

Gastric motor function
in health and diabetes –
implications for incretin hormone
release and postprandial blood glucose
regulation

A thesis submitted by

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THESIS SUMMARY

This thesis focuses on gastric motor function in patients with longstanding diabetes, and the role of gastric emptying and gastrointestinal hormones in the regulation of glycaemia in health and patients with type 2 diabetes mellitus.

Diabetes is a common chronic disorder worldwide, with the prevalence of type 2 diabetes escalating due to an increasingly sedentary lifestyle and rising rates of obesity. Diabetes is associated with micro- and macrovascular complications, particularly in the context of poor glycaemic control (1993, 1998). Another complication of type 1 and 2 diabetes is gastroparesis (Horowitz et al., 2001, Horowitz M, 1986, Horowitz et al., 1989, Horowitz et al., 1991) (delayed gastric emptying in diabetes) and there is limited information about the natural history and prognosis of this condition. While the prognosis of diabetic gastroparesis has been assumed to be poor, limited data in a small cohort followed for a mean period of 12 years suggest otherwise, with neither deterioration in the rate of gastric emptying (Jones et al., 2002) nor increased mortality due to the condition itself (Kong et al., 1999).

The study reported in Chapter 3 evaluated the longitudinal progression of gastric emptying in patients with longstanding diabetes over a 25 year period to determine if there is a progressive slowing of gastric emptying or whether

gastric emptying is relatively stable with a good prognosis from the outset, and to ascertain the potential impact of glycaemic control and/or autonomic function. The study concludes that gastric emptying and upper gastrointestinal symptoms are relatively stable over 25 years, while there was a deterioration in autonomic function and an improvement in glycaemic control. The study reported in Chapter 4 examined the prognosis of diabetic gastroparesis and its findings highlight that this condition is neither associated with a poor prognosis nor a higher rate of mortality.

There is increasing recognition that glycated haemoglobin (HbA1c), which is a measure of overall glycaemic control, is influenced more by postprandial, rather than fasting, blood glucose levels in the majority of patients with type 2 diabetes. This makes intuitive sense, because the majority of one's time is spent in a postprandial state, digesting the caloric load of the ingested meal, which in healthy subjects empties from the stomach in a tightly regulated process at a rate of 1-4kcal/minute (Khoo et al., 2009). Accordingly, good control of postprandial glucose excursions should be a priority for the treatment of diabetes. The rate of gastric emptying itself influences the magnitude of the initial rise in postprandial glycaemia in health as well as type 1 and 2 diabetes (Jones et al., 1996, Horowitz et al., 1993, Horowitz et al., 1986), whereby slower emptying is associated with diminished postprandial glucose excursions. The overall rate of gastric emptying is dependent on the integration of motor activity in each region of the stomach and slower gastric

emptying is associated with suppression of antral and duodenal contractions, and stimulation of phasic and tonic pyloric pressures, with the latter acting as a brake to gastric outflow (Horowitz et al., 1994).

When glucose is given by the oral/enteral route, the stimulation of insulin is markedly greater than with an isoglycaemic intravenous glucose infusion. This phenomenon is known as the 'incretin effect' and is mediated by the gastrointestinal hormones, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP), which are secreted from the small intestine in response to nutrients (Ma et al., 2009a). GLP-1 and GIP both stimulate insulin secretion from the pancreas in the setting of elevated blood glucose levels, and are responsible for ~70% of the postprandial insulin response in healthy humans (Horowitz and Nauck, 2006). GLP-1 analogues, such as exenatide, are now widely used in the management of type 2 diabetes, in whom the response to exogenous GIP is attenuated markedly (Holst and Gromada, 2004) but the insulin response to GLP-1 remains intact (Elahi et al., 1994). It appears that an important action of GLP-1 analogues in reducing postprandial glycaemia is by retardation of small intestinal motility modulating carbohydrate absorption (Linnebjerg et al., 2008, Little et al., 2006).

An alternative to the use of exogenous GLP-1 analogues in the management of type 2 diabetes is to develop dietary strategies which stimulate endogenous

GLP-1 release. Glutamine, which is widely used as a nutritional supplement, appears to be the most potent amino acid in inducing GLP-1 release (Reimann et al., 2004). It has been reported that 30g glutamine, given in 300mL water, stimulates GLP-1 release in both healthy subjects and patients with type 2 diabetes (Greenfield et al., 2009) and Samocha-Bonet et al (Samocha-Bonet et al., 2011) reported that 15g and 30g glutamine when given as a drink, before an oral glucose load in patients with type 2 diabetes, dose-dependently stimulate GLP-1 and diminish subsequent glycaemic excursion. However, the effect of glutamine on the rate of gastric emptying of glucose could potentially influence the observed effect on glycaemia as it is now appreciated that the rate of gastric emptying itself has a major influence on postprandial glucose levels in healthy subjects and patients with type 1 and 2 diabetes (Chang et al., 2010). The study reported in Chapter 5 examined the effects of intraduodenal glutamine on GLP-1, GIP and insulin release and the subsequent glycaemic response to an intraduodenal glucose load, in health and type 2 diabetes, of which the intraduodenal route of delivery of glutamine will bypass the stomach, thus, eliminating any influence of glutamine on the rate of gastric emptying of glucose. This study showed that intraduodenal glutamine has minimal effect on the glycaemic response to intraduodenal glucose, despite its ability to stimulate GLP-1, GIP and insulin release, and stimulate phasic pyloric contractions, suggesting that slowing of gastric emptying may play a major role for the glucose lowering effect seen with oral glutamine.

DECLARATION

Name: Jessica Chang

Program: Master of Philosophy

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Chang J, Wu T, Greenfield JR, Samocha-Bonet D, Horowitz M, Rayner CK. Effects of intraduodenal glutamine on incretin hormone release, the glycaemic response to an intraduodenal glucose infusion and antropyloroduodenal motility in health and type 2 diabetes. (Submitted for publication)

CHAPTER 1: DIABETIC GASTROPARESIS AND THE COMPLEX RELATIONSHIP BETWEEN GASTRIC EMPTYING AND GLYCAEMIA

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1.1 Introduction

Diabetic gastroparesis was once thought to be a rare condition, afflicting patients with longstanding type 1 (and not type 2) diabetes, associated with a poor prognosis, predictable on the basis of upper gastrointestinal symptoms, and solely attributable to irreversible autonomic (vagal) neuropathy (Rundles, 1945). The landmark study reported in 1986 (Horowitz et al., 1986) represented the first comprehensive evaluation of gastric emptying in type 1 diabetes and stimulated a substantial redefinition of these concepts. The study capitalised on the availability of radionuclide techniques to quantify gastric emptying and assessment of autonomic function using standardised cardiovascular reflex tests. By monitoring blood glucose concentrations during the measurements of gastric emptying, the potential impact of acute changes in the blood glucose concentration on gastric emptying was evaluated and upper gastrointestinal symptoms were assessed by a standardised questionnaire. During the last ~25 years, there has been a substantial redefinition of concepts relating to the prevalence, clinical significance, pathogenesis and management of disordered gastric emptying in diabetes, with the application of novel diagnostic techniques being fundamental to the achievement of these advances

in knowledge. This introductory chapter will elaborate on the substantial advances in knowledge gained over the last ~25 years relating to normal and disordered gastric emptying in diabetes, with particular emphasis on the prognosis of disordered gastric emptying in diabetes, the impact of gastric emptying on the regulation of blood glucose and consequent dietary and pharmacologic strategies to improve chronic glycaemic control by modulating gastric emptying.

1.2 Physiology of gastric emptying

It is now recognised that normal gastric emptying is dependent on the coordinated activity of the proximal and distal stomach, pylorus and the upper small intestine. It has been known since 1893 (Wingate, 1981) that in the fasting state, gastric motility undergoes a cyclical pattern, termed the 'migrating motor complex' consisting of phase I, motor quiescence (~40 minutes), phase II, irregular contractions (~50 minutes) and phase III, regular contractions at 3 per minute (~5-10 minutes) (Horowitz M, 2004). Larger, indigestible solids are usually emptied from the stomach into the small intestine during phase III and, accordingly, an absence or disordered phase III activity has the potential to result in gastric 'bezoar'. Following meal consumption, fasting motility is converted promptly to a postprandial pattern, with irregular antral contractions and an increase in tonic and phasic pyloric pressures (Horowitz and Dent, 1991).

The proximal stomach initially relaxes to 'accommodate' a meal while the antrum grinds solid food into particles <2mm in size and pumps chyme into the duodenum against pyloric resistance in a predominantly pulsatile manner. Contractions of the antrum and pylorus are controlled by electrical slow waves generated by the interstitial cells of Cajal (ICC), which are specialized pacemaker cells that result in approximately 3 slow waves per minute in the stomach. The emptying of nutrients from the stomach occurs at an overall rate of 1-4 kcal/min, primarily as a result of the interaction with receptors in the small intestine which generates inhibitory neurohumoral responses. The latter are mediated, at least in part, by cholecystinin (CKK) (Fried et al., 1991), glucagon-like peptide-1 (GLP-1) (Deane et al., 2010) and peptide YY (PYY) (Horowitz M, 2004), and are dependent on the length, and region, of small intestine exposed (Lin et al., 1989). Solids and liquids have different patterns of emptying, with solids emptying in an overall linear pattern after an initial lag phase, while the liquid emptying does not usually exhibit a significant lag phase and slows from an exponential to a linear pattern as the caloric content increases (Camilleri, 2006). The lag phase for solids reflects the time taken for meal redistribution from the proximal to the distal stomach and the grinding of solids into small particles by the antrum. When liquids and solids are consumed together, liquids empty preferentially.

1.3 Prevalence and natural history of disordered gastric emptying in diabetes

Gastroparesis refers to abnormal gastroduodenal motility characterized by delayed gastric emptying in the absence of mechanical obstruction. The aetiology is multifactorial and it is now recognised that diabetes is probably the most common cause.

1.3.1 A historical perspective

Gastric retention in diabetes was first noted by Boas in 1925 (Boas, 1925), with subsequent radiological findings by Ferroir in 1937 (Ferroir, 1937) noting that the stomach motor responses in diabetics are weaker than normal – ‘contractions are slow, lack vigour and die out quickly’ (Saltzman and McCallum, 1983, Ferroir, 1937). The first detailed description of the association between delayed gastric emptying and diabetes was by Rundles in 1945, who reported that gastric emptying of barium was abnormally slow in 5 of 35 type 1 patients with peripheral neuropathy (Rundles, 1945). In 1958, Kassander named the condition ‘gastroparesis diabetorum’ and commented that this syndrome was ‘more often overlooked than diagnosed’ (Kassander, 1958).

1.3.2 Prevalence of diabetic gastroparesis

While the prevalence of gastroparesis remains uncertain because of the lack of population-based studies, cross-sectional studies, which for the main part have employed radioisotopic methods, indicate that gastric emptying is abnormally delayed in 30-50% of outpatients with longstanding type 1 (as reported in the original study of 45 patients) (Horowitz et al., 1986) and type 2 diabetes (Horowitz et al., 1989, Samsom et al., 2003). The prevalence was clearly underestimated in early studies, which employed less sensitive diagnostic methods to quantify gastric emptying. The reported prevalence is highest when gastric emptying of both solids and nutrient-containing liquids is quantified, either concurrently or separately, reflecting the relatively poor correlation between gastric emptying of solids and liquids in diabetes (Horowitz and Dent, 1991, Jones et al., 1995). Symptoms attributable to gastroparesis are reported in 5-12% of patients with diabetes in the community, but much higher rates are evident in patients evaluated in tertiary referral centres (Parkman et al., 2010). Gastric emptying is not infrequently abnormally rapid in both type 1 and 2 diabetes (Bharucha et al., 2009).

In the study reported in 1986 (Horowitz et al., 1986), the patients were selected at random from an outpatient setting and, only patients with type 1 diabetes were included and while blood glucose levels were monitored, they were not stabilised. A subsequent study which evaluated a cohort of 20 unselected

outpatients with longstanding type 2 diabetes (Horowitz et al., 1989), indicated that the prevalence of gastroparesis was comparable to that observed in type 1 patients. Given that acute hyperglycaemia slows gastric emptying (discussed in Chapter 1.5.3 “Pathogenesis - Impact of Glycaemia”), the reported prevalence of gastroparesis in both studies (Horowitz et al., 1989, Horowitz et al., 1986) probably represents an overestimate.

1.3.3 Natural history of diabetic gastroparesis

Data from these initial studies (Horowitz et al., 1986, Horowitz et al., 1989) allowed subsequent evaluation of the impact of both upper gastrointestinal symptoms and gastroparesis on mortality (Kong et al., 1999) and the natural history of delayed gastric emptying in diabetes (Jones et al., 2002). The prognosis of diabetic gastroparesis had hitherto been assumed to be poor, however, when 20 subjects from the original cohort were re-evaluated after a mean period of 12 years, there was no major change in either the rate of gastric emptying, or symptoms over this time period (Jones et al., 2002) (Figure 1.1). While there was a deterioration in cardiovascular autonomic nerve function, there was a concomitant improvement in glycaemic control, as assessed by glycated haemoglobin (Jones et al., 2002), which is likely to be attributable to the increased attention given to the achievement of tight blood glucose control subsequent to the outcome of the DCCT study, which together may account for the lack of change in gastric emptying. A longer duration of follow-up into this

condition will provide important information regarding gastric emptying in diabetes to guide management of this common complication of a very common chronic disorder. The study reported in Chapter 3 represents the most prolonged longitudinal follow-up of patients with longstanding diabetes in which patients were followed over a mean period of ~25 years. This study aims to provide pivotal information into the natural history of this not-so-uncommon but little known condition.

1.4 Diagnosis of disordered gastric emptying

The decision of when to evaluate patients with diabetes for disordered gastric emptying is not straightforward. While upper gastrointestinal symptoms occur frequently, the original (Horowitz et al., 1986, Horowitz et al., 1989) and subsequent (Jones et al., 2002) studies have established that they are not strongly predictive of delayed gastric emptying, contrary to what was thought previously (Kassander, 1958). Furthermore, some patients with markedly delayed gastric emptying are asymptomatic. In any patient with diabetes who presents with upper gastrointestinal symptoms suggestive of delayed gastric emptying, reversible causes of gastroparesis must be excluded after endoscopy has been performed (Table 1.1). The diagnosis of gastroparesis is usually based on the presence of upper gastrointestinal symptoms in combination with objective evidence of delayed gastric emptying. The latter should be measured during euglycaemia, or at least with the blood glucose $> 4\text{mmol/L}$ and \leq

10mmol/L, given the effect of hyperglycaemia to slow emptying. Medications that may influence gastric emptying should ideally be withdrawn for 48-72 hours prior to the test (or for the half-life of the drug) (Camilleri et al., 1998) and smoking, which has been shown to slow gastric emptying, should be avoided on the day of investigation (Johnson et al., 1991). This section will summarise the various methods of assessing gastric emptying.

1.4.1 Scintigraphy

Scintigraphy, which is non-invasive and reproducible, remains the most sensitive and accurate method and is the 'gold standard' technique. Intra-gastric distribution of solid and/or liquid meal components, which is frequently abnormal in diabetics (Jones et al., 1995) can also be evaluated with scintigraphy. In an effort to standardize the test meal and technique between various centres, a recent consensus statement recommends the use of a low fat, egg white meal labelled with ^{99m}Tc -sulfur colloid (Abell et al., 2008), with measurement of gastric emptying for 4 hours. Despite this recommendation, scintigraphy is still not well standardized. Low nutrient liquids should not be used to quantify gastric emptying for diagnostic purposes since they do not stimulate small intestinal feedback mechanisms which retard gastric emptying. Contrary to what is generally assumed, there is little, if any, evidence that the use of high nutrient liquid, or semi-solid, meals is inferior to solids. Moreover, the concurrent measurement of solid and nutrient liquid emptying adds

diagnostic value, since, as shown in the study by Horowitz et al, the relationship between gastric emptying of solids and nutrient liquids is poor in diabetes (Horowitz et al., 1986). If carbohydrate is included in the meal, the relationship between glycaemic response with the rate of gastric emptying can be evaluated.

1.4.2 Stable isotope breath test

This non-invasive method of assessing gastric emptying uses ^{13}C -acetate or ^{13}C -octanoate as a label and, in contrast to scintigraphy, does not involve exposure to ionising radiation. It has good reproducibility and the results have been reported to correlate well with scintigraphy, with a sensitivity and specificity of 86% and 80% respectively for the presence of delayed gastric emptying (Viramontes et al., 2001), including in a diabetic population.

Following ingestion, the labelled meal passes through the stomach to the small intestine, where the ^{13}C -acetate or ^{13}C -octanoate is absorbed, metabolized into $^{13}\text{CO}_2$ in the liver and exhaled via the breath. $^{13}\text{CO}_2$ in breath samples is analysed by mass spectrometry. While this technique has advantages over scintigraphy, information relating to the validity of breath tests in patients with markedly delayed gastric emptying is limited.

1.4.3 Transabdominal ultrasound

Transabdominal ultrasound is a simple, non-invasive, inexpensive and convenient method to assess gastric distension, antral contractility, transpyloric flow and gastric emptying and is uniquely able to measure the latter three parameters simultaneously (Parkman et al., 2010). However, the necessity for considerable expertise, the technical limitations of obesity and abdominal gas, restrict its widespread use. While 2-dimensional ultrasonography provides an indirect measure of gastric emptying which is determined by changes in antral area over time (Haruma et al., 2008), the more recently applied 3-dimensional ultrasonography has the capacity to provide comprehensive imaging of the stomach, including information about intragastric meal distribution, and has been validated against scintigraphy to measure gastric emptying in both healthy subjects and patients with diabetic gastroparesis (Gentilcore et al., 2006b, Gilja et al., 1999, Hveem et al., 1996).

1.4.4 Magnetic resonance imaging (MRI)

Magnetic resonance imaging (MRI) has also been used to measure gastric emptying and motility with excellent reproducibility (Parkman et al., 2010). However, its use is limited to research purposes because of its high cost and limited availability.

1.4.5 Barium meal

Barium meal, involving a non-nutrient contrast load, has no role in quantifying gastric emptying and its use is limited to excluding mucosal lesions or obstructions.

1.4.6 Paracetamol (acetaminophen) absorption test

The paracetamol (acetaminophen) absorption test as a simple bedside test is limited to evaluation of the emptying of liquids and is not recommended as a diagnostic tool as its accuracy is variable at best (Willems et al., 2001).

1.4.7 Swallowed capsule telemetry ('SmartPill®')

Swallowed capsule telemetry ('SmartPill®') employs an indigestible capsule that has the capacity to measure intraluminal pH and pressure as the capsule travels through the digestive tract to determine the gastric emptying rate. The pressure measurements also provide information about the motor function of the stomach, small intestine and colon (Camilleri et al., 2008). This method has been reported to correlate relatively well with scintigraphy with good sensitivity (82%) and specificity (83%), but has not been used widely. Emptying of the capsule presumably usually occurs after that of digestible meal components.

