Studies Examining the Pathophysiology of Acid-induced Distal Oesophageal Squamous Mucosal Damage

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Thesis Aims

This thesis brings together a series of experiments to examine the aetiology of acid-induced distal oesophageal squamous mucosal damage in humans. Firstly, a review of the literature on gastro-oesophageal reflux disease, nitrite chemistry, the histology of the gastro-oesophageal junction and the role of obesity in reflux disease is undertaken. Subsequent chapters report studies of healthy adult volunteers investigating the effect salivary nitrite has on gastro-oesophageal reflux and gastric emptying. Following this, the effect of salivary nitrite on the post-prandial gastro-oesophageal morphology is described. Finally, studies examining the relationship between age and both the position and parietal cell density of the squamo-columnar junction are described.

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  Scandinavian Journal of Gastroenterology 2012;47(4):387-396.

- **The Effect Of Nitrite In Saliva On Gastric Emptying**

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  Gastroenterology 2010;138(5):S-344.

- **High Resolution pH and Manometry Confirms Distal Opening of the Lower Oesophageal Sphincter After Meals**

  John P. Seenan, Angela A. Wirz, Alan T. Clarke, Andrew W. Kelman, Kenneth .E.L. McColl

  Gastroenterology 2009;136(5):A-425.

- **Strong Correlation Between Squamocolumnar Junction Position and Age in Healthy Volunteers**

  John P. Seenan, Angela A. Wirz, Alan T. Clarke, Andrew W. Kelman, Kenneth .E.L. McColl

  Gastroenterology 2009;136(5):A-427.

- **Decrease in Parietal Cell Density at Squamo-Columnar Junction With Increasing Age in Asymptomatic Healthy Volunteers**

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  Gastroenterology 2010;138(5):S-558.

Abbreviations:

ATP - adenosine triphosphate

BMI - body mass index

BP - blood pressure

CCK - cholecystokinin

cGMP - cyclic-guanosine mono-phosphate

CLO - campylobacter-like organism

CT - computed tomography

DHPZ - distal high pressure zone

DNA - deoxyribonucleic acid

GI - gastro-intestinal

GOJ - gastro-oesophageal junction

GOPG - gastro-oesophageal pressure gradient

GORD - gastro-oesophageal reflux disease

HCl - hydrochloric acid

HPZ - high pressure zone

H_2A – Histamine-2 antagonist

IGP - intragastric pressure

L-NNA - N-nitroso-L-Arginine

L-NMME - N-nitroso-L-arginine-methyl-ester

LOS - lower oesophageal sphincter

MRI - magnetic resonance imaging

NO - nitric oxide

N_20_3 - dinitrite trioxide

NOSCN – nitrosothiocyanate

OR – odds ratio

PD_5 - type 5 phosphodiesterase

PHPZ – proximal high pressure zone

PPI - proton-pump inhibitor

RIP - respiratory inversion point

SAT - subcutaneous adipose tissue

SCJ - squamo-columnar junction

TLOSR - transient lower oesophageal sphincter relaxation

VAT - visceral adipose tissue

WHO - world health organisation

Summary

- Gastro-oesophageal reflux disease (GORD) is the commonest chronic disease in Western countries. Symptomatic GORD is the strongest risk factor for the development of oesophageal adenocarcinoma with obesity and male sex also linked to the development of neoplasia at this site. Recent decades have seen a significant increase in the incidence of this highly lethal cancer among Western populations with Scotland having the highest recorded incidence worldwide.

- Human saliva has a high nitrite content derived from the entero-salivary recirculation of nitrate in our diet which has resulted from the increased use of nitrogenous fertilisers over the past 50-60 years.

- The luminal chemistry produced at the gastro-oesophageal junction (GOJ) when swallowed salivary nitrite reacts with gastric acid, and most notably the production of nitric oxide (NO), may explain most of the physiological abnormalities that contribute to the pathogenesis of GORD. NO has been shown to reduce lower oesophageal sphincter (LOS) pressure, impair oesophageal clearance, delay gastric emptying and may be the final mediator of transient lower oesophageal sphincter relaxations (TLOSRs). Previous studies to investigate the role of this luminal chemistry in the pathogenesis of GORD show conflicting results.

- In addition to the distal oesophageal acidification produced by traditional trans-sphincteric reflux, previous studies suggest ‘splaying open’ of the distal lower oesophageal sphincter following a meal may expose the gastric cardia

and the most distal oesophageal squamous mucosa to the noxious effects of gastric acid.

- Although the gastric cardia is an important site of pathology in the upper gastrointestinal tract, it is a complex and poorly understood area. It has been proposed, from autopsy studies, that cardia mucosa itself may be pathological and in fact an ‘acquired cardia’ due to metaplasia of the most distal oesophageal squamous mucosa.

- A series of studies were designed to examine the effect of salivary nitrite on post-prandial GORD, gastro-oesophageal function and GOJ morphology in 20 healthy, asymptomatic adult volunteers using high-resolution pH manometry, an isotope gastric emptying breath testing and X-ray localisation of the squamo-columnar junction (SCJ).

- Despite an excellent range of salivary nitrite concentrations extending over and above the normal physiological range no effect of salivary nitrite on gastro-oesophageal reflux, function or morphology was demonstrated. However, the studies did confirm, for the first time using high-resolution manometry, that distal opening of the LOS occurs after a meal.

- The relationship of age and obesity to the SCJ position relative to the proximal border of the gastro-oesophageal high pressure zone (HPZ) was examined in 15 *Helicobacter Pylori* negative healthy volunteers. Strong negative correlations were seen between SCJ position relative to the proximal HPZ and increasing age, body mass index (BMI) and waist circumference (WC) respectively. These correlations were stronger in the male sub-group.

- In 25 healthy volunteers, parietal cell density was measured from endoscopic biopsies taken from the macroscopic SCJ, 1cm distal to the SCJ, the gastric body and the gastric antrum. Again, a strong negative correlation was seen between increasing age and parietal cell density at the SCJ. This effect was localised to the SCJ and not seen at the other biopsy sites.

- Our findings suggest that salivary nitrite does not alter gastro-oesophageal function, the integrity of the gastro-oesophageal barrier or gastro-oesophageal reflux in healthy volunteers. They confirm distal opening of the LOS after meals. The strong negative correlations between age and both SCJ position relative to the proximal HPZ and parietal cell density support the hypothesis of an 'acquired' cardia. The development of cardia mucosa may also be linked to obesity, visceral obesity and male sex.

- Future work could examine the carcinogenic effect of salivary nitrite and its luminal chemistry but this would require large scale epidemiological research. Further, larger clinical studies are needed to investigate the role of distal opening of the LOS after meals and to improve our understanding of the gastric cardia. Such studies should focus on the role of obesity and posture.

Chapter 1

An Introduction to Gastro-oesophageal Reflux Disease (GORD)

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1.1. Introduction

Gastro-oesophageal reflux disease (GORD) is the commonest chronic disease affecting Western populations. Heartburn and regurgitation are the cardinal symptoms of the disease (1) and pooled data from studies suggest approximately 25% have symptoms at least monthly, 12% have at least weekly symptoms and 5% suffer heartburn on a daily basis (2).

GORD is generally defined as the reflux of gastric contents into the oesophagus leading to oesophageal inflammation (oesophagitis), reflux symptoms sufficient to impair quality of life, or long-term complications (3). While most people experience some physiological gastro-oesophageal reflux after meals, such episodes are not usually associated with pathological signs or symptoms (4). GORD occurs due to an imbalance between the protective mechanisms in the distal oesophagus and the harmful effects of refluxed gastric acid, pepsin and bile on oesophageal mucosa.

1.2. The normal oesophagus and anti-reflux mechanisms

The oesophagus is a muscular tube approximately 25cm long, composed of inner circular and outer longitudinal muscle layers. It is lined with stratified squamous epithelium. The tight intercellular junctions of these squamous cells and the

production of a protective barrier of mucous and bicarbonate is designed to protect the epithelium from penetration by luminal molecules. However, unlike the columnar gastric mucosa, the squamous mucosa of the oesophagus is sensitive to damage from the highly acidic gastric juice (pH 2-3) contained in the stomach. Digestive enzymes, such as pepsin, and duodenal juices, including bile, which are contained in the gastric juice may also damage squamous oesophageal mucosa.

A pressure gradient exists between the abdomen and thorax, exacerbated by the negative intra-thoracic pressure and increased intra-gastric pressure during inspiration and straining. To prevent reflux into the oesophagus of these potentially damaging acidic gastric juices, an area of increased pressure must exist at the gastro-oesophageal junction. This ‘high pressure zone’ is the primary mechanism of defence the human body has against gastro-oesophageal reflux. It consists of the lower oesophageal sphincter (LOS) and the crural diaphragm and crucially, not only does it provide an area of increased resting pressure, but it is able to compensate for the elevations in intragastric pressure produced during inspiration and straining.

The LOS, or intrinsic sphincter, is a section of specialized smooth muscle in the distal oesophagus of approximately 4cm in length. LOS tone is thought to be maintained by excitatory neurones with cholinergic innervations which are under the control of the vagus nerve (5;6). In healthy individuals, the LOS tonically contracts, closing the distal oesophageal lumen and maintaining a pressure of at least 15mmHg above intra-gastric pressure (7). The sphincter is able to relax in response to oesophageal peristalsis to allow the passage of food, liquid or saliva into the stomach. This LOS relaxation is mediated by nitric oxide (NO) (8).

The human diaphragm is composed of two parts with separate embryological origins. The costal diaphragm originates from the ribs, while the crural diaphragm is attached to the vertebral column. The crural diaphragm encircles the proximal 2cm of the LOS. By contracting on inspiration and during straining, it augments LOS pressure and helps prevent reflux. However, studies also demonstrate that it prevents reflux during periods of absent LOS pressure, underlining its importance as an anti-reflux barrier (9;10). As a result, it is often referred to as the 'extrinsic sphincter'. In common with the rest of the diaphragm, the crura are innervated by the phrenic nerve. However, the crura contract a fraction of a second earlier than the costal diaphragm (11). This may be physiologically important to its role as an anti-reflux barrier, ensuring that pressure at the gastro-oesophageal junction increases before alterations in the gastro-oesophageal pressure gradient incurred by inspiration or straining.

The gastric sling fibres (oblique fibres) are located below the LOS and are also thought to contribute to the anti-reflux barrier. Muscle fibres are arranged on the greater curvature of the stomach with a gap in the fibres across the lesser curve. Due to this arrangement, pressure in the gastric fundus creates a flap that presses against the lower end of the oesophagus, again augmenting LOS pressure during periods of increased intra-gastric pressure. As a result, this area has been described as the gastro-oesophageal 'flap valve' (12).

Once gastric contents reflux into the oesophagus, rapid clearance of the refluxate must occur to prevent damage to the squamous oesophageal mucosa which is vulnerable to its noxious mixture of gastric acid, pepsin and bile. The normal mechanism of clearance is a two-stage process. Firstly, there is clearance of

refluxate volume by peristalsis. Peristalsis is a wave of oesophageal circular muscle contraction that propagates down the oesophagus to the level of the LOS and may be primary or secondary. Primary peristalsis is voluntary and occurs approximately 60 times per hour. During primary peristalsis, circular muscle contraction is accompanied by longitudinal muscle contraction which shortens the oesophagus by 2-2.5cm (13;14). Secondary peristalsis occurs without a pharyngeal swallow and may be provoked by distal oesophageal distension or acidification, both of which can occur during episodes of gastro-oesophageal reflux (15). During a reflux episode, one or two peristaltic waves will empty the distal oesophagus and only a fraction of the refluxate will persist. Residual acid can then be neutralized by swallowed saliva (16). The normal gastro-oesophageal anatomy and relevant anti-reflux structures are shown in Figure 1.1

1.3. The pathophysiology of GORD

A minority of GORD patients (20%) will have, as their primary disorder, LOS incompetence. This is either due to reduced resting LOS pressure, increased intra-abdominal pressure (e.g. secondary to obesity or pregnancy) or shortening of the LOS (15). Acid exposure in such patients is prolonged, especially when supine, and severe GORD with complications is often found (17).

However, many patients with GORD have a normal resting LOS pressure. This apparent contradiction was explained by Dodds et al who discovered that the majority of reflux events in both healthy volunteers (18) and reflux patients (19) occur during transient lower oesophageal sphincter relaxations (TLOSRs). TLOSRs are

defined manometrically as sudden, prolonged relaxations of the LOS not associated with swallowing (20). Gastric distension is a potent stimulus for the production of TLOSRs and studies have shown that distension with free air (21), a balloon (22) and, more importantly, a meal (23) all result in an immediate increase in TLOSRs. This helps to explain the increase in reflux events which occurs post-prandially. Significantly, during TLOSRs, the activity of the crural diaphragm is also inhibited (9), facilitating free reflux of gastric contents into the distal oesophagus.

The neural pathways underlying TLOSRs are now partly understood. By dividing the stomach into separate compartments, Franzi et al (24) were able to demonstrate that the cardia region has the lowest threshold for triggering TLOSRs. Furthermore, inhibition of the vagal nerve by cooling abolishes TLOSR production, suggesting a vago-vagal reflex (25). The afferent pathway in this reflex arc is therefore thought to consist of vagal afferents within the muscle layers of the proximal stomach, sensitive to stretching during gastric distension (26). These afferent fibres terminate in the nucleus tractus solitarius and the dorsal motor nucleus of the vagal nerve in the brainstem (27-29). From here, motor neurones project to the enteric nervous system of the LOS (30).

It would be assumed that GORD patients would have higher rates of TLOSRs but TLOSRs are not more frequent in GORD patients, simply a higher proportion are accompanied by reflux (31). However, the mechanisms behind this are poorly understood.

As previously described, the gastro-oesophageal ‘high pressure zone’, the primary anti-reflux barrier, consists of both the LOS and the crural diaphragm. A hiatus hernia occurs from spatial separation of these normally overlapping and

complimentary structures with the proximal stomach migrating above the level of the diaphragm in the thorax. This is a common endoscopic and radiological finding in patients with GORD. A large hiatus hernia certainly predisposes to GORD (32) and is associated with an increased risk of oesophagitis (33). Not only does it result in a reduction in peak LOS pressure (34) but the presence of a hiatus hernia predisposes to increased rates of TLOSRs (35), interferes with primary peristalsis leading to inadequate clearance of refluxate (36) and provides an opportunity for accumulation of gastric contents in the hiatal sac, facilitating reflux during swallow-induced LOS relaxations (37). Finally, in patients with a hiatus hernia, the absence of the gastro-oesophageal flap valve and loss of an intra-abdominal portion of the oesophagus may also contribute to reflux (38).

An unbuffered ‘pocket’ of acid in the proximal stomach and cardia region has also been described after a meal (39). Studies have shown it to be enlarged in reflux patients compared with health volunteers (40) and it may contribute to distal oesophageal exposure post-prandially (41).

Several studies have examined the influence of solid and liquid gastric emptying on GORD with conflicting results. Some studies show high rates of delayed gastric emptying in GORD patients compared to healthy controls (42;43) while in others (44-46) rates are equivalent. However, it is likely that in a small number of GORD patients delayed gastric emptying contributes both by increasing the amount of fluid available for reflux post-prandially and by increasing the rate of TLOSRs due to an associated increase in gastric distension.

Lifestyle factors and drugs may contribute towards the pathogenesis of GORD by causing reduced LOS pressures or inappropriate relaxation of the LOS. Obesity

leads to increased intra-abdominal pressure increasing the gradient across the gastro-oesophageal 'high pressure zone'. As a result it may disrupt the LOS (47), it may also lead to the development of a hiatus hernia due to mechanical pressures on the diaphragm and may increase the number of TLORSs (48). Systematic reviews (49) suggest an association between increased body mass index (BMI >25kg/m^2) and reflux symptoms (OR 1.43) while a similar association is seen with the risk of oesophagitis (OR 1.76). Many drugs (e.g. anticholinergics) reduce LOS pressure and similar effects are produced by smoking (50), alcohol (51), coffee (52) and chocolate (53). Fatty meals reduce LOS pressure as a result of increased CCK release in response to duodenal fat, can cause delayed gastric emptying and are also associated with increased rates of TLOSRs (22;54;55).

1.4 Complications of GORD

In addition to the typical symptoms of heartburn and regurgitation, GORD may be associated with more serious complications. Inflammation of the oesophagus (oesophagitis) can occur and ranges from mild erythema to the development of erosions and ulceration. More severe ulceration can progress to the formation of a peptic stricture and obstruction of the oesophagus. Barrett's oesophagus is defined as a metaplastic change in the oesophageal squamous mucosa to a specialized intestinal epithelium and occurs as a consequence of longstanding GORD (56). It is recognized as a premalignant condition and studies estimate the excess risk of oesophageal adenocarcinoma to be increased 30 to 60 fold relative to the general population risk (57-60). However, the actual risk of oesophageal adenocarcinoma may be as low as 0.4% per year (61).

Symptomatic gastro-oesophageal reflux is recognized as the strongest risk factor for the development of oesophageal adenocarcinoma. A large case-control study by Lagergren et al (62) found that the risk of oesophageal adenocarcinoma was nearly eight times higher in those experiencing symptoms of heartburn or regurgitation at least weekly than in asymptomatic individuals. Symptoms of reflux at night were associated with an almost 11-fold increased risk. Oesophageal adenocarcinoma continues to be a highly lethal cancer with 5-year survival rates of only approximately 10% across Europe (63).

1.5 Recent changes in upper gastrointestinal cancer epidemiology

The past four decades have seen a significant increase in the incidence of oesophageal adenocarcinoma among Western populations (64) with Scotland having the highest recorded incidence worldwide (65). In contrast, the incidence of carcinoma of the mid and distal stomach has progressively fallen (66).

Recent data from the Scottish Cancer Registry (2011) show that the incidence of oesophageal adenocarcinoma has increased by approximately 250% over the past 25 years, from a total of 179 cases in 1985 to 449 cases in 2009 – annual incidence rates of oesophageal adenocarcinoma are shown in Figure 1.2. At the same time there has been a 43.5% reduction in the number of cases of gastric cancer diagnosed, from 1328 in 1985 to 750 in 2008 – annual incidence rates of gastric cancer are shown in Figure 1.3. The 5-year survival rate in Scotland is only 10.8%.

The falling incidence of non-cardia gastric cancer may be explained by the falling incidence of *Helicobacter pylori* infection since *Helicobacter pylori* associated atrophic gastritis is a major risk factor for cancer at these site. Debate exists as to the aetiology of the increasing incidence of oesophageal adenocarcinoma but the reduction in *Helicobacter pylori* infection has also been proposed as an explanation for the increasing incidence of adenocarcinoma of the distal oesophagus. In earlier generations, acid secretion would diminish with increasing age due to *Helicobacter pylori*-induced gastric atrophy. However, *Helicobacter pylori* negative individuals maintain their ability to secrete gastric acid throughout their life with the potential for greater oesophageal acid exposure as a result. An additional explanation for the increase in distal oesophageal adenocarcinomas is the potential carcinogenic and refluxogenic effect of reactive nitrogen species generated at this anatomical site.

1.6 Summary

In summary, GORD is a common chronic condition. Its aetiology is multi-factorial and not fully understood. It is not only a significant burden on healthcare systems in terms of cost but causes significant morbidity and can be associated with more serious complications including oesophagitis, peptic strictures, Barrett's oesophagus and, ultimately, oesophageal adenocarcinoma. The incidence of oesophageal adenocarcinoma continues to rise, especially in our own country, where rates are the highest recorded globally. The cause for this increase in incidence remains to be confirmed.

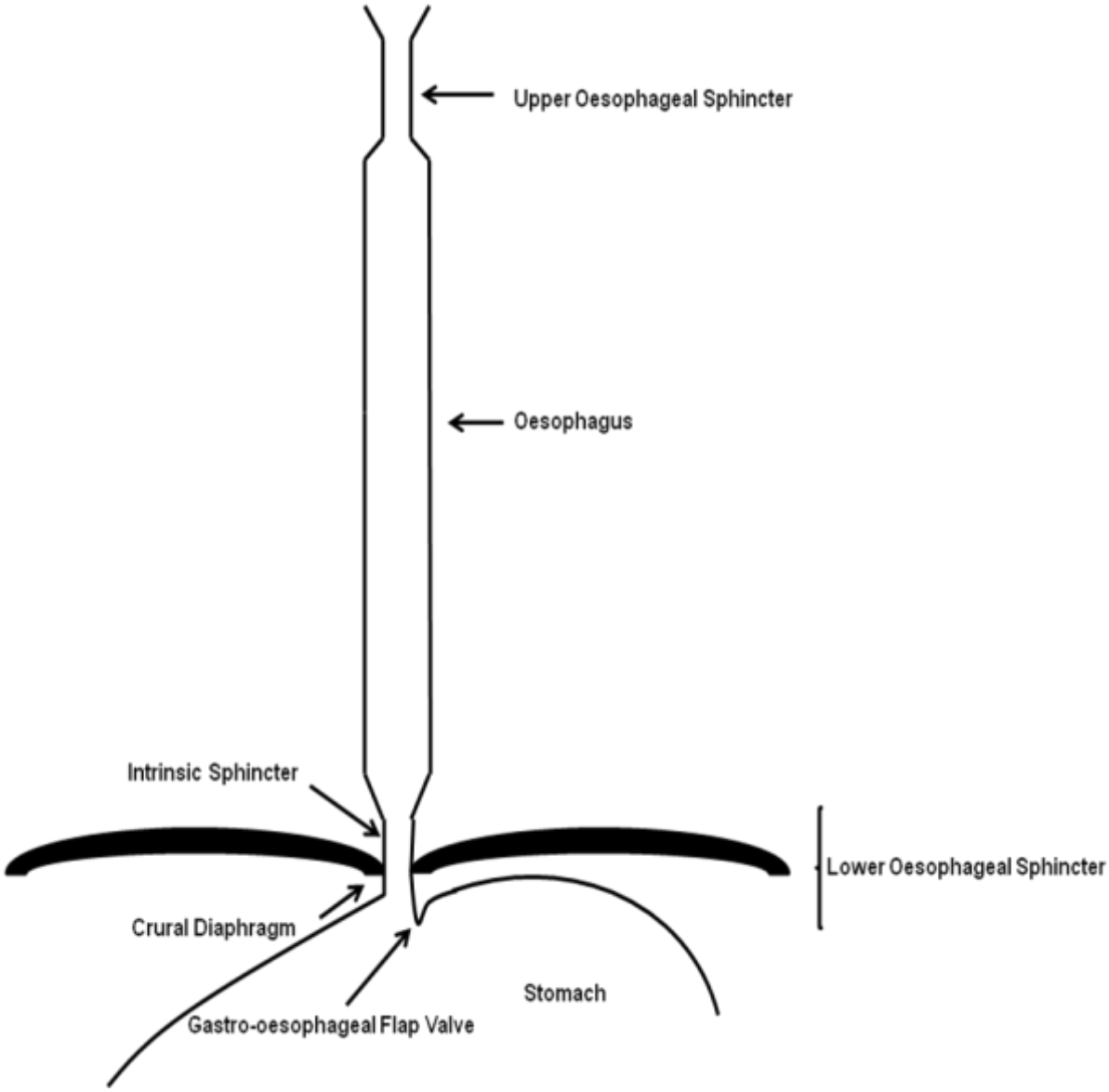

Figure 1.1 The normal oesophagus and gastro-oesophageal junction

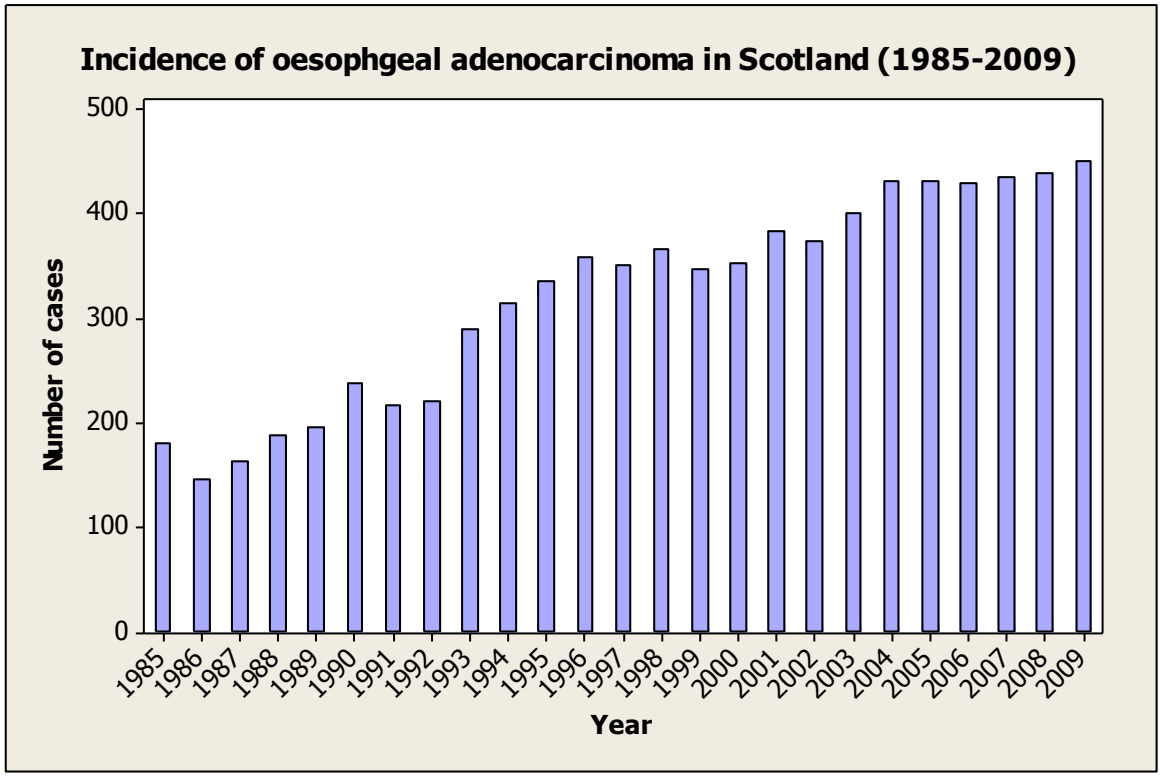

Figure 1.2. Incidence of oesophageal adenocarcinoma in Scotland (1985 -2009)

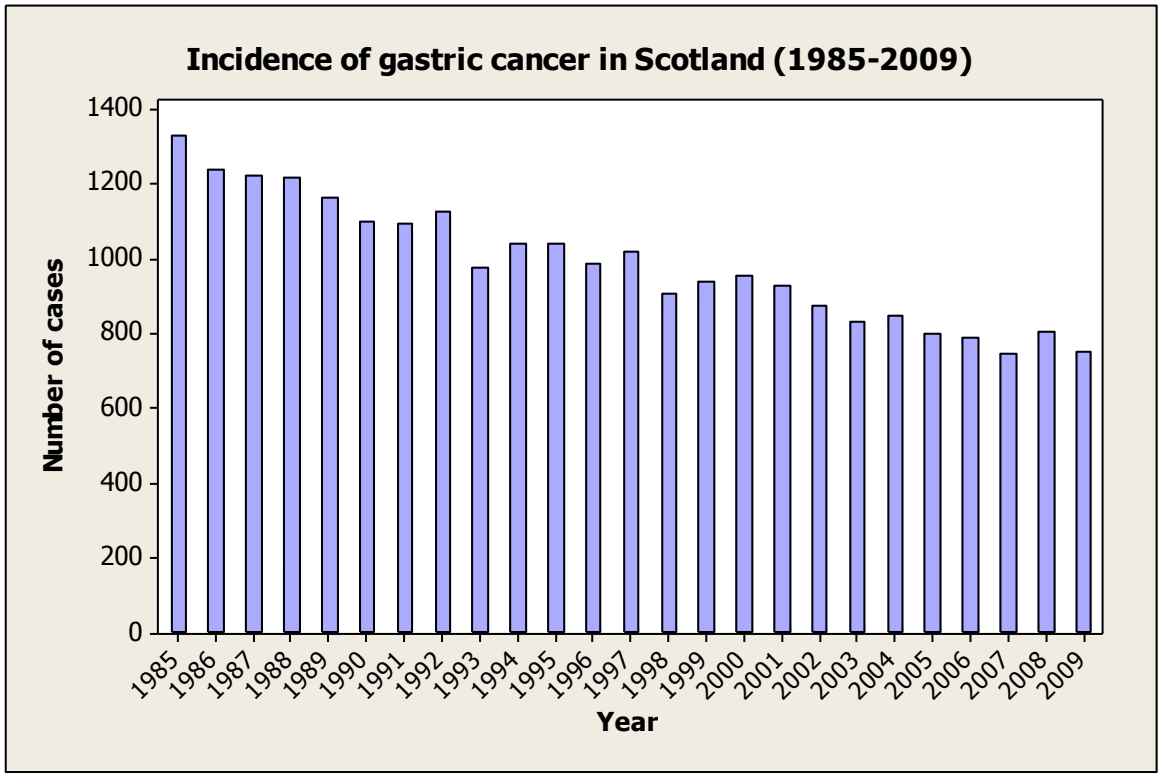

Figure 1.3. Incidence of gastric cancer in Scotland (1985 -2009)

Chapter 2

The Salivary Nitrite Hypothesis

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2.1 Salivary nitrite production from dietary nitrate

Human saliva has a high nitrite concentration, derived from the entero-salivary recirculation of nitrate. Once it is ingested, nitrate is absorbed into the bloodstream from the small intestine and supplemented by a small amount of endogenous nitrate. Entero-salivary recirculation results in 25% of this nitrate being actively secreted by the salivary glands (67). Bacteria in the mouth then enzymatically convert between 10 and 90% of this nitrate in saliva to nitrite (68). The production of nitrite from the entero-salivary recirculation of dietary nitrate is illustrated in Figure 2.1

Nitrate is present in substantial amounts in the modern diet and is mainly derived from nitrogenous fertilisers (69). Intensive farming, such as the growing of vegetables in low light intensity but high temperature glasshouses, also leads to increased nitrate concentrations (70). The use of nitrogenous fertiliser began to increase in Western countries from the middle of the 20th century (71) leading to increased nitrate deposition in soil. An increase in intensive farming techniques was seen at the same time. Following its uptake from the soil, nitrate is reduced to the ammonium ion (NH_4^+) and then incorporated rapidly into organic compounds by the plant. Leaching of fertiliser subsequently leads to a gradual increase in groundwater nitrate concentrations. As a result, the main sources of nitrate in the human diet are green leafy vegetables (such as lettuce and spinach), root vegetables (such as beetroot and potatoes) and water.

In the past few decades, increasing public health concerns developed regarding the association between nitrate and infantile methaemoglobinaemia (72) and the possible association between nitrate and gastric cancer (73). This prompted the introduction of regulations to reduce nitrate-based fertiliser use and limit nitrate concentrations in food and water. In 1990, the European Commission's (EC) Scientific Committee for Food (SCF) set an Acceptable Daily Intake (ADI) for the nitrate ion of 3.65 mg/kg body weight (equivalent to 219 mg/day for a 60 kg adult) (74). As a result of these measures, the use of nitrogenous fertilisers has plateaued towards the end of the 20^{th} century in most Western countries (75).

2.2 Gastric acidification of nitrite and its carcinogenic potential

When nitrite is swallowed it reacts with acidic gastric juice forming nitrous acid, the nitrosating species N_2O_3, NOSCN and by further reaction with ascorbic acid in gastric juice it is also converted to nitric oxide (NO). Nitrosating species are able to react with secondary amines and amides in gastric juice to form N-nitroso compounds (76). The endogenous production of these compounds is of interest due to their ability to induce cancer in animal models (77). Furthermore nitric oxide is itself known to be mutagenic at high doses.

Previous work by Moriya et al (78) using a model to simulate the interaction of salivary nitrite and gastric acid suggested the gastro-oesophageal junction to be the anatomical site of maximal acid catalysed generation of N-nitroso compounds following a nitrate load. Subsequently, using a luminal nitric oxide sensor, Iijima et al (79) demonstrated maximal generation of nitric oxide at the gastro-oesophageal

junction in normal healthy volunteers following nitrate ingestion. Similar work by Suzuki et al using microdialysis probes defined the anatomical location where chemical conditions favouring the acid catalysed generation of N-nitroso compounds was maximal. In normal volunteers this occurred at the level of the gastric cardia (80) while in Barrett's oesophagus, the maximal potential for nitrosation was found within the Barrettic segment itself (81). Therefore, in both normal volunteers and Barrett's patients the anatomical location of maximal acid catalysed luminal nitrosation corresponds to the interface between saliva and gastric acid. Winter et al subsequently demonstrated the *in situ* formation of N-nitrosocompounds from ingested dietary nitrate via NO formation. During episodes of reflux this occurred almost exclusively in the oesophagus (82).

These studies support the hypothesis that nitric oxide and N-nitroso compounds generated from salivary nitrite may be implicated directly in carcinogenesis at the gastro-oesophageal junction and within the distal oesophagus. However, they may also have a contributory role in potentiating gastro-oesophageal reflux disease.

2.3. Salivary nitrite in the pathogenesis of GORD

In addition to its mutagenic potential, excessive production of nitric oxide could explain most of the functional abnormalities associated with reflux disease. Nitric oxide is well recognised to induce smooth muscle relaxation, by elevation of tissue cyclic guanosine monophosphate (cGMP) levels (83), and a number of studies have described the effects of NO on upper gastrointestinal motility.

In vitro studies by Tottrup at al (84) on opossum lower oesophageal sphincter demonstrated that N-nitro-L-arginine (L-NNA) a substance known to inhibit NO formation inhibited lower oesophageal sphincter relaxation. This effect was attenuated by the NO substrate L-arginine. A similar study by Preksaitis et al (85) examining human lower oesophageal sphincter muscle, which also used L-NNA to inhibit NO, indicated that the same process was most likely involved in man.

The effect of NO on the production of TLOSRs has also been studied. Boulant et al (55) described a reduction in TLOSRs induced by gastric distension in dogs following infusion of the NO synthase inhibitor N-nitro-L-arginine-methyl-ester (L-NMME). Again, this effect was reversed by L-arginine. Hirsch et al (86) confirmed this effect on healthy, human volunteers undergoing oesophageal manometry also using gastric distension by balloon insufflation as the trigger for TLOSRs. L-NMMA or placebo was given in a randomised, double-blinded fashion and significantly inhibited the increase in TLOSRs brought about by gastric distension. A further study by Hirsch et al (87) looking at the physiological induction of TLOSRs by meal-induced gastric distension concluded that L-NMME infusion also inhibited TLOSRs post-prandially and reduced the total number of reflux episodes.

