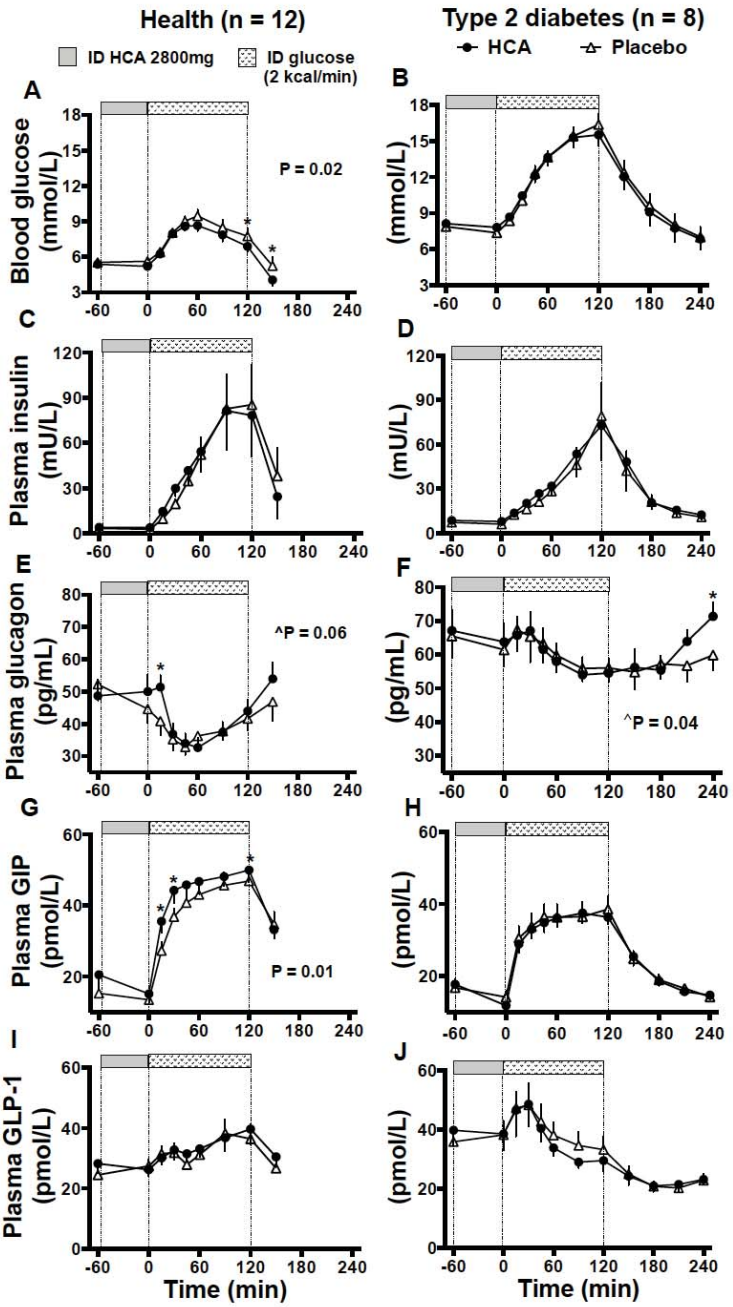


Figure 2:

Effect of intraduodenal infusion of hydroxycitric acid (HCA) [2800 mg dissolved in 420 ml water] compared to control [water alone], in health (n=12) and type 2 diabetes (n = 8), on blood glucose (A, B), plasma insulin (C, D), glucagon (E, F), GIP (G, H) and GLP-1 concentrations (I, J) during intraduodenal HCA infusion alone and in response to a subsequent intraduodenal glucose infusion. Two-factor repeated measures ANOVA, with treatment and time as factors, adjusted by Bonferroni correction, were used to compare these variables. Data are mean ± standard error of mean. *P < 0.05

In type 2 diabetes, fasting blood glucose concentrations (-60 min) did not differ between the two study days and remained unchanged after the intraduodenal HCA infusion (P= 0.23), but decreased slightly after the control infusion (P= 0.01), resulting in lower blood glucose concentrations at 0 min for control compared to HCA (P= 0.03). In response to intraduodenal glucose infusion, there was an increase in blood glucose concentrations on both days, followed by a decline thereafter, without any difference in AUC between HCA and control (P= 0.53).

7.4.2 Serum 3-OMG

In health, serum 3-OMG concentrations increased progressively during the intraduodenal infusion (0–120 min), followed by a decline thereafter, without any difference between HCA and control (P= 0.36). 3-OMG was not infused in type 2 diabetes (Table 1).

7.4.3 Plasma insulin

In health, fasting plasma insulin (-60 min) was similar between the two study days and was not affected by intraduodenal HCA or control infusion (0 min). In response to intraduodenal glucose infusion, plasma insulin increased on both days before declining thereafter, without any difference between HCA and control (P= 0.95). The insulin to glucose ratio was also not different between the treatments (P= 0.55) (Table 1 and Figure 2).

In type 2 diabetes, plasma insulin concentrations did not differ between the two treatments, either before or after the intraduodenal glucose infusion.

7.4.4 Beta cell function, insulin sensitivity and insulin resistance

In both health and type 2 diabetes, baseline (-60 min) β cell function, insulin sensitivity and insulin resistance, were similar between the study days. In health, β cell function at 0 min was greater after HCA compared to control ($P = 0.03$), without any effect of HCA on insulin sensitivity or insulin resistance. In type 2 patients, insulin sensitivity at 0 min was slightly less, and insulin resistance greater, after HCA compared to control ($P < 0.05$ for each), without any difference in β cell function.

In response to intraduodenal glucose infusion, there was no difference in insulin sensitivity between the study days, in either group (Matsuda index: 9.3 ± 1.6 vs 8.8 ± 1.6, $P = 0.58$ in health; 0.01 ± 0.00 for both in type 2 diabetes) (Table 2).

