OESOPHAGEAL MUCOSAL INTEGRITY IN NON-EROSIVE REFLUX DISEASE AND REFRACTORY GORD

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ABSTRACT

Background: 20 to 30% of patients with GORD respond inadequately to conventional therapy. Most of these patients belong to the non-erosive reflux disease group. Despite not having oesophagitis, in these patients oesophageal mucosal integrity appears to be impaired.

Aims: To study the dynamic *in vitro* and *in vivo* properties of oesophageal mucosal integrity in patients with non-erosive reflux disease, and to test the feasibility of a topical mucosal protectant therapy.

Methods: *In vitro* studies of mucosal integrity were done on human oesophageal biopsies using Ussing chambers. Change in transepithelial electrical resistance (TER) on exposure to acidic solutions was measured. Integrity was assessed *in vivo* by measuring impedance change and subsequent recovery after oesophageal acid perfusion in symptomatic patients. Proximal and distal oesophageal mucosal integrity was assessed *in vitro* and *in vivo*. The effect of *in vitro* topical application of an alginate-based solution on acid-induced changes in mucosal integrity was tested.

Results: *In vitro* exposure of biopsies to acidic and weakly acidic solutions caused a greater impairment of integrity in symptomatic patients than in controls. *In vivo* oesophageal acid perfusion causes a profound drop in distal oesophageal impedance that is slow to recover. Recovery is slower in patients with non-erosive reflux disease than in patients with functional heartburn, and a low baseline impedance is associated with painful perception of acid. Proximal oesophageal sensitivity appears unrelated to impaired mucosal integrity, but rather to a distinct sensory afferent nerve distribution. Topical pre-treatment with an alginate solution is able to prevent acid-induced changes in integrity *in vitro*.

Conclusion: Patients with non-erosive reflux disease have a distinct mucosal vulnerability to acidic and weakly acidic solutions that may underlie persistent symptoms. A topical therapeutic approach may be a feasible add-on strategy to treat GORD in the future.

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I dedicate this thesis to my mum and dad.

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PUBLICATIONS RELATING TO THIS THESIS

Original articles:

Woodland P, Al-Zinaty M, Yazaki E, Sifrim D. In vivo evaluation of acid-induced changes in oesophageal mucosa integrity and sensitivity in non-erosive reflux disease. *Gut*. Epub ahead of print 2012.

Woodland P, Lee C, Duraisamy Y, Farré R, Dettmar P, Sifrim D. Assessment and protection of esophageal mucosal integrity in patients with heartburn without esophagitis*. Am J Gastroenterol.* Epub ahead of print 2013.

Bravi I, Woodland P (joint 1st authors), Gill RS, Al-Zinaty M, Bredenoord AJ, Sifrim D. Increased prandial air swallowing and postprandial gas-liquid reflux among patients refractory to proton pump inhibitor therapy. *Clin Gastroenterol Hepatol*. Epub ahead of print 2013.

Woodland P, Aktar R, Mthunzi E, Lee C, Peiris M, Blackshaw LA, Sifrim D. Enhanced perception of proximal gastroesophageal reflux: Impaired mucosal integrity or distinct sensory innervation? Submitted Feb 2013.

Review articles:

Woodland P, Sifrim D. The refluxate: The impact of its magnitude, composition and distribution. *Best Pract Res Clin Gastroenterol* 2010, 24:861-71.

Roman S, Pandolfino JE, Woodland P, Sifrim D, Lenglinger J. Testing for gastroesophageal reflux in the 21st century. *Ann. N.Y. Acad. Sci*. 2011, 1232:358–364.

Triadafilopoulos G, Taddei A, Bechi P, Freschi G, Ringressi MN, Degli'Innocenti DR, Castiglione F, Masini E, Majewski M, Wallner G, Sarosiek J, Dillon JF, McCallum RC, Dvorak K, Goldman A, Woodland P, Sifrim D, Richter JE, Vieth M, Neumann H, Langner C, Ishimura N, Amano Y, Felix VN. Barrett's esophagus: proton pump inhibitors and chemoprevention I. *Ann. N.Y. Acad. Sci.* 2011, 1232:93–113.

Woodland P, Sifrim D. Management of gastroesophageal reflux disease symptoms that do not respond to proton pump inhibitors. *Curr Opin Gastroenterol.* In Press.

Abstracts:

Woodland P, Al-Zinaty M, Fikree A, Bravi I, Jafari J, Sifrim D. Clinical Evaluation of Esophageal Mucosal Integrity and Acid Sensitivity in Patients With NERD. A Study Using Baseline Impedance and Assessment of Mucosal Recovery After Acid Challenge. *Gastroenterology* 2012; 142, 5, (Suppl 1):S-203.

Woodland P, Lee C, Duraisamy Y, Dettmar PW, Sifrim S. Evaluation of Functional Esophageal Mucosal Integrity in Patients With NERD. Effect of Topical Mucosal Protection. *Gastroenterology* 2012; 142, 5(Suppl 1):S-753

Schaub N, Woodland P, Bravi I, Yazaki E, Kuo P, Jafari J, Sifrim D. Hiatal Hernia, Belching and Gas-Liquid Gastroesophageal Reflux. *Gastroenterology* 2012; 142, 5 (Suppl 1):S-285-S-286

Woodland P, Gill RS, Jafari J, Bravi I, Shimono R, Fikree A, Hayat J, Marunda T, Bredenoord AJ, Yazaki E, Sifrim D. Objective Assessment of Aerophagia During Meals in Normal Subjects and Patients With Post-Prandial Bloating and Belching. *Gastroenterology* 2011; 140, 5(Suppl 1):S-704

Woodland P, Bravi I, Gill RS, Bredenoord AJ, Jafari J, Sifrim D. Aerophagia during meals and postprandial gas-containing reflux in patients with GORD not responding to PPI. *Gut* 2012;61:Suppl 2 A40-A41.

Woodland P, Duraisamy Y, Gill RS, Farré R, Preston S, Sifrim D. Assessment of functional oesophageal mucosal integrity in biopsies of patients with refractory GORD. *Gut* 2011;60:Suppl 1 A178.

Shimono R, Yazaki E, Jafari J, Woodland P, Hayat J, Fikree A, Bravi I, Tsourali E, Sifrim D. Persistent dual high pressure zone, oesophageal body hypomotility and abnormal response to multiple rapid swallows are associated with reflux symptoms after fundoplication. *Gut* 2011;60:Suppl 1 A164.

PRESENTATIONS AT SCIENTIFIC MEETINGS

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Woodland P. Clinical evaluation of esophageal mucosal integrity and acid sensitivity in patients with NERD. *Oral presentation* at Digestive Diseases Week, Chicago, USA, May 2012.

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LIST OF ABBREVIATIONS

5-HT	5-Hydroxytryptophan
ASIC	Acid-sensing ion channel
ATP	Adenosine triphosphate
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CRH	Corticotropin-releasing hormone
DGOR	Duodeno-gastro-oesophageal reflux
DIS	Dilated intercellular spaces
FH	Functional heartburn
GOJ	Gastro-oesophageal junction
GORD	Gastro-oesophageal reflux disease
H_2RA	Histamine-2-receptor antagonist
IQR	Interquartile range
IR	Immunoreactive
LOS	Lower oesophageal sphincter
NERD	Non-erosive reflux disease
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
OR	Odds ratio
PAR	Proteinase activated receptor
PBS	Phosphate buffered saline
pK_a	Acid dissociation constant
PPI	Proton pump inhibitor
Ra	Apical membrane resistance
Rb	Basolateral membrane resistance
RS	Shunt/paracellular resistance
RT	Transepithelial resistance
SAP	Symptom associated probability
SEM	Standard error of the mean
SI	Symptom index
TER	Transepithelial electrical resistance
TLOSR	Transient lower oesophageal sphincter relaxation
TRPV	Transient receptor potential vanilloid

CHAPTER 1

Introduction

CHAPTER 1: INTRODUCTION

Gastro-oesophageal reflux disease (GORD) is a common and sometimes debilitating disease. Recent years have produced insights into the pathophysiology of the disease, and particularly the concept has developed that there may be subtle oesophageal mucosal injury in reflux disease without macroscopic erosions. This chapter outlines the current understanding of GORD epidemiology, pathogenesis and treatment. It describes the concept of oesophageal mucosal integrity, and its potential role in disease pathogenesis. Finally, it will outline the remaining questions, and specify the aims of the thesis.

1.1 Introduction to gastro-oesophageal reflux disease

Gastro-oesophageal reflux disease (GORD) has a global impact on health and quality of life, affecting much of the world's population[1]. Whilst over recent decades there have undoubtedly been important and successful advances in the treatment of GORD, the prevalence of the disease appears to be increasing, not only in the traditionally affected Western populations, but also in areas such as Asia. Advances in technologies to detect GORD have allowed a more detailed classification than was present perhaps even 20 to 30 years ago, when the words "hiatus hernia" and "oesophagitis" were often used as empirical and undoubtedly sometimes incorrect terms for reflux disease[2]. These advances have also made the problem of treatment-refractory GORD more apparent. This appears to be more common in certain subsets of GORD, including so-called non-erosive reflux disease (NERD)[3]. This increasingly encountered clinical problem, the association with

Barrett's oesophagus and oesophageal adenocarcinoma[4], and perhaps an increasing awareness of the adverse effects of proton pump inhibitor (PPI) therapy[5] drive a continued need to understand the pathophysiology of the disease.

1.1.1 Gastro-oesophageal reflux disease definition and classification

In past years the definition of GORD has become more concrete, encompassing the clinico-pathological consequences of material refluxing from the stomach into the oesophagus. A group of experts convened in Montreal to produce a consensus definition and classification of GORD that has become widely accepted. The definition has become "a condition which develops when the reflux of stomach contents causes troublesome symptoms and/or complications"[6]. Similarly, a consensus report from a workshop in Genval concluded "The term gastro-oesophageal reflux disease (GORD, reflux disease) should be used to include all individuals who are exposed to the risk of physical complications from gastro-oesophageal reflux, or who experience clinically significant impairment of health related well being (quality of life) due to reflux related symptoms, after adequate reassurance of the benign nature of their symptoms"[7]. Finally, another consensus meeting in Marrakech defined GORD by "the presence of reflux oesophagitis (Los Angeles grades A–D) and/or when it causes reflux symptoms that are sufficient to impair quality of life and/or when it is associated with a risk of long term complications"[8]. An important component to each of these definitions definitions is that reflux must cause symptoms and/or complications. This is because gastro-oesophageal reflux is a daily occurrence in healthy individuals. Such physiological reflux can occur up to 70 times per day[9]. It also recognises that some patients may be completely asymptomatic from gastro-oesophageal reflux, yet can develop complications such as silent oesophagitis and Barrett's oesophagus. The clinical

manifestations (whether symptoms or complications) can be divided into oesophageal syndromes, and extra-oesophageal syndromes (figure 1).

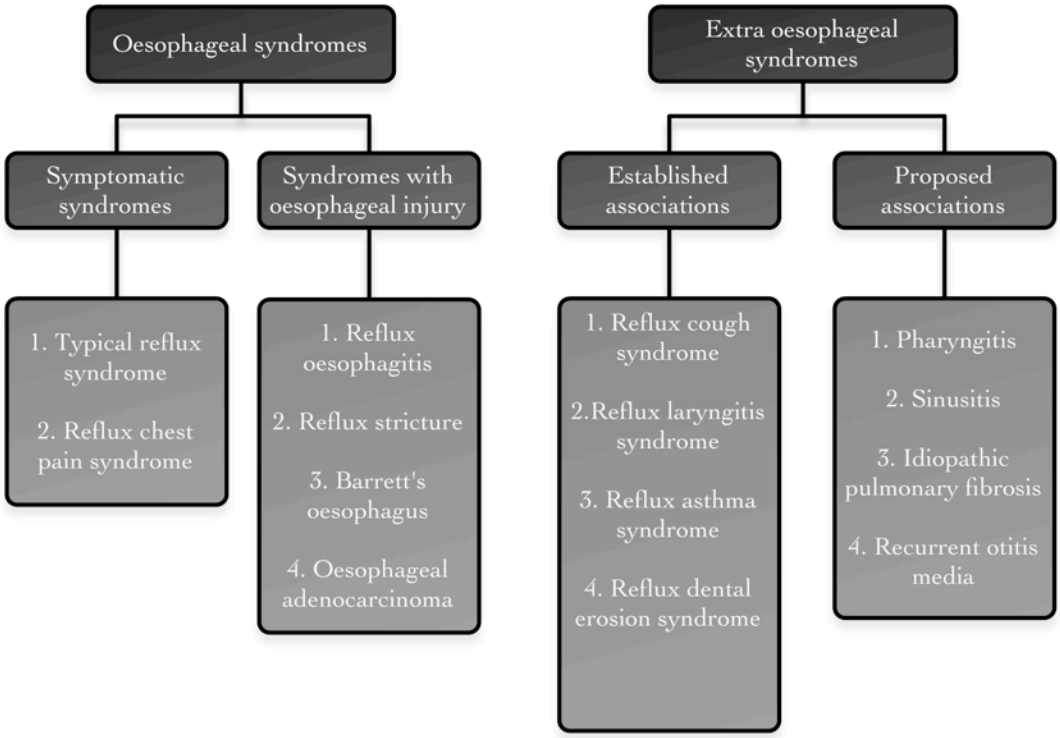


Figure 1: The Montreal Classification of GORD (from Vakil *et al*. 2006)

1.1.2 Symptomatic oesophageal syndromes

It can be seen that there are two symptomatic reflux oesophageal syndromes: typical reflux syndrome and reflux chest pain syndrome. The most established symptom associations with GORD are the so-called "typical symptoms": heartburn and regurgitation. Heartburn is a retrosternal burning sensation, and regurgitation is the perception of refluxed gastric content into the mouth or hypopharynx. Although heartburn is not specific for GORD, results of studies using acid suppression therapies for treatment of heartburn provide strong indirect evidence that GORD is the most common cause of heartburn[9-11]. When heartburn and

regurgitation are present as the only symptoms, they are specific but not sensitive in the diagnosis of GORD[12]. However the Diamond study, published in 2010, demonstrated that heartburn or regurgitation were the most troublesome symptoms in only 49% of patients with GORD (with dyspepsia being the next most frequent primary symptom)[13]. Typical reflux symptoms are characteristically worsened after eating, on bending, and on lying down (especially on the right side).

In chest pain reflux syndrome there are episodes of non-heartburn chest pain caused by gastro-oesophageal reflux. The pain can sometimes be indistinguishable from cardiac chest pain. Ambulatory oesophageal pH recordings have been used to document the direct association between reflux episodes and chest pain.

1.1.3 Syndromes with oesophageal injury

Reflux oesophagitis is defined endoscopically by visible breaks in the distal oesophageal mucosa. Such oesophagitis is seen in less than 50% of patients with GORD, and neither symptom pattern nor severity can predict its presence[2, 14, 15]. Oesophagitis is the most common macroscopic injury caused by GORD. In severe occasions it may result in an oesophageal stricture and thus cause dysphagia. Dysphagia can also occur in GORD in the absence of stricture[16]. This may be due to inflammatory damage to efferent (or perhaps afferent) neurones involved in the peristaltic process, leading to failure of bolus transit[17]. Chronic reflux may also cause metaplastic change of the squamous epithelium of the distal oesophagus to columnar epithelium: Barrett's oesophagus. It is included in the oesophageal injury category since it is associated with a risk of developing oesophageal adenocarcinoma.

1.1.4 Extra-oesophageal manifestations of GORD

GORD has been implicated in extra-oesophageal syndromes, notably chronic cough[18], laryngitis[19] and asthma[20]. Whilst these syndromes are widely accepted to be influenced by GORD, accurately identifying these patients and understanding the underlying mechanisms has proved difficult.

1.1.5 Erosive oesophagitis, non-erosive reflux disease and functional heartburn

It is important to realise that typical heartburn symptoms in GORD may occur in the presence or absence of oesophageal erosions. Indeed, 50-70% of patients with GORD have a normal endoscopic appearance of the oesophageal mucosa[21, 22]. Furthermore, using ambulatory oesophageal pH-monitoring, researchers have been able to establish normal values of oesophageal acid exposure, and determine whether a symptomatic patient has pathological oesophageal acid exposure. This, along with clinical response to PPI treatment, allows further definition of the GORD phenotypes. The Rome III consensus criteria[23] thus subdivides patients into: 1) erosive oesophagitis; 2) non-erosive reflux disease (NERD - symptoms with normal endoscopic appearance and pathological oesophageal acid exposure +/- positive symptom-reflux association); 3) hypersensitive oesophagus (symptoms with normal endoscopic appearance, physiological oesophageal acid exposure, but positive symptom-reflux association); or 4) functional heartburn (symptoms with normal endoscopic appearance, physiological oesophageal acid exposure, and no symptom-reflux association). All but functional heartburn are deemed to fit within the GORD umbrella: i.e. the symptoms are caused by reflux of gastric contents into the oesophagus. A diagnostic algorithm to explain this is demonstrated in figure 2.

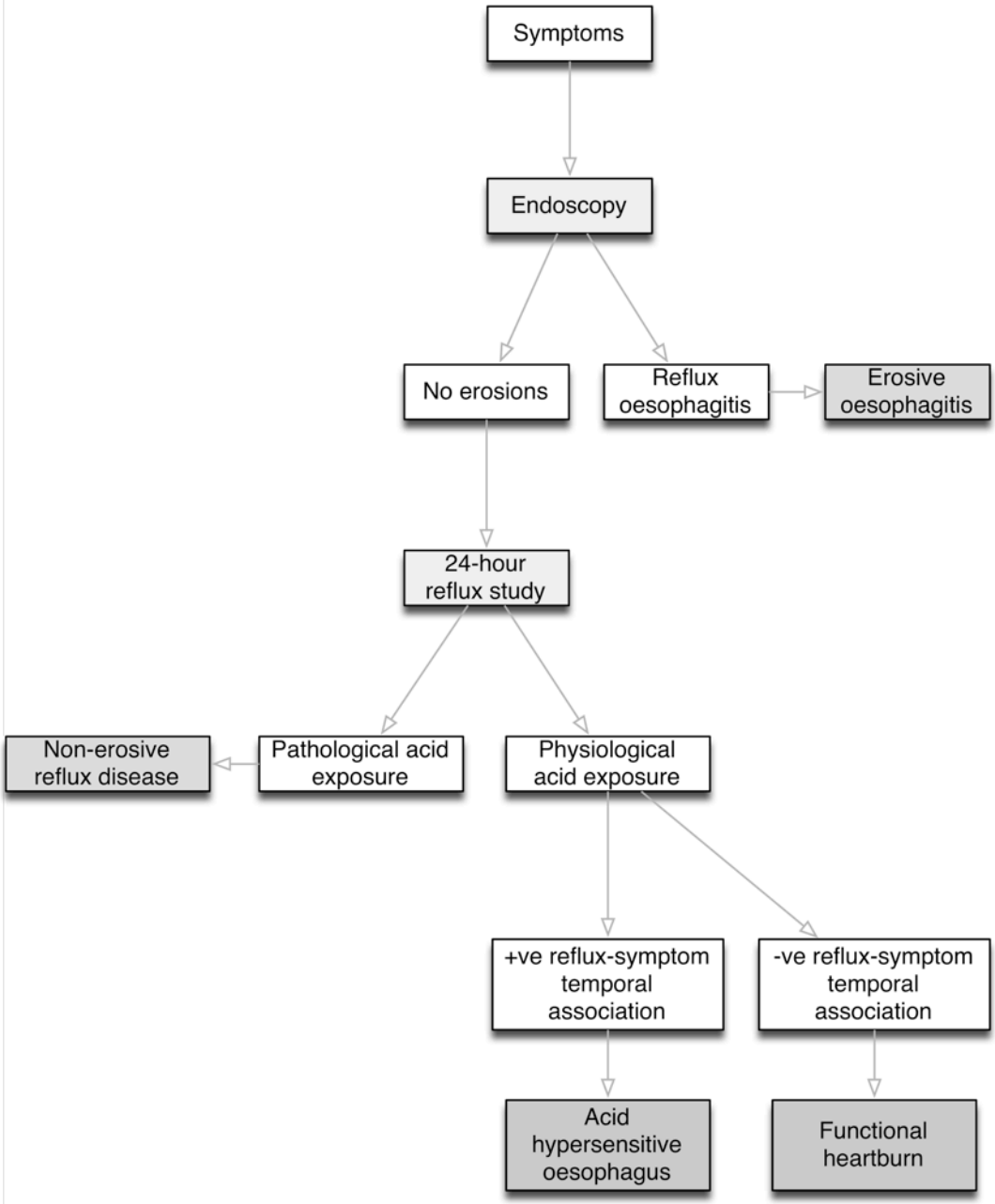


Figure 2: A diagnostic algorithm for patients with reflux symptoms

1.2 Epidemiology and risk factors for gastro-oesophageal reflux disease

We understand that GORD is a very common condition, but truly accurate quantification of its prevalence may be difficult since there is a relative scarcity of epidemiological data, and many people do not seek medical attention for their symptoms. When defined as the presence of at least weekly heartburn or regurgitation, there is an estimated prevalence of 10-20% in Western Europe and North America[24]. From a primary care database, the incidence of GORD in the UK has been estimated at 4.5-5.4 per 1000 person-years[25].

Differences may exist between ethnic groups. In one study in the US, a higher prevalence of reflux symptoms was found in Hispanic subjects when compared to Caucasian subjects[26]. A systematic review of the prevalence of GORD in Asia found a range of 2.5-6.7%, but high quality data is limited[27].

1.2.1 Risk Factors for GORD

There is some evidence to support genetic, demographic and behavioural associations with the development of GORD. Patients with GORD more frequently have relatives with frequent GORD symptoms[28]. The strongest evidence for a genetic link comes from a twins study in the UK where a significantly higher concordance of GORD prevalence was seen in monozygotic versus dizygotic twins[29]. A second twin study found that there was a stronger within-pair association of GORD (and IBS) within monozygotic compared to dizygotic twins. However, when these differences were controlled for the presence of depression and anxiety they lost significance[30]. A further study found a significant association of GORD symptoms between immediate relatives that was not seen in spouses, again suggesting a genetic influence that was separate from environmental

influences[31]. Clustering of hiatus hernia (a risk factor for GORD) has been described in 5 generations of a family[32]. The collagen type 3 alpha 1 (COL3A1) gene has been found to be a disease-associated gene in paediatric and adult GORD, and with the presence of hiatus hernia in males[33]. This gene encodes type 3 collagen, which has an important role in tissue strength and flexibility, and in the early phases of wound healing. More recently the 4-Amino-butyrate Aminotransferase (ABAT) gene has been found to be associated with the presence of GORD in children, and inhibition of ABAT in dogs causes inhibition of transient lower oesophageal sphincter relaxations and a reduction in the number of reflux episodes[34].

There has been much investigation into genetic influences on the development of Barrett's oesophagus and oesophageal adenocarcinoma. The relative risks of Barrett's oesophagus and oesophageal adenocarcinoma are increased by approximately 2- to 4-fold when one first degree relative is affected[35]. A complex segregation analysis of patients with Barrett's oesophagus and oesophageal adenocarcinoma has suggested an incomplete dominant inheritance with a polygenic component[36]. Finally, a recent genome-wide association study of 1852 patients with Barrett's oesophagus identified two genetic variants associated with increased risk[37].

Studies have repeatedly shown that there is no sex preponderance in GORD (excluding during pregnancy in females)[29, 31, 38-40].

There appears to be a very small increase in GORD symptom prevalence seen with increasing age[29, 39]. The UK GP database study found that prevalence increased until the age of 69, then decreased thereafter[25]. In a US study, this trend was also seen, but the reversal in prevalence occurred earlier, at 55[40]. It is important to note that these studies are based on symptoms alone, and so cannot take into account

the prevalence of complications such as oesophagitis and Barrett's oesophagus, nor do they consider extra-oesophageal manifestations.

Several studies have demonstrated an increasing prevalence of GORD with an excess body mass/higher body mass index (BMI). In the Olmsted County studies a high BMI was associated with an OR of 2.8 (95% CI 1.7-4.5) for the presence of GORD[31, 39, 41]. In the UK GP database study a BMI >25 was significantly associated with GORD (OR 1.3, 95%CI 1.2-1.5)[25], and a Georgia Medicaid study reported a positive relationship between obesity and GORD (OR 2.8, 95% CI 2.1-3.6)[40]. It has even been shown that modest weight gain, even in subjects with normal BMI, may cause or exacerbate GORD symptoms[42].

Some behaviours are frequently considered to be related to GORD, the most commonly discussed being cigarette smoking, alcohol consumption and caffeine consumption. Indeed, several cross-sectional studies have reported a significant positive association between GORD symptoms and smoking, with odds ratios of between 1.1 and 2.6[29, 31, 39]. The evidence for alcohol and caffeine consumption is less clear. A US study has reported a positive association between GORD diagnosis and alcohol consumption[40]. In contrast, two UK studies were able to find no such association[25, 29]. Although often anecdotally reported as a precipitant factor for reflux symptoms, three cross-sectional studies were unable to find a positive association between caffeine consumption and GORD[31, 43, 44]. The UK GP database found significant associations with a number of drug treatments (including nitrates, oral steroids and NSAIDs, but not aspirin)[25]. The Olmsted County survey found no such association with NSAIDs[40].

1.2.2 Comorbid factors in GORD

Further information from the UK GP database found that a diagnosis of irritable bowel syndrome or peptic ulcer disease is associated with an increased likelihood of GORD diagnosis[25]. There is also a significant overlap between GORD (both erosive and non-erosive) and dyspeptic symptoms, higher than would be expected due to chance alone[38]. This overlap may be driven particularly by patients with functional heartburn who have not been distinguished from non-erosive reflux disease in symptom-based studies[45]. Obstructive respiratory diseases may alter abdomino-thoracic pressure gradients, and indeed cough, COPD and asthma have been found to have a positive association with the presence of GORD symptoms[25, 40]. Furthermore, as mentioned above, there is likely to be a proportion of these respiratory diseases that are in fact caused *by* GORD.

Finally, there is an association of GORD with psychiatric disease. In China, anxiety and depression were both found to be more common in patients with GORD[46]. Two Western studies also reported a significant association of GORD with a psychosomatic checklist score[31, 44]. Within the spectrum of GORD, patients with NERD and functional heartburn are more likely to be affected by stress. Furthermore, patients with co-morbid psychological distress have an increased symptom burden, and poorer response to PPI[47].

1.3 The balance of aggressive and defensive factors in GORD

The human upper gastrointestinal tract manifests several properties that defend against the occurrence of gastro-oesophageal reflux. Since gastro-oesophageal reflux is a physiological phenomenon (indeed, in healthy individuals the oesophageal mucosa can be acidified for up to 4% of the day[48]) it follows that these properties do not completely inhibit reflux occurring. It also follows that the oesophagus itself must have properties that prevent damage occurring when reflux occurs, as in the vast majority this physiological reflux is asymptomatic. Thus, with gastro-oesophageal reflux there is a balance between the rigorousness of the defensive properties, and the aggressiveness of the reflux (in terms of amount and composition) that determines whether gastro-oesophageal reflux becomes pathological. Some understanding of these defensive and aggressive factors in GORD has led to the development of many therapies (both pharmacological and surgical). Greater understanding of the details of this relationship may allow future therapies to be developed.

1.3.1 Aggressive factors in GORD

The central aggressor in gastroesophageal reflux disease is the refluxate: the material that moves in a retrograde fashion from the stomach to the oesophagus during reflux episodes**.** The refluxate may contain varying concentrations of acid, pepsin, gas, or contents of duodenal reflux (such as bile acid and pancreatic enzymes). On one hand, the refluxate is defined and characterised by a multitude of pathophysiological variables. On the other hand, the contents and characteristics of the refluxate are essential for the pathological consequences of

GORD. Gastroesophageal reflux disease may manifest in different ways; in some the reflux episodes are only minimally (if at all) symptomatic and in others the symptoms are debilitating. Likewise, in many there is no macroscopic mucosal injury (NERD), and in others there is severe oesophagitis or even metaplasia and neoplasia. Amongst GORD sufferers there will be people who are mostly sensitive to acidic reflux and can be effectively treated with proton pump inhibitor (PPI) therapy. In others there is an apparent hypersensitivity of the oesophagus, and symptoms are perceived in response to weakly acidic or non-acid reflux. These observations serve to illustrate the heterogeneity of GORD, and as such there must be variables at play that determine whether the line between physiological and pathological reflux is crossed, whether by mucosal damage, by symptoms, or by both. The refluxate is one of these variables, and a vitally important one. This section will first discuss the aggressive components of the refluxate, and then outline the defensive factors that protect the healthy individual from symptomatic perception of reflux.

1.3.1.1 Acid reflux

The usual pH of the stomach is highly acidic at approximately 1.5–3.5. When this gastric juice refluxes into the oesophagus, the oesophageal mucosa is exposed to acid. In some situations the refluxate will be less acidic due to a higher gastric pH: most commonly after a meal, and in patients treated with proton pump inhibitors (PPIs). These powerfully block gastric acid secretion, and by doing so usually raise the gastric pH to above 4[49]. In the post-prandial state ingested food acts as a buffer to the gastric acid, and as such the majority of stomach contents during this period has a pH a little greater than 4. It has been noted that gastric fullness encourages post-prandial reflux, and one would therefore expect this reflux to be of a higher

pH than in the fasting state. However, during the post-prandial state conventional intragastric pH monitoring 5 cm below the lower oesophageal sphincter (LOS) often reveals discrepancies between gastric pH and that of the refluxate in the oesophagus, with a lower pH in the oesophagus than in the stomach during reflux episodes. These observations can be explained by the presence of an "acid pocket" in the proximal stomach in the 90 minutes post-prandially[50]. This area, that develops on top of ingested food after a meal, is a rich source of acid for reflux into the distal oesophagus during this period. The acid pocket is not something that is present during the fasting state, but can be detected in the proximal stomach (by pH pull-through techniques) for up to 90 minutes after a meal[51]. Although the acid pocket can be found in healthy volunteers, by comparison the acid pocket in patients with GORD has a greater distal extent, and extends proximally closer to the lower oesophageal sphincter. The size of the pocket is increased by the presence of a hiatus hernia[50]. Scintigraphy studies have indicated that acid reflux is more likely to occur when the acid pocket is located at or above the level of the diaphragm[52].

Acid reflux: role in symptom perception

Despite the increasing complexities that have been discovered, it holds true that acid exposure in the oesophagus is very important in symptoms genesis in GORD. In experimental conditions the infusion of hydrochloric acid solution into the mid-oesophagus is able to reproduce heartburn symptoms, and with increasing acid concentrations the duration of exposure required to cause symptoms decreases[53]. The generation of symptoms in these experiments is most consistent at pH 1–2, i.e. the pH of normal gastric juices.

Using oesophageal 24-hour pH recordings in GORD patients with erosive

oesophagitis a temporal relationship between pH falls and symptoms has been demonstrated[54], and using pH-MII monitoring symptomatic GORD patients have been seen to have more acid reflux events than normal subjects[55].

Weakly acid reflux: role in symptom perception

The studies outlined above have illustrated the ability of acid to cause symptoms in GORD. However, *in vivo*, heartburn is not specific to strong acid stimulus. During reflux in the "on" PPI condition, the oesophageal mucosa is exposed to gastric contents in the range pH 4-6.5[56]. The phrase "weakly acidic reflux" has been coined to describe acid reflux of pH >4.

Prolonged pH monitoring studies reveal a poor correlation between acid reflux events and heartburn sensation, and so it is possible that weakly acidic reflux events also have a role in symptoms perception. Even though the early acid perfusion experiments by Smith *et al*. led to a belief that heartburn was as a result of strongly acidic reflux, on closer examination they also demonstrate that higher pH solutions can cause heartburn[53]. Although symptoms took longer to develop, even with pH 6–7 perfusions symptoms occurred in 50% of subjects. Combined oesophageal pH and impedance measurement techniques have enabled further investigation in more physiological settings. This is because impedance techniques allow the detection of reflux events irrespective of the pH. By combining pH and impedance data from the same catheter one can ascertain the pH of reflux even when not acidic. Data from GORD patients has shown that up to 30% of symptoms may be associated with reflux episodes with a pH of 4–7[55, 56]. Emerenziani *et al*. showed that although most symptoms were related to acid, NERD patients in particular were sensitive to weakly acidic reflux events (accounting for 24% of their symptoms)[57]. Such observations may be important in explaining persistent

symptoms "on PPI", and the mechanisms of mucosal sensitivity to refluxates of weakly acidic pH should be explored in order to develop more effective therapies for PPI-refractory patients.

Acid reflux: role in mucosal injury

If acid and weak acid can be a cause of symptoms in GORD, what of their role in mucosal damage? For strongly acidic refluxate, the evidence is compelling. In animal studies, exposure to the oesophageal mucosa with acid alone (or in combination with pepsin) can induce oesophagitis[58]. Ambulatory oesophageal pH studies show that increasing levels of oesophageal acid exposure are associated with increasing severity of oesophageal lesions in human patients[59, 60]. Using 24-hour pH-impedance monitoring Savarino *et al*. demonstrated a higher acid (pH <4) exposure time, a higher total number of acid reflux events, and a higher mean acid clearance time for patients with erosive oesophagitis when compared to patients with NERD[61]. Taken together these studies would suggest that an increase in acid (pH <4) exposure is important for the development of mucosal damage in reflux disease. Perhaps most persuasive argument is the dramatic (>70%) rate of endoscopic healing when patients are treated for 8 weeks with PPIs[9].

Although weak acid is important for the generation of symptoms in GORD, it appears less able to cause macroscopic mucosal damage since there are no studies reporting an association between the amount of weakly acid reflux and oesophageal erosion formation.

1.3.1.2 Duodeno-gastro-oesophageal reflux

The aforementioned pH-impedance studies of reflux in GORD have illustrated that acid is undoubtedly important for symptoms and mucosal injury. However, not all

patients with acid reflux get symptoms or oesophagitis: do other properties of the refluxate also play a role? The refluxate consists of not just acid, but other components of gastric juice including pepsin, and elements of duodeno-gastro-oesophageal reflux (DGOR) such as bile acids.

It has long been known that DGOR occurs, but its accurate quantification has proven difficult, largely for technical reasons. Early studies of DGOR used pH monitoring for its detection, working on the assumption that reflux containing duodenal juice will have an alkaline pH. More recently an ambulatory bilirubin monitoring system has been used, and has offered clarification of this assumption. Ambulatory bilirubin monitoring enables spectrophotometric measurement of oesophageal luminal bilirubin concentrations, which closely correlates with DGOR[62]. Whereas previously the presence of alkaline or non-acid oesophageal reflux was considered to be a marker of DGOR, this is not the case: simultaneous measurement of oesophageal bilirubin spectrophotometry and pH-impedance have shown that biliary reflux and non-acid (pH > 4) reflux are not equivalent. Furthermore, most DGOR occurs in an acidic environment[63].

Reflux of duodenal contents into the oesophagus has been hypothesised to cause damage due to the toxic effects of components such as bile acids and pancreatic enzymes. Gastric bile acid concentrations may be between 0.3 mmol/l and 2 mmol/l[64-66]. Whereas conjugated bile acids are most commonly found in DGOR, in the "on" PPI condition there may also be significant presence of unconjugated bile acids due to gastric bacterial overgrowth and subsequent bacterial deconjugation in the stomach[67].

DGOR: role in symptom perception

A few studies have looked at the relevance of DGOR in symptoms perception in

GORD patients. Marshall *et al*. studied 59 patients with typical reflux symptoms and found that only 6% of symptomatic events were related to DGOR[68]. Similar ambulatory monitoring studies by Koek *et al*. in patients with GORD "off" acid-suppressive therapy again demonstrated that reflux symptoms are mainly related to acid reflux, or to mixed acid/bile–acid reflux events; fewer than 10% of reflux episodes are related to bile reflux alone[69]. Perhaps a more interesting group to consider is those patients with persistent symptoms "on" PPI, for in this group acidic reflux is unlikely to play such a prominent role. The role of DGOR in the generation of persistent symptoms in this group remains uncertain. Initial studies by a group in Leuven suggested a significant role for DGOR in PPI-refractory GORD. Tack *et al.* found DGOR alone to be rather important, being related to 18% of symptomatic episodes vs. 7% for acid and 10% for mixed reflux[70]. Conversely, other studies have suggested a less important role for DGOR in PPI-refractory GORD. Karamanolis *et al.* found DGOR alone to be related to 4% of symptomatic episodes vs. 10% for acid reflux and 17% for mixed reflux[71]. More recently, Gasiorowska *et al.* studied a similar group of patients, and again found DGOR alone to be of a relatively lesser relevance, being related to 9% of symptom events vs. 32% for acid, and 32% for mixed reflux[72]. In this study, treatment failure after PPI was found to be more associated with persistent acid reflux than with DGOR.