L-NMME has been found to reduce oesophageal peristalsis in animal models. A study by Anand et al (88) using adult opposums demonstrated a reduction in swallow induced contraction amplitude in the distal oesophagus following NO synthase inhibition. Studies in man using Sildenafil support the involvement of NO in oesophageal peristalsis. Sildenafil blocks type 5 phosphodiesterase (PD_5) which destroys NO stimulated cGMP. Rhee et al (89) performed oesophageal manometry on healthy volunteers before and after infusion of 50mg sildenafil directly into the

stomach. Oesophageal body peristaltic amplitudes gradually decreased and were subsequently abolished with a corresponding increase in peristaltic latency. Eherer et al (90) confirmed this in a randomised and double-blinded study of 50mg sildenafil or placebo given to healthy volunteers and patients with oesophageal hypercontractility disorders. Reduced oesophageal body propulsive forces were observed in both groups. Both these studies also confirmed a reduction in LOS pressure following sildenafil. Similarly, increasing NO bioactivity, by inhibiting phosphodiesterase, enhances gastric accommodation and delays gastric emptying (91).

Nitric oxide has therefore been demonstrated to lower the tone of the LOS, may impair oesophageal acid clearance through its effects on peristalsis, delays gastric emptying and is known to be the final mediator in the production of TLOSRs. However, can these effects be demonstrated post-prandially following an oral nitrate load?

2.4. Previous Studies

Bove et al (92) combined pH and manometry studies on both healthy volunteers and GORD patients following dietary nitrate supplementation. All volunteers received a nitrate deprived diet and were randomised to either potassium nitrate 200mg or placebo for 4 days with a two week wash-out period in between. They reported no significant effect on oesophageal reflux or oesophageal motility following nitrate supplementation. However there was a strong trend towards an increase in TLOSRs in the group receiving nitrate supplementation. Significantly,

salivary nitrite concentrations in the study were well below the levels that have regularly been described in studies by our own group (79). In contrast, by infusing nitrous acid and nitric oxide forming solutions directly into the distal oesophagus after a standardised meal, Manning et al (93) demonstrated that chemicals formed from the gastric acidification of salivary nitrite can influence oesophageal and gastric function which may be relevant to GORD. Using combined oesophageal pH and manometry studies they reported a reduction in LOS pressure and increase in frequency of TLOSRs post-prandially following infusion of the nitric oxide generating infusion. They also described an attenuation of the post-prandial increase in intra-gastric pressure following the nitrous acid infusion, possibly due to delayed gastric emptying.

2.5. Summary

Human saliva has a high nitrite concentration from the entero-salivary recirculation of dietary nitrate. Swallowed nitrite reacts with gastric acid to form nitrous acid, nitrosating species and nitric oxide (NO) at the gastro-oesophageal junction. In addition to being potentially mutagenic and carcinogenic, this luminal chemistry and in particular the generation of NO, might contribute to the pathogenesis of reflux disease. NO has been shown to reduce lower oesophageal sphincter (LOS) pressure, impair oesophageal clearance, delay gastric emptying and may be the final mediator in the production of transient lower oesophageal sphincter relaxations (TLOSRs). Existing studies that examine the effect of altering the luminal nitrite chemistry on gastro-oesophageal motility and whether or not this may potentiate gastro-oesophageal reflux show conflicting results.

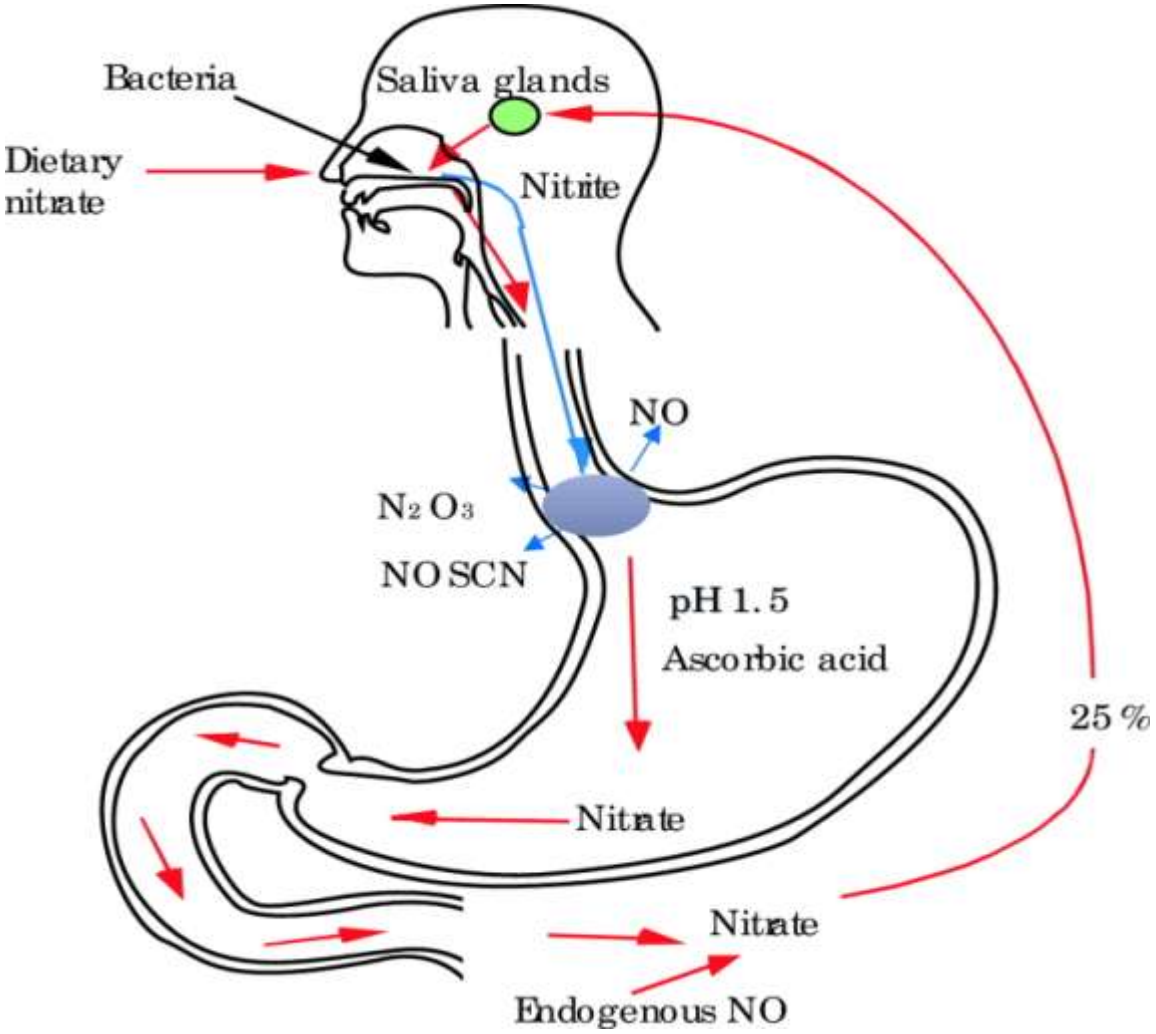

Figure 2.1. The entero-salivary recirculation of dietary nitrate (adapted from McColl KEL *Gut 2005;54:1-3*)

Chapter 3

The Changing Histology of the Gastro-oesophageal Junction (GOJ)

Chapter 3

The Changing Histology of the Gastro-oesophageal Junction

3.1. Oesophageal histology

The oesophagus is lined by non-keratinized stratified squamous epithelium with an average thickness of 500-800μm. The basal layer consists of cuboidal or rectangular cells and may be several cell layers thick, also containing scattered melanocytes and neuroendocrine cells. Above this, epithelial cells are larger and become more flattened as they approach the oesophageal lumen.

The oesophageal lamina propria is comprised of loosely arranged collagen fibres and fibroblasts embedded within an acellular matrix, with a scattering of lymphocytes, eosinophils, mast cells and plasma cells. The muscularis mucosa is of variable thickness, being thicker at its distal end where it approaches the GOJ.

Mucous glands are present within the oesophageal submucosa. Each gland has between 2 and 5 lobes, which drain into a short duct lined by stratified columnar epithelium. These ducts penetrate the muscularis mucosa, lamina propria and epithelium to open into the oesophageal lumen, allowing secretion of bicarbonate and mucous which help lubricate the oesophagus and protect against acid-related mucosal damage. Around the glands and their ducts, lymphocytes, plasma cells and eosinophils can be found while the oesophageal submucosa is also rich in blood vessels, lymphatics and ganglion cells.

3.2. Gastric histology

A key role of the gastric epithelium is to secrete hydrochloric acid and proteolytic enzymes to aid in the digestion of food. As a result, the gastric mucosa must be designed to withstand the effects of these potentially corrosive substances. The stomach also secretes mucous which lubricates food and helps protect the gastric mucosa from acid and pepsin. These secretions are produced from 3 main cell types:

- Mucous cells
- Acid-producing cells (oxyntic or parietal cells)
- Enzyme-producing cells (Chief or peptic cells)

In addition, there is a population of hormone-secreting enteroendocrine cells and stem cells from which other cell types are derived.

Gastric mucous cells are of 2 types. Surface mucous cells are tall and columnar. Neck mucous cells are smaller and less regular in shape, being compressed and distorted by adjacent cells.

The acid-secreting parietal cells are large pyramidal cells. The capacity of the stomach to secrete hydrochloric acid (HCl) is linearly related to parietal cell numbers. Parietal cells have a vast luminal surface area as a result of deep microvilli-lined invaginations called canaliculi. When stimulated, parietal cells secrete HCl at a concentration of approximately 160 millimoles (equivalent to a pH of 0.8). Acid is secreted into the canaliculi by a process of active transport via the H^+K^+ATPase proton pumps located in the canalicular membrane. Hydrogen ions are generated

within the parietal cell from the dissociation of water. Carbon dioxide diffuses across the basement membrane from blood capillaries into the cell where it links with water molecules. This reaction, which is catalysed carbonic anhydrase, produces carbonic acid (H_2CO_3) which immediately dissociates into a H^+ ion and $HCO3^-$ ion. The latter passes back into the blood while the H+ is pumped into the lumen via the canaliculi. Chloride ions are also actively transported across the cells into the canaliculi from capillaries in the lamina propria.

3.3. The histology of the gastric cardia

The gastric cardia is grossly defined anatomically as the area extending distally from the squamo-columnar junction at the lower end of the oesophagus into the proximal stomach. Conventional medical teaching states that cardiac mucosa is normally present in humans and extends a length of 2-3 centimetres. Cardiac mucosa has been defined histologically as mucosa in this region composed only of mucous cells. It is therefore distinct from the oxyntic mucosa typically seen elsewhere in the stomach which contains additional parietal cells and goblet cells. The endoscopic appearance (A) and histology (B) of the normal gastro-oesophageal junction are seen in Figure 3.1.

Cardiac mucosa is recognized as an area with a high incidence of inflammation, metaplasia and neoplasia. Oberg et al (94) studied patients with reflux symptoms or dyspepsia and found cardiac mucosa to be inflamed in 96%. Similarly Der et al (95) examined biopsied the cardia region in 141 patients. In all patients, cardiac mucosa showed significant chronic inflammation with 79% of patients

showing no evidence of gastritis on biopsies from the gastric antrum and body. Upper gastrointestinal endoscopy screening of patients attending for lower gastrointestinal endoscopy has also demonstrated the high prevalence of disease in this area. Gerson et al (96) performed upper gastrointestinal endoscopy and biopsy on 110 subjects attending for sigmoidoscopy as screening for colorectal cancer. In 16% of patients, intestinal metaplasia was found at the gastric cardia. Likewise, Rex et al (97) examined 961 patients attending for colonoscopy and found the detection rate of intestinal metaplasia at the gastric cardia to be 12.9%.

3.4. The 'acquired cardia' hypothesis

Despite the high rate of pathology at the gastric cardia, this complex area is poorly understood. Indeed controversy exists as to whether cardiac mucosa itself may be pathological. Previous autopsy studies show a near absence of cardia epithelium in neonates and an increase in length with increasing age (98;99). Similarly, in thoroughly sampling the squamocolumnar junction of endoscopically normal patients, Jain et al (100) and Marsman et al (101) reported an absence of cardiac mucosa in 65% and 38% respectively. Furthermore, in the study by Oberg et al (94), of the 334 patients studied only 246 (74%) were found to have cardiac mucosa while 88 (26%) did not. Those with cardiac mucosa were more likely to have abnormal acid exposure on 24 hour pH testing and were more likely to have abnormalities of the LOS manometrically. They concluded this was proof that cardiac mucosa is in fact an abnormal structure. They suggest it originates from acid-induced columnar metaplasia of the most distal oesophageal squamous mucosa with the only normal gastro-oesophageal mucosa being squamous oesophageal mucosa

and oxyntic gastric mucosa. It should be noted that *Helicobacter*-associated atrophy of the proximal gastric mucosa could cause a similar effect.

Although 30-50% of upper gastrointestinal tract adenocarcinomas occur at the gastro-oesophageal junction (GOJ) or gastric cardia (66), unlike oesophageal adenocarcinoma, the association of these cancers with symptomatic gastro-oesophageal reflux disease is weak (62). Studies using both 24 hour ambulatory pH monitoring and intraluminal impedance have shown a positive association between the proximal extent of reflux episodes and the perception of heartburn and regurgitation, the cardinal symptoms of reflux disease (102-105).

3.5. Distal opening of the lower oesophageal sphincter after meals

In studies using pull through pH and manometry, our own group have observed post-prandial shortening of the LOS. In studies by both Manning et al (93) and subsequently Clarke et al (40), this shortening was found to result from a loss of the distal segment of the lower oesophageal sphincter (LOS) with the pH step-up point moving closer to or even across the squamocolumnar junction (SCJ).

Fletcher et al had previously described an unbuffered postprandial ‘acid pocket’ occurring in the proximal stomach of healthy subjects after meals and observed that it extends across the SCJ into the distal oesophagus (39). The presence of a postprandial acid pocket in both healthy subjects and reflux patients was confirmed in the study by Clarke et al who found the ‘acid pocket’ to be located within the abdominal portion of the HPZ which ‘opens’ after a meal.

Therefore, this ‘distal opening’, which was first postulated by Oberg and DeMeester (94;106), may result in acidification of the most distal oesophageal squamous mucosa without complete loss of function of the LOS. Due to the limited proximal extent of acid exposure, such a process is likely to be asymptomatic and may explain the high incidence of pathology at the GOJ of otherwise ‘healthy’ subjects.

3.6. Summary

The gastric cardia and gastro-oesophageal junction is an important site of pathology in the gastrointestinal tract with a high incidence of inflammation, metaplasia and neoplasia even in asymptomatic people. It has been suggested that cardia mucosa itself may be pathological and represent acid-induced columnar metaplasia of the most distal oesophageal squamous mucosa. A possible mechanism for this could be distal opening of the LOS after meals in healthy subjects, exposing the distal oesophagus to the unbuffered post-prandial ‘acid pocket’.

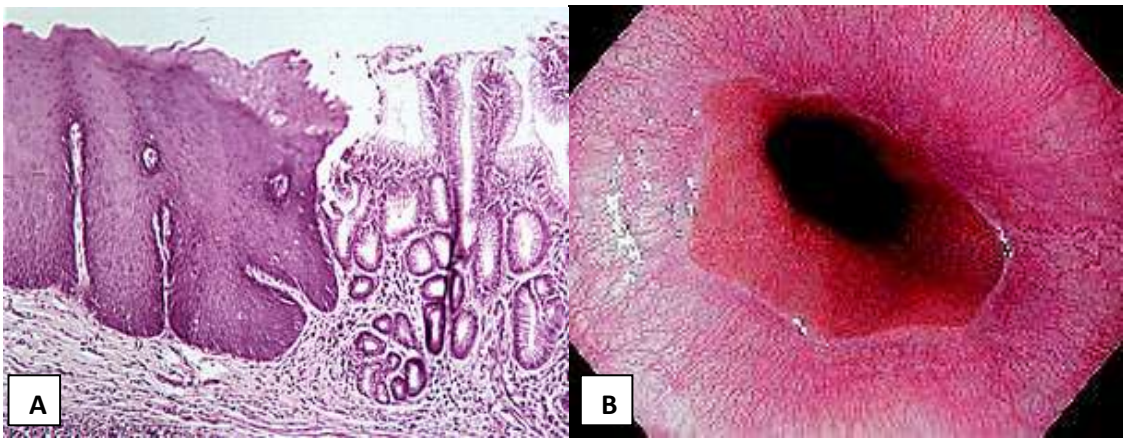

Figure 3.1. The normal gastro-oesophageal junction

Chapter 4

Obesity and Gastro-oesophageal Reflux Disease

Chapter 4

Obesity and Gastro-oesophageal Reflux Disease

4.1 The global obesity epidemic

In recent years global rates of obesity have dramatically increased, particularly in Western countries. As a result, healthcare systems are now dealing with what many describe as an epidemic of obesity and its complications, such as diabetes, heart disease and fatty liver disease.

The World Health Organisation (WHO) estimates that worldwide there are 1.7 billion people overweight (BMI ≥ 25 ≤ 30) while 300 million have a BMI >30 and would be classified as obese (107) according to the current WHO classification – see Table 4.1.

4.2. A Scottish perspective

In Scotland, a recent national survey (108) found that obesity rates have increased significantly over the past two decades. In adults between the ages of 16 and 64 years old, the percentage of men who are overweight or obese has risen from 55.6% in 1995 to 66.3% in 2009. A similar increase, from 47.2% to 58.4%, is seen among women while rates of morbid obesity (BMI > 40) have trebled from 0.5% to 1.8% and 1.3 to 3.6% in men and women respectively between 1995 and 2009. Measures of central, or visceral, obesity such as waist circumference or waist-to-hip ratio show similar increases over this period.

4.3. The rising Incidence of GORD

An increase in reflux disease has also been seen among Western populations over recent decades. A North American study by El-Serag et al (109) reported a four to seven-fold increase in presentations with reflux disease between 1970 and 1995. As a result, GORD is now the commonest chronic disease in Western populations. This raises the question of whether this increase in GORD can be explained due to the concomitant increase in obesity rates among the population?

4.4. The epidemiological link between obesity and GORD

Numerous studies have examined the link between obesity and GORD with almost all showing a significant association between obesity, measured by BMI, waist circumference or waist-to-hip ratio, and both the frequency and severity of reflux symptoms. This effect appears to be independent of differences in diet and exercise (110) and has been confirmed in two meta-analyses (49;111)

The largest epidemiological study to investigate this association was performed by Jacobson et al (112). Using a questionnaire, the authors investigated the frequency, severity and duration of symptoms of GORD among 10,545 randomly selected participants in the Nurses' Health Study. 2310 subjects (22%) had weekly symptoms of heartburn, acid regurgitation or both. They observed a dose-dependent relationship between increasing BMI and frequent reflux symptoms. Similar findings were documented when waist circumference was examined. Furthermore, in the group with a normal baseline BMI, an increase in BMI of greater than 3.5 was associated with an increased risk of frequent reflux symptoms (OR 2.8) when

compared with no weight gain. These results support the hypothesis that obesity increases the risk of GORD and suggest that even moderate weight gain in subjects of normal weight may cause or exacerbate reflux symptoms.

In the United Kingdom, similar work by Murray et al (113) studied 10,537 adult subjects as part of the Bristol *Helicobacter* Project. They confirmed this association in our own population, finding that overweight subjects were at increased risk of suffering GORD symptoms. The adjusted odds ratios for overweight subjects of suffering symptoms of heartburn and regurgitation were 1.82 and 1.5 respectively while the corresponding OR for obese subjects were 2.91 and 2.23.

The risk of reflux oesophagitis is also increased in overweight and obese subjects. In a study of 1224 Scandinavian patients referred for upper GI endoscopy, Stene-Larsen et al found that obesity, measured using the weight for height index, was associated with an increased risk of reflux oesophagitis (114). Following on from this, El-Serag et al found that obesity was an independent risk factor for severe (Los Angeles Grade C or D) oesophagitis with an OR of 1.21 (115).

These results clearly suggest an increased risk of both GORD symptoms and clinical manifestations in overweight and obese adults.

An interesting addition to the current evidence supporting this association is a randomised trial by Fletcher et al from our own group (116). In this study, which examines factors predicting response to proton pump inhibitor (PPI) therapy, 105 Helicobacter Pylori negative subjects with upper gastrointestinal symptoms but normal endoscopy were randomised to two weeks of treatment with PPI or placebo. Symptom severity scores were calculated before and during treatment with PPI or

placebo. Anthropometric measurements, 24 hour oesophageal pH studies and oesophageal manometry were also performed prior to randomisation. The investigators found that the only non-invasive predictor of response to PPI treatment was a patient's BMI. The BMI was noted to have a similar predictive value to 24h oesophageal pH or manometry suggesting a strong association between BMI and underlying reflux disease.

4.5. Mechanisms by which obesity may precipitate GORD

The epidemiological data clearly points to a significant positive association between obesity and GORD but if this is true, how does obesity lead to GORD?

A host of dietary and lifestyle factors relating to obesity share a common link to GORD but the effect of obesity has been shown to be independent of diet and exercise. It was previously suggested that obese patients may have a greater volume of acidic stomach contents than their leaner counterparts thus increasing the potential for harmful reflux. However, a study by Harter et al showed that, in fact, the opposite holds true with obese patients having a lower volume of gastric contents and a higher pH (117). Similarly, gastric acid secretion has actually been found to be lower in morbidly obese patients (118).

Despite this, there is good evidence that oesophageal acid exposure is increased in obese subjects. El-Serag et al and Cowell et al have both studied the effects of obesity on oesophageal acid exposure using conventional catheter-based 24 hour pH monitoring and 48 hour wireless capsule pH-metry respectively. In the first study, El Serag et al found that both increased BMI and waist circumference

were associated with a significant increase in reflux events, long reflux events (>5mins), time pH<4 and DeMeester Score (119). Subsequently Crowell et al confirmed these results over the more prolonged monitoring period, showing an increase in total acid exposure time and DeMeester scores in the obese subject group (120).

It has traditionally been postulated that obesity increases the mechanical stresses on the gastro-oesophageal junction. Increased abdominal girth could result in increased intra-abdominal pressure which in turn could promote gastro-oesophageal reflux by increasing intragastric pressure. Increased intragastric pressure potentially increases retrograde flow across the gastro-oesophageal junction by overcoming the lower oesophageal high pressure zone. However, as already described, the pathophysiology of GORD is multi-factorial and complex. As such, many possible mechanistic hypotheses exist to explain the role of obesity in reflux disease.

Increased intragastric pressure has been described in obese subjects. This results in an increase in the gastro-oesophageal pressure gradient (GOPG) in line with the traditional hypothesis above (121;122). However, in obesity, several morphological changes in the gastro-oesophageal junction have also been described. Since the GOJ provides the most important anti-reflux barrier, any change which results in a weakening of this barrier is likely to be important in the pathogenesis of reflux. Lower resting LOS pressure and shortening of the LOS length have been described in obesity (123). It has been suggested that the increased GOPG that exists in obesity may promote the development of hiatus hernia. Certainly several studies show a significant positive relationship between

obesity and the presence of a hiatus hernia, with an overall prevalence of 40% in obesity versus 12.6% in the general population (124). The association of hiatus hernia with GORD and oesophagitis is well described (33). Finally, Wu et al have previously demonstrated an association between obesity and post-prandial TLOSRs, the primary mechanism of reflux in patients with normal resting LOS pressure (125).

Contradictory evidence exists regarding the effect of weight loss on reflux disease. Studies by Fraser-Moodie et al (126) and Jacobson et al (112) both demonstrated a reduction in reflux symptoms with weight loss. Kjellin et al (127) studied the effects of weight loss on clinical and physiological correlates of reflux. Conversely, they found no effect on symptoms of GORD, pH or manometry. If weight loss does not reduce GORD, this could be explained by the hypothesis that obesity, through a subsequent increase in the GOPG, provokes the development of a hiatus hernia. This may be due to axial pressure strain through the phreno-oesophageal ligaments and crural diaphragm. Once a hiatus hernia has developed, the alteration in GOJ morphology and associated predisposition to reflux may be permanent regardless of weight loss.

4.6. Obesity and Barrett's oesophagus

Barrett's oesophagus, the metaplastic change in oesophageal mucosa from its usual squamous epithelial lining to a specialized columnar epithelium, occurs due to chronic reflux and is recognised as a strong risk factor for oesophageal adenocarcinoma. A number of recent studies show an association between obesity and the risk of Barrett's oesophagus. A retrospective cross-sectional study by Stein

et al (128) looked at 65 cases of Barrett's oesophagus compared with 385 controls. The risk of Barrett's oesophagus was increased approximately 2.5 times in subjects who were overweight or obese. While these results may be expected given the association between obesity and increased reflux, a study by Smith et al (129) suggests that it may not simply be by promoting acid reflux that obesity influences the development of Barrett's oesophagus. They performed a population-based case-control study of 167 Barrett's patients and 261 age and sex-matched controls, finding that obese subjects with reflux symptoms had a markedly higher risk of Barrett's oesophagus (OR 34.4) than subjects with reflux (9.3) or obesity (0.7) alone.

4.7. Male sex, visceral obesity, Barrett's oesophagus and adenocarcinoma of the oesophagus and GOJ

It is recognised that Barrett's oesophagus (130) and oesophageal adenocarcinoma (65) are significantly more common in males. A number of possible explanations for this gender difference exist. Barrett's oesophagus and oesophageal adenocarcinoma both arise from a background of chronic inflammation and mucosal damage. Oestrogen, has been shown to suppress the inflammatory response and cytokine production (131;132). Similarly, females have lower iron stores during their reproductive years which might modify the degree of DNA damage arising from chronic inflammation.

Gender differences are also well recognized in the distribution and type of adipose tissue. For any given body mass index (BMI), body composition differs between the sexes. Men have a higher lean mass while in women there is more

adiposity (133). Furthermore, the distribution of fat in males tends to be more central but predominantly peripheral in females. This altered fat distribution has been demonstrated using magnetic resonance imaging (MRI) and computerized tomography (CT) to correspond with greater visceral adipose tissue (VAT) and less subcutaneous adipose tissue (SAT) in men compared with women (134;135). Similarly, greater VAT is found in caucasian versus non-caucasian populations (136).

A higher risk of Barrett's oesophagus and oesophageal adenocarcinoma is seen in white males, in whom central (visceral) obesity predominates. Studies using CT measurement of visceral adiposity support the association between central obesity and both Barrett's oesophagus and oesophageal adenocarcinoma.

Fat distribution may act through a mechanical effect promoting the development of gastro-oesophageal reflux with a subsequent increased risk of Barrett's oesophagus and oesophageal adenocarcinoma. Alternatively, visceral adipose tissue is well recognised to be more metabolically active and secretes a variety of biological substances which may be pro-inflammatory and promote tumourigenesis (107). Potential mediators of carcinogenesis in obesity and biological pathways which have been implicated as contributory are listed in Table 4.2.

A number of possible mechanisms of tumourigenesis have also been described. Altered metabolism and increased production of sex hormones and glucocorticoids occurs in obesity (137). The resulting increases in insulin resistance induce compensatory hyperinsulinaemia with increased production of insulin-like growth factor 1. Increased cellular proliferation and impaired apoptosis may result, increasing the risk of metaplasia and neoplasia (138). Alternatively, Leptin may

stimulate tumour cell growth, migration and invasion as well as enhancing angiogenesis (139).

It is estimated that approximately 40% of patients with oesophageal adenocarcinoma have developed the disease due to being overweight or obese (140). Several large prospective epidemiological studies have demonstrated an increased risk of oesophageal adenocarcinoma and gastric cardia adenocarcinoma with increasing BMI (141-143). The strong positive relationship between obesity and these cancers persists when adjusted for meal size and physical activity (144) and obesity has also been found to be associated with the development of these cancers at an earlier age. Corley et al (145), using a nested case-control study design, have shown that increasing waist circumference is associated with an increased risk of oesophageal adenocarcinoma, independent of BMI. This data again supports a strong association between the risk of oesophageal adenocarcinoma and visceral obesity.

4.8. Obesity and the distal opening hypothesis

As previously discussed, 30-50% of upper gastrointestinal cancers occur at the GOJ, often in otherwise asymptomatic patients. Distal opening of the LOS after meals could expose the distal oesophageal squamous mucosa to the harmful and potentially carcinogenic effects of acidic reflux.

Previous studies in respiratory medicine have demonstrated that the diaphragm moves proximally in obesity, particularly when lying flat (146-149). Therefore, the migration of the squamous oesophageal lining into the stomach may

be more pronounced with increasing body mass index (BMI). This ‘distal opening’ may help to explain the increasing incidence of gastro-oesophageal junction cancers in western countries where there is also a trend to increasing BMI.

4.9. Summary

Western countries are currently experiencing an obesity epidemic. At the same time, gastro-oesophageal reflux disease and its complications are becoming more common in these populations. Clear links exist between symptomatic GORD, Barrett’s oesophagus and oesophageal adenocarcinoma. Furthermore, obesity could theoretically exacerbate distal opening of the LOS with resultant acidification of the distal oesophageal squamous mucosa leading to acid-induced inflammation, metaplasia and ultimately neoplasia.

Table 4.1 WHO BMI Classification

Classification	BMI (kg/m^2)
Underweight	<18.5
Normal	≥18.5 and <25
Overweight	≥25 and <30
Obese	≥30

Table 4.2. Potential mediators and pathways implicated the role of obesity in tumourigenesis

Mediator	Pathways
Adipokines	Complement Activation
Cytokines	Renin Angiotensin System
Tumour Necrosis Factor α (TNFα)	Altered Glucocorticoid Metabolism
Insulin	Altered Sex Hormone Metabolism
Plasminogen Activator Inhibitor 1	
Resistin	
Leptin	
Adiponectin	
Vascular Endothelial Growth Factor (VEGF)	

Chapter 5

Research Aims and Methods

Chapter 5

Research Aims and Methods

5.1. Research Aims

The aim of this series of experiments was to further investigate, in normal human subjects, the basic pathogenesis of gastro-oesophageal reflux and oesophageal columnar metaplasia.

In particular:

i. to examine the effect of nitrite delivered in saliva, and the associated luminal nitric oxide produced on reaction with acid at the gastro-oesophageal junction, on gastro-oesophageal function.

ii. to investigate anatomical and physiological changes at the GOJ following a meal

iii. to investigate the effect of age and obesity on the GOJ

5.2. Methods

5.2.1. Upper gastrointestinal endoscopy

Prior to the first study day, the participant attended fasted for an endoscopy. This was carried out using topical anaesthesia with xylocaine local anaesthetic spray. The anatomy of the upper gastrointestinal tract was documented. Biopsies were taken from both gastric antrum and body for rapid urease CLO test to

document Helicobacter Pylori status. Endoscopy was carried out a minimum of one day before the first study day.

5.2.2. pH studies

All pH studies were performed using a specially designed antimony 12 sensor high-resolution pH catheter (Synectics Medical Ltd, Enfield, UK) as previously described (150). The catheter was 2.1 mm in diameter, flexible and had 12 electrodes along its distal end with an external reference electrode for application to the upper arm – see Figure 5.1. The most distal pH electrode was located at the tip of the catheter and the other 11 electrodes were 30, 50, 61, 72, 83, 94, 105, 116, 127, 138 and 172 mm proximal to it – see Figure 5.2. Prior to nasal intubation, individual electrodes were calibrated in buffer pH 1.07 and 7.01. Data was collected and analysed using PolygramNet® software (Medtronic Inc., Fridley, Minnesota, USA) and was exported and processed using our own custom-designed software for further detailed analysis.

5.2.3. High-resolution manometry studies

Manometry was performed using a solid-state high-resolution manometry assembly (Manoscan®, Sierra Scientific Instruments Inc., Los Angeles, California, USA). The catheter comprises 36 circumferential pressure sensing elements spaced at 1cm intervals – see Figure 5.3. Each sensing element incorporates 12 radially dispersed pressure sensors able to detect pressure over a length of 2.5mm. Sector pressures are averaged to provide a circumferential pressure reading. The system

was calibrated to 0 and 300mmHg using externally applied pressure prior to use. Furthermore *in vivo* calibration was performed at body temperature (36-38°C). Immediately after extubation, while sensing-elements remained at body temperature and were exposed to atmospheric pressure a time for thermal re-calibration was selected. To correct for thermal drift, a change in measured pressure due to change in temperature, a linear correction from 0mmHg was applied to the exported manometry data. Robertson et al, from our own group, have previously characterized the thermal drift which occurs in prolonged studies using the Manoscan® high-resolution manometry (151). They have shown this to be a linear phenomenon and the above correction process significantly improves the accuracy of recordings using this system.

Manometry studies were analysed using specialized analysis software (Manoview®, Sierra Scientifics) – an example of a normal swallow viewed using the Manoview® analysis software is shown in Figure 5.4. In addition to this, manometric data was exported and processed using our own custom-designed software for further detailed analysis.

5.2.4. Combined pH and manometry apparatus

pH and manometry apparatus was attached using adhesive tape with pH sensor 7 adjacent to manometry sensor 29 – see Figures 5.5. and 5.6. This allowed accurate comparison of pH and manometry. The combined apparatus was passed per nasally and attached at the nose using adhesive tape. pH and manometry studies were synchronized electronically.

5.2.5. Data processing

Our pH and manometry studies were synchronized electronically by starting both recordings simultaneously. For all studies the raw manometry and pH data was extracted as text files from the Manoscan® and PolygramNet® software respectively. The 36 channel Manoscan® system records manometry data at a frequency of 40Hz while our high-resolution pH catheter records 8 readings per second for each of the 12 sensors (8Hz). Therefore, for a study consisting of 15 minute fasting data and 90 minute post-prandial data this generates 9,676,800 individual data points. The synchronized pH and manometry data was combined using our custom-designed 'ManpH' software. This allowed us to compress the manometry data into 8 readings per second allowing direct comparison with the pH data. To do this the mean of the manometry recordings taken over each 0.125s was calculated from the raw data. This reduced to 2,419,200 the number of individual data points. It also allowed selection of specific time periods and provided text files that could be exported into Microsoft Office Excel 2007 (Microsoft®, Redmond, Washington, USA) for further analysis.

5.2.6. Nitrite solutions

Nitrite solutions were pre-prepared as potassium nitrite by the Pharmacy Production Unit, Western Infirmary, Glasgow. Solutions were labelled A-D with the researcher blinded to the concentration of each solution until data analysis had been performed. Stock solutions were regularly monitored for stability of nitrite concentrations, again the researcher was blinded to these results.

The concentrations of potassium nitrite were 0, 0.286, 2 and 14mmol/l – details of the nitrite solutions are shown in Table 5.1. Each solution also contained 1mmol/l Potassium Thiocyanate. Thiocyanate is normally present at this concentration in non-stimulated saliva but levels have been found to be lower in studies involving orogastric tube insertion and with increased salivary flow rates.(80;152) Solutions were prepared to a pH expected of normal saliva (pH 6.12 – 6.38).