Table 1

	Health (n = 12; T = -60 to 150 min)			Type 2 diabetes (n = 8; T = -60 to 240 min)		
	HCA	**Control**	**P**	**HCA**	**Control**	**P**
Basal glucose (mmol. L^{-1})	5.4 ± 0.1	5.5 ± 0.1	0.34	8.1 ± 0.4	7.9 ± 0.3	0.28
Glucose at 0 min (mmol. L^{-1})	5.2 ± 0.2	5.6 ± 0.2	0.002	7.8 ± 0.3	7.4 ± 0.3	0.03
Peak glucose (mmol.L^{-1}.min)	9.0 ± 0.4	9.7 ± 0.6	0.12	16.0 ± 0.9	16.4 ± 0.9	0.31
Glucose AUC (mmol.L^{-1}.min)	1359 ± 68	1508 ± 84	0.02	3205 ± 199	3236 ± 184	0.62
Peak 3-OMG (mmol. L^{-1})	0.60 ± 0.0	0.60 ± 0.0	0.91	–	–	–
3-OMG AUC (mmol.L^{-1}.min)	49.3 ± 4.6	45.5 ± 3.4	0.99	–	–	–
Basal insulin (mU. L^{-1})	3.9 ± 0.8	3.4 ± 0.4	0.50	8.5 ± 1.7	7.2 ± 1.3	0.15
Insulin at 0 min (mU. L^{-1})	3.8± 0.6	2.7± 0.4	0.08	7.9 ± 1.6	6.0 ± 1.1	0.03
Peak insulin (mU.L^{-1})	90.9 ± 27.8	99.0 ± 26.7	0.72	73.65 ± 25.0	79.3 ± 24.2	0.57
Insulin AUC (mU.L^{-1}.min)	7924 ± 2306	7947 ± 2132	0.99	8682 ± 1952	8066 ± 1783	0.31
Basal glucagon (pg.mL^{-1})	48.7 ± 10.9	52.3 ± 11.1	0.43	67.15 ± 6.5	65.5 ± 6.9	0.71
Glucagon at 0 min (pg.mL^{-1})	50.0 ± 7.1	44.6 ± 5.9	0.25	63.8 ± 5.7	61.5 ± 5.3	0.48
Peak glucagon (pg.mL^{-1})	30.4 ± 3.0	29.7 ± 2.6	0.73	79.3 ± 3.7	73.2 ± 6.1	0.26
Glucagon AUC (pg.mL^{-1}.min)	9151 ± 778	8772 ± 720	0.40	18222 ± 1408	17886 ± 1484	0.62
Basal GIP (pmol.L^{-1})	24.5 ± 7.6	28.2 ± 8.4	0.22	17.8 ± 1.2	16.9 ± 2.4	0.70
GIP at 0 min (pmol.L^{-1})	15.2 ± 3.0	13.5 ± 2.4	0.40	11.9 ± 0.9	14.3 ± 1.8	0.11
Peak GIP (pmol.L^{-1})	51.5 ± 3.8	49.0 ± 4.2	0.23	39.1 ± 2.7	39.5 ± 3.9	0.86
GIP AUC (pmol.L^{-1}.min)	7561± 671	6806 ± 557	0.01	7483 ± 439	7657 ± 740	0.66
Basal GLP-1 (pmol.L^{-1})	24.5 ± 7.6	28.2 ± 8.4	0.22	39.7 ± 5.5	35.8 ± 3.1	0.26
GLP-1 at 0 min (pmol.L^{-1})	27.4 ± 3.4	26.2 ± 3.4	0.51	38.5 ± 5.8	38.2 ± 5.0	0.93
Peak GLP-1 (pmol.L^{-1})	45.6 ± 4.6	46.0 ± 4.9	0.92	56.3 ± 9.7	58.9 ± 6.9	0.69
GLP-1 AUC (pmol.L^{-1}.min)	6473 ± 458	6741 ± 524	0.50	9522 ± 970	9790 ± 782	0.52

Table 1: Values at baseline and after 60 min of an intraduodenal infusion of hydroxycitric acid (HCA) (2800 mg dissolved in 420 ml water) or control (water alone) and the peak values and area under the curves (AUC) in response to intraduodenal glucose infusion (2 kcal/min) for blood glucose, serum 3-O-methylglucose (3-OMG) and plasma insulin, glucagon, GIP and GLP-1 concentrations in health (T = -60 – 150 min; n = 12) and type 2 diabetes (T = -60 – 240 min; n = 8). Data are mean ± standard error of mean. Student's paired *t*-test was used to compare these variables.

Table 2

Beta cell function (%B), insulin sensitivity (%S) and insulin resistance (IR) before (-60 min) and after 60 min intraduodenal (intraduodenal) infusion of hydroxycitric acid (HCA) (2800 mg in 420 ml water) or control (water) alone (0 min), based on the HOMA2 model in health (n = 12) and in type 2 diabetes (n = 8). Data are mean ± standard error of mean. Student's paired *t*-test was used to compare these variables.

	Health (n = 12)						**Type 2 diabetes (n = 8)**					
	T = -60 min			**T = 0 min**			**T = -60 min**			**T = 0 min**		
	HCA	**Control**	**P**	**HCA**	**Control**	**P**	**HCA**	**Control**	**P**	**HCA**	**Control**	**P**
Beta cell function (%B)	50.5 ± 6.4	44.9 ± 4.3	0.35	57.7 ± 9.1	35.9 ± 3.5	0.03	10.4 ± 1.6	9.6 ± 0.9	0.51	10.6 ± 1.5	10.0 ± 1.2	0.26
Insulin sensitivity (%S)	2701± 7	260 ± 38	0.95	256 ± 8	365 ± 66	0.13	700 ±129	791 ± 119	0.43	780 ± 149	990 ± 169	0.01
Insulin resistance (IR)	0.5 ± 0.1	0.4 ± 0.1	0.66	0.5 ± 0.1	0.4 ± 0.0	0.10	0.2 ± 0.0	0.2 ± 0.0	0.14	0.2 ± 0.0	0.1 ± 0.0	0.03

7.4.5 Plasma glucagon

In health, fasting (-60 min) plasma glucagon concentrations did not differ between the two study days, and remained unchanged during intraduodenal HCA or control infusion (-60 – 0 min). In response to intraduodenal glucose, concentrations decreased rapidly, before slowly returning to baseline with control, whereas there was a delay in the suppression of glucagon with HCA, such that glucagon concentrations tended to be greater after HCA than control (treatment × time interaction, $P = 0.06$).

In type 2 diabetes, fasting glucagon was similar between the two study days, and was unaltered during intraduodenal HCA or control infusion (-60 – 0 min). In response to intraduodenal glucose, there was an initial modest increase in glucagon concentrations, followed by a progressive decline until the end of the glucose infusion (120 min). Thereafter, concentrations increased after HCA, but not control, such that the glucagon response was greater after HCA (treatment × time interaction, $P = 0.04$, with a significant difference at 240 min, $P < 0.05$) (Table 1 and Figure 2).

7.4.6 Plasma GIP

In both health and type 2 diabetes, fasting (-60 min) plasma GIP concentrations did not differ between the study days. Prior to intraduodenal glucose infusion (-60 – 0 min), there was a slight, but significant, reduction in GIP concentrations after HCA in both groups ($P = 0.01$ and < 0.001 respectively), and a tendency for reduction after control ($P = 0.06$ and 0.11 respectively), although concentrations at 0 min did not

differ between HCA and control. In response to intraduodenal glucose, GIP increased promptly on both days in health, and concentrations were higher after HCA than control (P= 0.007), with significant differences at 15 and 30 min (P = 0.002 and 0.007 respectively), whereas in type 2 diabetes, GIP responses to intraduodenal glucose did not differ between HCA and control (Table 1 and Figure 2).

7.4.7 Plasma total GLP-1

In both health and type 2 diabetes, fasting (-60 min) total GLP-1 concentrations did not differ between the two study days, and remained unchanged after intraduodenal HCA or control infusion (-60 – 0 min). In response to intraduodenal glucose infusion, GLP-1 increased modestly on both days before declining, without any difference between the two days in either group (Table 1 and Figure 2).