1.4.8 Electrogastrography

Electrogastrography measures the frequency of the gastric slow wave (~3 cycles/minute) using surface electrodes attached to the skin of the epigastrium (Koch, 2001). While it is clear that abnormalities in gastric electrical activity, particularly tachygastria, occur frequently in diabetic gastroparesis and may be induced by hyperglycaemia (Jebbink et al., 1994), the relationship is not sufficiently strong to be of diagnostic value.

1.4.9 Antropyloroduodenal manometry

Antropyloroduodenal manometry, using a water-perfused, or solid-state, catheter to measure intraluminal pressures in the stomach, pylorus, and small intestine, is only available in a few centres and remains primarily a research tool.

1.5 Pathogenesis

The pathogenesis of diabetic gastroparesis is now recognised to be complex and multifactorial; there has been recent awareness of cellular defects in various interacting cell types, in addition to the more established roles of autonomic neuropathy and acute hyperglycaemia.

1.5.1 Autonomic (vagal) neuropathy

The similarity in gastrointestinal symptoms experienced by surgically vagotomised patients and patients with longstanding diabetes led to the initial concept that irreversible vagal damage underlies disordered gastric emptying in diabetes (Rundles, 1945). Due to the difficulties of assessing gastrointestinal autonomic function directly, evaluation of cardiovascular autonomic function has been employed widely as a surrogate marker for the function of the abdominal vagus (Ewing and Clarke, 1982). Though the initial (Horowitz et al., 1986, Horowitz et al., 1989) and subsequent (Jones et al., 2002) studies established that the prevalence of disordered gastric emptying is higher in those patients with cardiovascular autonomic neuropathy, the relationship between disordered gastric emptying and abnormal cardiovascular autonomic function is relatively weak (Horowitz et al., 1991, Buysschaert et al., 1987).

1.5.2 Cellular dysfunction

Diabetic gastroparesis is associated with heterogeneous motor dysfunctions, including ‘incoordination’ of the motor activity of the proximal stomach, antrum, pylorus and duodenum (Ma et al., 2009a). Data from the NIH-funded Gastroparesis Clinical Research Consortium, based in the USA, have contributed substantially to knowledge of the role of cellular defects in the pathogenesis of gastroparesis. Recent insights gained from animal and human gastric tissue indicate a heterogeneous pathological picture, with abnormalities

in multiple, interacting cell types, including decreased numbers of ICC (Pasricha et al., 2008, He et al., 2001), deficiencies of inhibitory neurotransmission (Pasricha et al., 2008, He et al., 2001), reduced numbers of extrinsic autonomic neurons (Samsom et al., 1997), smooth muscle fibrosis (Pasricha et al., 2008) and abnormalities in the function of immune cells (Choi et al., 2010). Loss/dysfunction of ICC appears to be central to the pathogenesis of diabetic gastroparesis (Forster et al., 2005).

In animal models and humans with diabetic gastroparesis, a reduction in intraneuronal levels of nitric oxide, an important enteric neurotransmitter, has been observed reflecting loss of neuronal nitric oxide synthase (nNOS) expression within the myenteric neurons and, potentially, inhibition of nNOS by advanced glycation products (Watkins et al., 2000). Haeme-oxygenase-1, the enzyme which gives rise to carbon monoxide (CO), which protects the ICC from oxidative stress, has recently been shown to be reduced in non-obese diabetic (NOD) mice with delayed gastric emptying (Choi et al., 2008, Choi et al., 2010). Administration of haemin, which increases the expression of haeme-oxygenase-1 (Choi et al., 2010, Choi et al., 2008), and administration of CO (Kashyap et al., 2010), result in reversal of the loss of ICC and normalization of delayed gastric emptying. Haemin also increases plasma levels of haeme-oxygenase-1 when given intravenously to healthy humans (Bharucha et al., 2010) and may, accordingly, have a therapeutic role.

1.5.3 Impact of glycaemia

In the study by Horowitz et al, while there was no significant relationship between plasma glucose levels with the rate of gastric emptying, gastric emptying of liquids and the lag phase for solids were slower when the mean plasma glucose was $>15\text{mmol/L}$. It has subsequently been established, using the glucose 'clamp' technique, that acute variations in blood glucose impact significantly on gastric emptying in both healthy and diabetic subjects (Rayner et al., 2001), with marked hyperglycaemia (blood glucose $\sim 15\text{mmol/L}$) delaying gastric emptying of solids and liquids substantially (Fraser et al., 1990). Gastric emptying is also slower when the blood glucose is at the upper end of the physiological postprandial range ($\sim 8\text{mmol/L}$), when compared to a blood glucose of $\sim 4\text{mmol/L}$, in both healthy subjects and patients with uncomplicated type 1 diabetes (Schvarcz et al., 1997). The mechanisms by which acute hyperglycaemia slows gastric emptying include suppression of antral contractions (Rayner et al., 2001), stimulation of phasic and tonic pyloric contractions (Rayner et al., 2001), proximal stomach relaxation (Rayner et al., 2001) and induction of gastric electrical dysrhythmias (Jebbink et al., 1994). In the study by Horowitz et al, the duration of the lag phase for solids was apparently related to chronic blood glucose control, as assessed by glycated haemoglobin, but the relevance of long-term glycaemia to the pathogenesis of gastroparesis remains uncertain and is addressed in the study reported in Chapter 3.

In contrast to the effects of acute hyperglycaemia, insulin-induced hypoglycaemia accelerates gastric emptying in healthy subjects (Schvarcz et al., 1995), patients with uncomplicated type 1 diabetes (Schvarcz et al., 1993) and in type 1 diabetics with gastroparesis (Russo et al., 2005), probably serving as a counter-regulatory mechanism to hasten the delivery of nutrients for absorption.

1.6 Significance of upper gastrointestinal symptoms in diabetes and their aetiology

The prevalence of upper gastrointestinal symptoms such as nausea, vomiting, early satiety, postprandial fullness, bloating and abdominal pain, is higher in both type 1 and 2 diabetes in comparison to the general population (Bytzer, 2001, Schvarcz et al., 1996). What is contentious is the magnitude of this difference. It has been shown that gastrointestinal symptoms in patients with diabetes impact negatively on health-related quality of life and assessment of these symptoms should take into account potential psychological/psychiatric factors, along with other variables such as age, gender, body weight and use of drugs such as nicotine and alcohol (Talley et al., 2001a). Subsequent to the recognition that the relationship between upper gastrointestinal symptoms and the rate of gastric emptying is weak, studies have focused on other potential causes of symptom induction (Horowitz et al., 1991, Jones et al., 1996). In some patients, there is impaired proximal gastric relaxation and/or increased

perception of gastric distension, implicating the role of visceral hypersensitivity in the aetiology of symptoms (Samsom et al., 1995, Kumar et al., 2008, Rayner et al., 2000). Acute hyperglycaemia has been shown to increase the perception of gastrointestinal sensations eg nausea and fullness induced by gastric, or duodenal distension or small intestinal nutrient infusion, is greater during hyperglycaemia (blood glucose level ≥ 11 mmol/L) when compared to euglycaemia (Hebbard et al., 1996, Rayner et al., 2001, Lingens et al., 1999) and in diabetic patients the perception of postprandial fullness is greater, as the blood glucose increases (Rayner et al., 2001, Jones et al., 1997).

1.7 Impact of gastric emptying on incretin hormones and glycaemia

It is now recognised that the rate of gastric emptying impacts on blood glucose and this issue has assumed increasing importance. The presence of nutrients in the small intestine also stimulates the release of so-called ‘incretin’ hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) that stimulate insulin secretion (Ma et al., 2009a) and are responsible for ~70% of the postprandial insulin response in healthy humans (Horowitz and Nauck, 2006).

1.7.1 'Incretin' effect

The 'incretin effect' refers to the substantially greater insulin response to an oral, when compared to an isoglycaemic intravenous glucose load. GIP is secreted primarily from the proximal small intestine and GLP-1, predominantly from the distal small intestine and colon (Ma et al., 2009a). Exogenous (Willms et al., 1996) and endogenous (Deane et al., 2010) GLP-1 slows gastric emptying and decreases glucagon secretion in a glucose-dependent manner, whilst GIP probably has no effect on gastric emptying and may stimulate glucagon levels (Meier, 2009). In healthy subjects, exogenous GLP-1 slows gastric emptying markedly, but variably, with subsequent attenuation in postprandial insulin secretion (Little et al., 2006). In type 2 diabetes, the incretin effect is reduced, probably representing an epiphenomenon (Meier and Nauck, 2010) due to the inability of GIP to augment insulin secretion, partly attributable to hyperglycaemia, whilst the effects of GLP-1 are relatively intact (Nauck et al., 1993, Vilsboll et al., 2002).

1.7.2 The significance and determinants of postprandial glycaemia

Glycated haemoglobin, which is used widely as a measure of 'overall' glycaemic control, is influenced by both fasting and postprandial glucose levels, with a greater contribution of the latter, especially as glycaemic control improves (Monnier et al., 2003). This is not surprising – as emptying of nutrients from the stomach occurs at an overall rate of ~1-4kcal/minute in

health (and frequently slower than this in diabetes), only a few hours each day, prior to breakfast, are truly reflective of the 'fasting' glycaemic state. Thus, the management of postprandial blood glucose excursions has in recent years attracted increasing interest (Monnier et al., 2003, Ceriello et al., 2004).

Postprandial glycaemia is potentially influenced by several factors, including preprandial glycaemia, the carbohydrate content of a meal, the rate of small intestinal delivery and absorption of nutrients, insulin and glucagon secretion and peripheral insulin sensitivity. While the relative contribution of these factors is variable, it is now appreciated that gastric emptying accounts for at least a third of the variance in peak postprandial levels after oral glucose in both healthy subjects (Horowitz et al., 1993) and patients with type 1 (Horowitz et al., 1986) and type 2 diabetes (Jones et al., 1996). In type 1 patients with gastroparesis, less insulin is initially required to maintain euglycaemia postprandially when compared to those with normal gastric emptying (Ishii et al., 1994). Gastric emptying also accounts for a substantial amount of variation in glycaemic response to carbohydrate of variable glycaemic indices (Rayner et al., 2001).

1.7.3 Glutamine and GLP-1 release

With the advent in knowledge regarding the role of GLP-1 in reducing postprandial glycaemia, dietary strategies have been employed to stimulate the

release of endogenous GLP-1. Glutamine, a commonly used nutritional supplement, appears to be the most potent amino acid in inducing GLP-1 release (Reimann et al., 2004). Oral glutamine has been shown to stimulate GLP-1 secretion in both healthy and diabetic individuals (Greenfield et al., 2009) (Figure 1.2), and oral loads of 15g and 30g, given before oral glucose in patients with type 2 diabetes, dose-dependently stimulated GLP-1 release and reduced the subsequent glucose excursion (Samocho-Bonet et al., 2011). It is however, uncertain whether the latter is mediated by stimulation of insulin and/or slowing of gastric emptying. To address this, Chapter 5 reports a study examining the effects of glutamine, delivered at a fixed rate into the duodenum to eliminate any potential influence of glutamine on the rate of gastric emptying, on GLP-1, GIP and insulin concentrations, antropyloroduodenal motor activity, and the glycaemic response to a subsequent intraduodenal glucose load, in both healthy humans and patients with type 2 diabetes.

1.7.4 Impact of variation in rate of glucose delivery on glycaemia and incretin hormones

It has only recently been appreciated is that the relationship of glycaemia with small intestinal glucose delivery is non-linear, as evidenced by the glycaemic response to intraduodenal infusion of glucose at rates within the normal range for gastric emptying in both healthy (Pilichiewicz et al., 2007) and type 2 diabetic subjects (Rayner C K, 2009). For example, at an intraduodenal

glucose infusion rate of 1kcal/min, there is only a modest elevation in blood glucose, but a substantial elevation in blood glucose occurs in response to an infusion rate of 2kcal/min. However there is minimal further increase when the rate is increased to 4 kcal/min (Figure 1.3) (Pilichiewicz et al., 2007). These discrepant blood glucose responses are likely to reflect the substantially increased plasma insulin response to the 4kcal/min infusion, which is probably accounted for by greater GLP-1 (and perhaps GIP) secretion (Pilichiewicz et al., 2007). At 1kcal/min, there is minimal, transient, stimulation of GLP-1 compared with sustained elevation of GIP. In contrast at 4kcal/min, there is a substantial increase in GLP-1 secretion, with a further increase in GIP (Pilichiewicz et al., 2007, Ma et al., 2009a). Thus, the marked increase in insulin secretion at higher rates of intraduodenal glucose infusion is likely to be attributable to GLP-1 secretion (Ma et al., 2009a), which increases in a non-linear fashion whilst GIP rises linearly (Meier et al., 2003). In both healthy and type 2 diabetic subjects, an initially more rapid delivery of glucose to the small intestine results in higher GIP, GLP-1 and insulin responses when compared to constant delivery of an identical glucose load (Figure 1.4) (O'Donovan et al., 2004). However this early greater insulin response is unable to compensate for the greater amount of absorbed glucose, so that there is no improvement in the overall glycaemic control, rendering this an unsuitable therapeutic strategy (O'Donovan et al., 2004, Chaikomin et al., 2005).

1.8 Modulation of gastric emptying to improve glycaemic control

The novel insights relating to the impact of gastric emptying on glycaemia have stimulated the development of dietary and pharmacological strategies to improve glycaemic control by modulating gastric emptying. Such strategies differ between patients with type 1 diabetes and patients with type 2 diabetes who are using insulin, as opposed to those with type 2 diabetes who are treated with oral hypoglycaemic agents and/or lifestyle modifications. In the former, treatment should aim to coordinate the delivery of nutrients - potentially by either slowing or accelerating gastric emptying with insulin delivery – but it is essential that gastric emptying is predictable to achieve a more stable glycaemic profile with less fluctuation. Thus in a select group of insulin-treated patients with recurrent postprandial hypoglycaemia, delayed gastric emptying can potentially be the cause of low blood glucose levels, and drugs which accelerate emptying may be of therapeutic benefit in these individuals. Certainly, measurement of gastric emptying is indicated in patients with potential ‘gastric’ hypoglycaemia (Horowitz and Nauck, 2006). In contrast, in type 2 patients who are not on insulin, a slower rate of nutrient delivery would be beneficial given the delay in insulin release and/or insulin resistance.

1.8.2 Dietary strategies

Non-pharmacological approaches for the management of type 2 diabetes include dietary strategies to slow gastric emptying by increasing dietary fibre

(Chandalia et al., 2000), addition of guar gum (Russo et al., 2003) and, more recently, the use of fat (Gentilcore et al., 2006a, Cunningham and Read, 1989) or protein ‘preloads’ taken before a meal (Ma et al., 2009b). The rationale of the latter strategy is to slow gastric emptying by stimulating small intestinal neurohumoral feedback mechanisms and stimulate the release of GIP and GLP-1 before the main meal (Gentilcore et al., 2006a, Ma et al., 2009b). Fat, a potent inhibitor of gastric emptying, when consumed in small amounts before or with a meal, has been shown to slow gastric emptying of other meal components and thus minimize the postprandial rise in blood glucose (Cunningham and Read, 1989). However only a modest suppression of the peak postprandial blood glucose level was observed (Gentilcore et al., 2006a), as opposed to the effects of an acute whey protein preload (Ma et al., 2009b), which in addition to delaying gastric emptying and stimulating GIP and GLP-1, also increases insulin secretion markedly, possibly via amino acids (Figure 1.5) (Ma et al., 2009b).

1.8.3 Pharmacological strategies

Pharmacological agents known to modify gastric emptying have been shown to affect glycaemic control acutely in patients with type 1 and 2 diabetes, including prokinetics and agents which slow emptying. There is evidence that erythromycin, in addition to accelerating gastric emptying as a result of its

motilin agonist properties, may stimulate insulin secretion, thus improving glycaemic control in type 2 diabetes (Ishii et al., 1997, Ueno et al., 2000).

Pramlintide, an amylin analogue, slows gastric emptying in healthy subjects (Samsom et al., 2000) and in type 1 and 2 diabetes (Vella et al., 2002), and its long term use is associated with an improvement in glycaemic control (Thompson et al., 1998, Thompson et al., 1997). GLP-1 analogues, such as exenatide and liraglutide, are now used therapeutically in the management of type 2 diabetes, and may augment the postprandial insulin response, as well as suppressing glucagon secretion and appetite. However, the main mechanism leading to the reduction in postprandial glycaemic excursions, at least in the case of exogenous GLP-1, exenatide, may be via retardation of gastric emptying (Little et al., 2006, Linnebjerg et al., 2008) with a significant correlation between the magnitude of the slowing of gastric emptying and the pre-existing rate of gastric emptying ie. the magnitude of the reduction in glycaemic excursions is less when there is pre-existing delay in gastric emptying (Linnebjerg et al., 2008). It has been suggested that there may be tachyphylaxis with sustained use of longer-acting GLP-1 analogues (such as exenatide LAR, as opposed to exenatide), resulting in diminution of their effects in slowing of gastric emptying (Drucker and Nauck, 2006).

Dipeptidyl-peptidase-4 (DPP-4) inhibitors increase plasma concentration of active GLP-1 and thus would be expected to slow gastric emptying, but the data to date are inconsistent and any effect on gastric emptying appears to be modest (Drucker and Nauck, 2006). This may potentially be accounted for by the effects of DPP-4 inhibitors on other gut hormones, such as PYY or ghrelin, which neutralize the effect of active GLP-1 elevation (Vella et al., 2008).

1.9 Management of symptomatic gastroparesis

The management of patients with symptomatic diabetic gastroparesis should focus on the relief of gastrointestinal symptoms, improvement in nutritional status, and optimization of glycaemic control. The latter is, of course, pivotal to a reduction in the risk of development, and progression, of micro and macrovascular complications.

1.9.1 Manipulation of pharmacological treatment for diabetes

Patients with type 2 diabetes frequently need insulin therapy in place of, or in addition to, oral hypoglycaemic agents, and type 1 patients may benefit from insulin pump therapy (O'Donovan et al., 2003, Sharma et al., 2011).

1.9.2 Dietary manipulation

Dietary recommendations include increasing the liquid content of meals, restricting fat and fibre intake, and eating a vitamised diet with small, frequent (4-6 per day) meals (Olausson et al., 2008), as well as avoiding alcohol, but none of these measures have been evaluated formally so their use is empirical.

1.9.3 Pharmacological agents

At present, prokinetic agents, including metoclopramide, erythromycin and domperidone, form the mainstay of treatment. These drugs accelerate gastric emptying by increasing antral contractility and improving the organisation of antropyloroduodenal motility (Khoo et al., 2009). The acceleration of gastric emptying by prokinetics is, in general, greater when the emptying at baseline is more delayed and, at least in the case of erythromycin (Jones et al., 1999), this effect is attenuated during acute hyperglycaemia (Sturm et al., 1999, Jones et al., 2001). In a systematic analysis of clinical trials of prokinetics, erythromycin appeared to be superior in accelerating gastric emptying and in relieving symptoms (Sturm et al., 1999), but its long term efficacy is limited by tachyphylaxis due to the down-regulation of motilin receptors, gastrointestinal adverse effects and, possibly, an increased risk of cardiac arrhythmias and death. Metoclopramide, when administered subcutaneously, appears to generate plasma concentrations comparable to those achieved via the intravenous route and is an option for those who cannot tolerate oral

medications. Central nervous system adverse effects are common and tardive dyskinesia, which may be irreversible, is a rare complication with its use. The FDA has recently issued a 'black box' warning in relation to the latter.