Concentration of nitrite in saliva in the fasting state ranges between 50 and 600µmol/l and rises several fold following ingestion of nitrate in the diet (79). The concentration of nitrite used in our highest dose was greater than that occurring in swallowed saliva after a nitrate–rich meal. It is equivalent to the total nitrite load in the study by Manning et al which demonstrated an increase in post-prandial TLOSRs following infusion of a NO-generating solution into the gastric cardia (93).

In normal circumstances, the minimal total unstimulated salivary flow rate is defined as 0.1 ml per minute, and the minimal stimulated flow rate is 0.2 ml per minute. Maximal stimulated flow rate is 7 ml per minute. The 24-hour volume of salivary secretion has been estimated to be 500 to 1,500 ml, or an average flow of 1 ml per minute.

Table 5.2. shows the estimated potassium concentrations of saliva and gastric secretions compared with our solutions over the 90 minute study period. The maximum safe nitrite dose for a healthy volunteer has been estimated by the World Health Organisation (WHO) as between 290 - 370 mg of nitrite (153). The maximum cumulative nitrite dose used in our study, over all 4 study days, was less than 20% of this value at 50.57mg.

5.2.7. Controlling for variability in dietary nitrate intake

To control for variability in dietary nitrate intake volunteers were instructed regarding measures to reduce their endogenous salivary nitrite content in the 24 hours prior to each study day. This included avoiding foods recognised as having a high nitrate concentration, such as salads and green leafy vegetables, as well as the twice daily use of 0.2% chlorhexidine antibacterial mouthwash. Antibacterial mouthwash has been shown to reduce salivary nitrite concentrations by reducing bacterial reduction of salivary nitrate to nitrite.(154)

5.2.8. Intra-oral nitrite delivery

On each of the nitrite study days, the volunteers' salivary nitrite concentrations were modified to a different level post-prandially by intra-oral infusion of potassium nitrite solutions. Following consumption of our standardised meal, a fine bore (1mm) catheter (Vygon (United Kingdom) Ltd, Cirencester, Gloucestershire, United Kingdom) perforated at its distal end, was placed in the mouth for infusion of test solutions to modify salivary nitrite concentration – see Figure 5.7. Solutions were infused over 90 minutes at a rate of 0.5ml per minute.

5.2.9. Quantification of salivary nitrite concentration

Immediately after sampling, 1ml of saliva was mixed with 50µl of 1mmol/l NaOH. This rendered the sample alkaline, preventing nitrite being converted to nitric acid and lost. Samples were stored at 4ºC, then centrifuged on the same day at 3000 rpm for 10 minutes. 250µl aliquots were subsequently removed from the supernatant. These were frozen at -20ºC for later analysis. Analysis of thawed

samples was performed on 96-well microplates using a modified Griess reaction. Colorimetric analysis was performed 30 minutes after the addition of the Griess reagents using a 540nm filter. Analysis was performed at CBP laboratories, Western Infirmary and results blinded to researcher until after data analysis was complete.

5.2.10. X-ray screening

X-ray screening of the gastro-oesophageal junction was carried out during normal respiration using a portable C-arm fluoroscope (Philips BV Pulsera). Screening was performed both in the fasting state and following the meal for approximately 30 seconds and synchronized with pH manometry recordings using an event marker. Images were recorded using a digital video recorder (JVC SR-DVM600E). Further analysis was performed using image analysis software (Scion Image, Scion Corporation, Frederick, Maryland, USA). The position of the clip relative to the pH and manometry catheters was calculated with the measured distance of one pH sensor (known to be 1cm) used as an internal scale. An example of an X-ray screening still obtained during the study is shown in Figure 5.8.

5.3. Statistical Analysis

Statistical analysis was performed using MiniTab version 16 (Minitab, State College, PA, USA) or SPSS version 18 (IBM, New York, NY, USA) statistical software.

Table 5.1 Details of nitrite solutions

Nitrite Concentration (mmol/l)	Total Nitrite Load (µmol)	Randomisation
0	0	B
0.26	12.87	C
2	90	A
14	630	D

Table 5.2 Estimated electrolyte levels in stimulated saliva and post-prandial secretions and comparison with nitrite solutions

Electrolyte	Range in normal stimulated saliva (mg)*	Range in post-prandial gastric secretions (mg) †	Maximum present in solutions infused (mg)
Potassium	35.2 -105.6	71.0 – 699.6	26.56

*Calculated from stimulated saliva flow rate of 1ml/min = 90ml over study period

†Calculated from estimated 90-minute post-prandial gastric secretion volume of 506.9ml (155)

Figure 5.1. 12-sensor pH catheter

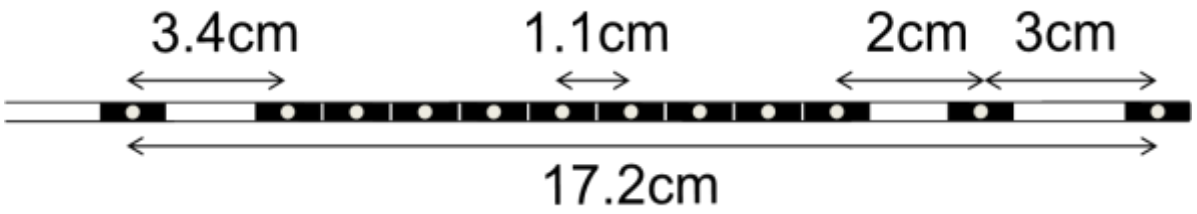

Figure 5.2. pH catheter sensor spacing

Figure 5.3. Manoscan® 36-sensor high-resolution manometer

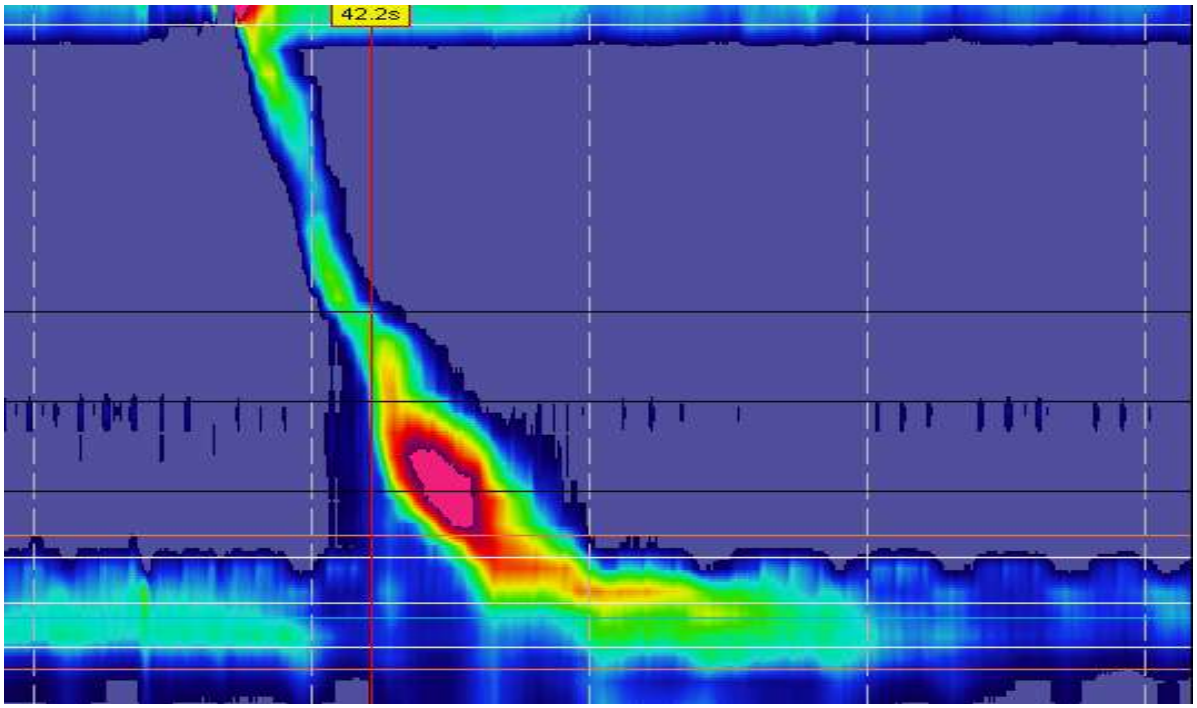

Figure 5.4. Manoview® analysis software – normal swallow

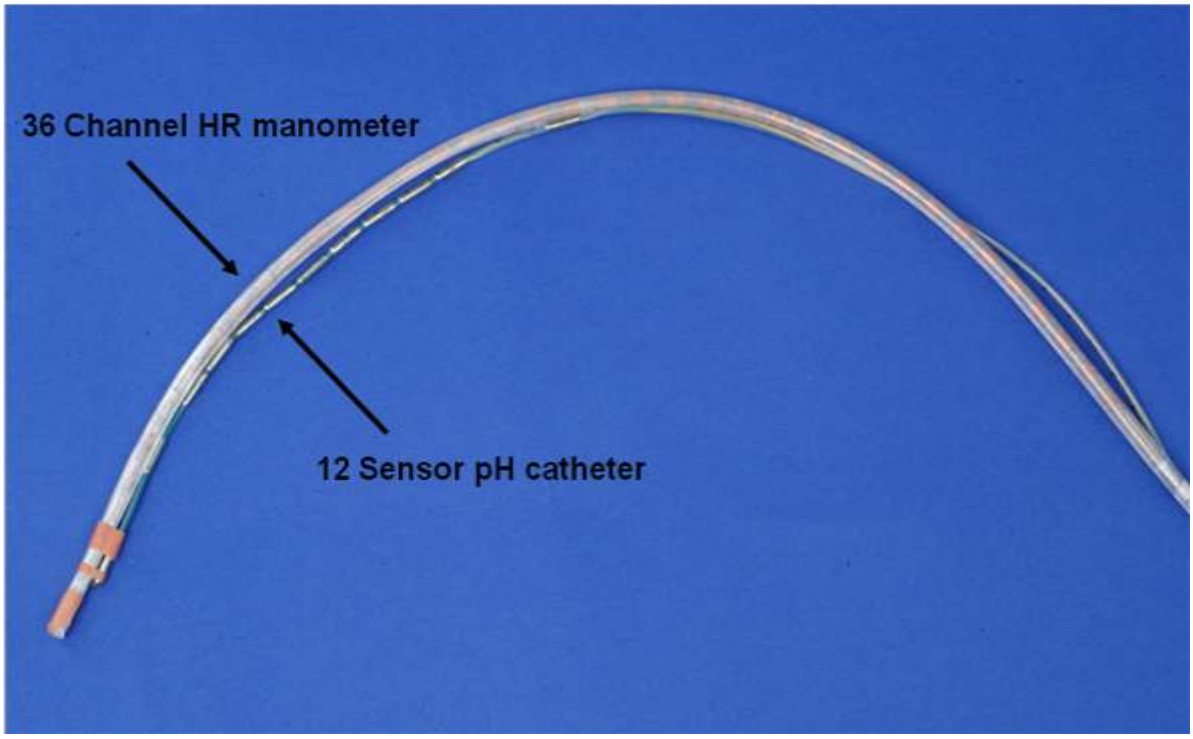

Figure 5.5. Combined pH manometry apparatus

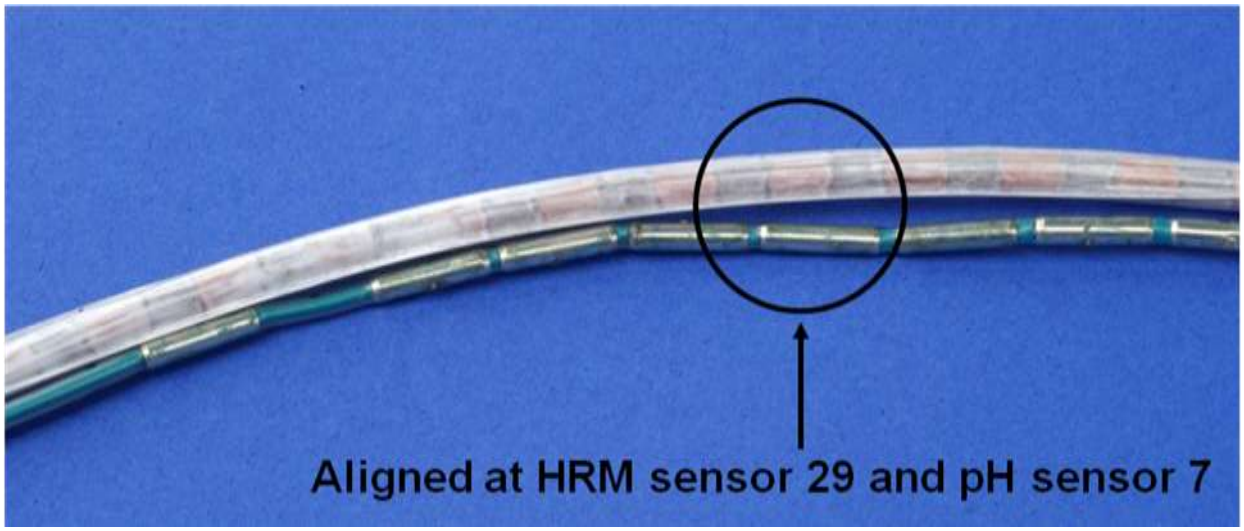

Figure 5.6. Combined pH manometry apparatus alignment

Figure 5.7. Intra-oral nitrite infusion catheter

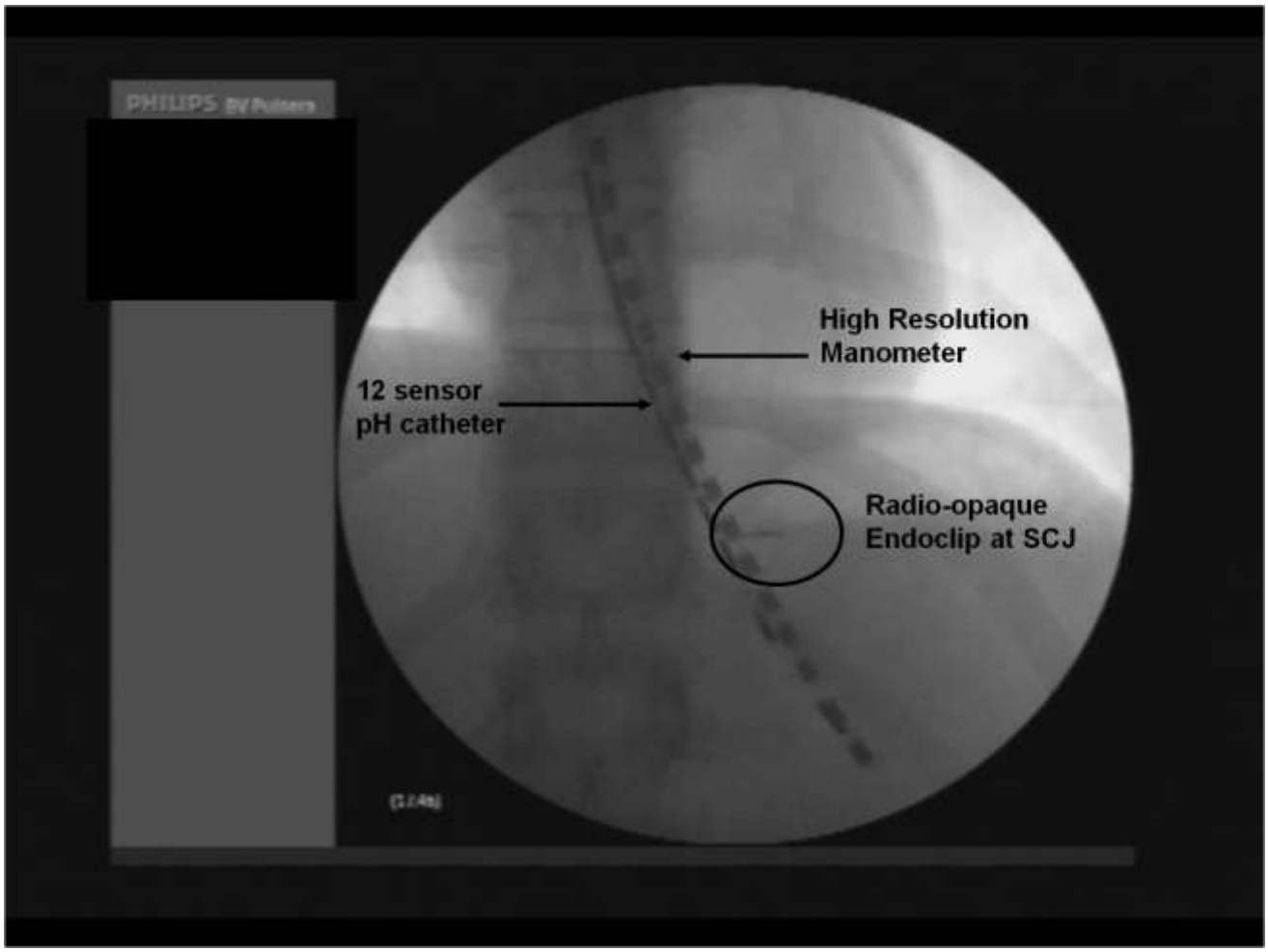

Figure 5.8. Fluoroscopic screening image

Chapter 6

The Effect of Nitrite in Saliva on Gastro-oesophageal Reflux

Chapter 6

The Effect of Nitrite in Saliva on Gastro-oesophageal Reflux

6.1. Introduction

Human saliva has a high nitrite concentration from the entero-salivary recirculation of dietary nitrate. Swallowed nitrite reacts with gastric acid to form nitric oxide (NO) at the gastro-oesophageal junction. This may contribute to the pathogenesis of reflux disease. NO has been shown to reduce lower oesophageal sphincter (LOS) pressure, impair oesophageal clearance and may be the final mediator in the production of transient lower oesophageal sphincter relaxations (TLOSRs).

6.2. Aims

The aims of this study were:

i. to determine whether nitrite in swallowed saliva increases oesophageal acid exposure

ii. to establish whether nitrite in swallowed saliva modifies the manometric characteristics of the lower oesophageal sphincter

iii. to examine how any of these effects relate to the range of nitrite present in human saliva.

6.3. Subjects

Twenty-five non-smoking healthy subjects were recruited. In 2 subjects we were unable to pass the pH manometry apparatus per nasally while a further 3 subjects withdrew due to discomfort during the procedure. 20 subjects (11 male) completed the full study protocol with a mean age of 30.8 years (Range 19 – 59). The baseline characteristics of our study population are shown in Table 6.1.

6.4. Methods

The subjects underwent studies of gastro-oesophageal function on 4 separate days with their saliva nitrite concentration modified to a different level on each of the days. The study day outline is illustrated in Figure 6.1. Two days prior to the first study day, upper gastrointestinal (GI) endoscopy was performed in order to document the anatomy of their upper GI tract. Antral and body biopsies were taken for *H. pylori* rapid urease test.

On each of the four study days, subjects reported fasted at 0900h and a combined high resolution solid state manometer probe and high resolution pH catheter were passed per nasally to allow detailed continuous recording of pressure and pH throughout the oesophagus, gastro-oesophageal junction and proximal stomach. The high resolution manometer has 36 pressure sensors 1cm apart and records the pressure at each site 40 times per second as previously described (156). The custom-made pH catheter has 12 antimony sensors, with the nine electrodes in the region of the squamocolumnar junction 11 mm apart, and records the pH at each site eight times per second as previously described (150). A fine bore (1mm)

catheter was placed in the mouth and secured to the teeth for infusion of test solutions into the mouth to modify salivary nitrite concentration. The studies were performed with the patients sitting semi-upright.

After recording pH and manometry over a 15 min fasting period, the subjects consumed a standardized meal over 20 minutes and the pH and manometer recordings continued for a further 90 minutes. The meal consisted of two fishcakes and one rolled oats flapjack with 150ml water. (Total calorie content = 526.35kcal). Infusions of the test solutions (0.5ml/min) into the mouth commenced immediately after completion of the meal and continued throughout the 90min postprandial recording period. The buccal infusions were given in randomised double blind fashion containing 0, 0.286, 2 or 14 mmol/l potassium nitrite on the four separate study days. All participants were instructed regarding measures to reduce their endogenous salivary nitrite content over the 48 hours prior to each test. This consisted of avoiding high nitrate containing foods and twice daily use of chlorhexidine antibacterial mouth wash. Samples of saliva were obtained for determining the actual nitrite concentration during the fasting period and at 30 minute intervals following the commencement of the nitrite infusion on each study day. The concentrations were not revealed until all the data were complete.

Blood pressure and pulse rate were recorded on each study day prior to and following completion of the nitrite infusion.

6.5. Analysis

6.5.1 Oesophageal acid exposure

Acid exposure was calculated as %time pH<4 and recorded both at 5 - 6cm (traditional reflux) and 1 - 2cm (short-segment reflux) proximal to the fasting pH step-up point. Individual reflux episodes were identified using PolygramNet® software (Medtronic Inc., Fridley, Minnesota, USA). These were defined at 5-6cm according to the following criteria; i) pH fall to <4 with a minimum pH fall of 1 unit, ii) a minimum duration of event of 3 seconds and iii) a minimum interval of 10 seconds between events (157). The duration (in seconds) and nadir pH of each reflux event was also recorded.

6.5.2. Manometry of the lower oesophageal sphincter

Transient Lower oesophageal Sphincter Relaxations (TLOSRs) were identified using the Manoview analysis software and defined according to the Holloway criteria (20) which is detailed in Table 6.2.

For more detailed analysis, the 15 minute period immediately before the meal and the 6 consecutive 15 minute periods after the meal were identified. During the last 10 minutes of each period, 6 inspiratory and 6 end-expiratory points were selected using a custom-made computer programme. The lower border of the LOS was defined as the most distal position where the pressure rose to > 2mmHg above gastric baseline. Similarly, the upper border of the LOS was defined by a fall in pressure to within 2mmHg of intragastric pressure. The expiratory and inspiratory pressures were defined as the highest recorded pressures within the LOS at each

time-point. The respiratory inversion point (RIP) was defined as the point at which the manometry pressure wave decreases on inspiration. Total LOS length, intra-thoracic sphincter length and intra-abdominal sphincter length were calculated in expiration.

To evaluate oesophageal peristalsis, using the Manoview® analysis software, a single completed swallow was identified from each of the 9 consecutive 10 minute periods following completion of the meal. The mean peristaltic wave velocity was calculated using measurements from 9cm and 5cm proximal to the mid-point of the lower oesophageal high pressure zone (HPZ). Mean onset velocity (cm/s) was calculated between 13cm and 5cm proximal to the mid-point of the high pressure zone.

6.5.3. Intragastric pressure

Intragastric pressure was defined, relative to atmospheric pressure, as the median pressure in the 3 sensors immediately distal to the LOS.

6.6. Power calculations

The primary outcome was oesophageal acid exposure. Calculations indicated that recruiting 20 subjects would have power to detect a doubling (100% change) in values. This assumes standard deviation of the percentage change in oesophageal acid exposure to be 105 and utilizes a significance level of 1% to account for the

multiple comparisons that will be made with a two-sided test. The standard deviation of the percentage change was derived from published data (158).

6.7. Statistical analysis

All results are given as medians and interquartile ranges unless otherwise stated. The Wilcoxon signed-rank test was used to analyse the effect of the meal on the control day. To test the changes of variables against different nitrite solutions, the nonparametric Friedman's test or Kruskal-Wallis test were used. The Wilcoxon-signed rank test or Mann-Whitney U test was used as post-hoc test in case of a significant (p value < 0.05) result of the Friedman's or Kruskal-Wallis tests.

6.8. Ethics

The study was approved by West Glasgow Research Ethics Committee and informed consent was obtained from each participant.

6.9. Results

Of the 25 healthy volunteers recruited, 5 could not tolerate the nasal intubations and withdrew from the study. Twenty (11 male, mean age 30.8 years, range 19-59; 18 *H. pylori* negative) completed the full study protocol.

6.9.1. Effect of the meal on gastro-oesophageal function on the control day

We and others have reported previously that changes occur in the gastro-oesophageal junction in response to a meal. These include (i) increased oesophageal acid exposure, (ii) increased frequency of TLOSRs, (iii) reduction in peak LOS pressure and (iv) reduction in overall LOS length due to loss of pressure in its most distal component (40;93). In order to confirm the validity of our recording equipment and experimental set-up, we analysed data obtained on the control infusion day to ensure that the expected changes were recorded in response to the meal. Manometry data obtained over the 15 minute initial fasting period was compared with that over each six consecutive 15 min periods after commencement of the standardised meal. Results and p values are displayed in Table 6.3.

6.9.1.a. Oesophageal acid exposure

In the conventionally positioned sensor (5 - 6cm above fasting pH step-up point) the median percentage time oesophageal pH < 4 increased from 0% (0 - 1.89) during fasting to 0.49% (0 - 19.96) post-prandially (p=0.002). Similarly at the sensor located 1 - 2cm above the fasting pH step-up point, acid exposure increased significantly after the meal from 1.21% (0 – 7.75) fasting to 6.01% (0.89 – 74.36) post-prandially (p<0.001). These results are illustrated in Figure 6.2.

6.9.1.b. Lower oesophageal sphincter

There was an increase in the rate of TLOSRs following the meal from 0/hour (0 - 4) during fasting to 7.3 (4 - 11.3) post-prandially ($p<0.001$) – see Figure 6.3. Expiratory LOS pressure was reduced following the meal from 29.37mmHg (11.83 – 51.19) to 19.18mHg (8.18 – 34.53; $p=0.001$) relative to atmospheric pressure and from 17.29mmHg (4.9 – 37.76) to 12.04mmHg (4 – 23.79; $p<0.001$) relative to intragastric pressure. Inspiratory LOS pressure was also reduced from 39.47mmHg (16.9 – 60.17) to 30.42mmHg (14.45 – 53.09; $p=0.02$) relative to atmospheric pressure and 25.35mmHg (10.11 – 43.9) to 18.13mmHg (6.50 – 40.25; $p=0.035$) relative to IGP.

Although the absolute reduction in LOS pressure following the meal was similar in both expiration and inspiration, proportionally, the post-prandial fall in expiratory LOS pressure was greater than the reduction in inspiratory LOS pressure relative to both atmospheric pressure and IGP. The post-prandial reductions in expiratory LOS pressure and inspiratory LOS pressure, relative to atmospheric pressure, were 27.92% (-9.15 – 71.76) and 22.67% (-139.86 – 59.3; $p=0.005$) respectively. Similarly, when taken relative to intragastric pressure, the end-expiratory reduction in LOS pressure was greater, being 39.66% (-30.74 – 78.8) versus a reduction in the inspiratory LOS pressure of 33.3% (-185.1-63.9; $p=0.004$). The result for each individual 15 minute post-prandial period is displayed in Figure 6.4.

Following the meal there was significant shortening of the LOS length, from 5cm (3 – 6cm) to 4cm (1 – 6cm; $p=0.001$) during expiration, due to loss of the distal component of the LOS. While intrathoracic length was unchanged, median difference

0cm (-1 – 2), intra-abdominal sphincter length was reduced, median difference 1cm (0 – 2; p<0.001) – see Figures 6.5 – 6.7.

6.9.1.c. Intragastric pressure

There was a trend towards a reduction in intragastric pressure following the meal. Inspiratory IGP fell from 11.99 (6.02 – 20.02) fasting to 10.25 (9.52 – 21.79;p=0.059) after the meal. Expiratory IGP fell from 10.51mmHg (4.15 – 19.07) fasting to 8.93mmHg (3.22 – 20.4: p=0.089) after the meal. These results are shown in Figure 6.8.

6.9.3. Effect of alteration in salivary nitrite on gastro-oesophageal function

The analysis of salivary nitrite demonstrated that there was a significant difference in the salivary nitrite concentrations on each of the four study days. At 60 minutes following the meal the concentrations of salivary nitrite recorded were 24.3µmol/l (3.8 – 65.4), 80.4µmol/l (20.8 – 158.7), 372.8µmol/l (118.2 – 726.6) and 2398.5µmol/l (600 – 8087) for the solutions containing 0mmol/l, 0.286mmol/l, 2mmol/l and 14mmol/l potassium nitrite respectively. The difference in salivary nitrite concentrations at 60 minutes is shown in Figure 6.9.

To save space in presenting results, the four different study days will be referred to as 1, 2, 3, 4 with one being the lowest nitrite concentration (control) and 4 the highest. Results and p values are displayed in Table 6.4.

6.9.2.a Oesophageal acid exposure

The percentage time pH<4 at 5-6cm above the fasting pH step-up point during the 90 min postprandial period was similar on each study day, being 0.43 (0 - 19.96), 1.09 (0 - 35.8), 1.02 (0 - 28.09) and 0.57 (0 - 31.45) on days 1-4 respectively – see Figure 6.10. At 1-2cm above fasting pH step-up point, the corresponding values were also not different from each other, being 6.01 (0.84 – 74.36), 6.38 (0.81 – 86.63), 9.67 (0.02 – 93.2) and 5.72 (0.08 – 68.64) – see Figure 6.11. The number of reflux events was also similar on each study day being 2.5 (0 – 11), 5 (0 – 16), 4 (0 – 17) and 2.5 (0 – 13) for study days 1-4 respectively– see Figure 6.12. Duration of reflux events was also similar being 12.81 seconds (3.09 - 264.12), 14.98 seconds (3.24 – 301.15), 17.65 seconds (3.08 – 353.31) and 13.34 seconds (3.11 – 473.4) – see Figure 6.13.

6.9.2.b. Lower oesophageal sphincter function

There was no difference in the number of TLOSRs recorded during the 90 minute postprandial period between the different study days being 11 (6 - 17), 13 (6 - 17), 12 (6 - 17) and 12 (7 - 18) for solutions 1-4 respectively – see Figure 6.14.

Peak expiratory LOS pressure decreased following the meal on each study day relative to both atmospheric and intragastric pressure. Comparing the fasting and postprandial values for each individual showed that the change in LOS pressure following the meal was similar on each of the study days, being –8.03mmHg (-26.12 – 2.08), -7.83mmHg (-37.56 – 7.72), -5.67mmHg (-42.37 – 12.82) and -8.33mmHg (-55.57 – 4.13) relative to atmospheric pressure and -7.14mmHg (-22.9 – 3.15), -

4.88mmHg (-37.04 – 5.75); -6.76mmHg (-42.08 – 14.7) and -8.54mmHg (-51.83 – 2.15) relative to IGP for solutions 1-4 respectively.

The changes in the lower oesophageal sphincter profile following the meal were similar on each study day including total length, intra-thoracic sphincter length and intra-abdominal sphincter length.

6.9.2.c. Oesophageal peristalsis

There was no difference in the peristaltic wave amplitude during the 90 min postprandial period on the four study days being 36.5mmHg (18.5 – 98.7), 33.5mmHg (19.4 – 83.6), 33.4mmHg (21.1 – 79), 32.4mmHg (19.8 – 93.1) on days 1-4 respectively – see Figure 6.15. Peristaltic wave velocity was also similar on the four different study days being 5.2cm/s (2.6 – 11.3), 5.3cm/s (2.9 – 15.7), 4.8cm/s (2.8 – 26.5) and 4.8cm/s (3.1 – 17.9) on days 1-4 respectively – see Figure 6.16.

6.9.2.d. Intragastric pressure

Intragastric pressure fell following the meal on each study day. Comparing the median fasting and median postprandial value for each individual showed that there was no difference in the change in intragastric pressure following the meal between the 4 study days when measured in either inspiration or expiration. The median difference being -1.78mmHg (-9.46 – 5.6), -1.4mmHg, (-6.1 – 3.5), -0.1mmHg, (-5.4 – 5.7) and -0.42mmHg, (-9.2 – 7.7; $p>0.032$) in inspiration and -

1.57mmHg (-7.2 – 4.81), -1.83mmHg (-5.21 – 2.05), -0.49mmHg (-5.45 – 4.54) and -1.5mmHg (-9.19 – 6.15; p>0.112)) in expiration with solutions 1-4 respectively.

6.9.3. Effect of alteration in salivary nitrite on blood pressure and pulse

There was a significant difference in the change in systolic blood pressure (pre to post infusion) between the 4 study days (p= 0.007, by Friedman's test). However, on post-hoc analysis, only the solution containing 0.286mmol/l nitrite showed a significant change in systolic blood pressure compared with the control solution. The change in systolic blood pressure was -4 mmHg (-29 – 18), 2.5 mmHg (-17 - 30; p<0.05 vs. control), -1 mmHg (-15 -24; p=0.85 vs. control) and -12 (-25 6; p= 0.17 vs. control) for solutions 1-4 respectively – see Figure 6.17.

Diastolic blood pressure and pulse rate were similar following infusions on each of the four study days – see Figures 6.18 and 6.19.

6.10. Discussion

The above studies demonstrate that the range of nitrite likely to be present in human saliva does not modify gastro-oesophageal function or predispose to gastro-oesophageal reflux.

There are several reasons why we believe our findings are robust and that it is very unlikely that a clinically relevant effect has been missed. Firstly, we studied the effect of nitrite over a 100-fold range in saliva nitrite concentrations which extended from 50% of the normal lowest fasting concentrations to approximately ten

times the peak concentrations reported following human nitrate ingestion. Secondly, we confirmed with strong significance all the previously reported changes in gastro-oesophageal function following a meal indicating that our study protocol was sensitive and robust (32,33). These changes included a fall in LOS pressure, loss of distal component of LOS, increase in frequency of TLOSRs and increased oesophageal acid exposure.

On the control day we observed that the fall in LOS pressure following the meal was similar during inspiration and expiration when expressed as absolute values but greater during expiration when expressed as percentage fall. The LES comprises two main components, the first being the intrinsic sphincter within the wall of the distal oesophagus and proximal stomach and the second being the extrinsic sphincter formed by the crura of the diaphragm. The rise in LOS pressure on inspiration is due to the increase in pressure exerted by the diaphragmatic crura. The intrinsic sphincter makes a greater contribution to the total pressure during expiration than inspiration. The overall LOS pressure is thought to represent the sum of the crural pressure plus intrinsic sphincter pressure (34). The fact that the fall in LOS pressure after the meal was proportionally greater during expiration suggests that it is due to a fall in the pressure exerted by the intrinsic sphincter. This may be explained by the effects of cholecystokinin released after a fatty meal (159;160).

We also observed a shortening of the length of the LOS following the meal during expiration mainly affecting the abdominal segment. This is again consistent with relaxation of the intrinsic component.