7.4.8 Gastrointestinal sensations

Sensations of hunger, fullness and nausea did not differ between HCA and control, in health or in type 2 diabetes (data not shown).

7.5 Discussion

Our study showed that, in healthy humans, acute small intestinal exposure to HCA (2800 mg) resulted in a modest reduction of blood glucose during fasting and following an intraduodenal glucose load. The latter was not accounted for by changes in glucose absorption or stimulation of GLP-1 or insulin, but was associated with

enhanced GIP and glucagon responses to intraduodenal glucose. However, in patients with type 2 diabetes, small intestinal infusion of HCA neither lowered blood glucose nor affected plasma insulin, GIP or GLP-1 during fasting or in response to intraduodenal glucose infusion, but was associated with a slight, late increase in glucagon.

The intraduodenal route of administration of HCA and glucose in this study avoided the potentially confounding effects of differences in the rate of gastric emptying between individuals, or in response to treatment with HCA (Wielinga et al., 2005). The cause of the observed modest reduction in blood glucose in healthy subjects after HCA is uncertain. A direct stimulatory effect of HCA on pancreatic beta cell function (assessed by HOMA2) could be implicated for the former effect, although there was no increase in serum insulin. In contrast to our hypothesis, the glucose lowering by HCA in response to intraduodenal glucose was neither associated with suppression of glucose absorption nor stimulation of GLP-1. This contrasts with rodent studies, where intragastric administration of HCA markedly suppressed glucose absorption (Wielinga et al., 2005); the discrepancy might possibly relate to species-specific differences in the regulation of glucose transporters (Margolskee et al., 2007, Ma et al., 2010). As proximal glucose absorption was not affected by HCA in our study, it is not surprising that the GLP-1 responses also did not differ between HCA and control, in both groups.

In healthy subjects, HCA increased plasma GIP concentrations compared to control, in response to intraduodenal glucose, an effect more evident in the earlier period of the glucose infusion, suggesting enhanced stimulation of K-cells in the more

proximal small intestine (Loe et al., 2001). Furthermore, hyperglycemia, even within the physiological range, is known to enhance glucose-induced GIP secretion in health (Kuo et al., 2010b), and the lower blood glucose concentrations observed with HCA, especially in the later stages of glucose infusion, may to some extent have counterbalanced the stimulatory effect on GIP release. On the other hand, in type 2 diabetes, HCA failed to exert any effect on GIP secretion, possibly due to a relatively reduced sensitivity of the K-cells to HCA stimulation.

Despite the capacity for GIP to induce glucose-dependent stimulation of insulin secretion (Vilsboll et al., 2003) and enhance insulin sensitivity in healthy lean individuals (Ceperuelo-Mallafre et al., 2014), we did not observe any insulinotropic effect or difference in insulin sensitivity (assessed by the Matsuda index) in response to intraduodenal glucose infusion between the two study days. Furthermore, the increase in plasma GIP associated with HCA in the healthy group mirrored the changes in plasma glucagon; the latter hormone would be expected to attenuate any glucose-lowering effect of the former. Taken together, the modest stimulation of GIP is unlikely to have contributed substantially to the reduction in blood glucose after HCA.

There is recent evidence that HCA acts directly to increase postprandial glycogenesis in skeletal muscle of healthy lean humans, but not in the obese (Cheng et al., 2012), which may account for the glucose-lowering effect observed before and after the intraduodenal glucose infusion in our healthy subjects, and might also explain the loss of glucose-lowering by HCA in the patients with type 2 diabetes, who were either overweight or obese. However, further studies would be needed in overweight

or obese non-diabetic subjects to differentiate the effects of obesity from those of diabetes.

Given that HCA is marketed as a weight loss agent, our finding that it augmented glucose-induced GIP secretion seems surprising, given that GIP is regarded as a "fat-promoting" hormone. However, the evidence for the latter is based mainly on rodent and in vitro studies which do not clearly dissect out the individual contributions of GIP and insulin secretion to adipogenesis (Ugleholdt, 2011). Although animal data indicate that HCA could suppress appetite (Ohia et al., 2001) , we did not observe any effect of HCA on appetite scores during the current study.

Our study has several limitations. First, the sample size of both groups was relatively small; however, increasing the sample size would be unlikely to alter our conclusions. Second, only a single dose of HCA (2800 mg) was evaluated, although this dose has been shown to be efficacious in at least a few previous studies concerning weight loss (Chuah et al., 2012). Third, we focussed only on the acute effects of HCA, administered in advance of an enteral glucose load; the effect of a continued HCA infusion during the intraduodenal glucose infusion as well as the long-term effects of regular use of HCA before or along with a meal remain to be studied. Fourth, we did not quantify endogenous glucose production, which would require a double- or triple-tracer technique. Such measurements would have provided further insights into the mechanism of glycemic reduction in health; however, the lack of a clinically relevant glycemic reduction makes such additional studies difficult to justify.

In summary, intraduodenal administration of HCA in advance of a small intestinal glucose load resulted in a modest reduction in glycemia in healthy subjects, but not in patients with type 2 diabetes. The mechanism of glycemic reduction in the former group was apparently unrelated to inhibition of small intestinal glucose absorption or stimulation of insulin secretion, despite an increase in GIP concentrations. Our findings do not support the use of HCA for the management of postprandial hyperglycemia in patients with type 2 diabetes.

CHAPTER 8:

Thazhath, S.S., Wu, T., Bound, M.J., Checklin, H.L., Standfield, S., Jones, K.L., Horowitz, M. and Rayner, C.K. (2016). Effects of 5 weeks resveratrol supplementation on GLP-1 secretion, gastric emptying, and postprandial glycemia in patients with Type 2 diabetes. *American Journal of Clinical Nutrition,* 103(1), 66-70.

Published article is titled:
'Administration of resveratrol for 5 weeks has no effect on glucagon-like peptide 1 secretion, gastric emptying, or glycemic control in type 2 diabetes: a randomized controlled trial'.

CHAPTER 9: COMPARATIVE EFFECT OF INTRADUODENAL AND INTRAJEJUNAL GLUCOSE INFUSION ON THE GUT-INCRETIN AXIS RESPONSE IN HEALTHY MALES

(Adapted from Nutr Diabetes. 2015 May 18; 5:e156.)

(Wu et al., 2015b)

9.1 Abstract

The region of enteral nutrient exposure may be an important determinant of postprandial incretin hormone secretion and blood glucose homoeostasis. We compared responses of plasma glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), insulin and glucagon, and blood glucose to a standardised glucose infusion into the proximal jejunum and duodenum in healthy humans. Ten healthy males were evaluated during a standardised glucose infusion (2 kcal min(-1) over 120 min) into the proximal jejunum (50 cm post pylorus) and were compared with another 10 healthy males matched for ethnicity, age and body mass index who received an identical glucose infusion into the duodenum (12 cm post pylorus). Blood was sampled frequently for measurements of blood glucose and plasma hormones. Plasma GLP-1, GIP and insulin responses, as well as the insulin:glucose ratio and the insulinogenic index 1 (IGI1) were greater ($P<0.05$ for each) after intrajejunal (i.j.) than intraduodenal glucose infusion, without a significant difference in blood glucose or plasma glucagon. Pooled analyses revealed direct relationships between IGI1 and the responses of GLP-1 and GIP (r=0.48 and

0.56, respectively, P<0.05 each), and between glucagon and GLP-1 (r=0.70, P<0.001). In conclusion, i.j. glucose elicits greater incretin hormone and insulin secretion than intraduodenal glucose in healthy humans, suggesting regional specificity of the gut-incretin axis.