It can be concluded that DGOR alone may be responsible for a significant minority of symptoms in GORD patients "on" or "off" PPI treatment, although its importance is less than that of acid.

DGOR: role in oesophagitis

As with acid, it is of clinical importance not only to consider the role of DGOR in symptom perception in GORD, but also its role in producing mucosal injury. Animal

experiments have suggested a likely role. For example, canine bile is capable of producing oesophagitis in a dog model with biliary diversion and a jejunal conduit anastomosing directly to the oesophagus[73]. Harmon *et al.* examined the effect of pH on oesophageal injury in rabbit mucosa[74]. Using hydrogen ion permeability as a marker of mucosal injury, they found that the addition of bile acids to acidic and weakly acidic solutions greatly increased mucosal injury proportional to their concentration (0 to 5 mM). Furthermore, they demonstrated that taurine conjugated bile acids (taurocholic acid and taurodeoxycholic acid) significantly increased injury at pH 2, but the unconjugated bile acids increased injury at pH 7 (owing to the different pK_a of conjugated and unconjugated bile acids).

Clinical studies of the importance of bile acids in human oesophageal injury have been contradictory. Two different intra-oesophageal aspiration studies have demonstrated conflicting results regarding the association between the degree of oesophageal injury and bile acid concentrations in GORD patients[75, 76]. In summary, it seems likely that DGOR plays a role in the development of mucosal erosion in GORD, but as with symptoms its influence appears less than that of acidity.

Role of bile acids in Barrett's oesophagus and oesophageal adenocarcinoma

The increasing prevalence of oesophageal adenocarcinoma despite widespread use of proton pump inhibitors has led to speculation that other reflux factors such as bile acids may have an important role in Barrett's oesophagus and associated tumours. Barrett's-like intestinal metaplasia of the oesophagus and oesophageal adenocarcinoma occur in rat models of bile reflux (via surgical oesophagojejunostomy). This occurs with or without total gastrectomy suggesting it is the bile reflux causing the effect[77-79]. In addition, patients with Barrett's oesophagus appear to have greater oesophageal concentration of bile acids than

patients with GORD but without Barrett's[80]. Mechanistic support is added by the observations that bile acids are able to cause *in vitro* and *in vivo* damage to epithelial DNA in Barrett's cell lines and in Barrett's biopsies[81].

1.3.1.3 Pepsin

Pepsin is an enzyme whose precursor, pepsinogen, is released by chief cells in the stomach. Its proteolytic activity has long established it as a possible candidate in mucosal injury in GORD, particularly in association with acid reflux (pepsin causes the most damage at its optimal pH activity range of pH 2–3). Some evidence exists for its role. *Ex vivo* studies in rabbits showed that acid infusion only caused oesophageal mucosal injury when combined with pepsin[82], and feline studies showed that mucosal damage could occur at higher pH if pepsin was present[58]. Finally, Nagahama *et al*. found that experimentally-induced oesophagitis (caused by pyloric ligation) could be prevented by the intra-gastric administration of pepstatin, a pepsin inhibitor[83].

It is likely that pepsin plays a synergistic role with acid in the development of mucosal injury in GORD.

1.3.2 Defensive factors in GORD

The human body has a variety of defence mechanisms against the noxious aggressors of gastro-oesophageal refluxate. These range from gross anatomical features to microscopic and molecular physiological properties that guard against injury from the noxious aggressors of the refluxate. The following summary discusses these anatomical and macro-physiological features (figure 3) at the stomach, gastro-oesophageal junction, and oesophageal levels.

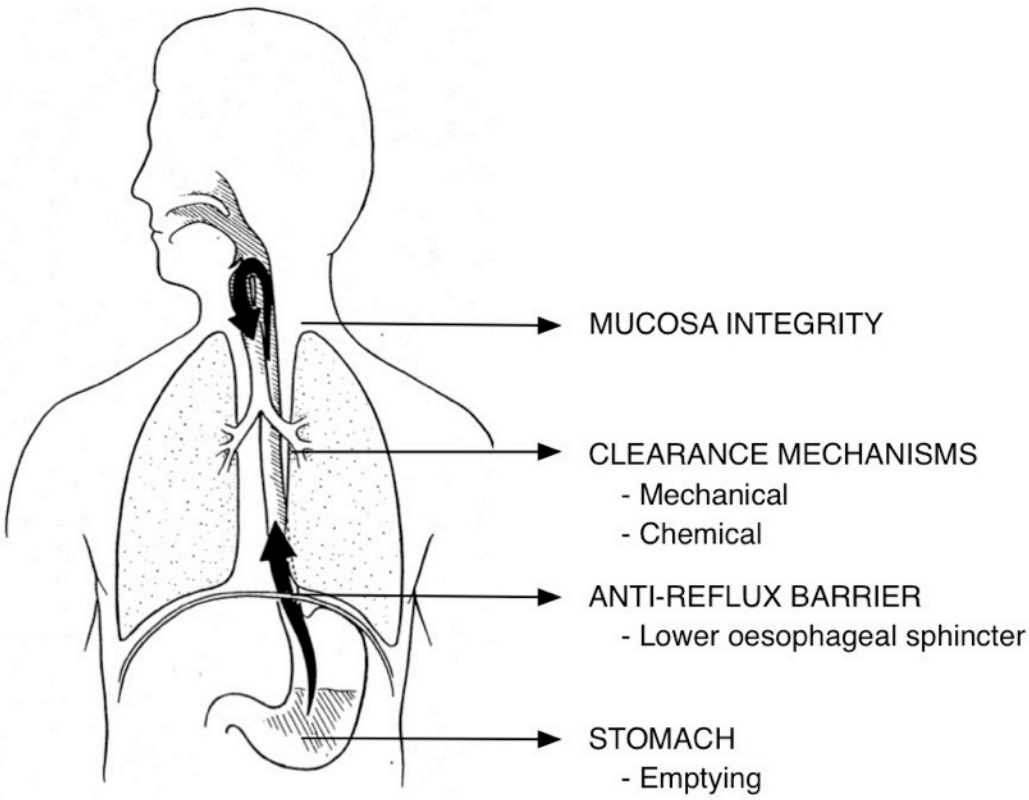


Figure 3: Defence mechanisms against GORD

1.3.2.1 Stomach

It is believed that effective gastric emptying is important in reducing gastro-oesophageal reflux, since most reflux occurs in the post-prandial situation when the stomach is full of material and gastro-oesophageal pressure gradients are greatest. Indeed, several studies (although not all) have shown that gastric emptying is delayed in a variable proportion of patients with GORD[84-87]. However, a problem with these studies may be the prevalence of "abnormal" gastric emptying studies in healthy volunteers. For example, one study found delayed gastric emptying (by scintigraphy) in 37% of GORD patients and in 44% of controls[87].

More recent studies have focused on the proximal stomach particularly, since this distinct area of the stomach may be more relevant to reflux events. Two studies

have indeed shown that delayed recovery of proximal gastric tone after a meal is delayed in GORD patients compared to controls[88, 89]. In agreement with this, Stacher *et al.* found a correlation between slow proximal, but not slow distal or total gastric emptying, with 24-hour oesophageal acid exposure in patients with symptoms of GORD and delayed gastric emptying[86]. The proximal stomach motility also appears to be vitally important in modulating formation of the proximal stomach acid pocket after a meal. Treatment with the prokinetic azithromycin reduces number of acid reflux events, seemingly by causing the acid pocket to form in a more distal position[52].

From the above observations, it appears likely that normal gastric motility and emptying can play a role in guarding against pathological gastroesophageal reflux.

1.3.2.2 The antireflux barrier

The gastro-oesophageal junction (GOJ) is a specialised anatomical complex that is designed to allow passage of swallowed boluses from the oesophagus into the stomach, while at the same time control reflux of gastric contents back into the oesophagus. It consists of two structures: the lower oesophageal sphincter (LOS) and the crural diaphragm.

The GOJ is situated at the interface between the thoracic and abdominal cavities, across which there is a pressure gradient that varies throughout respiration. During inspiration there is a decrease in intra-thoracic pressure and increase in intra-abdominal pressure, a situation that favours the occurrence of gastro-oesophageal reflux. The GOJ forms a high-pressure zone between these cavities that, under normal conditions, prevents reflux of contents.

The LOS is a specialised part of the oesophageal smooth muscle and is approximately 4 cm long. In healthy individuals it exerts a tonic (but variable)

pressure of 15-30 mmHg above the intragastric pressure[90, 91]. This accounts for approximately 90% of the basal pressure of the GOJ. The remaining 10% is provided by the crural diaphragm, which overlaps the LOS for approximately 2 cm (unless there is a hiatal hernia, where there are two distinct pressure zones, with the LOS found in the thorax above the level of the diaphragm). This provides an essential compensatory mechanism that maintains pressure, particularly during inspiration and straining (when it contracts). This compensation can prevent reflux even in times of absent LOS pressure[92, 93]. Thus the LOS and crural diaphragm act in a coordinated, supplementary fashion to prevent gastro-oesophageal reflux occurring. The presence of a hiatus hernia means that the two mechanisms are not working effectively in tandem, and the basal gastro-oesophageal junction pressure is lower, leading to increased risk of reflux[94]. The hernia sac can also serve as a reservoir of acid contents that are readily available for reflux during LOS relaxations. This mechanism may prolong oesophageal acid exposure, and indeed most patients with severe oesophagitis have a hiatus hernia[95].

Transient lower oesophageal sphincter relaxations (TLOSRs) are a physiological mechanism whereby the LOS and crural (but not costal) diaphragm involuntarily relax to vent excess gastric gas (belching). They can be defined as an abrupt decrease in LOS and crural pressure to the level of intragastric pressure that are not triggered by a swallow[96]. Most reflux events, whether in healthy controls or in patients, occur during a TLOSR[97, 98]. In healthy volunteers, 70-100% of reflux episodes occur during TLOSRs[98, 99]. In patients, 63-74% of reflux events are due to TLOSRs[99, 100]. The lower percentage in patients is because swallow-induced reflux, extremely low basal LOS tone and straining are likely to play a slightly stronger role. TLOSRs are triggered by distension of the proximal stomach, and serve as a

prolonged (typically 10-45 seconds) weakness in the anti-reflux capabilities of the GOJ. Meals are associated with an increase in post-prandial TLOSR frequency[101], and an increase in frequency of TLOSRs is associated with reflux events in patients[91, 101, 102]. In humans, TLOSRs only occur during the awake state. The current belief on the neural activation of TLOSRs is that they are a vagally-mediated event predominantly stimulated by activation of proximal gastric stretch receptors. Obliteration of the vagus in dogs ceases TLOSRs[103], and absence of TLOSRs in achalasia suggests a similar neural mechanism of TLOSRs to that of swallow-induced relaxations (which are vagally-mediated)[104].

1.3.2.3 Oesophageal clearance mechanisms

Oesophageal body motility is a key factor in maintaining defence against gastro-oesophageal reflux. Peristalsis is important both for clearance of the refluxed material back into the stomach, and for the delivery of buffering bicarbonate in saliva to the distal oesophagus.

Peristalsis in response to a reflux event can either be swallow-induced (primary peristalsis) or due to a distension-induced reflex not related to a swallow (secondary peristalsis). Primary peristalsis is the most frequent response to a gastro-oesophageal reflux event in both healthy subjects and patients with GORD[105]. However, primary peristalsis may be more often impaired in GORD patients compared to healthy volunteers. A study by Dodds *et al*. showed incomplete peristalsis in 27% of GORD patients versus 7% of controls, and oesophageal acid clearance was inversely related to the rate of intact swallow-induced peristalsis[106]. A more recent study has shown that severe ineffective oesophageal motility carried an independent risk of prolonged oesophageal acid clearance (OR 2.9)[107]. Indeed, peristaltic function appears to worsen as

oesophagitis severity increases[108], and impaired oesophageal motility is more prevalent in erosive oesophagitis patients than in patients with NERD[107].

Secondary peristalsis is a reflex mechanism triggered by oesophageal body distension, usually either due to a retained swallowed bolus or a gastro-oesophageal reflux event. Deficient secondary peristalsis has been implicated in GORD. It has been demonstrated that the frequency of secondary peristalsis events is lower in patients with GORD than in healthy individuals[106]. Triggering of secondary peristalsis requires intact afferent signalling. There is evidence that this may be defective in oesophagitis, where the time to triggering of secondary peristalsis is delayed compared to healthy controls[106, 109].

Of course, it is not yet entirely clear whether ineffective oesophageal motility in GORD is a primary event promoting oesophageal acid exposure, or a phenomenon that is secondary to the reflux disease itself. Studies in cats where oesophagitis was experimentally produced and then healed suggest that, at least in the case of severe oesophagitis, impaired oesophageal motility can be a reversible response to oesophageal inflammation[17]. In contrast, a number of human studies have shown that treatment of oesophagitis with anti-secretory drugs does not improve effectiveness of primary or secondary peristalsis[110, 111].

Mechanical clearance by way of peristalsis is the major mechanism for clearance of refluxed acid, but an important secondary role is played by the chemical effect of saliva that is delivered to the distal oesophagus during swallowing. Overall it takes 7 to 10 swallows to restore normal pH to the distal oesophagus after acidification with a 15 ml bolus of acid, and stimulation of saliva secretion reduces the time to acid clearance[112]. Saliva production is increased on acidic stimulation of the oesophagus (causing symptoms of waterbrash). It has been shown that acid perfusion of the healthy oesophagus induces parotid salivary secretion in a pH-

dependent manner[113]. This salivary response is more profound when the proximal rather than distal oesophagus is exposed to acid[114].

1.3.3 Epithelial defensive mechanisms

The aforementioned macroscopic features are undoubtedly important in defence against GORD. However, perhaps the most important reason why physiological acid reflux does not cause mucosal inflammation and erosion in healthy individuals is the fact that the human oesophageal epithelium has an array of protective mechanisms and characteristics to prevent damage occurring.

As a generalisation, the epithelial defence mechanisms can be split into three separate (but not necessarily mutually exclusive) parts: pre-epithelial, epithelial, and post-epithelial defence (figure 4).

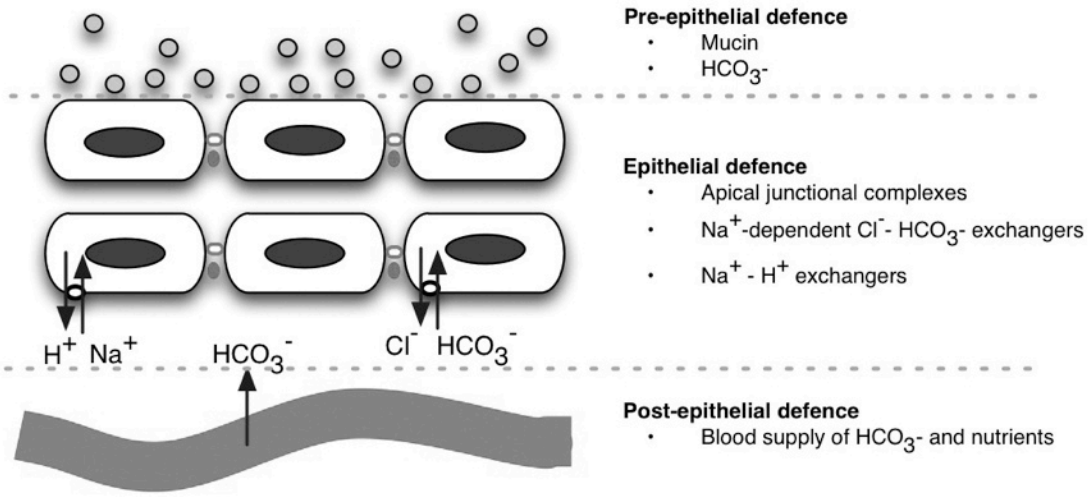


Figure 4: Epithelial mechanisms of defence against GORD

1.3.3.1 Pre-epithelial defence

Pre-epithelial defences are those that act as a barrier to limit contact of the gastro-oesophageal refluxate with the oesophageal epithelium. These include the mucous layer, and bicarbonate ions.

Oesophageal submucosal glands lie within the oesophageal submucosa. Each gland culminates in a single duct that collects acinar secretions and delivers them to the oesophageal lumen. The predominant cell type in the submucosal gland acinus is the chief cell, which secretes mucous. In contrast to the mucous layer of the stomach (which contains the mucoproteins MUC5AC and MUC6[115]), the mucous layer of the oesophagus is relatively ineffective as a barrier. The mucous secreted by the oesophageal submucous glands contains a water-soluble mucoprotein (MUC5B) that serves well as a lubricant, but not well as a protectant[116]. The mucous layer does not block diffusion of hydrogen ions. As such, the pH at the oesophageal epithelium falls rapidly to 2-3 when the luminal pH is 2.0 (a typical pH of the gastroesophageal refluxate)[117]. In the submucous gland acini there are also subsidiary cells that produce a watery, bicarbonate-rich secretion[118]. This bicarbonate secretion can be stimulated, in humans, by vagal excitation and oesophageal acid perfusion[119, 120]. In the supine position whilst sleeping (eliminating the effects of gravity and swallowing) this bicarbonate secretion into the lumen appears to be able to raise the oesophageal pH at a rate of approximately 1 pH unit per 10 minutes[97], and thus may be very important particularly in nocturnal reflux when primary peristalsis occurs infrequently.

1.3.3.2 Epithelial defence

Oesophageal epithelial defence is required when pre-epithelial mechanisms are not sufficient to prevent the noxious components of the refluxate coming into

contact with the epithelium. The oesophageal epithelium is a multilayered, non-keratinised stratified squamous epithelium. It consists of the stratum corneum, stratum spinosum and the stratum germinativum. The stratum corneum is closest to the lumen and consists of multiple layers of flat cells in various stages of desquamation. It acts as a barrier to the passage of ions and aqueous molecules from lumen to deeper epithelium. This permeability barrier is formed by apical cell membranes and apical junctional complexes that prevent the diffusion of luminal acid into the cells or intercellular spaces. The apical cell membranes prevent this diffusion by their hydrophobic nature, and due to the fact that their cation channels are inhibited by luminal acidity. The apical junctional complexes are essential for maintenance of mucosal integrity. They are formed by tight junctions, adherens junctions and desmosomes. These structures greatly limit the rate of paracellular ion diffusion[121, 122], which is very important since transcellular ion diffusion from lumen to basolateral aspect of the cell is extremely limited in the oesophagus. Each of these components has an extracellular, transmembrane and intracellular domain responsible for cell signalling and barrier function. Their barrier function is provided by component proteins bridging the intercellular space. The tight junction is positioned at the boundary of the apical and basolateral plasma membrane domains. The gate function of the tight junction controls the paracellular pathway for ion movement in between cells in an epithelial layer. For tight junctions the major proteins are members of the claudin and occludin families. At least 19 claudins have been found in the oesophageal mucosa, but claudin 1 and 4 are the most prominent. The structure of claudin-based tight junctions is yet to be fully resolved, but the primary role of claudins appears to be related to the regulation of paracellular selectivity to small ions[123]. The heterologous expression in monolayers of the majority of claudin isoforms leads to

an experimental increase of the transepithelial electrical resistance (see later), predominantly due to a selective decrease in cation permeability[124-128]. In a rat model it was shown that rats with reflux oesophagitis have an increased expression of claudin 1, a decreased expression of claudin 3, and an altered localisation of claudin 4 compared to control rats[129].

Occludin is becoming increasingly recognised as an important transmembrane protein localising at the tight junction. It comprises four transmembrane domains, a long carboxyl-terminal cytoplasmic domain, a short amino-terminal cytoplasmic domain, two extracellular loops, and one intracellular turn[130]. It is directly associated with the cytoplasmic, tight junction constitutive protein ZO-1[131]. The barrier role of occludin is not yet fully understood, but it may perform a regulatory role for claudins. ZO-1 binds to occludin (and other tight junction proteins such as claudin-1) and is essential for the integrity of the tight junction[132]. ZO-1 has been proposed to be a scaffolding protein between transmembrane and cytoplasmic proteins, and possibly to form a link between the adherens and tight junctions.

Along with the more apically located tight junction, an intact adherens junction is also required for integrity of the epithelial barrier[133]. The adherens junction performs important roles in cell-cell adhesion and regulation of the actin cytoskeleton. Cadherins, especially E-cadherin, are the major protein components of the adherens junction. They initiate cellular contacts through pairing between cadherins on opposing cells. They can also bind to cytoplasmic proteins (catenins) which locally regulate actin cytoskeleton organisation, cadherin stability and intracellular signalling pathways that control gene transcription[134]. Formation of the adherens junction leads to tight junction formation, but after assembly E-cadherin is not required to maintain tight junction organisation[135].

Unlike adherens and tight junctions, desmosomes do not fully encircle the cell. They do, however, contribute to cell-cell apposition by acting as spot weld-like adhesions arranged around the cell plasma membranes. They act as anchors for intermediate filaments that project into the cell cytoplasm. Desmosomes appear to be more important in structural rather than ionic integrity of cells, and they act to resist against shearing forces.

The barrier formed by tight junctions, adherens junctions and desmosomes is not perfect, and sometimes acid is able to penetrate. Consequently there must be further epithelial defences in place to protect the tissue. Intracellular proteins, phosphates and bicarbonate are able to buffer the pH when hydrogen ions diffuse into the oesophageal epithelial cells. Bicarbonate can readily diffuse from the blood into the intracellular space, and can also be produced *de novo* in the cytosol via carbonic anhydrase[136].

Excess acid can also be transported actively out of the epithelial cells. This is done on the basolateral membrane by sodium-dependent chloride-bicarbonate exchangers, and by sodium-hydrogen anion exchangers[137, 138].

1.3.3.3 Post-epithelial defence

The blood supply to the epithelium forms the basis of most post-epithelial defence against reflux. Tissue acid-base balance is preserved by delivery of bicarbonate from the blood to the epithelium to neutralise acid pH shifts. In cases of increased tissue acid load the blood flow is able to increase to deliver more bicarbonate (and remove more carbon dioxide). The blood is, of course, also a source of nutrients to aid repair of damaged epithelium.

1.4 Oesophageal mucosal integrity

The integrity of the epithelial defences already described are likely to be of paramount importance in protection against gastro-oesophageal reflux induced symptoms and complications. Impairment of these epithelial defences are increasingly being realised in GORD, and are an exciting area for research into pathogenesis and, potentially, new therapeutic interventions.

The morphological and functional barrier effectiveness of the oesophageal mucosal epithelium may otherwise be termed as the oesophageal mucosal integrity. Clearly in erosive oesophagitis there is a breakdown in the epithelial integrity that may allow penetration of noxious refluxate deep into the mucosa. However, in non-erosive reflux disease the integrity of the mucosa is less easy to assess. A number of methods have now been devised to enable expression of epithelial defence properties in non-erosive reflux disease. Thus, the integrity of the mucosa can be expressed in terms of:

1) Functional integrity

- The integrity of barrier function as demonstrated by permeability (e.g. to ions or small molecules).

1) Morphological integrity

- Macroscopic (oesophageal erosions, as in erosive oesophagitis).
- Microscopic (epithelial changes on light or electron microscopy).

1.4.1 Functional measurements of oesophageal mucosal integrity

1.4.1.1 In vitro studies

Functional demonstration of mucosal integrity and its impairment in GORD is not a new concept. Electrical potential differences across the wall of the gastrointestinal tract were first described in 1834[139]. Over 60 years ago it was realised that, on measuring the transmural potential difference, the charge of the luminal surface of the stomach was negative in relation to the serosal surface[140, 141]. Subsequently it was demonstrated that there is a positive change in the transmural potential difference on transition from the gastric columnar epithelium to the oesophageal squamous epithelium[142].

It was found in 1964 that, in the stomach, areas of mucosal damage were associated with reduced transmucosal potential difference[143]. In 1969 Beck and Hernandez used a "through the oesophagoscope" electrode to measure variations in potential difference over lesions where the mucosal integrity of the oesophagus was destroyed by erosions. They found that there was a profound drop (less negative) in potential difference measured over the ulcerated area compared to the surrounding oesophageal mucosa[144]. As such, it was proposed that measurements of potential difference could be a surrogate of the oesophageal mucosal integrity.

Khamis *et al.* proposed potential difference as a possible tool in diagnosis of GORD in 1978[145]. They studied 19 patients with upper gastrointestinal symptoms (dysphagia, restrosternal discomfort, heartburn, and epigastric pain) by gastroscopy and biopsy. They used a "through the endoscope" electrode, referenced to the skin, to measure transmural potential difference during the procedure. Potential difference was measured at the distal oesophagus, and a mucosal biopsy taken from the same area for histological analysis. Nine patients

fulfilled the authors' histological criteria (basal cell hyperplasia and extension of the papillae) for gastro-oesophageal reflux disease, and ten patients had normal biopsies. The mean value of the oesophageal potential difference in those with normal biopsies was −14.4mV, whereas in those with "reflux" changes the value was +9.4mV. Consequently the authors suggested that the potential difference might aid in diagnosis of GORD. It should be realised that such a histological method is a non-specific approach (probably with low sensitivity) to diagnosis of GORD, and this will have had an impact on the validity of the findings.

The possibility of measuring oesophageal potential difference as a diagnostic tool was revisited by Orlando *et al.* in 1982[146]. They measured potential difference and took mucosal biopsies from the oesophagus of 103 patients with symptoms of heartburn, chest pain or dysphagia. The potential difference was measured in a station pull-through manner from distal to proximal oesophagus. They again found that a low (less negative) potential difference is found in areas of macroscopically inflamed mucosa. It was postulated that the oesophageal potential difference may become lower due to a decrease in tissue resistance. However, the potential difference was less sensitive at detecting less severe, microscopic, inflammation. This is important since the diagnostic challenge is the distinction of non-erosive reflux disease from functional heartburn, which could not be met by this approach.

The same group investigated the pathophysiological sequence further using an animal model in which potential difference was measured[147]. They developed a model of progressive acid damage in the oesophageal mucosa of rabbits by intra-oesophageal catheter perfusion of acid and pepsin. The *in vivo* oesophageal potential difference was measured before, during and after the perfusion. At specific periods related to changes in potential difference, the rabbits were sacrificed, the oesophagus removed, and the mucosa placed in Ussing chambers for

studies of sodium and chloride transport. They found that medium to high concentrations of acid perfusion caused a reduction in transmural potential difference *in vivo*. The studies identified the mechanism for acid movement across the epithelium. This can potentially be paracellular, transcellular, or both. The Ussing chamber experiments show a reduced electrical resistance and increased bidirectional chloride transport on acid exposure, suggesting paracellular movement is predominantly occurring.

Tobey *et al.* investigated the implications of this possibility on permeability in the rabbit oesophagus when exposed to acid *in vitro*[148]. They placed strips of rabbit mucosa in Ussing chambers and exposed them to acid and acid-pepsin solutions, conducting circuit analysis and permeability studies. The circuit analysis was done to calculate the RT (transepithelial resistance), Ra (apical membrane resistance), Rb (basolateral membrane resistance), and RS (shunt, or paracellular resistance). The RT can be calculated according to Ohm's law (V=IR). The resistance is calculated from knowing the open circuit transepithelial voltage potential, and the current required to clamp the potential to a constant (e.g. zero). The calculated resistance multiplied by the surface area of the preparation is the RT (equivalent to transepithelial electrical resistance, TER). It has previously been demonstrated that RT = (Ra + Rb).RS / (Ra + Rb + RS)[149]. The investigators used nystatin in the basal chamber to effectively eliminate the Rb component of RT (nystatin permeabilises the basolateral membrane of the cell epithelium), and allow approximation of the RT to Ra + RS. After treatment with nystatin both chamber solutions were filled with a sodium- and potassium-free solution to abolish sodium and potassium diffusion across the apical membrane through sodium channels (eliminating Ra). RT was recorded again as an approximation of RS. They were able to demonstrate that in a physiological solution, RT very closely approximates

RS: i.e. almost all ion transit occurs via the paracellular route. This is why, in more recent studies, the RT (TER) has been used in preference to the potential difference in measurement of oesophageal mucosal integrity: it offers a better reflection of the paracellular barrier, and it also offers a correction according to the surface area of the epithelium being studied.

During 30 minutes exposure of the oesophageal tissue to acid (pH 1) and acid-pepsin (pH 2 + 1 mg/ml pepsin) solutions in the "basal" Ussing chamber, the RT of the mucosa fell by approximately 50%. They were further able to demonstrate that the decline in RT is almost completely caused by a decline in RS, i.e. is caused by an increase in paracellular tissue permeability. Having established increased shunt permeability on exposure of oesophageal epithelium to acid, the authors investigated the size of this shunt leak by measuring the permeability to various sized dextran molecules. They found little or no dextran permeability (and no reduction in RT) on exposure of the epithelium to a control (pH 7.4) solution, but acid and acid-pepsin exposure resulted in significantly increased permeability to dextrans up to 20 kD in size. Furthermore, they found an inverse and linear relationship between RT and dextran permeability.

Thus far it had been established that, in rabbits, strong acids at pH 1 or pH 2 are able to cause an increased paracellular permeability of the oesophageal epithelium. However, there had also been clinical observations that patients taking proton pump inhibitors can also perceive weakly acidic (pH 4-6) reflux events as symptomatic[56, 150]. In addition, as previously mentioned, there has long been consideration that components of DGOR (especially bile acids) are involved in GORD pathogenesis[70, 151, 152]. Farré *et al.* investigated the *in vitro* effects of acidic and weakly acidic solutions containing pepsin and bile acids on RT and permeability to fluorescein molecules[153]. Again they used rabbit oesophageal

mucosal in an Ussing chamber model. Again, they found that exposure of the mucosa to a neutral (pH 7.4) solution had no effect on epithelial RT. However they found that exposure of rabbit oesophageal mucosa to acidic and weakly acidic solutions containing low (0.5 to 5 mM) concentrations of bile acids and pepsin (1 mg/ml) caused a fall in the RT and an increase in the permeability to fluorescein. The most striking effect was seen in the case of strongly acidic (pH 2) solutions containing bile acid and pepsin. Weakly acidic solutions containing bile acid and pepsin caused a smaller, yet significant, fall in RT and increase in fluorescein permeability. Bile acids in neutral (pH 7.4) solutions with pepsin caused no increase in permeability to fluorescein. In this study there was a strong correlation (r=0.83) between the TER values and and permeability to fluorescein, suggesting that the TER is a good reflection of the tissue permeability.

Data on functional integrity in human subjects is much more sparse. Tobey *et al.* have published data of baseline electrical properties of squamous epithelium and Barrett's columnar epithelium from biopsies taken at upper gastrointestinal endoscopy[122]. They found that the transepithelial potential difference is lower in squamous epithelium than in Barrett's columnar epithelium. Using electrical parameter measurements at baseline and after bathing solution ionic replacement, they again found that the potential difference in squamous epithelium reflects a low level of active ion transport combined with a high level of tissue shunt resistance. In Barrett's columnar epithelium they demonstrated that the potential difference reflects a high level of active transport and a low level of resistance. This was interpreted as showing that the Barrett's epithelium has a greater capacity for cation (including hydrogen) and bicarbonate secretion than squamous epithelium, a potential protective mechanism.

Finally, also in human subjects, Jovov *et al.* investigated oesophageal biopsies of 20 patients with GORD, and 23 healthy controls. In Ussing chambers they bathed the biopsies in neutral solutions, but did not perform exposures to acidic solutions. They found that the baseline RT was significantly lower at baseline in GORD patients than in controls, and that fluorescein flux across the epithelium was significantly greater over 2 hours in GORD patients than in controls[133].

The above, *in vitro*, studies suggest that the contact of acid and perhaps weak acid (plus or minus bile acid and pepsin) with animal oesophageal mucosa is able to produce an impairment of mucosal functional integrity in terms of failure of the barrier mechanism against paracellular passage of ions and small molecules. What is unknown is how the functional integrity (in terms of RT, or TER) of human oesophageal mucosa dynamically responds to acid and bile acid exposure, or, importantly, whether the mucosa of patients with reflux symptoms is more vulnerable to the exposure than other subjects.

1.4.1.2 In vivo studies

Efforts have recently been made to test oesophageal mucosal integrity *in vivo*, in humans. Multichannel oesophageal intraluminal impedance is a technique that has been developed to complement measurements of pH in reflux studies[154]. It has recently been highlighted as an interesting surrogate tool for *in vivo* assessment of oesophageal mucosal integrity. The technique allows detection of the movement of a bolus through the oesophagus. It does this by measuring the change of current flow between a pair of electrodes. The current is not able to pass directly along the catheter, so it must pass through a material external to the catheter that bridges the gap between the electrode pair. Liquids (containing ions, such as gastro-oesophageal refluxate) are excellent electrical conductors and cause a fall in

impedance as it bridges the electrode pair. In the empty oesophagus it is the oesophageal mucosa that bridges the space between impedance electrode pairs, and thus it is the mucosa that offers the resistance to direct current flow (figure 5). If the mucosa is more permeable to ionic flow the baseline impedance will be seen to be lower. As such, the baseline impedance may offer insight into the barrier integrity of the oesophageal mucosa. Indeed, a study of baseline impedance in patients GORD found that baseline impedance was significantly lower in subjects with Barrett's oesophagus and erosive oesophagitis than in those with non-erosive reflux disease[155].

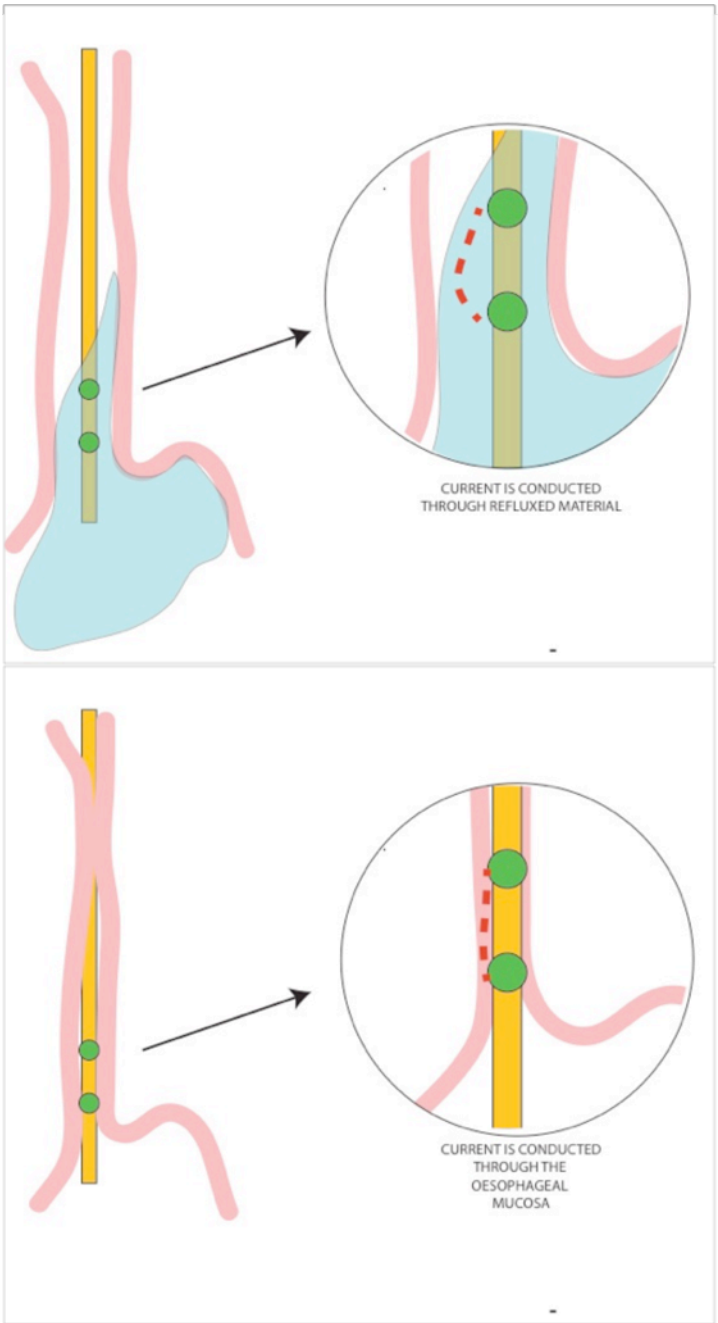


Figure 5: In the top diagram there is a liquid reflux event, and the impedance to current flow between electrodes in an impedance segment is produced by the refluxate. On the bottom, in the empty, collapsed oesophagus it is the mucosa that offers impedance to current flow

Further evidence for the use of oesophageal impedance as a technique to investigate mucosal integrity came from a study by Kessing *et al.*[156]. This group studied baseline oesophageal impedance in 24 GORD patients with, and 24 GORD patients without pathological acid exposure on 24-hour ambulatory reflux monitoring (those without pathological exposure were defined as GORD due to a

positive reflux-symptom association, i.e. acid hypersensitive oesophagus). The patients were compared to 10 healthy volunteers. Baseline impedance was highest in the control group, lower in the GORD patients without pathological acid exposure, and lowest in those with pathological acid exposure (2827Ω vs. 2090Ω vs. 781Ω respectively). There was a significant negative correlation between 24-hour oesophageal acid exposure time and baseline impedance. A further 20 patients with refractory GORD were tested twice, once "on" PPI and once "off" PPI therapy. Median distal baseline impedance "off" PPI was significantly lower than "on" PPI (886 Ω vs. 1372 Ω).