Metoclopramide appears to be less effective than cisapride, which has been largely withdrawn from clinical use due to its capacity to prolong the QT interval and lead to ventricular arrhythmias (Tonini et al., 1999). Domperidone is also effective at relieving symptoms, whilst not crossing the blood-brain barrier in significant quantities and may now be regarded as the current 'first-line' agent. Several drugs, including the motilin agonist, mitemincal (Takanashi and Cynshi, 2009), ghrelin receptor agonists (Ejskjaer et al., 2009, Murray et al., 2005), 5-HT₄-receptor agonists and the muscarinic antagonist, acotiamide (Parkman et al., 2010), are being investigated for their potential use.

1.9.4 Non-pharmacological therapy

A number of non-pharmacological treatments for diabetic gastroparesis have been explored. Intrapyloric botulinum toxin has been shown in randomized, controlled trials to have little, if any, effect to improve gastric emptying or symptoms (Arts et al., 2007, Friedenbergl et al., 2008) despite promising outcomes in earlier, uncontrolled studies (Lacy et al., 2002, Miller et al., 2002). Gastric electrical stimulation (GES) employs the use of electrodes implanted in the smooth muscle layer of the gastric wall, which are connected

to a subcutaneously located pulse generator. Two types of stimulation have been evaluated in humans, one using low frequency, long duration pulses at, or just above, the frequency of gastric slow wave of 3 pulses per minute, and the other using high frequency, short duration, pulses at about four times the slow wave frequency (12 per minute) (Rayner and Horowitz, 2005). The latter mode is commercially available as the Enterra™ device and benefits have been reported in several uncontrolled case series (McCallum et al., 1998, O'Grady et al., 2009, Abell et al., 2003). However, a recent double-blind trial with GES in diabetic gastroparesis showed initial improvement in the run-in 'on' phase, but no significant difference when the subsequent phase was randomized to 'on' or 'off' (McCallum et al., 2010), indicating the need for further evaluation before it can be recommended.

1.9.5 Surgical therapy

Benefits of surgical therapy for intractable gastroparesis remain uncertain, as case series have been uncontrolled and involve small numbers (Ejskjaer et al., 1999, Jones and Maganti, 2003). There are also uncontrolled observations of the benefit of pancreatic transplantation on gastric emptying (Gaber et al., 1991).

1.10 Conclusions

There have been major advances in knowledge about diabetic gastroparesis, of which a number were stimulated by the publication of the pivotal paper in 1986 (Horowitz et al., 1986) which has allowed a longitudinal evaluation of the prognosis and natural history of gastric emptying in diabetes after 12 years (Jones et al., 2002, Kong et al., 1999) and, subsequently, 25 years as reported in Chapters 3 and 4. With the recognition of the role gastric emptying plays in regulating postprandial glycaemia and the novel therapeutic use of exogenous GLP-1 in the treatment of type 2 diabetes, studies have suggested the use of glutamine as a potent stimulator of endogenous GLP-1 release to reduce postprandial glycaemia in patients with type 2 diabetes (Greenfield et al., 2009, Samocha-Bonet et al., 2011). However, it is not certain if the observed glucose lowering effect with glutamine is due to GLP-1 stimulated insulin release or to a slowing of gastric emptying and thus the study in Chapter 5 aims to provide some answers to the mechanism of the effects of glutamine. While numerous novel diagnostic and therapeutic strategies have been evaluated and implemented, there is still much to be understood about the complex and beguiling nature of disordered gastric emptying, which is now recognised to be inextricably linked to glycaemic control. The search for more effective treatments for diabetic gastroparesis represents an area of major research activity as therapy is frequently suboptimal.

Table 1.1 Adapted from Rayner, Horowitz 2005.

NOTE:

This table is included on page 45 of the print copy of the thesis held in the University of Adelaide Library.

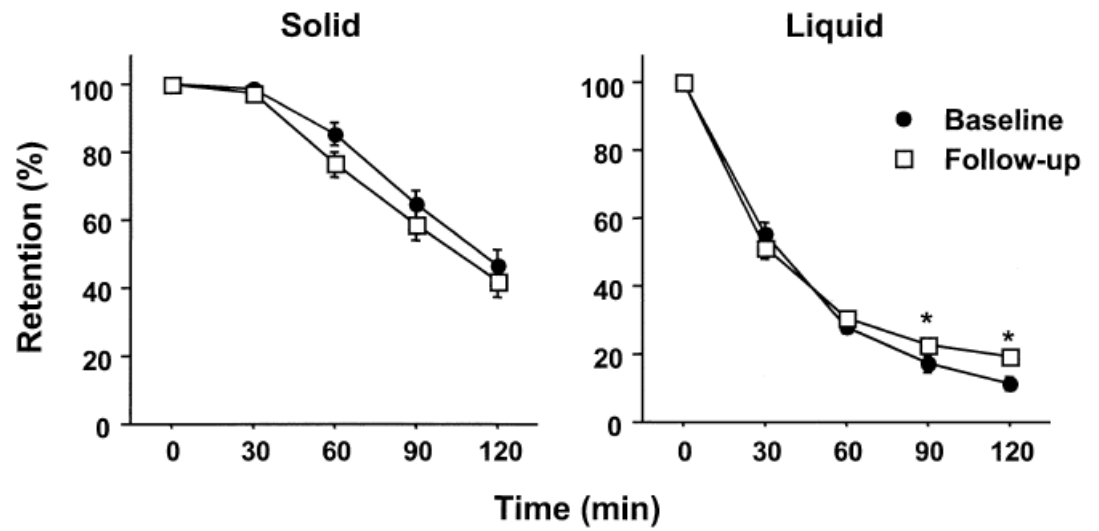


Figure 1.1 Gastric emptying (mean \pm SEM) of solid and liquid meal components measured at baseline and follow-up (12.3 ± 3.1 years) in 20 patients with diabetes mellitus. * $P < 0.05$ for baseline vs. follow-up by analysis of variance. Adapted from Jones et al., 2002 (Jones et al., 2002).

NOTE:

This figure is included on page 47 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 Plasma glucagon-like peptide 1 (GLP-1) concentrations after the ingestion of glucose (black circles), glutamine (white circles), and water (black squares) in (A) 8 lean subjects, (B) 8 obese nondiabetic control subjects, and (C) 8 obese individuals with type 2 diabetes or impaired glucose tolerance. Data are mean \pm SEM. * $P < 0.05$, † $P < 0.01$, and ‡ $P < 0.001$ compared with water (paired t test). Adapted from Greenfield et al., 2009 (Greenfield et al., 2009).

NOTE:

This figure is included on page 49 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.3 Blood glucose (A), plasma insulin (B), GLP-1 (C) and GIP (D) in response to intraduodenal glucose (25%, 1390 mOsmol/L) infused over 120 minutes at rates of 1 (G1), 2 (G2) or 4 (G4) kcal/min, or saline (4.2%, 1390 mOsmol/L) control (C) in 10 healthy males. (A) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (B) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (C) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. (D) * vs. control: $P < 0.05$, # vs. G1: $P < 0.05$, § vs. G2: $P < 0.05$. Data are means \pm SEM. Adapted from Pilichiewicz et al., 2007 (Pilichiewicz et al., 2007).

NOTE:

This figure is included on page 51 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4 Effects of initially more rapid intraduodenal glucose infusion (3kcal/min between t=0 and 15 min and 0.71kcal/min between t=15 and 120 min) (closed symbols) compared to constant infusion (1kcal/min between t=0 and 120 min) (open symbols) in healthy subjects (squares) and patients with type 2 diabetes (circles) on blood glucose, plasma insulin, plasma GLP-1 and plasma GIP. Each pair of curves differs between 0 and 30 min for variable vs. constant intraduodenal infusion ($P < 0.05$). Adapted from O'Donovan et al., 2004 (O'Donovan et al., 2004).

NOTE:

This figure is included on page 53 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.5 Gastric emptying (A), concentrations of blood glucose (B), plasma insulin (C), plasma GLP-1 (D) and plasma GIP (E) in response to a mashed potato meal in eight type 2 diabetic patients. On each study day, subjects ingested 350ml beef-flavoured soup 30min before a radiolabeled mashed potato meal; 55g whey protein was added to either the soup (whey 'preload') or no whey was given (no whey). Data are means \pm SEM. *P<0.05: whey 'preload' vs. whey in meal; #P<0.05: whey in meal vs. no whey; §P<0.05: whey 'preload' vs. no whey. Adapted from Ma et al 2009. (Ma et al., 2009b)

Dr Jessica Chang - Preparation of manuscript

A/Prof Chris Rayner – Correction of manuscript

Prof Karen Jones – Correction of manuscript

Prof Michael Horowitz – Correction of manuscript

I give permission for the review paper to be included in the thesis.

(Dr Jessica Chang)

(A/Prof Chris Rayner)

(Prof Karen Jones)

(Prof Michael Horowitz)

CHAPTER 2: METHODOLOGIES

2.1 Introduction

Various techniques were employed in the studies reported in this thesis, including measurement of gastric emptying by scintigraphy, intubation of the upper gut to deliver intraduodenal infusions and assess antropyloroduodenal motility, evaluation of cardiovascular autonomic nerve function by standardised tests, quantification of upper gastrointestinal symptoms and appetite by validated visual analogue scales and assays for measurement of glucose and gut hormones.

2.2 Subjects

For the study reported in Chapter 3, subjects were patients with longstanding diabetes who participated in initial studies conducted between 1984-9, who consisted of both male and female patients, who were firstly contacted by mail and followed by telephone contact if agreeable to participate in the study. In the study reported in Chapter 4, subjects consisted of both male and female patients with longstanding diabetes, who participated in the initial studies on gastric emptying conducted between 1984-1989. In the study reported in Chapter 5, healthy, male subjects were recruited by advertisement on University and Hospital notice boards whilst the patients with diabetes, were of either gender, had a shorter duration of diabetes, were well controlled on diet

alone and were recruited by advertisements on University and Hospital notice boards as well as diabetes magazines.

2.3 Gastric emptying

As discussed (1.4.1), scintigraphy is the ‘gold standard’ technique to quantify the rate of gastric emptying and intragastric distribution of solid and/or liquid meal components. In the studies described in Chapters 3 and 4, the solid component of the meal comprised of chicken liver labelled with 20 MBq of ^{99m}Tc sulphur colloid added to 100g minced beef that was subsequently grilled, to reproduce the method that was used in the baseline study performed in 1984-9 (Horowitz et al., 1986, Horowitz et al., 1989). The liquid phase was 150mL 10% dextrose (~60 kcal), labelled with 8 MBq ⁶⁷Ga-ethylene-diamine-tetraacetic acid (EDTA) in the study reported in Chapter 3 and labelled with 25-37 MBq of ^{113m}In-diethylene-triaminepentaacetic acid in the study reported in Chapter 4. The test meal was ingested between 0900-1000h, after an overnight fast, and the solid component was eaten within a 5-minute period followed by the dextrose, which was consumed within 30 seconds. The patient was seated, with the gamma camera positioned posteriorly. Data were acquired for 120 minutes and time zero (t=0) was defined as the time of meal completion. Upon completion of the dynamic acquisition, a static lateral image of the stomach was acquired to derive correction factors for gamma ray attenuation (Jones et al., 1997, Collins et al.,

1983). Radionuclide data were also corrected for subject movement, Compton scatter and decay (Collins et al., 1983). A region-of-interest was drawn for the total stomach and gastric emptying curves, representing percentage retention over time, were derived (Collins et al., 1983, Jones et al., 1997). For solids and liquids, the amount (%) remaining in the stomach at 30, 60, 90, and 120 minutes, the percentage remaining in the stomach at 100 minutes for solids (T100min), and the time taken for 50% of the liquid to empty (T50%), were quantified (Collins et al., 1983). Gastric emptying results were classified as normal or abnormal (accelerated or delayed) according to an established control range in healthy volunteers (solid retention at 100 minutes [12% to 61%] and liquid T50 [8 to 31 minutes]) (Horowitz et al., 1991, Collins et al., 1983).

2.4 Intraduodenal infusion and antropyloroduodenal pressure

Manometry is the most commonly utilised tool for assessing motility in the upper gut and was utilised in the study described in Chapter 5. The technique used involved the insertion of a multi-channel manometry catheter (Dentsleeve International Ltd, Ontario, Canada) through an anesthetized nostril into the stomach which was then allowed to move across the pylorus by peristalsis (Hedde et al., 1989). The catheter consisted of 7 sideholes positioned in the antrum at 1.5cm intervals, 6 sideholes positioned in the duodenum at 1.5cm intervals and a 4.5cm sleeve sensor, with 2 channels on the side opposite the

sleeve. The catheter also incorporated a channel that allowed for infusion of test solutions directly into the duodenum, opening ~12cm distal to the sleeve sensor. Correct positioning of the catheter was maintained by continuous measurement of the transmucosal potential difference (TMPD) at the most distal antral channel (~ -40mV) and the most proximal duodenal channel (~0mV) (Heddle et al., 1989). For this purpose, a catheter filled with sterile saline was placed subcutaneously in the forearm and was used as a reference electrode (Heddle et al., 1989). All manometry channels were perfused with degassed 0.9% saline (Heddle et al., 1989).

Manometry pressures were digitised and recorded on a computer-based system running commercially available software (Flexisoft, Oakfield Instruments of Oxford, UK), and stored for subsequent analysis. Manometric data were analysed using custom-designed software (Prof AJ Smout, University Medical Centre, Utrecht, The Netherlands) using accepted definitions (Samsom et al., 1998, Heddle et al., 1988) to determine the number of isolated pyloric pressure waves (IPPWs), and antral and duodenal pressure waves (sum of all waves recorded in the antral and duodenal channels respectively). In addition, pressure waves in the pylorus that occurred in isolation (isolated pyloric pressure waves or IPPWs), detected by either the sleeve sensor or one of the side holes between the antral and duodenal channels, were counted manually on the basis that they were (i) not associated with a pressure wave of any amplitude within 5 seconds in the most distal antral or most proximal duodenal

channels, and (ii) not recorded by more than one of the sideholes between the antral and duodenal channels (Hedde et al., 1988).

2.5 Autonomic nerve function

Autonomic nerve function was evaluated by standardised cardiovascular reflex tests (Ewing and Clarke, 1982). Parasympathetic function was evaluated by the variation (R-R interval) in the heart rate during deep breathing and the immediate heart rate response to standing (“30:15”). Sympathetic function was assessed by the fall in systolic blood pressure in response to standing. The result of each test was scored as 0 (normal), 1 (borderline), or 2 (abnormal). A total score of ≥ 3 was taken to indicate definite autonomic nerve dysfunction (Ewing and Clarke, 1982).

2.6 Upper gastrointestinal symptoms and appetite

Upper gastrointestinal symptoms were assessed by a validated questionnaire before commencement of gastric emptying measurement (Horowitz et al., 1991, Jones et al., 1995). ‘Gastric’ symptoms (anorexia, nausea, early satiation, abdominal bloating/fullness, vomiting, abdominal pain) and ‘oesophageal’ symptoms (dysphagia, heartburn, acid regurgitation) were graded as 0 (none), 1 (mild; the symptom could be ignored), 2 (moderate; the symptom could not be ignored, but did not influence daily activities), or 3

(severe; the symptom influenced daily activities). A total symptom score was calculated as the score for both 'gastric' and 'oesophageal' symptoms, with a potential maximum score of 27.

Visual analogue scale (VAS) questionnaires were used to assess appetite and gastrointestinal sensations in the study reported in Chapter 5. Each consisted of 13 different questions, followed by a 100mm horizontal line (Parker et al., 2004). For each question, the subject was asked to place a vertical mark along the line to indicate the strength of that sensation eg. 'How hungry do you feel?' within the range from 'weak' (to the left) to 'strong' (to the right) and scores were determined by measuring the distance from the starting point (on the left) to the mark. Sensations evaluated using VAS were hunger, desire to eat, fullness, nausea and projected amount of food that could be eaten.

2.7 Biochemistry/Hormones

For analysis of hormone concentrations, blood samples were collected into ice-chilled tubes containing EDTA (for GLP-1 and GIP) and ice-chilled serum tubes (for insulin). Plasma and serum were obtained by centrifugation at 3200rpm for 15 min at 4°C. Samples were then frozen at -80°C until analysis (Wishart et al., 1998, MacIntosh et al., 1999).

2.7.1 Blood glucose

Blood glucose concentrations were analysed immediately using a portable glucose meter (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). The accuracy of this method has been validated against the hexokinase technique (Horowitz et al., 1993).

2.7.2 Plasma GLP-1

Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK, Millipore, Billerica, MA). The minimum detectable limit was 3 pmol/L, and intra- and inter-assay coefficients of variation were 6.2% and 11.6% respectively (Ma et al., 2009b).

2.7.3 Plasma GIP

Plasma GIP was measured by radioimmunoassay modified from a previously published method (Wishart et al., 1992). The standard curve was prepared in buffer rather than extracted charcoal-stripped serum and the radioiodinated label was supplied by Perkin Elmer (Boston, MA). The minimum detectable limit was 2pmol/L, and intra- and inter-assay coefficients of variation were 7.7% and 16.3% respectively (O'Donovan et al., 2004).

2.7.4 Serum insulin

Serum insulin was measured by Enzyme-Linked Immunosorbent Assay (ELISA) (10-1113, Merckodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L and the intra- and inter-assay coefficients of variation were 1.6% and 9.3% respectively (O'Donovan et al., 2004).

2.8 Statistical analysis

All data in Chapter 3 and 5 are presented as mean \pm standard error of mean (SEM) whilst in Chapter 4, data are presented as median values and range. In Chapter 3, changes in gastric emptying and blood glucose levels between baseline and follow-up were evaluated using repeated measures analysis of variance. Linear regression analysis was used to assess relations between changes in gastric emptying and other variables. In Chapter 4, the Mann-Whitney U test was used to evaluate results in those who had died compared with those who were alive. The χ^2 test was used to evaluate the prevalence of delayed gastric emptying in these two groups. The Cox proportional hazards model for multivariate analysis was used when considering all-cause mortality (Cox, 1972). In Chapter 4, the incremental area under the curve (iAUC) was calculated using the trapezoidal rule (Wolever, 2004), subtracting baseline values for blood glucose, GLP-1, GIP and insulin. Blood glucose, GLP-1, GIP, insulin and antropyloroduodenal pressure waves were compared using one-factor analysis of variance (ANOVA) for the healthy subjects and paired t-tests

for the patients with diabetes, and comparisons between healthy and diabetic subjects were made using unpaired t tests. Visual analogue scores were evaluated using repeated measures ANOVA with treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant effects (Dunn, 1961). All analyses were performed using SPSS version 19 (IBM Corporation, Armonk, NY, USA) and significance calculated using the Dunn-Bonferroni correction (Dunn, 1961). A P value < 0.05 was considered significant.