9.2 Introduction

Roux-en-Y gastric bypass leads to remarkable improvements in glycemic control in type 2 diabetes, associated with an enhanced incretin effect (the phenomenon of an amplified insulin response to enteral versus intravenous glucose, mediated by glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP)) (Laferrere et al., 2008, Laferrere et al., 2007). This may relate to diversion of nutrients more distally in the small intestine, as similar benefits are observed with the endoluminal sleeve device (de Jonge et al., 2013). We hypothesised that bypassing the duodenum would elicit a greater response of the gut-incretin axis to small intestinal glucose infusion, and compared plasma GLP-1, GIP, insulin and glucagon, and blood glucose responses to a standardised glucose infusion into the proximal jejunum and duodenum in healthy humans.

9.3 Subjects and methods

Ten healthy males received an intrajejunal (IJ) glucose infusion; data regarding blood glucose, and plasma insulin, glucagon and GLP-1 have been reported previously (Wu et al., 2013). These, together with plasma GIP, were compared with 10 healthy males who received an intraduodenal (ID) glucose infusion (**Table 1**). All subjects

provided written, informed consent. Protocols were approved by the Royal Adelaide Hospital Human Research Ethics Committee.

On the evening before each study (~1900), each subject consumed a standardised beef lasagne meal (McCain, Victoria, Australia), and then fasted from solids and refrained from liquids after 2200. Subjects attended the laboratory at ~0800 hours the following day, when a multilumen silicone catheter (Dentsleeve International, Ontario, Canada) was positioned transnasally in either the duodenum or proximal jejunum (infusion port: 12 cm vs. 50 cm beyond the pylorus) by peristalsis, with monitoring of antral and duodenal transmucosal potential difference (Wu et al., 2013). In the IJ study, a balloon was inflated 30cm beyond the pylorus to exclude the duodenum (Wu et al., 2013). Enteral glucose was then infused at 2 kcal/min for 120 min (t = 0-120 min). An intravenous cannula was inserted into a forearm vein for blood sampling. Blood samples were collected at frequent intervals into ice-chilled EDTA tubes and immediately centrifuged at 3200 rpm for 15 min at 4 ºC. Plasma was separated and stored at -70 ºC until analysed.

Blood glucose was measured by glucometer (Medisense Precision QID; Bedford, Massachusetts). Plasma total GLP-1 was measured by radioimmunoassay (GLP1T-36HK; Linco Research, St Charles, Missouri) with a sensitivity of 3 pmol/L, and intra- and inter-assay coefficients of variation (CV) of 6.8% and 8.5%, respectively. Plasma total GIP was measured by radioimmunoassay modified from a previously published method (Wishart et al., 1992a), with a sensitivity of 2 pmol/L, and intra- and inter-assay CVs of 5.1% and 8.8%, respectively. Plasma insulin was measured by ELISA (10-1113; Mercodia, Uppsala, Sweden) with a sensitivity of 1 mU/L and

intra- and inter-assay CVs of 2.7% and 7.8%, respectively. Plasma glucagon was measured by radioimmunoassay (GL-32K; Millipore, Billerica, MA) with a sensitivity of 20 pg/mL, and intra- and inter-assay CVs of 15% and 10.5%, respectively.

Student's unpaired t-test was used to compare subject demographics, fasting biochemical measures, and insulinogenic index 1 (IGI_1), which was calculated from insulin (I) and glucose (G) concentrations, as $(I_{30}-I_0)/(G_{30}-G_0)$, to evaluate β-cell responsiveness (Herzberg-Schafer et al., 2010). Two-way ANOVA, with treatment and time as factors, was used to compare responses between the two studies. Pearson's correlation was used to assess relationships between integrated area under the curve (ΔAUC), calculated using the trapezoidal rule, for incretin hormones and both IGI_1 and ΔAUC for glucagon. Analyses were performed using Prism 6.0 software (GraphPad, CA). Data are means ± standard error; $P < 0.05$ (two-sided) was considered statistically significant.

9.4 Results

Demographics and fasting values did not differ between the two studies (Table 1). During enteral glucose infusion, plasma GLP-1 increased substantially with IJ administration (time effect: $P < 0.001$), but minimally with intraduodenal delivery (time effect: $P = 0.003$), and was greater for intrajejunal than intraduodenal glucose (treatment effect: $P = 0.037$). Plasma GIP increased promptly on both days (time effect: $P < 0.001$), and concentrations were also greater with intrajejunal glucose (treatment effect: $P = 0.017$). Blood glucose concentrations increased to ~8 mmol/L

on both days, and were numerically, but not significantly, lower with intrajejunal glucose. However, plasma insulin, the insulin:glucose ratio and IGI_1 (12.0 ± 1.4 vs. 5.6 ± 1.3 mU/mmol), were all greater with intrajejunal glucose (treatment effect: $P < 0.05$ for all). Plasma glucagon did not change with intrajejunal glucose, but fell slightly with intraduodenal glucose (time effect: $P < 0.001$), without significant difference between the two (Figure 1).

On pooling data from all 20 subjects, IGI_1 was related directly to ΔAUC for total GLP-1 and GIP ($r = 0.48$, $P = 0.036$ and $r = 0.56$, $P = 0.012$, respectively). ΔAUC for glucagon was related directly to ΔAUC for GLP-1 ($r = 0.70$, $P < 0.001$), but not GIP.