The ability to detect a change in the intrinsic component of the LOS in our study protocol was important as an effect of nitric oxide derived from the luminal

nitrite would be on the intrinsic and not extrinsic sphincter. Previous investigators have also demonstrated the ability to detect changes limited to the intrinsic sphincter while measuring overall LOS pressure. For example, atropine relaxes the intrinsic sphincter but does not affect the diaphragmatic crura and a fall in lower oesophageal sphincter pressure can be readily demonstrated following atropine during expiration (161).

Could an effect of nitrite be limited to subjects with erosive oesophagitis where the damage to the epithelium might facilitate the nitric oxide reaching the intrinsic muscle layer of the oesophagus. It is probable that such subjects would be more sensitive to the presence of luminal nitric oxide than healthy volunteers. However, in our study, the luminal nitrite concentration on one study day was 10 times higher than the upper range of normal and an effect would have been expected at this very high level even in subjects with an intact epithelium.

Webb et al have previously described a significant reduction in systolic blood pressure following dietary nitrate supplementation (162). In our own study, the only significant change in blood pressure or pulse was an increase in systolic blood pressure following infusion of the solution containing 0.286mmol/l nitrite. However, with increasing dose of nitrite, there was a trend toward decreasing systolic blood pressure. In the study by Webb et al, depending on bacterial conversion of the re-circulated nitrate, the amount of nitrite delivered may have been up to 9 times higher than the maximum nitrite load in our study. Also, unlike the study by Webb et al where regular blood pressure measurements were recorded using an automated machine for up to 24 hours, BP and pulse were only recorded before initial nasal intubation of pH manometry apparatus and after completion of the infusion.

Therefore, a transient effect of the administered nitrite during infusion could easily have been missed. Similarly a delayed effect on BP, as occurs in the study by Webb et al, would not have been detected. The possibility we may have missed a transient BP effect during the infusion of nitrite is supported by their observations that the *in vivo* half life of nitrite (~1.5 hours) was significantly longer than the *ex vivo* half life (< 2 minutes), suggesting that nitrite was being continuously produced from nitrate (half life of ~ 8 hours) by entero-salivary recirculation. Without recirculation of dietary nitrate, in our own study the *in vivo* half life of the delivered nitrite is likely to be short.

In another previous study by our own group, Manning et al examined the effect of infusing a nitrous acid solution and a nitric oxide generating infusion into gastric cardia. Results were compared with the infusion of a control solution containing hydrochloric acid (pH 1.0). They described an increase in the rate of TLOSRs and acid exposure with the NO-generating solution versus control. In addition, there was an attenuation of the postprandial increase in IGP following infusion of the nitrous acid infusion. In our study neither of these changes were demonstrated. However, differences in methodology between the two studies may be important. In the study by Manning et al infusions were delivered directly into the gastric cardia while, in our study, nitrite is continually infused into the oral cavity. In our study therefore, nitrite is not delivered to the GOJ until saliva is swallowed and therefore delivery of nitrite is intermittent which is analogous to the physiological delivery of nitrite in normal saliva. By continuously exposing the distal oesophagus, LOS and stomach to nitric oxide or nitrous acid without time to ‘recover’ Manning et al may have provoked an increase in TLOSRs which would not be induced by the physiological delivery of nitrite. In addition it is possible that following the infusion of

the NO generating infusion into the gastric cardia, the increase in TLOSRs may have been triggered by distension of the proximal stomach. This may have occurred due to the production of NO gas which would have had a similar effect to the instillation of air into the proximal stomach which is recognised as a potent stimulator of TLOSRs. Any increase in the rate TLOSRs could explain the increase in acid exposure recorded. Finally as well as infusing nitrous acid and hydrochloric acid into the gastric cardia, Manning et al infused ascorbic acid as part of the NO-generating solution. As a result, the GOJ was exposed to super-physiological levels of ascorbic acid potentially leading to greater than physiological NO production. In our study no additional ascorbic acid was given, thus NO production is likely to be more in the physiological range and may therefore be reduced when compared with Manning et al.

6.11 Conclusion

Our study demonstrates that salivary nitrite and its associated nitric oxide production on reaction with acid at the gastro-oesophageal junction does not influence integrity of the gastro-oesophageal barrier in healthy volunteers.

Table 6.1. Baseline characteristics of study group

Parameter	Value	
Total Subjects Recruited	25	
Completed Study	20 (80%)	
Sex	Male 11 (55%)	Female 9 (45%)
Helicobacter Status	Positive 3 (15%)	Negative 17 (85%)
Age (y)	26.5 (range 19 – 59)	
Height (m)	1.72 (range 1.55 – 1.84)	
Weight (kg)	75.0 (range 55 – 110)	
BMI (kg/m^2)	23.7 (range 18.6 – 35.5)	
Waist Circumference (cm)	83.5 (range 67 – 110)	
Baseline Systolic BP (mmHg)	125 (range 114 – 161)	
Baseline Diastolic BP (mmHg)	75 (range 61 – 90)	
Baseline Pulse Rate (bpm)	70 (range 48 – 98)	

Values are medians (range) unless otherwise stated

Table 6.2. Holloway criteria for definition of transient lower oesophageal sphincter relaxations (TLOSRs) (20)

Criteria
1. Absence of swallowing for 4s before to 2s after *r*, the onset of LOS relaxation AND 2. Relaxation rate of ≥1mmHg/s AND 3. Time from onset to complete relaxation of ≤10 seconds AND 4. Nadir pressure ≤2mm OR **5.** Any pressure falls fulfilling criteria 2-4 but with duration >10 seconds (excluding those associated with multiple swallows)

Table 6.3. Changes in gastro-oesophageal function following the meal

	Fasting	Post-prandial	p value
5-6cm Acid Exposure (% time pH<4)	0 (0–1.9)	0.5 (0-20)	**p = 0.002**
1-2cm Acid Exposure (% time pH<4)	1.2 (0-7.8)	6.0 (0.9-74.4)	**p < 0.001**
Rate of TLOSRs (number/h)	0 (0-4)	7.3 (4-11.3)	**p = 0.001**
Expiratory LOS Pressure vATM (mmHg)	29.4 (11.8-51.2)	19.2 (8.2-34.5)	**p < 0.001**
Expiratory LOS Pressure vIGP (mmHg)	17.3 (4.9-37.7)	12 (4-23.8)	**p < 0.001**
Inspiratory LOS Pressure vATM (mmHg)	39.5 (16.9-60.2)	30.4 (14.5-53.1)	**p = 0.02**
Inspiratory LOS Pressure vIGP (mmHg)	25.4 (10.1-43.9)	18.1 (6.5-40.3)	**p = 0.035**
LOS Length (cm)	5 (3-6)	4 (1-6)	**p = 0.001**
Inspiratory Intragastric Pressure (mmHg)	12 (6-20)	10.2 (4.5-21.8)	p = 0.059
Expiratory Intragastric Pressure (mmHg)	10.5 (4.5-19.1)	8.9 (3.2-20.4)	p = 0.089

TLOSRs, transient lower oesophageal sphincter relaxations; LOS, lower oesophageal sphincter; vATM, relative to atmospheric pressure; vIGP, relative to intragastric pressure.

Results are expressed as medians (range). Significant p values (p <0.05) are highlighted in bold.

Table 6.4. Salivary nitrite concentrations and gastro-oesophageal and cardiovascular function on the four study days

	0mmol/l	0.286mmol/l	2mmol/l	14mmol/l	p value
Nitrite Concentration (mmol/l)*	24.4 (3.9–65.5)	80.4 (20.9-158.8)	358.4 (118.2-726.6)	2694 (600-8087)	**p < 0.001†**
5-6cm Acid Exposure (% time pH<4)	0.4 (0.0 – 20.0)	1.1 (0.0-35.8)	1.0 (0.0-28.1)	0.6 (0.0-31.5)	p = 0.214
1-2cm Acid Exposure (% time pH<4)	6.0 (0.8-74.4)	6.4 (0.8-86.6)	9.7 (0-93.2)	5.7 (0.1-68.6)	p = 0.981
No. of TLOSRs	11.0 (6.0-17.0)	13.0 (6.0-17.0)	12.0 (6.0-17.0)	12.0 (7.0-18.0)	p = 0.117
Change in Expiratory LOSP vATM (mmHg)	– 8.0 (-26.1–2.1)	-7.8 (-37.6-7.7)	-5.7 (-42.4-12.8)	-8.3 (-55.6-4.1)	p = 0.776
Change in Expiratory LOSP vIGP (mmHg)	-7.1 (-22.9-3.2)	-4.9 (-37-5.8)	-6.8 (-42.1-14.7)	-8.5 (-51.8-2.2)	p = 0.73
Change in Inspiratory IGP (mmHg)	-1.8 (-9.5-5.6)	-1.4 (-6.1-3.5)	-0.1 (-5.4-5.7)	-0.4 (-9.2-7.7)	p = 0.13
Change in Expiratory IGP (mmHg)	-1.6 (-7.2-4.8)	-1.8 (-5.2-2.1)	-0.5 (-5.5-4.5)	-0.2 (-9.2-6.6)	p = 0.312
No. of Reflux Events	2.5 (0.0-11.0)	5.0 (0.0-16.0)	4.0 (0.0-17.0)	2.5 (0.0-13.0)	p = 0.429
Nadir pH of Reflux	1.6 (0.6-3.7)	1.5 (0.7-3.3)	1.6 (0.7-3.2)	1.6 (0.8-3.1)	p = 0.681
Duration of Reflux (secs)	12.8 (3.1-264.1)	15 (3.2-301.2)	17.7 (3.1-353.3)	13.3 (3.1-473.4)	p = 0.614
Peristaltic Amplitude (mmHg)	36.6 (18.5-98.7)	33.6 (19.4-83.6)	33.4 (21.1-79.0)	32.4 (19.8-93.1)	p = 0.936
Peristaltic Velocity (cm/s)	5.2 (2.6-11.3)	5.3 (2.9-15.7)	4.8 (2.8-26.5)	4.8 (3.1-17.9)	p = 0.093
Change in SBP (mmHg)	-4.0 (-29.0-18.0)	2.5 (-17.0-30.0)	-1.0 (-38.0-29.0)	-12.0 (-25.0-6.0)	**p = 0.007‡**
Change in DBP (mmHg)	-1.0 (-18.0-11.0)	-4.0 (-27.0-17.0)	1.0 (-15.0-24.0)	-3.0 (-27.0-6.0)	p = 0.316
Pulse (bpm)	-2.5 (-20.0-14.0)	-2.0 (-23.0-6.0)	-3.5 (-22.0-18.0)	1.5 (-22.0-6.0)	p = 0.903

TLOSRs, transient lower oesophageal sphincter relaxations; LOSP, lower oesophageal sphincter pressure; vATM, relative to atmospheric pressure; vIGP, relative to intragastric pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure. *Median nitrite concentrations at 60minutes post-prandial. Results are expressed as medians (range).

Significant p values (p <0.05) are highlighted in bold.

† Post-hoc test: (0.286 vs. 0), (2 vs. 0), and (14 vs. 0), p value for each was <0.001; ‡ Post-hoc test: (0.286 vs. 0) p value <0.05, (2 vs. 0) p value = 0.850, and (14 vs. 0) p value = 0.170.

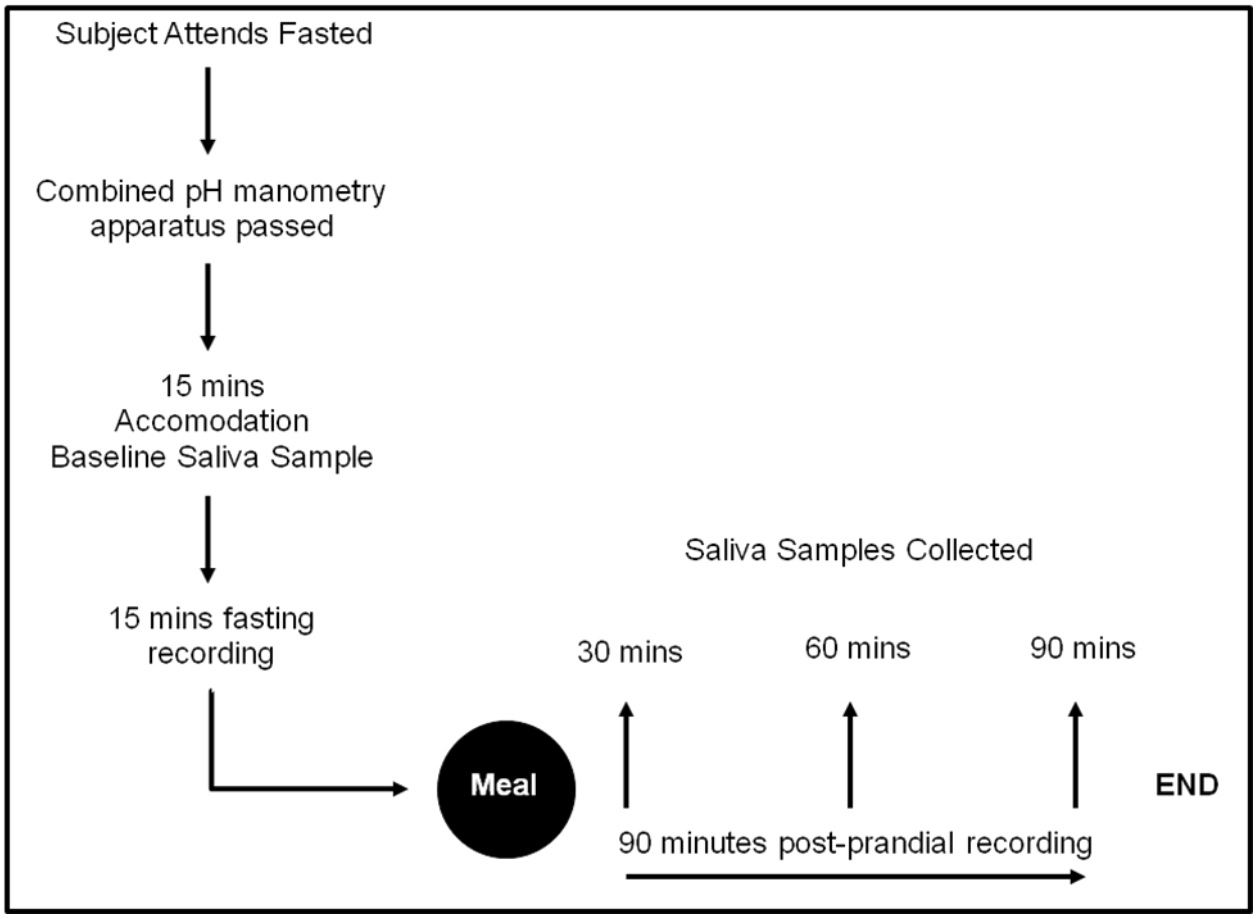

Figure 6.1. The effect of nitrite in saliva on gastro-oesophageal reflux – study day outline

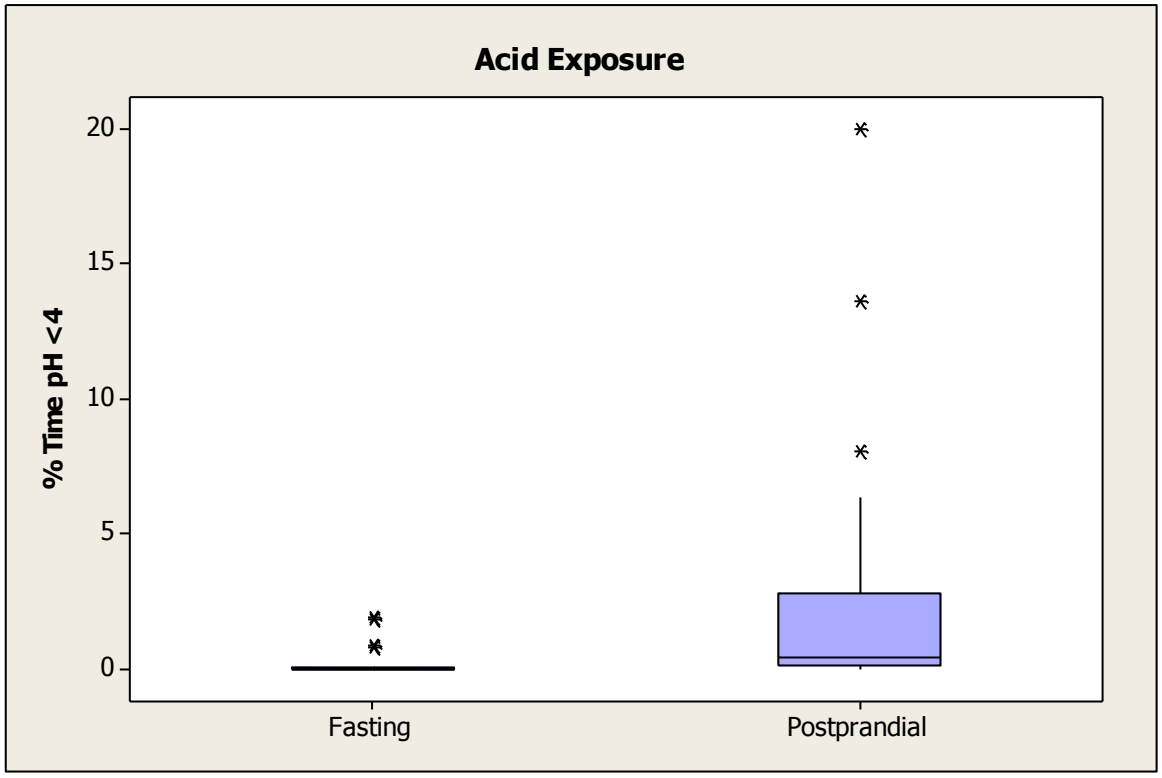

Figure 6.2. Box-plot showing the effect of the meal on oesophageal acid exposure

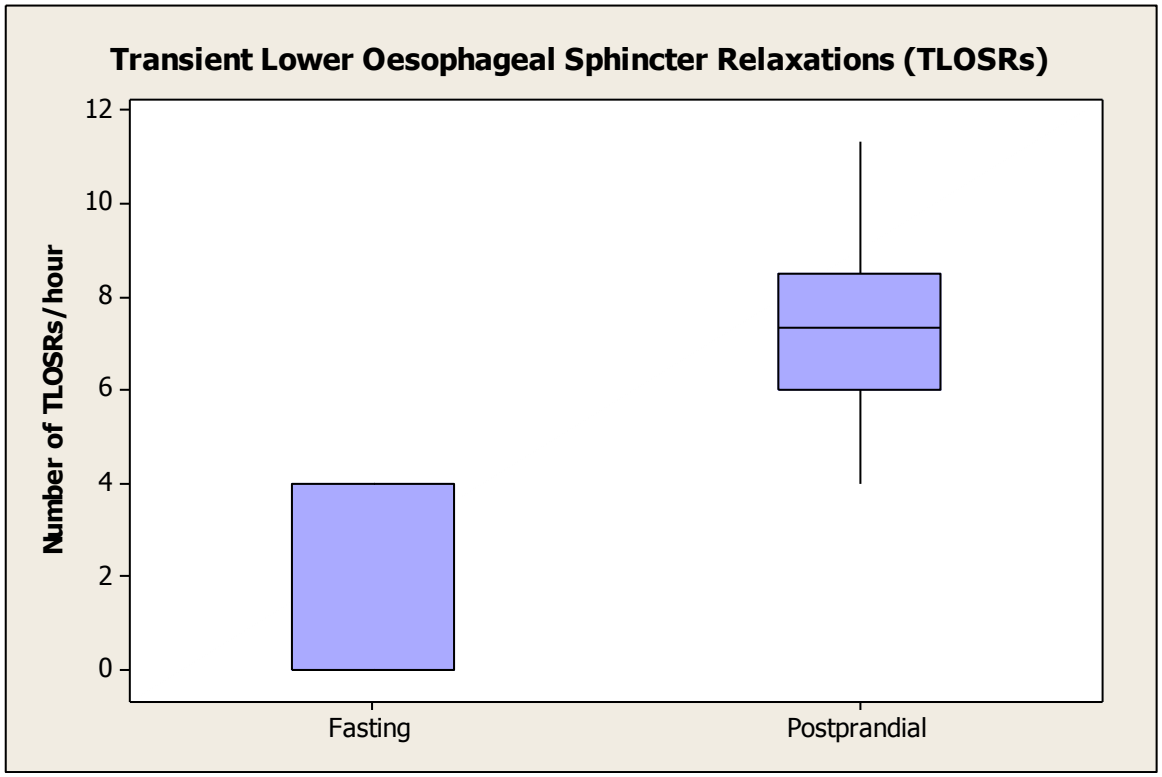

Figure 6.3. Box-plot showing the effect of the meal on the rate of transient lower oesophageal relaxations (TLOSRs)

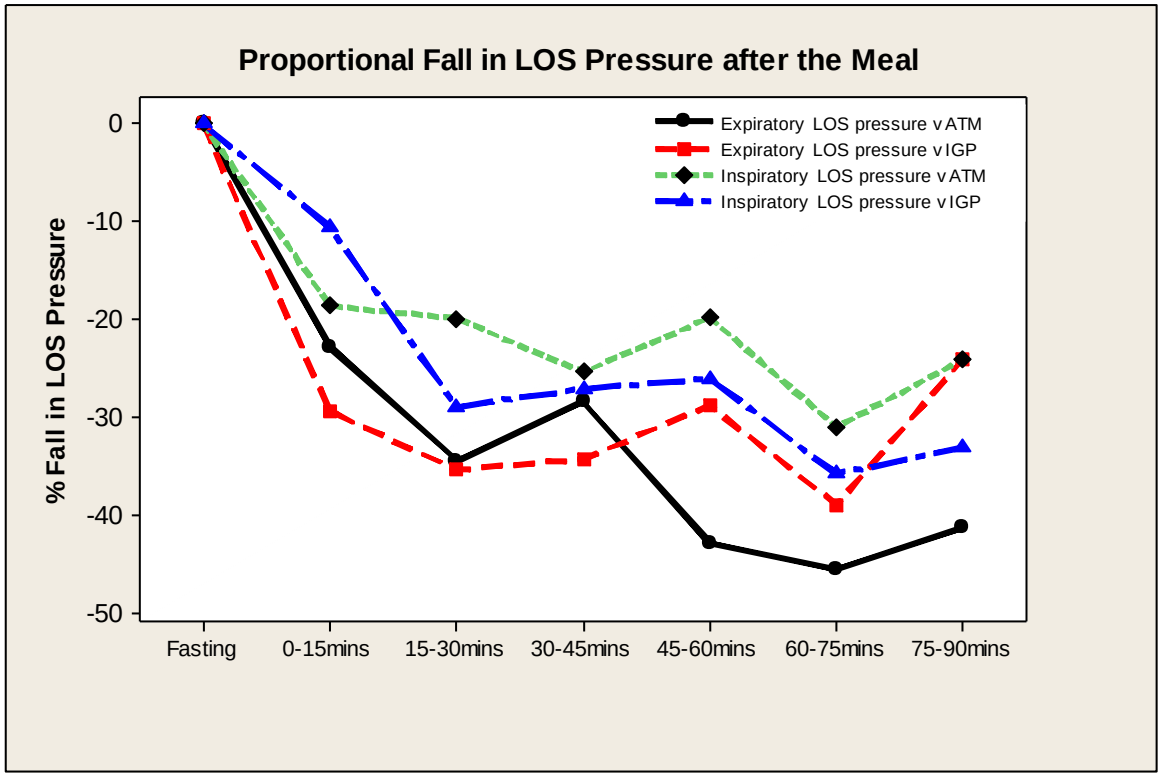

Figure 6.4. Line graph illustrating the proportional fall in LOS pressure after the meal

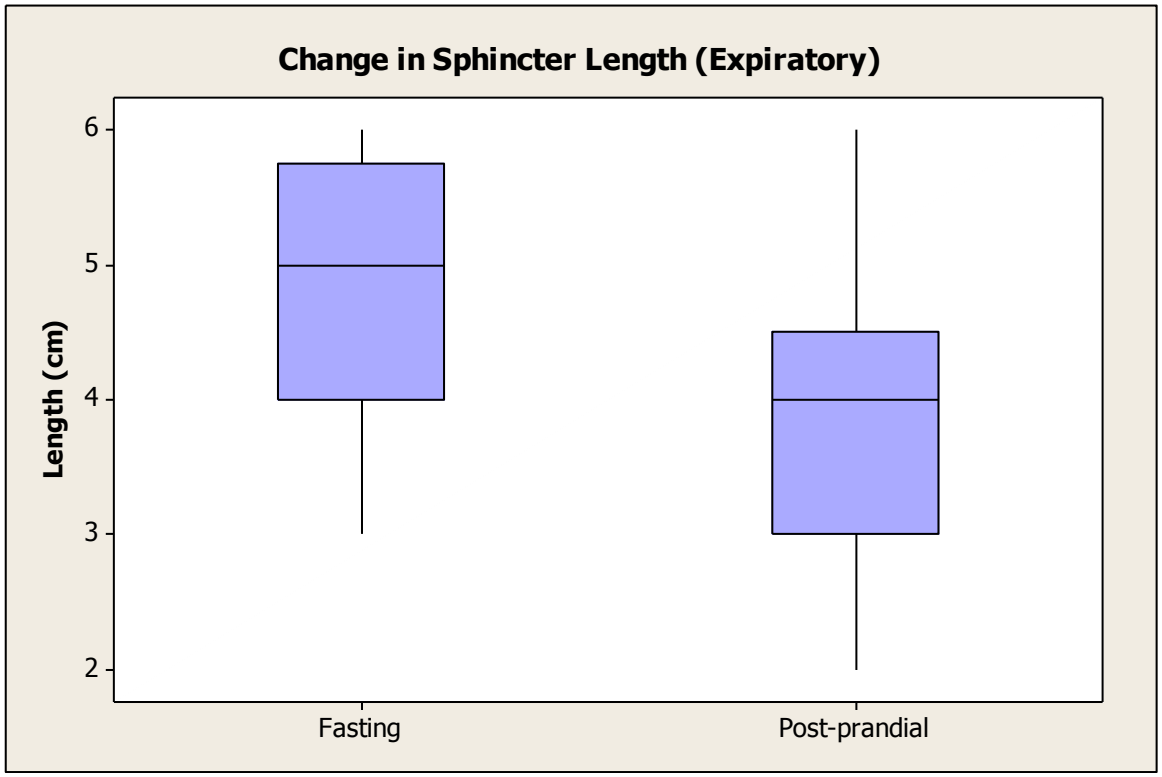

Figure 6.5. Box-plot showing the change in lower oesophageal sphincter length after the meal

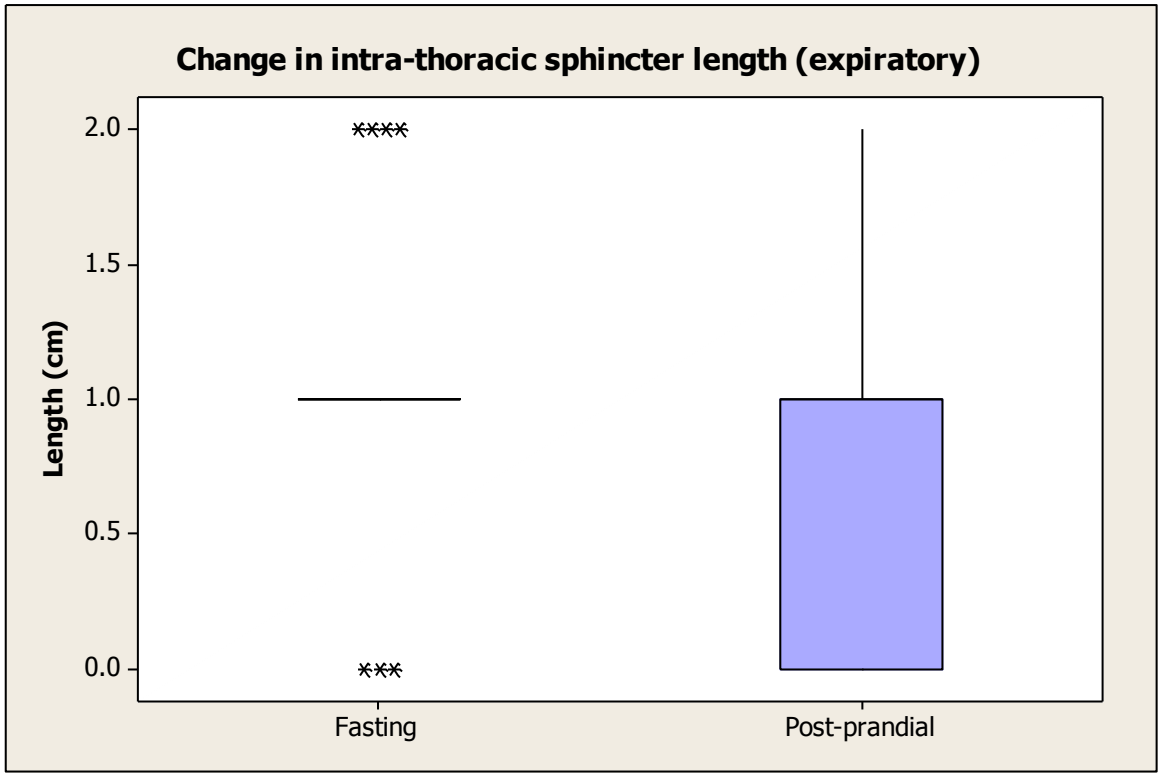

Figure 6.6. Box-plot showing the change in intra-thoracic sphincter length after the meal

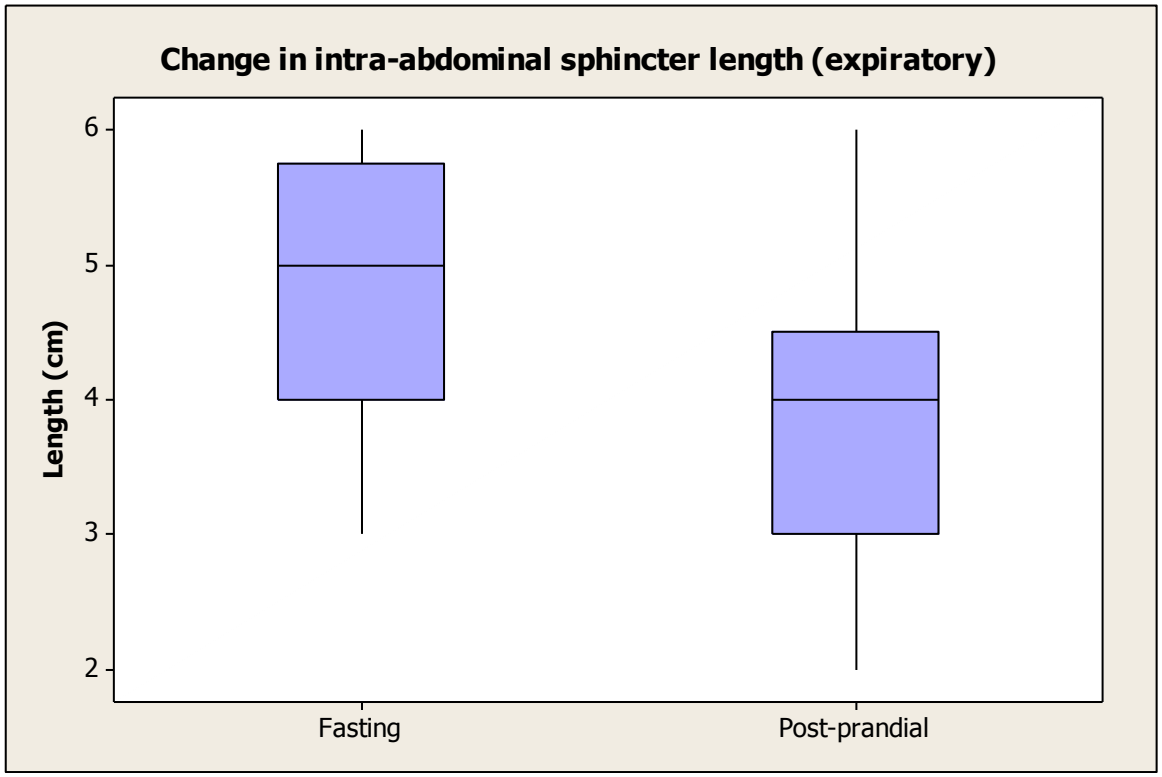

Figure 6.7. Box-plot showing the change in intra-abdominal sphincter length after the meal

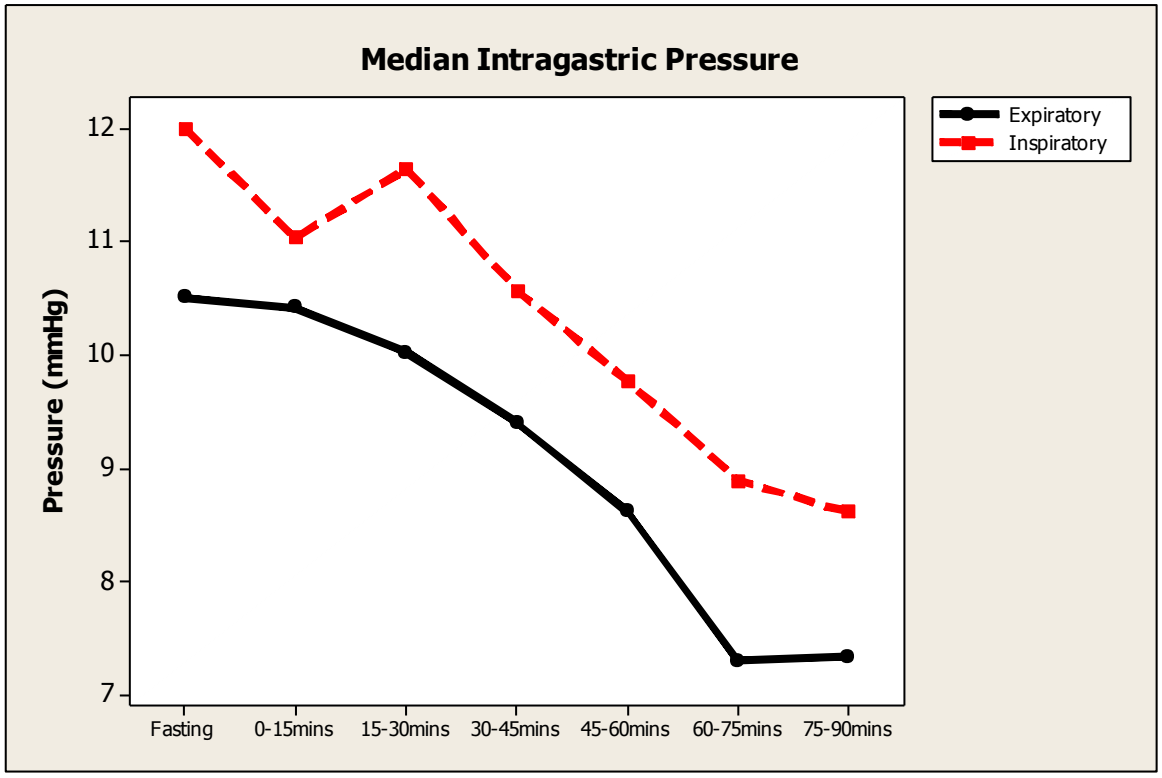

Figure 6.8. Line graph showing the change in intragastric pressure during study – control day