2.9 Conclusions

All methods used in this thesis are well-established and validated, and are well tolerated by subjects. They represent the optimal techniques to address the hypotheses underlying each study.

CHAPTER 3: A 25 YEAR LONGITUDINAL EVALUATION OF GASTRIC EMPTYING AND GASTROINTESTINAL SYMPTOMS IN DIABETES MELLITUS

3.1 Summary

There is limited information about the natural history of gastric emptying and gastrointestinal symptoms in patients with longstanding diabetes and the author re-examined a selection of patients who had gastric emptying measurements 25 years ago. 13 patients (9 female, 4 male) with diabetes mellitus (12 type 1, 1 type 2) had measurements of gastric emptying of a mixed solid (100g minced beef)/ liquid (150mL 10% dextrose) meal using scintigraphy, mean blood glucose levels during the gastric emptying measurement, glycated haemoglobin, upper gastrointestinal symptoms (questionnaire) and autonomic nerve function (cardiovascular reflexes) at baseline and after a mean follow-up of 24.7 ± 1.5 years. There was no change in gastric emptying of either solids (% retention at 100 minutes) (baseline $58.5 \pm 5\%$ vs. follow-up $51.9 \pm 8\%$; $P=0.35$) or liquids (50% emptying time) (baseline 29.8 ± 3 minutes vs. follow-up 34.3 ± 6 minutes; $P=0.37$). 8 patients (62%) had delayed gastric emptying of solids at baseline and 5 (39%) at follow-up. Gastric emptying of solid at follow-up was related to emptying at baseline ($r=0.56$, $P<0.05$). Mean blood glucose concentrations during the gastric emptying measurement were lower at follow-up (baseline 17.7 ± 1.1 mmol/L vs. follow-up 12.8 ± 1.0 mmol/L; $P=0.006$), while there was a deterioration in autonomic function (baseline score 1.3 ± 0.4 vs. follow-up 3.2

± 0.4 ; $P=0.02$). There was no change in the score for upper gastrointestinal symptoms (baseline 3.1 ± 0.7 vs. follow-up 4.6 ± 1.3 ; $P=0.23$). In conclusion, in unselected patients with diabetes, both gastric emptying and upper gastrointestinal symptoms are stable over a period of 25 years.

3.2 Introduction

Disordered gastric emptying was once thought to be a relatively rare complication of diabetes, affecting predominantly patients with type 1 diabetes, that was predictable on the basis of upper gastrointestinal symptoms, associated with a poor prognosis and caused by irreversible, extrinsic autonomic neuropathy (Rundles, 1945). These concepts are now recognised to be incorrect. Gastric emptying is delayed in 30-50% of patients with longstanding type 1 and 2 diabetes (Horowitz et al., 1991, Horowitz et al., 1996, Horowitz et al., 2001, Jones et al., 1995, Samsom et al., 2003), and is sometimes accelerated (Bharucha et al., 2009), the relationship between upper gastrointestinal symptoms and the rate of gastric emptying is weak (Horowitz et al., 1991, Horowitz et al., 2002), and the pathogenesis of gastroparesis is complex and multifactorial (Chang et al., 2010, Kashyap and Farrugia, 2010, Camilleri et al., 2011). There is limited information about the natural history of gastric emptying, or gastrointestinal symptoms in diabetes. Our group reported that there was little, if any, change in either gastric emptying or symptoms over a period of 12 years, possibly because a deterioration in autonomic function

was counteracted by an improvement in glycaemic control (Jones et al., 2002). The author has now examined a selection of patients from the same cohort after a period of 25 years.

3.3 Methods

3.3.1 Subjects

13 patients (9 female, 4 male) with longstanding diabetes mellitus (12 with type 1 and 1 with type 2 diabetes) who had measurements of gastric emptying and other variables performed in 1984-9 (Horowitz et al., 1991, Horowitz et al., 1986, Horowitz et al., 1989) were studied. The time between the two measurements (mean \pm SD) was 24.7 ± 1.5 years. The patients were part of a cohort of 86 ambulatory outpatients (66 type 1, 20 type 2; 40 male, 46 female) who had attended the Royal Adelaide Hospital for management of diabetes mellitus in 1984-1989. Baseline (Horowitz et al., 1991, Horowitz et al., 1989, Horowitz et al., 1986) and longitudinal (Jones et al., 2002) measurements in this cohort have been reported, most recently in 2002 (Jones et al., 2002). Of the 86 patients, 29 were known to be dead, 53 were known to be alive and 4 had been lost to follow up. Of the 53 patients known to be alive, contact was made with 30 and, of these, 13 agreed to participate in the study, whilst the remaining 17 declined to participate mainly due to ill health, or having relocated geographically. None of the patients was taking medication known to affect gastrointestinal motility, or had a history of gastrointestinal surgery. Age

at baseline was 36.9 ± 8.6 years and body mass index was 25.0 ± 2.6 kg/m². At follow up, the mean age was 61.4 ± 7.9 years and the mean body mass index was higher at 28.3 ± 6.1 kg/m² ($P < 0.05$). The duration of known diabetes was 13.5 ± 7.5 years at baseline and 38.4 ± 7.8 years at follow-up.

3.3.2 Protocol

After an overnight fast of 14 hours for solids and 12 hours for liquids, each patient underwent measurements of gastric emptying of a mixed solid/liquid meal, upper gastrointestinal symptoms, glycaemic control and autonomic nerve function. Smoking was prohibited for 12 hours prior to, and on the study day, and in the one patient who had type 2 diabetes, oral hypoglycaemic medication was withheld until after the completion of the gastric emptying measurement. The patients with type 1 diabetes administered long acting insulin at the usual dose and time prior to the study, and injected their usual dose of short acting insulin immediately prior to the commencement of the gastric emptying measurement. An intravenous cannula was inserted into an antecubital vein for blood sampling. Written, informed consent was obtained from all subjects, and the study protocol for the follow-up measurements was approved by the Ethics Committee of the Royal Adelaide Hospital.

3.3.3 Measurements

Gastric emptying

A dual isotope test that measures emptying of solid and liquid meal components simultaneously was used to quantify gastric emptying (Horowitz et al., 1991, Jones et al., 1995, Collins et al., 1983) with the methodology described in Chapter 1.4.1. In the baseline (ie. 1984-1989) measurement, the liquid phase was labelled with 25-37 MBq of ^{113m}In -diethylene-triaminepentaacetic acid and at follow-up, with 8 MBq ^{67}Ga -ethylene-diamine-tetraacetic acid (EDTA). The test meal was ingested between 0900-1000h, and the solid component was eaten within a 5-minute period followed by the dextrose, which was consumed within 30 seconds. Each study was performed with the patient seated, with the gamma camera positioned posteriorly. Data were acquired for 120 minutes and time zero ($t=0$) was defined as the time of meal completion. Upon completion of the dynamic acquisition, a static lateral image of the stomach was acquired to derive correction factors for gamma ray attenuation (Jones et al., 1997, Collins et al., 1983). Radionuclide data were also corrected for subject movement, Compton scatter and decay (Collins et al., 1983). A region-of-interest was drawn for the total stomach and gastric emptying curves, representing percentage retention over time, were derived (Collins et al., 1983, Jones et al., 1997). For solids and liquids, the amount (%) remaining in the stomach at 30, 60, 90, and 120 minutes, the percentage remaining in the stomach at 100 minutes for solids (T100min), and the time taken for 50% of the liquid to empty (T50%), were quantified (Collins et al.,

1983). Gastric emptying results were classified as normal or abnormal (accelerated or delayed) according to an established control range in healthy volunteers (solid retention at 100 minutes [12% to 61%] and liquid T50 [8 to 31 minutes]) (Horowitz et al., 1991, Collins et al., 1983).

Glycaemic control

At baseline and follow-up, glycated haemoglobin (HbA1c) as well as plasma creatinine was measured using the initial venous sample. Blood glucose levels were determined using a portable blood glucose meter immediately prior to ingestion of the test meal and subsequently at 30, 60, 90 and 120 minutes (Jones et al., 2002).

Upper gastrointestinal symptoms

Upper gastrointestinal symptoms were assessed by a validated questionnaire before commencement of gastric emptying measurement (Horowitz et al., 1991, Jones et al., 1995). ‘Gastric’ symptoms (anorexia, nausea, early satiation, abdominal bloating/fullness, vomiting, abdominal pain) and ‘oesophageal’ symptoms (dysphagia, heartburn, acid regurgitation) were graded as 0 (none), 1 (mild; the symptom could be ignored), 2 (moderate; the symptom could not be ignored, but did not influence daily activities), or 3 (severe; the symptom influenced daily activities). A total symptom score was

calculated as the score for both ‘gastric’ and ‘oesophageal’ symptoms, with a potential maximum score of 27.

Autonomic nerve function

Autonomic nerve function was evaluated by standardised cardiovascular reflex tests, which were performed immediately after the gastric emptying measurement (Horowitz et al., 2002, Collins et al., 1983, Horowitz et al., 1991). Parasympathetic function was evaluated by the variation (R-R interval) in the heart rate during deep breathing and the immediate heart rate response to standing (“30:15”). Sympathetic function was assessed by the fall in systolic blood pressure in response to standing. The result of each test was scored as 0 (normal), 1 (borderline), or 2 (abnormal). A total score of ≥ 3 was taken to indicate definite autonomic nerve dysfunction (Horowitz et al., 1991, Horowitz et al., 2002, Jones et al., 1995).

3.3.4 Statistical analysis

Data were analysed by a professional biostatistician using SPSS version 19 and significance calculated using the Dunn-Bonferroni correction (Dunn, 1961). Changes in gastric emptying and blood glucose levels between baseline and follow-up were evaluated using repeated measures analysis of variance. Linear regression analysis was used to assess relations between changes in gastric

emptying and other variables. Data are shown as mean \pm SEM, unless otherwise stated. A P value < 0.05 was considered significant.

3.4 Results

All patients tolerated the study well, and none became hypoglycaemic during the measurements. There was no change in plasma creatinine between baseline and follow-up (0.085 ± 0.03 mmol/L vs. 0.082 ± 0.03 mmol/L, $P=0.52$) and creatinine was above the normal range in two patients at follow-up. In one patient, liquid was ingested as part of the test meal, but gastric emptying of liquid was not evaluated at follow-up because of unavailability of $^{67}\text{Ga-EDTA}$

3.4.1 Gastric emptying

Gastric emptying of solid was delayed (% retention at 100 minutes $> 61\%$) at baseline in 8 of 13 patients (62%), and in 5 of 13 patients (39%) at follow-up. Emptying of liquid ($T_{50} > 31$ minutes) was delayed in 6 of 12 patients (50%) at baseline, and in 8 of 12 patients (66.7%) at follow-up. One patient had abnormally rapid gastric emptying of solid at both baseline and follow-up and gastric emptying of solid was rapid at follow-up only in another. One subject exhibited abnormally rapid gastric emptying of liquid at follow-up. There was no change in gastric emptying of either solid, $n=13$, ($P= 0.35$), or liquid, $n=12$, ($P=0.37$) (Figure 3.1). There was a relationship between the baseline and

follow-up measurements of gastric emptying of solid ($r=0.56$, $P<0.05$), with a trend for the liquid component ($r=0.49$, $P=0.11$). The latter was significant ($r=0.82$, $P=0.002$) if one subject who had abnormally rapid emptying at follow-up was removed from the analysis (Figure 3.2).

3.4.2 Glycaemic control

Mean fasting blood glucose level was lower at follow-up (baseline 16.2 ± 1.0 mmol/L vs. follow-up 10.7 ± 1.3 mmol/L, $P=0.005$), as was the mean blood glucose level during the gastric emptying measurement (baseline 17.7 ± 1.1 mmol/L vs. follow-up 12.8 ± 1.0 mmol/L, $P=0.006$) (Figure 3.3). Glycated haemoglobin (HbA1c) was non-significantly lower at follow-up (baseline $8.1 \pm 0.7\%$ vs. follow-up $7.2 \pm 0.3\%$, $P=0.25$). There was a relationship between HbA1c and the mean blood glucose level during measurement of gastric emptying at baseline ($r=0.64$, $P=0.02$), but not at follow-up ($r=0.31$, $P=0.31$).

3.4.3 Upper gastrointestinal symptoms

11 patients (84.6%) had upper gastrointestinal symptoms at baseline and 12 patients (92.3%) at follow-up. There was no difference in either 'total' ($P=0.23$), 'gastric' ($P=0.28$) or 'oesophageal' ($P=0.61$) symptoms between baseline and follow-up (Table 3.1).

3.4.4 Autonomic nerve function

At follow-up, autonomic nerve function was not assessed in 1 patient who had atrial fibrillation. 3 of the 12 patients had evidence of autonomic neuropathy at baseline and 8 at follow-up; the total score was lower at baseline than follow-up (1.3 ± 0.4 vs. 3.2 ± 0.4 , $P=0.02$). There was a significant relationship between gastric emptying of solids and liquids and the score for autonomic function at baseline (solid retention $r=0.76$, $P=0.003$; liquid T50 $r=0.67$, $P=0.01$), but not at follow-up (solid $r=0.09$, $P=0.79$; liquid $r=-0.09$, $P=0.80$).

3.5 Discussion

This study represents the most prolonged longitudinal evaluation of gastric emptying and upper gastrointestinal symptoms in diabetes. After ~ 25 years, there was no change in gastric emptying of either solids and liquids, or upper gastrointestinal symptoms. There was a substantial deterioration in cardiovascular autonomic nerve function, but an improvement in glycaemic control, and a relationship between measurements of gastric emptying at baseline and follow-up. These observations are consistent with those derived from a previous longitudinal study (Jones et al., 2002) and suggest that in patients with diabetes, both gastric emptying and gastrointestinal symptoms are usually relatively stable over time.

The pathogenesis of delayed gastric emptying in diabetes has long been attributed to irreversible autonomic (vagal) neuropathy (Rundles, 1945). However, its aetiology is now recognised to be more complex and heterogeneous as discussed in Chapter 1.5. Studies have characterised the potential impact of both hyperglycaemia (Fraser et al., 1990, Rayner et al., 2001, Schvarcz et al., 1997) and hypoglycaemia (Russo et al., 2005) on gastric emptying – gastric emptying is slower during hyperglycaemia, including elevations in blood glucose that are within the normal postprandial range (Rayner et al., 2001, Fraser et al., 1990, Schvarcz et al., 1997), and accelerated during insulin-induced hypoglycaemia (Russo et al., 2005). Recent data, particularly those derived from the Gastroparesis Clinical Research Consortium (Grover et al., 2011), have demonstrated that in patients with intractable diabetic gastroparesis, there are heterogeneous cellular abnormalities, particularly involving the myenteric plexus. These include marked loss/dysfunction of interstitial cells of Cajal (He et al., 2001), reduced numbers of extrinsic autonomic neurons, deficiency of inhibitory neurotransmission (He et al., 2001), particularly nNOS (He et al., 2001), smooth muscle fibrosis (Ejskjaer et al., 1999) and disordered function of immune cells, with an abnormal immune infiltrate (Grover et al., 2011). Dysfunction of the interstitial cells of Cajal appears to be central to the pathogenesis of severe diabetic gastroparesis (Forster et al., 2005) and may be caused by increased oxidative stress (Chandrasekharan et al., 2011, Kashyap and Farrugia, 2011), reflecting a reduction in haeme-oxygenase-1 which

protects against oxidative injury (Choi et al., 2008, Kashyap et al., 2010). In the current study acute glycaemia was improved at follow-up, which would favour more rapid gastric emptying (Fraser et al., 1990, Schvarcz et al., 1997, Rayner et al., 2001) and, if sustained, as suggested by the mean fall in glycated haemoglobin, also a reduction in oxidative stress. Extrinsic autonomic impairment may also contribute to the pathogenesis of diabetic gastroparesis. The use of cardiovascular reflexes is, of course, only a surrogate marker of the function of the abdominal vagus (Ewing and Clarke, 1982) that has been shown to correlate weakly with the rate of gastric emptying in diabetes (Horowitz et al., 1991, Horowitz M, 1986) as was the case with the measurements at baseline. The absence of a relationship between autonomic dysfunction and gastric emptying at follow-up, in contrast to baseline, may reflect the high prevalence of the former.

It is of interest that gastric emptying at baseline and follow-up were related, certainly for solids and probably also for liquids. Hitherto, there has been limited information about the 'reproducibility' of gastric emptying in diabetes (Lartigue et al., 1994). Our observations suggest that, as is the case with healthy subjects (Collins et al., 1983), the inter-individual variation in gastric emptying in diabetes is much greater than the intra-individual variation. This may have particular relevance to the impact of gastric emptying on glycaemia (Chang et al., 2010), including the effects of drugs, such as the GLP-1

analogue exenatide, which improve glycaemic control, at least in part, by modulating gastric emptying (Linnebjerg et al., 2008).

It is now well-recognised that upper gastrointestinal symptoms in diabetes, while representing a substantial source of morbidity, correlate poorly with delayed gastric emptying and frequently remit and relapse (Horowitz et al., 1991, Jones et al., 1995, Horowitz et al., 1986, Talley et al., 2001b, Quan et al., 2008, Talley et al., 2002). As is the case with gastroparesis, the pathogenesis of symptoms is complex and heterogeneous. Specific symptoms may potentially relate to impairment in gastric relaxation, hyperglycaemia, intrinsic and extrinsic neural abnormalities, and gastric dysrhythmias (Owyang, 2011).

The limitations of this study should be recognised. The number of subjects studied was relatively small and, while selection bias cannot be excluded, the results appeared clear-cut ie. there was no trend for a change in either gastric emptying or upper gastrointestinal symptoms, autonomic function clearly deteriorated and the blood glucose level during the gastric emptying measurement was unequivocally less at follow-up. The latter is likely to reflect the increased attention given to optimisation of glycaemic control in diabetes subsequent to the outcome of the DCCT/EDIC (1993) and UKPDS (1998) studies. The non-significant reduction in glycated haemoglobin at follow-up (a significant fall was evident in the previous study) (Jones et al., 2002) is,

however, likely to represent a type 2 error. Gastric emptying in diabetes may be influenced by gender (Jones et al., 2001, Kashyap and Farrugia, 2010) but the size of the cohort studied was insufficient to evaluate this.

In summary, this prospective study indicates that gastric emptying and upper gastrointestinal symptoms in patients with long-term diabetes are relatively stable over time.