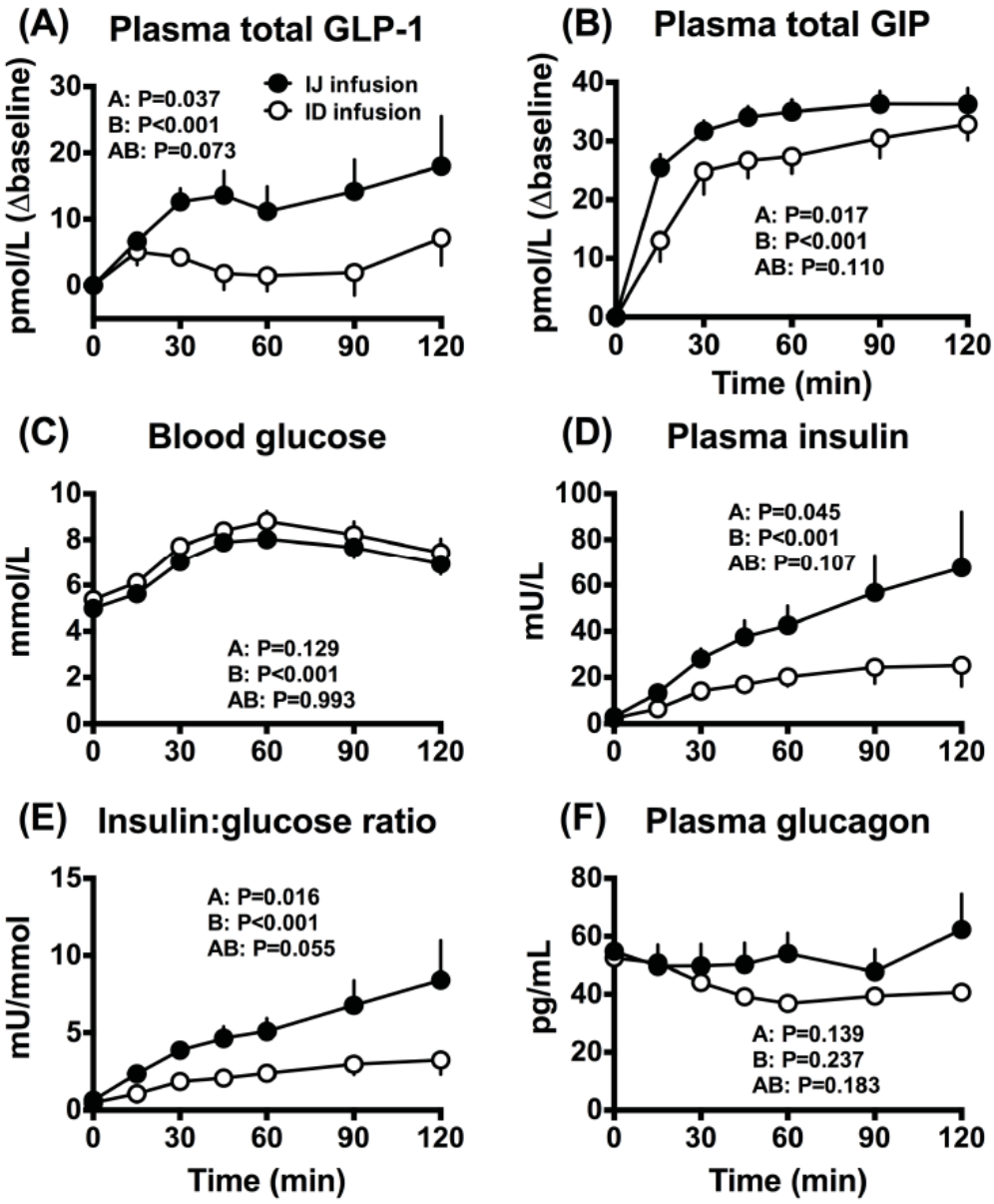
Table 1

Demographics and fasting biochemical measures of subjects in the intrajejunal (IJ) vs. intraduodenal (ID) study. [a]

	IJ study	**ID study**
Subjects	10 healthy males (8 Caucasians and 2 Asians)	10 healthy males (8 Caucasians and 2 Asians)
Age (years)	33.4 ± 6.0	33.4 ± 5.3
BMI (kg/m^2)	24.5 ± 1.1	25.0 ± 1.0
Fasting glucose (mmol/L)	5.0 ± 0.1	5.4 ± 0.1
Fasting insulin (mU/L)	3.1 ± 0.4	2.5 ± 0.5
Fasting insulin:glucose ratio (mU/mmol)	0.6 ± 0.1	0.5 ± 0.1
Fasting GLP-1 (pmol/L)	19.2 ± 2.0	24.1 ± 2.4
Fasting GIP (pmol/L)	14.0 ± 1.6	15.3 ± 2.7
Fasting glucagon (pg/ml)	54.9 ± 7.9	52.5 ± 2.6

Data are means ± standard error; Student unpaired t-test was used to determine the statistical significance. $P < 0.05$ was considered statistically significant.

Figure 1



Comparative responses of plasma total GLP-1 (A), total GIP (B), blood glucose (C), plasma insulin (D), the insulin:glucose ratio (E) and plasma glucagon (E) during an intrajejunal (IJ) and intraduodenal (ID) glucose infusion at the rate of 2 kcal/min (t = 0-120 min) in healthy males (n = 10 each). P values are for (A): differences by experiment, (B) differences over time and (AB): differences due to the interaction of experiment and time. Data are means ± standard error; $P < 0.05$ was considered statistically significant.

9.5 Conclusion

We showed that intrajejunal glucose elicited greater incretin and insulin release than intraduodenal glucose in healthy males, supporting the concept that directing nutrients more distally in the small intestine could ameliorate type 2 diabetes. Blood glucose did not differ significantly, probably because of the modest glycemic excursion in these healthy individuals. However, we cannot rule out a type 2 error due to the small size of each group.

Enteral glucose was delivered at 2 kcal/min on both days, which is within the physiological range of gastric emptying (Brener et al., 1983). The infusion site was 38 cm more distal in the intrajejunal study; given that the small intestine can absorb glucose at 2 kcal/min per 30 cm in health (Duchman et al., 1997, Modigliani and Bernier, 1971), this would have allowed substantially greater interaction of glucose with more distal gut regions where GLP-1-releasing L-cells are more abundant. This is consistent with observations of enhanced GLP-1 secretion after implantation of a duodenal-jejunal sleeve (de Jonge et al., 2013). Alternatively, exclusion of the duodenum may have a role in ameliorating diabetes (the "foregut hypothesis" (Rubino and Marescaux, 2004)). The relative contribution of more distal gut exposure versus duodenal exclusion should be evaluated in subsequent studies. The greater GIP response to intrajejunal glucose may also reflect a higher density of GIP secreting K-cells in the proximal jejunum than duodenum in humans, as seen in pigs (Mortensen et al., 2003). Moreover, the expression of sodium glucose co-transporter-1 may be of relevance (Wu et al., 2012) – this was reported to be greater in the jejunum than duodenum in rodents (Woyengo et al., 2011).

Plasma glucagon decreased during intraduodenal glucose infusion, but remained unchanged during intrajejunal glucose infusion. This may be partly accounted for by the interplay between GLP-1 and GIP on pancreatic α-cells; the glucagonostatic effect of GLP-1 is blunted (Nauck et al., 2002), and the glucagonotropic effect of GIP is potentiated (Meier et al., 2003), in the context of relatively low blood glucose concentrations. Surprisingly, a direct relationship between glucagon and GLP-1 was observed, which might imply a contribution of GLP-2, a hormone co-secreted with GLP-1 and potent at stimulating glucagon (Meier et al., 2006). Differences in glucagon between the intraduodenal and intrajejunal studies may have contributed to the lack of difference in blood glucose concentrations.