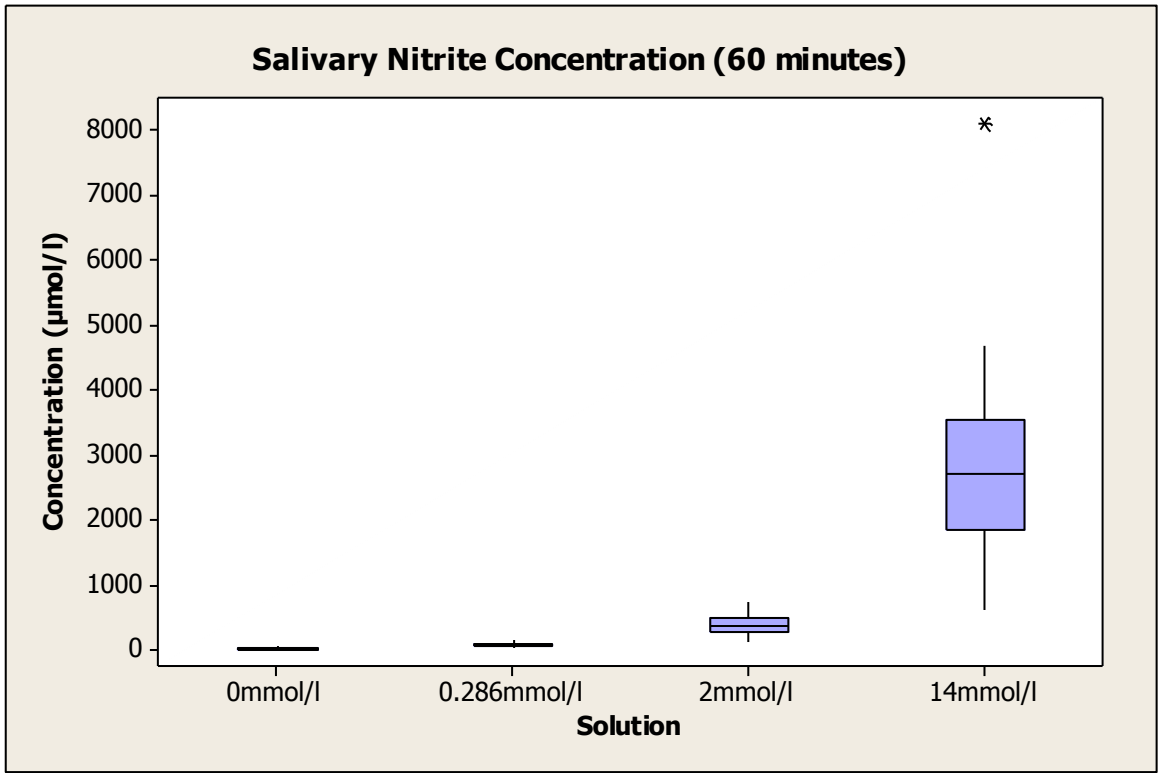

Figure 6.9. Box-plot of salivary nitrite concentrations between solutions - measured 60 minutes into study

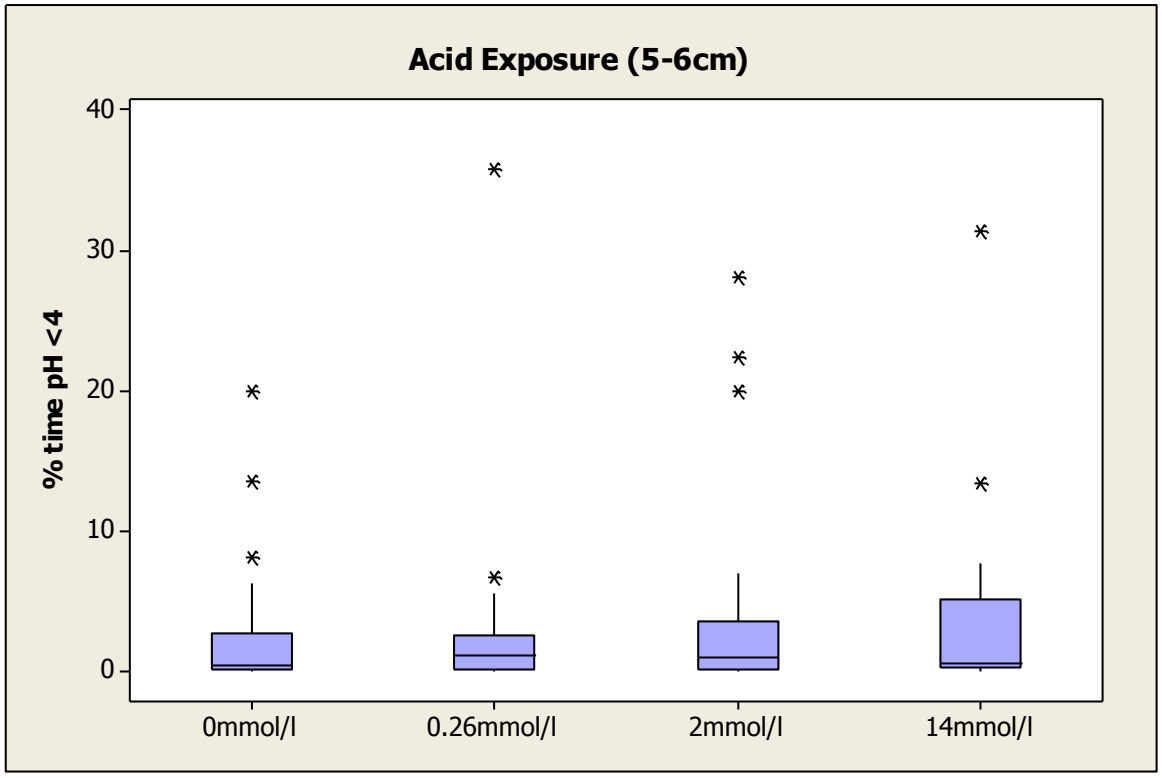

Figure 6.10. Box-plot of post-prandial acid exposure between solutions - measured 5-6cm above the fasting pH step-up point

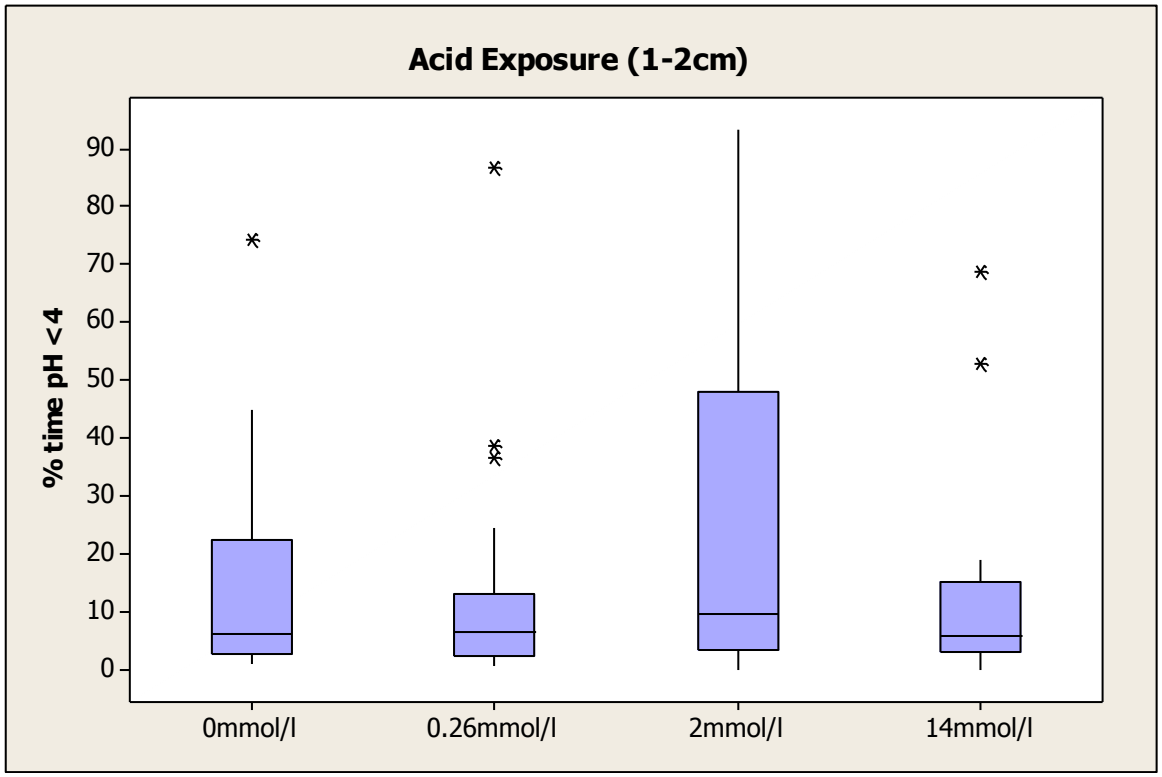

Figure 6.11. Box-plot of post-prandial acid exposure between solutions - measured 1-2cm above the fasting pH step-up point

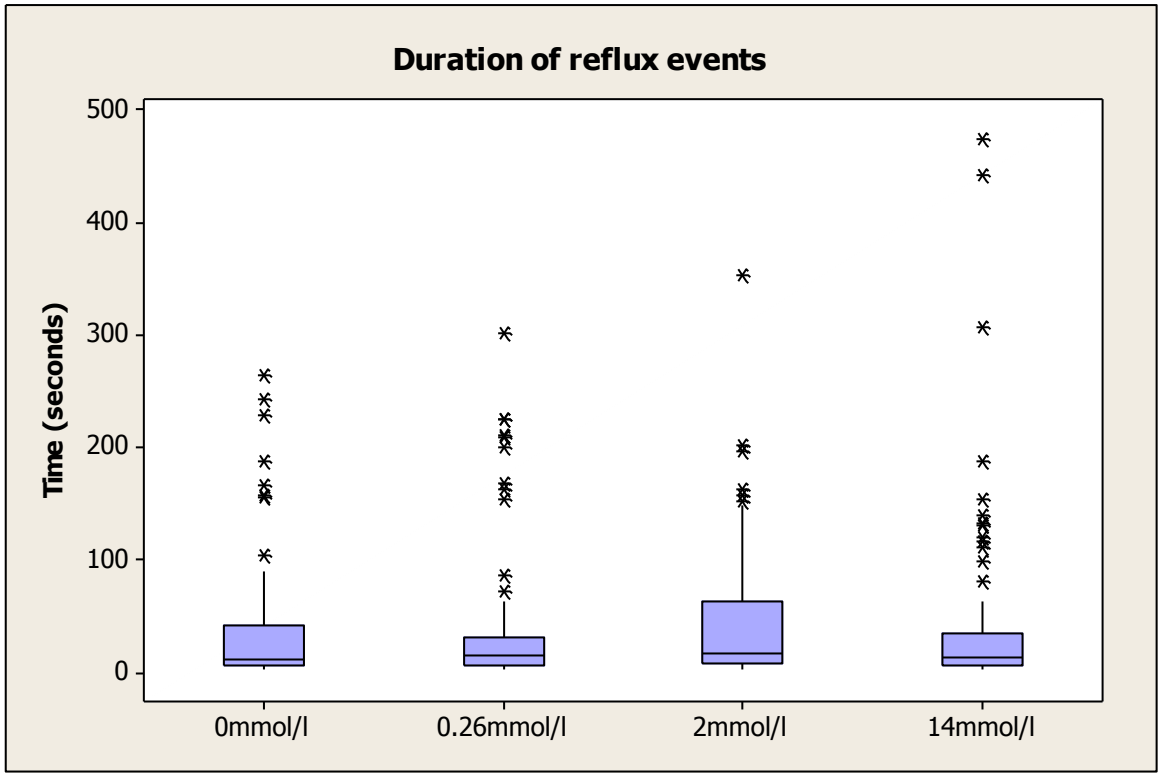

Figure 6.12. Box-plot showing the duration of individual post-prandial reflux events between solutions

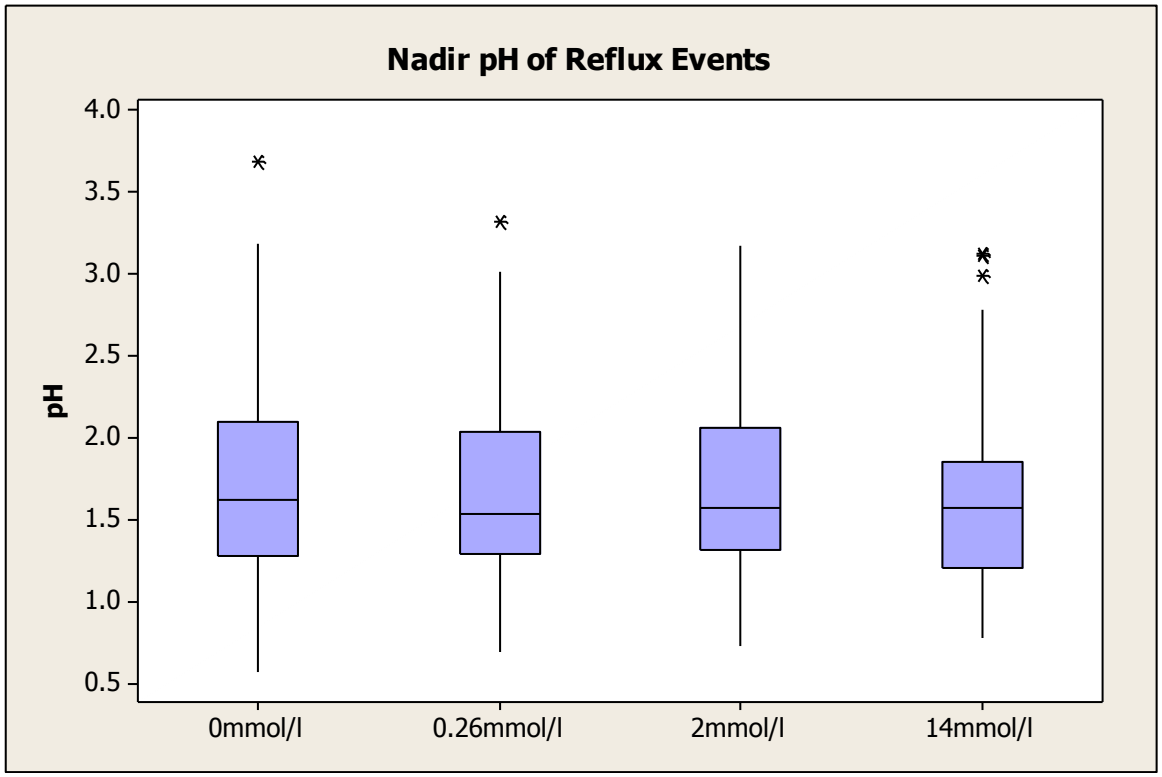

Figure 6.13. Box-plot showing the nadir pH of individual post-prandial reflux events between solutions

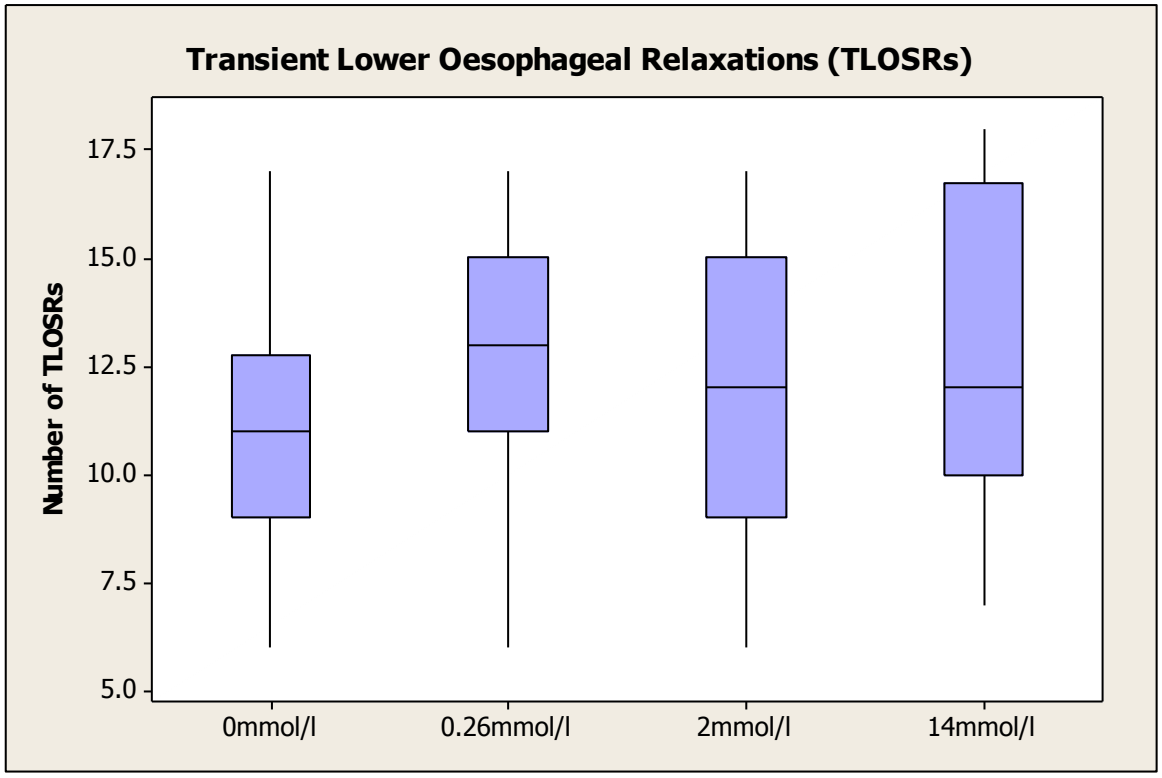

Figure 6.14. Box-plot showing the number of post-prandial transient lower oesophageal sphincter relaxations (TLOSRs) between solutions

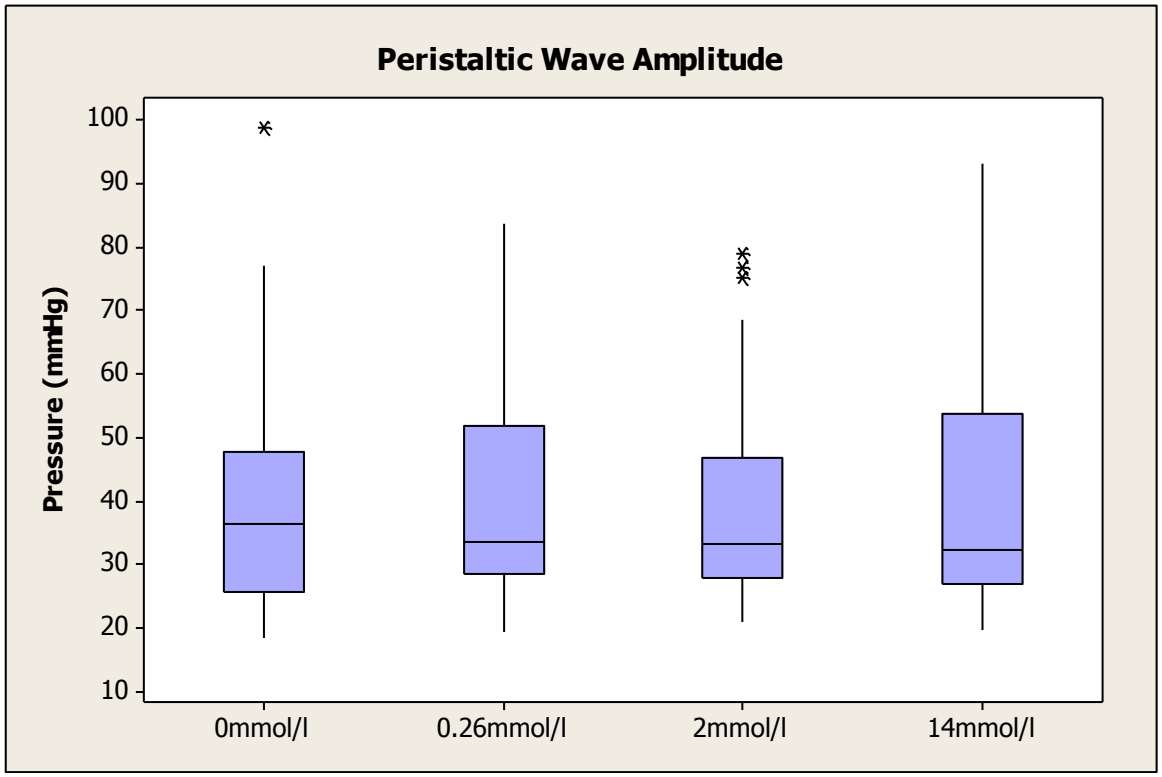

Figure 6.15. Box-plot showing the post-prandial peristaltic wave amplitude between solutions

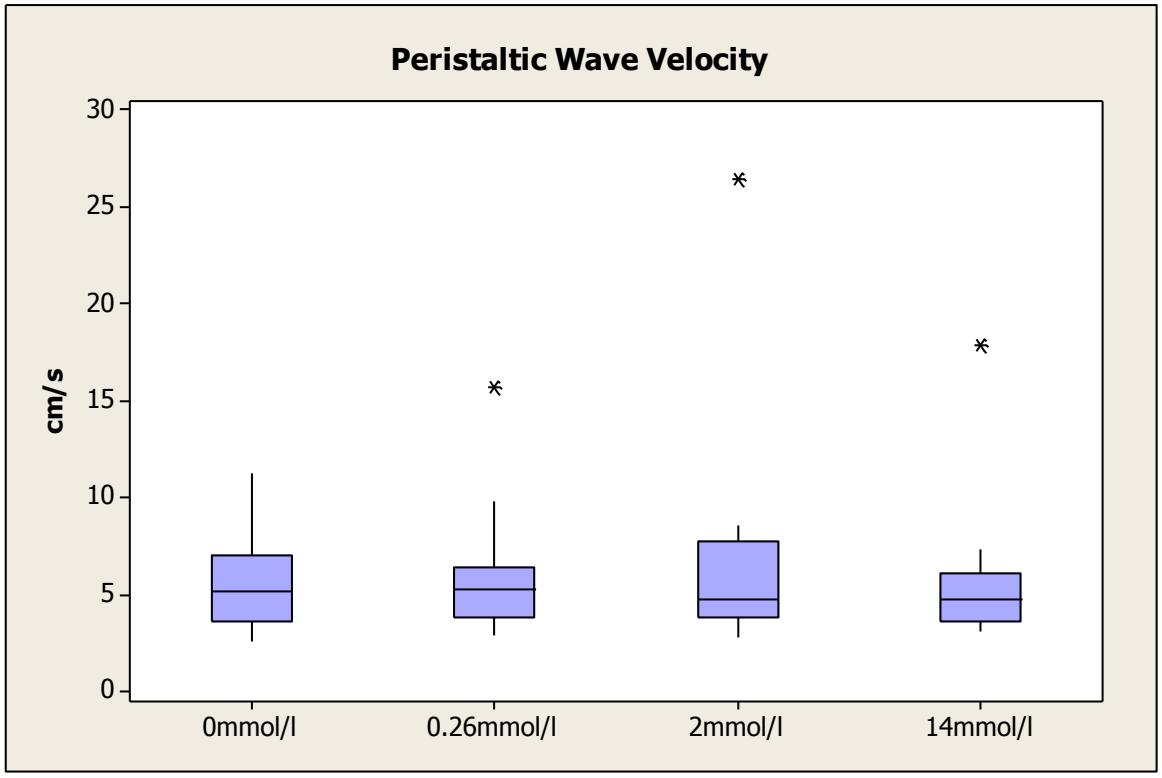

Figure 6.15. Box-plot showing the post-prandial peristaltic wave velocity between solutions

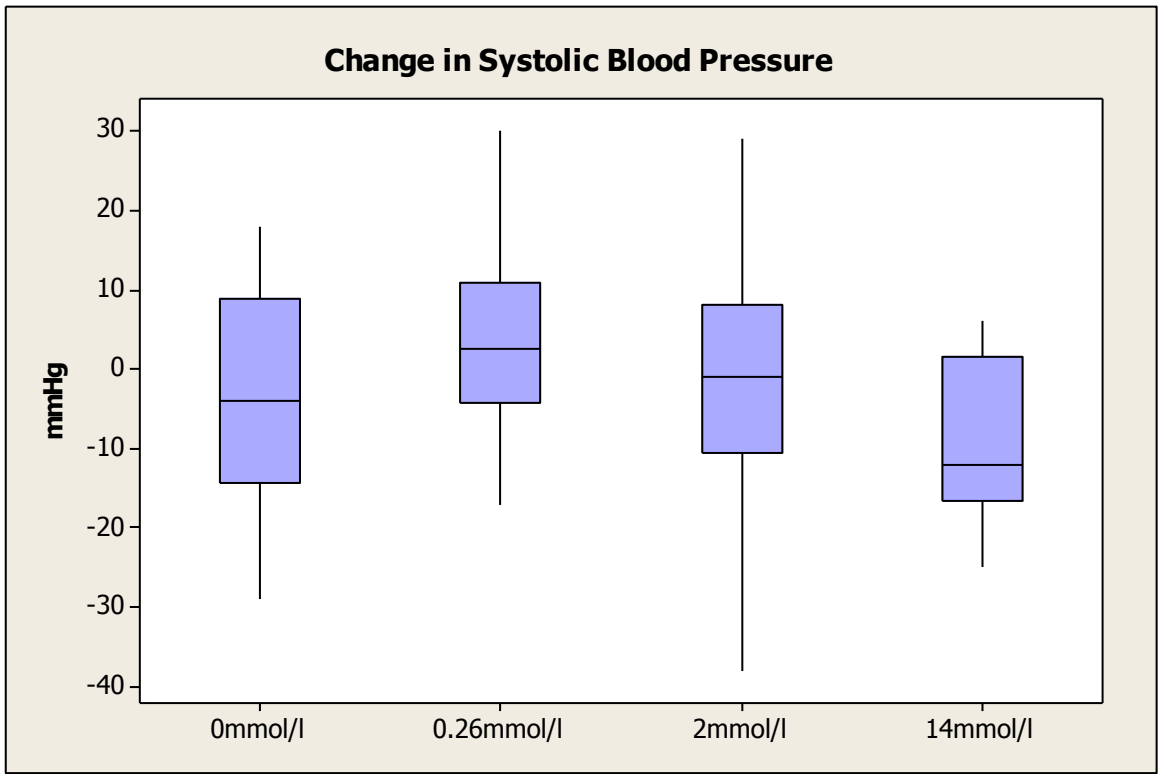

Figure 6.17. Box-plot showing the change in systolic blood pressure between solutions

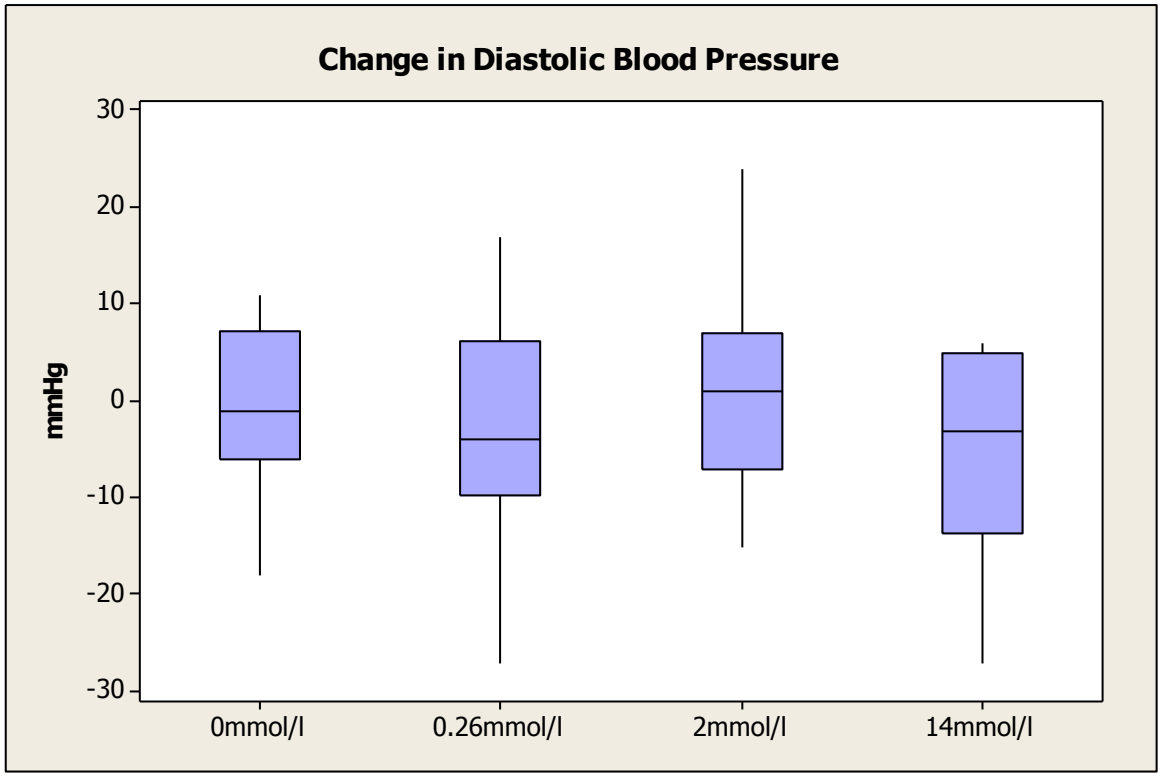

Figure 6.18. Box-plot showing the change in diastolic blood pressure between solutions

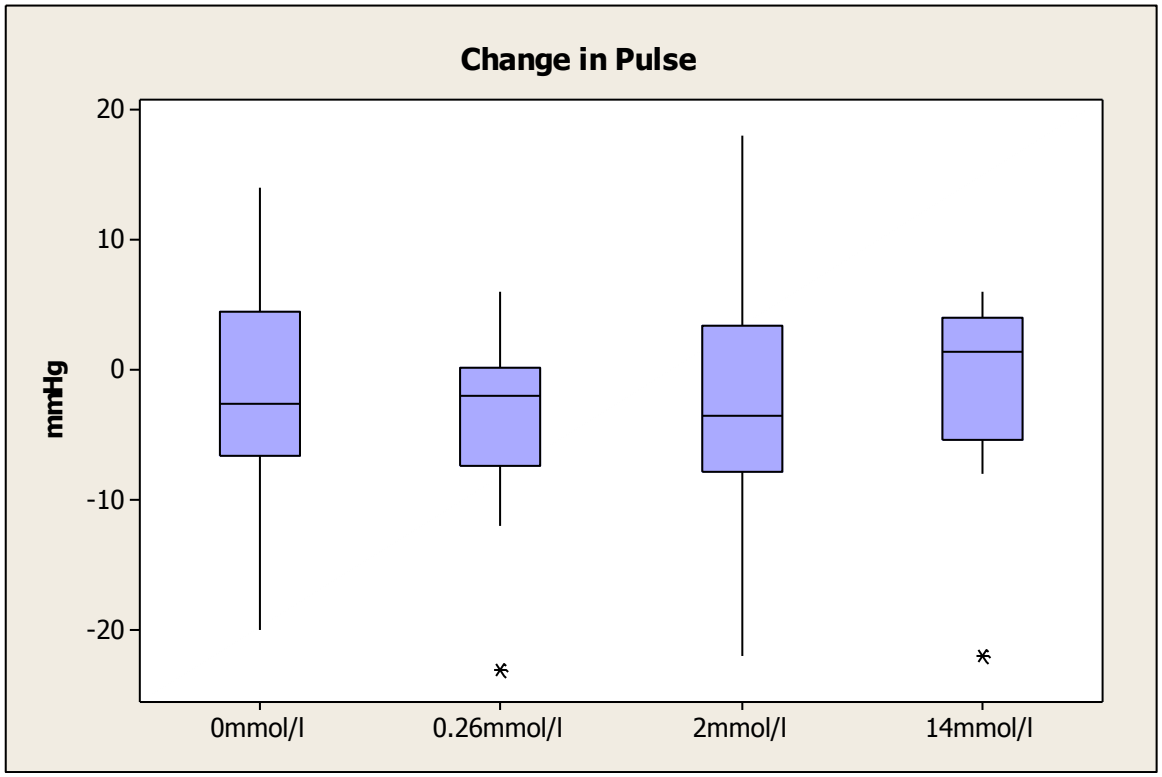

Figure 6.19. Box-plot showing the change in pulse rate between solutions

Chapter 7

The Effect of Nitrite in Saliva on Gastric Emptying

Chapter 7

The Effect of Nitrite in Saliva on Gastric Emptying

7.1 Introduction

Human saliva has a high nitrite concentration derived from the entero-salivary recirculation of dietary nitrate. Swallowed nitrite reacts with acidic gastric juice forming nitrous acid, nitrosating species and nitric oxide (NO). Increasing NO bioactivity, by inhibiting phosphodiesterase, enhances gastric accommodation and delays gastric emptying (91). Recently, our group described attenuation of the postprandial rise in intragastric pressure (IGP) following intragastric nitrous acid infusion (93). We now wish to examine the effect of salivary nitrite on gastric emptying.

7.2. Aims

The aims of this study were:

i. to investigate whether nitrite in saliva affects gastric emptying

ii. to determine whether nitrite in saliva alters postprandial IGP

7.3. Subjects

20 subjects (11 male) with a mean age of 30.8 years (Range 19 – 59) were studied. *Helicobacter* status was determined using rapid urease test with 17 testing

negative and 3 positive for *Helicobacter pylori* infection. All subjects were free of symptoms of gastro-oesophageal reflux and were not on any regular anti-secretory medications.

7.4. Methods

Gastric emptying studies following modification of salivary nitrite concentration were performed on 4 separate study days – the study day outline is shown in Figure 7.1. Each patient acted as their own control. Volunteers attended for each study day fasted from the night before. To measure changes in intragastric pressure, high-resolution manometry studies were also performed on each study day.

7.4.1. High-resolution manometry

High-resolution manometry was performed using the Manoscan® system (Sierra Scientific Instruments Inc). At the beginning of each study, the manometry apparatus was passed per nasally and attached at the nose using adhesive tape. Volunteers were given 15 minutes to get used to the apparatus. Manometry recording was then performed for 15 minutes in the fasting state prior to the consumption of a standardised meal, and continued for 90 minutes post-prandially.