Table 3.1 Gastric emptying and upper gastrointestinal symptoms in 13 patients with diabetes at baseline and after ~25 years. Data are mean \pm SEM

Measurement	Baseline (n=13)	Follow-up (n=13)	P value
Gastric emptying			
Solid retention at 100 minutes (%)	58.5 \pm 5.2	51.9 \pm 8.0	0.35
Liquid T50 (min)	29.8 \pm 3.2*	34.3 \pm 5.5*	0.37
Gastrointestinal symptoms (scale)			
‘Gastric’ (0-18)	2.2 \pm 0.7	3.5 \pm 1.0	0.28
‘Oesophageal’ (0-9)	0.9 \pm 0.4	1.2 \pm 0.4	0.61
Total (0-27)	3.1 \pm 0.7	4.6 \pm 1.3	0.23

*n=12

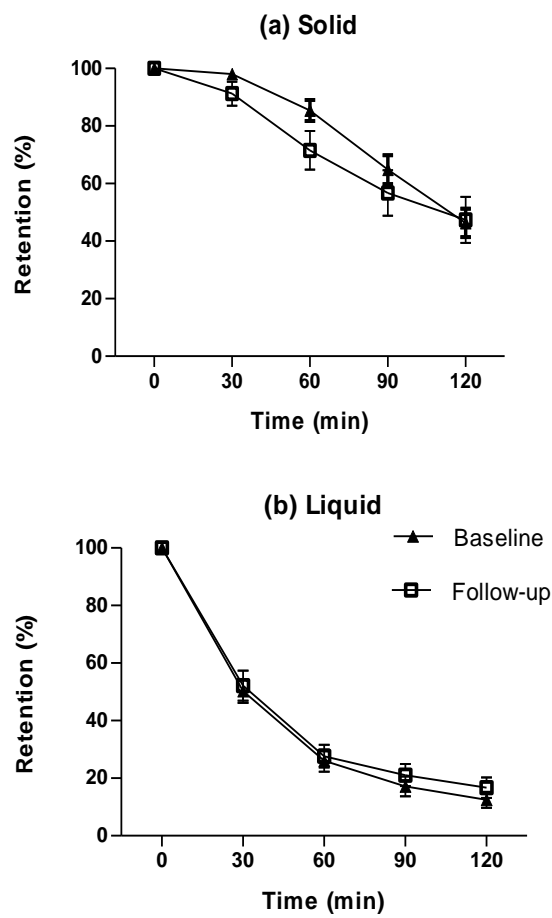


Figure 3.1 Gastric emptying of (a) solid (n=13) and (b) liquid (n=12), meal components measured at baseline and at follow-up (24.7 ± 1.5 years) in patients with diabetes mellitus. Data are mean \pm SEM.

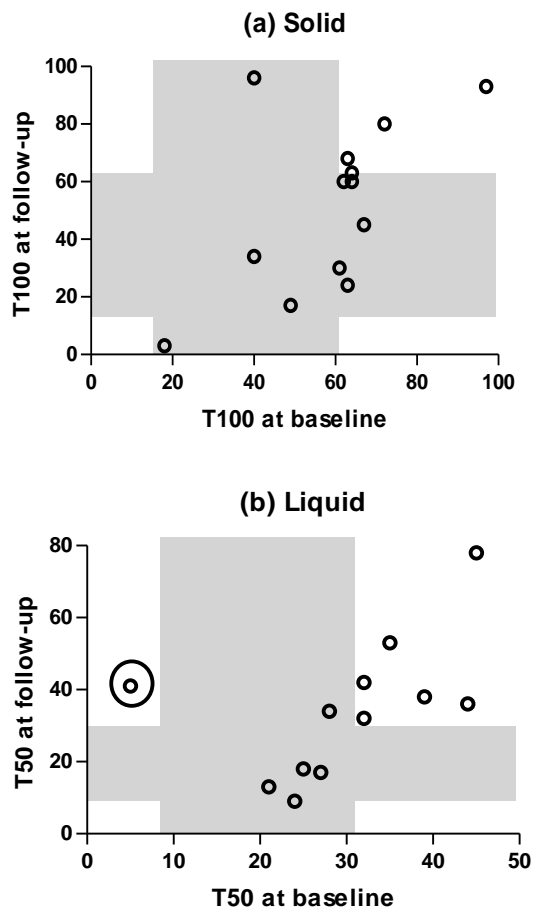


Figure 3.2 Relationships between gastric emptying of (a) solid ($r=0.56$, $P<0.05$), ($n=13$) and (b) liquid ($r=0.49$, $P=0.11$), ($n=12$) components at baseline and follow-up. The normal ranges are shown in the shaded areas. The relationship is significant for liquid emptying ($r=0.82$, $P=0.002$) if one data point (circled) is removed.

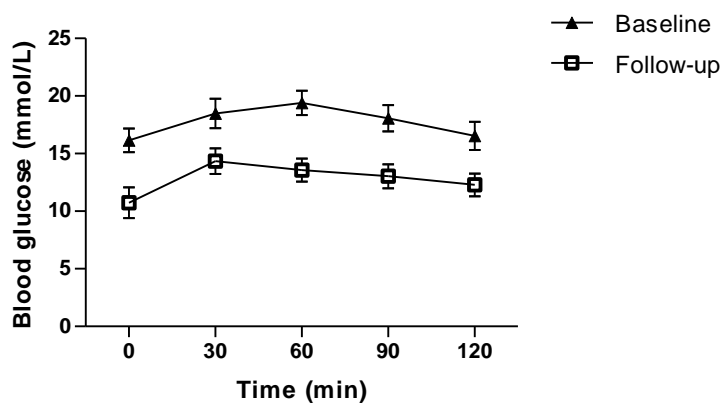


Figure 3.3 Blood glucose levels during measurement of gastric emptying at baseline and follow-up in 13 patients with diabetes mellitus. Data are mean \pm SEM, $P < 0.01$ for baseline versus follow-up by analysis of variance.

Dr Jessica Chang - Conducted study, including preparation of protocol, analysis of data and preparation of manuscript

Ms Antonietta Russo – Assisted in performance of study, including preparation of protocol and analysis of gastric emptying data

Ms Michelle Bound – Assisted in performance of study

A/Prof Chris Rayner – Supervision, including preparation of protocol, correction of manuscript

Prof Karen Jones – Supervision, including preparation of protocol, analysis of gastric emptying data, correction of manuscript

Prof Michael Horowitz – Supervision, including preparation of protocol, correction of manuscript and assumes overall responsibility for the manuscript

I approve the final draft submitted and give permission for the paper to be included in the thesis.

(Dr Jessica Chang)

(Ms Antonietta Russo)

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(Prof Karen Jones)

(Prof Michael Horowitz)

PROGNOSIS OF DIABETIC GASTROPARESIS – A 25 YEAR EVALUATION

4.1 Summary

There is limited information about the prognosis of diabetic gastroparesis. The author examined 86 patients (46 female, 40 male) with diabetes (66 type 1, 20 type 2) who had measurements of gastric emptying of a mixed solid (100g minced beef)/ liquid (150mL 10% dextrose) meal using scintigraphy, mean blood glucose levels during the gastric emptying measurement, glycated haemoglobin, upper gastrointestinal symptoms (questionnaire) and autonomic nerve function (cardiovascular reflexes) performed in 1984-1989. These patients were followed up in 2011, after a mean period of ~25 years. Of the 86 patients, gastric emptying of solid (percentage retention at 100 min, T100min) was delayed in 35 (41%) and of liquid (50% emptying time, T50) in 38 (44%). In 2011, 53 patients were known to be alive, 29 had died and 4 were lost to follow-up. In those who had died, both age at baseline ($P<0.001$) and the score for autonomic nerve dysfunction ($P<0.001$) were greater than those who were alive but there was no difference in either the solid T100min or liquid T50 between the two groups. When patients who had delayed gastric emptying were divided accordingly to the median value (ie. 'delayed' and 'markedly delayed'), mortality tended to be greater in the 'markedly delayed' group for both solids ($P=0.12$) and liquids ($P=0.09$). Of the 82 patients that could be followed-up, 23 of the 35 patients (66%) with delayed gastric emptying of solid and 25 of 38 (66%) with delayed gastric emptying of liquid were alive.

After adjustment for factors which were associated with the risk of dying, there was no association between either gastric emptying of solid or liquid and death. The author concludes that over a period of ~25 years, diabetic gastroparesis is usually not apparently associated with a poor prognosis, or a higher rate of mortality.

4.2 Introduction

Delayed gastric emptying in longstanding diabetes was once incorrectly presumed to be a rare condition, predictable on the basis of upper gastrointestinal symptoms and associated with a very poor prognosis (Rundles, 1945). Diabetic gastroparesis is now recognised to occur frequently and have a complex and multifactorial aetiology, which is not attributable solely to irreversible autonomic (vagal) neuropathy (Chang et al., 2011, Kashyap and Farrugia, 2010, Camilleri et al., 2011) and that the association between upper gastrointestinal symptoms with the rate of gastric emptying is weak (Horowitz et al., 1991, Horowitz et al., 2002). A study by Kong et al (Kong et al., 1999) followed-up a cohort of outpatients with longstanding type 1 or type 2 diabetes for an average of 9 years after they had their gastric emptying measured and found that diabetic gastroparesis was not associated with a higher rate of mortality, suggesting that diabetic gastroparesis is not associated with a poor prognosis. To further clarify this issue, we have now re-examined the same cohort after a mean period of 25 years.

4.3 Methods

4.3.1 Subjects

86 patients (46 female, 40 male) with longstanding diabetes mellitus (66 with type 1 and 20 with type 2 diabetes) and a median age of 46 years (range 18-77), BMI of 24.7 kg/m² (19.9-35.9) and duration of known diabetes of 15 years (1-49) were studied (Horowitz M, 1986, Horowitz et al., 1991, Horowitz et al., 1989). All patients were ambulatory and selected randomly from patients attending the Royal Adelaide Hospital for management of diabetes between 1984-1989 (Horowitz et al., 1986, Horowitz et al., 1989, Horowitz et al., 1991). None was taking medication known to affect gastrointestinal motility, or had a history of gastrointestinal surgery.

4.3.2 Protocol

Each patient underwent measurements of gastric emptying of a mixed solid/liquid meal, upper gastrointestinal symptoms, glycaemic control and autonomic nerve function in 1984-9. In 2011, each of the 86 patients was determined to be alive or dead by a combination of telephone contact, postal contact, review of electoral rolls and the Registry of Births, Deaths and Marriages. The study protocol was approved by the Ethics Committee of the Royal Adelaide Hospital.

4.3.3 Measurements

Gastric emptying

A dual isotope test that measures emptying of solid and liquid meal components simultaneously was used to quantify gastric emptying (Horowitz et al., 1991, Jones et al., 1995, Collins et al., 1983). The solid component of the meal comprised chicken liver labelled with 20 MBq of 99m technetium sulphur colloid added to 100g minced beef that was subsequently grilled. The liquid phase was 150mL 10% dextrose (~60 kcal) labelled with 25-37 MBq of 113m In-diethylene-triaminepentaacetic acid. Radionuclide data were corrected for subject movement, radionuclide gamma ray attenuation, Compton scatter and decay (Collins et al., 1983). For solids, the percentage remaining in the stomach at 100 minutes (T100min), and the time taken for 50% of the liquid to empty (T50%), were quantified (Collins et al., 1983). Gastric emptying results were classified as normal or abnormal (accelerated or delayed) according to an established control range (solid T100min [12% to 61%] and liquid T50% [8 to 31 minutes]) (Horowitz et al., 1991, Collins et al., 1983).

Glycaemic control

An indwelling cannula was inserted into an antecubital vein for blood sampling. Glycated haemoglobin (HbA1c) was measured on the initial venous sample and blood glucose levels determined using a portable blood glucose

meter immediately prior to ingestion of the test meal and subsequently at 30, 60, 90 and 120 minutes (Jones et al., 2002).

Upper gastrointestinal symptoms

Upper gastrointestinal symptoms were assessed by a validated questionnaire before commencement of the gastric emptying measurement (Horowitz et al., 1991, Jones et al., 1995). ‘Gastric’ symptoms (anorexia, nausea, early satiation, abdominal bloating/fullness, vomiting, abdominal pain) and ‘oesophageal’ symptoms (dysphagia, heartburn, acid regurgitation) were graded as 0 (none), 1 (mild; the symptom could be ignored), 2 (moderate; the symptom could not be ignored, but did not influence daily activities), or 3 (severe; the symptom influenced daily activities). A total symptom score was calculated as the score for both ‘gastric’ and ‘oesophageal’ symptoms, with a potential maximum score of 27.

Autonomic nerve function

Autonomic nerve function was evaluated by standardised cardiovascular reflex tests, which were performed immediately after the gastric emptying measurement (Horowitz et al., 2002, Collins et al., 1983, Horowitz et al., 1991). Parasympathetic function was evaluated by the variation (R-R interval) in the heart rate during deep breathing and the immediate heart rate response to

standing (“30:15”). Sympathetic function was assessed by the fall in systolic blood pressure in response to standing. The result of each test was scored as 0 (normal), 1 (borderline), or 2 (abnormal). A total score of ≥ 3 was taken to indicate definite autonomic nerve dysfunction (Horowitz et al., 1991, Horowitz et al., 2002, Jones et al., 1995).

4.3.4 Statistical analysis

Data were analysed by a professional biostatistician using SPSS version 19 (IBM Corporation, Armonk, NY, USA). The Mann-Whitney U test was used to compare data in those who had died compared with those who were alive. The χ^2 test was used to compare the prevalence of delayed gastric emptying in these two groups. To evaluate whether mortality was associated with the magnitude of delay in gastric emptying, patients with delayed emptying were divided at the median value into ‘delayed’ and ‘markedly delayed’ groups. In considering all-cause mortality, the Cox proportional hazards model for multivariate analysis was used (Cox, 1972). Data are shown as median values and range. A P value < 0.05 was considered significant.

4.4 Results

Of the 86 patients, gastric emptying of solid (percentage retention at 100 min) was delayed in 35 (41%) patients and of liquid (50% emptying time) in 38

(44%) patients. In 2011, of the 86 patients, 53 were known to be alive, 29 were known to be dead and 4 were lost to follow up. At the time of writing this thesis, the date of death was not available in all patients. In those who had died, the age at baseline ($P < 0.001$) and the scores for autonomic neuropathy ($P < 0.001$) were greater than those who were alive. There were no significant differences between the two groups in the other parameters, including gastric emptying of solid or liquid (Table 4.1). When gastric emptying of solid and liquid were classified as either normal or delayed, there was no association between the presence of delayed gastric emptying and death (solid: χ^2 0.031, $P = 0.86$; liquid: χ^2 0.041, $P = 0.84$). Mortality tended to be greater in patients with 'markedly delayed' compared to 'delayed' gastric emptying for both solids ($\chi^2 = 2.39$, $P = 0.12$) and liquids ($\chi^2 = 2.92$, $P = 0.09$). In the total group of 82 patients, 23 of the 35 patients (66%) with delayed emptying of solid and 25 of 38 patients (66%) with delayed emptying of liquid were still alive. After adjustment for other factors that were associated with the risk of dying, there was still no significant relationship between either gastric emptying of solid or liquid and the risk of death.

4.5 Discussion

This study establishes that delayed gastric emptying of solid or liquid, at least when the latter is not marked, is not associated with a higher likelihood of death in patients with longstanding diabetes. Over a period of ~25 years, the

majority of patients with delayed gastric emptying were still alive. There has hitherto been limited information about the natural history of diabetic gastroparesis and these observations are consistent with those of a previous study in which the same cohort was evaluated after 9 years (Kong et al., 1999).

The rate of death was predictably higher in those patients who were older and had cardiovascular autonomic dysfunction. The latter is known to be associated with increased mortality, perhaps particularly from cardiac arrhythmias, has been reported, even in those patients who are ‘asymptomatic’ (Vinik et al., 2003). The use of cardiovascular reflexes is, of course, only a surrogate marker of the function of the abdominal vagus (Ewing and Clarke, 1982) that has been shown to correlate weakly with the rate of gastric emptying in diabetes (Horowitz et al., 1991, Horowitz et al., 1986).

It should be recognised that the cohort studied was derived from a tertiary referral centre and cannot be considered representative of patients with diabetes living in the community. Moreover, acute hyperglycaemia is known to slow gastric emptying and mean blood glucose concentrations during the gastric emptying measurement were ~15mmol/L (Fraser et al., 1990). Hence, the prevalence of gastroparesis may have been overestimated (Choung et al., 2012).

However, at the time the initial studies were performed, the impact of glycaemia on gastric emptying had not been recognised. It should also be recognised that in many patients the magnitude of the delay in gastric emptying, while significant, was relatively modest, as has been documented in other studies (Ziegler et al., 1996, Samsom et al., 2003). It is of interest that mortality tended to be higher in those patients with ‘markedly delayed’ emptying and it would not be surprising if this was associated with a worse prognosis. It should also be recognised that our group did not include patients with intractable, symptomatic gastroparesis, which is known to be associated with substantial morbidity, including hospitalisation (Hyett et al., 2009).

Along with the established roles of irreversible autonomic (vagal) neuropathy (Rundles, 1945) and effect of hyperglycaemia (Schvarcz et al., 1997) in the pathogenesis of diabetic gastroparesis, recent data particularly from the Gastroparesis Clinical Research Consortium are indicative of heterogeneous cellular abnormalities, particularly involving the myenteric plexus, in the pathogenesis of this condition (Parkman et al., 2011, Grover et al., 2011). These include marked loss/dysfunction of interstitial cells of Cajal (He et al., 2001), reduced numbers of extrinsic autonomic neurons, deficiency of inhibitory neurotransmission (He et al., 2001), smooth muscle fibrosis (Ejskjaer et al., 1999) and disordered function of immune cells, with an abnormal immune infiltrate (Grover et al., 2011). Hence, it is not surprising

that the association of autonomic impairment with death was apparently independent of gastric emptying.

In summary, diabetic gastroparesis is not associated with a poor prognosis or a higher rate of mortality, at least in the majority of cases.

Table 4.1. Results at baseline (1984-1989) in 82 outpatients with diabetes known to be alive or deceased in 2011. Data are median values (range).

	Alive	Deceased	P value
n	53	29	-
Age at baseline (yr)	42 (18-66)	60 (24-77)	<0.001
Duration of diabetes (yr)	15 (1-32)	13 (2-49)	NS
HbA1c (%)	9.1 (4.2-16)	9.7 (3.6-13)	NS
Mean glucose concentration (mmol/L)	17.7 (7.7-29.7)	14.9 (6.0-24.2)	NS
Gastrointestinal symptoms (score)	2 (0-13)	3 (0-15)	NS
Autonomic nerve dysfunction (score)	1 (0-6)	4 (0-6)	<0.001
Solid retention at 100 minutes (%)	59 (0-97)	59 (28-100)	NS
Liquid 50% emptying time	30 (4-57)	30 (9-120)	NS

Dr Jessica Chang - Conducted study, including preparation of protocol, analysis of data and preparation of manuscript

A/Prof Chris Rayner – Supervision, including preparation of protocol, correction of manuscript

Prof Karen Jones – Supervision, including preparation of protocol, correction of manuscript

Prof Michael Horowitz – Supervision, including preparation of protocol, correction of manuscript and assumes overall responsibility for the manuscript

I approve the final draft submitted and give permission for the paper to be included in the thesis.