Thus, the baseline impedance appears to be able to give an insight into the integrity of the oesophageal mucosa. To test this hypothesis further, Farré *et al.* performed *in vivo* perfusions of pH 1 and pH 1.5 solutions in rabbits whilst simultaneously measuring oesophageal impedance[157]. After completion of a 30-minute perfusion, impedance measurements were continued for a further 30 minutes, following which the animals were sacrificed. The oesophageal mucosa was then mounted in Ussing chambers to measure the transepithelial electrical resistance. A positive correlation was found between the post-infusion baseline impedance and the subsequent transepithelial electrical resistance (TER) ($r=0.72$, $p=0.002$), suggesting that the impedance does indeed reflect the paracellular permeability of the mucosal epithelium. A recent study from a group in Peking has added further weight to the association between impedance and mucosal integrity by finding a significant negative correlation between the baseline impedance and intercellular space diameter (see later)($r=-0.64$, $p<0.001$)[158]. In the aforementioned study by Farré *et al.* an interesting phenomenon was shown from intra-oesophageal perfusions of neutral, and acidic solutions in healthy humans. There was no fall in oesophageal impedance that occurred after perfusion with the

neutral solution. However, after perfusion with acidic (pH 1) solution a significant reduction in impedance from baseline could be seen (a 53% decrease on average) that outlasted the duration of the acid exposure (figure 6).

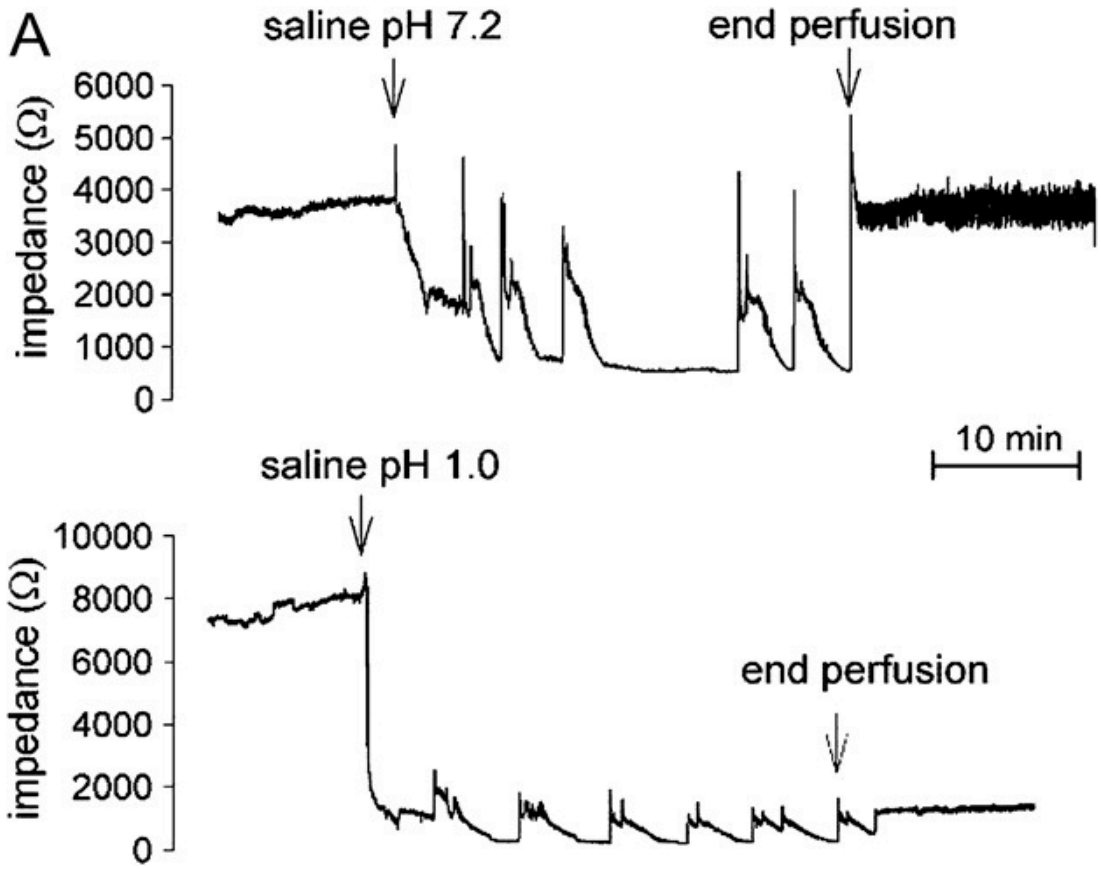


Figure 6: Impedance baselines recovered almost immediately after cessation of the saline perfusion, but remained low after cessation of the acid perfusion in the study by Farré *et al.* 2011

Furthermore, this impedance had not recovered to baseline 2 hours after cessation of the perfusion (remaining at a mean 48% reduction). This suggests that the mucosal integrity changes effected by acid do not rapidly reverse.

This recovery of impedance has only been tested in healthy subjects without symptoms, not in patients. Even in the healthy subjects there was some important inter-individual variability in the recovery of the impedance after acid perfusion in

subjects. It is possible that variability in mucosal recovery could have an impact on susceptibility to GORD. It is thus far unknown whether the recovery capacity of mucosal integrity after acid damage is variable amongst patient phenotypes.

1.4.2 Morphological measurements of oesophageal mucosal integrity

It had been noted during some of the earlier functional studies that are mentioned above that, as the epithelial electrical resistance decreased in rabbit oesophagus on exposure to acid, a change in morphological appearances of the mucosa appeared. When Orlando *et al.* perfused the rabbit oesophagus with acid, they found that at a time of early acid damage (where potential difference had fallen by 40-50%), dilated intercellular spaces (DIS) could be seen under electron microscopy, but not light microscopy (figure 7). As acid perfusion continued, light and electron microscopy revealed intraepithelial cellular necrosis, oedema and vesicle formation, predominantly in the mid-zone of the stratum spinatum. All of these changes occurred in the absence of macroscopic erosion or exudate[159]. In addition, the presence of DIS in these experimental models correlated with a decrease in TER and an increase in transepithelial mannitol flux. This observation was subsequently reproduced in other animal studies of oesophageal acid exposure[147, 160]. Similarly, in the Ussing chamber studies of Farré *et al.* mentioned in the previous section, the fall in TER and increase in fluorescein permeability seen on exposure of rabbit oesophageal mucosa to weakly acidic solution containing bile acids was accompanied by the development of DIS[153].

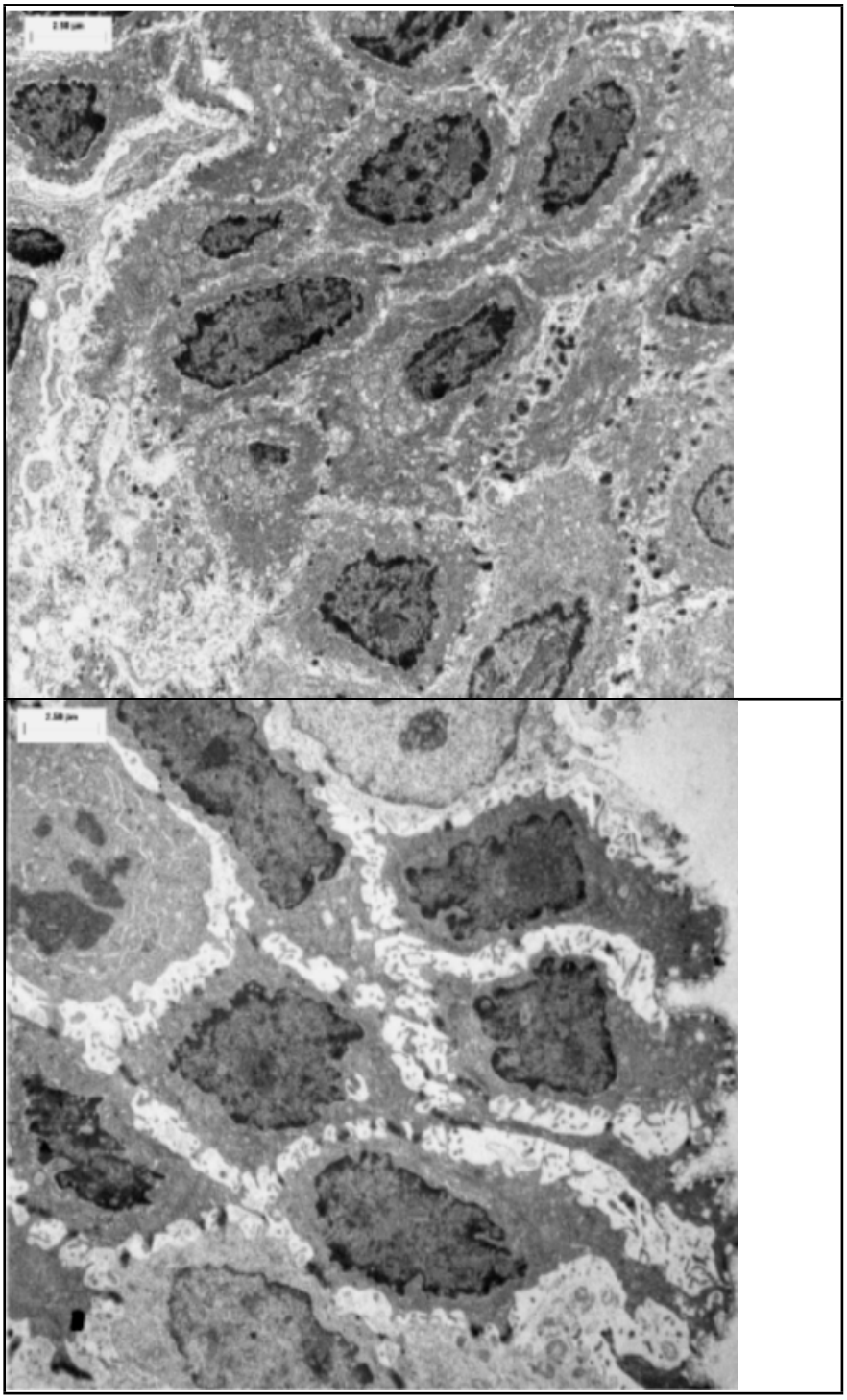


Figure 7: Above, an electron photograph of normal oesophageal epithelium. Below, abnormal epithelium displaying dilated intercellular spaces.

The formation of DIS appears to be an refluxate-induced phenomenon since the same group were able to demonstrate that acid and weak acid perfusion of the healthy human oesophagus *in vivo* is able to cause formation of DIS[161]. Oesophageal biopsies were taken from healthy volunteers before and after

perfusion with a neutral (pH 7.2), weakly acidic (pH 5.5), or acidic (pH 2) solution containing pepsin and glycocholic acid. Intercellular spaces were normal at baseline, and remained normal after exposure to the neutral solution. It was found that perfusion of weakly acidic or acidic solution for 30 minutes was able to induce DIS in these subjects.

As a result of these findings, interest was generated in the relevance of DIS as an early marker of reflux disease. Furthermore, it seemed possible that the presence of DIS was a morphological marker of an impaired oesophageal mucosal integrity: that the presence of DIS was the defect that allowed passage of ions (including hydrogen) and molecules across the acid-damaged epithelium, and as such may be responsible for symptoms in NERD[162].

Tobey *et al.* were the first to investigate whether DIS was present in human subjects with reflux symptoms. They took oesophageal biopsies from 11 patients with, and 13 patients without recurrent heartburn symptoms[163]. Six symptomatic patients had erosive oesophagitis, and 5 had no endoscopic erosions. They found that the intercellular space diameter on electron microscopy was significantly greater in patients with reflux than in asymptomatic subjects. This was true regardless of whether the patient had erosive or non-erosive reflux disease (mean ±SEM; controls 0.46 ± 0.06 μm, erosive reflux 0.80 ± 0.12 μm, non-erosive reflux 1.00 ± 0.15 μm).

Caviglia *et al.* followed this up by taking oesophageal biopsies from 33 patients with NERD, 6 patients with erosive oesophagitis, and 12 asymptomatic controls. They again found that intercellular space was increased in patients compared to controls (by a factor of 3 times), irrespective of whether the patients had NERD or erosive oesophagitis[164]. The same group also demonstrated that DIS was present in patients with heartburn symptoms but normal oesophageal acid exposure. It is

unclear whether these patients had functional heartburn or hypersensitive oesophagus since reflux-symptom association data was not given[165].

Weight was added to the association between DIS and symptoms in reflux disease by the study by Calabrese *et al*.[166]. They took oesophageal biopsies of 38 patients with GORD (22 NERD, 16 erosive reflux disease) at baseline, and after 3 and 6 months of 40 mg omeprazole daily. At baseline, all patients were deemed to have DIS (>74 μm, as determined by the 95th percentile value of normal subjects). After 3 and 6 months of treatment, 92.1% and 97.4% of cases displayed resolution of normal intercellular spaces respectively. Recovery of DIS was accompanied by regression of heartburn in all cases. The three patients with persistent symptoms after 3 months of PPI therapy, and the single patient with persistent symptoms after 6 months of therapy, showed incomplete healing of DIS. This perhaps suggests a strong association with DIS and reflux symptoms, if not a causal role. This concept was strengthened by a study specifically looking at patients with reflux symptoms refractory to PPI therapy[167]. In this study, oesophageal biopsies were taken from 15 patients with GORD not responding to, but taking, PPI therapy, and also from 11 patients with functional heartburn, and from 11 healthy control subjects. The mean intercellular space in epithelium viewed under electron microscopy was significantly greater in those with refractory GORD than in patients with functional heartburn or controls (0.87 vs. 0.42 vs. 0.32 μm respectively, figure 8).

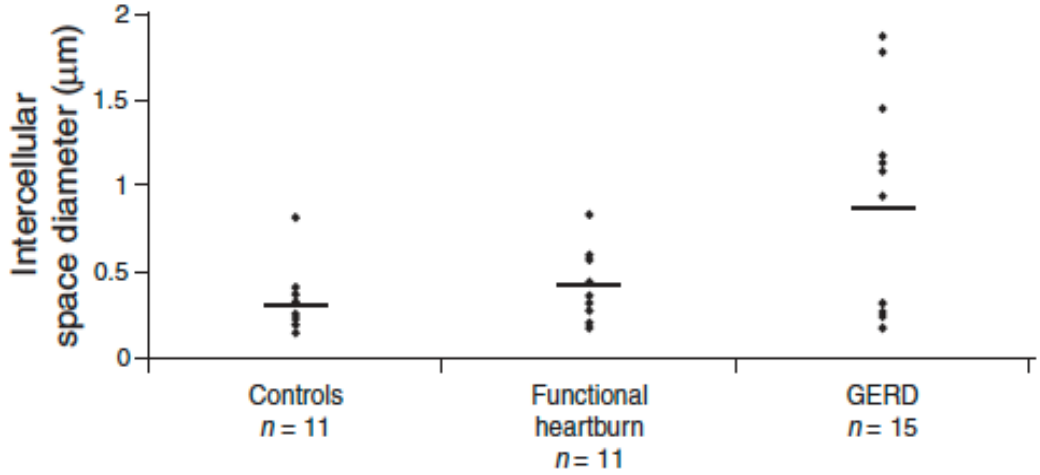


Figure 8: Persistence of DIS in patients with refractory gastro-oesophageal reflux disease, but not in controls or patients with functional heartburn. From Vela *et al.* 2011

There are some limitations of measuring DIS in patients with reflux symptoms. First, it is not specific to GORD and is also found in other oesophageal mucosal inflammatory diseases. Second, it is a difficult to perform a truly *random* measurement of DIS. In any one electron microscopy image there are wide variations of intercellular space diameter, and how the measurement points are chosen has not been clearly defined in the studies. Finally, DIS serves as a "snapshot" of one point in time. It tells us little about how the mucosa responds to acid challenges in a dynamic situation.

In summary, the integrity of the oesophageal mucosa can be studied by morphological or functional means. The *dynamic* functional changes in integrity (e.g. during an acid challenge) have not been studied in patients. Further investigation of the integrity behaviour of mucosa in different human phenotypes (e.g. healthy controls and heartburn phenotypes) may allow insight into the mucosal pathophysiology of GORD, and lead towards development of novel therapies.

1.5 Sensory mechanism in heartburn perception

The mechanisms of symptom perception in oesophageal disease are not fully elucidated, but are clearly relevant to GORD pathogenesis. Symptom phenomena are formed by a complex interaction of noxious oesophageal stimuli, oesophageal nociceptor activation, afferent nociceptive nerve fibres, and central processing.

It is known that experimental perfusion of acid into the human mid-oesophagus is able to reproduce heartburn symptoms, and with increasing acid concentrations the duration of exposure required to cause symptoms decreases[53]. Furthermore, reflux studies using transnasal catheters have shown distal oesophageal acid exposure to be higher in patients with heartburn[48, 168] and have been able to show a temporal relationship between acid reflux episodes and heartburn perception[169-171]. Finally, gastric acid suppression therapy is often an effective treatment for heartburn[10]. As such, it can be implied that, at least in a proportion of patients, gastric acid reflux into the oesophagus is a cause of heartburn. During such an acidic reflux event stimulation of acid-sensitive receptors on nerves in the oesophageal wall is likely to be important event in perception.

1.5.1 Nociceptive sensory fibres and receptors in the oesophagus

Unlike somatic nociception, visceral nociception from the gut is enacted by two extrinsic innervations as well as an intrinsic innervation. The extrinsic innervation is formed of vagal and spinal visceral afferent nerve fibres, and both types have nerve endings in all layers of the gut wall. Most afferent axons are unmyelinated C-fibres, with a minority being myelinated Aδ-fibres[172].

Vagal afferent fibres project to the vagus nerve via the superior laryngeal nerves, recurrent laryngeal nerves, and vagal branches within the oesophageal plexus. Vagal afferent cell bodies are located in the jugular and nodose ganglia with central projections to the nucleus of the tractus solitarius. Spinal afferent cell bodies are located in the cervical and thoracic dorsal root ganglia. The spinal levels of visceral preganglionic afferents are significantly fewer than somatic afferents, and are spread out across a range of dorsal root ganglia, and this probably explains the relative poor localisation of visceral pain[173]. First-order neurones synapse with second-order neurones in the dorsal horn of the spinal cord, which ascend via the spinoreticular and spinothalamic tracts to the reticular nuclei and thalamus. The latter tract transmits conscious sensation, whereas the former mostly activates unconscious responses to visceral sensory input.

Spinal visceral afferents represent 10-20% of nerve fibres in the splanchnic nerves[174]. It is suggested that spinal fibres are the most important afferent innervation in visceral nociception[172]. However, it has also been demonstrated that (at least in guinea pigs) vagal nerves supply the oesophagus with nociceptors as well as tension mechanoceptors[175, 176].

Afferent fibres projecting to the oesophagus can be excited by the presence of acid, most probably due to a direct action on the neurones[177, 178]. Indeed, vagal and spinal afferent nerves have been shown to express cation channels that act as molecular acid sensors. There is no single acid-sensitive receptor that modulates acid sensitivity, and so far several candidate channels have been identified. Acid-sensitive ion channels (ASICs) belong to the voltage-insensitive, amiloride-sensitive family of epithelial cation channels[179]. ASIC1, ASIC2 and ASIC3 are acid-gated and as such may be involved in nociception during an acid reflux event. They are also likely to have a role in mechanosensitivity[180]. Their role in GORD is

unknown, but in a mouse model deletion of ASIC3 caused reduced response of acid-sensitive nociceptors[181].

Transient receptor potential vanilloid receptors (TRPV receptors) are also important candidate receptors for acid-induced oesophageal nociception. As a group of over 30 proteins, TRPV channels serve a diverse array of sensory functions including hearing, touch, osmolality and pain[182]. TRPV1 and TRPV4, in particular, are able to respond to acidosis. At a pH of less than 6, these cation channels are activated forming a sustained channel current[179, 183]. Besides acid, heat and vanilloids such as capsaicin can also gate TRPV1 channels. TRPV1 is expressed in the mucosa, musculature and enteric nerve plexuses in the rat gut, and by vagal and spinal afferents throughout the gastrointestinal tract[184, 185]. The transduction threshold of TRPV1 is reduced by phosphorylation via protein kinases A and C. These protein kinases are activated in response to injury in a cAMP-dependent manner and are modulated by signals from other G-protein-coupled receptors including those to 5-HT and proteases (especially protease-activated receptor 2, PAR_2, which may be important since reflux events often contain pepsin)[186, 187].

P2X purinoceptors are ligand-gated membrane cation channels that open when extracellular ATP is bound[188]. $P2X_2$-containing purinoceptors are sensitised by acid in the presence of ATP (which is liberated from the cells in response to various physiological and pathological stimuli). ATP has been shown to sensitise vagal afferents to mechanical stimuli in the ferret oesophagus[189]. Although these findings make purinoceptors attractive candidates in reflux-induced nociception, their role in gastrointestinal nociception is thus far unclear.

1.5.2 Oesophageal sensitisation

Heightened visceral sensitivity (visceral hypersensitivity) is a hallmark of functional gastrointestinal disorders. This hypersensitivity may be due to excessive sensory transmission from the viscera to the brain (peripheral sensitisation), aberrant central processing (central sensitisation), or a combination of both.

In peripheral sensitisation there is a decreased threshold and exaggerated magnitude of sensory response to a given stimulus. This is usually affected by local injury and inflammation. An easily relatable example is the increased sensitivity of skin in the area surrounding a burn.

Sensitising mediators are potentially numerous, and chemical mediators are likely to include various amines, protanoids, purines, proteases and cytokines. These may act by direct activation of receptors coupled to the opening of ion channels on afferent nerve terminals, causing depolarisation and firing. Alternatively they may act indirectly by sensitisation in the absence of direct activation, for example by G-protein-coupled alterations in second messenger systems that in turn lead to phosphorylation of membrane receptors and ion channels that control excitability of afferent endings. Finally they may cause changes in the genetic phenotype of the mediators, channels and receptors expressed by the afferent terminals[190]. Peripheral sensitisation can be rapid and short-lasting, but in the case of prolonged or repetitive injury or inflammation it is the changes in genetic expression that lead to prolonged peripheral sensitisation.

Repetitive firing of nociceptive signals from the periphery is able to alter the amount and pattern of neurotransmitters released from the sensory nerve terminals in the spinal cord and brain, and thus can alter the central processing of visceral sensory information[191]. Such central sensitisation may contribute to

visceral hypersensitivity in the oesophagus, particularly in functional disorders such as functional heartburn. It is also the mechanism believed to underlie secondary hyperalgesia: a phenomenon whereby there is increased responsiveness to stimuli distant to the site of injury or inflammation. Altered synaptic transmission in the spinal cord leads to a decrease in threshold, increased responsiveness, and widening of spinal nociceptive neuronal fields[192]. Indeed, it has been shown that patients with NERD have not only increased sensitivity of the oesophagus[88, 193], but also increased somatic sensitivity of the chest wall[194]. This suggests that central sensitisation is likely to play at least a part in acid and mechanosensitivity in NERD. The secondary hyperalgesia can be attenuated by prostaglandin E2 receptor-1 antagonism and by the NMDA receptor antagonist, ketamine[195, 196].

A final probable component to oesophageal pain is psychoneuroimmune modulation. Many patients with heartburn report that psychological stress worsens their symptoms[197]. Acute experimental stress is known to reduce pain thresholds to oesophageal acid perfusion[198]. Whilst this is likely to be, at least in part, a central phenomenon, it is noteworthy that acute stress is able to induce oesophageal mucosal changes of dilated intercellular spaces in rats[199]. It is tempting to wonder if this phenomenon is due to release of mast cell inflammatory mediators in response to stress, since we know that mast cells express corticotrophin-releasing hormone (CRH) receptors, and we also know that CRH receptors can be located in the rat oesophageal mucosa[200].

1.5.3 Link between mucosal integrity and oesophageal sensitisation

By allowing increased access of noxious components through the epithelial barrier to areas of dense nociceptor presence, the reflux-induced impairment of

oesophageal mucosal integrity will, in effect, produce a state of peripheral oesophageal sensitisation. If this nociceptor activity is allowed to continue it can lead to an additional state of central sensitisation by mechanisms documented above. As such, protection of oesophageal mucosal integrity is an important therapeutic consideration in treatment of reflux disease.

1.6 Differences between the distal and proximal oesophagus in GORD

The average human oesophagus is approximately 20-22 cm in length (with a range of approximately 17-30 cm), from upper to lower oesophageal sphincters. Physiologically and anatomically the proximal and distal oesophagus are quite distinct. The upper 5% of the oesophagus, including the upper oesophageal sphincter, is composed of striated muscle. The distal 50-60% is composed of smooth muscle. Between these two distinct zones is a transition zone where the change from striated to smooth muscle progressively occurs.

There is a myenteric plexus of ganglion cells found in both upper and lower oesophagus, but it appears to be more dense in the smooth muscle portion. The submucosal plexus is sparse[201].

Knowledge of the mucosal innervation of the human oesophageal mucosa is limited, and most research has focused on the animal oesophagus. In animals, vagal mucosal afferent innervation appears to be unevenly distributed through the oesophagus. Vagal sensitivity and innervation appears to be concentrated mostly in the upper third of the oesophagus in the rat, cat and monkey oesophagus[202-204]. In cats at least, this appears to be associated with a functional differentiation, whereby the proximal oesophagus appears to be more sensitive to mechanical and chemical stimulation than the distal oesophagus[205].

Historically, most investigation of gastro-oesophageal reflux disease has been focused on the distal oesophagus. Of course, this is perhaps not surprising since most exposure to the refluxate occurs at the distal oesophagus. Consequently it is at the distal, not the proximal, oesophagus where erosive oesophagitis, Barrett's oesophagus and oesophageal adenocarcinoma occur.

Whilst it does not appear to be frequently affected by mucosal damage in GORD, there is increasing realisation of the importance of the proximal oesophagus in the perception of oesophageal symptoms in humans. There is now data to suggest that, in some cases, the proximal oesophagus may be even more important to perception than the distal oesophagus. It has been suggested that this may be a protective mechanism, whereby the presence of reflux in the upper oesophagus is a threat to the respiratory system and its recognition is essential. There is experimental data to suggest a difference in perception at the distal and proximal oesophagus in humans. Patel and Rao investigated sensitivity to oesophageal distension at different levels of the oesophagus in a group of healthy volunteers[206]. Intensity of oesophageal sensation to intra-oesophageal balloon distension was measured at 1) the lower oesophageal sphincter; 2) 5 cm above the lower oesophageal sphincter; 3) 10 cm above the lower oesophageal sphincter; 4) 5 cm below the upper oesophageal sphincter. At the lower oesophageal sphincter level all subjects perceived the distension, but not as pain or discomfort. At all other levels a painful stimulus was felt with increasing distension. They found that the proximal oesophagus was the most sensitive region to distension, and the sensitivity decreased the more distal it was tested.

These findings were reproduced more recently by Krarup *et al.* They compared sensitivity to balloon distension at 4 cm and 14 cm above the lower oesophageal sphincter in healthy volunteers and patients with Barrett's oesophagus. In both groups sensory and pain thresholds were lower in the proximal oesophagus than in the distal oesophagus[207]. Interestingly, in the same study no difference was found in thermal sensitivity between the distal and proximal oesophagus. There is some data to suggest that, in patients with non-erosive reflux disease (but possibly not controls), the proximal oesophagus is more sensitive to experimental acid

perfusion than the distal oesophagus[208]. It is, of course, very difficult to perfuse the proximal oesophagus without simultaneously perfusing the distal oesophagus, and a robust model to test acidification of the proximal oesophagus in isolation has not been described.

The potential clinical relevance of a difference in sensitivity of the distal and proximal oesophagus has been highlighted in gastro-oesophageal reflux disease by a number of reflux physiology studies. We know from both oesophageal pH[209] and impedance[210] studies that patients with gastro-oesophageal reflux have more reflux events reaching the proximal oesophagus than healthy subjects. More interestingly, it has also become apparent that patients are more likely to perceive a reflux event if it reaches the proximal oesophagus (usually determined as 15 cm above the lower oesophageal sphincter). Cicala *et al.,* using oesophageal impedance, discovered that a reflux event of a given duration is more likely to be perceived if it reaches the proximal oesophagus than if it reaches only the distal oesophagus[211].

The characteristics of perceived reflux events have also been investigated in patients taking proton pump inhibitors. This is of clinical relevance since up to 40% of patients with non-erosive reflux disease have a sub-optimal response to therapy, representing the main unmet need in GORD therapy. Two impedance studies in patients "on" PPI found that a reflux event is more likely to be perceived if it reaches the proximal oesophagus. In one of these, proximal extent of reflux was one of only two factors associated with an increased odds ratio of perception[212]. In the other study proximal extent of reflux was the only factor associated with an increased chance of reflux perception[213]. It is notable that in both of these studies factors such as residual acid reflux and patient age or sex did not influence perception "on" PPI. Supporting the concept of proximal reflux

events being perceived in the "on" PPI condition, a further impedance study by Emerenziani *et al*. showed that not only acid, but also weakly acidic reflux events are more likely to be perceived in the proximal oesophagus[214].

Although the clinical significance of the proximal oesophagus appears clear, the mechanisms underlying its apparent relative sensitivity compared to the distal oesophagus are not. There is no data published on the distribution of oesophageal mucosal afferent nerves in humans. There is also no data on the mucosal integrity of the proximal oesophagus in health and disease.

1.7 Treatment of GORD

A relatively small proportion of patients with gastro-oesophageal reflux disease develop complications, in the form of strictures, Barrett's oesophagus, and oesophageal adenocarcinoma. In treating the disease, it is sensible to aim for mucosal healing in patients with erosive oesophagitis (especially if severe) since chronic macroscopic inflammation is likely to predispose to complication. High acid exposure[215, 216] over a prolonged period of time[217] appear to predispose to Barrett's oesophagus and adenocarcinoma. However, other factors such as bile acids also appear to be important[81, 215, 218, 219], and at present it is unknown whether pharmacological or surgical intervention modifies the risk. As such, presently, apart from in a minority (perhaps less than 25%), the treatment of gastro-oesophageal reflux disease is symptomatic and can take the form of lifestyle modification, medical therapy, or surgery.

1.7.1 Lifestyle modifications

Many patients with gastro-oesophageal reflux symptoms begin to address their symptoms by making lifestyle modifications. Some changes are based on good outcome evidence, more are based on hypothetical benefit, and other interventions are sometimes dubious in their efficacy. Most informed changes are based on physiological data that certain foods, drugs and body properties (such as body mass index and body position) may influence reflux, either by modifying reflux content or amount, or modifying transient lower oesophageal sphincter relaxation frequency.

Several foods can reduce lower oesophageal sphincter pressure (such as chocolate, coffee and onions)[220, 221]. Chocolate and caffeinated coffee can also augment gastric acid production[222, 223] and therefore avoidance is often suggested. A high-fat meal increases reflux frequency in patients with GORD[224], but it is unclear whether this is a a fat-specific effect (perhaps due to delay of gastric emptying), or whether it is due to meal size (since a large meal will lead to fundic distension and transient lower oesophageal sphincter relaxation).

Symptomatic reflux does appear to have a relationship with body mass index (BMI). A meta-analysis found odds ratios of 1.43 and 1.94 for risk of GORD symptoms in overweight and obese patients respectively[225]. A BMI of greater than 30 kg/m^2 is associated with a greater risk of failure of anti-reflux surgery[226]. Reflux events appear to reach a more proximal oesophageal extent in GORD patients with a larger waist circumference[227]. It is also clear that increasing BMI is a risk for development of adenocarcinoma in patients with Barrett's oesophagus[228]. Although outcome data suggesting improvement of symptoms with weight loss in GORD is lacking, it is of course likely to be beneficial to overall health, and may reduce reflux symptoms in obese patients.

Alcohol can reduce lower oesophageal sphincter pressure by acting as a muscle relaxant. White wine may have a more profound effect than red wine on sphincter pressure, and beer may be worse than both types of wine[229]. Avoiding beer may, therefore, be beneficial for reflux symptoms. Perhaps tempering the need for advice against drinking wine is some epidemiological evidence associating wine (but not beer) with a reduced risk of oesophageal adenocarcinoma[230-232].

Probably the most logical dietary advice to reduce reflux is to avoid eating in the 2 hours before sleep. Most reflux occurs in the first 4 hours after going to bed, and proximal acid reflux is most common during sleep. Patients eating in the 1 to 2

hours before sleep are more likely to experience excess nocturnal gastro-oesophageal reflux[233]. Many people advocate elevation of the head of the bed at night to reduce nocturnal reflux. This does appear to speed up oesophageal clearance[234], and there is some evidence to suggest it can improve reflux symptoms[235]. Perhaps of greater benefit may be left lateral posture whilst sleeping. Acid exposure time and number of reflux episodes has been found to be reduced in this position when compared to the right lateral, supine and prone positions[236, 237]. This observation may be partly due to a reduction in transient lower oesophageal sphincter relaxations in the left lateral position[238].

Several cross-sectional studies have found a positive association between GORD symptoms and smoking[29, 31, 39], and so smoking cessation would seem sensible. A recent abstract presentation indicated that reduction of smoking improves symptoms in patients with severe symptoms taking anti-secretory medication[239].

1.7.2 Pharmacological therapy

Pharmacological treatment of gastro-oesophageal reflux disease has been dominated by anti-secretory drugs (i.e. drugs that reduce gastric acid secretion), and with good reason for in many patients they have excellent efficacy and good tolerability. There are, however, other pharmacological therapies (some older, some newer) that are used or have been trialled. The need for alternative therapies reflects the failure of anti-secretory drugs in some cases, and increasing concerns about long-term safety of standard therapies.

1.7.2.1 Anti-secretory drugs (H_2-receptor antagonists and proton pump inhibitors)

Two drug classes are available to reduce gastric acid production in GORD: H_2-receptor antagonists (H_2RAs), and proton-pump inhibitors (PPIs).

Approximately two litres of gastric acid is produced by the parietal cells of the human stomach each day. Three main stimuli are able to act on the basolateral aspect of the parietal cell to promote acid production. Gastrin is secreted by G cells in the gastric antrum in response to food in the stomach, and reaches the parietal cells by blood. Acetylcholine is released by the vagus nerve, probably in response to the sight, smell and taste of food. Gastrin and acetylcholine are able to stimulate enterochromaffin-like cells to release histamine, which is then able to bind to histamine receptors on parietal cells. Histamine binding effects a second messenger pathway (predominantly via cAMP) which activates the parietal cell proton pump. Activation of the proton pump causes exchange of a H^+ for a K^+ at the secretory canaliculus, driven by a H^+/K^+-ATPase. It follows that inhibition of gastrin, acetylcholine or histamine would lead to a reduction in gastric acid production. Only histamine antagonists are approved for use in GORD, and H_2RAs are indeed able to cause a rise in gastric pH and be used in the treatment of GORD. It also follows that blocking the proton pump itself blocks the final component of the acid secretion pathway and will lead to a greater degree of acid suppression, and this is indeed seen in proton-pump inhibitors. For healing of erosive oesophagitis, it can be seen that the degree of healing is related to the time gastric acid is above a pH of 4[240]. Below this pH direct acid exposure appears able to cause oesophagitis, and pepsinogen is activated to pepsin, which is likely to further contribute to oesophageal injury. As this thesis repeatedly emphasises, symptoms in GORD are more complicated than the presence or absence of erosions, and there is no conclusive data correlating duration of intragastric pH control and symptoms relief. However, clinical data on efficacy is available and will be discussed below.