7.4.2. Gastric emptying test

Gastric emptying was assessed by means of a specially designed breath test which was based on the method described by Meier- Augenstein et al (163). This

uses ^{14}C labelled sodium acetate incorporated into a flapjack consisting of butter, syrup and rolled oats during the baking process. The flapjack was prepared in batches and constituted part of our standardised meal.

Following consumption of the flapjack, breath samples were taken at regular intervals to assess gastric emptying. Samples are taken at baseline then 10 minute intervals for 1 hour followed by 15 minute intervals for the subsequent 2 hours. $^{14}CO_2$ breath samples were collected in hyoscine hydroxide and ethanol scintillation medium. Each sample contains 1mmol of CO_2 and is analyzed by beta scintillation counting.

7.4.2.a. *In vitro* validation study of gastric emptying test

Prior to the study, the radio-labelled flapjack was evaluated as an accurate and reproducible marker of the solid phase of a meal by assessing retention of the ^{14}C labelled sodium acetate in the solid phase under gastric simulation. 2g aliquots of the flapjack were added to 10mls of gastric juice (pH 1.44) obtained from a healthy volunteer and 1ml of fresh human saliva. Samples were immersed in a water bath at 37ºC and mixed at regular intervals to simulate gastric motor activity. 4 individual samples were analysed at 30, 60, and 180 minutes. Samples were taken from the liquid phase to measure release of the tracer. The results were expressed as the mean percentage of the initial radioactivity that remained in the solid phase.

7.4.3. Standardised meal

Each volunteer was given a standardised meal, consisting of two haddock and potato fishcakes and one rolled oats flapjack with 150ml of water (Total calorie content: 526.35 kcal). 20 minutes was allocated for consumption of the meal.

7.4.4. Nitrite infusions

Infusions of the test solutions into the mouth commenced immediately after completion of the meal and continued for 90 minutes post-prandially.

7.5. Analysis

7.5.1. Gastric emptying

Total CO_2 production was taken to be 300 millimoles per square metre of body surface per hour. Body surface area was calculated by the formula previously described by Haycock et al (164). The quantity of ^{14}C appearing in breath per unit time (µmol/minute) was calculated. The mathematical model described by Viramontes et al was used to estimate gastric half-emptying times (T½). This used only 4 breath samples at 45, 90, 105 and 120 minutes (165). In this method, the gastric emptying T½ is estimated directly as $T½ = 1/LP_{½}$, where the linear predictor ($LP_{½}$) is given by:

$$LP_{½} = 0.0025 – 0.0039 \times {}^{14}C_{45} + 0.0088 \times {}^{14}C_{90} – 0.0063 \times {}^{14}C_{105} + 0.0024 \times {}^{14}C_{120}$$

7.5.2. Intragastric pressure

Intragastric pressure was defined, relative to atmospheric pressure, as the median pressure in the 3 sensors immediately distal to the LOS.

7.6. Statistical analysis

All results are given as medians and interquartile ranges unless otherwise stated. To test the changes of variables against different nitrite solutions, the nonparametric Friedman's test was used.

7.7. Ethics

The study was approved by West Glasgow Research Ethics Committee and informed consent was obtained from each participant.

7.8. Results

7.8.1. *In vitro* validation study of gastric emptying test

The bench top study confirmed the retention of the majority of tracer in the solid phase of the meal under gastric simulation. At 30, 60 and 180 minutes the mean percentage ^{14}C retention in the solid phase was 83.9% (range 73.7 – 89.6%), 74.6% (range 65.3 – 79.2%) and 62.44% (range 52.1 – 66.4%) respectively - see Table 7.1.

7.8.2 Salivary nitrite concentrations

There was a significant difference in the median salivary nitrite concentrations during infusion of each solution. At 60 minutes following the meal the median concentrations of salivary nitrite recorded were 24.35µmol/l (range 3.86 – 65.46), 80.42µmol/l (range 20.88 – 158.79; $p<0.001$ v control), 372.86µmol/l (range 118.2 – 726.6; $p<0.001$ v control) and 2398.53µmol/l (range 600 – 8087; $p< 0.001$ v control) for the solutions containing 0mmol/l, 0.286mmol/l, 2mmol/l and 14mmol/l potassium nitrite respectively. The salivary nitrite concentration results are shown in Table 7.2.

To save space in presenting results, the four different study days will be referred to as 1, 2, 3, 4 with one being the lowest nitrite concentration (control) and 4 the highest.

7.8.3. Gastric emptying – control v nitrite solutions

There was no significant difference in the rate of gastric emptying of the test meal between the different study days with the T½ being 39.79 minutes, (range 10.94 – 136.69), 32.25 minutes, (range 15.69 – 108.36); 33.98 minutes, (range 24.91 – 121.96) and 37 minutes, (range 19.7 – 363) for solutions 1 - 4 respectively. Results are shown in Figure 7.2.

7.8.4. Intragastric pressure – control v nitrite solutions

Intragastric pressure fell following the meal on each study day. Comparing the median fasting and median postprandial value for each individual showed that

there was no difference in the change in intragastric pressure following the meal between the 4 study days when measured in either inspiration or expiration. Results are shown in Figures 7.3. and 7.4.

7.9. Discussion

Our initial *in vitro* results support the previous observation by Meier-Augenstein et al that the sodium acetate-labelled 'radioactive flapjack test' is a reliable measure of solid-phase gastric emptying (163). The sodium acetate breath test had previously been described as an accurate, reliable and non-invasive tool for the analysis of liquid phase gastric emptying rates (166). Meier-Augenstein's work subsequently demonstrated retention of a majority of the tracer in the solid phase of the meal if incorporated in the baking process of a rolled-oats flapjack. Similar results were found in our own study with a majority of the tracer retained in the solid phase under simulated intragastric conditions out to 180 minutes. Confirmation of tracer retention in the solid phase was important as measurement of solid gastric emptying is recognised to be more sensitive than measurements of liquid gastric emptying in detecting individuals with delayed gastric emptying (167)

We calculated the gastric half-emptying time (T½) as a measure of the rate of gastric emptying. Initially we used the mathematical model previously described by Ghoos et al (168;168), by fitting the percentage ^{14}C recovery per minute by non-linear regression to the formula $y = a^b te^{-ct}$ and the cumulative recovery of ^{14}C to the formula $y = m(1-e^{-kt})^{\beta}$, the inverse of the scintigraphic decay curve. Through mathematical

curve fitting, it is then possible to calculate the gastric half-emptying time (T½) using the equation ($-1/k)ln(1-2^{-1/\beta}$).

However, using the Ghoos method, we frequently observed an atypical double-peak in our excretion curves. This has previously been described by Sanaka et al and inevitably results in poor curve-fitting. Such a phenomenon was seen in at least one gastric emptying study in 14/20 subjects (70%). One possible cause for the frequency of this 'double-peak' phenomenon in our own study may have been that the nitrite infusion and manometry studies were only continued for 90 minutes, not the full 180 minutes over which breath testing was performed. Even in the absence of a direct effect of salivary nitrite on gastric emptying these changes, including removal of the nasogastric manometry catheter may have an impact upon gastric emptying. As a result of this finding, the mathematical model described by Viramontes et al, and detailed in the methods section above, was preferred for estimation of gastric half-emptying times (T½).

Despite an excellent range of modified salivary nitrite concentrations extending over and beyond the normal physiological range, we were unable to demonstrate any effect of salivary nitrite modification on gastric emptying or postprandial changes in IGP.

In previous work by Manning et al from our own group, the effect of infusing a nitrous acid solution and a nitric oxide generating infusion into gastric cardia was studied. The researchers found that there was an attenuation of the postprandial increase in IGP following infusion of the nitrous acid infusion. It was therefore postulated that this may have been due to a delay in gastric emptying. In our study neither an attenuation in IGP nor a delay in gastric emptying was observed.

However, as detailed in the previous chapter, differences in methodology between the two studies may be important. In particular the mode of delivery of infusions and the excess ascorbic acid delivered to the GOJ and stomach may produce results which would not be seen with the more physiological delivery of nitrite we have employed.

While our in vitro gastric simulation confirms that the majority of tracer is retained in the solid phase of the meal throughout our study, the actual amount of tracer retained in the solid phase is lower than either Meier-Augenstein (74.6% v 95.2% at 1 hour) or Ghoos (62.4% v 86.8% at 3 hours) have described (163;168). Similarly, the median gastric half-emptying times we obtained are significantly shorter than those obtained by Ghoos et al in their study using a ^{14}C-octanoic acid solid emptying breath test meal correlated with radioscintigraphy. Indeed our results more closely resemble their median values for the lag phase of solid gastric emptying. No lag phase is present in liquid emptying and the lower in vitro retention and shorter emptying times may suggest that we have in fact measured liquid rather than solid gastric emptying. Although solid emptying may be more sensitive in detecting delayed gastric emptying, the majority of patients with gastric emptying disorders have delayed emptying of both liquids and solid food. The use of subjects as their own control makes it unlikely any significant effect of salivary nitrite on gastric emptying would have been missed.

It is also possible that liquid gastric emptying is more relevant to GORD than solid emptying. The rate of liquid emptying is largely determined by the degree of fundic relaxation (accommodation) while the emptying of solids depends more on antral motility (169). Abnormalities in gastric accommodation are more likely to lead

to increased pressure in the proximal stomach and, through the generation of TLOSRs, to increased gastro-oesophageal reflux. Importantly, in the study by Sarnelli et al where they examined the impact of increasing NO bioavailability by using the phosphodiesterase inhibitor Sildenafil, they demonstrated an increased in gastric accommodation and a delay in liquid rather than solid gastric emptying (91).

7.10 Conclusion

Despite an excellent range of salivary nitrite concentrations, extending over and beyond the normal physiological range, no difference in the rate of gastric emptying or postprandial rise in IGP was seen. This suggests salivary nitrite does not affect gastric emptying.

Table 7.1. *In vitro* study of tracer retention in the solid phase

Time (Minutes)	^{14}C Retention (%)
30	83.9 (73.7 – 89.6)
60	74.56 (65.3 – 79.2)
180	62.44 (52.1 – 66.4)

Values are medians (range)

Table 7.2. Salivary nitrite concentrations (mmol/l) at baseline and during 90 minute post-prandial infusion

Time	0mmol/l Solution	0.286mmol/l Solution	2mmol/l Solution	14mmol/l Solution
0 minutes	18.77	22.73	19.01	25.25
30 minutes	28.61	60.95	349.68	2776.28
60 minutes	24.35	80.42	358.44	2693.83
90 minutes	26.49	32.84	138.56	886.45

Results shown are median values.

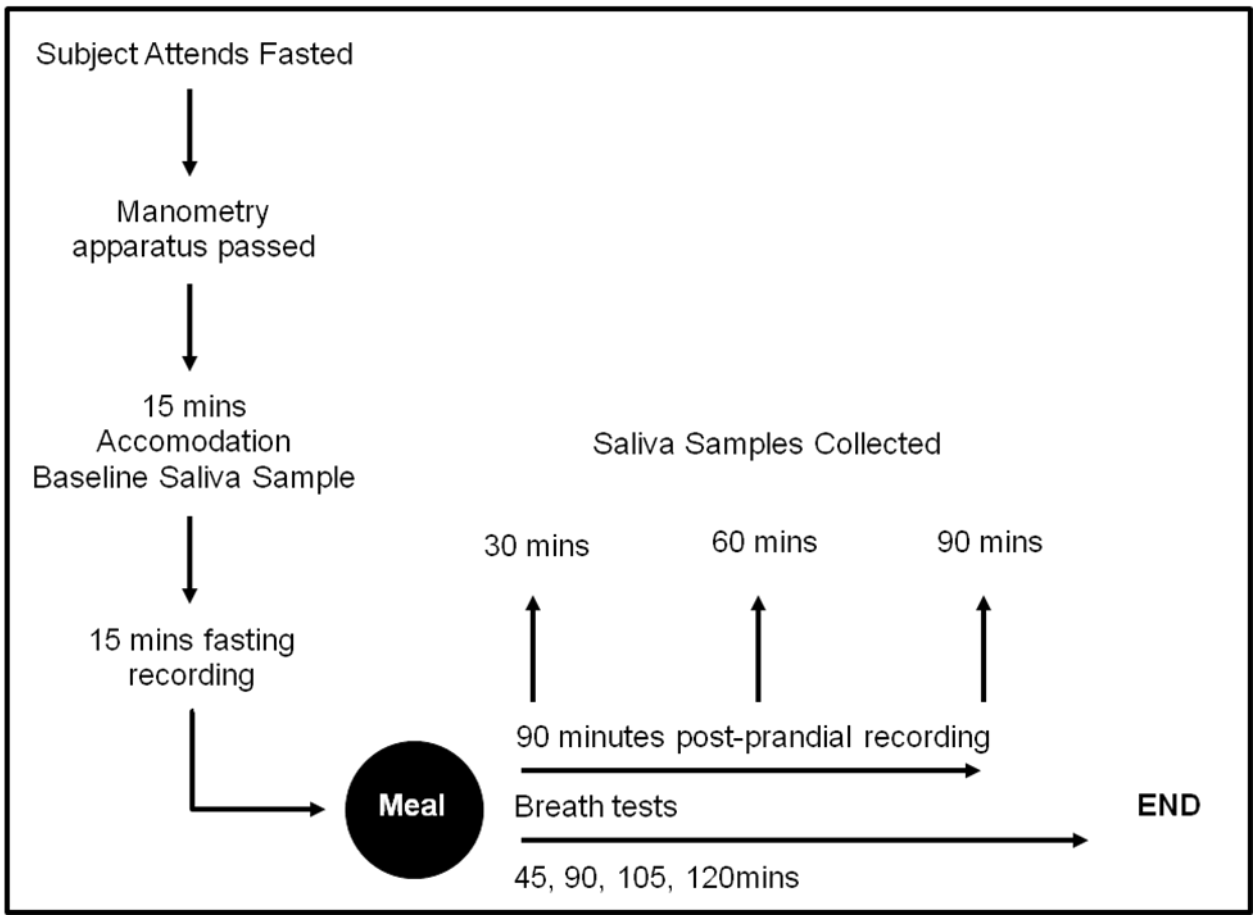

Figure 7.1. The effect of nitrite in saliva on gastric emptying – study day outline

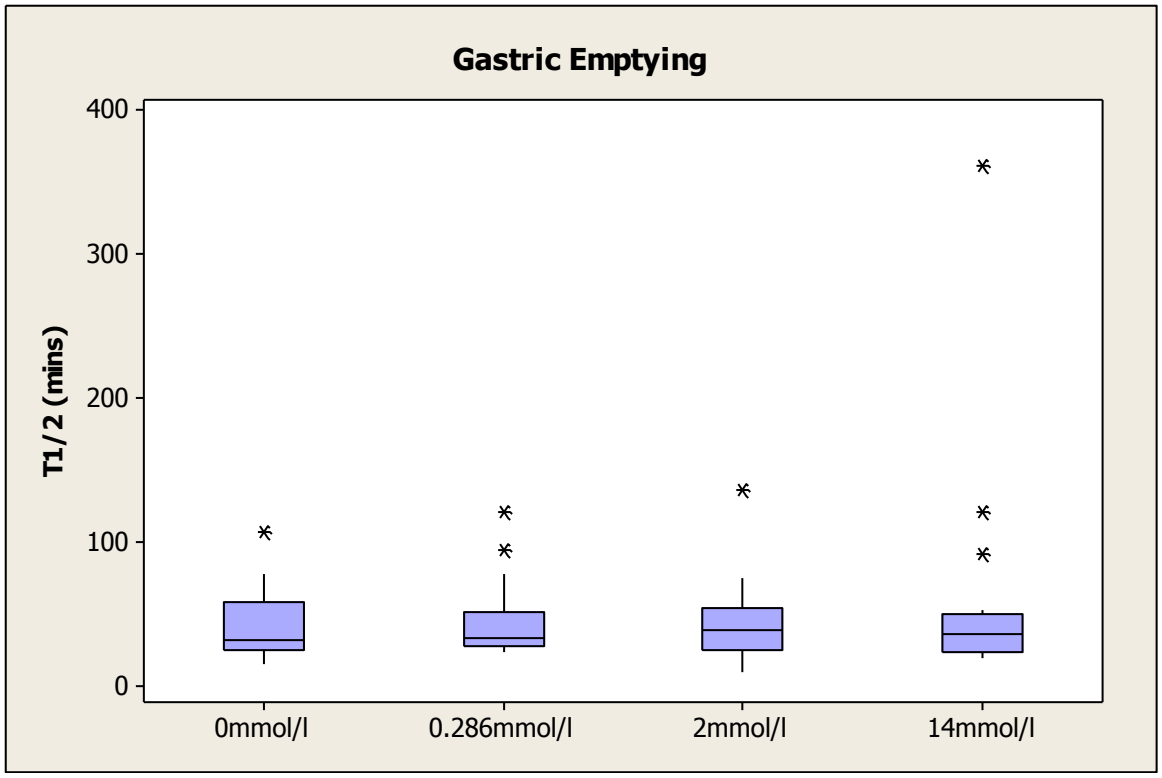

Figure 7.2. Box-plot showing gastric half-emptying time between solutions

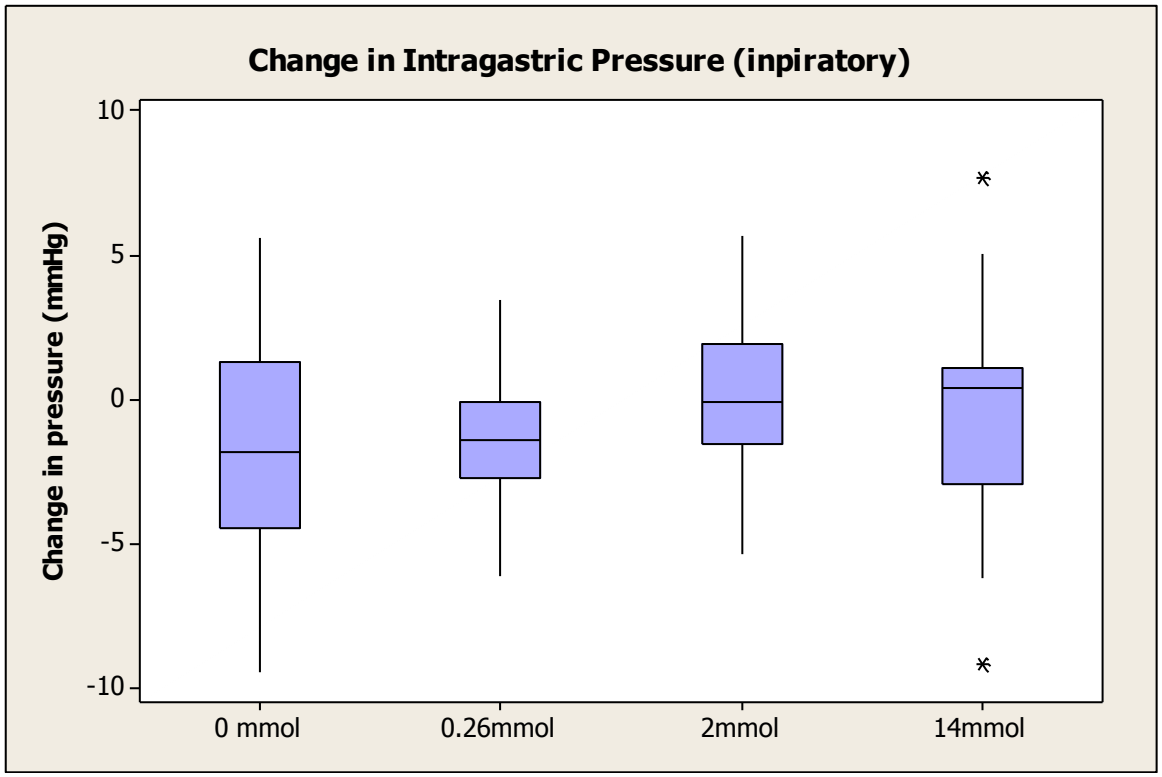

Figure 7.3. Box-plot showing the change in intragastric pressure (inspiratory) between solutions

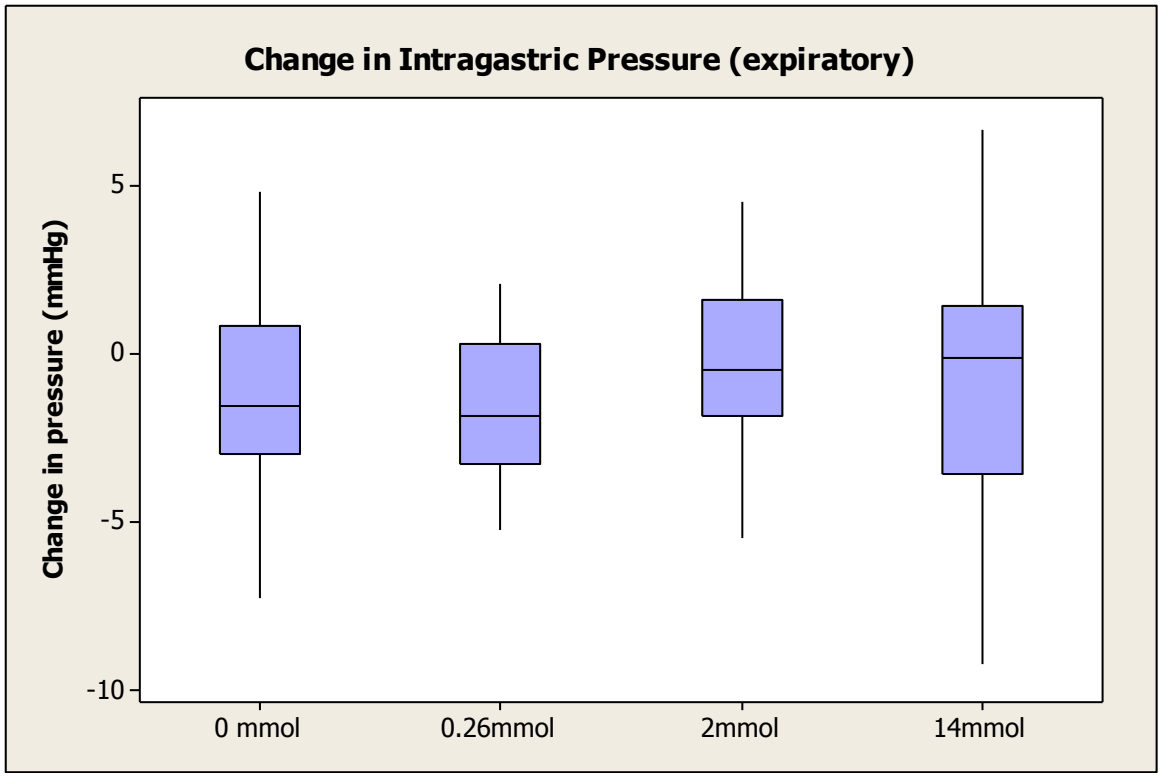

Figure 7.4. Box-plot showing the change in intragastric pressure (expiratory) between solutions

Chapter 8

Detailed Analysis of Post-prandial Changes in Gastro-oesophageal Morphology and the Effect of Salivary Nitrite

Chapter 8

Detailed Analysis of Post-prandial Changes in Gastro-oesophageal Morphology and the Effect of Salivary Nitrite

8.1. Introduction

30-50% of upper gastrointestinal tract adenocarcinomas occur at the gastro-oesophageal junction (GOJ) or gastric cardia. Unlike oesophageal adenocarcinoma, the association with reflux is weak. In studies using pull through pH and manometry, we have observed loss of the distal segment of the lower oesophageal sphincter (LOS) with the pH step-up point moving closer to or even across the squamocolumnar junction (SCJ). This distal opening may explain the high incidence of pathology at the GOJ in asymptomatic subjects.

8.2. Aims

The aims of this study were to investigate, in healthy subjects using static high resolution manometry

i. the effect of a meal on manometric changes in the gastro-oesophageal high pressure zone (HPZ), the location of the pH step-up point and the position of the SCJ

ii. whether nitrite in saliva influences the effect of a meal on manometric changes in the gastro-oesophageal high pressure zone (HPZ), the location of the pH step-up point and the position of the SCJ

8.3. Subjects

20 healthy subjects (11 males, 17 *Helicobacter Pylori* negative) were studied over 4 separate study days.

8.4 Methods

8.4.1. Endoscopic placement of radio-opaque clip at the squamocolumnar junction (SCJ)

A minimum of 24 hours prior to the first study day, subjects attended for upper gastrointestinal endoscopy. At endoscopy, a radio-opaque 11mm stainless steel endoclip (Olympus HX-600-090) was placed at the macroscopic squamo-columnar junction using an endoscopic clip-fixing device (Olympus HX-5LR-1). Placement of this clip allowed subsequent fluoroscopic localisation of the SCJ.

8.4.2. pH manometry

On each study day, combined high-resolution pH manometry apparatus was passed per nasally as previously described. The apparatus was secured at the nose using adhesive tape and 15 minutes allowed for subject accommodation to the apparatus. Following this, synchronized recording of pH and manometry was performed for 15 minutes fasting, during our standardized meal and for 90 minutes post-prandially.

8.4.3. Standardized meal

Our standardized meal consisted of two fishcakes and one rolled oats flapjack with 150ml water. (Total caloric content = 526.35kcal).

8.4.4. Nitrite infusions

Infusions of the test solutions into the mouth commenced immediately after completion of the meal and continued for 90 minutes post-prandially.

8.4.5. X-ray localization of the SCJ

X-ray screening of the gastro-oesophageal junction was carried out during normal respiration using a portable C-arm fluoroscope (Philips BV Pulsera). Screening was performed both in the fasting state and following the meal for approximately 30 seconds and synchronized with pH manometry recordings using a radio-opaque event marker. Images were recorded using a digital video recorder (JVC SR-DVM600E).

8.5. Analysis

8.5.1. Detailed Analysis of the Gastro-oesophageal Junction

To examine the gastro-oesophageal junction in more detail, 10 second periods of stable sphincter tone out with sphincter relaxations were identified using the Manoview Analysis software during fasting and for each of the 6 consecutive 15

minute periods following completion of the meal. The raw data was extracted and analysed to determine the mean positions of the distal border of the lower oesophageal high pressure zone (DHPZ), the proximal border of the lower oesophageal high pressure zone (PHPZ), the respiratory inversion point (RIP) and the pH step-up point. The DHPZ was defined as the most distal point where there was an increase of >2mmHg in pressure above the sensor recording gastric baseline pressure (defined as the lowest mean pressure in the most distal 6 manometry sensors). The PHPZ was identified as the point where there was a step-down in pressure to intra-oesophageal pressure. The RIP was defined as the point at which the manometry pressure wave decreases on inspiration. The pH step-up point was identified as the point where the mean pH increased to >4. The mean length of the high pressure zone (HPZ), the intra-abdominal component of the HPZ and the intra-oesophageal component of the HPZ were calculated.

To correct for errors in measuring the position of the PHPZ, DHPZ and RIP using 1cm increments, 0.5cm was added to the distance from the nares for each measurement. Similarly to correct for errors in measurement of the pH step-up point using 1.1cm increments, 0.55cm was added to the mean position of the pH step-up point.

8.5.2. Measurement of SCJ position

10 second periods of stable sphincter tone, out with sphincter relaxations, were identified during screening episodes using the Manoview analysis software. Corresponding still X-ray images at 2 second intervals were analysed using image

analysis software (Scion Image, Scion Corporation, Frederick, Maryland, USA). The position of the clip relative to the pH and manometry catheters was calculated with the measured distance of one pH sensor (known to be 1cm) used as an internal scale. The mean SCJ position for the 10 second period was calculated.

8.6. Statistical Analysis

Statistical analysis was performed using the one-sample Wilcoxon test or Mann Whitney U unless specified otherwise. The Bonferroni method was used to correct for the use of multiple comparisons with value of $p<0.0083$ considered significant.

8.7. Ethics

The study was approved by West Glasgow Research Ethics Committee and informed consent was obtained from each participant

8.8. Results

8.8.1. Salivary Nitrite Concentrations

There was a significant difference in the median salivary nitrite concentrations during infusion of each solution.

8.8.2. Changes after the meal – control solution

8.8.2.a. Detailed analysis of the gastro-oesophageal junction

Following the meal the PHPZ was unchanged from its fasting state but there was significant shortening of the HPZ, a maximum of 2.08cm (-0.48 – 5.59; p<0.001) see Figure 8.1. There was, therefore, significant proximal movement of the DHPZ relative to the nares, a maximum of 2.14cm (-1.37 – 4.23; p<0.001). In addition there was proximal movement of the RIP relative to the PHPZ, a maximum 1.09cm (0 - 1.43; p<0.001), as a result there was shortening of both the intra-oesophageal (maximum 1.11cm, range -0.55 – 4.36; p<0.001) and intra-abdominal (maximum 1.15cm, range -1.43 – 3.53; p=0.007) components of the HPZ. The pH step-up point also moved proximally, a maximum of 1.61cm (-1.49 – 5.15; p<0.001). The changes occurring at the GOJ following the meal are illustrated in Figure 8.2.

8.8.2.b. SCJ position

18 subjects underwent X-ray screening on the day of the control infusion. In the remaining 2 subjects, videofluoroscopy equipment was unavailable at the time of study. In 5 of the subjects screened the endoclip had spontaneously dislodged and they were excluded from further analysis. SCJ position was calculated in the remaining 13 subjects. Following the meal the DHPZ moved significantly closer to the SCJ, a median 1.72cm (-0.37-2.93, p=0.004). However, there was no difference in the position of the pH step-up point relative to the SCJ between the fasting and postprandial screening episodes with the median position being 0.62cm distal to SCJ (-2.25–5.93, p>0.4). These results are illustrated in Figure 8.3.

An illustration of the changes at the GOJ following the meal is shown in Figure 8.4.

8.8.3. Effect of Salivary Nitrite on GOJ Morphology

8.8.3.a. Detailed Analysis of the Gastro-oesophageal Junction

The position of the PHPZ following the meal was similar between all the solutions ($p>0.08$). There was no difference in the position of the RIP following the meal between the control solution and the nitrite-containing solutions ($p>0.05$). With all solutions, the median length of the HPZ and the length of both the intra-oesophageal HPZ and the intra-abdominal HPZ was reduced following the meal with no significant difference in lengths between the control solution and the nitrite-containing solutions ($p>0.05$, $p>0.01$ and $p>0.13$) respectively. Finally, the median distance of the pH step-up point relative to the PHPZ was reduced following the meal with all solutions, again there was no difference in the position of the pH step-up point relative to the PHPZ between the control solution or the nitrite-containing solutions ($p>0.04$). These results are shown in Figures 8.5. – 8.7.

8.8.3.b. SCJ position

Due to lack of availability of equipment or spontaneous clip dislodgement, only 7 subjects had suitable X-ray screening images available for comparison between all solutions. There was no significant change in the position of the pH step up point relative to the SCJ following the meal between the control solution (0.69cm, -0.26-2.97) and those containing 0.286mmol/l (-0.69cm, -5.15 – 0.15; $p>0.03$ v control), 2mmol/l (-0.24cm, -2.08 – 2.86; $p>0.1$ v control) and 14mmol/l potassium nitrite (-1.07cm, -2.39 - 4.9; $p>0.15$ v control). With all solutions, following the meal

the DHPZ moved closer to the SCJ but there was no difference between the control solution (-0.26cm, -2.7 - 0.37) and those containing 0.286mmol/l (-0.8cm, -3.21 – 0.5; p>0.93 v control), 2mmol/l (-0.89cm, -3.42 – 0.51; p>0.35 v control) and 14mmol/l potassium nitrite (-0.38cm, -3.64 – 1.15; p>0.67 v control) when the change in DHPZ position relative to the SCJ was compared.

8.9. Discussion

This study combines, for the first time, high resolution pH manometry with X-ray localization of the SCJ to provide detailed analysis of the GOJ during fasting and in the post-prandial state. By analysing the GOJ in more detail, we have demonstrated that following the meal there is no difference in the position of the PHPZ but significant proximal movement of the DHPZ. As a result there is significant shortening of the HPZ. Proximal movement of the RIP occurs which would be consistent with cephalic displacement of the diaphragm by a distended stomach. With X-ray screening the DHPZ is seen to move significantly closer to the SCJ after the meal and the pH step up point migrates proximally. These changes are consistent with distal opening of the LOS. This mechanism was first proposed by Oberg and DeMeester (94;106) and has recently been demonstrated using pull-through manometry (93;170). It has been proposed as a mechanism by which distal oesophageal exposure may occur (intra-sphincteric reflux) and as a possible explanation for the recently described paradox of postprandial acidification of the gastric cardia (171). This is the first study using static high-resolution manometry which confirms distal opening of the LOS after meals. The fact that we were unable to demonstrate any change in the position of the pH step-up point relative to the SCJ

after the meal, consistent with intra-sphincteric reflux, is likely to be due to the methods used in our study and the subjects examined. Studies were performed with subjects semi-recumbent at 45º to the horizontal, while the screening group also had a median body mass index (BMI) in the normal, healthy range of 23.92. Intra-sphincteric reflux is presumably more likely to occur in obese subjects in whom cephalic displacement of the diaphragm and raised intragastric pressures are well documented (121;146-148). Similarly posture is likely to be important with cephalic displacement of the diaphragm being well documented to occur on lying flat. (149).

8.10. Conclusion

This study confirms, using high-resolution pH manometry, that distal opening of the LOS and proximal migration of the pH step-up point occurs after a meal. The results suggest that this may in part be mediated by proximal movement of the diaphragm. This mechanism may be important in the pathogenesis of disease at the GOJ. However, no effect on post-prandial gastro-oesophageal morphology was seen with modification of salivary nitrite concentrations.