We inflated a balloon in the intrajejunal study to exclude the duodenum; this *per se* would be unlikely to enhance incretin secretion (Little et al., 2006a). Exclusion of bile in the intrajejunal study would not affect GIP secretion (Bjornsson et al., 1982), and if anything, would favour a reduced GLP-1 response (Wu et al., 2013). In the intraduodenal study, it is possible that some of the infused glucose could have refluxed into the stomach, but this should have been minimised by the increased pyloric tone associated with intraduodenal glucose infusion (Rayner et al., 2004).

Our study has limitations, which should be recognised. First, our observations were made in a parallel study design (a paired design would provide even stronger evidence), with a relatively small number of subjects in each group; however, the subjects were well matched and the differences in plasma incretin hormones and insulin were consistent between the two studies. Therefore, increasing the sample

size in a crossover study is unlikely to alter the study conclusions. Furthermore, small intestinal glucose was delivered into two sites at a single rate. It would be of interest to employ different rates of glucose infusion into various sites in order better to characterise the regional specificity of the gut-incretin axis. Finally, the balance of evidence seems to suggest alterations in secretion and/or action of incretin hormones in obesity and type 2 diabetes (Deacon and Ahren, 2011). For example, the secretion of GLP-1 is reportedly impaired in obesity, while GIP secretion may be enhanced (Deacon and Ahren, 2011). In the case of type 2 diabetes, the insulinotropic effect of GIP is largely diminished, although that of GLP-1 is better preserved. These pathophysiological features warrant further evaluation of the gut-incretin physiology in the presence of obesity and/or type 2 diabetes.

In summary, our observations indicate that intrajejunal glucose elicits greater incretin hormone and insulin secretion than intraduodenal glucose in healthy humans, suggesting regional specificity of the gut-incretin axis.

CHAPTER 10: MECHANISM OF INCREASE IN PLASMA INTACT GLP-1 BY METFORMIN IN TYPE 2 DIABETES: STIMULATION OF GLP-1 SECRETION OR REDUCTION IN PLASMA DPP-4 ACTIVITY?

(Adapted from Diabetes Res Clin Pract. 2014 Aug 12 pii: S0168-8227(14)00328-3.)
(Wu et al., 2014c)

10.1 Abstract

Metformin was reported to increase plasma intact glucagon-like peptide-1 (GLP-1) concentrations in type 2 diabetes. This is, at least partly, attributable to stimulation of GLP-1 secretion. A reduction in soluble dipeptidyl peptidase-4 activity may also make a modest contribution.

10.2 Introduction

Metformin has been shown to increase plasma intact glucagon-like peptide-1 (GLP-1) concentrations in health and type 2 diabetes (Migoya et al., 2010, Wu et al., 2014b). However, it remains unclear whether this reflects an enhancement of GLP-1 secretion and/or a reduction in plasma DPP-4 (dipeptidyl peptidase – 4) activity, since the latter has been inconsistently reported in different *in vitro* and *in vivo* experimental conditions (Hinke et al., 2002, Cuthbertson et al., 2009, Green et al., 2006, Lindsay et al., 2005, Migoya et al., 2010). We retrospectively evaluated the effects of metformin on plasma DPP-4 activity, and total and intact GLP-1

concentrations, before and during an intraduodenal glucose infusion in patients with type 2 diabetes.

10.3 Subjects and methods

We evaluated plasma DPP-4 activity in 12 type 2 patients (**Table 1**), treated with metformin 850mg or placebo each for 7 days (first dose in evening of day 1) in a randomised, crossover design (14 days 'washout' between). Subjects were evaluated on days 5 or 8 of each treatment (6 each). Plasma total and intact GLP-1 data were reported previously (Wu et al., 2014b). All subjects provided written, informed consent. The protocol was approved by the Royal Adelaide Hospital Human Research Ethics Committee.

After a standardised evening meal (~1900h) and an overnight fast, an intraduodenal catheter (Dentsleeve International, Ontario, Canada) was positioned as previously described (Wu et al., 2014b). The morning dose of either 850mg metformin or placebo was administered orally with 30mL water (t=-30min), followed 30min after by an intraduodenal glucose infusion (60g glucose dissolved in water to 240mL infused during t=0-120min; 2kcal/min). Venous blood was sampled at t=-30, 0, 15, 30, 45, 60, 90 and 120min, into ice-chilled EDTA tubes, with DPP-4 inhibitor (DPP4-010; Linco Research, MO, USA; 10μL/mL) added to tubes for total and intact GLP-1 measurement. A small aliquot of blood was sampled at t=-30, 0, 60 and 120min into ice-chilled lithium heparin tubes for the measurement of DPP-4 activity. Plasma was separated and stored at –70 °C.

Biochemical measurements, including HbA1c, cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), alkaline phosphatase (ALP) and creatinine, were obtained using standard clinical analytical methods (SA Pathology, Australia). Plasma total GLP-1 (C-terminally directed assay) and intact GLP-1 (sandwich ELISA) were determined as previously described (Wu et al., 2014b). Plasma DPP-4 activity was determined by measuring p-nitroaniline (pNa) liberated from a DPP-4 substrate, H-Gly-Pro-pNa (Bachem L1880, Switzerland), with modification of previously described methods (Nagakura et al., 2003, Durinx et al., 2000), and is expressed as U/L, where each unit produces 1 μmol pNa per min. Plasma DPP-4 activity during intraduodenal glucose infusion is expressed as percentage activity relative to baseline.

The parameters were analysed using repeated measures ANOVA, with treatment and time as factors, with Bonferroni's correction for post hoc comparisons. Student's paired t-test was used to compare the basal parameters. Within-subject correlation analysis was used to assess the relationships of the increment in plasma intact GLP-1 (ie. difference in the area under the curve (AUC) after metformin versus placebo) with that of total GLP-1 and reduction in plasma DPP-4 activity (Bland and Altman, 1995). Pearson's correlation analysis was used to assess the relationships of demographic parameters with plasma fasting DPP-4 activity. All analyses were performed using SPSS 21 (IBM, NY, USA). Data are means ± standard error; $P<0.05$ was considered significant.

10.4 Results

Plasma fasting DPP-4 activity was lower (18.4±1.6 vs. 20.5±1.7U/L, P=0.014) after metformin than placebo. Prior to intraduodenal glucose (t=0min), plasma intact GLP-1 was greater (2.0±0.3 vs. 1.0±0.3pmol/L, P=0.005), after metformin than placebo, without significant difference in total GLP-1 (19.2±1.4 vs. 17.3±2.3pmol/L). During intraduodenal glucose (t=0-120min), there was a small, but significant, reduction in plasma DPP-4 activity on both days (P<0.001), without any difference between metformin and placebo, whereas plasma total and intact GLP-1 were greater after metformin (P=0.014 and 0.006, respectively) (Figure 1).

Plasma DPP-4 activity was related directly to serum cholesterol, ALT and AST, but not GGT or ALP. In addition, plasma DPP-4 tended to be inversely related to age, but not creatinine, BMI, fasting plasma glucose or HBA1c. Plasma basal intact GLP-1 tended to be inversely related to plasma DPP-4 activity (Table 1). The increase in plasma intact GLP-1 after metformin compared to placebo was related directly to total GLP-1 (r=0.61, P=0.028), and tended to be inversely related to plasma DPP-4 activity (r=-0.48, P=0.098).