Before the advent of PPIs, H_2RAs (histamine receptor-2 antagonists) were the mainstay of GORD therapy. At peak action they block gastric acid production by

60-70%[241]. Endoscopic healing oesophagitis was found in a meta-analysis to be 31-82% (27-45% where there is only grade 1 or 2 oesophagitis)[242]. Report of symptoms resolution has been similarly varied, with figures between 31% and 88% quoted[243]. Overall H_2RAs are very well tolerated. Some patients complain of nausea, abdominal pain and nausea. Some H_2RA drugs can interact with the cytochrome P450 system, and so can cause clinically significant interactions with drugs such as warfarin, phenytoin and theophylline. Cimetidine can also cause gynaecomastia in men. Another important problem with H_2RAs is tachyphylaxis. A study showed that addition of nighttime ranitidine (an H_2RA) to PPI therapy caused an initial improvement in nocturnal pH control, but this effect is significantly decreased after 1 week of regular dosing. After 1 month there was no benefit at all seen by the addition of ranitidine[244]. It would seem that intermittent, as required dosing of H_2RAs is likely to be more beneficial than continuous dosing.

PPIs are currently the most efficacious medical therapy for GORD, and suppress gastric acid secretion to a significantly greater extent than H_2RAs[245]. Examples of PPIs are omeprazole, lansoprazole, rabeprazole, prantoprazole, esomeprazole and dexlansoprazole. All PPIs are weak bases that highly selectively accumulate in the secretory canaliculi at pH less than 4. Here the inactive benzimidazole of the PPI is converted to a cationic sulphonamide which binds to and blocks the proton pump[246]. Since PPIs bind to actively secreting pumps, they are most efficacious when given before a meal (ideally the first meal of the day). Dexlansoprazole may be an exception to this rule, since it has a two-phase absorption at 90 minutes and 4 to 5 hours after ingestion, meaning that accuracy of meal timing may be somewhat less important. All PPIs bind to proton pumps irreversibly, and so to regain secretory activity after PPI administration new pumps must be synthesised. Complete acid suppression is not achieved since not all pumps are active at the

same time, and there is continuous re-synthesis of pumps resulting in a steady-state situation. Nevertheless PPIs usually suppress 70-80% of gastric acid secretion[247, 248]. PPIs are metabolised by enzymes in the cytochrome P450 system. In terms of gastric pH control, a once-daily morning dose of PPI gives a between 11 and 15 hours of gastric pH >4 during a 24 hour period. Esomeprazole at a 40 mg once daily dose gives approximately an additional 2 hours of pH >4 per 24 hours (15.3 hours) than omeprazole 20 mg once daily[249]. It has been claimed that the newer PPI preparation, dexlansoprazole, may allow 16 hours gastric pH >4 per day[250].

Along with their excellent gastric acid suppression, PPIs also have proven benefits over H_2RAs for healing of oesophagitis. A double-blind study comparing omeprazole 40 mg daily with ranitidine 150 mg twice daily found omeprazole to heal oesophagitis faster, and achieve mucosal healing more frequently than ranitidine (oesophagitis healing rates at 12 weeks were 91% for omeprazole and 54% for ranitidine in subjects with grade 2 or 3 oesophagitis)[251]. In various studies, all available PPIs are very effective at healing reflux oesophagitis, and there is likely to be little real-life difference between them in their relative efficacy[252-257].

Outcome studies of symptom improvement with PPI treatment will inevitably be more subjective than those addressing oesophagitis healing. Nevertheless, they are important since healing of oesophagitis does not necessarily correlate with symptom relief, a fact that is reinforced by the presence of symptoms in non-erosive reflux disease. There are a number of studies that have looked at the efficacy of PPIs in treating heartburn in patients with suspected GORD, and each PPI provides a figure of approximately 70-80% heartburn-free days[252-256]. Dexlansoprazole 60 mg once daily has achieved a diary report of 96% heartburn-

free days after 6 months of treatment in patients with erosive oesophagitis, which was significantly higher than placebo (29%, $p<0.0025$)[258], but head-to-head studies with other PPIs are not yet published.

1.7.2.2 Antacids

Over the counter antacid preparations are often used in cases of mild heartburn, and as an early course of action by symptomatic subjects usually before consulting a doctor. Antacids neutralise gastric juice acidity and increase the pH. Examples of "true" antacids are sodium bicarbonate, magnesium hydroxide, calcium carbonate, and aluminium hydroxide. Although very commonly used, study data on antacid efficacy is relatively sparse. Studies have offered conflicting results as to whether antacids demonstrate a benefit of antacid over placebo in terms of heartburn improvement[259-261]. There is no evidence that antacids are beneficial in healing of oesophagitis. The most common regime of antacid use is on an as required basis for symptomatic improvement only. More frequent use may be harmful since magnesium-containing antacids can cause diarrhoea, and aluminium-containing antacids may cause constipation, and both can accumulate to toxic levels, particularly in the presence of renal failure. Excess calcium ingestion can lead to hypercalcaemia, and milk-alkali syndrome.

1.7.2.3 Sucralfate and alginates

Since damage to the epithelium may occur in non-erosive reflux, there may be potential for development of topical therapies that protect the mucosa locally from the noxious refluxate (such as in the way topical sun cream protects the skin from ultraviolet light). The two main topical agents with this property are sucralfate, and alginate-containing therapies.

Sucralfate, is a sucrose sulphate-aluminium complex. It is believed to bind to the oesophageal mucosa, particularly in ulcerated areas (by adhering to positively charged proteins at the ulcer base), perhaps protecting against further diffusion of acid, pepsin and bile acids[262]. It has been found to have equivalent heartburn resolution and oesophagitis healing to H_2RAs in two studies[263, 264].

Alginate preparations have been added to antacids in products such as Gaviscon and Gaviscon Advance, and this addition appears to act in a manner unique to simple antacid formulations, via physical rather than chemical properties. Alginates are natural polysaccharide polymers isolated from brown seaweed. Chemically they are copolymers of α-L-guluronic and β-D-mannuronic acid residues connected by 1:4 glycosidic linkages. In an acidic environment alginic salts and alginic acids precipitate within minutes to form a viscous gel. This gel is then able to form a physical raft on top of the gastric juice. This floating capability is often enhanced by the inclusion of bicarbonate, which facilitates the production of CO_2 in the acid stomach environment, which is proposed to turn the raft into a foam that aids buoyancy[265]. It has also been proposed that the alginate may promote adherence to the oesophageal mucosa, where it may be able to protect against reflux locally. As yet, this potential is untested. Several studies have investigated the efficacy of alginate-antacids in treatment of gastro-oesophageal reflux symptoms. Randomised double-blind trials of Gaviscon (tablets or liquid) have found these alginates to be superior to placebo for heartburn control[266, 267].

In comparison with omeprazole, alginate-antacids are predictably inferior to PPI in symptomatic relief in patients with heartburn[268]. Similarly, Gaviscon tablets were found to be inferior to the H_2RA famotidine in preventing post-prandial heartburn when given before a meal[269].

1.7.2.4 Prokinetic therapies

Prokinetic agents are often considered in patients not responding to standard medical therapy. Theoretically this is attractive, given their potential to increase the lower oesophageal sphincter pressure, to speed gastric emptying, and to enhance oesophageal acid clearance. In the UK the most commonly prescribed agents are domperidone and metoclopramide (both dopamine antagonists). The antibiotic erythromycin acts as a motilin agonist and increases gastric emptying. Metoclopramide is a potent dopamine antagonist with peripheral and central effects, stimulating gastrointestinal smooth muscle and acting as a powerful centrally acting anti-emetic. It has been found to be equally effective as cimetidine and better than placebo in treatment of reflux symptoms[270, 271], but not in healing erosive oesophagitis. Its use is limited by central side-effect such as anxiety, motor restlessness, hallucinations and drowsiness. Domperidone is another dopamine antagonist that stimulates oesophageal peristalsis, increases lower oesophageal sphincter pressure, and speeds gastric emptying[272]. It has also been found to be superior to placebo and equivalent to H_2RAs in symptom reduction in GORD[273, 274]. Since there is little central activity of domperidone it is generally well tolerated with a good side-effect profile. Perhaps the most problematic side-effect is hyperprolactinaemia.

1.7.2.5 Surgery

Surgical intervention for GORD was often inadequate until Nissen discovered (serendipitously) that creating a wrap of the proximal stomach around the lower oesophageal sphincter resulted in a functioning anti-reflux barrier, and a potential cure for the disease[275]. Over the years since there has been a waxing and waning of the popularity of anti-reflux surgery. Although they were far superior to available

treatments (even with the arrival of H_2RAs, surgery offered superior symptom control[276]), surgical procedures were general reserved for severe and complicated GORD since a laparotomy was required. When highly effective PPI therapy was introduced in 1989, the result was a sharp decline in anti-reflux procedures. Then, throughout the 1990s the increasing use of laparoscopic techniques caused a resurgence in surgery again. However, since 2000 surgical therapy has been on the decline again. Partly this has been because the costs of PPIs decreased as they became generically available, but also because there has been some doubt as to the long-term efficacy of anti-reflux surgery. A follow-up to the initial study by Spechler that compared H_2RAs and anti-reflux surgery showed that, at 10 years, 62% of patients in the surgery group were using anti-reflux medications regularly[277]. It appears likely that both centre expertise[278] and patient selection is of paramount importance. There is still likely to be a place for surgery, especially when one considers that, on survey, 30% of GORD patients are either marginally satisfied or totally dissatisfied by their PPI therapy[279, 280]. PPI therapy is often needed lifelong, and as such patient concordance with therapy can influence effectiveness of therapy. There are also recent concerns about the safety of PPI therapy[5] that may be influencing patients' satisfaction with the drugs. One of the most common reasons for referral for surgery is refractoriness to PPI therapy, but it is known that, along with the presence of typical reflux symptoms (heartburn and regurgitation) and abnormal pH exposure on reflux testing, good symptom response to PPI is an associated with a positive outcome from anti-reflux surgery[281, 282]. At least in part this is because these factors often distinguish true reflux symptoms from non-reflux heartburn (functional heartburn) and alternative problems such as dyspepsia. Hence perhaps the ideal patient for anti-reflux

surgery is the one with a good symptomatic response to PPI but who is unwilling to take long-term medication.

1.8 PPI-refractory GORD

Although the advent of proton pump inhibitors (PPIs) has revolutionised the treatment of gastro-oesophageal reflux disease in recent decades, it has been estimated that 10 to 40% of patients with GORD symptoms have an incomplete response to treatment[22, 283], a significant clinical problem given the high prevalence of the disease.

The treatment response in non-erosive reflux disease (NERD) is controversial, having historically been considered inferior to response in erosive disease, in the region of 40%[22, 284]. A recent study has reported that patients with NERD are more likely to only partially respond to PPI therapy[283]. However, a recent meta-analysis has questioned this inferior response, suggesting that in well-defined (with symptom, endoscopic and objective reflux analysis) patients with NERD, the treatment response may be as high as 70%[284]. Nevertheless it is clear that there are a significant number of patients with GORD who are inadequately treated with PPI (in fact a recent systematic review of subjective opinions of patients revealed that only 34% were extremely satisfied with their PPI therapy[283]). As such there is an unmet need to develop new therapies for PPI non-responders. Recent attempts to treat refractory GORD by inhibiting transient lower oesophageal sphincter relaxations have been met with disappointing clinical response[285, 286]. An alternative approach to such patients may be required. This chapter has illustrated the potential role of impaired oesophageal mucosal integrity in disease pathogenesis. It may be that a topical treatment that can “protect” the mucosa from damage to its barrier integrity could be an interesting future strategy for refractory patients. It is possible that, due to their bioadhesive properties, alginate

compounds could form a basis of such a therapy. Such a possibility requires further experimental evaluation.

1.9 Remaining questions and aims of thesis

After reviewing the current literature, it can be proposed that a better understanding of human oesophageal mucosa physiology and pathophysiology may contribute to a) better understanding of refractory GORD, and b) new treatment strategies.

To move forward in these fields the following remaining questions have been identified:

1) How does human oesophageal mucosa compare with animal oesophageal mucosa previously described in experimental work?
2) How does the normal human oesophageal mucosa respond when it is exposed to reflux (experimentally and *in vivo*)?
3) Is the oesophageal mucosa different or more vulnerable to reflux in different disease phenotypes?
4) Is the regional difference in oesophageal sensitivity observed in humans due to distinct oesophageal mucosal characteristics?
5) What is the relationship between human oesophageal sensitivity to acid and oesophageal mucosal status and functional behaviour?
6) Can the human oesophageal mucosa integrity be protected with a topical agent?

To answer these questions the **aim** of this PhD research project was to:

1) Assess the *in vitro* functional behaviour of human oesophageal mucosa in biopsies from asymptomatic controls and patients with reflux symptoms.

2) Evaluate, *in vivo,* the integrity of oesophageal mucosa in basal conditions and during exposure to acid using oesophageal impedance monitoring.

3) Compare the aforementioned oesophageal mucosa functional behaviour *in vivo* between patients with functional heartburn and non-erosive reflux disease.

4) Characterise, *in vitro* and *in vivo,* the differences in basal and functional behaviour of distal and proximal human oesophageal mucosa.

5) Test the *in vitro* feasibility of a topical protection of oesophageal mucosal integrity with an alginate solution.

CHAPTER 2

Materials and methods

CHAPTER 2: METHODS AND MATERIALS

The methods and materials used in the studies presented in this thesis will briefly be described here, and specific methods will be presented in greater detail in the relevant chapter.

2.1 *In vitro* studies

The *in vitro* general methods are applicable to the studies presented in Chapter 3: *In vitro* assessment of oesophageal mucosal integrity in patients with heartburn without oesophagitis; Chapter 5: *in vivo* and *in vitro* assessment of mucosal integrity in the distal and proximal oesophagus, and; Chapter 6: protection of human oesophageal mucosal integrity.

2.1.1 Endoscopy and oesophageal mucosal biopsy

All endoscopic biopsies were taken at the endoscopy unit of the Royal London Hospital. Endoscopy was performed per-orally using Olympus video gastroscopes with a 2.8 mm working channel. The gastro-oesophageal junction was identified as the location of the proximal extent of the gastric folds. The presence of Barrett's oesophagus was excluded by ensuring that the squamo-columnar junction corresponded to the this level. The level of the gastro-oesophageal junction was measured relative to the external aspect of the mouth guard using the 'on the endoscope' measurements. Distal oesophageal biopsies were taken at 5 cm above, and proximal biopsies at 20 cm above the gastro-oesophageal junction. Boston Scientific Radial Jaw 3 biopsy forceps (with 2.2 mm jaws) without needle were used to take biopsies. Biopsies were removed one "bite" at a time, and as

tangentially as possible with use of suction and angulation. Biopsies were placed immediately (with a blunt needle) into pre-oxygenated Krebs-Henseleit physiological buffer at pH 7.4 (for Ussing chamber studies) or 4% paraformaldehyde in 1mM phosphate buffer (for immunohistochemistry studies).

2.1.2 Ussing chamber technique: measurements of mucosal transepithelial electrical resistance

The technique involves placing an epithelial tissue across an aperture that separates two halves of a chamber. In the cases of the studies presented in this thesis, the epithelial tissue was a human oesophageal mucosal biopsy. The two halves of the chamber can be filled with (usually physiological) solutions, and electrodes are placed in each chamber that are able to measure and induce current and voltage across the tissue. By placing the tissue across two separated halves of the chamber and filling each half with an identical volume of an identical electrolyte solution, osmotic and hydrostatic gradients for ion movement are eliminated. Voltage electrodes are placed close to the mucosa on both sides of the chamber to allow measurement of the transepithelial voltage. Current-passing electrodes are placed laterally to the voltage electrodes, forming a circuit that can pass current across the epithelium for the purpose of voltage clamping. The current required for voltage clamping is calculated from the inherent transepithelial voltage and the resistance of the mucosa and circuit ($I = V/R$).

In using the Ussing chamber for physiological studies, the orientation of the epithelium placed across it is important. The tissue is placed so that the mucosal membrane and “basal” membrane each face one half of the chamber, so producing a “luminal” and “basal” chamber that are filled independently with solutions. Typically (and in the case of the experiments in Chapters 3, 5 and 6) the chambers

are filled with a physiological Krebs bicarbonate Ringer solution. The composition of the Krebs' solution was (mM): NaCl (118.1), KCl (4.69), $MgSO_4.7H_2O$ (1.18), KH_2PO_4 (1.18), D-glucose (11.1), $NaHCO_3$ (25.0) and $CaCl_2.6H_2O$ (2.5).

This solution was continuously perfused by carbogen gas (95% O_2, 5% CO_2). This mixture provides a high partial pressure of oxygen to the tissue, which is required to overcome the lack of haemoglobin delivery. The pCO_2 provided is similar to that of venous blood and helps maintain the buffer at a physiological pH. The system is heated by a water bath system to body temperature.

Before starting electrical measurements across the epithelium, the system was calibrated. The solution was placed in the chambers without the tissue in place, and electrical bias eliminated by zeroing the voltage difference between the voltage electrodes and the inherent resistance of the solution. This, and subsequent measurement was done using the proprietary software, VCC Clamp (Mussler Scientific Instruments, Aachen, Germany).

The voltage clamp can be used to calculate the transepithelial resistance (the reciprocal of the conductance). This is done by pulsing a small command voltage and measuring the resulting change in current (conductance = Δcurrent / pulsed voltage). More than 90% of intestinal conductance occurs through the paracellular pathway, which is regulated by tight junctions and the apposition of basolateral membranes of adjacent epithelial cells. Therefore changes in conductance (or resistance) can indicate changes in the mucosal integrity.

The solution in the "basal" chamber was replaced with a solution that represented refluxate-like material (acid + pepsin + bile acid). The test exposure solutions used in the experiments of this thesis (acid pH 2, and weakly acidic pH 5) were prepared by adjusting the pH of the Kreb's physiological solution using HCl and NaOH. Porcine pepsin was added at a concentration of 1 mg/ml. Deoxycholic acid 1 mM

was added to the weakly acidic solution (it is not soluble at pH 2) and taurodeoxycholic acid 1 mM was added to the acidic solution. The change in transepithelial electrical resistance (TER) that occurs during a 30 minute "luminal" exposure was calculated.

2.1.3 Immunohistochemical studies: assessment of mucosal afferent nerve fibres

Proximal and distal oesophageal biopsies were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer overnight. This was followed by cryoprotection in 30% sucrose in phosphate-buffered saline (PBS) for 24-hours at 4^0C, followed by 30% sucrose PBS:OCT embedding compound (1:1) at 4^0C. Sections were embedded in OCT at -25^0C and 10 µm sections were cut on a cryostat and mounted on positively charged glass slides. Sections were then air-dried for 1 hour. 400 µl per slide of 10% horse serum in PBS (blocking agent to prevent non-specific binding) + 0.3% Triton-X100 (detergent to destroy cell membranes and increase antigen penetration) was applied and left at room temperature for 1 hour. Sections were then incubated with a primary antibody to calcitonin gene-related peptide (CGRP) (1:500 Monoclonal mouse anti-human, Pierce Antibodies ABS 026-05-02) at 4^0C overnight. The primary antibody was made up in 10% horse serum in PBS and 0.3% Triton-X100. Sections were then washed three times for 10 minutes in PBS + Triton-X100, followed by incubation with the secondary antibody (donkey anti-mouse Invitrogen, labeled with green-fluorescent Alexa Fluor 488 dye) and incubated for 4 hours in darkness. Sections were then washed again three times for 10 minutes, and mounted with Vectashield HardSet™.

Fluorescence was visualised using an epifluorescent microscope (Olympus BX61). All images were obtained with a 40x oil immersion lens under the 488 nm excitation setting.

2.2 In vivo studies

2.2.1 Oesophageal high resolution manometry

Studies were performed at the upper gastrointestinal physiology unit of the Royal London Hospital. Before impedance measurements, the lower oesophageal sphincter location was determined using oesophageal manometry. After pressure calibration, a 4.2 mm diameter, solid-state high resolution oesophageal manometry catheter (ManoScan™ catheter, Given Imaging, USA) with 36 circumferential pressure channels was placed transnasally. Manometry images were observed real-time using proprietary software (Manoview™, Given Imaging, USA). Correct placement was deemed when the catheter had traversed the crural diaphragm, and an image was obtained that included pressure recordings from pharynx, oesophagus and proximal stomach simultaneously. The lower oesophageal sphincter (LOS) was identified as a high pressure area at the distal margin of the oesophageal body (see figure 9). Recording the catheter measurement at the nares enabled calculation of the LOS position relative to this point.

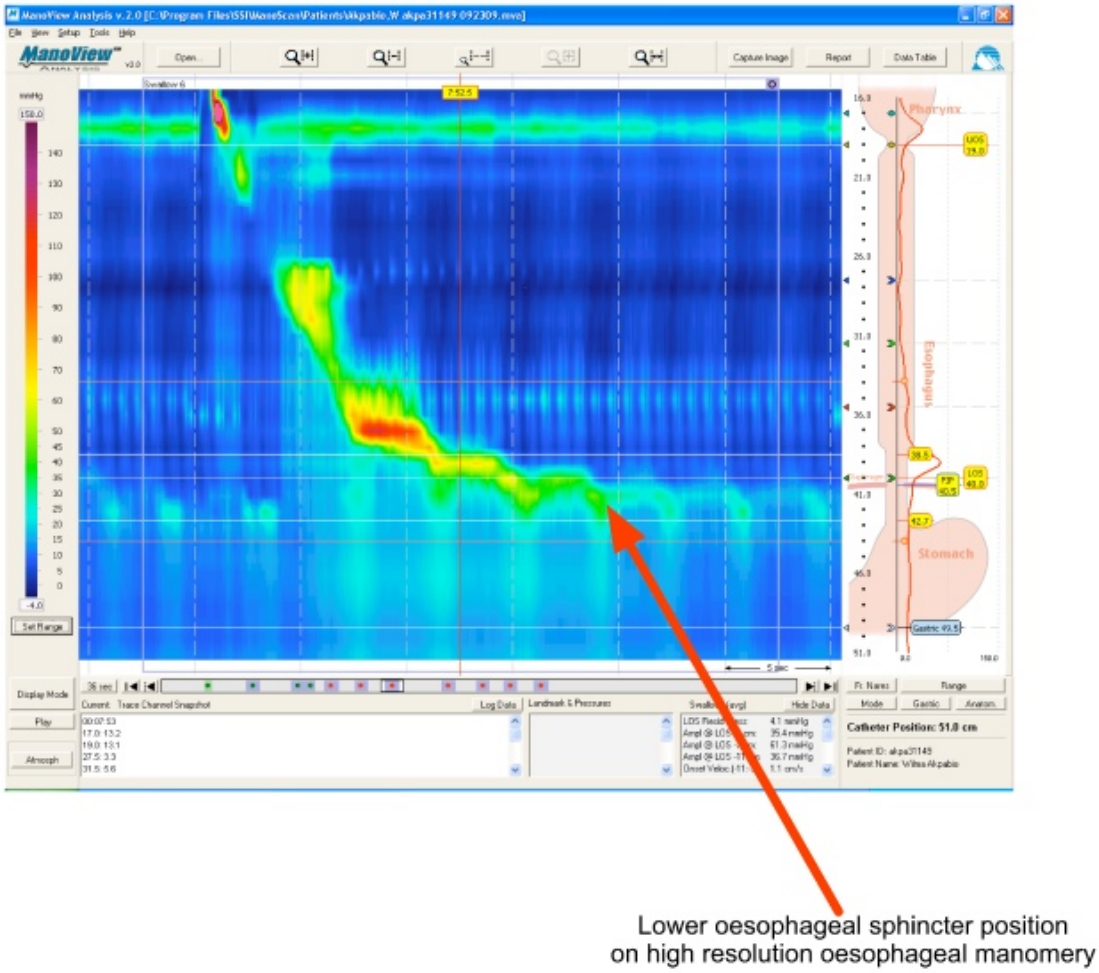


Figure 9: Demonstration of the LOS on a normal high resolution manometry plot

2.2.2 Reflux monitoring and impedance baseline measurements

After oesophageal manometry testing, the manometry catheter was removed, and impedance monitoring was performed. To this end a combined oesophageal impedance-pH catheter (Comfortec®Z/pH ZAI-BG -44, Sandhill Scientific, USA) was used. This is a 2.13 mm diameter catheter containing 6 impedance segments and 2 pH channels. When correctly placed with reference to the manometrically-determined LOS position, the impedance channels are located at 3, 5, 7, 9, 15 and 17 cm above the LOS, and the pH sensors 5 cm above and 10 cm below the LOS. This catheter also has an integrated sphincter locator port at 11 cm above the LOS.

This can be water perfused to allow pressure measurement and location of the sphincter by manometry, but it can also be used to perfuse the oesophagus with liquids. Prior to insertion into the nasal cavity, the pH probes (which are internally referenced) are calibrated in pH 7 and pH 4 solutions.

After insertion, the patient was asked to remain in a seated position for baseline and perfusion studies. Perfusions were by way of a peristaltic pump attached to a 3-way tap, which in turn was connected to neutral (normal saline buffered to pH 6.7 with phosphate buffer) and acid (HCl at pH 1). Rate of perfusion was checked *ex vivo* before each perfusion experiment.

Any symptoms during perfusions were measured on a 0-10 visual analogue scale, where 0 is no pain and 10 is the worst pain imaginable.

24-hour pH-impedance studies were analysed according to a consensus report of detection and definitions of reflux studies[287].

2.3 Research ethics committee approval

The *in vitro* and *in vivo* studies of patients and healthy controls presented in this thesis were approved by the East London and the City research ethics committee.

Ethics committee reference number: 07/H0705/57 (and amendments)

QMUL reference number: ICMS/PR/07/029

CHAPTER 3

In vitro assessment of oesophageal mucosal integrity in patients with heartburn without oesophagitis

CHAPTER 3: IN VITRO ASSESSMENT OF OESOPHAGEAL MUCOSAL INTEGRITY IN PATIENTS WITH HEARTBURN WITHOUT OESOPHAGITIS

3.1 Introduction

In erosive gastro-oesophageal reflux disease it is relatively easy to understand how exposure of the defective, inflamed oesophageal mucosa to noxious components of the reflux can lead to unpleasant sensation and hence reflux symptoms. However, the mechanism of heartburn perception in patients without oesophageal erosions is much less well understood. The majority of patients with gastro-oesophageal reflux disease have normal macroscopic oesophageal findings on endoscopy[22]. In patients with such non-erosive reflux disease symptoms are still being caused by the exposure of the "normal" mucosa to components of gastro-oesophageal reflux; whether associated with excessive gastro-oesophageal refluxate exposure, or by hypersensitivity of the oesophagus to normal amounts of reflux. Of likely relevance to the pathophysiology of non-erosive reflux disease is that, although macroscopically normal, the mucosa may still have microscopic and/or functional abnormalities[163, 165, 167]. The oesophageal mucosal integrity is a critical protective mechanism against gastroesophageal reflux. Impairment of oesophageal mucosa integrity may lead either to significant inflammation and erosive reflux disease[288], or permeation of noxious components of the refluxate (e.g. H^+, bile acids, pepsin) that stimulate release of epithelial cell mediators[289] or directly activate nociceptors[162], producing typical reflux symptoms without significant inflammation.

Impairment of oesophageal mucosal integrity in macroscopically normal tissue can

be demonstrated as ultrastructural microscopic changes (i.e. dilated intercellular spaces, DIS) and/or in functional terms. *In vitro* Ussing chamber experiments can demonstrate decreased transepithelial electrical resistance (TER) and increased permeability of the mucosa to passage of small molecules[122, 153, 290].

The relationship between the ultrastructural and functional changes in oesophageal mucosa integrity is probably complex. DIS has been suggested to be caused by an initial increased permeability to ionic flow through the epithelium. Movement of chloride ions is then followed osmotically by water which enters the intercellular spaces and causes dilation[291]. It is therefore probable that changes in TER would be seen before the development of DIS, since the movement of ions (detected by TER) would occur before the water enters the intercellular spaces.

TER appears to be a good marker of mucosal permeability. In experiments using humans and animals, TER has been shown to correlate well with other measures of permeability, and is decreased when DIS is present[153, 157]. Thus far, human oesophageal mucosal integrity has been measured in static conditions: i.e. in terms of DIS or baseline TER. In fact, it is possible to evaluate oesophageal mucosal integrity in human biopsies both in static conditions, and in response to a stress stimulus (i.e. acid exposure). The use of continuous measurement of TER allows evaluation of dynamic changes in oesophageal mucosal integrity on exposure to noxious solutions. Unlike determination of DIS (an “all or nothing” phenomenon), continuous measurement of TER allows a quantification of relative changes in mucosal integrity over time. This dynamic response of oesophageal mucosa has not yet been tested in human tissue, but may allow evaluation of the degree of integrity change that occurs in different populations of human patients. As such it allows assessment of integrity response to a “stress test” of noxious exposures.

The most common phenotype of GORD patients currently evaluated by

gastroenterologists is the patient referred with reflux symptoms and negative endoscopy[292]. It is known that patients with non-erosive reflux disease "off PPI" and with refractory non-erosive reflux disease "on PPI" have persistent DIS[167]. It is also known that successful PPI treatment reduces intercellular space diameter[166]. These findings suggest that an impaired oesophageal mucosal integrity may have an important role in the pathogenesis of ongoing symptoms. It is of interest to know how the oesophageal mucosal integrity of these patients responds to a "stress test" of acidic solutions, and whether it displays an abnormal handling of acid that may be important in disease pathogenesis.

The hypothesis of this study is that in patients with heartburn and a macroscopically normal oesophageal mucosa there is persistent underlying mucosal vulnerability that can predispose to ongoing symptoms, hypersensitivity to normal or low amount of acid or weakly acidic exposure, or early relapse after PPI treatment withdrawal. Such vulnerability might be expressed as a subtle impairment of mucosal handling of acid that might be detected during continuous TER measurements in human biopsies.

The aim of the study is to assess the *in vitro* functional behaviour of human oesophageal mucosa in biopsies from asymptomatic controls and patients with reflux symptoms.

3.2 Methods

3.2.1 Study design and population

The study was a prospective comparison of dynamic mucosal integrity in patients with typical reflux symptoms and asymptomatic controls.

All subjects were recruited for the study at the gastrointestinal endoscopy unit of the Royal London Hospital.

Oesophageal biopsies were taken from patients having upper gastrointestinal endoscopy for heartburn (troublesome, daily retrosternal ascending burning). For comparison, biopsies were also taken from control subjects with no upper gastrointestinal symptoms (having endoscopy for iron deficiency anaemia or diarrhoea).

Subjects with oesophageal erosions or Barrett's oesophagus were excluded from the study.

Overall 78 subjects were recruited for the study. From 9 subjects biopsies were used for reproducibility validation, from 5 subjects biopsies were used to assess orientation, from 11 subjects biopsies were used to compare functional and morphological findings, and biopsies from the remaining 53 subjects were used for the main study.

3.2.2 Endoscopy

Endoscopic procedures were performed under midazolam sedation or with pharyngeal local anaesthetic spray. Three oesophageal mucosal biopsies were taken (Radial Jaw 3 forceps, Boston Scientific, USA) from 3 cm above the squamo-columnar junction, and immediately placed in a pre-oxygenated Krebs-Henseleit

buffer solution at pH 7.4. The biopsies were rapidly transported to the laboratory for Ussing Chamber study. All biopsies for the following studies were taken by the same endoscopist (Dr Woodland) using the same technique.

3.2.3 Orientation of biopsies

Oesophageal biopsies were first orientated under stereo-microscopy to determine the luminal and basal sides.

Accurate orientation is essential for the conduct of this study. Alongside a consultant gastrointestinal pathologist (Dr J Chin-Aleong), the criteria for determining orientation was first established.

The following indicators of biopsy orientation were used:

1) Macroscopically, the biopsies form a curved shape due to the pinching action of the biopsy forceps. The convex surface of the curve is thus usually the luminal aspect of the biopsy (figure 10).

2) Microscopically (under high powered stereomicroscope, figure 11), the following features are able to indicate the orientation:
    - The papillae are visible as pale dots on the luminal aspect of the biopsy.
    - A very thin, flat layer of tissue (superficial flat squamous layer) can often be seen originating at the edges of the luminal aspect.
    - The basal aspect of the biopsy can be identified by an irregular appearance of connective tissue.

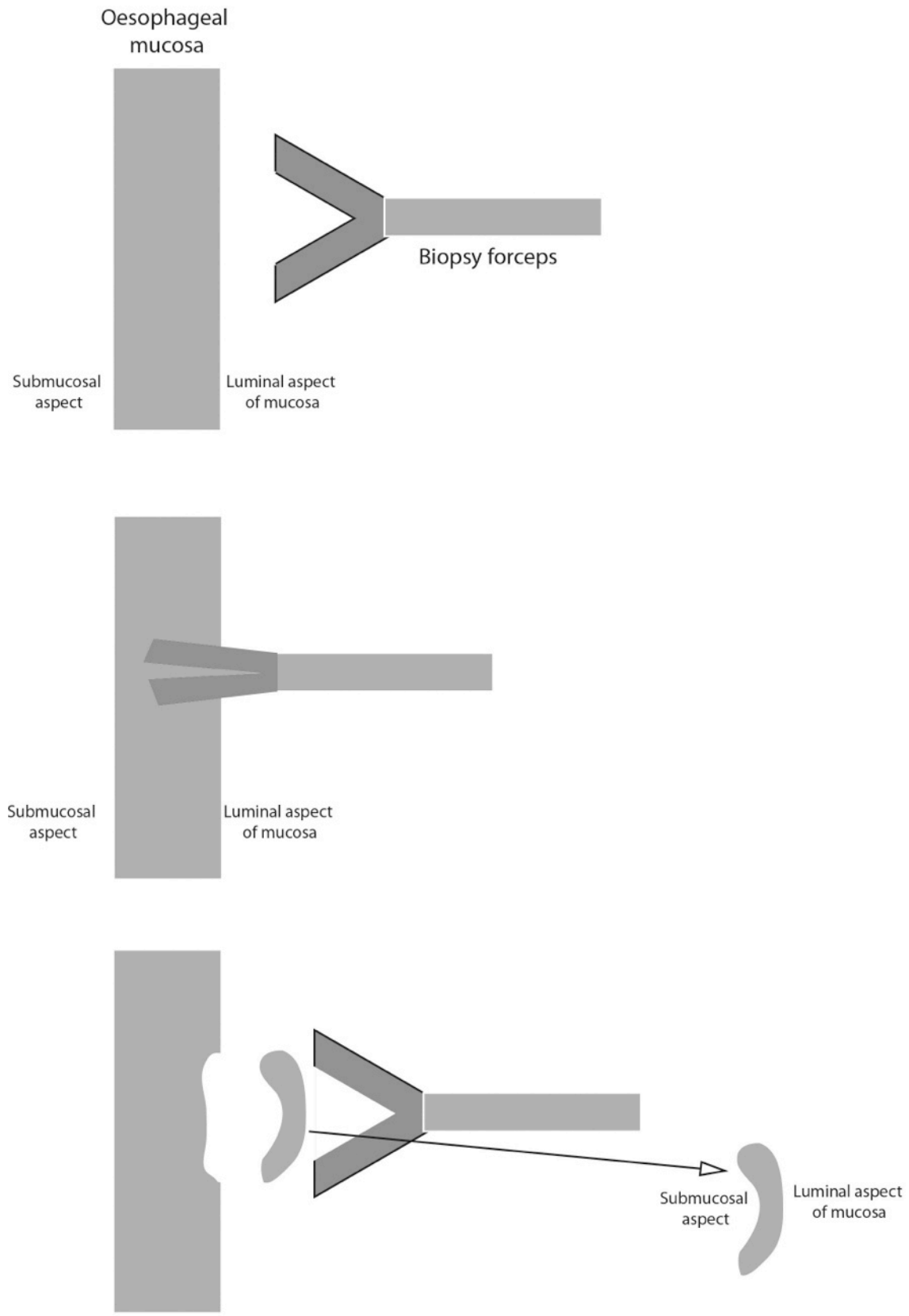
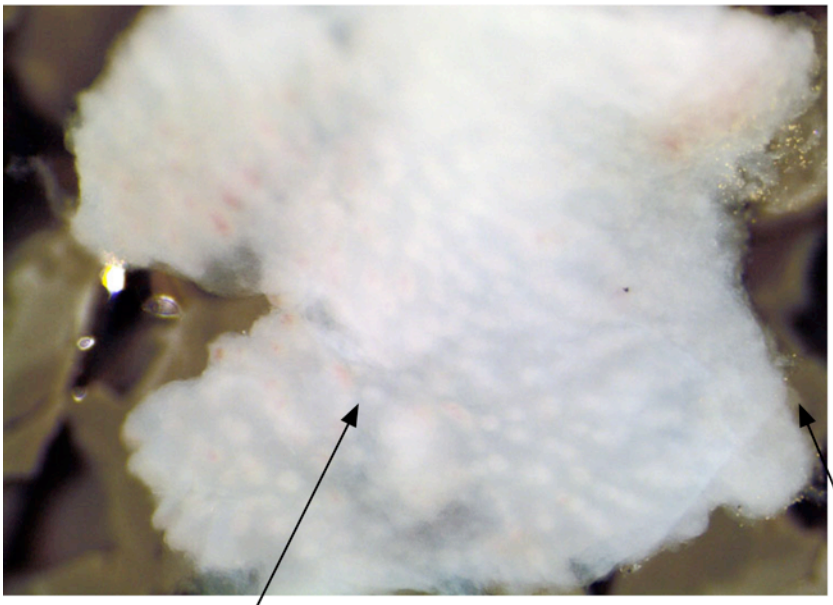


Figure 10: Illustration of mucosal biopsy technique resulting in biopsy shape

Luminal aspect of biopsy



Basal aspect of biopsy

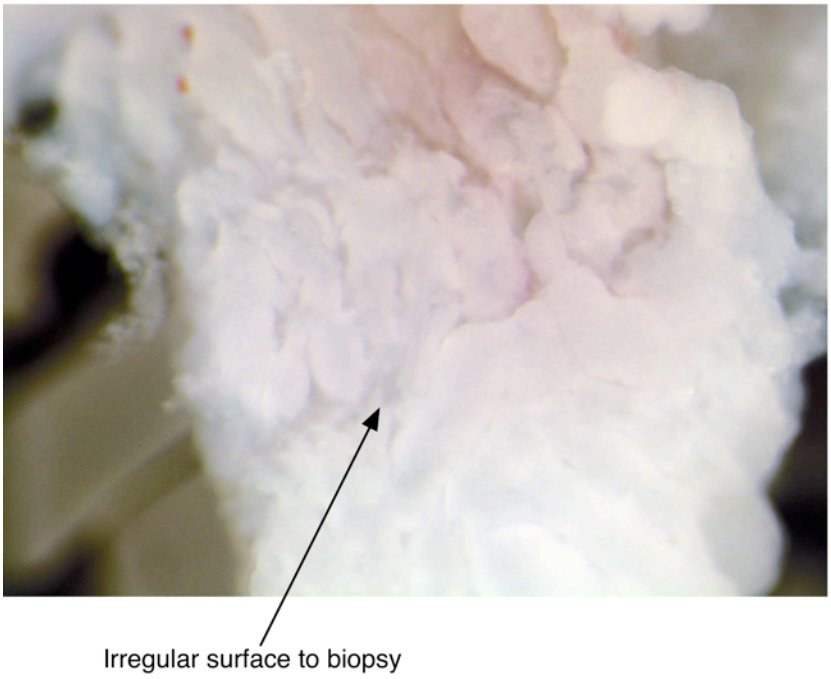


Figure 11: Stereo-microscopy image of oesophageal biopsy highlighting features of the luminal and basal surfaces

3.2.3.1 Validation of biopsy orientation technique

The validity of this orientation process was tested in 10 biopsies from 5 patients. Biopsies were mounted within the adaptors (see below) with the surface deemed the basal aspect facing upwards. A small volume of toludine blue dye was applied to this surface, thus selectively staining the expected "basal" aspect of the biopsy. The stained biopsy was then fixed immediately in formalin and stained with haematoxylin and eosin. Sections were embedded, cut, and light microscopy was used to confirm whether the correct orientation had been identified.