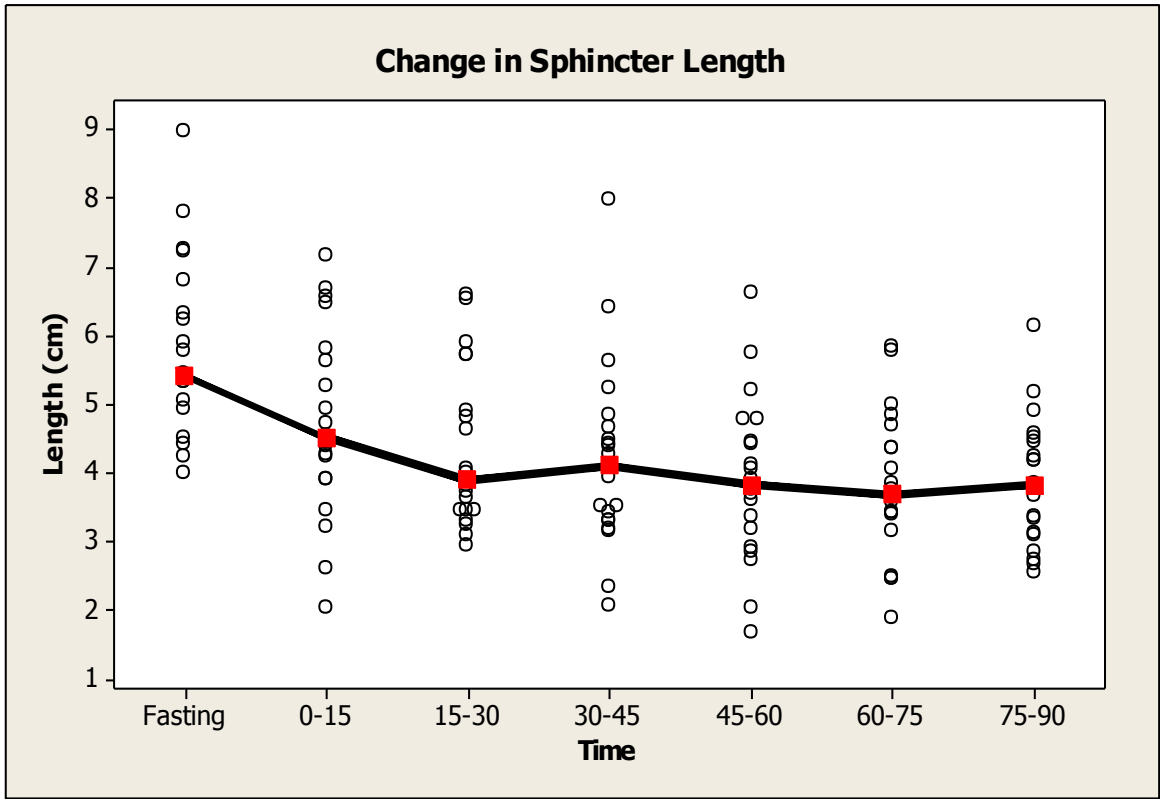

Figure 8.1. Line graph showing significant shortening of the LOS postprandially

***maximum 2.08cm (range -0.48-5.59cm) at 60-75 mins (p<0.001)**

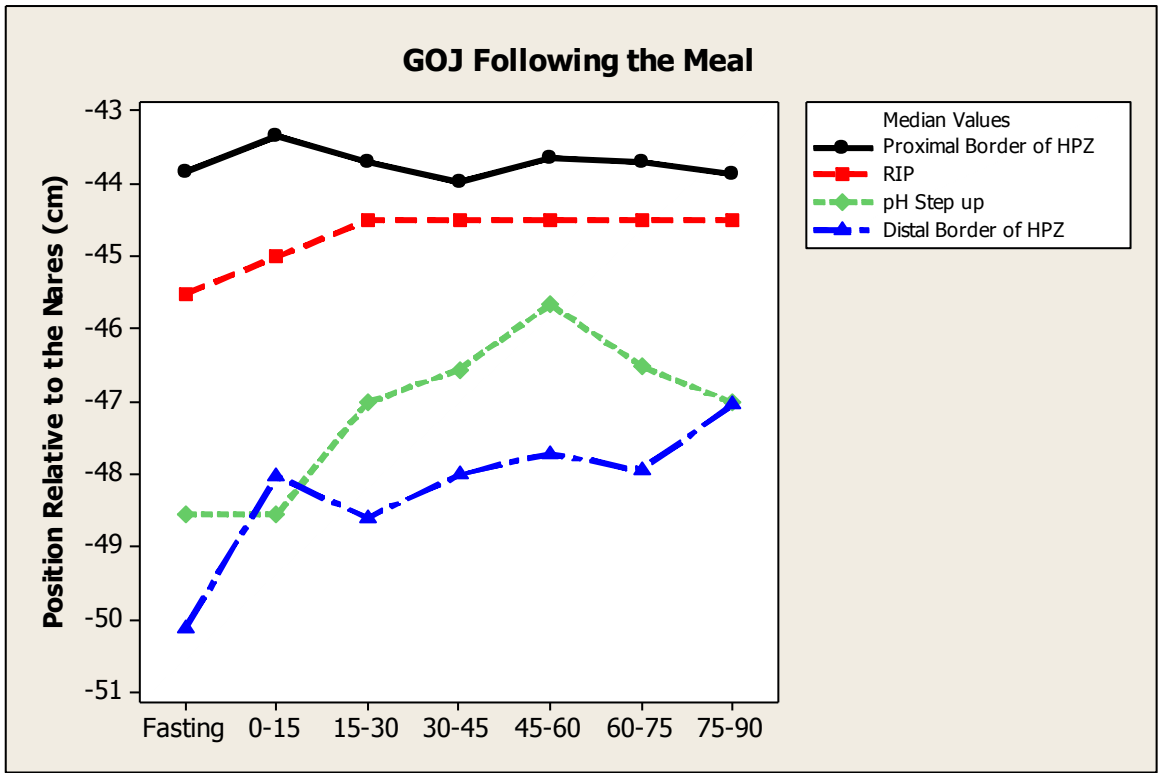

Figure 8.2. Line graph showing changes occurring at the GOJ following the meal

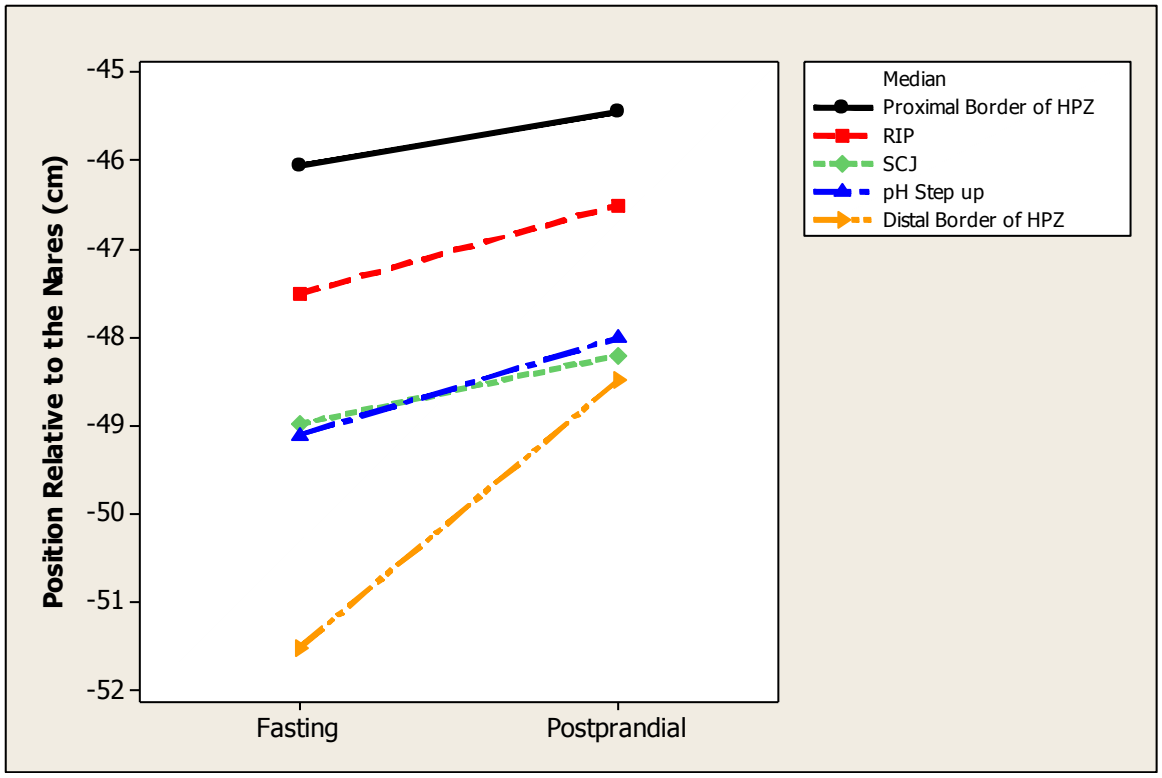

Figure 8.3. Line graph showing the change in the position of the SCJ and pH step-up point post-prandially

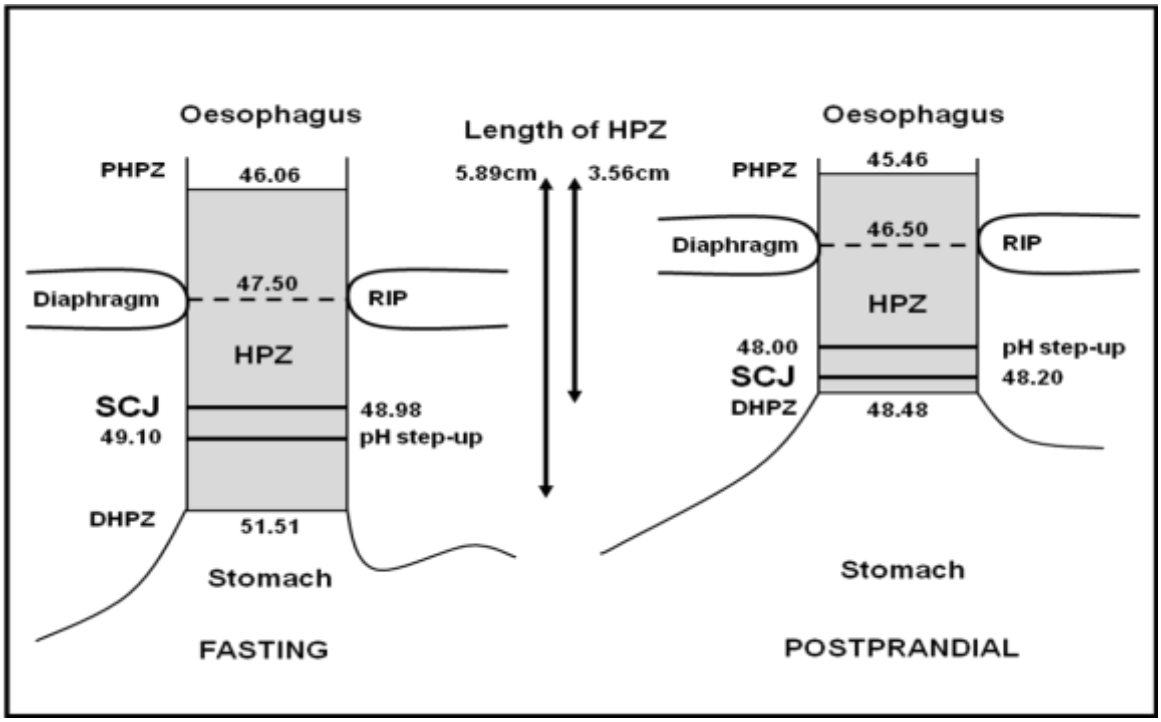

Figure 8.4. Illustration of changes occurring at the GOJ following a meal. Results are median values at 90 minutes post-prandially. (Note: the median pH step-up point was 0.2cm proximal to the median SCJ position but this did not reach statistical significance)

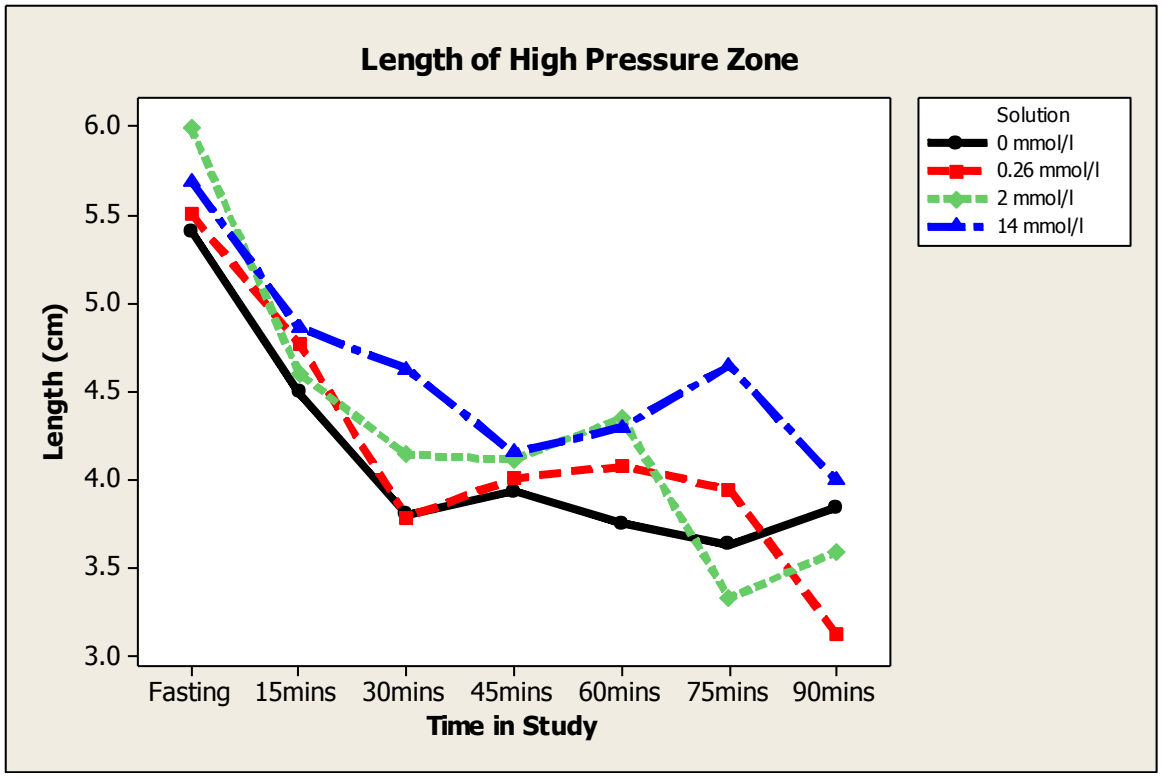

Figure 8.5. Line graph showing shortening of HPZ following the meal

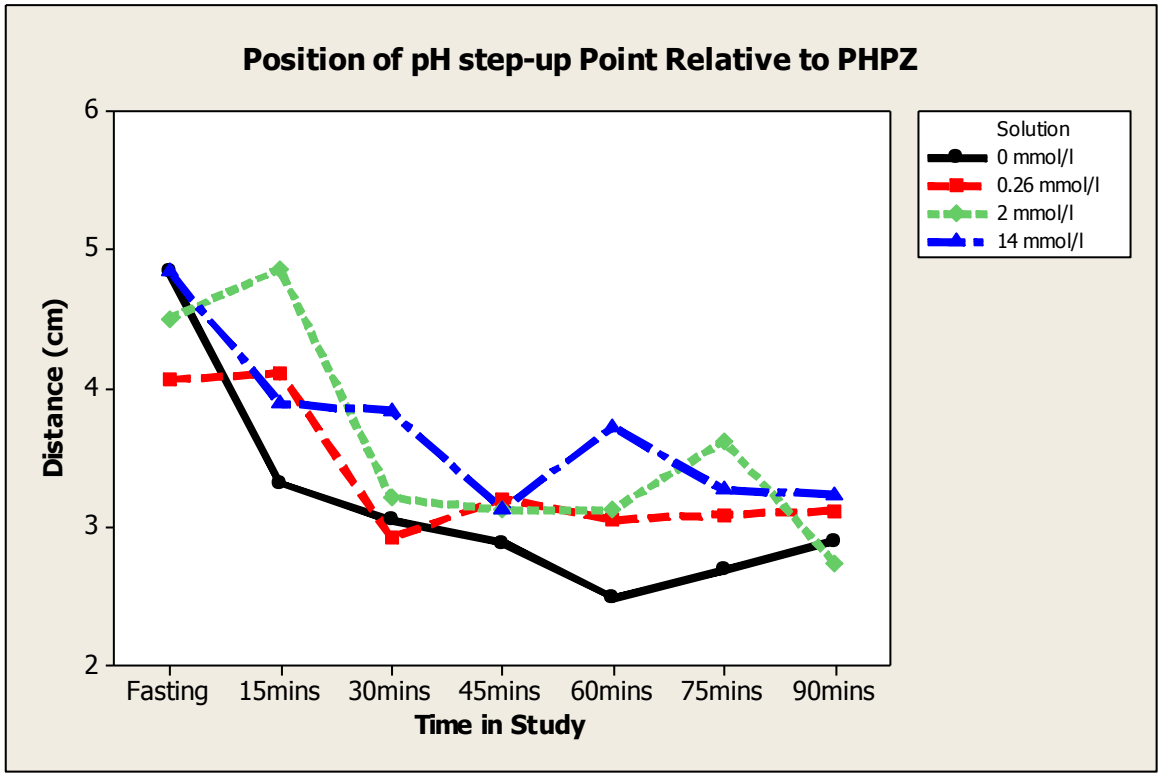

Figure 8.6. Line graph showing pH step-up point moves proximally after the meal

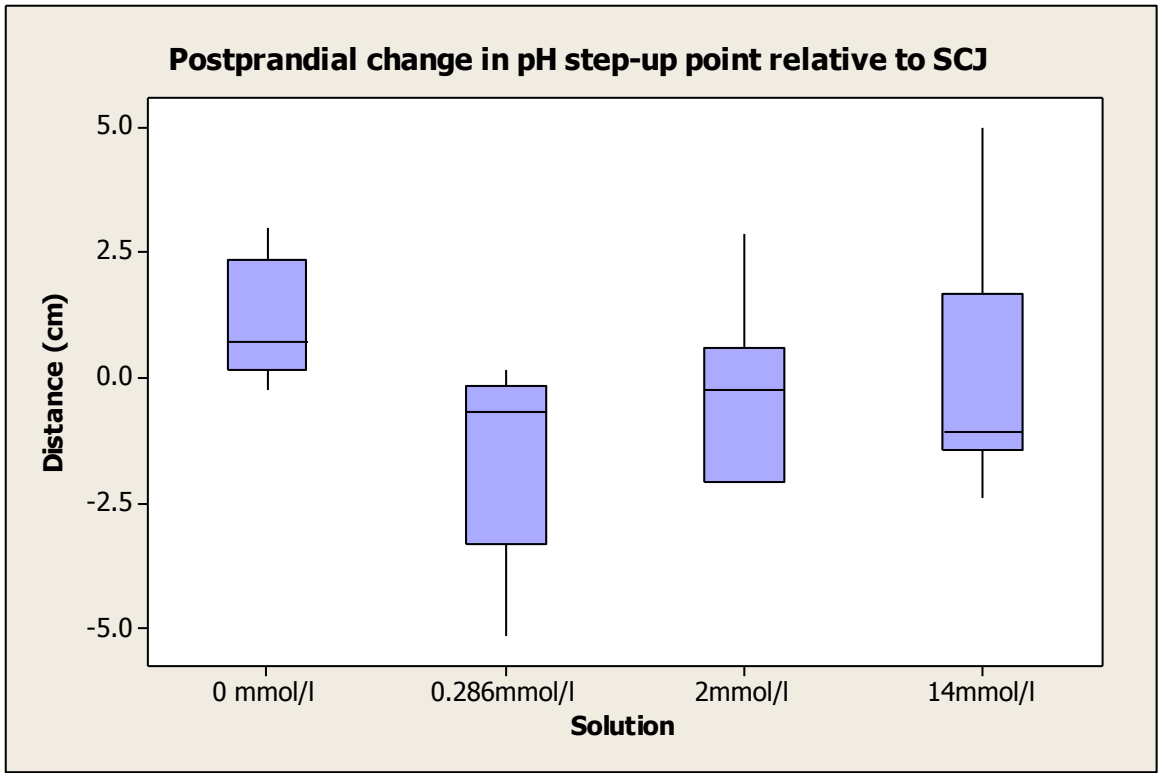

Figure 8.7. Box-plot showing the post-prandial position of the pH step-up point relative to the SCJ

Chapter 9

Increasing Age and Obesity is Associated with Proximal Migration of the SCJ in Healthy Volunteers

Chapter 9

Increasing Age and Obesity is Associated with Proximal Migration of the SCJ in Healthy Volunteers

9.1. Introduction

The gastric cardia is an important site of pathology with a high incidence of inflammation, metaplasia and neoplasia. However this complex area is poorly understood and it has been proposed that cardia mucosa itself may be pathological, arising from columnar metaplasia of the distal oesophageal squamous mucosa. Previous autopsy studies show a near absence of cardia epithelium in neonates and an increase in length with age (98;99).

9.2. Aims

The aims of this study were:

i. to determine if the position of the squamocolumnar junction (SCJ) within the gastro-oesophageal high pressure zone changes with age in healthy subjects

ii. to examine the influence of waist circumference (WC) and body mass index (BMI) on squamocolumnar position within the gastro-oesophageal high pressure zone.

9.3 Subjects

15 *Helicobacter pylori* negative subjects (9 males) who were free of any gastrointestinal symptoms were studied.

9.4. Methods

Each subject underwent upper GI endoscopy with clipping of a radio-opaque endoclip at the squamocolumnar junction. On a separate study day, synchronized 36 sensor high resolution manometry and videofluoroscopy was performed with subjects fasted and in a semi-recumbent position. Age, WC (cm) and BMI (m/kg^2) were recorded for each individual

9.5. Analysis

A 10 second analysis period of stable sphincter tone (excluding sphincter relaxations) was selected. The proximal high pressure zone (PHPZ) was identified from manometry data as a step-down in pressure to the intrathoracic pressure. The location of the SCJ was determined using image analysis software (Scion Image, Scion Corporation, Frederick, Maryland, USA) with the measured distance of a pH sensor (known to be 1cm) used as a scale. The distance between the position of the SCJ and the proximal HPZ was calculated for each individual.

9.6. Statistical Analysis

Spearman's rank correlation coefficient was used for analysis of non-parametric data. Results are medians and ranges unless otherwise stated.

9.7. Ethics

The study was approved by West Glasgow Research Ethics Committee and informed consent was obtained from each participant.

9.8. Results

The median age of the subjects was 25 years (20-59 years). The median BMI was 22.7 kg/m^2 (18.6 - 34.7 kgm^2) while the median waist circumference was 82cm (66 - 110cm). The median distance between the SCJ and PHPZ was 3.59cm (0.31 - 4.46cm). The baseline characteristics of our study population are shown in Table 9.1.

9.8.1. Effect of age on SCJ position

There was a strong negative correlation between age and distance between SCJ and PHPZ (R = -0.624; p = 0.013) – results shown in Figure 9.1. This correlation was even stronger when the male sub-group was analysed (R = -0.814; p = 0.008) – results shown in Figure 9.2.

9.8.2. Effect of BMI on SCJ position

There was a strong negative correlation between age and distance between SCJ and PHPZ (R = -0.596; p = 0.019) – results shown in Figure 9.3. This correlation was even stronger when the male sub-group was analysed (R = -0.750; p = 0.02) – results shown in Figure 9.4.

9.8.3. Effect of waist circumference on SCJ position

There was a strong negative correlation between age and distance between SCJ and PHPZ (R = -0.525; p = 0.044) – results shown in Figure 9.5. This correlation was even stronger when the male sub-group was analysed (R = -0.767; p = 0.016) – results shown in Figure 9.6.

9.8.4. Multiple regression analysis

In multiple regression analysis the combination of both increasing age and body mass index was significantly associated with movement of the SCJ closer to the proximal HPZ (R^2=73.6%, P<0.001) with a strong trend towards significance for both individual variables (p=0.053 and p=0.088 respectively).

9.9. Discussion

Our results indicate that the position of the squamocolumnar junction relative to the proximal high pressure zone in Helicobacter Pylori negative individuals is

strongly correlated to age. Furthermore the association is stronger in males and similar associations can be seen with increasing BMI and waist circumference. This is a cross-sectional study and while it confirms that increasing age and obesity are associated with proximal migration of the SCJ it cannot determine cause and effect. Nevertheless, these findings would be in keeping with previous autopsy studies which suggest cardia mucosa may be acquired over time and could be due to columnar metaplasia of the most distal oesophageal squamous mucosa.

If this effect is independent of age-related gastric atrophy, it would support the hypothesis that this is due to acid-induced metaplasia. It would suggest that obesity and more specifically the central (visceral) obesity seen in males, which correlates with waist circumference, may potentiate acidification of the most distal squamous oesophageal mucosa. This could be explained either by traditional gastro-oesophageal reflux or by 'intra-sphincteric reflux' where the pH step-up point migrates across the SCJ due to distal opening of the LOS following meals.

9.10. Conclusion

The position of the squamocolumnar junction relative to the proximal high pressure zone correlates strongly with age, BMI and waist circumference in healthy subjects. This supports proximal migration of the squamocolumnar junction due to columnar metaplasia of the distal oesophageal squamous mucosa.

Table 9.1. Baseline characteristics of study participants

Parameter	Value		
Number of Subjects	15		
Sex	Male 9 (60%)	Female 6 (40%)	
Age (y)	25 (20 – 59)		
Age Range	<30 y 9 (60%)	>30y 6 (40%)	
Weight (kg)	75.0 (55 – 110)		
BMI (kg/m^2)	22.7 (18.6 – 34.7)		
BMI Range	Normal (<25) 11 (68.8%)	Overweight (25-30) 2 (12.5%)	Obese (>30) 3 (18.8%)
Waist Circumference (cm)	82 (66 – 110)		
Distance from SCJ to PHPZ (cm)	3.59 (0.31 – 4.46)		

Values are medians (ranges) unless stated

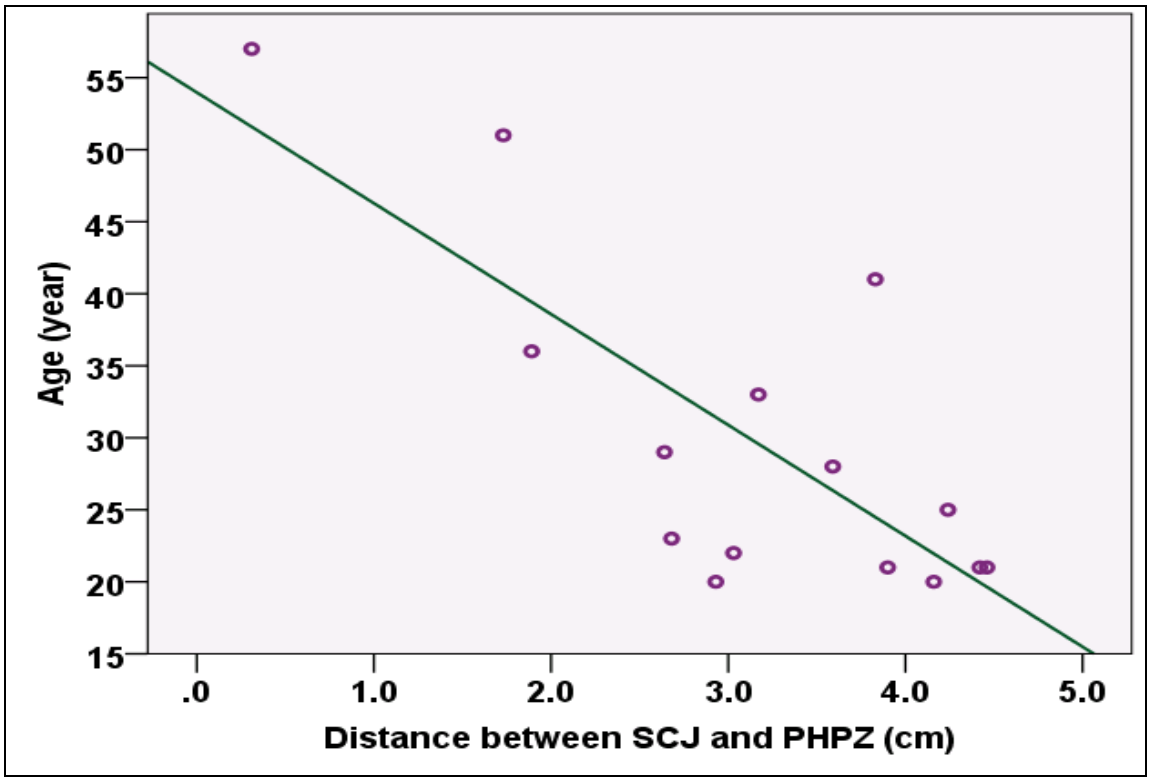

Figure 9.1. Fitted line plot of age versus distance between SCJ and PHPZ

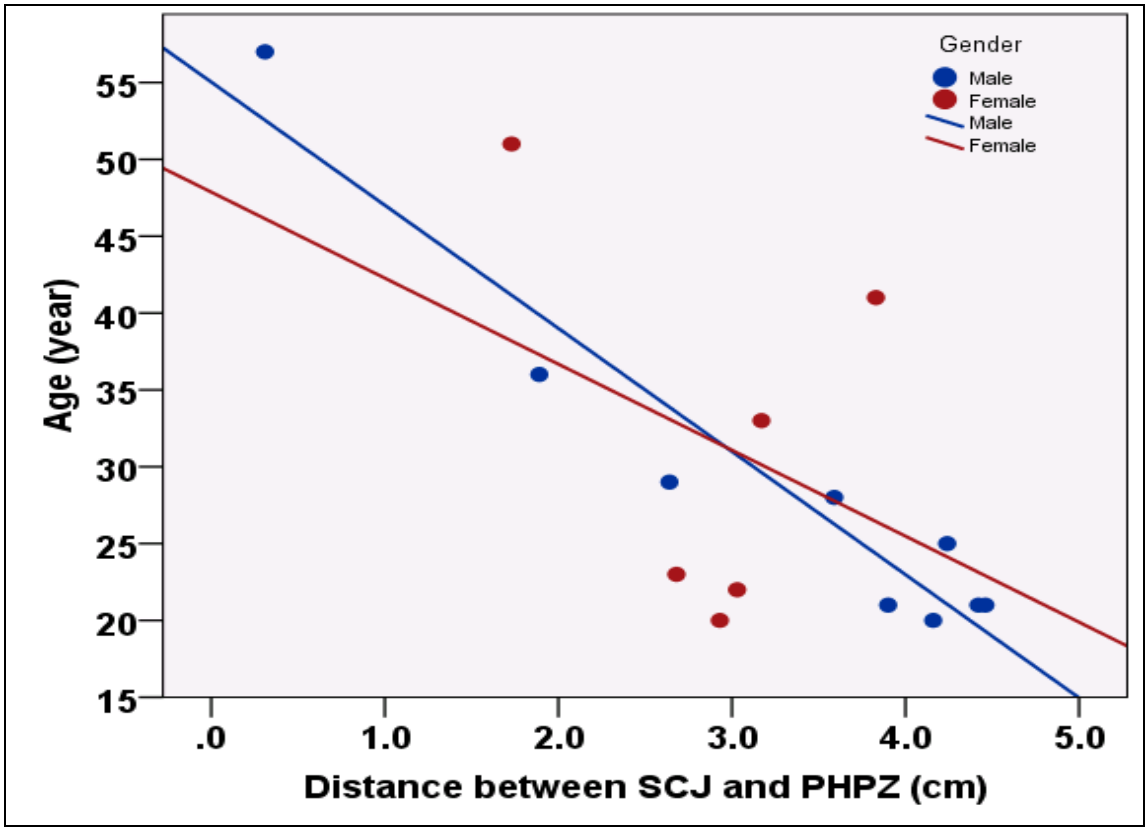

Figure 9.2. Fitted line plot of age versus distance between SCJ and PHPZ – males versus females

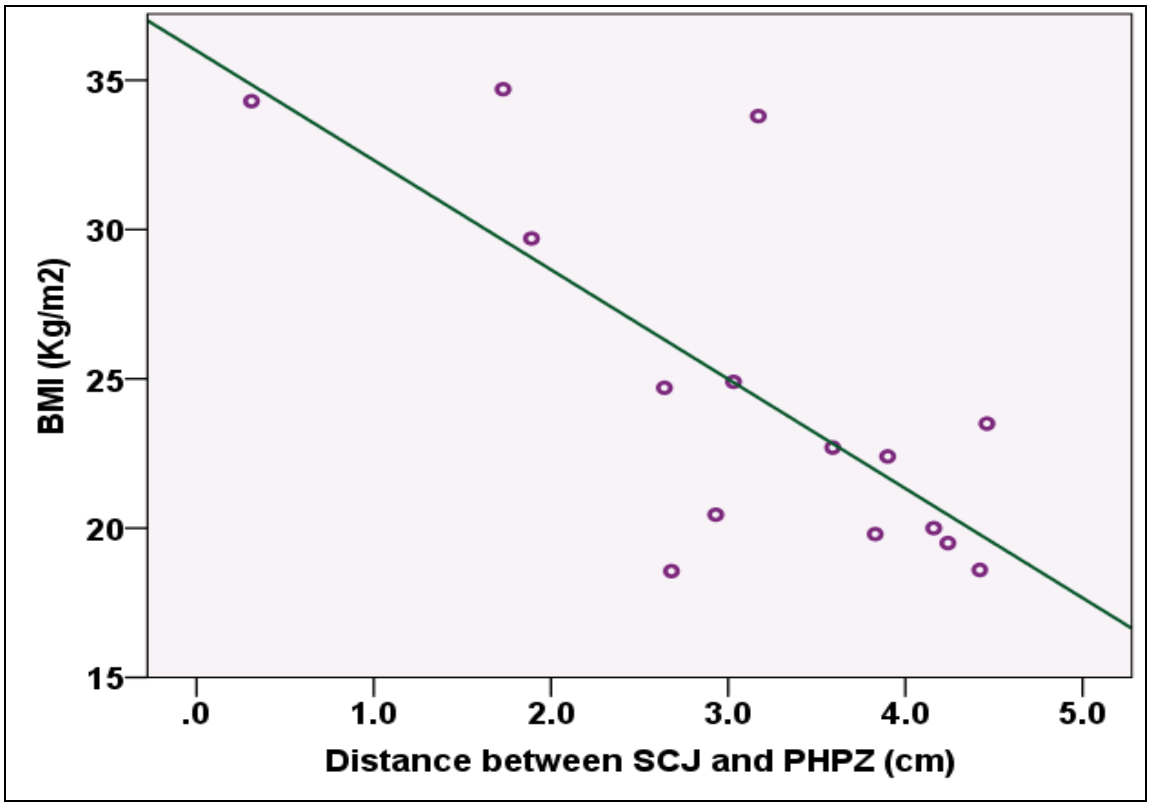

Figure 9.3. Fitted line plot of body mass index versus distance between SCJ and PHPZ