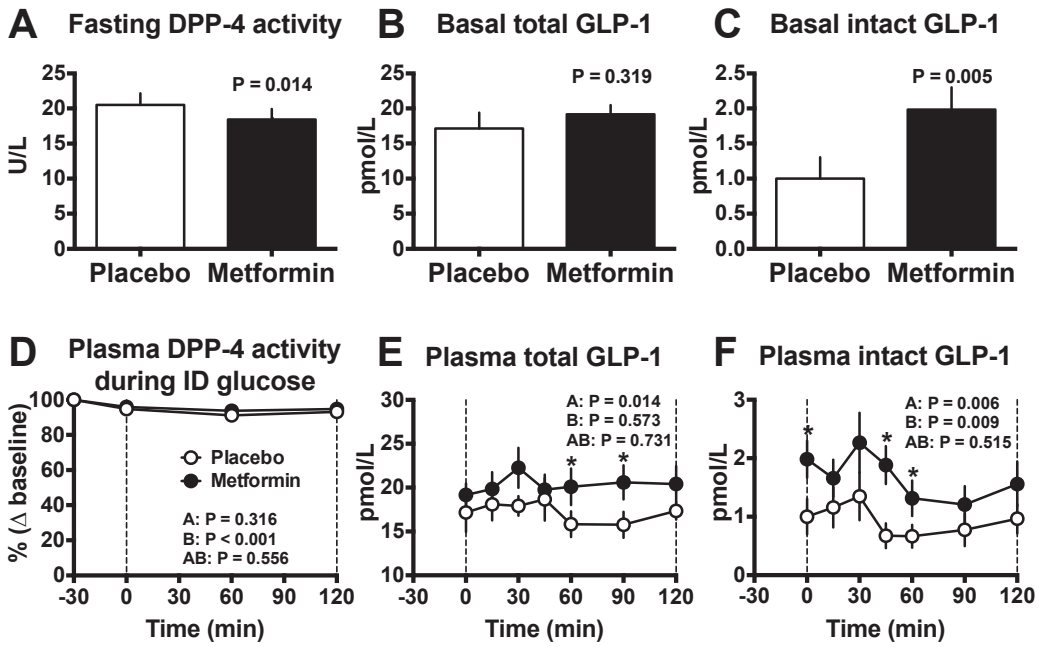


Figure 1

Plasma fasting dipeptidyl peptidase 4 (DPP-4) activity (A), basal total glucagon-like peptide-1 (GLP-1) (B), basal intact GLP-1 (C), plasma DPP-4 activity relative to baseline during intraduodenal (ID) glucose infusion (C), plasma total GLP-1 (E) and intact GLP-1 (F) after metformin versus placebo in patients with type 2 diabetes (n = 12). (A-C) paired Student t-test; (D-F) repeated measures ANOVA, with treatment and time as factors. Results of ANOVA are reported as *P*-values for (A): differences by treatment, (B) differences over time and (AB): differences due to the interaction of treatment and time. Post hoc comparisons, adjusted by Bonferroni's correction, were performed, if ANOVAs were significant. * $P < 0.05$ for each. Data are means ± SEM.

Table 1

Subject demographics and their relationships with plasma fasting dipeptidyl peptidase 4 (DPP-4) activity at baseline (n = 12) [1]

Demographics of the study participants		Relationship with DPP-4 activity
Plasma DPP-4 activity (U/L)	20.5 ± 1.7	
Gender	male	
Ethnicity	Caucasian	
Anti-diabetic medication	none	
Gastrointestinal disorder	none	
Smokers	none	
Alcohol intake	< 20g/day	
Age (years)	63.7 ± 1.9	r = -0.52, P = 0.087
BMI (kg/m^2)	29.9 ± 1.2	r = 0.40, P = 0.195
Basal intact GLP-1 (pmol/L)	1.0 ± 0.3	r = -0.56, P = 0.057
Basal total GLP-1 (pmol/L)	17.2 ± 2.3	r = -0.16, P = 0.613
Fasting plasma glucose (mmol/L)	6.7 ± 0.3	r = 0.10, P = 0.752
HbA1c (%)	6.5 ± 0.1	r = 0.14, P = 0.662
HbA1c (mmol/mol)	47.6 ± 1.4	
Cholesterol (mmol/L)	4.8 ± 0.4	r = 0.77, P = 0.018
ALT (U/L)	42.5 ± 9.1	r = 0.72, P = 0.008
AST (U/L)	36.8 ± 8.5	r = 0.76, P = 0.004
GGT (U/L)	39.8 ± 6.2	r = 0.51, P = 0.107
ALP (U/L)	81.9 ± 7.4	r = -0.27, P = 0.387
Creatinine (µmol/L)	85.2 ± 3.3	r =0.31, P = 0.327

[1] Data are means ± standard error; inter-subject correlation analyses were used to determine the relationships. P < 0.05 was considered statistically significant.

10.5 Conclusion

We have demonstrated that administration of metformin for 4 or 7 days modestly suppressed plasma fasting DPP-4 activity and increased basal intact GLP-1, without any effect on basal total GLP-1, in patients who had relatively well-controlled type 2 diabetes managed by diet alone. During intraduodenal glucose infusion, metformin had no acute effect (2.5 hours) on plasma DPP-4 activity, but increased plasma total and intact GLP-1. Within individuals, there was a direct relationship between the increments of total and intact GLP-1 during intraduodenal glucose infusion, and a tendency for a relationship between the increase in intact GLP-1 and decrease in plasma DPP-4 activity.

That metformin has been reported not to stimulate L-cell secretion (Mulherin et al., 2011) or suppress the enzymatic effect of DPP-4 *in vitro* (Lenhard et al., 2004, Hinke et al., 2002) suggests that both effects observed *in vivo* are indirect. Indeed, metformin may mediate GLP-1 secretion, at least partly, via neural pathways (Mulherin et al., 2011) and/or altering intestinal glucose or bile acid transport (Cho and Kieffer, 2011). A reduction in the production of plasma soluble DPP-4 is also possible; plasma DPP-4 activity correlates well with plasma soluble DPP-4 concentrations (Busso et al., 2005).

We have observed direct relationships between fasting plasma DPP-4 activity and serum cholesterol and liver enzymes, which indicate a hepatic origin of plasma DPP-4 and are consistent with findings that patients with non-alcoholic fatty liver disease or non-alcoholic steatohepatitis have increased hepatic expression and serum activity of DPP-4 (Itou et al., 2013, Firneisz et al., 2010, Balaban et al., 2007). Whether the

observed effect of metformin on plasma DPP-4 activity was related to suppression of hepatic DPP-4 over-production warrants further evaluation.

In conclusion, the increase in plasma intact GLP-1 concentrations with metformin is, at least partly, attributable to stimulation of GLP-1 secretion, while a reduction in soluble DPP-4 activity may also make a modest contribution.