3.2.4 Ussing chamber experiments

The biopsies were placed into the Ussing chamber (Mussler Scientific Instruments, Aachen, Germany) using specially made adaptors (figure 12). The adaptors were cut from radiographic film, with a central aperture of 1.5 mm diameter (0.017 cm^2 area). The adaptors were scored on the side in contact with the biopsy to reduce the chance of biopsy slipping from covering the aperture. Biopsies were only studied further if they were clearly seen to be covering the aperture (overlapping all edges) under stereo-microscopy.

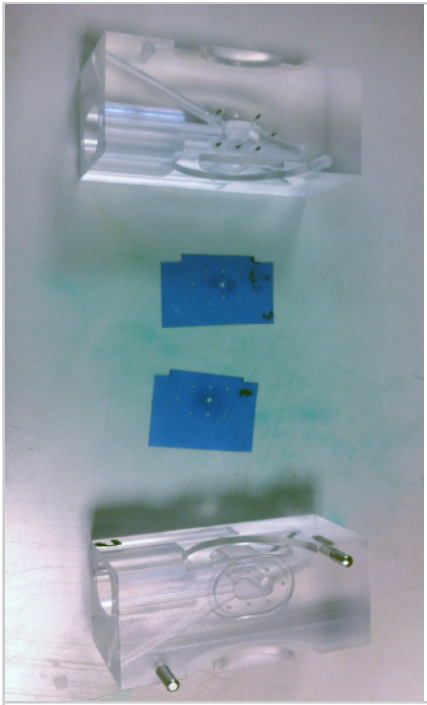
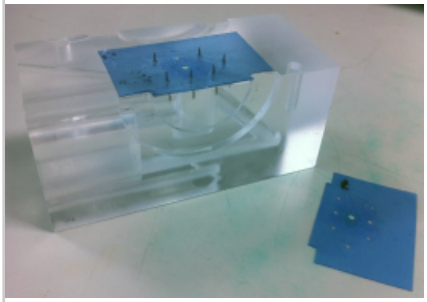
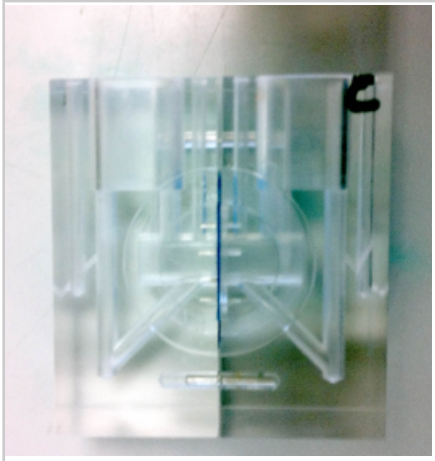
	The two halves of the Ussing chamber are seen with the biopsy adaptors.
	The biopsy adaptor fits over the half of the Ussing chamber, and the biopsy is placed to cover the aperture. The second adaptor is placed over the biopsy, and finally the two halves of the chamber are joined and fastened with metal "O" rings.
	The chamber and biopsies are seen placed together. In this orientation the luminal aspect of the biopsy will be facing the right half of the chamber as viewed in the picture.

Figure 12: Description of biopsy placement in Ussing chamber

Immediately on mounting the biopsies they were bathed on both luminal and basal sides with Krebs-Henseleit buffer at pH 7.4, 37°C, and the solution was continuously bubbled with carbogen gas. After making a correction for fluid and circuit resistance, transmucosal potential difference was continuously monitored with Ag/AgCl electrodes. The basal transepithelial resistance (TER) was calculated according to Ohm's law from the voltage deflections induced by bipolar current pulses of 50 μA, duration 200 ms every 6 seconds applied through platinum wires. All experiments were conducted in open-circuit conditions. The system was equilibrated at 37^0C until a stable TER baseline was established (typically 20 minutes). Biopsies with a baseline TER of less than 50 $\Omega.cm^2$ were excluded from further analysis since these were deemed to be unsatisfactory. Biopsies were also excluded if they did not demonstrate the characteristic curve to plateau of TER increase during the equilibration (pilot studies had indicated to us that if there was a leak, i.e. a hole due to incomplete covering of the chamber aperture by the biopsy, the TER pattern over the initial equilibration period was a flat line rather than the usual gradual increase to a plateau over 15 to 20 minutes).

After a stable baseline was achieved the solution in the "luminal" bath of the chambers was replaced with the "test solution", either:

1) Neutral solution: Krebs-Henseleit at pH 7.4, or
2) Weakly acidic solution: Krebs-Henseleit at pH 5 + 1 mg/ml porcine pepsin + 1 mM deoxycholic acid, or
3) Acidic solution: Krebs-Henseleit at pH 2 + 1 mg/ml porcine pepsin + 1 mM taurodeoxycholic acid (figure 13A).

Each subject had one biopsy exposed to one of the test solutions (i.e. with each

subject having three biopsies, each test solution could be studied for each subject). The biopsies were exposed to the test solution for 30 minutes.

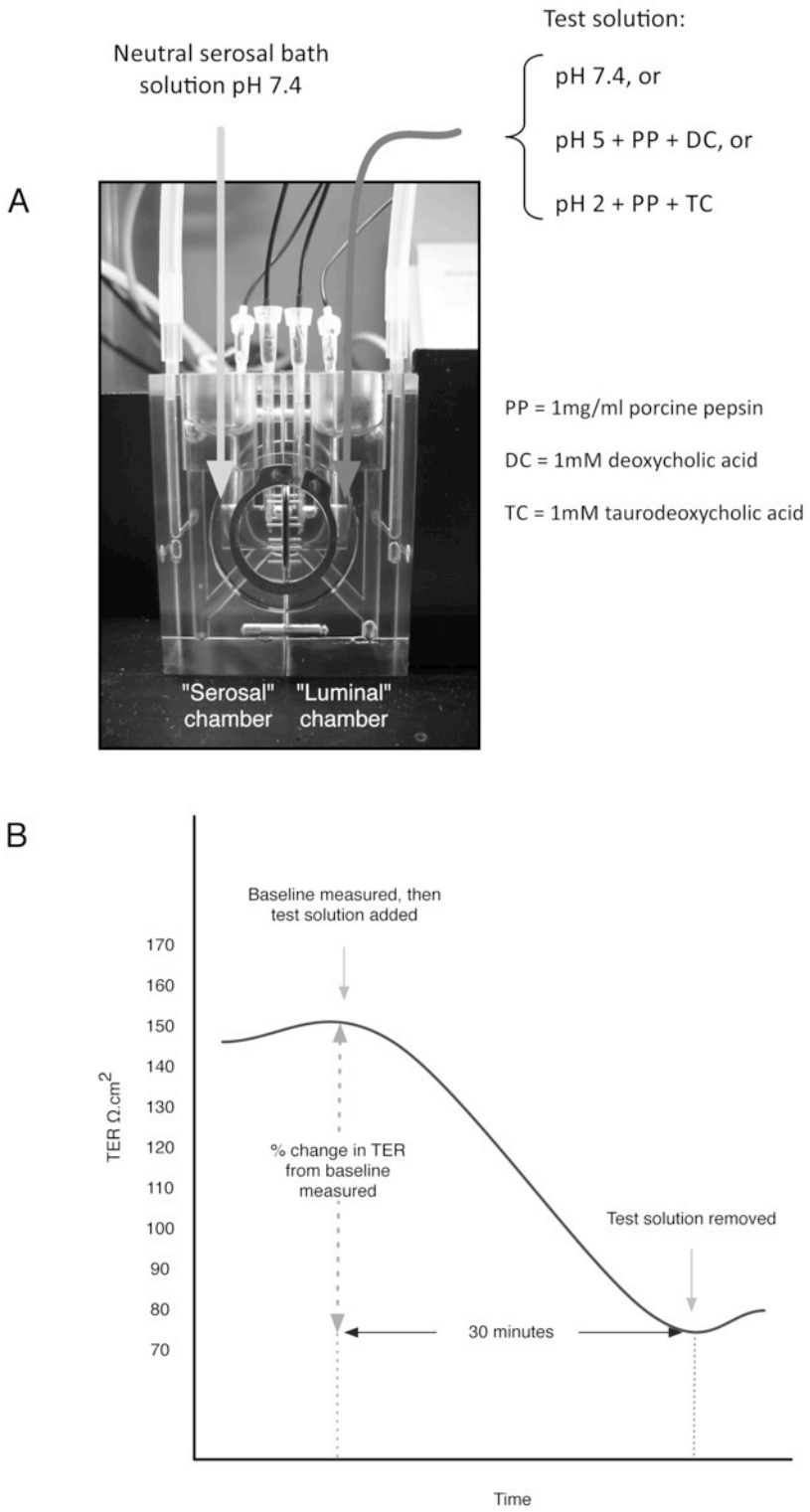


Figure 13: Study scheme **A** demonstrating placement of test solutions in Ussing chamber, and, **B** a representative TER recording

The TER was measured continuously throughout the exposure. The baseline TER was determined as the TER after equilibration, immediately before the "test solution" was placed in the luminal bath.

The change in TER caused by the test solution was expressed as a percentage change at the end of 30 minutes exposure, relative to the TER at the beginning of exposure (figure 13B).

After the exposure, 1 mg/ml fluorescein (375 Da) was placed in the basal chamber to exclude obvious leak across the biopsy into the opposite chamber (which would require results to be discarded).

3.2.5 Reproducibility study

It is important to assess the reproducibility of the methods used in this study, i.e. do two biopsies taken from the same patient respond to an acidic exposure in the same way? To investigate this 9 subjects were studied to assess repeatability of the Ussing chamber results. Biopsies were taken from subjects attending the Royal London Hospital endoscopy department for various upper gastrointestinal complaints, but without oesophagitis or Barrett's oesophagus. Two biopsies from the same subject were taken from 3 cm above the gastro-oesophageal junction, were immediately placed into Krebs-Henseleit solution at pH 7.4, then placed into two separate Ussing chambers. After equilibration, the "luminal" bath of each chamber was replaced with Krebs-Henseleit at pH 5 + 1mg/ml porcine pepsin + 1mM deoxycholic acid. Each biopsy was exposed to the weakly acidic solution for 30 minutes and percentage change in TER after 30 minutes was calculated. Consistency between the two biopsies' results was assessed by calculating Cronbach's alpha. Weakly acidic solution was chosen to test reproducibility since these solutions do not cause a fall in TER in all subjects, so consistency of response

within patients on exposure to this solution was deemed of particular importance.

3.2.6 Assessment of biopsy thickness and relationship with basal TER and change in TER on acid exposure

It is possible that the thickness of an oesophageal biopsy may influence the basal TER, and the percentage change on exposure to acid. To quantify biopsy thickness, fixation for histological analysis is needed. Unfortunately, if a biopsy is fixed for histology purposes, it cannot subsequently be studied physiologically in the Ussing chamber. Similarly, a biopsy fixed for histology after Ussing chamber study is unlikely to be representative of the biopsy pre-exposure.

To attempt to address this issue to an acceptable extent, a further study of 11 patients with typical reflux symptoms was conducted. The aim of this study was to:

1) Assess the consistency of the biopsy thickness (expressed as the number of epithelial layers) in biopsies taken by the study endoscopist (Dr Woodland).

2) Assess, in pairs of biopsies taken in parallel (i.e. 2 per subject from 3 cm above the gastro-oesophageal junction) the relationship between biopsy thickness, baseline TER, and drop in TER when exposed to the acidic solution.

From each subject one biopsy was fixed immediately in formalin, and the other biopsy was placed in Krebs-Henseleit solution at pH 7.4. The biopsy placed in Krebs-Henseleit was transferred to an Ussing chamber, and the basal TER and change in TER from baseline after 30 minutes exposure to an acidic solution (Krebs-Henseleit at pH 2 + 1mg/ml porcine pepsin + 1mM taurodeoxycholic acid) was calculated as described above.

The biopsy in formalin was placed in a frozen block and cut into frozen sections of 10 μm thickness using a cryostat. Biopsies were stained with haematoxylin and eosin, and sections from the centre of the biopsy were assessed by light

microscopy. The number of epithelial layers from basal to luminal aspect of the biopsy were counted.

The number of epithelial layers was correlated with the basal TER and percentage change in TER from baseline in the corresponding paired biopsy.

3.2.7 Statistical methods

All data are expressed as mean ± standard deviation unless otherwise stated. Normality of distributions was assessed using a D'Agostino and Pearson omnibus normality test. Comparison of basal TER between groups was done using a Mann Whitney U test. Differences in response to test solutions was assessed using ANOVA followed by Bonferroni's multiple comparison test. Reproducibility was tested by calculating Cronbach's alpha. Correlations were assessed using a Pearson r test. Significance was declared at $p<0.05$.

3.3 Results

3.3.1 Subjects

Of the 53 subjects from the main Ussing chamber study, 28 were from symptomatic patients, 25 were asymptomatic controls. 6 symptomatic and 3 control subjects were excluded due 2 or more biopsies being considered inadequate (by inadequate chamber aperture coverage or basal TER less than 50 $\Omega.cm^2$). 15 of the remaining 22 control subjects, and 17 of the remaining 22 patients had 3 biopsies able to be studied with each of the test solutions. The remaining 7 controls and 5 patients had 2 adequate biopsies and were tested only with the neutral and weakly acidic test solutions. None of the studied biopsies displayed evidence of fluorescein leakage when tested at the end of the experiment. As such, 22 symptomatic (mean age 49, range 20-76) and 22 control subjects (mean age 47, range 18-78) were studied in final analysis. All patients except for one were taking PPI at the time of endoscopy. No control subjects were on PPI. The further demographic and medical data of each group is displayed in table 1 below.

	Age	**Sex**	**Comorbidity**	**PPI therapy**	**Other therapy**	**Smoker**
PATIENTS						
1	49	M	Nil	Omeprazole 20mg[1]	Gaviscon Advance	Y
2	35	M	Asthma	Lansoprazole 30mg[1]	Salbutamol	N
3	45	M	Chronic pancreatitis	Omeprazole 20mg[1]	Loperamide, creon	Y
4	58	M	Diabetes mellitus, asthma	Lansoprazole 30mg[2]	Gliclazide, metformin, simvastatin, salbutamol	N
5	53	M	Diabetes mellitus	Omeprazole 20mg[1]	Simvastatin, metformin	N
6	51	F	Nil	Lansoprazole 30mg[1]	Nil	N
7	66	F	Hypothyroidism, hypertension	Lansoprazole 30mg[2]	Thyroxine, amlodipine	Ex

8	46	M	Nil	Lansoprazole 30mg[2]	Nil	Y
9	76	F	Polymyalgia rheumatica	Omeprazole 20mg[1]	Prednisolone, adcal	N
10	40	F	Nil	Esomeprazole 20mg[2]	Ranitidine	N
11	28	M	Asthma	Omeprazole 20mg[1]	Salbutamol, becotide	Y
12	54	M	Nil	Omeprazole 20mg[2]	Nil	Y
13	38	M	Hypothyroidism	Omeprazole 20mg[1]	Thyroxine	Ex
14	48	M	Nil	Omeprazole 20mg[2]	Nil	N
15	63	F	Nil	Lansoprazole 30mg[1]	Nil	Ex
16	20	F	Nil	Lansoprazole 30mg[1]	Nil	N
17	68	F	Epilepsy, hypertension	Omeprazole 20mg[1]	Epilim chrono, bendrofluazide	N
18	52	M	Hypercholesterolaemia	Nil	Simvastatin	N
19	68	M	Benign prostatic hypertrophy	Lansoprazole 30mg[1]	Tamsulosin	N
20	68	M	GIST	Omeprazole 20mg[1]	Nil	Ex
21	32	F	Nil	Lansoprazole 30mg[1]	Ranitidine	N
22	48	M	Ischaemic heart disease	Omeprazole 20mg[2]	Aspirin, atorvastatin, lisinopril, GTN	Ex
CONTROLS						
1	78	M	Hypertension, ischaemic heart disease	Nil	Aspirin, simvastatin, amlodipine, isosorbide mononitrate	Ex
2	32	F	Nil	Nil	Nil	Y
3	42	F	Hypertension	Nil	Ramipril	Ex
4	65	F	Diabetes mellitus, asthma	Nil	Gliclazide, ramipril	N
5	58	F	Migraine	Nil	Sumatriptan	N
6	40	F	Hypertension	Nil	Ramipril	N
7	43	F	Nil	Nil	Nil	N
8	25	F	Nil	Nil	Nil	N
9	54	F	Nil	Nil	HRT	N
10	20	F	Nil	Nil	Nil	N
11	39	M	Hay fever	Nil	Cetirizine	Y
12	18	M	Nil	Nil	Loperamide	N
13	72	M	Diabetes mellitus	Nil	Metformin	Ex
14	21	F	Nil	Nil	Nil	N
15	39	F	Nil	Nil	Nil	N
16	78	M	COPD	Nil	Seretide	Ex
17	52	M	Nil	Nil	Nil	Ex
18	59	M	Nil	Nil	Nil	Y
19	53	M	Diabetes mellitus, hypertension	Nil	Gliclazide, metformin, simvastatin, ramipril	Y
20	69	F	Hypercholesterolaemia	Nil	Atorvastatin, loratidine	N
21	41	M	Nil	Nil	Nil	Y

22	59	F	COPD	Nil	Seretide	Ex

Table 1: Study patient characteristics. M=male; F=female; COPD=chronic obstructive pulmonary disease; GIST=gastrointestinal stromal tumour; [1]=once daily; [2]=twice daily; GTN=glyceryl trinitrate

In summary the mean age in the symptomatic patient group was 50 (range 20 – 76), and in the control group was 48 (range 18 – 78). There were 8 females in the patient group and 13 females in the control group. All symptomatic patients except one were taking current PPI. The one who had stopped had been off PPI for 3 months due to perceived lack of response.

3.3.2 Validation of biopsy orientation technique

10 biopsies were assessed for accuracy of orientation using the criteria mentioned. On haematoxylin and eosin staining, all biopsies were confirmed to be correctly orientated. An example of a correctly stained specimen is seen in figure 14.

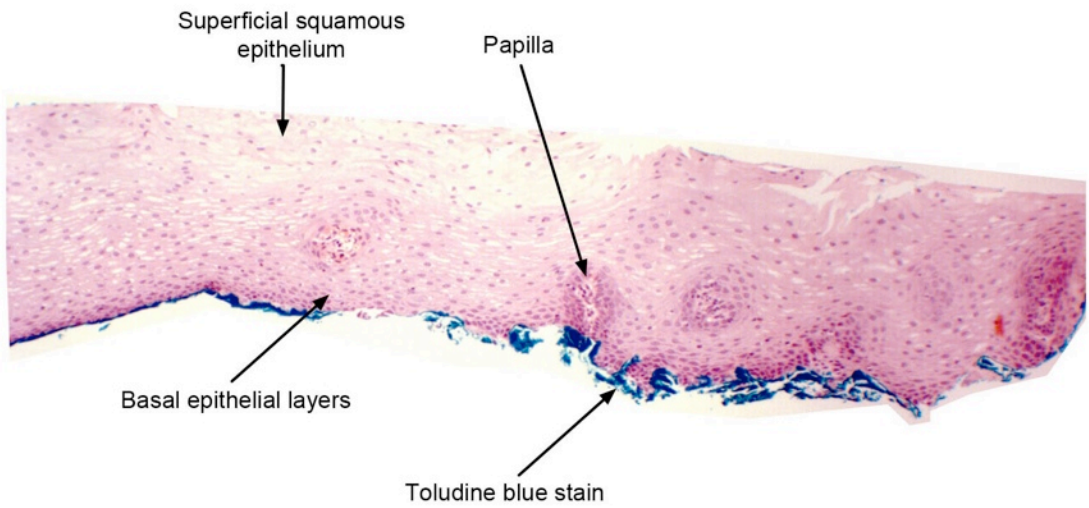


Figure 14: A haematoxylin and eosin stained oesophageal mucosal biopsy demonstrating additional toludine blue staining at the basal surface

3.3.3 Ussing chamber studies

3.3.3.1 Baseline transepithelial electrical resistance

Oesophageal biopsies in symptomatic patients had a mean TER baseline of 115 ± 30.1 Ω.cm^2. In controls the mean TER baseline was 107 ± 49.8 Ω.cm^2. There was no significant difference between these values (p=0.15, figure 15).

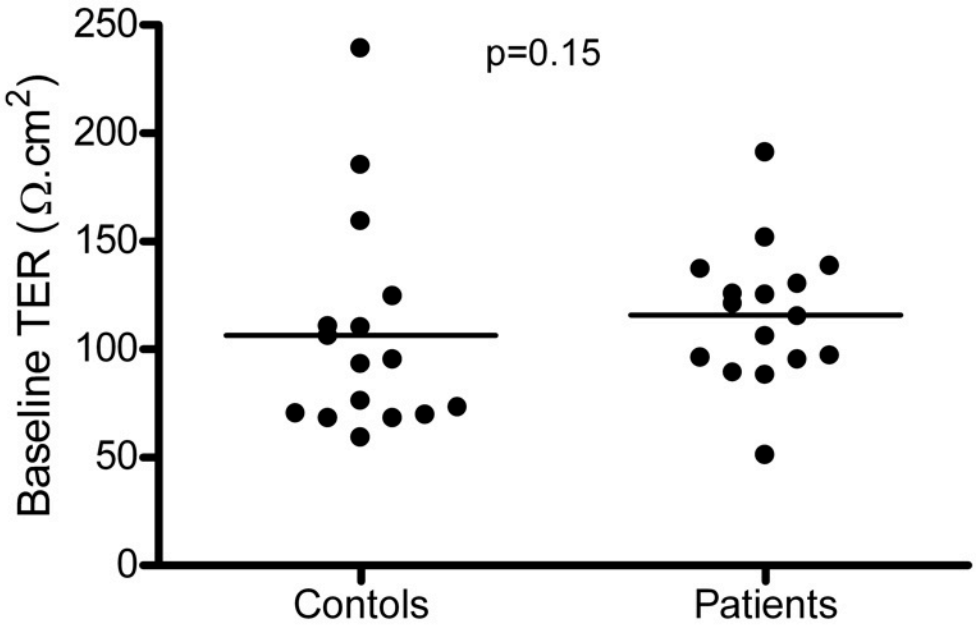


Figure 15: Baseline TER in control subjects with no upper gastrointestinal symptoms, and in patients with symptomatic heartburn

3.3.3.2 TER response to test solution exposure

After 30 minutes exposure to the neutral test solution the mean percentage change from baseline was 3.0 ± 7.3% in symptomatic patients, and was 7.0 ± 7.3% in controls. There was no significant difference in TER change on neutral exposure between the two groups (p=0.14).

When all subjects are taken into account (symptomatic patients and controls), 30-

minute exposure to the weakly acidic test solution caused a very small decrease in TER from baseline (-1.6 ± 10.1%, n=41). Exposure to the acidic test solution caused a larger decrease in TER (-14.4 ± 15.3%, n=32) than seen for neutral and weakly acidic solutions (p<0.0001 for both comparisons). Figure 16 demonstrates this.

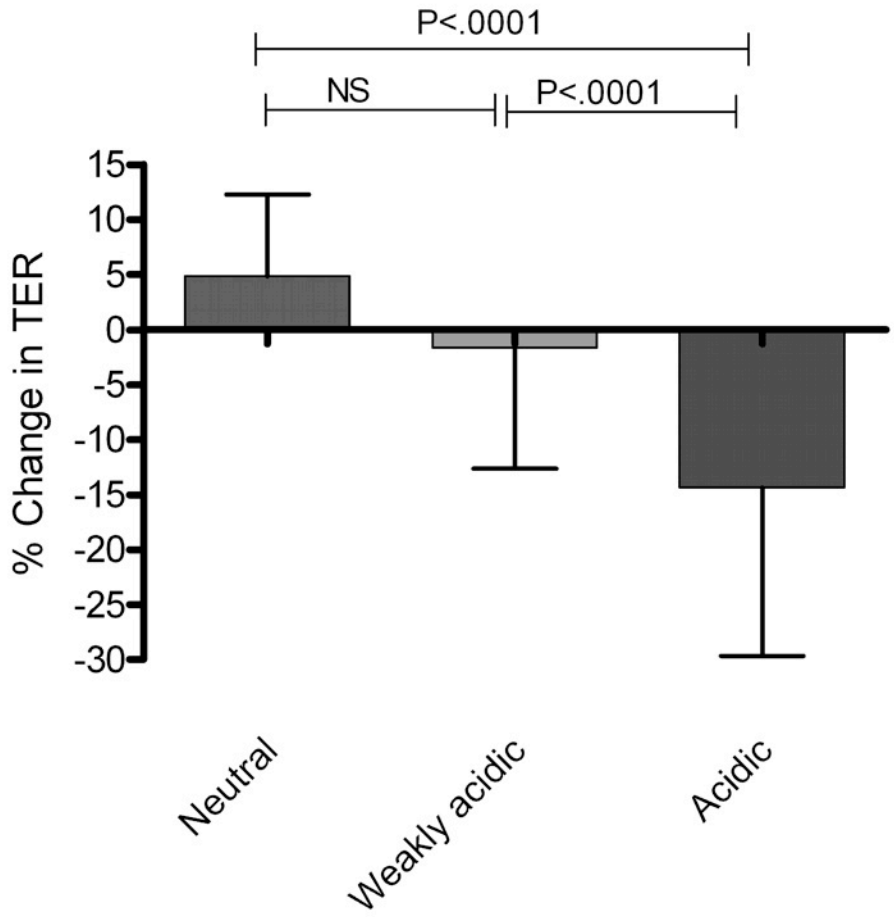


Figure 16: Percentage change in TER from baseline in all subjects (symptomatic patients and controls) on biopsy exposure to neutral, weakly acidic and acidic solutions

When comparing the change in TER that occurs in response to test solutions in symptomatic patients and control subjects, one can see a differential effect. Thirty minutes exposure to the weakly acidic test solution caused a greater fall in TER in symptomatic patients than in controls (-7.2 ± 5.5%, n=19 vs. 3.2 ± 7.3%, n=22

p<0.05). Likewise 30 minutes exposure to the acidic test solution caused a greater fall in TER in symptomatic patients than in controls (-22.8 ± 11.9%, n=15 vs. -9.4 ± 15.1%, n=17, p<0.01, figure 17).

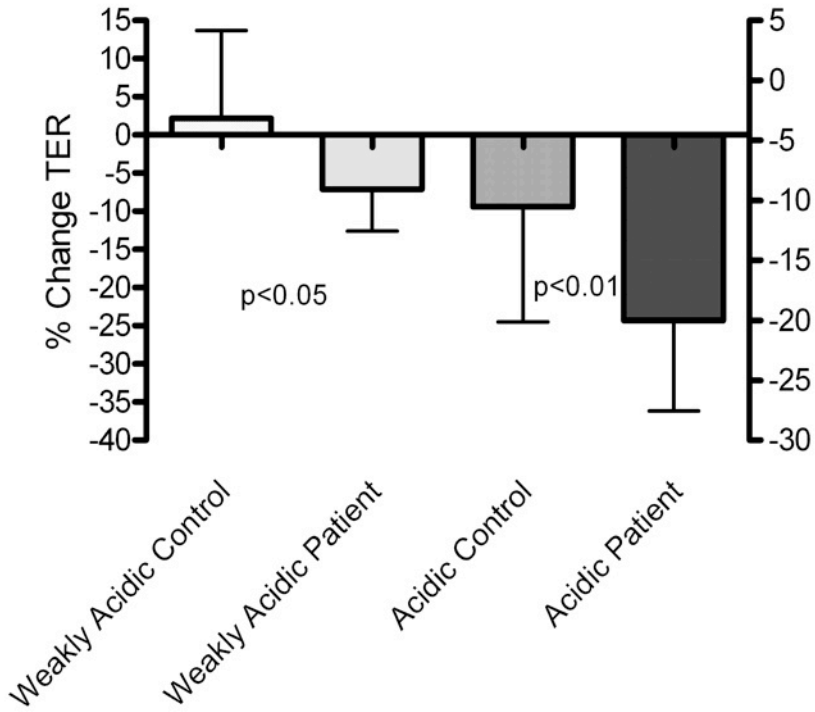


Figure 17: Differential TER response on exposure to weakly acidic solution (left) and acidic solution (right) in control subjects and symptomatic patients

3.3.4 Reproducibility study

The calculated Cronbach's alpha for the biopsy pairs in this study was 0.81, representing good test-retest reliability with this methodology (table 2). It is acknowledged that the patient phenotype studied was more heterogeneous than for the other studies, but it is expected that re-test consistency should not be affected by this.

	Percentage change in TER after 30 mins exposure to weakly acidic solution	
Subject	**Biopsy 1**	**Biopsy 2**
1	-20.6	-8.1
2	-2.3	-5.4
3	2.3	7.4
4	0.0	1.2
5	-6.2	-7.1
6	6.7	9.0
7	-6.4	-11.2
8	0.2	-1.5
9	-2.4	-11.1

Table 2: Results of reproducibility study

3.3.5 Assessment of biopsy thickness and relationship with basal TER and change in TER on acid exposure

The results from the 11 sets of paired histology/Ussing chamber studies are presented in table 3.

Biopsy pair	Number of epithelial layers	TER baseline ($\Omega.cm^2$)	Change in TER from baseline on exposure to acidic solution (%)
1	27	95	-1.2
2	29	280	-52.7
3	29	81	-22.9
4	30	68	-63.2
5	31	70	-12.9
6	31	119	-2.7
7	35	155	-22.7
8	36	119	-23.5
9	37	285	-7.5
10	41	167	-20.4
11	46	233	-23

Table 3: Assessment of relationship between biopsy thickness and TER

Thus the median number of epithelial layers in the biopsies was 31, with a

standard deviation of 4.8.

There was no significant correlation between the number of epithelial cell layers and the baseline TER in the corresponding paired biopsy (r=0.4, p=0.28, figure 18). In addition, there was no significant correlation between the number of epithelial cell layers and the change in TER on exposure to the acidic solution in the corresponding paired biopsy (r=0.57, p=0.11, figure 19).

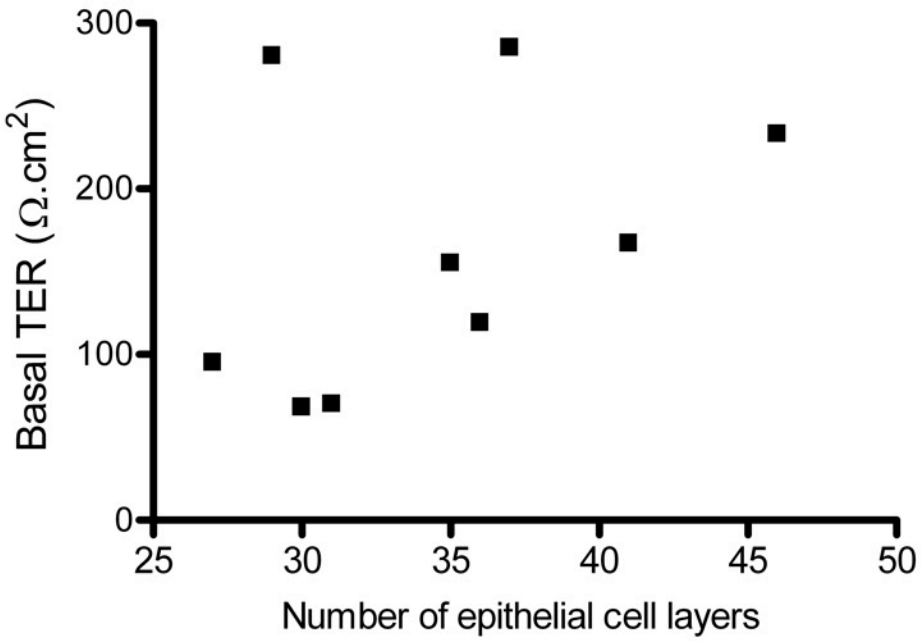


Figure 18: Correlation of number of epithelial layers on histological specimen with the baseline TER of the corresponding biopsy.