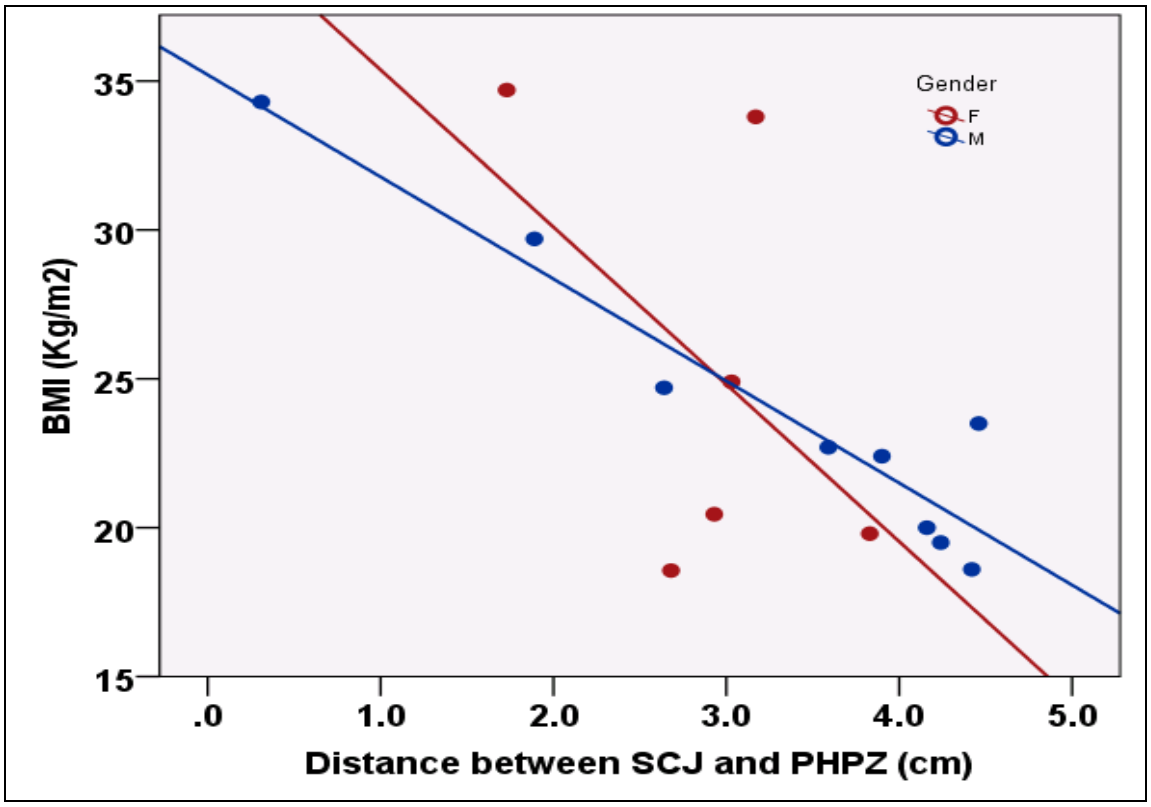

Figure 9.4. Fitted line plot of body mass index versus distance between SCJ and PHPZ – males versus females

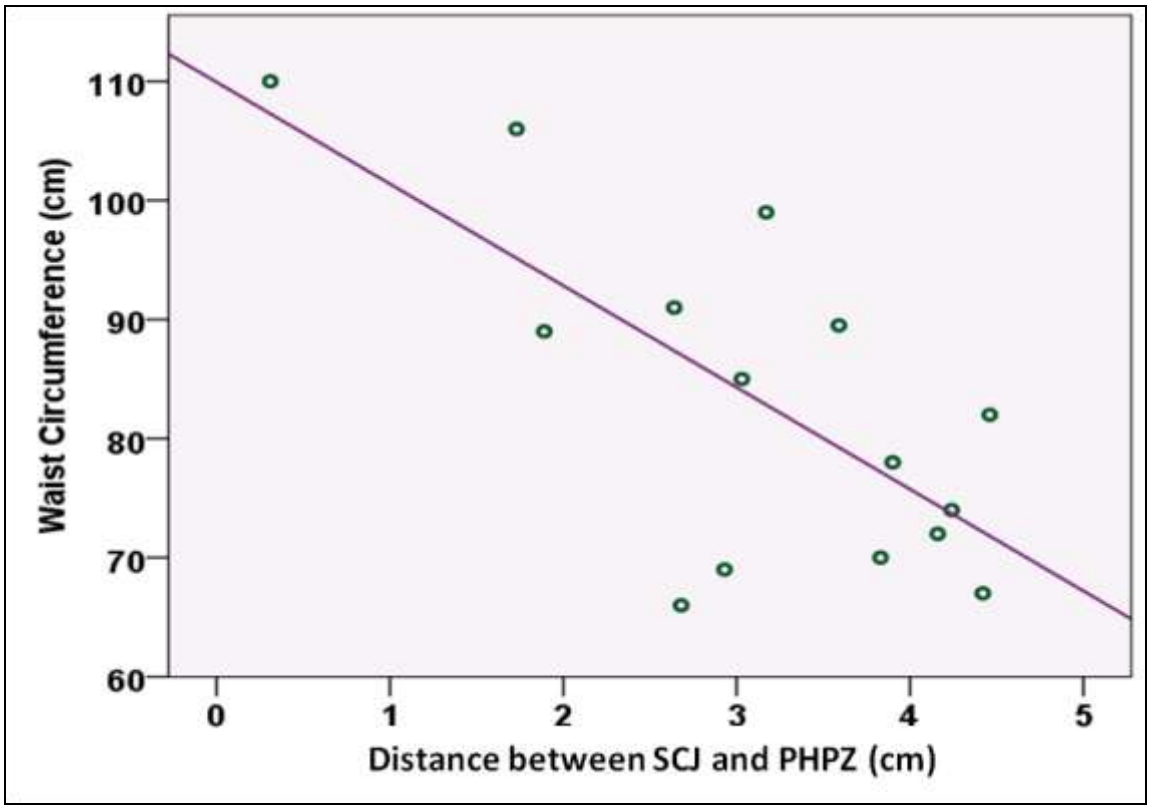

Figure 9.5. Fitted line plot of waist circumference versus distance between SCJ and PHPZ

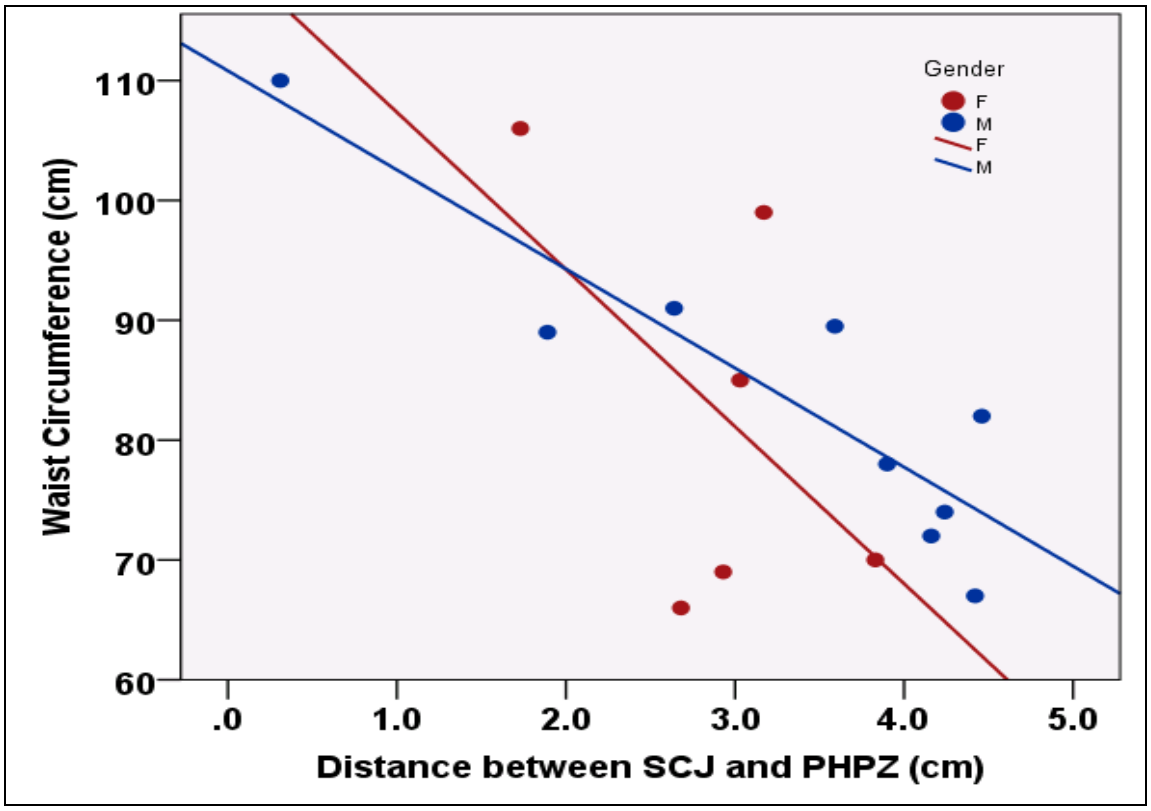

Figure 9.6. Fitted line plot of waist circumference versus distance between SCJ and PHPZ – males versus females

Chapter 10

A Localised Decrease in Parietal cell Density Occurs at the SCJ with Increasing Age in Asymptomatic Healthy Volunteers

Chapter 10

A Localised Decrease in Parietal Cell Density Occurs at the SCJ with Increasing Age in Asymptomatic Healthy Volunteers

10.1. Introduction

It has been hypothesized that gastric cardia mucosa may be pathological and represent acid-induced columnar metaplasia of the most distal oesophageal mucosa. We have shown that the squamocolumnar junction moves closer to the proximal border of the lower oesophageal high pressure zone with increasing age. This suggests proximal migration of the SCJ with progressive enlargement of the gastric cardia may occur over time. This would be expected to be accompanied by a reduction in parietal cell density at the SCJ but how much of this is due to age-related gastric atrophy and is it associated with distal oesophageal acid exposure?

10.2. Aims

Our aims in this study were:

i. to investigate whether parietal cell density in the columnar mucosa at the squamocolumnar junction (SCJ) changes with age

ii. to determine whether this is a localised phenomenon or associated with more generalised gastric atrophy

iii. to examine the role of *Helicobacter pylori* infection

iv. to investigate the association of SCJ parietal cell density with distal oesophageal acid exposure

10.3. Subjects

25 healthy volunteers (14 males, 5 *Helicobacter pylori* positive) were studied.

10.4 Methods

During upper GI endoscopy, biopsy samples were taken from the macroscopic SCJ, 1cm distal to the SCJ, the gastric body and the gastric antrum. Biopsies were embedded in paraffin blocks and 5 micron thick sections cut for immunohistochemistry. Parietal cells were stained and visualised using a standardised monoclonal anti-H^+K^+ATPase antibody as shown in Figure 10.1. All slides were scanned via a digital scanner (Olympus, UK) and uploaded onto a slide server. A tissue image analysis programme (SlidePath, Dublin, Ireland) was used to quantify parietal cell density in each cell which was reflected as intracellular positive pixels. To allow correlation with acid exposure, pH studies were performed in 20 subjects before and for 90 minutes after our standardised meal of two fishcakes and one rolled oats flapjack with 150ml water (Total calorie content = 526.35kcal).

10.5. Analysis

The average parietal cell density in a minimum of 4 sections of columnar mucosa per slide was reported as a percentage of the total cells per section. Acid

exposure was calculated 5 - 6cm above the level of the fasting pH step-up point and calculated as % time pH<4.

10.6. Statistical analysis

Spearman's rank correlation coefficient and the Mann-Whitney U test were used for analysis of non-parametric data. Results are medians and ranges unless otherwise stated.

10.7. Ethics

The study was approved by West Glasgow Research Ethics Committee and informed consent was obtained from each participant.

10.8. Results

10.8.1. Parietal cell density

Median parietal cell density results are shown in Table 10.2.

10.8.2. Age and parietal cell density

There was a significant negative correlation between increasing age and parietal cell density in the columnar mucosa at the level of the SCJ ($R = -0.530$, $p = 0.008$). There was no significant correlation between age and parietal cell density

1cm distal to the SCJ (R = 0.153, p=0.5), in the gastric body (R = 0.386, p = 0.08) or antrum (R = 0.117, p = 0.6). These results are displayed in Figure 10.2.

In the *Helicobacter pylori* negative subgroup, parietal cell density at the SCJ of volunteers under 30 years old was significantly greater than those aged 30 years and over (6.8% (range 0 – 16.3%) v 0.4% (range 0 – 6.3%); p = 0.05). In contrast, gastric body parietal cell density was lower in the younger group (30.0% (range 19.9 – 35.7%) v 34.5% (range 30.5 – 42.7%); p = 0.019. These results are shown in Figure 10.3.

10.8.3. Distal oesophageal acid exposure and parietal cell density

The median % time post-prandial oesophageal pH < 4 was 0.49% (0 - 19.96). There was no significant correlation between post-prandial distal oesophageal acid exposure and parietal cell density (R = 0.223, p = 0.3) – see Figure 10.4.

10.9. Discussion

Parietal cell density decreases with age in the columnar mucosa at the SCJ. This effect appears to be localised to the SCJ and is not seen 1cm distal to this or within the stomach suggesting it is not due to generalized age-related parietal cell atrophy. Furthermore, when the Helicobacter negative subgroup was analysed separately, while SCJ parietal cell density significantly decreased in the group over 30 years of age, gastric body parietal cell density actually increased. There was no

correlation between parietal cell density and conventional acid reflux, measured as %time pH <4 at 5 - 6cm above the fasting pH step-up.

These results suggest a progressive, localised decrease in parietal cell density at the GOJ with increasing age which is due to a mechanism other than traditional 'trans-sphincteric' gastro-oesophageal reflux. While parietal cell atrophy due to *Helicobacter pylori* infection could explain a reduction in parietal cell density, this would be expected to cause more generalized parietal cell atrophy affecting the gastric body. In addition, only 5 (20%) of our subjects were Helicobacter Pylori positive and sub-group analysis confirmed a reduction in SCJ parietal cell density with increasing age in the Helicobacter Pylori negative group. These findings therefore support the hypothesis that the gastric cardia is an acquired structure due to acid-induced columnar metaplasia. This may occur due to 'intra-sphincteric reflux' with the pH step-up point migrating across the SCJ due to distal opening of the LOS as we, and others in our group, have already described.

10.10. Conclusion

Parietal cell density decreases with age in the columnar mucosa at the SCJ, an effect localised to the GOJ and independent of, conventionally measured, distal oesophageal acid exposure. These results support the hypothesis that cardiac mucosa may be acquired and due to mechanisms other than traditional gastro-oesophageal reflux.

Table 10.1 Median parietal cell density results

	Location of Biopsies			
Group	**SCJ**	**1cm Distal to SCJ**	**Gastric Body**	**Gastric Antrum**
Total*	5.4 (0-16.3)	26.5 (6.7-34.7)	31.2 (19.9-42.7)	2.5 (0-21.3)
<30 years old	6.3 (0-16.3)	22.3 (6.7-34.7)	30.0 (19.9-35.7)	3.2 (0-21.3)
≥30 years old	0.2 (0-6.3)	26.9 (12.1-34.7)	34.0 (30.5-42.7)	2.5 (0-20.0)

*Median (range in brackets) parietal cell densities are shown as % of total number of cells per biopsy section

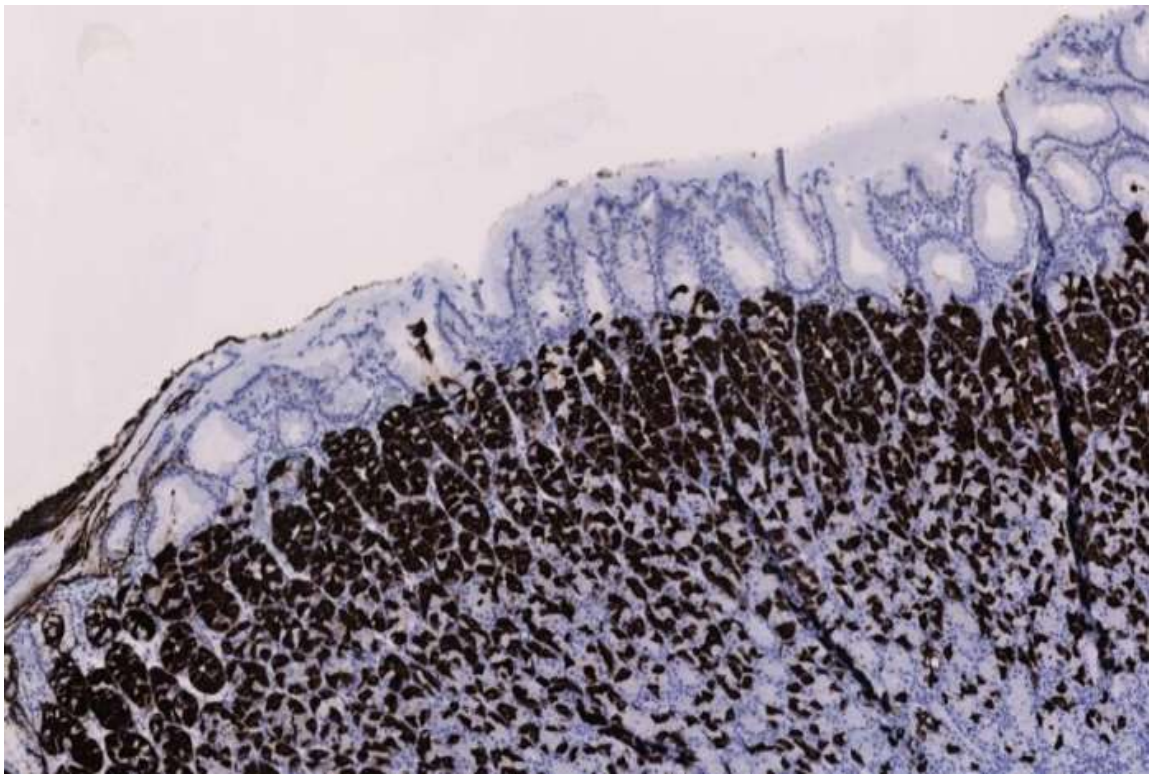

Figure 10.1 Histology Slide showing parietal cells stained with monoclonal anti-H+/K+ATPase antibody

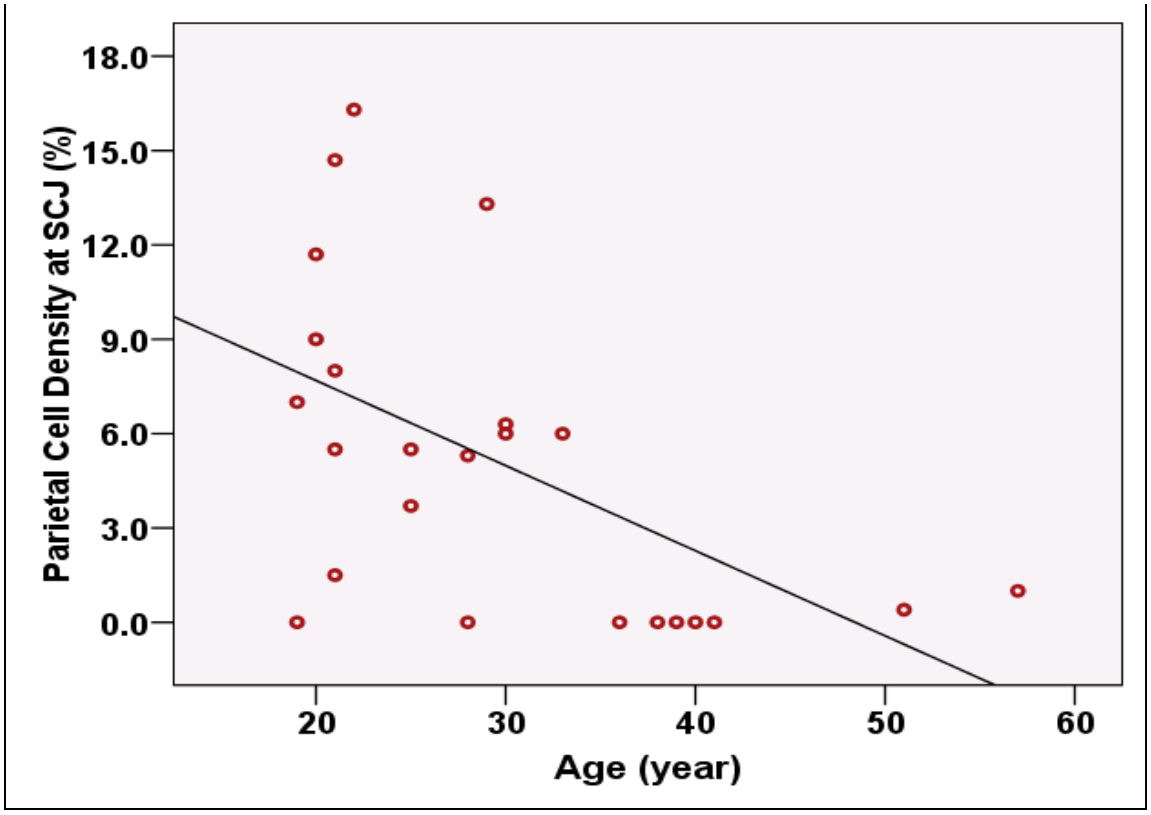

Figure 10.2 Fitted line plot of SCJ parietal cell density versus age

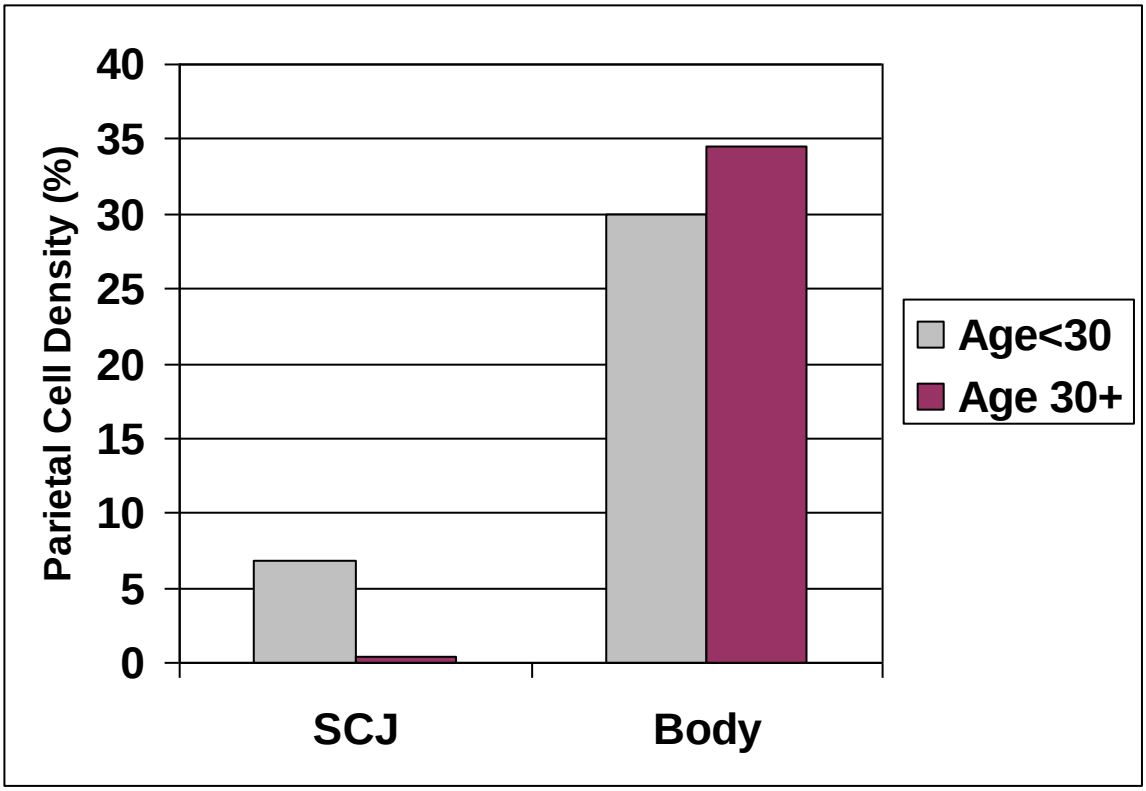

Figure 10.3. Bar chart of parietal cell densities at the SCJ and gastric body of *Helicobacter Pylori* negative subjects below and above 30 years of age

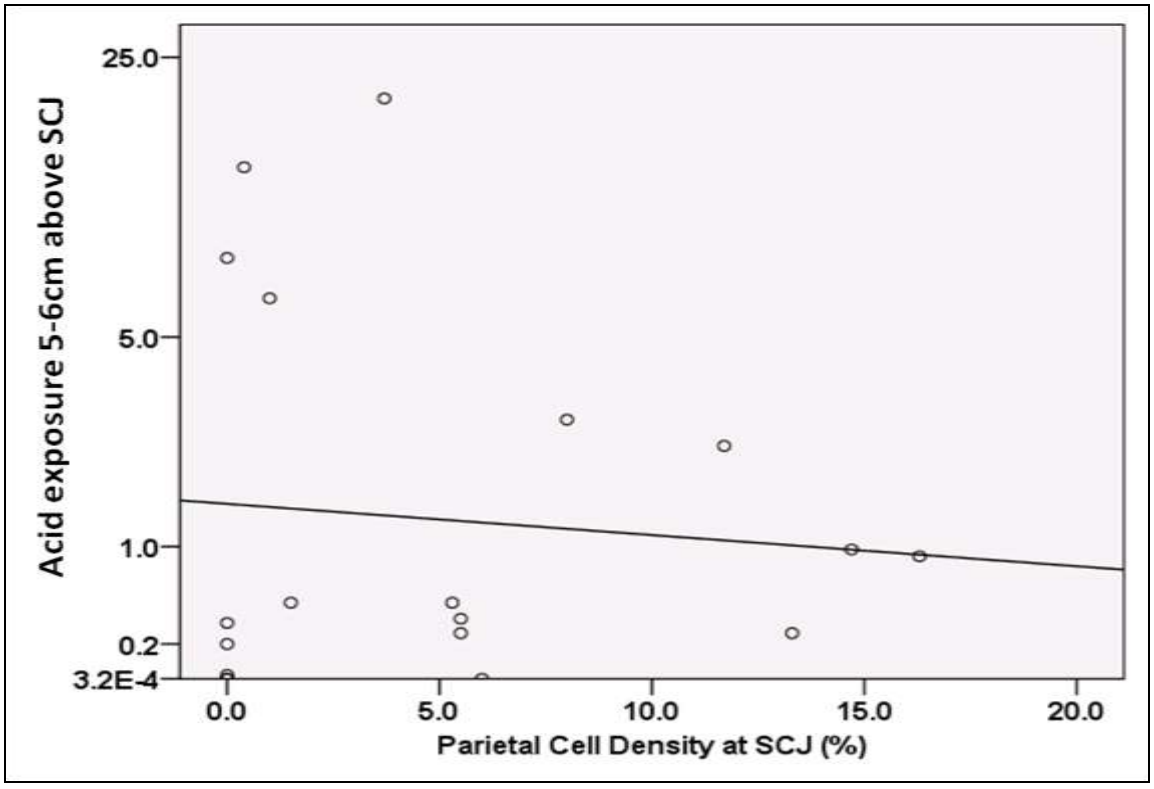

Figure 10.4. Fitted line plot of distal oesophageal acid exposure versus SCJ parietal cell density

Chapter 11

Discussion and Future Work

Chapter 11

Discussion and Future Work

7.1. Discussion

This thesis describes a number of studies which provide further insight into the possible aetiological mechanisms which underpin the development of acid-induced distal oesophageal squamous mucosal damage.

In examining the role of salivary nitrite in the pathogenesis of GORD using healthy volunteers (Chapters 6, 7 and 8), we have produced the most robust study in this area to date. We were able to effectively control for variability in dietary nitrate consumption, as demonstrated by the negligible basal salivary nitrite concentrations. Furthermore, we produced an excellent range of salivary nitrite concentrations throughout these studies, over and above the normal physiological range. The total nitrite load was equivalent to that used in the previous study by Manning et al in which an increase in TLOSRs and acid exposure was seen following the infusion of a NO-generating infusion. However, in contrast to the study by Manning et al, by infusing nitrite solutions intra-orally without the addition of supra-physiological amounts of NO-promoting ascorbic acid, our study is more physiological in design. Despite this, these studies show no effect of salivary nitrite on gastro-oesophageal function, GOJ morphology or gastro-oesophageal reflux – either conventional or short-segment. This suggests that salivary nitrite does not alter gastro-oesophageal function, the integrity of the gastro-oesophageal barrier or gastro-oesophageal reflux.

A criticism of these studies could be that only healthy volunteers were examined. This raises the possibility that an effect of salivary nitrite, and the luminal

chemicals it generates at the GOJ following ingestion, that is isolated to reflux patients may have been missed as this group has not been evaluated. Theoretically, the epithelial damage produced by erosive oesophagitis might facilitate NO reaching the intrinsic muscle layers of the oesophagus making reflux patients more sensitive to the presence of luminal NO than our healthy volunteers. However, given that in these studies the luminal nitrite concentrations following infusion of the highest concentration solution were 10-fold the upper limit of the normal range, it is unlikely an effect has been missed even in subjects with an intact epithelium.

The heterogeneous nature of reflux patients – from those with abnormal 24 hour acid exposure but non-erosive disease to patients with large hiatus hernia, long-segment Barrett's oesophagus and severe (LA grade D) oesophagitis – makes them a difficult population to study. Moreover, as the majority of patients with confirmed reflux disease are likely to be receiving acid suppressing medical treatment with either a proton-pump inhibitor (PPI) or histamine-$_2$ antagonist (H_2A), attempts to study this population may be complicated by the phenomenon of rebound acid hypersecretion. This occurs after discontinuation of anti-secretory therapy and previous work by Gillen et al (172) and Fossmark et al (173) have shown this effect to be prolonged, lasting at least 8 weeks in *Helicobacter Pylori* negative individuals following PPI withdrawal. In symptomatic patients it is impractical to request discontinuation of acid suppression for this duration. As a result, being able to study 'true' reflux patients without pharmacological influence to either reduce or, through rebound hypersecretion, increase acid secretion is increasingly difficult.

These studies are also the first studies examining the effect of salivary nitrite on GORD to utilise high-resolution pH and manometry techniques. While this provides high-frequency and detailed steady state analysis of gastro-oesophageal function, the volume of data produced and the complexity of data analysis is challenging and time-consuming. It has necessitated the development of our own in-house custom-made analysis programmes and the development of a specific thermal drift correction to ensure the most accurate data analysis (151). However, being able to confirm, with a high level of significance, previously published physiological meal-induced changes at the GOJ confirm our study protocols and methods are both sensitive and robust.

A further question is whether the effect of salivary nitrite on gastro-oesophageal function, morphology and reflux could be masked by the effect of the meal itself? It is well recognised that fatty meals, as result of increased CCK release secondary to duodenal fat, reduce LOS pressure, delay gastric emptying and may stimulate TLOSRs (22;54;55). Similarly, high volume meals may stimulate TLOSRs through gastric distension (23). We purposefully selected a low-fat meal to minimise the risk of this occurring. Importantly, the meal selected for these studies is likely to be both a lower volume and lower fat meal than the battered fish and chips meal used in the study by Manning et al (93) where an increase in TLOSRs and acid exposure is described following infusion of their NO-generating solution. Accordingly, it is unlikely that the meal used in our studies would mask any effect of salivary nitrite especially in view of the range of salivary nitrite concentrations produced.

The semi-recumbent position was used in all studies, however, this is not physiological following a meal as most patients are either mobile or sitting upright.

Therefore, the question remains whether these results are applicable to ambulant subjects or would be seen in subjects sitting upright following a meal.

The manometry studies in Chapters 6 and 8 also confirm, for the first time using high-resolution manometry, distal opening of the LOS after meals. This is a possible mechanism of distal oesophageal acidification independent of traditional 'trans-sphincteric' reflux. Although in our detailed studies of GOJ morphology (Chapter 8) we did not see significant proximal migration of the pH step-up point across the SCJ this may be the result of examining a non-obese population in a semi-recumbent position.

In our study examining the effect of age and obesity on SCJ position in healthy volunteers (Chapter 9) it could be argued that the results, showing a significant negative correlation between SCJ position relative to the proximal margin of the HPZ and age, BMI and waist circumference respectively, provide *in vivo* evidence to support the 'acquired cardia' hypothesis. Moreover, these results, which are more significant in the male sub-group, highlight a potential link to obesity and more specifically the visceral obesity which predominates in white males. This may be important in the pathogenesis of GOJ adenocarcinoma especially in the asymptomatic population. These results are underlined by the study in Chapter 10 where a localised decrease in parietal cell density is seen at the SCJ. This appears to be independent of traditional trans-sphincteric reflux measured as distal oesophageal acid exposure.

However, both these studies have obvious limitations with small number of subjects examined and limited age, BMI and waist circumference ranges. Importantly, both studies are cross-sectional and although they may highlight an

association they are unable to prove cause and effect. Despite this, the highly significant correlations deserve further investigation.

7.2. Future Work

Although our studies suggest that salivary nitrite does not promote gastro-oesophageal reflux this does not mean that the luminal chemistry produced by swallowed nitrite does not still have a role to play in carcinogenesis at the GOJ. The nitrosating species N_2O_3 and NOSCN are able to react with secondary amines and amides in gastric juice to form N-nitroso compounds which induce cancer in animal models while nitric oxide is itself known to be mutagenic at high doses. To investigate whether a causal relationship exists between salivary nitrite produced from dietary nitrate and neoplasia at the GOJ will require large scale epidemiological studies.

The GOJ and, in particular, the highly mobile SCJ remains a difficult area to study in great detail. In these studies, like many others, we used X-ray localisation with fluoroscopy to determine the location of the SCJ. While this can currently be considered the 'gold standard' its use in prolonged studies and appropriateness for detecting episodic changes in GOJ morphology (e.g. TLOSRs) is significantly limited by the radiation exposure involved. More work is needed to develop alternative techniques for investigating this dynamic and highly clinically important region.

Further work is also required to clarify the contribution of distal opening of the LOS to acidification, inflammation, metaplasia and neoplasia of the most distal oesophageal squamous mucosa. Future studies should be larger with greater range

in age, BMI and waist circumference. They should examine the effect of both obesity and posture. In particular, the supine posture which promotes cephalic displacement of the diaphragm and may precipitate distal opening. It would also be important to formally quantify visceral adiposity by utilising CT or MRI scanning and to investigate the effect of this visceral adiposity on the structures involved in maintaining the normal anti-reflux barrier such as the position of the crural diaphragm relative to the GOJ. Finally, further *in vivo* evidence is required to support the 'acquired cardia' hypothesis and subsequent studies may wish to examine the histology across the SCJ and gastric cardia in greater detail than simply quantifying parietal cell density.

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