(Dr Jessica Chang)

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(Prof Michael Horowitz)

CHAPTER 5: EFFECTS OF INTRADUODENAL GLUTAMINE ON
INCRETIN HORMONE RELEASE, THE GLYCAEMIC RESPONSE TO AN
INTRADUODENAL GLUCOSE INFUSION AND
ANTROPYLORODUODENAL MOTILITY IN HEALTH AND TYPE 2
DIABETES

5.1 Summary

In patients with type 2 diabetes, oral glutamine stimulates glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), with a corresponding increase in insulin, and reduces postprandial glycaemia when given before a meal. However, it is uncertain whether the latter is mediated by stimulation of insulin and/or slowing of gastric emptying. The author aims to determine the effects of glutamine, delivered at a fixed rate into the duodenum, on GLP-1, GIP and insulin concentrations, antropyloroduodenal motor activity, and the glycaemic response to a subsequent intraduodenal glucose load, in healthy humans and patients with type 2 diabetes. 10 healthy subjects were studied on 3 days each in single-blind, randomised order. A multilumen manometry catheter was passed transnasally and positioned across the pylorus. Glutamine (7.5g or 15g) or saline control was infused into the duodenum over 30 min, followed by intraduodenal glucose (75g over the following 100min). Blood was sampled frequently to measure blood glucose, serum insulin, and plasma GLP-1 and GIP concentrations, and phasic pressures in the antrum, pylorus, and duodenum were monitored. 10 patients with diet-controlled type 2 diabetes

(T2DM) were studied in similar fashion on 2 days each (15g glutamine or saline control). Intraduodenal glutamine was associated with stimulation of GLP-1 (healthy: $P<0.05$; T2DM: $P<0.05$), and GIP (healthy: $P=0.098$; T2DM: $P<0.05$), and modest stimulation of insulin (healthy: $P=0.05$; T2DM: $P<0.01$). There was a trend for a reduction in glycaemia with 15g glutamine in the healthy subjects ($P=0.077$) but not in the patients with diabetes ($P=0.5$). Glutamine increased phasic pyloric pressure waves (healthy: $P<0.05$; T2DM: $P<0.05$), and suppressed antral waves in the patients with diabetes ($P<0.05$). In summary, intraduodenal glutamine in doses of 7.5g and 15g has minimal, if any, effect on the glycaemic response to intraduodenal glucose, despite its capacity to stimulate GLP-1, GIP and insulin release, but does stimulate phasic pyloric contractions. These observations suggest that delayed gastric emptying may be a major mechanism for the lowering of blood glucose evident when glutamine is given before an oral glucose load.

5.2 Introduction

Good glycaemic control, as assessed by glycated haemoglobin (HbA1c), is fundamental to reducing the incidence and progression of microvascular, and probably macrovascular, complications of diabetes mellitus (1993, Nathan et al., 2005, Stratton et al., 2000, 1998). It is now recognised that in most patients with type 2 diabetes, postprandial blood glucose makes a greater contribution to overall glycaemic control than fasting glucose and there has, accordingly,

been increasing interest in strategies to limit postprandial blood glucose excursions (Monnier et al., 2003, Ceriello et al., 2004).

Both the rate of gastric emptying (Chang et al., 2011, Ceriello et al., 2004), and the release of ‘incretin’ hormones subsequent to the exposure of the small intestine to nutrient (Horowitz and Nauck, 2006), are important determinants of postprandial glycaemic excursions. There is a substantial inter-, but relatively low, intra-individual variation in the rate of gastric emptying – in healthy subjects, glucose empties from the stomach at an overall rate of 1-4kcal/minute, as a result of inhibitory small intestinal feedback. Differences in gastric emptying account for about a third of the variation in peak postprandial blood glucose levels after oral glucose in both healthy individuals (Horowitz et al., 1993) and patients with type 1 (Horowitz M, 1986) and type 2 diabetes (Jones et al., 1996). Moreover, in type 2 patients, interventions that slow gastric emptying, such as the administration of morphine, reduce the glycaemic excursion after a standardised meal (Gonlachanvit et al., 2003). While the overall rate of gastric emptying depends on the integration of motor activity in each region of the stomach, the stimulation of pyloric motility may be the most important mechanism given that isolated pyloric pressure waves result in cessation of transpyloric flow (Tougas et al., 1992). Slower gastric emptying is also associated with suppression of antral and duodenal contractions (Horowitz et al., 1994).

When glucose is given by the oral route, the stimulation of insulin is markedly greater than with an isoglycaemic intravenous glucose infusion (Perley and Kipnis, 1967). This phenomenon is termed the 'incretin' effect and is attributable to the release of glucagon-like peptide-1 (GLP-1) from enteroendocrine L cells, and glucose-dependent insulintropic polypeptide (GIP) from K cells, which stimulate insulin secretion from the pancreas in the setting of elevated blood glucose levels (Holst and Gromada, 2004) and are responsible for ~70% of the postprandial insulin response in healthy humans (Horowitz and Nauck, 2006). The insulintropic effect of GIP is blunted in patients with diabetes, while that of GLP-1 remains relatively intact (Nauck et al., 1993, Vilsboll et al., 2002). Therefore, GLP-1 has hitherto been the focus of incretin-based therapies for diabetes, such as the GLP-1 analogues, exenatide and liraglutide, and dipeptidyl peptidase-4 inhibitors, such as vildagliptin and sitagliptin (Chang et al., 2010).

An alternative to the use of exogenous GLP-1 analogues is to develop dietary strategies to stimulate endogenous GLP-1 release. Using GLUTag cells as a model of the L cell, glutamine has been found to be the most potent amino acid for inducing GLP-1 release (Reimann et al., 2004). Glutamine is widely used as a nutritional supplement and is thought to maintain the integrity of the small intestinal mucosa (van der Hulst et al., 1993, Tremel et al., 1994). Oral

glutamine stimulates GLP-1 secretion in both healthy and diabetic individuals (Greenfield et al., 2009), and oral glutamine in doses of 15g and 30g, when given before oral glucose in patients with type 2 diabetes, dose-dependently stimulates GLP-1 release and reduces the subsequent glucose excursion, with a greater effect seen with 30g than 15g glutamine (Samocha-Bonet et al., 2011).

The beneficial effect of glutamine on the blood glucose profile may also be attributable to slowing of gastric emptying, regardless of any insulinotropic effect (Karamanlis et al., 2007). Samocha-Bonet et al noted in patients with type 2 diabetes that the improvement in blood glucose after glutamine preceded any elevation in serum insulin. Furthermore, since C-peptide concentrations were not increased by glutamine, the relevance of the increase in insulin was uncertain (Samocha-Bonet et al., 2011). Moreover, Lobo et al have reported that the addition of 15g glutamine slowed the rate of emptying of a carbohydrate drink (Lobo et al., 2009), and we have shown that slowing of gastric emptying is an important mechanism, at least acutely, in the lowering of postprandial glycaemia induced by protein 'preload' drinks (Ma et al., 2009b).

The purpose of the current study was to determine the effects of intraduodenal administration of glutamine on GLP-1, GIP and insulin secretion, and whether glutamine retains its capacity to lower glycaemia in response to a glucose load

delivered directly into the duodenum, thereby removing any influence of gastric emptying. We concurrently evaluated the effects of glutamine on antropyloroduodenal motor activity, in order to assess its potential to slow gastric emptying.

5.3 Methods

5.3.1 Subjects

Ten healthy volunteers and ten patients with diet-controlled type 2 diabetes were recruited. No subject was taking medication known to influence gastrointestinal function, was a smoker, or had a history of gastrointestinal disease. Demographic, anthropometric and fasting metabolic parameters of the study participants are shown in Table 5.1. The patients with diabetes were older and had a higher body mass index than the healthy subjects, but had good glycaemic control as measured by HbA1c. The study was approved by the Royal Adelaide Hospital Research Ethics Committee and conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983, and all subjects gave written, informed consent.

5.3.2 Protocol

All subjects attended the laboratory at 0830h following an overnight fast. The healthy subjects were studied on 3 days, and the diabetic patients on 2 days,

each separated by a minimum of 4 days. The order of the studies was randomised and single-blinded. An intravenous cannula was inserted into an antecubital vein for repeated blood sampling.

A multi-channel manometry catheter (Dentsleeve International Ltd, Ontario, Canada) was inserted through an anesthetized nostril into the stomach and allowed to move across the pylorus by peristalsis (Heddle et al., 1989). The catheter incorporated 7 side-holes positioned in the antrum at 1.5cm intervals, 6 side-holes positioned in the duodenum at 1.5cm intervals and a 4.5cm sleeve sensor, with 2 channels on the side opposite the sleeve. The catheter also incorporated a channel that allowed for infusion of test solutions directly into the duodenum, opening ~12cm distal to the sleeve sensor. Correct positioning of the catheter was maintained by continuous measurement of the transmucosal potential difference (TMPD) at the most distal antral channel (~ -40mV) and the most proximal duodenal channel (~0mV) (Heddle et al., 1989). For this purpose, a catheter filled with sterile saline was placed subcutaneously in the left forearm and used as a reference electrode (Heddle et al., 1989). All manometry channels were perfused with degassed 0.9% saline (Heddle et al., 1989).

Once the catheter was positioned correctly, the healthy subjects received an intraduodenal (ID) infusion containing either 15g or 7.5g glutamine in 350mL

aqueous solution (both matched for osmolality, ~ 300 mOsm/L, by the addition of sodium chloride), or 350mL of 0.9% saline (~ 300 mOsm/L) as a control, over 30min (t = 0 to 30 min). This was followed by an intraduodenal glucose infusion at 3 kcal/min over 100min (ie. t = 30 to 130 min) (3 mL/min of 25% solution, total load 75g). Blood was sampled at t = -2, 15, 30, 45, 60, 75, 90 and 130 min, and at the same intervals, subjects completed 100mm visual analogue questionnaires, to assess sensations of hunger, desire to eat, fullness, nausea and projected amount of food that could be eaten (Parker et al., 2004).

The patients with type 2 diabetes were studied under an identical protocol, except that only one dose of glutamine (15g) was given.

5.3.3 Measurements

Blood glucose

Blood glucose concentrations were analysed immediately using a portable glucose meter (Medisense Precision QID; Abbott Laboratories, Bedford, MA, USA). The accuracy of this method has been validated against the hexokinase technique (Horowitz et al., 1993).

Serum insulin, plasma GLP-1 and GIP concentrations

For analysis of hormone concentrations, blood samples were collected into ice-chilled serum tubes (for insulin), or tubes containing EDTA (for GLP-1 and GIP). Plasma and serum were obtained by centrifugation at 3200rpm for 15 min at 4°C. Samples were then frozen at -80°C until analysis.

Serum insulin was measured by ELISA immunoassay (10-1113, Merckodia, Uppsala, Sweden). The sensitivity of the assay was 1.0 mU/L and the intra- and inter-assay coefficients of variation were 1.6% and 9.3% respectively (O'Donovan et al., 2004).

Plasma total GLP-1 was measured by radioimmunoassay (GLPIT-36HK, Millipore, Billerica, MA). The minimum detectable limit was 3 pmol/L, and intra- and inter-assay coefficients of variation were 6.2% and 11.6% respectively (Ma et al., 2009b).

Plasma GIP was measured by radioimmunoassay modified from a previously published method (Wishart et al., 1992). The standard curve was prepared in buffer, rather than extracted charcoal-stripped serum and the radioiodinated label was supplied by Perkin Elmer (Boston, MA). The minimum detectable

limit was 2pmol/L, and intra- and inter-assay coefficients of variation were 7.7% and 16.3% respectively (O'Donovan et al., 2004).

Manometric analysis

Manometric pressures were digitised and recorded on a computer-based system running commercially available software (Flexisoft, Oakfield Instruments of Oxford, UK), and stored for subsequent analysis. Manometric data were analysed using custom-designed software (Prof AJ Smout, University Medical Centre, Utrecht, The Netherlands) using accepted definitions (Samsom et al., 1998, Hedde et al., 1988) to determine the number of isolated pyloric pressure waves (IPPWs), and antral and duodenal pressure waves (sum of all waves recorded in the antral and duodenal channels respectively) (Andrews et al., 2001).

Calculation of insulin sensitivity

The homeostasis model assessment - insulin resistance (HOMA-IR), an empirical mathematical formula based on fasting plasma glucose and fasting plasma insulin levels, was used to estimate insulin sensitivity (Matthews et al., 1985).

5.3.4 Statistical analysis

All data were analysed for two time periods - the duration of ID glutamine or saline infusion (t = 0-30min), and the duration of ID glucose infusion (t = 30-130min). The incremental area under the curve (iAUC) was calculated using the trapezoidal rule (Wolever, 2004), subtracting baseline values for blood glucose, GLP-1, GIP and insulin. Values were compared using one-factor analysis of variance (ANOVA) for the healthy subjects and paired t-tests in the patients with diabetes, and comparisons between healthy and diabetic subjects were made using unpaired t tests. Visual analogue scores were evaluated using repeated measures ANOVA with treatment and time as factors. Post hoc comparisons, adjusted for multiple comparisons by Bonferroni's correction, were performed if ANOVAs revealed significant effects (Dunn, 1961). All analyses were performed using SPSS version 19 (IBM Corporation, Armonk, NY, USA) and data are presented as mean values \pm standard error of mean. Statistical significance was accepted at $P < 0.05$.

5.4 Results

All subjects tolerated the study well, other than for one patient with diabetes who experienced marked nausea soon after the onset of the glutamine infusion; data from this patient were excluded from the analysis.

5.4.1 Blood glucose concentrations

Baseline blood glucose concentrations did not differ between the study days in either the healthy or diabetic subjects.

In the healthy subjects, blood glucose concentrations were unchanged during ID glutamine/saline infusion ($t = 0-30\text{min}$). During ID glucose infusion ($t = 30-130\text{min}$), blood glucose concentrations increased with all treatments to $\sim 9\text{mmol/L}$. There was a trend for the increment to be less after 15g glutamine than either 7.5g glutamine or saline ($P=0.077$) with the trend for modest glucose lowering effect occurring early, ie. $\sim 60-90\text{min}$ (Table 5.2, Figure 5.1A).

In the patients with diabetes, blood glucose concentrations were not affected by ID glutamine, and increased during ID glucose infusion to $\sim 15\text{mmol/L}$, but without any difference between treatments (Table 5.2, Figure 5.2A).

Both the fasting blood glucose concentrations ($P<0.01$) (Table 1), and the iAUC during ID glucose infusion ($t=30-130\text{min}$) ($P<0.01$) (Table 5.2) were greater in the patients with diabetes than the healthy subjects, after both saline and 15g glutamine.

5.4.2 Plasma GLP-1 concentrations

In the healthy subjects, plasma GLP-1 concentrations increased during ID glutamine infusion (t = 0-30min), with a greater increment observed with 15g glutamine than with 7.5g glutamine or saline (iAUC, P=0.05), evident at t = 30min, but no difference between the latter two. During ID glucose infusion (t=30-130 min), plasma GLP-1 concentrations increased further, with a greater increment observed with 15g glutamine than 7.5g glutamine (iAUC, P<0.05), but no significant difference between either glutamine dose and saline (Table 5.2, Figure 5.1B).

In the patients with diabetes, plasma GLP-1 concentrations increased during ID glutamine infusion (t = 0-30min), so that the increment was greater than for saline (iAUC, P<0.05), evident at t = 30min. During ID glucose infusion (t = 30-130 min), plasma GLP-1 concentrations increased after both treatments, with a trend for a greater increment after 15g glutamine (iAUC, P=0.056) (Table 5.2, Figure 5.2B).

Fasting GLP-1 concentrations were slightly higher in the patients with diabetes than the healthy subjects (P< 0.05) (Table 5.1), but there was no difference in iAUC between healthy and diabetic subjects during ID glutamine/saline

infusion (t = 0-30min) or ID glucose infusion (t = 30-130min), for either saline or glutamine studies (Table 5.2).

5.4.3 Plasma GIP concentrations

In the healthy subjects, there was a non-significant trend for an increase in plasma GIP during ID glutamine infusion (t = 0-30min), (iAUC, P=0.098).

During ID glucose infusion (t=30-130min), plasma GIP concentrations increased with all treatments, without any difference between them (Table 5.2, Figure 5.1C).

In the patients with diabetes, plasma GIP concentrations increased during ID glutamine infusion (t = 0-30min), so that the increment was greater than for saline (iAUC, P<0.05). During ID glucose infusion (t = 30-130min), plasma GIP increased in both treatment groups, without any difference between them (Table 5.2, Figure 5.2C).

Fasting GIP concentrations were higher in the patients with diabetes than the healthy subjects (P<0.01) (Table 5.1), but there was no difference in the iAUC between the healthy subjects and the patients with diabetes for GIP during ID glutamine/saline infusion (t = 0-30min) or ID glucose infusion (t = 30-130min), for either saline or glutamine studies (Table 5.2).

5.4.4 Serum insulin

In the healthy subjects, serum insulin increased slightly during ID glutamine infusion (t = 0-30min) (iAUC, P=0.05), with no difference between either 15g or 7.5g glutamine and saline, or between the latter two. During ID glucose infusion (t=30-130min), insulin concentrations increased in all studies, without any difference between any treatment (Table 5.2, Figure 5.1D). Nor was there any difference in the insulin: glucose ratio between any treatment during this period.

In the patients with diabetes, serum insulin increased very slightly during ID glutamine infusion (t = 0-30min) (iAUC, P<0.01), most evident at t = 30min. During ID glucose infusion (t = 30-130min), serum insulin and the insulin: glucose ratio increased during both treatments, but with a greater increment after 15g glutamine (iAUC, P<0.05 for each) (Table 5.2, Figure 5.2D).

Fasting insulin concentrations were higher in the patients with diabetes than the healthy subjects (P<0.05) (Table 5.1), but there was no difference in the iAUC during ID glutamine/saline infusion (t = 0-30min) or ID glucose infusion (t = 30-130min) between the healthy and diabetic subjects, for either the saline or glutamine studies (Table 5.2). HOMA-IR scores were higher in the patients with diabetes than in the healthy subjects (2.1 ± 0.4 vs. 0.7 ± 0.1 ; P<0.01) (Table 5.1).

5.4.5 Isolated pyloric pressure waves (IPPWs)

In the healthy subjects, there were more IPPWs during 15g glutamine infusion than during either 7.5g glutamine or saline, with no difference between the latter two ($t = 0-30\text{min}$, treatment effect $P < 0.05$). During ID glucose infusion ($t = 30-130\text{ min}$), the number of IPPWs increased on each study day, without any difference between the treatments (Figure 5.3A).

Similarly, in the patients with diabetes, there were more IPPWs during 15g glutamine infusion than during saline infusion ($t = 0-30\text{min}$, $P < 0.05$), and no difference between treatments during the ID glucose infusion ($t = 30-130\text{min}$) despite a stimulation of IPPWs on both study days during the latter period (Figure 5.4A).

There was no difference in the number of IPPWs between the healthy subjects and the patients with diabetes during ID glutamine/saline infusion ($t = 0-30\text{min}$), for either the saline or 15g glutamine studies, but there were more IPPWs in the patients with diabetes than the healthy subjects during ID glucose infusion ($t = 30-130\text{min}$), for both the saline ($P < 0.05$) and 15g glutamine ($P < 0.05$) studies (Table 5.2).

5.4.6 Antral pressure waves

In the healthy subjects, there was no difference in the number of antral pressure waves between the treatments during ID glutamine/saline infusion (t = 0-30min). During ID glucose infusion (t = 30-130min), there were fewer antral waves than during glutamine/saline infusion on each study day, without any difference between the treatments (Figure 5.3B).