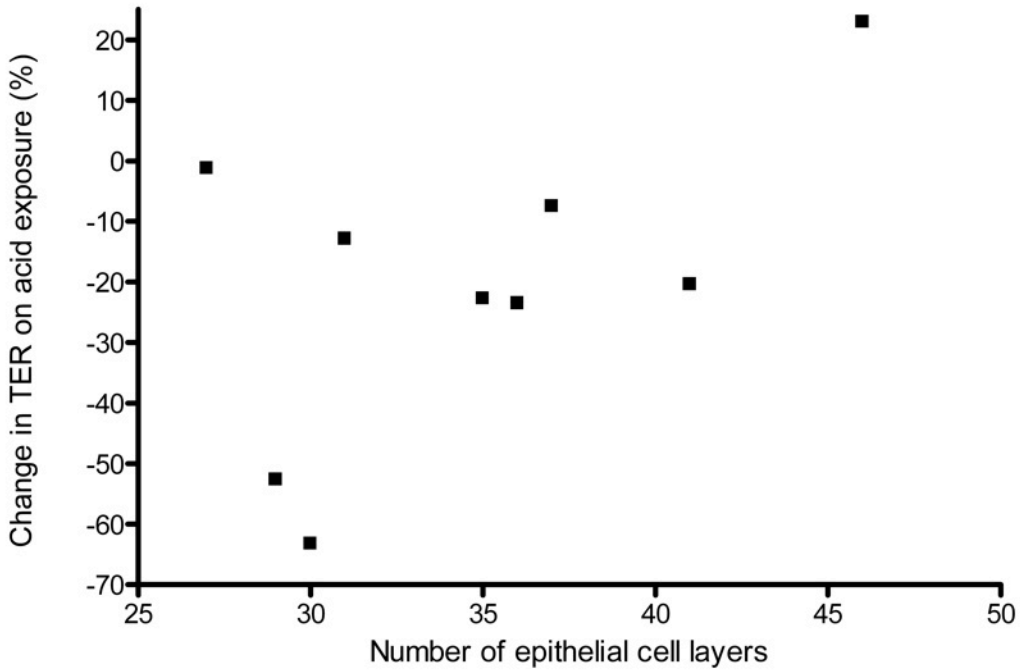


Figure 19: Correlation of number of epithelial layers on histological specimen with the change in TER on exposure of the corresponding paired biopsy to an acidic solution.

3.4 Discussion

The hypothesis of this study was that patients with heartburn without endoscopic mucosal erosions have a persistent mucosal vulnerability to acid that can be detected during continuous TER measurements in their biopsies.

The study results were the following:

1) Exposure of human oesophageal mucosal biopsies to acidic solutions (containing pepsin and bile acids) provokes a decrease in transepithelial electrical resistance (TER).
2) The fall in TER observed in biopsies from patients with symptoms is significantly more pronounced than that observed in biopsies from asymptomatic controls.
3. In symptomatic patients (but not controls), even exposure to weakly acidic solutions provokes a significant drop in TER.

In healthy subjects, a degree of gastro-oesophageal reflux is physiological and asymptomatic. In contrast, in patients with gastro-oesophageal reflux disease, contact with the oesophageal mucosa by gastric contents results in symptoms such as heartburn. The reason for a symptomatic perception of a reflux event may be due to a variety of factors including the chemical and physical properties of the refluxate, the sensitivity of nociceptors and afferent nerve fibres, and central sensitivity. However a significant contributor may be the barrier integrity of the oesophageal mucosa. The human stratified squamous epithelium forms a tight protective barrier against the noxious components of gastroesophageal refluxate.

Both injurious factors (e.g. duration of acid exposure[60], and presence of pepsin and bile acids[80, 82]) and defensive factors (e.g. acid buffering, acid transport and tissue repair[293]) determine mucosal integrity. The oesophageal epithelial apical cell membranes and apical junctional complexes provide a permeability barrier that prevents the permeation of noxious substances into the cells and intercellular spaces[121]. The apical junctional complexes are formed by tight junctions, adherens junctions and desmosomes, and act as an effective barrier to paracellular ion movement[293].

The oesophageal mucosa of symptomatic patients may not be completely normal, and often demonstrates dilated intercellular spaces (DIS). DIS usually resolves in parallel with symptom resolution on treatment with PPIs[166]. A proportion of patients with remaining symptoms in spite of PPI treatment show persistence of DIS in oesophageal biopsies[167]. It is possible that patients with symptomatic reflux disease have an excess vulnerability to acid or weak acid that means their barrier integrity is further impaired on exposure compared to control subjects. Furthermore, a significant group of patients not responding well to PPI, including NERD (40%) and functional heartburn (80%) have no DIS[167]. It is possible that these symptomatic patients without DIS still have a functional mucosal impairment that might potentially underlie their symptoms. That is why in this study a method was used that allows detection of subtle differences in *dynamic* mucosal behaviour when exposed to a noxious solution regardless of the mucosal basal status. Indeed, the results showed that patients and controls had similar basal mucosal electrical resistance, but they differed in their response to a “stress” test i.e. exposure to acid and weak acid solutions containing pepsin and bile acids.

The use of TER as a functional marker of mucosal integrity might have advantages over the “static” measurement of DIS. It allows dynamic measurement of changes

in integrity e.g. during an exposure to acid and assessment of pharmacological intervention over time. Previous studies of dynamic properties of oesophageal resistance in response to acid challenge have been limited mostly to animals[148, 153]. The results of this study enable us to make a comparison between TER measurements in human oesophageal tissue, and that found in previous animal studies. An immediately noticeable finding is that the baseline TER in the human mucosa is much lower than that seen in other animals. In Ussing chamber studies of rabbit oesophageal mucosa, Farré *et al.* found baseline TER values in the range of approximately 1500 to 2500 $\Omega.cm^2$ [153]. The separate group of Tobey *et al*. also measured baseline rabbit oesophageal mucosal TER to be approximately 2000 $\Omega.cm^2$ [148]. However, our human data appears in keeping with other groups who have looked at baseline TER of biopsies in Ussing chamber models. Jovov *et al*. in the USA have measured baseline human oesophageal mucosal TER to be between approximately 70 to 300 $\Omega.cm^2$ [133], and Weijenborg *et al.* in the Netherlands have found values between approximately 70 to 125 $\Omega.cm^2$ [294]. Our human oesophageal baseline TER values were between 68 and 285 $\Omega.cm^{2,}$ concurring with the other groups' findings. A part of the reason for this discrepancy between rabbit and human baseline TER may be due to size of the tissue sample. In the aforementioned studies rabbit oesophagus was cut in sections and mounted in chambers with an aperture of 0.3 to 1.2 cm^2. In the human studies an aperture of 0.017 cm^2 was used. There is considerably more "edge effect" at smaller aperture sizes: i.e. there is inevitably damage at the edge of the biopsy due to pressure from the apposing halves of the chamber, and for a smaller aperture the circumference where this damage occurs is a higher overall proportion of the tissue being studied. This effect should be a constant for all of the experiments. However, we have conducted comparative studies using human oesophageal mucosal sections

taken from surgical resections and mounted in chambers with a 0.5 cm^2 aperture. These still had a much lower baseline TER than seen in rabbits (approximately 300 to 400 $\Omega.cm^2$). This suggests an inherent difference in the baseline integrity characteristics of rabbit and human oesophageal mucosa. Since the TER is formed almost entirely from characteristics of paracellular ion diffusion, it suggests that this pathway is more ionically permeable in humans than in rabbits. Differences in resistance in this pathway are likely to be due to differences in the tight junction-apical membrane morphology and/or function between species, but this is as yet untested.

Another noticeable feature of the baseline TER in our study, and in that of others, is that there is a large (almost 5-fold) variability in values from the lowest to the highest, even in healthy subjects. This appears to be a wide variability for a physiological measurement and is thus far unexplained. It is possible that it in part is reflective of variations that are inherent to the technique (e.g. size of biopsy, , trauma during biopsy process, degree of edge effect), and as such is illustrative of limitations of thus method of study. We also know that variations in tight junction expression can result in wide variations in TER in cell culture lines[124, 125]. It would be very interesting to examine tight junction expression in the context of this TER variability. The focus of the measurements in this study is on dynamic changes in TER on exposure to acid, and the use of percentage changes is an attempt to control for the variability in baseline TER. As such we hope that some of the effect of the variability is mitigated by the use of these measurements.

The dynamic changes in TER that occur on acid challenge in this study reveal an apparent distinct vulnerability of the mucosa in patients with typical reflux symptoms. The reason for this increased vulnerability is unclear. It is possible that the chronic effects of acid exposure causes a fragility to the normal oesophageal

barrier mechanisms (e.g. tight junctions) against the refluxate, or it is possible that the vulnerability is the initial (perhaps genetic) pathology that favours symptomatic perception of reflux events. The reason for heartburn in the absence of mucosal erosions is incompletely understood. It is highly probable that the mucosal barrier integrity is only part of a complex interaction between the refluxate, epithelial cells secretion, the activity of oesophageal nociceptors, and sensitivity of afferent nerves[295-297]. Thus, whilst the small difference in mucosal integrity seen between patients and controls in this study appears clear, this difference may only explain a proportion of the symptom pathogenesis in non-erosive gastro-oesophageal reflux disease. The barrier dysfunction observed might be an initial event that can be further amplified by the other mechanisms and, therefore, the initial weakening of the mucosal barrier may be an essential pathophysiological event.

This study assessed the *in vitro* oesophageal mucosal handling of solutions simulating both the "off" and "on" PPI condition. During reflux in "on" PPI conditions, the oesophageal mucosa is exposed to gastric contents in the range pH 4-6.5[56]. Gastric bile acid concentrations can be between 0.3 and 2 mM[64-66]. We used different bile acids for our weakly acidic and acidic solutions. Whereas taurodeoxycholic acid is present in oesophageal aspirates in gastro-oesophageal reflux patients not taking PPIs, unconjugated bile acids such as deoxycholic acid are seen in higher concentrations in patients "on" PPI. This is likely to be a result of gastric bacterial overgrowth and subsequent bacterial bile acid deconjugation in the less noxious gastric pH environment that occurs "on" PPI[67]. Previous animal experiments have assessed the effect of different bile acids on oesophageal mucosal integrity[161]. These studied taurodeoxycholic acid, deoxycholic acid, and glycocholic acid in acidic (pH 2) and weakly acidic (pH 5) solutions using an Ussing

chamber model. They found that there was a dose-dependent reduction in TER (at 0.5, 2 and 5 mM concentrations), however most profound reductions were seen with taurodeoxycholic acid and glycocholic acid in acidic conditions, and taurodeoxycholic acid and deoxycholic acid in weakly acidic conditions.

As expected, in the present study the effect of acid solutions on mucosa integrity was significantly stronger than that of weakly acidic solutions. However, the latter also showed a differential mucosal behaviour between symptomatic patients and controls. It caused a fall in mucosal TER in symptomatic patients, but not in controls. This suggests that, in patients, mucosal vulnerability is such that weakly acidic refluxates may produce changes underlying clinical observations of weakly acidic reflux-symptom association in some patients with refractory gastro-oesophageal reflux disease[298].

It is noteworthy that, at baseline, there were no differences in integrity seen between patients and controls. This is in keeping with recent studies that have demonstrated no difference between baseline TER in patients and controls[294], or between PPI-refractory and PPI-responsive reflux patients[299]. In contrast, studies using *in vivo* impedance have demonstrated lower impedance in gastro-oesophageal reflux disease patients than in patients with functional heartburn (Chapter 4 of this thesis) or controls[156]. There are a number of possible explanations for this. First, the size of the tissue studied in an Ussing chamber is much smaller than studied using impedance. Second, there are other factors that may affect resistance (e.g. saliva, blood flow, bicarbonate secretion) *in vivo* that are not seen *in vitro*. Finally, it is likely that there are inherent inconsistencies in the biopsy technique that are not present in impedance. This study has found that there is a variation of biopsy thickness that occurs, even when the same endoscopist takes the biopsies (range 27 to 41 epithelial cell layers). Although we

found that there was no significant correlation between epithelial thickness and baseline TER, it is appreciated that the lack of statistical significance does not rule out an association, particularly since the sample size is fairly small. In addition, impedance measures a circumferential area of mucosa, but a biopsy is only a sample of a few millimetres of a mucosal region. We know that acid exposure can vary circumferentially at the distal oesophagus (e.g. more exposure in the furrows of the folds), and this cannot be easily controlled for in the biopsy technique. It is possible that these variable in baseline TER add greater weight to the importance of the tissue response to acid challenge that is seen in the current study, rather than relying on static baseline measurements.

Within the group of patients with heartburn without oesophagitis in this study, there were probably patients with erosive disease healed by previous PPI treatment, "real" non-erosive reflux disease patients, and functional heartburn patients. Those with erosive disease healed by PPI had troublesome heartburn symptoms at the time of (normal) endoscopy. We would expect that 25-30% of our patients had functional heartburn[45, 300, 301]. It is possible that the difference of *in vitro* mucosal behaviour between symptomatic subjects and controls is accounted for entirely by the GORD-NERD subgroup, and the functional heartburn group responded in the same way as controls. This would mean that the differences observed between patients and controls could have even been slightly underestimated. An alternative interpretation could be that the mucosa in functional heartburn is not entirely normal. Although functional heartburn patients do not display DIS on electron microscopy[167], changes in TER may still occur and contribute to these patients' symptoms. Patients with functional heartburn have been shown to be more sensitive to oesophageal acid perfusion than controls[208]. Whilst this may represent a central phenomenon, it is possible

that this hypersensitivity may be in part due to subtle mucosal integrity impairment. This possibility deserves further investigation.

In conclusion, the present study showed that there is oesophageal mucosal vulnerability to refluxate-like solutions in patients with heartburn without oesophagitis when compared to controls. This apparent impaired acid handling offers new insight into the pathophysiology of symptomatic gastro-oesophageal reflux disease.

CHAPTER 4

In vivo evaluation of acid-induced changes in oesophageal mucosal integrity and acid sensitivity in non-erosive reflux disease

CHAPTER 4: IN VIVO EVALUATION OF ACID-INDUCED CHANGES IN OESOPHAGEAL MUCOSA INTEGRITY AND SENSITIVITY IN NON-EROSIVE REFLUX DISEASE

4.1 Introduction and aims

Most studies of oesophageal integrity thus far have involved "static" measures of integrity in the form of morphological changes (DIS) and baseline measures of ionic permeability, often in animals. Human oesophageal mucosal integrity is unlikely to be a static phenomenon, but more likely is a dynamic phenomenon reflecting damage from gastro-oesophageal reflux events, and the mucosal capacity to recover its integrity after this damage. Dynamic changes in human oesophageal mucosal integrity on exposure to reflux-like solutions *in vitro* have been described in Chapter 3. However, to better understand the pathophysiology of the mucosa in non-erosive reflux disease, *in vivo* studies are desirable, and measurement of the recovery capacity of the mucosa would be of interest.

Multichannel oesophageal intraluminal impedance is a technique that has been developed to complement pH measurements in reflux studies[154]. It allows detection of the movement of a bolus through the oesophagus. It does this by measuring the change of current flow between a pair of electrodes. The current is not able to pass directly along the catheter, so it must pass through a material external to the catheter that bridges the gap between the electrode pair. Liquids (containing ions, such as gastro-oesophageal refluxate) are excellent electrical conductors and cause a fall in impedance as it bridges the electrode pair. In contrast, air is a very poor conductor and so when it bridges the electrode pair (as

happens during a belch) there is a very sharp rise in impedance. Most modern impedance catheters have several (usually six) pairs of impedance electrodes spanning the oesophagus. This allows assessment of the direction of bolus flow in the oesophagus (figure 20). Most catheters incorporate pH sensors allowing assessment of the acidity of this bolus movement. Combined pH-impedance is a very sensitive tool for reflux measurement[298], and unlike conventional pH-monitoring it allows detection of non-acidic reflux, and is able to distinguish refluxed from swallowed acid (e.g. as found in some drinks such as orange juice and cola).

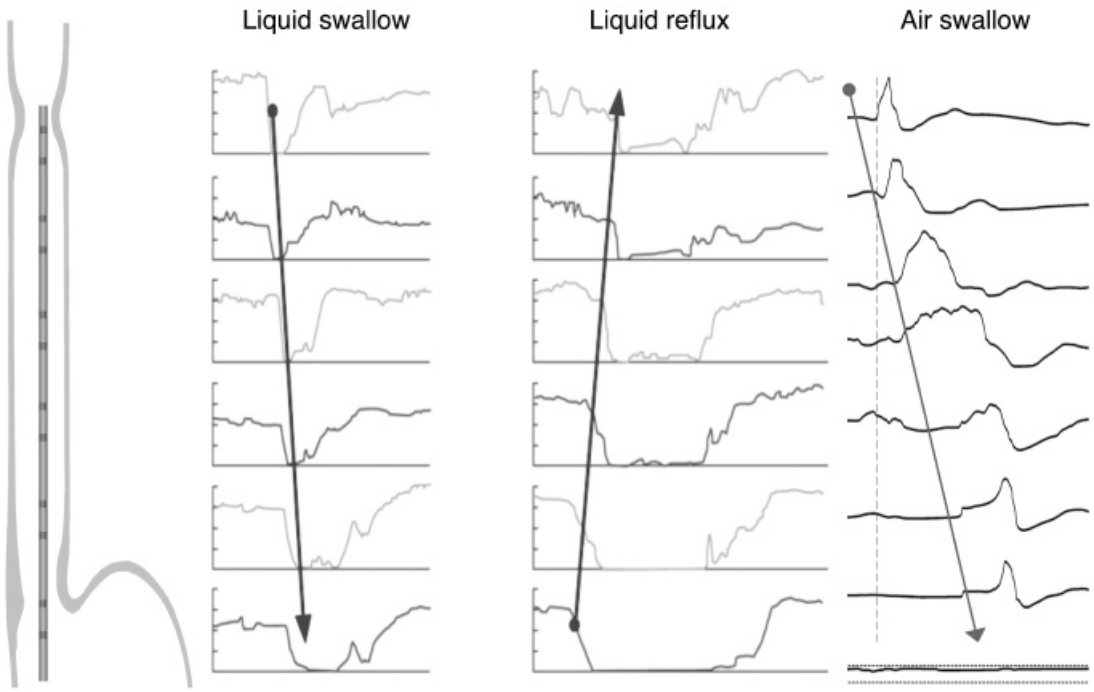


Figure 20: Illustration of impedance during liquid and air passage in the oesophagus. In the left panel there is a liquid swallow causing anterograde passage of low impedance liquid from proximal to distal catheter. The middle panel shows a liquid reflux event, where passage of low impedance liquid occurs in anterograde direction. In the right panel there is an air swallow characterised by anterograde passage of high impedance air from proximal to distal catheter

The empty oesophagus, as found in between swallows and reflux events, is collapsed. In the empty oesophagus it is the oesophageal mucosa that bridges the space between impedance electrode pairs, and thus it is the mucosa that offers the resistance to direct current flow. It would follow that a more electrically tight (less permeable to ionic flow) mucosa should offer higher impedance than a reflux-damaged mucosa with disruption of epithelial tight junctions. Indeed, oesophageal impedance (when measured at baseline, figure 21) has recently been highlighted as a potential surrogate tool for *in vivo* assessment of oesophageal mucosal integrity.

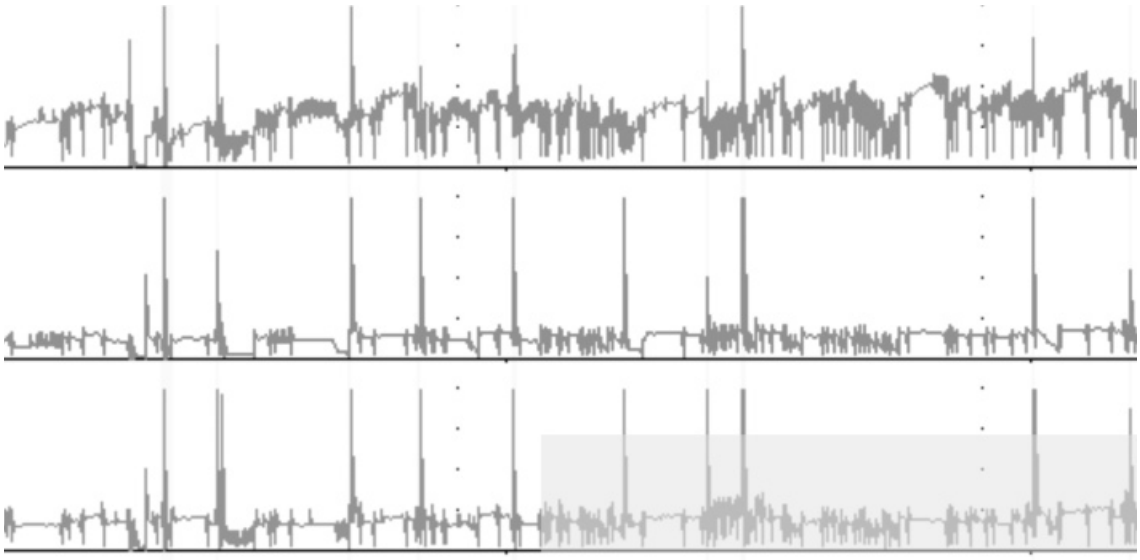


Figure 21: The baseline impedance can be calculated by taking a mean impedance measurement from an impedance segment (usually the most distal) over a period of time (e.g. 10 minutes – shaded area). When measured for such a period with a high sample frequency (e.g. 50 Hz) the mean impedance is a good representation of the correct baseline

It has previously been shown that patients with overtly damaged mucosa (Barrett's oesophagitis and erosive oesophagitis) have significantly lower distal oesophageal mucosal impedance than patients with non-erosive reflux disease[155].

It has also been shown that, within patients with non-erosive reflux disease, those with higher oesophageal acid exposure have lower baseline distal oesophageal impedance values (correlation between baseline oesophageal impedance and 24-hour oesophageal acid exposure (%): r=-0.7, p<0.001, figure 22). Furthermore this impedance increases after treatment with proton pump inhibitors[156].

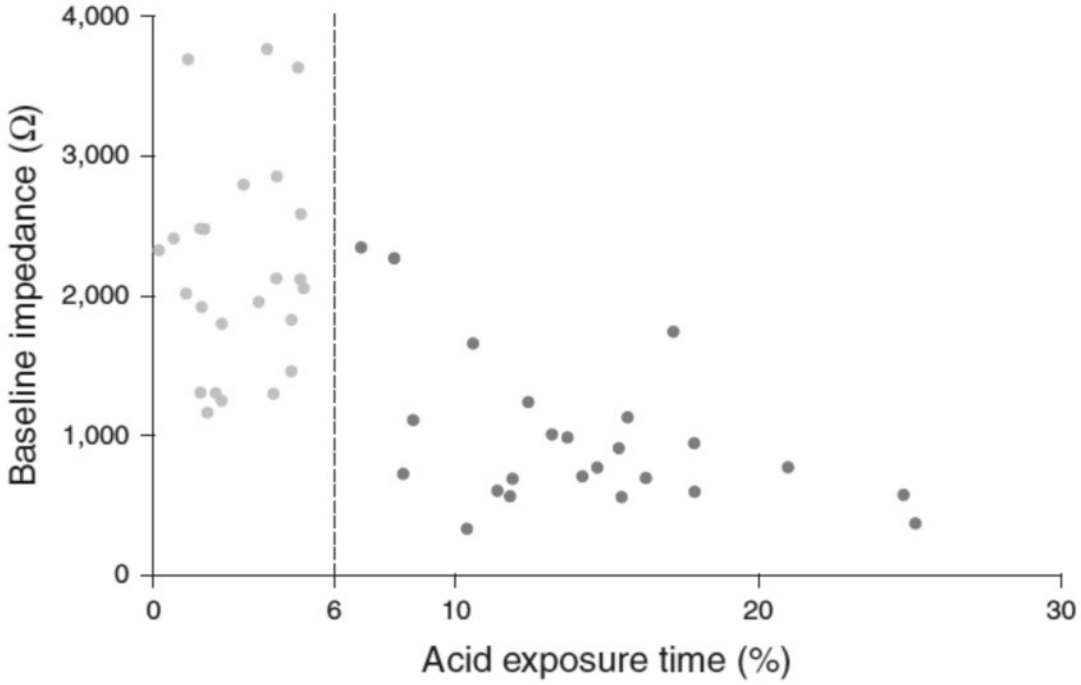


Figure 22: Correlation between baseline oesophageal impedance and 24-hour oesophageal acid exposure time. From Kessing *et al*. Am J Gastroenterol 2011

Further support for a relationship between impedance and oesophageal mucosal integrity came from a study in 2011 by Farré *et al*.[157]. This study included animal and human data, and showed that there was a positive correlation between *in vivo* baseline impedance and *in vitro* TER measurements (r=0.72, p=0.002). Furthermore, it demonstrated that when the oesophagus is infused with acid in healthy human subjects a drop in impedance was observed followed by an

incomplete recovery to baseline. In this study the mean impedance baseline before acid infusion was 3256 ± 1165 Ω, and after completion of the acid perfusion (pH 1 solution at 2 ml per minute for 30 minutes) this had fallen to 1378 ± 291 Ω. There was incomplete impedance recovery to baseline even at 2 hours post-perfusion (to a mean of 1550 Ω).

It is possible that the speed of this recovery of the impedance back towards baseline may reflect the health of the oesophageal mucosa. In addition, it may be of pathophysiological significance. The barrier hypothesis of non-erosive reflux disease suggests that an impaired barrier function will leave the subject vulnerable to symptomatic perception of a reflux event. If, after an acid reflux event, the mucosal barrier integrity is impaired (as suggested in chapter 3), and then remains impaired for a period of time, the subject may remain vulnerable to symptomatic reflux events until integrity is restored. Perhaps a clinical correlate of this is the finding that a prior recent acid reflux burden is associated with an increased likelihood of symptomatic reflux perception[210, 213].

Thus far, the *in vivo* dynamic properties of mucosal integrity in patients with reflux symptoms have not been studied. Oesophageal mucosal integrity, as expressed by baseline impedance, is probably a dynamic process reflecting 1) the damaging effect of repeated acid reflux events and 2) the mucosal capacity to recover integrity.

Historically the acid perfusion test has been used in assessment and diagnosis of gastro-oesophageal reflux disease. It was first introduced in 1958 by Bernstein and Baker as an objective method to identify chemosensitivity to acid[302]. It uses a nasogastric tube to deliver first a control solution of 0.9% sodium chloride and then pH 1 hydrochloric acid into the mid-oesophagus. The test was used to establish if acid infusion reproduces the patient's symptoms (and originally to

distinguish between chest pain of cardiac and oesophageal origin). It is still used in clinical practice by some centres, and continues to be used as a research tool to assess oesophageal chemosensitivity to acid. It also serves as a potential tool to deliver a standardised acid provocation challenge to the oesophagus.

The hypothesis of the study presented in this chapter is that there is a relationship between slow recovery of mucosal integrity after acid exposure, mucosal vulnerability (low baseline impedance) and increased perception of reflux episodes.

The aim of the study is to evaluate, *in vivo,* the integrity of oesophageal mucosa in basal conditions and during exposure to acid using oesophageal baseline impedance monitoring. Furthermore, it aims to compare the aforementioned oesophageal mucosa functional behaviour *in vivo* between patients with non-erosive reflux disease and patients with functional heartburn.

4.2 Material and methods

4.2.1 Patients

50 patients (25 male and 25 female, mean age 44, range 20 to 68) were studied at the upper gastrointestinal physiology unit at the Royal London Hospital. All patients had undergone prior upper gastrointestinal endoscopy either at the Royal London or at their local hospital, and all patients had oesophageal manometry at the unit prior to reflux testing.

Patients were selected consecutively on fulfilling the entry criterion, which was that the predominant complaint for investigation was of typical reflux symptoms (i.e. heartburn and/or regurgitation).

Exclusion criteria were 1) The presence of erosive oesophagitis or Barrett's oesophagus on endoscopy. 2) The presence of major oesophageal motility abnormality (achalasia, absent peristalsis) on oesophageal manometry.

Oesophageal reflux monitoring and acid sensitivity testing was done as part of the patients' clinical assessment for reflux disease. All patients gave written informed consent. PPIs were stopped for a minimum 5 days prior to testing.

4.2.2 Questionnaires

Before the study each subject completed an reflux disease questionnaire (RDQ). This is a self-reported questionnaire assessing heartburn, regurgitation and upper abdominal pain. This is achieved by scoring twelve items on a six-point modified Likert scale. The presence of an RDQ score above fifteen is associated with GORD in over 75% of subjects (figure 23)[13].

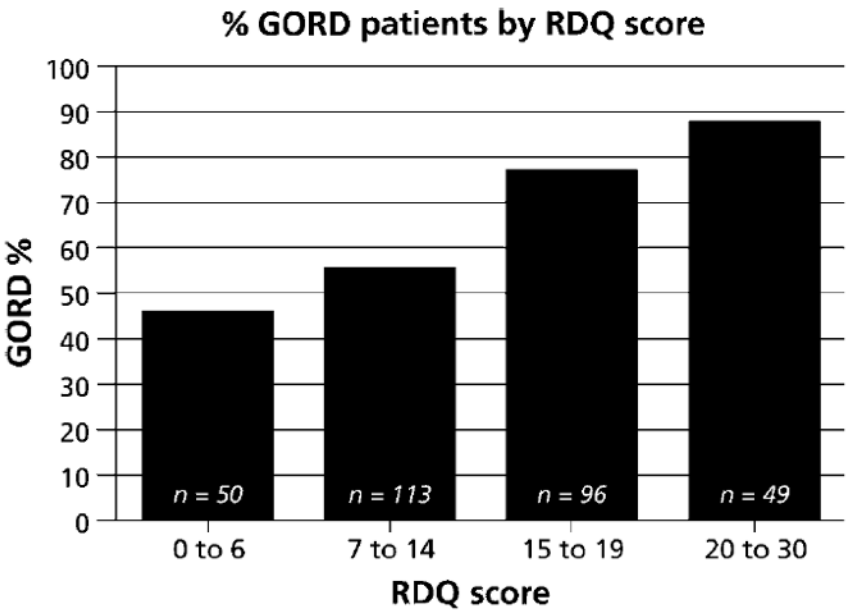


Figure 23: The accuracy of the RDQ score in identifying patients with reflux disease. It can be seen that a score above 15 is associated with a >75% detection of true GORD. This is similar to the subjective clinical opinion of a gastroenterologist. From Dent *et al.* Gut 2010

Subjects were also asked to indicate on a visual analogue scale their overall perception of historical heartburn severity (scored from 0 to 100, where 0 is no symptoms, 100 is the maximum severity imaginable).

4.2.3 Impedance measurements

An intraluminal combined pH-impedance catheter (Sandhill Scientific, Highlands Ranch, CO, USA) was used for performing oesophageal mucosal impedance (figure 24). This catheter incorporates a single water-perfused manometry channel (sphincter locator port) that can also be used for perfusion. This enables perfusion of the mid-oesophagus whilst measuring oesophageal impedance with the use of only one catheter.

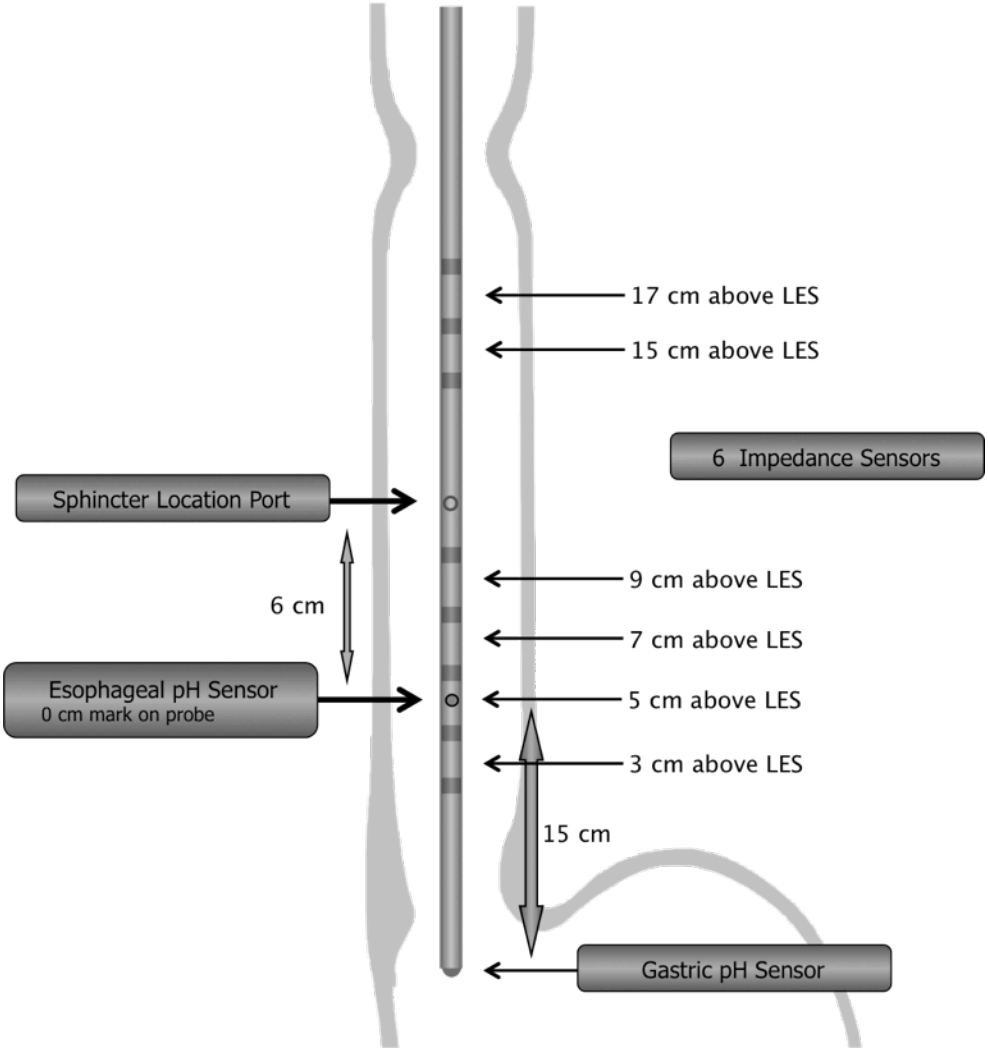


Figure 24: The combined pH-impedance catheter with integrated sphincter location port (used as a perfusion channel). LES = lower (o)esophageal sphincter

The lower oesophageal sphincter position was located using high resolution or water perfused manometry. After oesophageal manometry the pH-impedance catheter was lubricated and passed transnasally into the oesophagus such that the pH sensor was placed 5 cm above the manometrically-defined lower oesophageal sphincter. This placed the perfusion port at 11 cm above the lower oesophageal sphincter. Throughout the study impedance was measured at a frequency of 50 Hz in the distal impedance segment at 3 cm above the lower oesophageal sphincter (i.e 8 cm below the perfusion port). The data was recorded on a portable digital

data logger (Sandhill Scientific), and analysed on proprietary pH-impedance analysis software (Bioview Analysis, Sandhill Scientific).

The experimental protocol was as follows (and shown in figure 25).

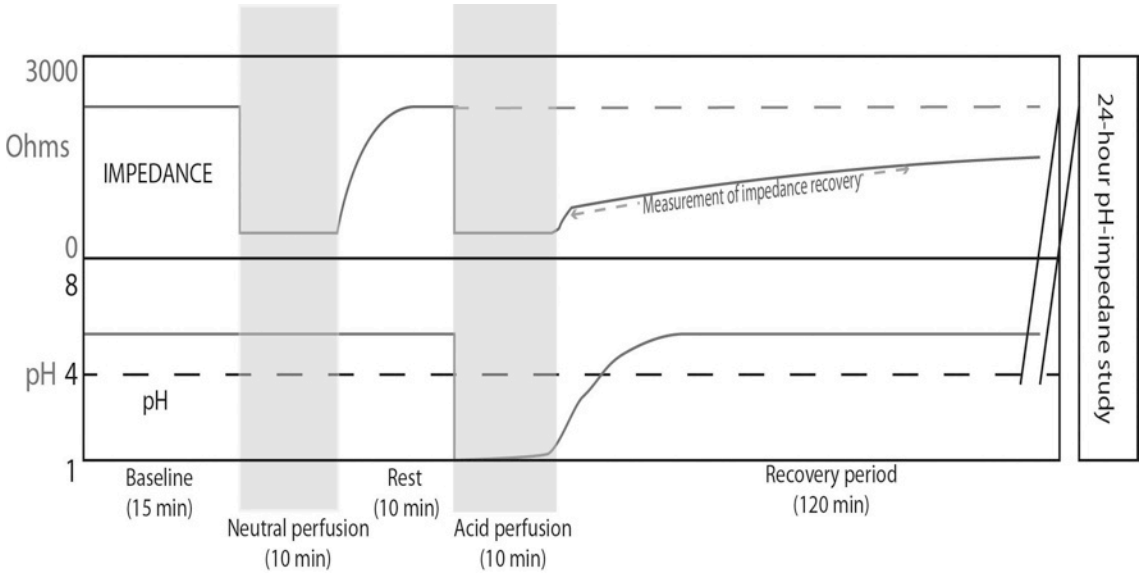


Figure 25: Schematic representation of the experimental protocol. The upper panel represents distal oesophageal impedance. The lower panel represents distal oesophageal pH

After placement of the pH-impedance catheter a baseline distal oesophageal impedance measurement was made with the subject in an upright sitting position for 15 minutes. Following this the subject was told they were to receive two perfusions, one neutral and one acid. They were not told in which order the perfusions were performed. First, a 0.9% sodium chloride solution (buffered to pH 6.7 with phosphate buffer) was perfused through the catheter perfusion port via a peristaltic pump at a rate of 10 ml per minute for 10 minutes (the rate was calibrated before beginning the experiment).