CHAPTER 11: CONCLUSION

The experimental studies detailed in this thesis provide a deep understanding of various aspects of upper gastrointestinal function, and further establish the central role of the gut in glucose homeostasis; not only is the gut the primary absorptive site for glucose, but it also triggers neurohumoral responses that regulate the pre- and post-absorptive phases of glucose metabolism. My projects demonstrate how various pharmacological and non-pharmacological interventions, which modify gut function, affect the postprandial glycemic and cardiovascular risk profiles in humans.

The experimental study reported in chapter 4 supports the concept that manipulation of gastrointestinal function by dietary means can modulate postprandial endothelial function. We showed that, when compared to adding guar gum (which slows the rate of gastric emptying), consuming a meal more slowly attenuates the postprandial glycemic excursion as well as the postprandial decline in endothelial function. This information could be of significance in designing dietary strategies for the management of high-risk patients with type 2 diabetes and cardiovascular disease at presentation. This study has also raised the possibility that as yet unknown factors could be responsible for a sustained suppression of endothelial function following certain "low-glycemic index" meals; an improvement in the postprandial glycemic profile based on 2 hour-postprandial blood glucose concentrations following such meals may not always be a useful predictor of cardiovascular risk. Though we do not dispute the long-term benefits of fiber rich food, our study points to the possibility of an acute detrimental effect on postprandial endothelial function of adding guar gum to

a high carbohydrate meal, which could potentially be of clinical significance in high-risk patients with pre-existing cardiovascular disease.

Apart from the use of dietary fiber, pharmacological methods have also been used to slow gastric emptying in order to achieve a lower postprandial glycemic profile. This is a well-established effect of the GLP-1 receptor agonist, exenatide; however, the effect of this drug on small intestinal function has not been well studied to date. In chapter 5, we demonstrated the inhibitory effects of exenatide on small intestinal motor function. An acute intravenous infusion of exenatide markedly suppressed duodenal motility and flow events, slowed small intestinal transit, decreased glucose absorption, and delayed and suppressed the glycemic excursion in response to an intraduodenal glucose infusion, in both healthy subjects and patients with type 2 diabetes. Glucose absorption and the glycemic response were both related to duodenal motility and the frequency of small intestinal flow events. We also demonstrated that in the initial phase of the intraduodenal glucose infusion, inhibition of small intestinal motility and glucose absorption outweighed the insulinotropic effects of exenatide in contributing to lowering of glycemia, This is similar to the predominant effect reported previously for the slowing of gastric emptying of a liquid meal by an intravenous GLP-1 infusion (Nauck et al., 1997b). It is now evident that inhibition of small intestinal motility may be an important mechanism, in addition to the slowing of gastric emptying, in the reduction in postprandial glycemia observed with the use of GLP-1 receptor agonists, such as exenatide.

In the same study, we observed that intravenous exenatide was associated with increases in the mean arterial blood pressure and heart rate during an intraduodenal

glucose infusion; for heart rate, the increase was directly related to the decrease in the small intestinal motility index induced by exenatide. These findings, which are discussed in chapter 6, suggest that GLP-1 receptor agonists could be beneficial for patients with postprandial hypotension.

Extracts from the fruit of *Garcinia cambogia*, which have hydroxycitric acid (HCA) as the active ingredient, have been widely marketed to assist with weight loss, while findings from rodent studies also indicated that HCA might inhibit intestinal glucose absorption and stimulate GLP-1 release from the distal gut. However, in chapter 7, we observed that intraduodenal administration of 4800mg of HCA, in advance of an intraduodenal glucose infusion, resulted in only a slight and probably clinically irrelevant reduction in glycemia in healthy subjects, and had no effect on blood glucose in patients with type 2 diabetes. Glucose lowering in the healthy group was unrelated to inhibition of glucose absorption, or to stimulation of insulin secretion, even though an increase in GIP concentrations was noted, the relevance of which is uncertain. In the relatively well-controlled group of patients that we studied, acute administration of HCA appeared to have no benefit from a glycemic perspective.

In chapter 8, we evaluated another nutritional supplement, resveratrol, which is found in the red grapes. Various animal and human studies have suggested possible beneficial effects of resveratrol in type 2 diabetes and obesity, but its mechanism of action has not been completely elucidated. Based on a rodent study, we hypothesized that resveratrol would increase both fasting and postprandial GLP-1 concentrations and, accordingly, lower fasting and postprandial blood glucose concentrations and slow gastric emptying in patients with type 2 diabetes. However, we observed that,

administration of 500 mg resveratrol twice daily for 5 weeks in non-obese patients with type 2 diabetes that was well-controlled with life-style management alone, had no effect on GLP-1 secretion, glycemic control, gastric emptying, body weight or energy intake, compared to placebo. Therefore, our observations do not support stimulation of GLP-1 release as a mechanism by which resveratrol acts in humans, and suggest that resveratrol is unlikely to improve glycemic control in patients such as those enrolled in this study. The inconsistencies between ours and other studies could at least partly be related to species differences, patient selection criteria, the duration of the intervention, and/or the dose of resveratrol used.

In chapter 9, we examined the regional specificity of incretin hormone responses to an enteral glucose load. We demonstrated that intrajejunally administered glucose elicited greater GLP-1, GIP, and insulin release than intraduodenally administered glucose in healthy males, supporting the concept that directing nutrients more distally in the small intestine could ameliorate type 2 diabetes. Such an effect is achieved by some bariatric procedures, such as Roux-en-Y gastric bypass, and might also be achieved by dietary strategies that delay carbohydrate absorption to the more distal gut.

Having recognised the importance of GLP-1 in glucose homeostasis, we examined the effects of the widely used anti-diabetic medication, metformin, on total and intact GLP-1 concentrations and the enzymatic activity of plasma DPP-4, before and during an intraduodenal glucose infusion in patients with type 2 diabetes. We reported, in chapter 10, that administration of metformin for 4 or 7 days resulted in modest suppression of plasma fasting DPP-4 activity, and increased basal intact GLP-1,

without any effect on basal total GLP-1. During a 2-hour intraduodenal glucose infusion, metformin had no acute effect on plasma DPP-4 activity, but plasma total and intact GLP-1 were increased. Within individuals, there was a tendency for a relationship between the increase in intact GLP-1 and decrease in plasma DPP-4 activity. The increase in plasma intact GLP-1 concentrations with metformin is, at least in part, attributable to stimulation of GLP-1 secretion, possibly via indirect means such as neural pathways and/or modulation of intestinal glucose or bile acid transport. However, a reduction in soluble DPP-4 activity may also make a modest contribution towards increases in GLP-1 concentrations. Taken together, it is reasonable to suggest that metformin has multiple mechanisms of action, which are of benefit in the management of type 2 diabetes.

In summary, the experimental studies outlined in this thesis have highlighted the central role of the gastrointestinal system in the mediation of glucose homeostasis and postprandial cardiovascular risk. These studies are likely to help improve the range of strategies available for the management of type 2 diabetes and its complications, and will stimulate further research in this field.

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