In the patients with diabetes, there were fewer antral pressure waves during glutamine than saline infusion (t = 0-30min) ($P < 0.05$). During ID glucose infusion (t = 30-130min), once again there was a suppression of antral waves on each study day, without any difference between the treatments (Figure 5.4B).

There was no difference in the number of antral waves between healthy subjects and patients with diabetes during ID glutamine/saline infusion (t = 0-30min) or ID glucose infusion (t=30-130min), for either the saline or 15g glutamine studies (Table 5.2).

5.4.7 Duodenal pressure waves

In the healthy subjects, there was no difference in the number of duodenal pressure waves between any of the treatments during ID glutamine/saline infusion (t = 0-30min). During ID glucose infusion, there were fewer duodenal waves than during glutamine/saline infusion on each study day, without any difference between the treatments (Figure 5.3C).

In the patients with diabetes, there was no difference in the number of duodenal pressure waves during either the ID glutamine/saline infusion (t = 0-30min) or ID glucose infusion (t = 30-130min), despite a suppression in the number of duodenal waves during ID glucose infusion for both studies (Figure 5.4C).

There was no difference in the number of duodenal waves during ID glutamine/saline infusion (t = 0-30min) or ID glucose infusion (t = 30-130min) between the healthy subjects and the patients with diabetes, for either the saline or 15g glutamine studies (Table 5.2).

5.4.8 Gastrointestinal sensations

In the healthy subjects, there were no differences in nausea, desire to eat, fullness, hunger or the projected amount that could be eaten between the different treatments during ID glutamine/saline infusion (t = 0-30min) or during ID glucose infusion (t = 30-130min) (Figure 5.5A).

In the patients with diabetes, 15g glutamine was associated with suppression of hunger during ID glutamine infusion (t = 0-30min) when compared with saline (treatment effect, $P < 0.05$), but there was no difference between the treatments during ID glucose infusion (t = 30-130min), and no difference between treatments for any other sensation (Figure 5.5B).

5.5 Discussion

In this study, we demonstrated that 15g of glutamine delivered intraduodenally stimulates GLP-1 secretion in both healthy subjects and patients with type 2 diabetes, associated with modest stimulation of insulin secretion. There was, however, no effect of glutamine on the blood glucose response to a subsequent intraduodenal glucose load in either healthy subjects or patients with type 2 diabetes. Intraduodenal glutamine stimulated phasic pyloric pressure waves, indicating that it is capable of generating feedback on gastric motor function that would delay gastric emptying. The effects of intraduodenal glutamine on

hormone secretion and motility appear to be dose-dependent, as in healthy subjects a dose of 7.5g had no effect.

The duration of glutamine infusion in the current study was selected on the basis that GLP-1 release after oral glutamine was maximal at $t = 30\text{min}$ (Greenfield et al., 2009). In pilot studies, we found that 30g glutamine infused intraduodenally over this period was poorly tolerated in healthy volunteers due to nausea and we therefore used a maximum dose of 15g in the current study. The amount of glucose infused was identical to that used by Greenfield et al in their study relating to the effects of oral glutamine.

Our finding of GLP-1 stimulation with glutamine in healthy subjects and patients with type 2 diabetes is consistent with reports from Greenfield et al (Greenfield et al., 2009) and Samocha-Bonet et al (Samocha-Bonet et al., 2011), and in vitro studies of Reimann et al, in which glutamine stimulated the release of GLP-1 from GLUTag cells (Reimann et al., 2004). The magnitude of the GLP-1 response to 15g intraduodenal glutamine in our study (peak $\sim 50\text{pmol/L}$) appeared somewhat greater than after 15g oral glutamine ($\sim 35\text{pmol/L}$) reported in patients with type 2 diabetes (Samocha-Bonet et al., 2011), while the onset of stimulation occurred after a similar interval (~ 30 minutes). The latter appears a little longer than the interval seen for GLP-1 stimulation after glucose (~ 15 minutes) (Pilichiewicz et al., 2007), suggesting

that exposure of L cells in more distal regions of the small intestine may be required. We found that fasting GLP-1 concentrations were slightly higher in patients with type 2 diabetes, but the incremental increase in GLP-1 concentrations were comparable in patients with type 2 diabetes and healthy subjects, implying that the GLP-1 response to glutamine and glucose is not impaired in patients with well-controlled type 2 diabetes, managed by diet alone, consistent with our previous observations (Ma et al., 2011). This is in contrast to studies which reported a 20-30% reduction in postprandial GLP-1 concentrations after a mixed meal in patients with type 2 diabetes compared with healthy subjects (Toft-Nielsen et al., 2001, Vilsboll et al., 2001), although not all postprandial studies have indicated such a deficit (Ryskjaer et al., 2006, Vollmer et al., 2008).

Fasting GIP concentrations were higher in patients with type 2 diabetes, but the incremental increases in GIP concentrations in response to glutamine and glucose were comparable to those seen in healthy subjects, implying that the secretion of GIP is not impaired in patients with type 2 diabetes who are well controlled on diet alone. We also observed that glutamine-stimulated GIP release reached statistical significance only in the patients with diabetes. This finding is consistent with other studies which have shown glutamine to be a relatively weak stimulus of GIP secretion (Greenfield et al., 2009), and that its release is stimulated predominantly by carbohydrate and fat (Meier and Nauck, 2004).

In both the healthy subjects and the patients with diabetes, we observed a statistically significant, but quantitatively modest, stimulation of insulin in response to glutamine. In the healthy subjects, any stimulation of insulin did not extend beyond the period of glutamine infusion, so that the insulin: glucose ratio during intraduodenal glucose administration was not increased over the control day. In the patients with diabetes, both serum insulin and the insulin: glucose ratio were higher after glutamine than control, during the period of intraduodenal glucose infusion. This might reflect the fact that the patients had higher blood glucose concentrations than the healthy subjects, increasing the potential for GLP-1 to stimulate insulin secretion. Despite this, there was no significant effect of glutamine to lower blood glucose concentrations compared to control, even in the diabetic patients. As noted previously by Samocha-Bonet et al (Samocha-Bonet et al., 2011), it is possible that raised insulin levels following glutamine in patients with type 2 diabetes reflect, at least in part, an effect on insulin clearance, rather than stimulation of insulin secretion, although unlike Samocha-Bonet et al, we did not measure C-peptide to support this assertion. As expected, our patients with diabetes had evidence of insulin resistance in that their fasting insulin concentrations were substantially higher than those in the healthy subjects, with correspondingly higher HOMA-IR scores.

We observed no effect on blood glucose excursions after glutamine in either the healthy subjects or the patients with diabetes, despite the fact that glutamine stimulated GLP-1 release in both groups. This could be due to the fact that incretin stimulation by glutamine was relatively short-lived, being evident only during the administration of glutamine, and was, therefore, insufficient to suppress the glycaemic excursion following subsequent intraduodenal glucose. Furthermore, amino acids including glutamine have been shown to potentiate glucagon release in dogs (Barrett et al., 1986) and humans (Greenfield et al., 2009, Samocha-Bonet et al., 2011), and any stimulation of glucagon secretion would counterbalance the potential glucose-lowering effect of GLP-1. A limitation of the current study is that we did not measure glucagon concentrations.

The most important difference from previous studies, which have demonstrated a substantial glucose-lowering effect of glutamine, was that in our design, glucose was infused directly into the duodenum at a fixed rate, thereby removing any influence on the outcome of the potential of glutamine to slow gastric emptying. Indeed, our evaluation of antropyloroduodenal motility during intraduodenal glutamine infusion established both stimulation of phasic pyloric contractions and suppression of antral motility, a pattern that is associated with slowing of gastric emptying (Hedde et al., 1988). This motor pattern in response to intraduodenal glutamine could be mediated by GLP-1, or other hormones such as cholecystokinin or peptide YY, which we

did not measure, or could reflect stimulation of neural feedback mechanisms from the small intestine. During intraduodenal glucose infusion, the patients with diabetes had more IPPWs than the healthy subjects; this might be due to their higher blood glucose concentrations (Fraser et al., 1991), although the number of subjects in the current study was too small to evaluate this hypothesis statistically. Our findings are in agreement with the observation that in healthy humans, the addition of glutamine slows gastric emptying of carbohydrate (Lobo et al., 2009). A limitation of previous studies demonstrating a reduction in postprandial glycaemia with oral glutamine in patients with diabetes (Samocha-Bonet et al., 2011) is that they have not measured, or controlled for, the rate of gastric emptying. We would postulate that individuals who exhibit the greatest slowing of gastric emptying after glutamine also have the greatest reduction in postprandial glycaemia.

Our study has several limitations. The number of subjects studied was relatively small, with a different gender distribution between the healthy subjects and the patients with diabetes; however, the effects examined were consistent between subjects, and it is unlikely that an increase in the number of subjects would have yielded substantially different outcomes. Secondly, it is unclear whether the effects of glutamine are specific for that amino acid, and it would be of interest to make a comparison with another amino acid that has little, if any, effect on incretin hormone release. Thirdly, as discussed, the measurement of plasma glucagon concentrations would be helpful to confirm

the potential for this hormone to have counterbalanced the effects of GLP-1, GIP and insulin on blood glucose concentrations. Fourthly, it is possible that a higher dose of glutamine may be more efficacious in lowering blood glucose, given previous reports of the effects of 30g doses (Samocha-Bonet et al., 2011, Greenfield et al., 2009), and the fact that the effects appeared to be dose-dependent in our study. However, as mentioned above, it would have been difficult to infuse a substantially higher dose given that intraduodenal administration of 30g glutamine over 30 minutes induced nausea. While the mechanism underlying this phenomenon is uncertain, it could potentially be due to the caloric rate of infusion (4kcal/min) being at the extreme of the physiological range. It is unclear whether the observed GLP-1 stimulation by glutamine represents direct or indirect stimulation of L cells, and it would therefore also be of interest to infuse glutamine more distally into the small intestine, where the density of L cells responsible for GLP-1 secretion is greater. Finally, our patients with diabetes were well controlled and managed by diet alone. Further studies would be required to determine whether glutamine maintains its capacity to stimulate endogenous incretins in patients with poorly controlled diabetes.

In summary, we have demonstrated that intraduodenal glutamine in a dose of 15g stimulates both GLP-1 and GIP secretion in patients with well controlled type 2 diabetes, but that its ability to lower subsequent blood glucose concentrations substantially is likely to be dependent on slowing of the

emptying of a subsequent glucose load. Further studies are indicated to establish the optimum dosing of glutamine, its effects in type 2 patients with less well controlled diabetes, and whether it has a place in the long-term management of this condition.

Table 5.1 Demographic, anthropometric and fasting metabolic parameters of the study participants. Data are mean \pm SEM.

	Healthy	Diabetic
Gender	10 males	5 males, 5 females
Age (yr)	29.5 \pm 3.8	68 \pm 1.1
BMI (kg/m ²)	22.6 \pm 0.7	28.9 \pm 1.1
HbA1c (%)	not measured	6.7 \pm 0.2
Fasting blood glucose concentration (mmol/L)	5.5 \pm 0.1	6.8 \pm 0.3 **
Fasting plasma GLP-1 concentration (pmol/L)	19.6 \pm 2.6	31.8 \pm 4.0 *
Fasting plasma GIP concentration (pmol/L)	11.2 \pm 1.1	17.1 \pm 1.7 **
Fasting serum insulin concentration (mU/L)	3.0 \pm 0.4	6.7 \pm 1.3 *
HOMA-IR	0.7 \pm 0.1	2.1 \pm 0.4 **

*P<0.05 for comparison of healthy subjects vs. patients with type 2 diabetes

**P<0.01 for comparison of healthy subjects vs. patients with type 2 diabetes

Table 5.2 Incremental areas under the curve for blood glucose, plasma GLP-1, plasma GIP and serum insulin concentrations for t = 0-30 min and t = 30-130 min in healthy subjects (n=10) and patients with type 2 diabetes mellitus (T2DM) (n=9). Data are mean \pm SEM.

	saline		7.5 g glutamine		15 g glutamine	
	Healthy	T2DM	Healthy	Healthy	T2DM	
Glucose iAUC0-30 (mmol/L \times min)	2.6 \pm .7	4.4 \pm 1.3	2.6 \pm 1.7	1.0 \pm 0.6	5.7 \pm 4.3	
Glucose iAUC30-130 (mmol/L \times min)	278.4 \pm 42.5	535.2 \pm 37.9 ⁹	251.9 \pm 43.4	221.5 \pm 43.5 ¹	473.2 \pm 57.6 ⁹	
GLP-1 iAUC0-30 (pmol/L \times min)	51.4 \pm 23.1	37.0 \pm 27.1	45.1 \pm 12.7	127.1 \pm 33.1 ²	84.6 \pm 19.3 ⁵	
GLP-1 iAUC30-130 (pmol/L \times min)	1242 \pm 369	1596 \pm 534	1099 \pm 354	1715 \pm 440 ³	1673 \pm 496 ⁶	
GIP iAUC0-30 (pmol/L \times min)	45.9 \pm 39.6	10.8 \pm 7.0	64.0 \pm 16.9	74.3 \pm 16.9 ⁴	110.3 \pm 36.6 ⁵	
GIP iAUC30-130 (pmol/L \times min)	2570 \pm 372	2741 \pm 280	2251 \pm 316	2176 \pm 227	2599 \pm 361	
Insulin iAUC0-30 (mU/L \times min)	8.3 \pm 3.9	5.7 \pm 3.4	20.1 \pm 8.4	32.3 \pm 9.4 ²	62.8 \pm 19.9 ⁷	
Insulin iAUC30-130 (mU/L \times min)	4463 \pm 897	4689 \pm 1553	4505 \pm 931	4454 \pm 885	5994 \pm 1783 ⁵	
IPPWs 0-30 min (number)	3.6 \pm 1.6	5.5 \pm 1.8		19.5 \pm 6.7	16.1 \pm 5.1	
IPPWs 30-130 min (number)	15.9 \pm 4.8	60.5 \pm 14.7 ⁸		28.4 \pm 8.7	57.9 \pm 9.4 ⁸	
Antral waves 0-30 min (number)	30.5 \pm 7.7	32.9 \pm 10.3		26.5 \pm 10.7	10.6 \pm 5.6	
Antral waves 30-130 min (number)	22.9 \pm 8.7	14.3 \pm 9.2		18.9 \pm 15.2	10.5 \pm 2.3	
Duodenal waves 0-30 min (number)	295.4 \pm 62.0	213.9 \pm 39.6		289.7 \pm 50.4	215.3 \pm 63.2	
Duodenal waves 30-130 min (number)	128.3 \pm 33.8	84.9 \pm 32.9		160.5 \pm 72.6	75.1 \pm 24.7	

¹ P = 0.077, for three treatments in healthy subjects (one-factor ANOVA); ² P = 0.05, for three treatments in healthy subjects (one-factor ANOVA); ³ P < 0.05, for three treatments in healthy subjects (one-factor ANOVA); ⁴ P = 0.098, for three treatments in healthy subjects (one-factor ANOVA).

⁵ P < 0.05, for the comparison of two treatments in T2DM (two-tailed paired Student's t-test); ⁶ P = 0.056, for the comparison of two treatments in T2DM (two-tailed paired Student's t-test); ⁷ P < 0.01, for the comparison of two treatments in T2DM (two-tailed paired Student's t-test).

⁸ P < 0.05 for comparison of healthy subjects vs. patients with type 2 diabetes (two-tailed unpaired Student's t-test); ⁹ P < 0.01 for comparison of healthy subjects vs. patients with type 2 diabetes (two-tailed unpaired Student's t-test).

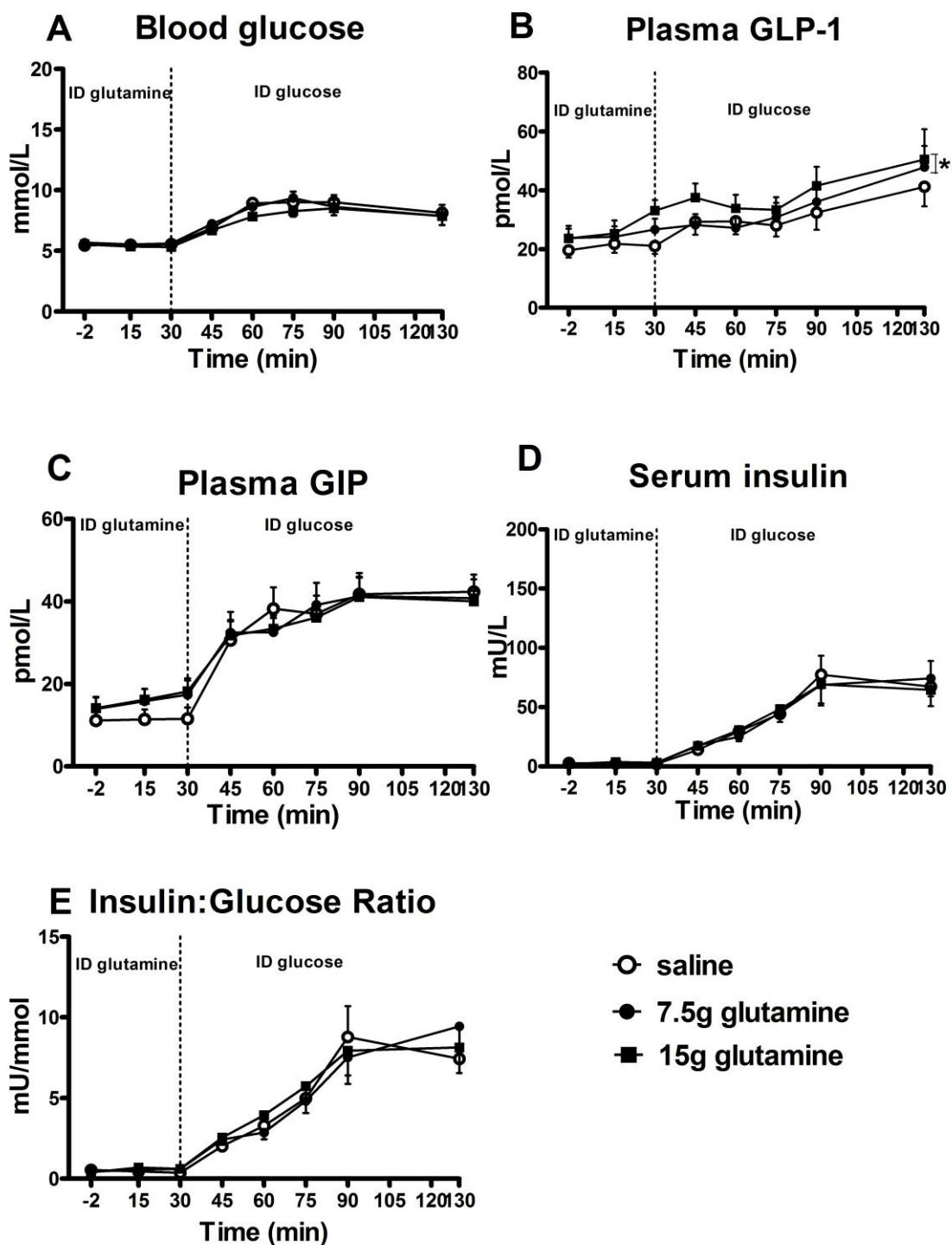


Figure 5.1 Effects of saline (control), 7.5g glutamine and 15g glutamine on blood glucose (A), plasma glucagon-like peptide-1 (GLP-1) (B), plasma glucose-dependent insulintropic polypeptide (GIP) (C), serum insulin (D) and insulin: glucose ratio (E) in healthy subjects. Incremental areas under the curves were compared by one-way ANOVA, with post hoc comparisons adjusted for Bonferroni's correction. (B) There was a significant treatment effect for GLP-1 iAUC₃₀₋₁₃₀ ($P < 0.05$), such that GLP-1 iAUC₃₀₋₁₃₀ was greater after 15g glutamine than 7.5g glutamine (* $P < 0.05$). Data are means \pm SEM.