This neutral perfusion was followed by a 10 minute rest period where no perfusion was performed. The second infusion (of hydrochloric acid at pH 1.0) was

then performed, also at 10 ml per minute for 10 minutes.

The subject was asked to report whether or not heartburn was perceived during each perfusion. If heartburn was perceived, they were asked to rate the maximal symptoms severity on a scale of 0 – 10 (where 0 is no pain, 10 is the maximum imaginable pain).

After the perfusion period subjects were free to ambulate, but were asked not to eat or drink, and to remain upright during the next 120 minutes.

Patients subsequently completed their clinical 24-hour pH-impedance reflux study.

4.2.4 Data analysis

4.3.4.1 Baseline impedance

The baseline impedance was calculated as the average impedance between 5 and 15 minutes after catheter placement. The first 5 minutes were excluded from the calculation to allow for acclimatisation to the catheter. Reflux episodes but not swallows were excluded from this calculation, or from subsequent measurements of mean impedance.

4.2.4.2 Perfusion and recovery periods

Mean impedance was calculated during the perfusion periods, and in the 10 minute rest period post-neutral perfusion. The acid clearance time (time to oesophageal pH > 4 after the acid perfusion) was calculated (in seconds) for each subject.

During the 120 minute recovery period mean impedance was measured at 10-minute intervals by measuring the mean impedance during the 5 minutes leading up to the time point (e.g. mean impedance at 50 minutes was measured as the mean impedance between 45 and 50 minutes). At the same intervals mean

oesophageal pH was also measured. The impedance recovery after perfusion was calculated as the rate (Ω/minute) of impedance increase between minutes 5 and 90 after cessation of the acid perfusion. Minutes zero to five were not considered in order to allow for acid bolus clearance from the distal oesophagus. The rate of impedance recovery between 5 and 90 minutes was chosen because non-experimental retrospective analysis of impedance data from acid sensitivity tests suggested that this is the most linear part of the recovery process. The rate of impedance recovery as a percentage of baseline impedance increase per minute was also calculated.

4.2.4.3 Reflux study

The 24-hour reflux study was analysed according to our standard reflux protocol, with the exception that the first 3 hours of recording (corresponding to the perfusion and recovery phase of the study protocol) were excluded from analysis. Patients were requested to press buttons on the data logger to indicate mealtimes, movement to the recumbent or upright position, and the presence of symptoms. Meal periods were excluded from reflux analysis according to standard protocol. Oesophageal acid exposure was defined as the percentage time of oesophageal pH <4 during the analysed study. Pathological acid exposure was considered as over 4.2%[48]. Reflux-symptom correlation was determined using the symptom index (SI)[170] and symptom associated probability (SAP)[303]. An SI >50% and SAP >95% were considered a positive test result. In this study a positive reflux-symptom correlation was defined as when symptom index was >50% *and* SAP was >95%. The patient was considered to have non-erosive reflux disease if there was pathological oesophageal acid exposure and/or positive reflux-symptom association. If there was neither pathological oesophageal acid exposure nor

positive symptom-reflux association the patient was considered to have functional heartburn.

4.2.5 Statistical methods

All data are expressed as mean ± standard deviation unless otherwise stated. Normality of distributions was assessed using a D'Agostino and Pearson omnibus normality test. Correlations were tested using the Pearson r test. Comparison of baseline impedance values was tested with a Mann-Whitney U test. Comparisons of baseline impedance and acid exposure time between slow and fast impedance recovery groups were also tested by a Mann-Whitney U test. Fisher's exact test was used to test proportional differences. Significance was declared at $p<0.05$.

4.3 Results

4.3.1 Subjects

The median RDQ score for all subjects was 27 (interquartile range 17 to 36). The median VAS score for heartburn was 70 (interquartile range 40 – 85).

14 patients were current smokers. All patients had taken proton pump inhibitor therapy for their reflux symptoms, however 15 patients had ceased therapy due to perceived poor response. All other patients were on at least once daily proton pump inhibitor therapy (lansoprazole, pantoprazole, omeprazole or esomeprazole) which had been stopped only in order to have reflux investigation. All patients had undergone prior upper gastrointestinal endoscopy and none had evidence of erosions or Barrett's oesophagus. None of the research participants needed to be excluded due to the presence of severe motility disorder on oesophageal manometry. 11 patients were found to have a hiatus hernia on this manometric investigation.

4.3.2 24-hour clinical reflux monitoring

The median 24-hour oesophageal acid exposure was 2.25% (interquartile range 1.05 – 6.25%). According our stated criteria, 20 patients were classified by reflux testing as having non-erosive reflux disease, 30 as functional heartburn. Within the non-erosive reflux group, 15 were defined on criteria of excessive oesophageal acid exposure (with or without positive reflux-symptoms association), 5 were defined on a positive reflux-symptom association alone. Eight of the patients with non-erosive reflux disease were female, and 17 of the patients with functional heartburn were female. Five of the subjects with hiatus hernia were in the non-

erosive reflux group. The median age in the non-erosive reflux group was 48 (range 41 - 59). The median age in the functional heartburn group was 44 (35 – 66).

4.3.3 Acid sensitivity

No subjects perceived heartburn during the neutral perfusion. Thirty-one of the 50 (62%) patients experienced heartburn during the acid perfusion. The mean maximum symptom intensity perception in subjects perceiving heartburn was 7.3 out of 10 (range 2 – 10). All patients completed 10 minutes of acid perfusion.

4.3.4 Baseline oesophageal mucosal impedance

In all study subjects the mean baseline impedance at 3 cm above the gastro-oesophageal junction was 2098 Ω (range 466 – 5388 Ω).

There was a weak but significant negative correlation between baseline impedance and 24-hour oesophageal acid exposure (r=-0.38, p=0.01, figure 26).

The median post-perfusion acid clearance time was 8 minutes (interquartile range 5.5 – 13.5 minutes). The median number of pharyngeal swallows taken to achieve oesophageal pH of greater than 4 was 4 (interquartile range 3 – 7).

There was no correlation between post-perfusion acid clearance time or number of swallows to pH4 and baseline impedance (r=0.04, p=0.8; r=0.2, p=0.11 respectively).

Baseline impedance was lower in patients who perceived the acid perfusion as heartburn than in those who did not (1736 ± 784 Ω vs. 2741 ± 1256 Ω, p<0.01, figure 27).

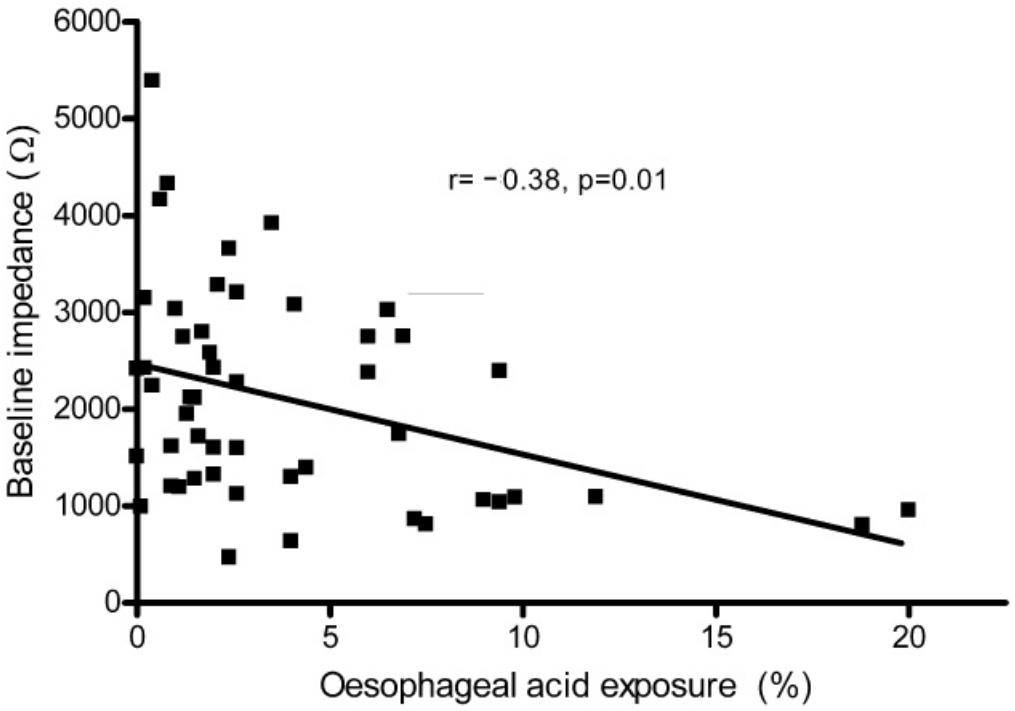


Figure 26: Correlation between distal oesophageal baseline impedance and 24-hour oesophageal acid exposure time (%)

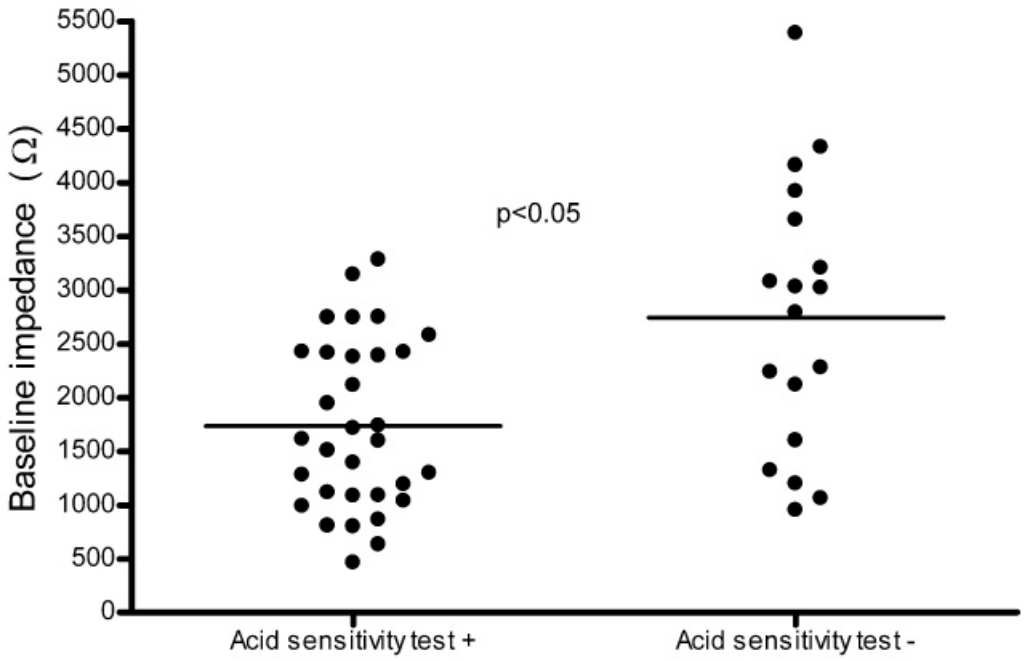


Figure 27: Baseline impedance according to whether the subject perceived the acid perfusion as heartburn (acid sensitivity test +) or did not (acid sensitivity test -)

Even when only patients with functional heartburn were considered, baseline impedance was lower in those who perceived heartburn on acid perfusion (1927 ± 814 Ω vs. 3018 ± 1241 Ω, p=0.01).

4.3.5 Perfusion with neutral solution

Perfusion with neutral solution provoked a fall in impedance to 675 ± 375 Ω in all patients, occurring as the conductive saline surrounded the impedance electrodes. After cessation of the neutral perfusion there was a very fast recovery of impedance to baseline (within 10 minutes impedance was 98 ± 28% of baseline, mean increase rate 203.7 ± 83 Ω/min).

4.3.6 Perfusion with acidic solution

During the perfusion with acidic solution there was a fall in impedance to 349 ± 141 Ω in all patients as the conductive solution passed the impedance electrodes.

After acid perfusion there was a much slower recovery of impedance compared to the recovery post-neutral perfusion. The median impedance recovery rate measured between 5 and 90 minutes post-acid perfusion was 6.5 Ω/min (25th-75th percentile = 3.3 - 12.0 Ω/min). The mean percentage of baseline increase rate was 0.4%/min. Baseline impedance correlated well with post-perfusion impedance recovery rate (r=0.7, p<0.01, figure 28).

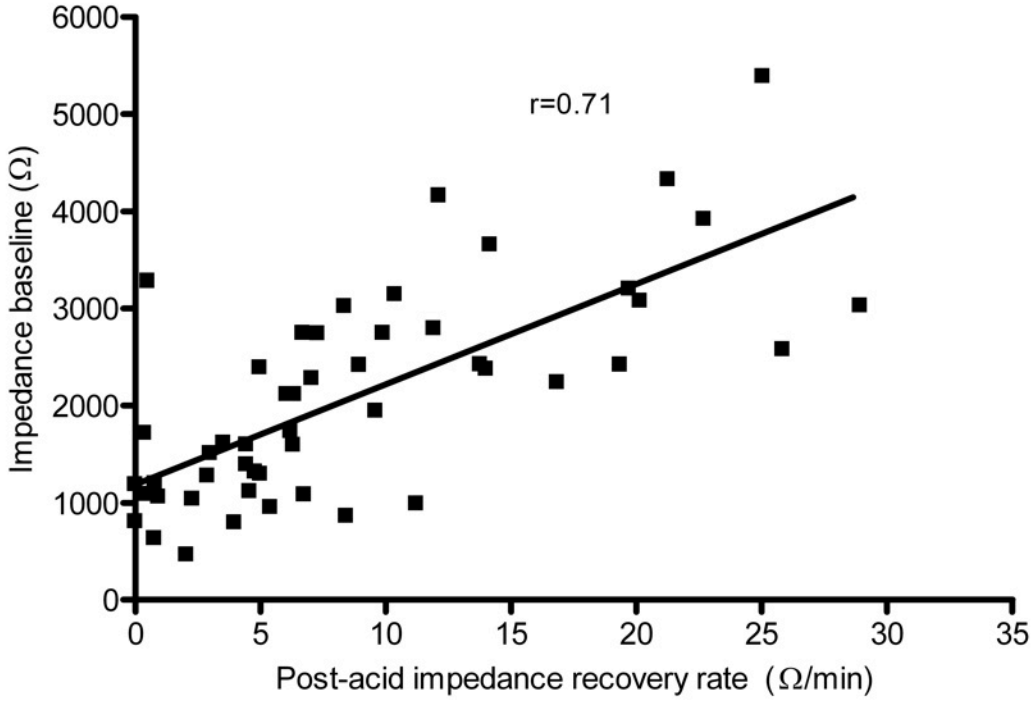


Figure 28: Correlation between baseline oesophageal impedance and impedance recovery rate after cessation of acid perfusion

Impedance recovery expressed as absolute values (Ω/min) and as percentage baseline increase (% baseline/min) showed a significant positive correlation ($r=0.73$, $p<0.01$). At 90 minutes after perfusion the median impedance was 73% (IQR 67-92%) of baseline. There was no correlation between impedance recovery rate and post-perfusion acid clearance time to pH4 ($r=-0.02$, $p=0.85$).

The post-acid perfusion impedance recovery rate demonstrates a significant inter-individual variability. If one takes subjects with a recovery rate greater than the 75th percentile (12 Ω/min) and lower than the 25th percentile (3.3 Ω/min), then we may consider two groups: one with fast, and one with slow post-acid perfusion impedance recovery (figure 29).

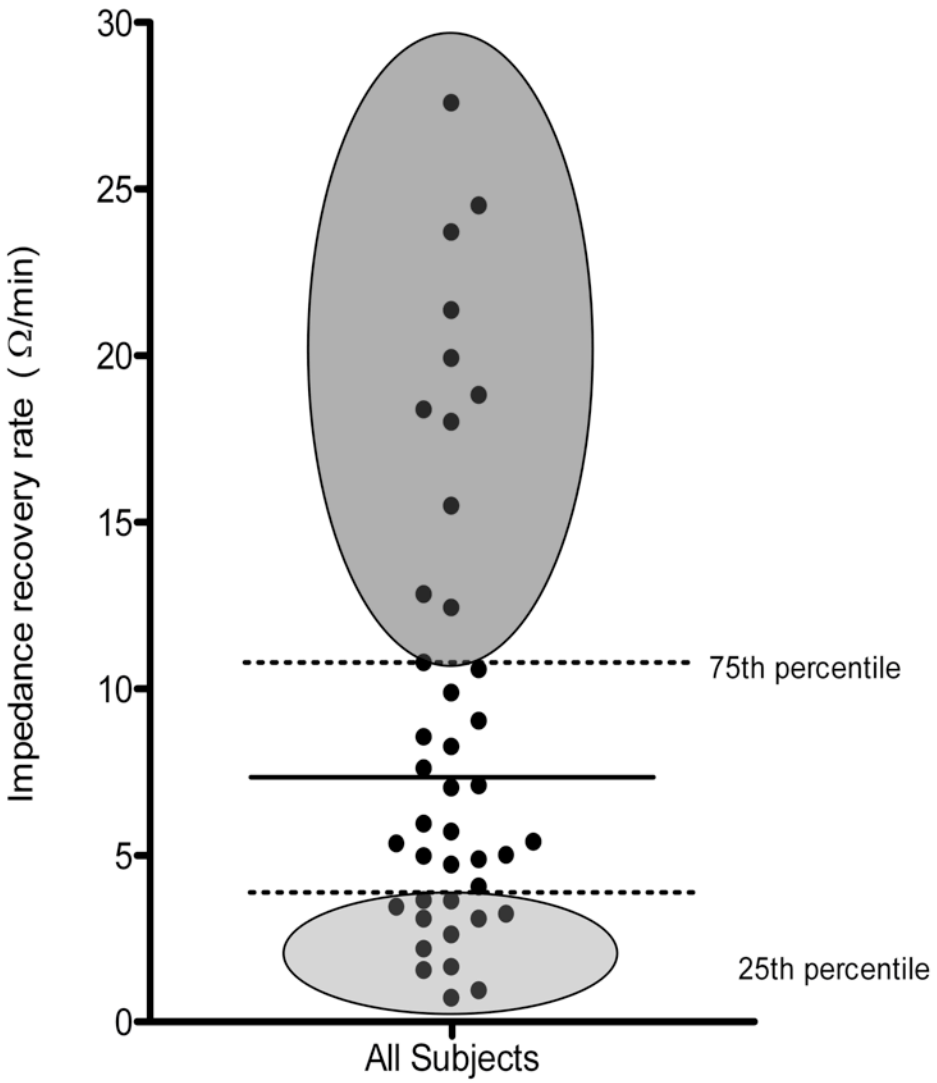


Figure 29: Inter-individual variability of post-acid impedance recovery rate. Those subjects within the light grey circle may be considered to have a "slow" recovery rate, and those within the dark grey circle a "fast" recovery rate

In considering these two groups, patients with slower impedance recovery (n=12) had lower baseline impedance than those with fast recovery (n=11) (1273 Ω ± 720 vs. 3220 Ω ± 954, $p<0.01$, figure 30).

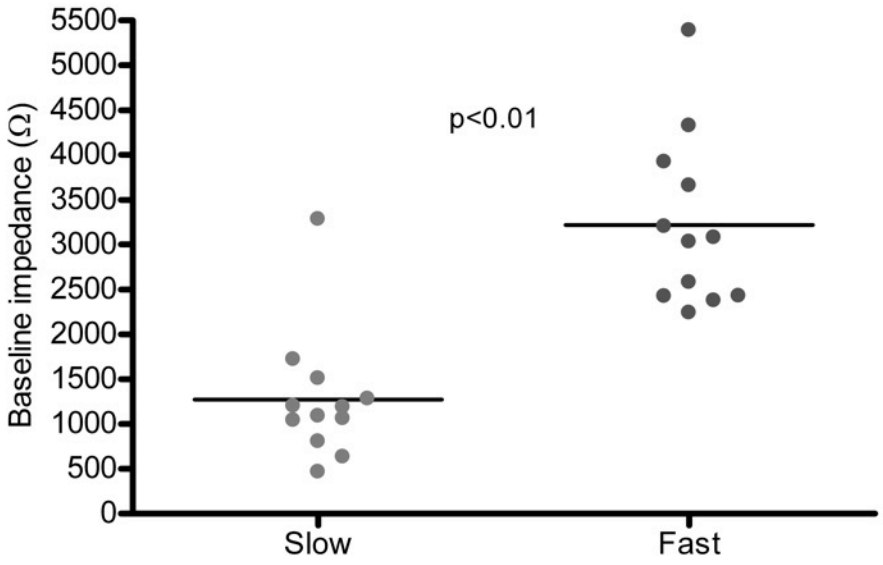


Figure 30: Baseline impedance according to "slow" and "fast" post-acid impedance recovery rate

Patients with slow impedance recovery also demonstrated a higher 24-hour oesophageal acid exposure than those with fast recovery (4.3 ± 4.0% vs. 1.7 ± 1.3%, p=0.04, figure 31).

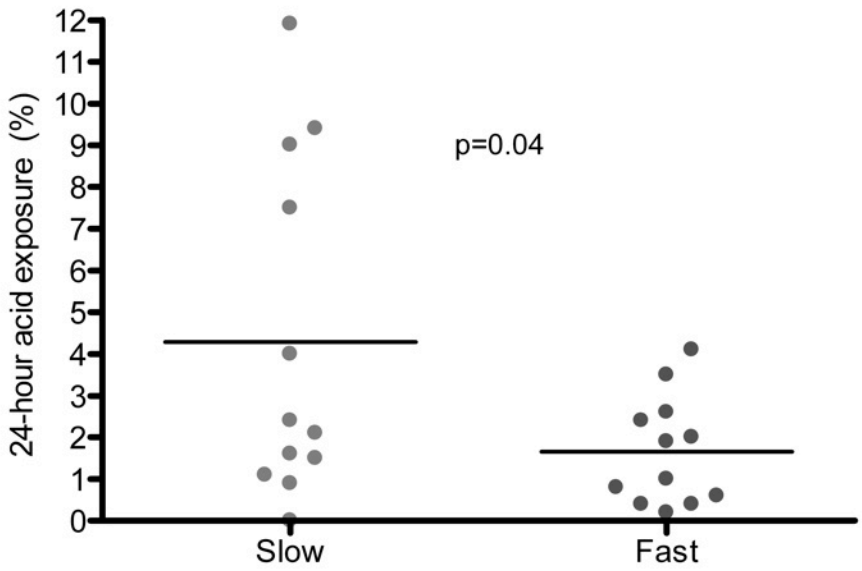


Figure 31: 24-hour oesophageal acid exposure according to "slow" and "fast" post-acid impedance recovery rate

Furthermore, patients with slow impedance recovery more often perceived the acid perfusion as heartburn than those with fast impedance recovery (10/12 vs. 4/12, p=0.03, figure 32).

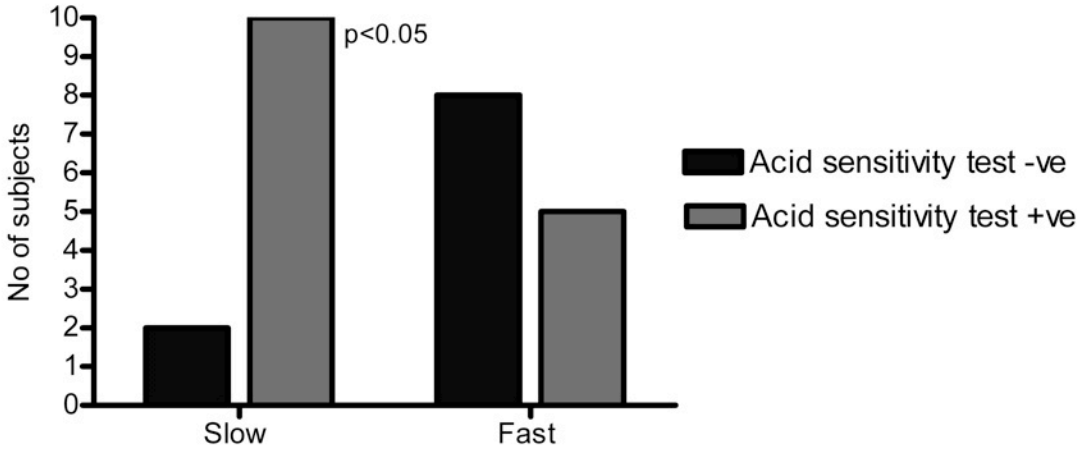


Figure 32: Perception of the acid perfusion test as heartburn according to "slow" and "fast" post-acid impedance recovery rate. Acid sensitivity test –ve means heartburn was not felt during acid perfusion. Acid sensitivity test +ve means heartburn was felt

4.3.7 Comparison of patients with non-erosive reflux disease and functional heartburn

In analysis of the characteristics of the 24-hour reflux monitoring, as expected, patients with non-erosive reflux disease had significantly higher acid exposure, greater percentage of acid (vs. weakly acidic) reflux events, and longer acid clearance time than patients with functional heartburn. The characteristics are expressed as median (interquartile range) in table 4 below.

	Functional heartburn	Non-erosive reflux disease	
Oesophageal acid exposure (%)	1.3 (0.5 - 2.4)	6.9 (4.2 - 9.4)	p<0.001
Total number of reflux events	22 (8 - 37)	27 (20 - 46)	NS
% acid reflux events	54 (26 - 71)	75 (63 - 81)	p<0.01
Acid clearance time (s)	55 (37 - 92)	129 (88 - 196)	p<0.05

Table 4: Reflux characteristics of functional heartburn and non-erosive reflux disease patients

In patients with non-erosive reflux disease baseline impedance was significantly lower than those with functional heartburn (1669 ± 814 Ω vs. 2384 ± 1156 Ω, p=0.02, figure 33).

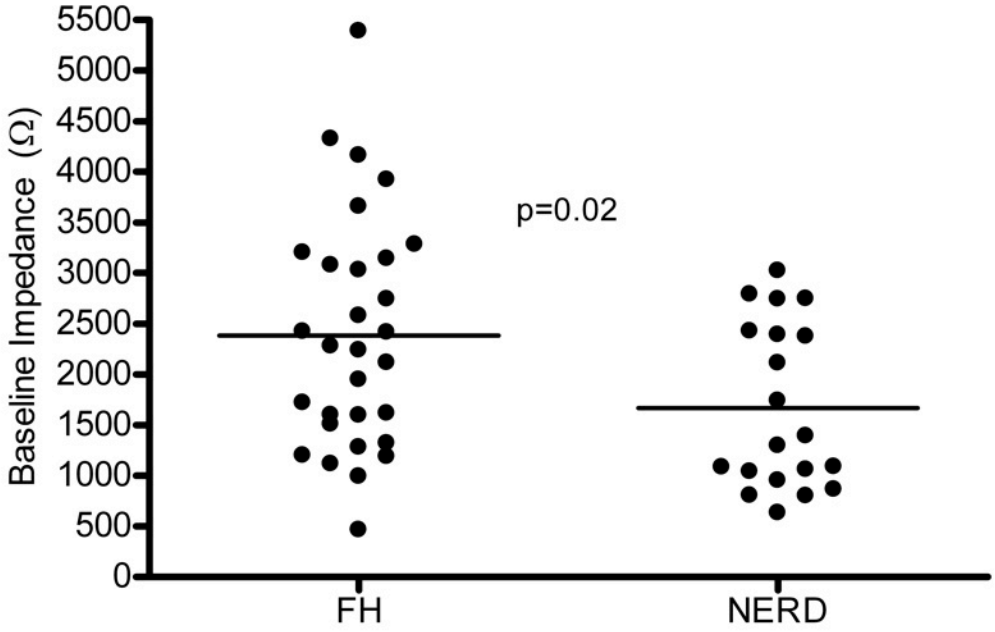


Figure 33: Baseline impedance according to patient disease phenotype

Patients with non-erosive reflux disease had a slower rate of impedance recovery compared to patients with functional heartburn (6.0 ± 4.2 Ω vs. 10.7 ± 8.6 Ω,

p=0.03, figure 34).

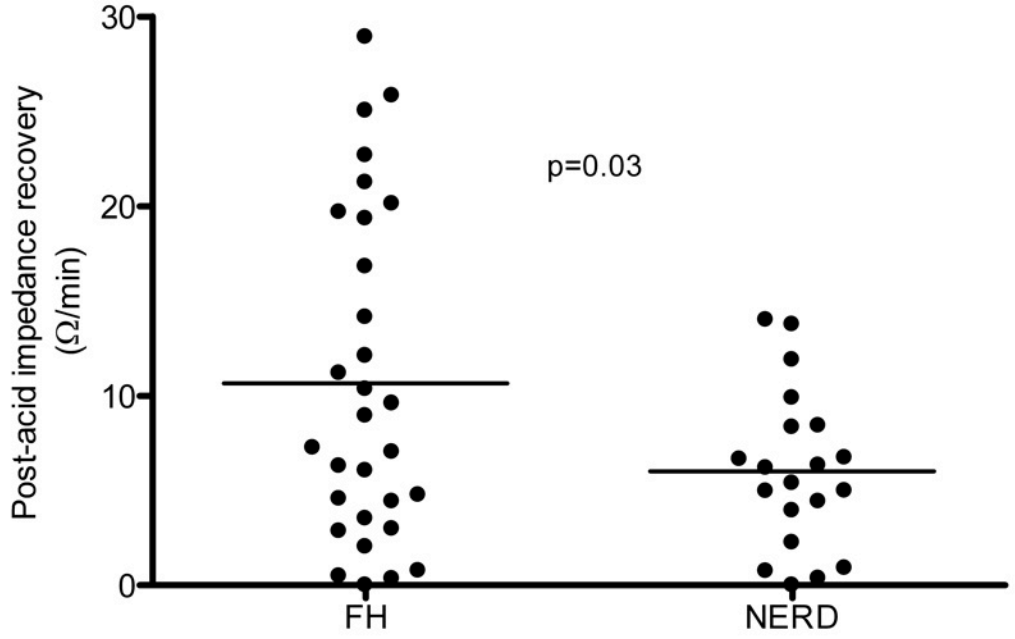


Figure 34: Post-acid perfusion impedance recovery rate according to patient disease phenotype

The reason for the different rate of impedance recovery was not due to differences in acid exposure or number of swallows needed to clear the acid during the recovery period. The acid exposure (pH<4) during the recovery period in functional heartburn and non-erosive reflux disease patients was 9.4 ± 9.3% and 13.4 ± 12.1% respectively (not statistically significant). There was no correlation between recovery period acid exposure and impedance recovery rate (r=-0.17, p=0.24). The number of swallows needed to clear acid after perfusion was 5.0 ± 2.7 and 5.5 ± 3.1 respectively (not statistically significant). Patients with non-erosive reflux disease more often perceived the acid perfusion as heartburn than patients with functional heartburn (16/20 vs. 15/30, p=0.04 for comparison).

4.4 Discussion

This study examined the relationship between baseline oesophageal impedance, and its dynamic response to an oesophageal acid challenge in patients with heartburn symptoms. It tested the hypothesis that there is a relationship between slow recovery of mucosal integrity after acid exposure, mucosal vulnerability (low baseline impedance) and increased perception of intra-oesophageal acid. It used impedance as a surrogate marker of mucosal integrity at baseline, and as a marker of progression of restitution of the integrity after an acid insult.

The study results show:

1) Patients with low baseline impedance are more sensitive to perception of an acid perfusion as heartburn.
2) A mid-oesophageal perfusion of a neutral solution causes a drop in distal oesophageal impedance (due to the conductance of the liquid) that restores to normal almost immediately on cessation of the perfusion.
3) A mid-oesophageal perfusion of an acidic solution in patients with reflux symptoms causes an abrupt fall in impedance that recovers slowly, displaying a significant inter-individual variability in recovery rate.
4) There is a relationship between rate of impedance recovery and baseline impedance i.e. the slower the recovery, the lower baseline impedance.
5) A group of subjects can be identified who display slow recovery of impedance after acid perfusion, low baseline impedance, high 24-hour acid exposure, and high acid sensitivity.
6) Compared to patients with functional heartburn, patients with non-erosive reflux disease have slower recovery of mucosal integrity after acid perfusion, lower baseline impedance, and increased acid sensitivity.

In contrast to the rest of the gastrointestinal tract, the oesophageal mucosa is characterised by tightly apposed non-keratinised stratified squamous epithelium. Under physiological conditions the oesophageal epithelium forms an effective barrier against the passage of noxious substance such as acid from the oesophageal lumen into the deep epithelium[304]. *In vivo* and *in vitro* experimental mucosal exposure to acid impairs the barrier properties as assessed by morphological and permeability studies[148, 153, 161, 163]. This failure of normal barrier function may allow the passage of acid or other noxious components of the refluxate (e.g. pepsin or bile acid) such that they can stimulate submucosal nociceptors and provoke symptoms in the absence of macroscopic erosions[162]. Studies thus far have been in terms of static phenomena (such as the baseline impedance in relation to 24-hour acid exposure[156]), but the dynamic *in vivo* response of the oesophageal mucosal integrity to acid exposure has not been characterised in patients with reflux symptoms. Mucosal integrity is determined by dynamic circumstances, with repeated reflux events over the course of a day interacting with the mucosa, which in turn responds to and recovers from the damage caused by the exposures. As such, baseline impedance is likely to be a function of mucosal restoration capacity after repeated acid exposure. *In vivo* mucosal impedance measurement may allow an assessment of oesophageal mucosal integrity in these dynamic circumstances, and thus enables us to evaluate properties of oesophageal mucosal integrity in patients during and after a standardised acid challenge.

As always with a new technique, it is important to recognise the potential limitations, and these should be remembered when making interpretations. During the introduction to this chapter supporting evidence (the lower baseline impedance in higher acid exposure patient groups and the good correlation with TER in animal studies) was given for the use of oesophageal impedance as a

measure of mucosal integrity. This suggests that the technique is a valid representation of integrity, but this has not been definitively demonstrated. For example, it is possible that a low impedance occurs on acid exposure, not because of mucosal changes, but instead due to an increased liquid/mucous layer lining the mucosa. This could be due to increased salivary production, or due to submucous gland secretion. As yet, this possibility is untested.

In addition, although impedance in vivo correlates with TER in vitro, this has been demonstrated in animals, and as such cannot be translated to humans with complete confidence. Studies comparing this in humans are not performed, although a recent study using a similar technique (electrical tissue impedance spectroscopy) has demonstrated a correlation (r=-0.65) between impedance measurements and TER in biopsies in humans[305]. This perhaps offers some more support for the application of impedance techniques in mucosal measurement, but cannot be interpreted as direct evidence for the technique used in our studies.

The fall in impedance that occurs during acid perfusion is predominantly due to the conductance of the acid solution itself lying in contact with the impedance segment. However, this study demonstrates that a relatively low impedance persists for a long time after the clearance of the acid from the oesophageal lumen. The mucosal behaviour on acid exposure *in vivo* is in accordance with *in vitro* findings when human oesophageal mucosal biopsies are exposed to acid, as seen in Chapter 3. The long lasting fall of the impedance implies that, not only is the barrier integrity of the mucosa disrupted when it is exposed to acid, but it remains impaired for some time after an acid exposure.

The speed of recovery of the impedance after cessation of acid perfusion displays a significant inter-individual variability. This observation is of interest since it suggests that patients with acid-induced mucosal damage do not reconstitute their

mucosal integrity at the same rate. As such, one can hypothesise that patients with a slower recovery of integrity will be more likely to have long-lasting vulnerability of their mucosa to exposure from subsequent gastro-oesophageal reflux events. Indeed this study found a close association between slow recovery of integrity after acid challenge and a low baseline impedance value. It is therefore possible that low baseline impedance is partly a consequence of the impaired ability of the mucosa to rapidly reconstitute its barrier function after acid damage. We should also be aware that the excellent correlation between baseline impedance and rate of recovery could be interdependent in the other direction: that a lower baseline *results* in a slower recovery. The complexities of this relationship have not been resolved in this study.

A hypothesis formed in this thesis is that a patient whose oesophageal mucosa displays impaired integrity should be more sensitive to oesophageal acid exposure. This is supported in the current study by the finding that patients with low baseline impedance (more impaired integrity) have more sensitivity to acid perfusion than those with high baseline impedance. This is true even when only patients with functional heartburn are included in the calculation. This second point is of importance. It could be argued that patients with non-erosive reflux disease have a lower impedance and more acid sensitivity than patients with functional heartburn, but that these two observations are unrelated. The finding of an association between lower baseline impedance and acid sensitivity within *only* functional heartburn patients makes a stronger argument for a pathophysiological relationship.