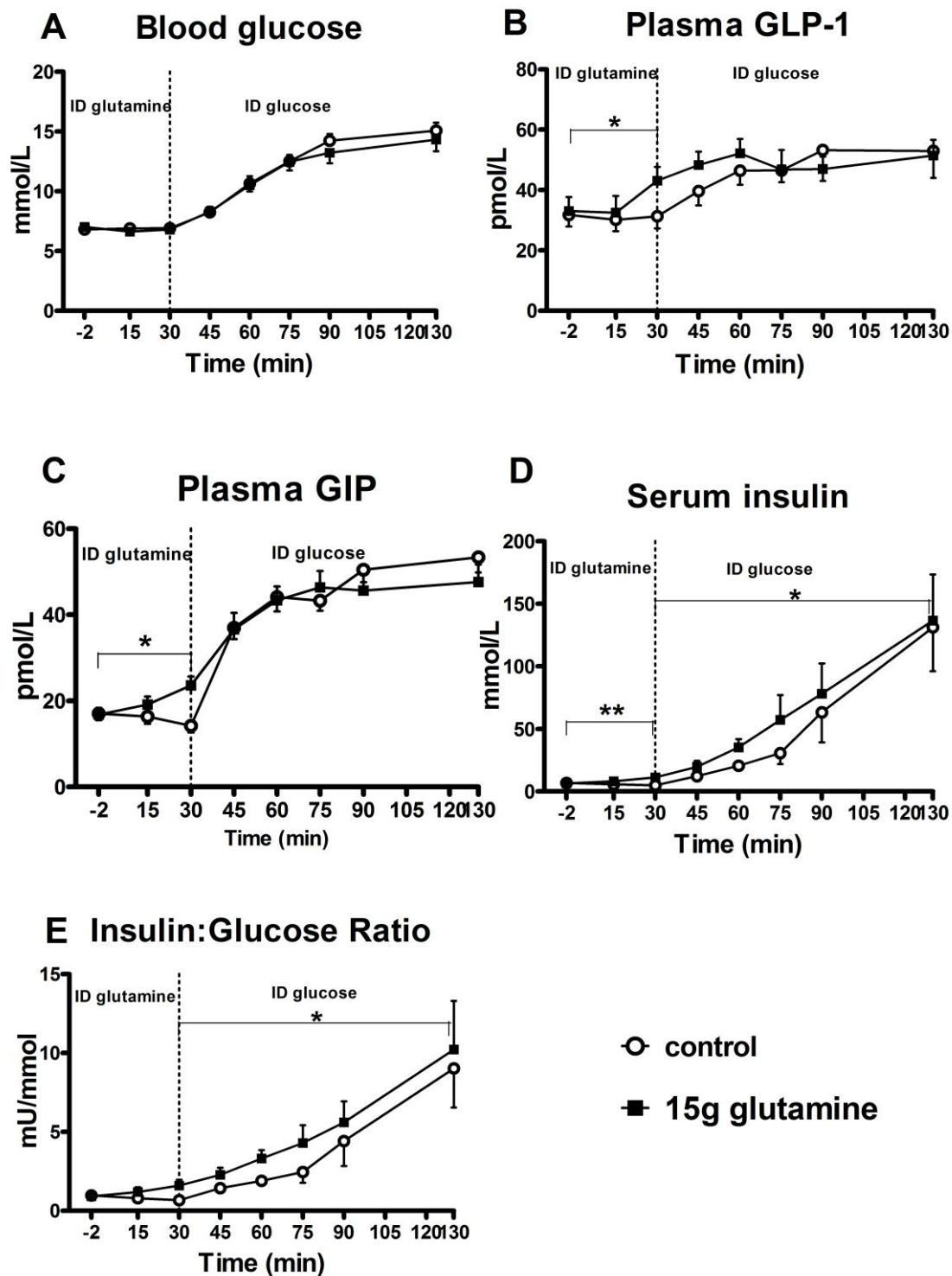


Figure 5.2 Effects of saline (control) and 15g glutamine on blood glucose (A), plasma glucagon-like peptide-1 (GLP-1) (B), plasma glucose-dependent insulinotropic polypeptide (GIP) (C), serum insulin (D), and insulin: glucose ratio (E) in patients with type 2 diabetes mellitus. Incremental areas under the curves were compared by two-tailed paired Student's t-test. There were significant differences for iAUC0-30 of GLP-1 (B), GIP (C) and insulin (D); and iAUC30-130 of insulin (D) and insulin: glucose ratio (E) (*P< 0.05, ** P<0.01). Data are means \pm SEM.

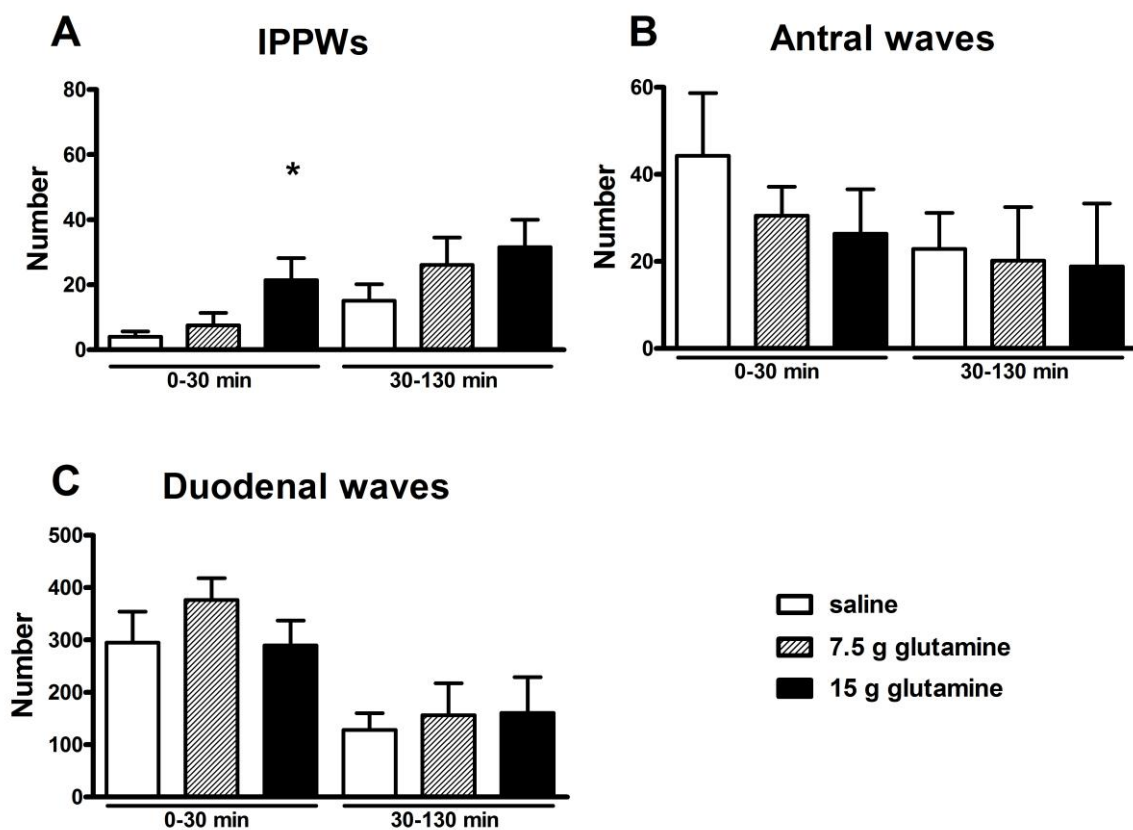


Figure 5.3 Effects of saline (control), 7.5g glutamine or 15g glutamine on isolated pyloric pressure waves (IPPWs) (A), number of antral waves (B) and number of duodenal waves (C) in healthy subjects. One-way ANOVA was significant with post hoc comparisons adjusted for Bonferroni's correction. (A) 15g glutamine vs. saline, * $P < 0.05$. Data are means \pm SEM.

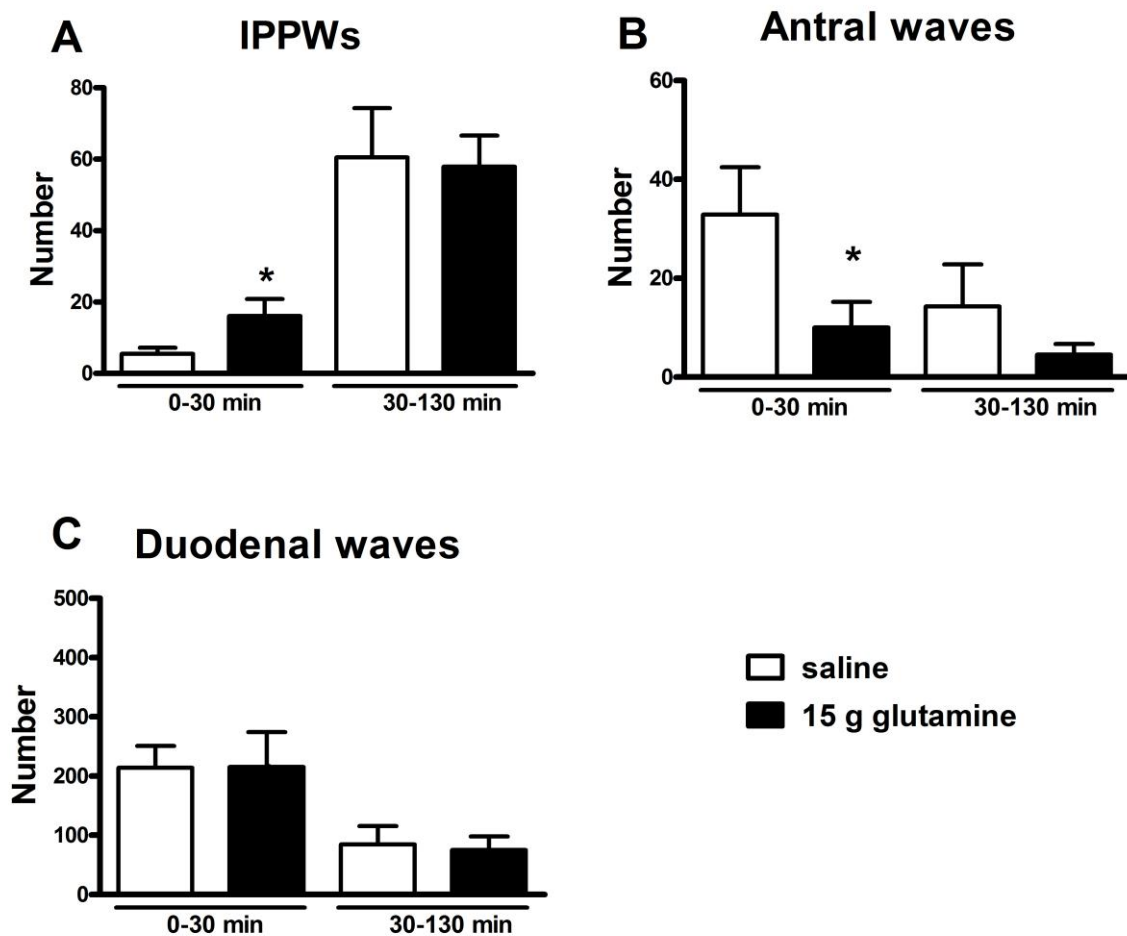


Figure 5.4 Effects of saline (control) or 15g glutamine on isolated pyloric pressure waves (IPPWs) (A), number of antral waves (B) and number of duodenal waves (C) in patients with type 2 diabetes mellitus. Two-tailed paired Student's t-test was used to determine statistical significance. (A) 15g glutamine vs. saline, * $P < 0.05$; (B) 15g glutamine vs. saline, * $P < 0.05$. Data are means \pm SEM.

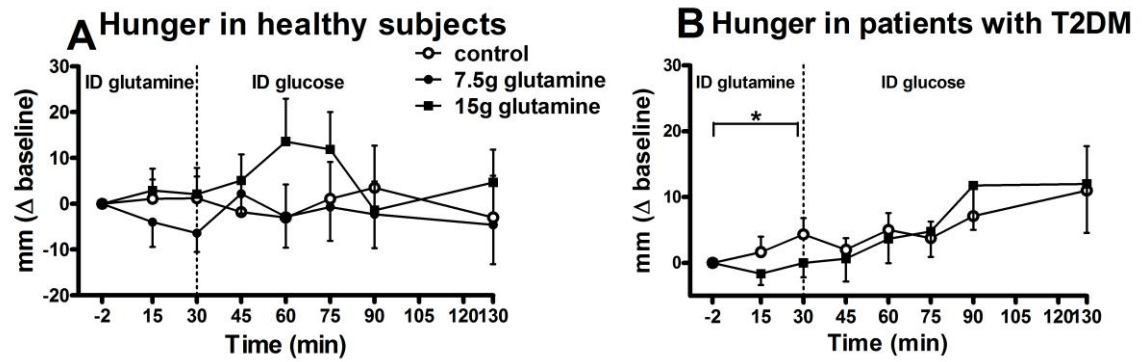


Figure 5.5 Effects of saline (control), 7.5g glutamine or 15g glutamine on hunger in (A) healthy subjects and (B) effects of saline (control) or 15g glutamine on hunger in patients with type 2 diabetes mellitus. Repeated measures ANOVA was used to determine statistical significance. 15g glutamine vs. saline, * $P < 0.05$. Data are means \pm SEM.

The authors' responsibilities were as follows-

Dr Jessica Chang: study design and preparation of protocol, subject recruitment, conducted research, data collection, data and statistical analyses, data interpretation and preparation of manuscript

Dr Tongzhi Wu: assisted in conduct of research, statistical analysis; graphic assistance in manuscript

Dr Jerry R Greenfield: involved in study concepts, data interpretation, drafting of manuscript

Dr Dorit Samocha-Bonet: data interpretation, drafting of manuscript

Professor Michael Horowitz: involved in study concepts, data interpretation and drafting of manuscript

A/Professor Chris Rayner: study design and preparation of protocol, data interpretation, overall supervision of the study and responsible for final content of manuscript

All authors read and approved the final manuscript, and give permission for the paper to be included in the thesis.

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CHAPTER 6: CONCLUSIONS

The studies reported in this thesis have provided novel insights into gastric motor function in patients with longstanding diabetes and the relevance of gastric emptying and incretin hormones to the regulation of glycaemia in health and patients with type 2 diabetes.

Disordered gastric emptying occurs frequently in patients with type 1 and type 2 diabetes; the latter is itself a chronic condition which has reached epidemic proportions worldwide. The study reported in Chapter 3 evaluated the natural history of gastric emptying in patients with longstanding diabetes and the mean follow-up of 25 years represents the most prolonged assessment of gastric emptying in this group. The results provide reassurance that, at least in most cases, delayed gastric emptying is a minimally progressive condition in diabetes and, as in healthy subjects, there is a substantially greater inter-, than intra-individual, variation in gastric emptying, with a high degree of reproducibility in gastric emptying. That autonomic nerve function had deteriorated but gastric emptying remained stable, is consistent with recent findings that the aetiology of disordered gastric emptying is complex and not attributable simply to autonomic (vagal) neuropathy. Much emphasis has been placed on the optimisation of glycaemic control in the management of diabetes to prevent the development of microvascular complications and this potentially could have contributed to the stabilisation of gastric emptying in these patients. To 'exclude' improved glycaemia, a confounder in the relatively stable gastric emptying, ideally blood glucose during the gastric emptying

measurement should be ‘clamped’ to be identical to the initial study, particularly as these averaged at ~15mmol/L, and were also variable between individuals. The study in Chapter 4 provides additional reassurance that diabetic gastroparesis is usually not associated with a poor prognosis or a higher rate of mortality. Given that the study cohort was derived from a tertiary referral centre and the prevalence of gastroparesis was high, it is likely that prognosis may be even better in population-based studies, which are indicated. It should be recognised that the number of patients with severe gastroparesis (ie. markedly delayed emptying) was small and it is possible that the prognosis differs in this group and in those patients who are hospitalised frequently because of intractable gastroparesis. The apparently benign prognosis in the majority of cases also does not discount the importance of achieving more effective symptomatic management.

With much emphasis placed on optimising glycaemic control in diabetes, it is now appreciated that postprandial glucose levels play an integral role, possibly greater, than fasting glucose levels, which have hitherto been the focus of management. Accordingly, there is increased emphasis in strategies to minimise postprandial glucose excursions. Much has also been learnt about the role of the incretin hormones, GLP-1 and GIP in the regulation of postprandial glycaemia and dietary strategies have been employed to stimulate the endogenous production of these hormones. Oral glutamine has been shown to stimulate the release of GLP-1, GIP and insulin in both healthy humans and patients with diabetes and to reduce postprandial glycaemia in patients with

type 2 diabetes. However, it is uncertain if the observed effects of oral glutamine are attributable to increased insulin secretion either directly or as a result of glutamine induced stimulation of endogenous GLP-1 or reflects an effect of glutamine to slow gastric emptying of carbohydrate.

The outcome of the study reported in Chapter 5, in which glutamine was administered intraduodenally to healthy subjects and type 2 patients, supports the concept that slowing of gastric emptying contributes to the glucose lowering effect of oral glutamine, and thus warrants exploration. Undoubtedly, glutamine, when administered orally or intraduodenally, does stimulate the release of GLP-1, GIP and insulin but this may not be its primary mechanism of action in reducing postprandial glycaemia. The observed reduction in postprandial glycaemia does support the use of glutamine in the management of type 2 diabetes. Fat and whey protein, when given as a preload in advance of a meal, have been shown to reduce postprandial glycaemia and it would be of interest to examine if oral glutamine when included as a preload may have a more significant effect on postprandial glycaemia, perhaps with the addition of a DPP IV inhibitor. It would also be of interest to determine whether the effect of glutamine on GLP-1 release is greater if it was infused into the distal small intestine, where the density of the enteroendocrine cells responsible for GLP-1 release are the greatest. The design makes it uncertain the effect observed in the study reported in Chapter 5 reflect the direct effect of glutamine on GLP-1 release or the effect of hyperglycaemia secondary to the glucose infusion on

GLP-1 stimulation. It would be of interest to make this distinction and define the duration of stimulation of incretin hormone release by glutamine.

In summary, the studies reported in this thesis have addressed some pertinent questions regarding the natural history and prognosis of diabetic gastroparesis and provided insights into the mechanism of glucose-lowering induced by glutamine.

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