This study could have used statistical analyses of reflux-symptom correlation (such as SAP) from the 24-hour reflux study to assess acid sensitivity. This was not done, and instead perception of a standard acid challenge was used. Although the

former method seems to be more physiological, most studies on oesophageal chemosensitivity rely on standard acid perfusion techniques due to large inter-individual and day-to-day variability in symptom perception and patient behaviour during ambulatory reflux monitoring. As such it was felt that the administration of a fixed concentration and volume acid challenge would result in more robust comparison of patients.

Patients with a slow impedance recovery had a lower baseline impedance and more acid sensitivity than patients with fast impedance recovery. The slower recovery group also had higher 24-hour acid exposure than the faster recovery group. This is important as one can consider the following paradigm: if a patient has a reflux episode, the oesophageal mucosal integrity is impaired. This mucosal integrity slowly recovers. During this time of low integrity, the patient is more vulnerable to symptoms from reflux episodes. As more reflux events (and more oesophageal acid exposure occurs), the integrity is further impaired, delays further the adequate reconstitution of barrier function, and renders the patient yet more vulnerable to reflux perception (a "multiple-hit" hypothesis). A clinical correlate of this has previously been documented, whereby it was noted a reflux event is more likely to be perceived if there was a previous burden of acid exposure[210, 213].

On comparison of patients with functional heartburn and non-erosive reflux disease it can be seen that patients with non-erosive reflux disease have a lower baseline impedance. This would be in keeping with previous observations of morphological changes (dilated intercellular spaces) in the distal oesophagus of patients with non-erosive reflux disease, but not in patients with functional heartburn[167]. According to the paradigm presented in this Chapter, the lower baseline impedance in non-erosive reflux disease would be more likely to occur if

the recovery of impedance is slower than in functional heartburn patients, and indeed this is the case. Correspondingly, it was also demonstrated that perception of oesophageal acid challenge is more frequent in the non-erosive reflux disease patient group.

The mechanism of symptom perception in functional heartburn is unclear. By definition, *true* functional heartburn is not due to the contact of the oesophageal mucosa with gastro-oesophageal refluxate[6]. It is already known that patients with functional heartburn display a high "positive" rate when the acid sensitivity test is used as a diagnostic tool[306]. Within the functional heartburn group in this study, patients who perceived acid perfusion had lower baseline impedance than those who did not, suggesting that peripheral factors may still play a role in their acid perception. Indeed, one can identify a subgroup of functional heartburn patients who, despite having a normal reflux study, have a mucosal integrity behaviour phenotype that is very similar to non-erosive reflux disease patients. This would be of interest to explore further. It is highly likely that a proportion of patients during their 24-hour study do not have a "typical" day (i.e. the presence of the catheter may alter behaviour in terms of meals and activities), and some may forget to press the event markers every time they perceive symptoms. Indeed it is known that a prolonged reflux study (48 or 72 hours) can "convert" some patients previously determined as functional heartburn on a 24-hour study into patients with pathological gastro-oesophageal reflux disease[307]. It would be interesting to investigate whether the mucosal characteristics of patients can aid in the phenotyping process.

A limitation of this study is that impedance recovery was only measured for 2 hours post-acid perfusion. Ideally this period would be longer since most subjects had not re-attained baseline levels over 2 hours. Food and drink was not

permitted during this recovery period as it creates significant impedance artefact. As such the 2-hour timeframe was considered a satisfactory compromise since a recovery rate can be reasonably calculated in this time period, without the need for patients to undergo an even longer period of uncomfortable fasting.

It is possible that the classification of non-erosive reflux disease patients was not completely accurate. The reason for this is that patients had their reflux study as part of their clinical evaluation for refractory reflux symptoms. All had undergone prior endoscopy which demonstrated no erosive disease. However, some patients may previously have erosive oesophagitis that was "converted" to non-erosive reflux disease by PPI therapy. However, all the patients had ongoing symptoms despite normal endoscopic mucosa, and we are interested in the physiological properties of this non-eroded mucosa in persistent symptom generation.

The perfusions were performed before knowing the patient phenotype (functional heartburn or non-erosive reflux disease). For the objective of the study it was not necessary to know the phenotype before the test. Indeed, it ensured investigator blinding during the acid sensitivity test.

It could have been very informative if the study could have incorporated a corresponding *in vitro* assessment of mucosal integrity by analysing oesophageal biopsies in Ussing Chambers (such as in Chapter 3). To achieve this an experiment whereby serial endoscopic biopsy over a 90 minute period would be required, but would be unfeasible and distressing for the participant. However, it is known from previous animal studies that *in vitro* measures of mucosal permeability do correlate well with *in vivo* impedance measurements[157], and as such the impedance findings are still of interest on their own strength.

In summary this study indicates that impaired mucosal integrity can be induced by acid, and maintenance of this impaired status can be promoted by slow

reconstitution after acid exposure. This situation appears to favour symptomatic acid perception. These findings add another layer to our understanding of the mucosal integrity behaviour in non-erosive reflux disease.

CHAPTER 5

In vitro and in vivo assessment of mucosal integrity in the distal and proximal oesophagus

CHAPTER 5: IN VITRO AND IN VIVO ASSESSMENT OF MUCOSAL INTEGRITY IN THE DISTAL AND PROXIMAL OESOPHAGUS

5.1 Introduction and aims

Historically the distal oesophagus has been the focus of investigation into pathogenesis of GORD. However, it has been increasingly documented that the proximal oesophagus may have a greater importance than was previously realised. The use of pH impedance techniques has allowed more spatial definition of reflux events. The most commonly used pH-only probes have only one or two pH sensors and allow only detection of a pH drop in the distal oesophagus during a reflux event. This tells us little or nothing about the more proximal movement of the refluxate. Multiple sensor pH probes or combined pH-impedance catheters commonly have several measurement segments spanning from distal to proximal oesophagus. Studies with such techniques have enabled characterisation of not only the pH, but also the proximal movement of reflux events. Using such techniques it has been shown that, in patients with GORD, reflux events reaching the proximal oesophagus (defined as 15 cm above the lower oesophageal sphincter) are more likely to be perceived than those reaching only the distal oesophagus[209, 210]. As has been regularly emphasised in this thesis, there exists a significant minority of patients with gastro-oesophageal reflux disease who remain refractory to proton-pump inhibitor therapy[3]. The majority of gastro-oesophageal reflux events in this group of patients are weakly acidic (pH 4 to 6) in nature. In patients taking PPI therapy, impedance-pH studies have indicated that a high proximal extent of reflux events is the most important factor in determining whether a reflux episode will be perceived by the patient[212, 213].

Whilst distal reflux events can be symptomatic (and can result in significant complications ranging from erosions to adenocarcinoma), the distal reflux event is not immediately threatening to the individual. In contrast, a reflux event reaching the proximal oesophagus is in danger of reaching the pharynx and being aspirated into the airways. It is therefore likely that a heightened perception is needed to initiate subconscious (secondary peristalsis) and conscious (swallowing) clearance mechanisms, and to initiate fast oesophago-sphincteric reflexes (which result in an abrupt increase in upper oesophageal sphincter pressure during an increase in intra-oesophageal pressure)[308, 309].

The physiological mechanism behind this increased perception to proximal reflux events remains unclear. There are indications that the proximal oesophagus appears more sensitive to acid during experimental perfusion than the distal oesophagus. Thus far a study by Thoua *et al.* is the only study to compare sensitivity to intra-oesophageal acid perfusions of the distal and proximal oesophagus[208]. This was done in patients with GORD and controls by catheter perfusions of saline and pH 1 solutions at 5 cm and 15 cm above the lower oesophageal sphincter, and it was found that all subjects (particularly those with non-erosive reflux disease and functional heartburn) perceived more discomfort during the proximal acid perfusion. Such an experimental design should be interpreted with caution, however, since the proximal acid perfusion will also simultaneously perfuse the distal oesophagus and so there may be cumulative sensitivity effect from proximal and distal oesophagus. We could only truly make interpretation of relative chemosensitivity from such a study if the segments of the oesophagus were isolated. It is possible that the mechanism of increased proximal oesophageal sensitivity is not due to a chemosensitivity effect, but perhaps another mechanism such as distension caused by the refluxate in the proximal oesophagus

(or a combination). For example, experimental studies have demonstrated that, in control subjects and in patients with Barrett's oesophagus, the proximal oesophagus is more sensitive to balloon distension than the distal oesophagus[207].

The mechanism of increased sensitivity of the proximal oesophagus is unknown, but may originate via a mucosal abnormality. In patients with non-erosive reflux disease mucosal dilated intercellular spaces (DIS) are present in not only the distal oesophagus, but also in the proximal oesophagus[164]. Furthermore, there is also the interesting suggestion that proximal oesophageal acid exposure is not required for impairment of mucosal integrity in the proximal oesophagus. It is possible to induce DIS both in the distal (exposed) and proximal (non-exposed) oesophagus in healthy subjects by way of experimental distal oesophageal acid perfusion[161]. Dilated intercellular spaces may allow easier access of noxious components of the gastro-oesophageal refluxate into the epithelium where they can stimulate nociceptors. How this spread of DIS (all over the length of the oesophagus) occurs is yet to be elucidated, but it does raise the possibility that distal acid exposure can sensitise the mucosa of the proximal oesophagus. This thesis has demonstrated that mucosal integrity can be assessed not only by morphological, but also by functional means. As yet the mucosa of the proximal oesophagus has not been investigated in these terms.

It is also possible that the enhanced sensitivity of the proximal oesophagus is related to a distinct sensory neural innervation. Data on human oesophageal mucosal regional innervation is lacking, but there are animal data supporting an unequal innervation of the oesophagus. In the rat, density of nerve fibres is most prominent in the upper cervical region of the oesophagus, and decreases in the

lower cervical and thoracic oesophagus before slightly increasing again in the abdominal portion[310-312].

A differential distribution of sensory afferent fibres in the human oesophageal mucosa may contribute to proximal oesophageal hypersensitivity.

The study presented in this chapter examines the status of mucosal integrity in the proximal oesophagus in patients with heartburn. It also investigates the distribution of sensory afferent mucosal nerves in the oesophagus.

We hypothesise that the proximal oesophageal mucosal integrity is more vulnerable to acid injury than the distal oesophagus, and that this may underlie proximal oesophageal chemosensitivity. It is also hypothesised that there may be an increased density of mucosal sensory afferent nerve fibres in the proximal oesophagus compared to the distal.

The aims of the current study are:

To investigate in patients with heartburn without oesophagitis:

1) The proximal oesophageal mucosa integrity *in vivo* and *in vitro.*

2) The density and distribution of afferent mucosal nerve fibres in the proximal and distal oesophagus.

5.2 Methods

5.2.1 Subjects

Overall 66 patients were recruited for this study. The 50 patients described in Chapter 4 were also studied for *in vivo* impedance investigation of the proximal oesophagus. They had presented to the Royal London Hospital upper gastrointestinal physiology unit with typical symptoms of gastro-oesophageal reflux disease. Exclusion criteria for this aspect of the study were 1) The presence of erosive oesophagitis or Barrett's oesophagus on endoscopy. 2) The presence of major oesophageal motility abnormality (achalasia, absent peristalsis) on oesophageal manometry. 3) Proximal oesophageal impedance fall of >5% during the neutral perfusion section of the protocol (which was taken as being possible proximal oesophageal contamination during perfusion, and as such further interpretation of "spread" of impairment of mucosal integrity to the distal oesophagus could not be considered reliable).

10 healthy subjects were also investigated with proximal oesophageal impedance measurements.

A further 16 patients were recruited from the gastrointestinal endoscopy department of the Royal London Hospital for participation in the *in vitro* study. Entry criteria were the presence of daily, troublesome reflux symptoms (heartburn and/or regurgitation). Subjects were excluded if there was subsequent evidence of oesophagitis or Barrett's oesophagus on endoscopy. In 9 of these patients one further distal and proximal biopsy was placed immediately into paraformaldehyde 4% in 0.1M PBS for subsequent histological evaluation.

5.2.2 In vivo impedance study of proximal mucosal integrity

5.2.2.1 Impedance measurements

As in chapter 4, an intraluminal combined pH-impedance catheter (Sandhill Scientific, Highlands Ranch, CO, USA) with integrated water-perfused channel was used for performing oesophageal mucosal impedance and performing mid-oesophageal perfusions. The lower oesophageal sphincter position was located using high resolution or water perfused manometry. After oesophageal manometry the pH-impedance catheter was lubricated and passed trans-nasally into the oesophagus such that the pH sensor was placed 5 cm above, and the perfusion port 11 cm above the manometrically-defined lower oesophageal sphincter. Throughout the study impedance was measured at a frequency of 50 Hz in the proximal impedance segment at 17 cm above the lower oesophageal sphincter (i.e 6 cm above the perfusion port). The data was recorded on a portable digital data logger (Sandhill Scientific, CO, USA), and analysed on proprietary pH-impedance analysis software (Bioview Analysis, Sandhill Scientific, CO, USA).

5.2.2.2 Experimental protocol

After placement of the pH-impedance catheter a baseline proximal oesophageal impedance measurement was made with the subject in an upright sitting position for 15 minutes. Following this the subject was told they were to receive two perfusions, one neutral and one acid. They were advised that they would not be told which order the perfusions will be performed. First, a 0.9% sodium chloride solution (buffered to pH 6.5) was perfused through the catheter perfusion port via a peristaltic pump at a rate of 10 ml per minute for 10 minutes. An acidic perfusion (of hydrochloric acid at pH 1.0) was then performed at 10 ml per minute for 10 minutes.

The subject was asked to report whether or not heartburn was perceived during each perfusion. If heartburn was perceived, they were asked to rate the maximal symptoms severity on a scale of 0 – 10 (where 0 is no pain, 10 is the maximum imaginable pain).

Patients subsequently completed their clinical 24-hour pH-impedance reflux study, allowing accurate phenotype as having either non-erosive reflux disease or functional heartburn.

Healthy volunteers had only baseline proximal impedance recorded, and change in proximal impedance during distal acid perfusion measured. The catheter was then removed after acid perfusion.

5.2.2.3 Data analysis

Baseline impedance

The baseline proximal oesophageal impedance was calculated as the average impedance between 5 and 15 minutes after catheter placement. Swallows were not excluded from this analysis since they are short lasting and make little difference to mean baseline over 10 minutes. Reflux episodes (if any) were excluded from baseline analysis.

Proximal impedance measurements during perfusions

The impedance in the proximal impedance segment was first measured during the neutral perfusion. The mean impedance during the second 5 minutes of the 10-minute perfusion was measured, and the percentage change (if any) compared to the baseline impedance. If there was a more than 5% drop in impedance in the proximal impedance channel during the neutral perfusion the subject was excluded from further study. This is because *in vivo* and *in vitro* neutral exposures

of the oesophagus to neutral solutions do not induce morphological changes in integrity (DIS), and as such a fall in impedance during the neutral perfusion was taken to be due to proximal contamination of the oesophagus by the perfusion fluid.

In those subjects who did not have a fall in proximal impedance during the neutral perfusion, the mean impedance during the second 5 minutes of the 10-minute acid perfusion was measured, and the percentage change (if any) from baseline was calculated (figure 35).

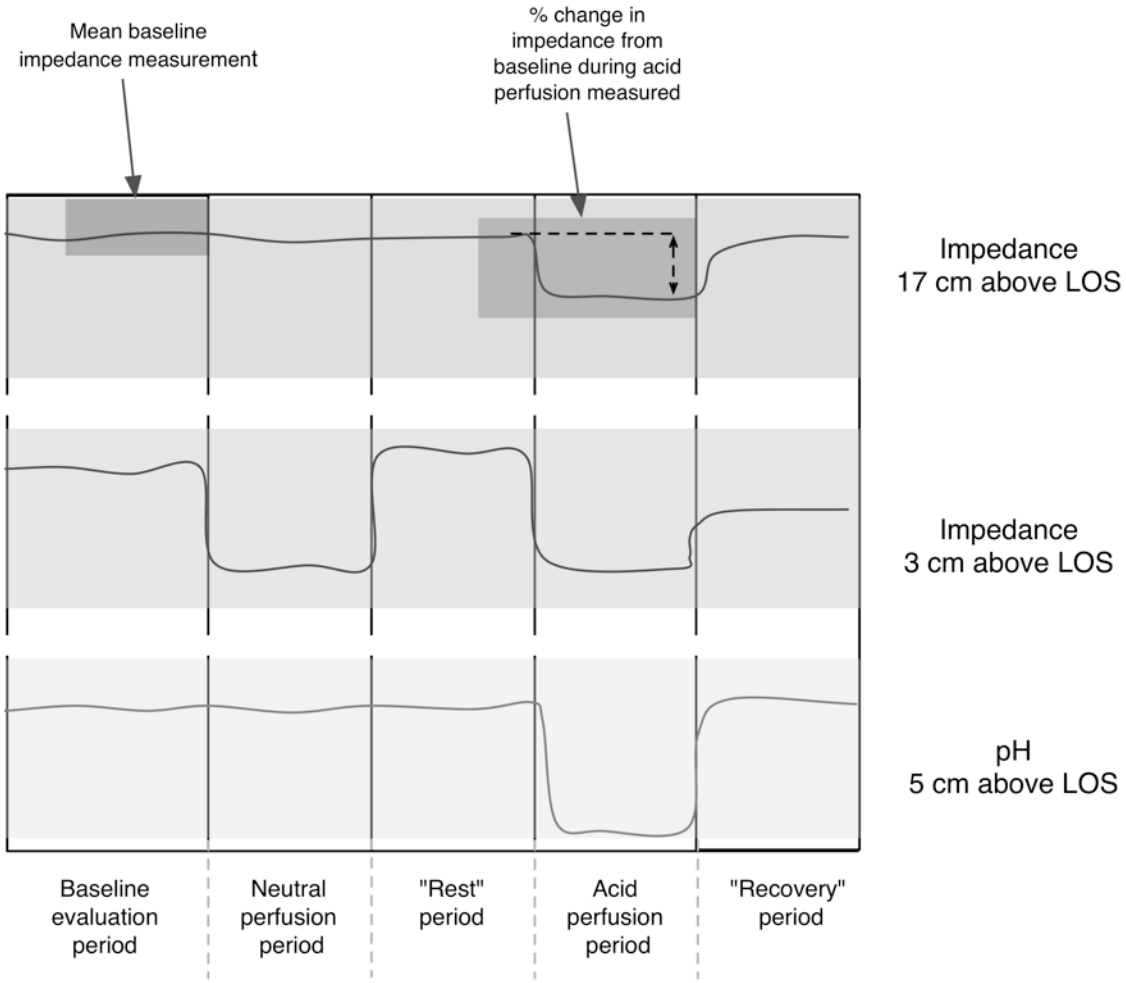


Figure 35: Experimental protocol for *in vivo* study of proximal impedance

Reflux study

The subsequent 24-hour reflux study was analysed as described in Chapter 4, with subsequent classification of patients into non-erosive reflux disease or functional heartburn.

5.2.3 In vitro assessment of proximal oesophageal integrity

5.2.3.1 Endoscopy

Endoscopic procedures were performed either under midazolam sedation or with pharyngeal local anaesthetic spray. In each subject two oesophageal mucosal biopsies were taken (Radial Jaw 3 forceps, Boston Scientific, USA), one from 3 cm above the squamo-columnar junction (distal oesophageal biopsy), and one from 20 cm above the squamo-columnar junction (proximal oesophageal biopsy). Biopsies were immediately placed in a pre-oxygenated Krebs-Henseleit buffer solution at pH 7.4 and at 4°C, and rapidly transported to the laboratory for Ussing Chamber study.

5.2.3.2 Ussing chamber studies

Biopsies were orientated and mounted into the adapted Ussing Chambers as described in chapter 3. Immediately on mounting the biopsies they were bathed on both luminal and basal sides with Krebs-Henseleit buffer at pH 7.4, 37^{0}C, and the solution was continuously bubbled with carbogen gas. After making a correction for fluid and circuit resistance, basal transepithelial resistance (TER) was calculated according to Ohm's law from the voltage deflections induced by bipolar current pulses of 50μA, duration 200 ms every 6 seconds applied through platinum wires. All experiments were conducted in open-circuit conditions. The system was equilibrated at 37^{0}C until a stable TER baseline was established (typically 20

minutes). Biopsies that did not adequately cover the chamber aperture on visualisation under stereo-microscopy, or with a baseline TER of less than 50 $\Omega.cm^2$ were excluded from further analysis since these were deemed to be unsatisfactory. After a stable baseline was achieved the solution in the "luminal" bath of the chambers was replaced with an acidic solution (Krebs-Henseleit at pH 2 + 1 mg/ml porcine pepsin + 1 mM taurodeoxycholic acid). The exposure to the acidic solution was for 30 minutes, and TER was continuously measured during this time.

The baseline TER was determined as the TER after equilibration, immediately before the “test solution” was placed in the luminal bath.

The change in TER caused by the test solution was expressed as a percentage change at the end of 30 minutes exposure, relative to the TER at the beginning of exposure, immediately after placing the test solution in the “luminal bath of the chamber.

5.2.3.3 Immunohistochemical studies

Proximal and distal oesophageal biopsies were fixed in 4% paraformaldehyde overnight. This was followed by cryoprotection in 30% sucrose in phosphate-buffered saline (PBS) for 24-hours at 4^0C, followed by 30% sucrose PBS:OCT embedding compound (1:1) at 4^0C. Sections were embedded in OCT at -25^0C and 10 µm sections were cut on a cryostat and mounted on positively charged glass slides. Sections were then air-dried for 1 hour. 400 µl per slide of 10% horse serum in PBS (blocking agent) + 0.3% Triton-X100 was applied and left at room temperature for 1 hour. Sections were then incubated with a primary antibody to calcitonin gene-related peptide (CGRP) (1:500 monoclonal mouse anti-human, Pierce Antibodies ABS 026-05-02) at 4^0C overnight. The primary antibody was

made up in 10% horse serum in PBS and 0.3% Triton-X100. Sections were then washed three times for 10 minutes in PBS + Triton-X100, followed by incubation with the secondary antibody (donkey anti-mouse Invitrogen, labeled with green-fluorescent Alexa Fluor 488 dye) and incubated for 4 hours in darkness. Sections were then washed again three times for 10 minutes, and mounted with Vectashield HardSet™. Negative controls were prepared with the primary antibody omitted, and showed no labelling. Some sections were incubated instead with a primary antibody to neurofilament (Dako: monoclonal mouse anti-human neurofilament protein clone 2F11, 1 : 500) to confirm that neural structures were being labelled. Fluorescence was visualised using an epifluorescent microscope (Olympus BX61). All images were obtained with a 40x oil immersion lens under the 488nm excitation setting. Where CGRP-immunoreactive (IR) fibres were seen on microscopy, their position relative to the luminal surface of the section was analysed (as number of cells from most superficial location of the fibre to the luminal surface). An estimate of the relative quantity of fibres in the distal and proximal oesophagus was made by calculating the number of positive (with CGRP-IR fibres) sections relative to the total number of sections analysed.

5.2.4 Statistical analysis

Data are presented as mean ± standard deviation. Normality of distributions was assessed using a D'Agostino and Pearson omnibus normality test. Changes in proximal impedance from baseline, to during neutral perfusion and to during acid perfusion were tested using repeated measures ANOVA. Bonferroni's multiple comparison test was used to test significance of differences. Comparison of mean change in impedance during acid perfusion between patients with non-erosive reflux disease and functional heartburn was tested by an unpaired t test. Baseline

impedance between patients with non-erosive reflux disease and functional heartburn was compared using an unpaired t test. Baseline TER and change in TER on exposure to acidic solution between proximal and distal oesophageal biopsies from the same patient were tested with a paired t test. Comparison of mean number of cells between CGRP-IR fibres and the luminal surface in proximal and distal biopsies were compared with an unpaired t test. The relative frequency of appearance of fibres in analysed histological sections in proximal and distal biopsies was compared using Fisher's exact test. Significance was declared at $p<0.05$.

5.3 Results

5.3.1 In vivo impedance study of proximal mucosal integrity

5.3.1.1 Subjects

The details of the 50 subjects studied are found in Chapter 4. Although 50 patients with heartburn were studied, of these only 23 subjects were included in analysis of change of impedance during acid perfusion since the other 27 had a fall in proximal impedance of more than 5% during the neutral perfusion. In the remaining 23 subjects there was a mean age of 48 (range 20-75). pH-impedance monitoring subsequently identified that 12 of these had non-erosive reflux disease, 11 had functional heartburn. All 10 healthy volunteers had proximal impedance tracings that were able to be used in analysis.

5.3.1.2 Mean baseline proximal impedance versus distal impedance

In all subjects, baseline proximal impedance was significantly higher than baseline distal impedance (2949 ± 1103 Ω. vs. 1945 ± 1661 Ω; $p < 0.001$, figure 36).

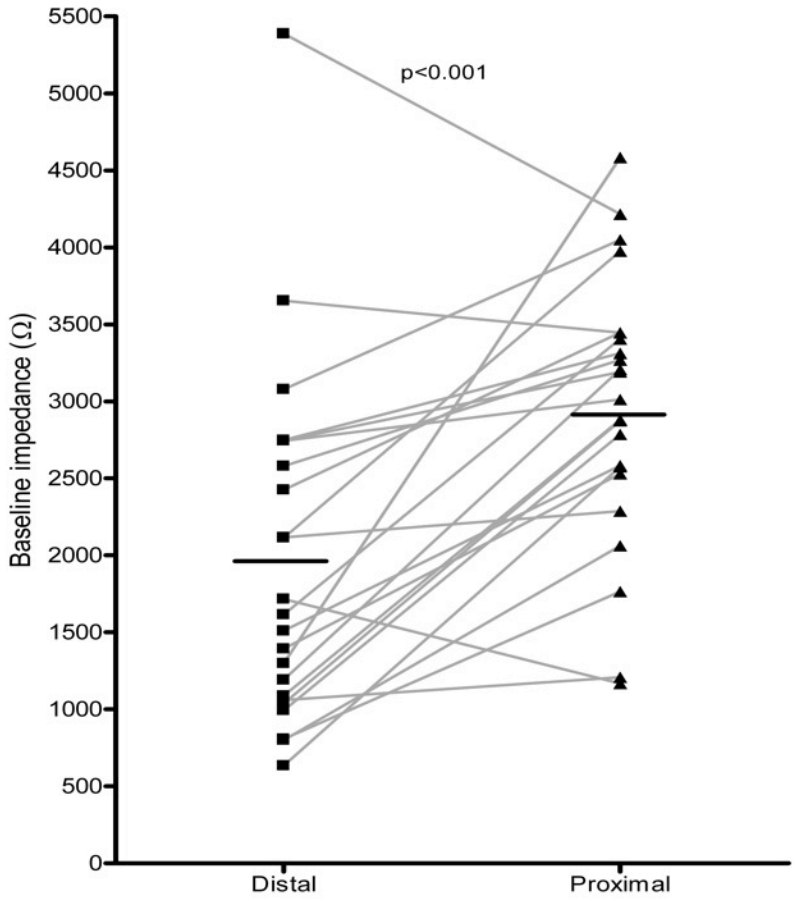


Figure 36: Baseline impedance in the distal and proximal oesophagus. The lines connect paired impedance measurements from the same patient. Horizontal lines represent the mean.

5.3.1.3 Mean baseline proximal impedance in non-erosive reflux disease, functional heartburn and healthy volunteers

There was no significant difference in mean proximal oesophageal baseline impedance between subjects with non-erosive reflux disease, functional heartburn or in healthy volunteers (2867 ± 935 Ω vs. 3039 ± 844 Ω vs. 2950 ± 765 Ω, $p>0.05$ for all comparisons, figure 37).

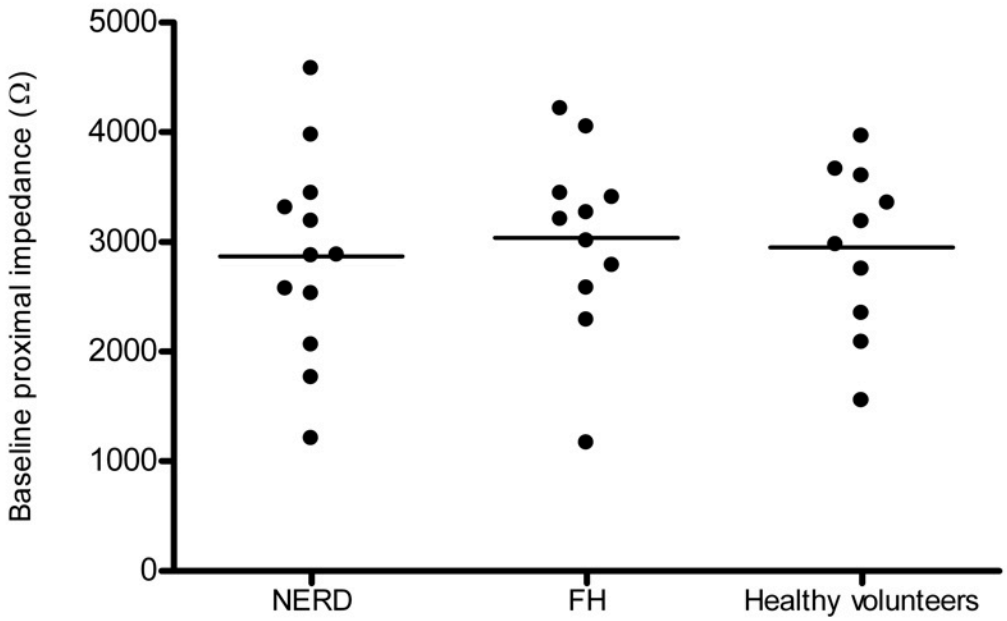


Figure 37: Mean baseline proximal oesophageal impedance in subjects with non-erosive reflux disease (NERD), functional heartburn (FH), and healthy volunteers

5.3.1.4 Change in proximal oesophageal impedance during distal oesophageal acid perfusion

All subjects

Distal oesophageal perfusion with the neutral solution caused a mean *proximal* impedance change from baseline of 182 Ω ($p>0.05$). During distal oesophageal acid perfusion there was a mean change in *proximal* impedance from baseline of -633 Ω, a 22% fall ($p<0.001$, figure 38).

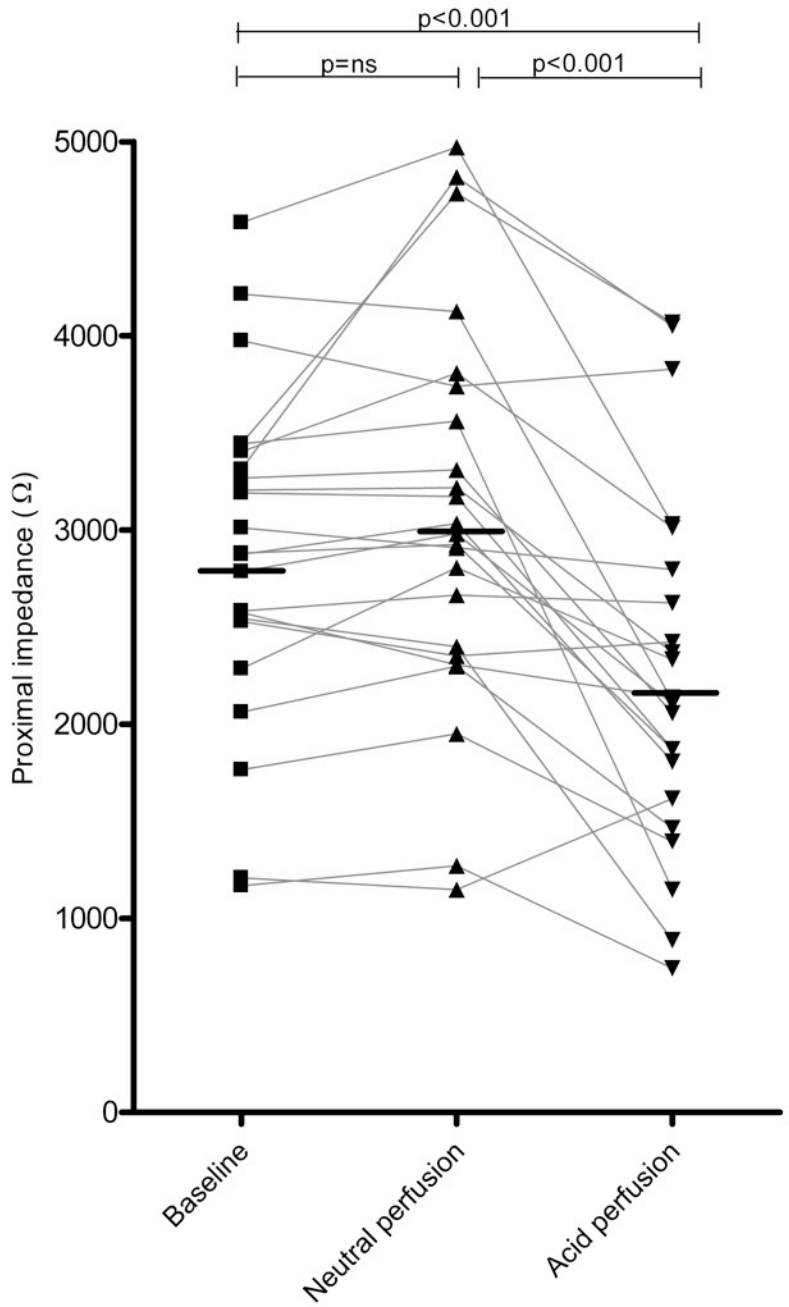


Figure 38: Mean proximal oesophageal impedance at baseline, during distal neutral perfusion, and during distal acid perfusion. The lines connect serial impedance measurements from the same patient. ns = not significant

Comparison between functional heartburn and non-erosive reflux disease

There was no difference in change of *proximal* impedance during distal acid perfusion between subjects with NERD, functional heartburn, or healthy volunteers (-397 ± 750 Ω vs. -663 ± 920 Ω vs. -591 ± 660 Ω, p>0.05 for all comparisons, figure 39).

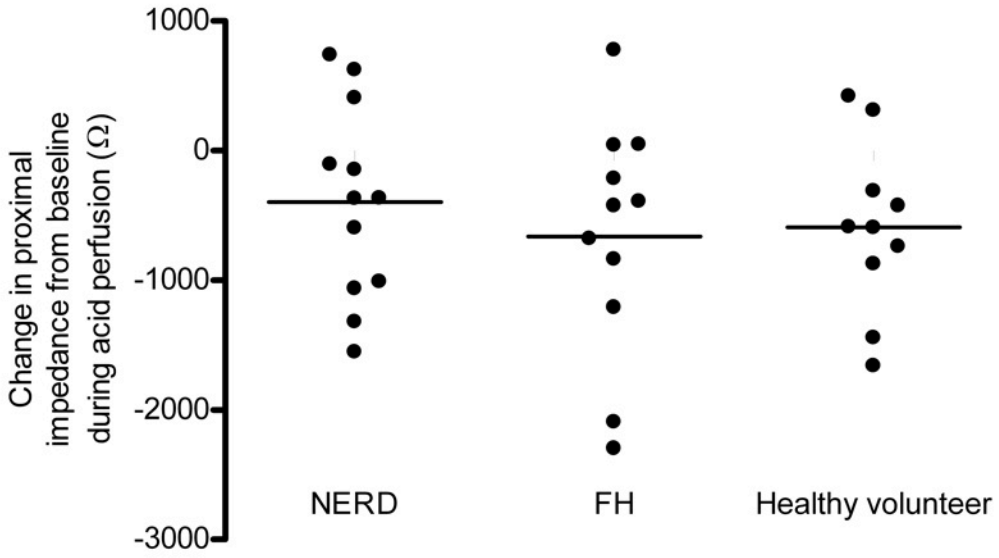


Figure 39: Proximal oesophageal fall in impedance from baseline during distal oesophageal acid perfusion in patients with non-erosive reflux disease (NERD), functional heartburn (FH), and in healthy volunteers

Comparison according to acid sensitivity

Those subjects who perceived the acid perfusion as heartburn (n=18) did not have a significantly different baseline distal or proximal impedance compared to those who did not (n=5) (2867 ± 842 Ω vs. 2979 ± 1218 Ω, p=0.8). In addition, those subjects who perceived the acid perfusion as heartburn did not have significantly

different nadir proximal impedance during the acid perfusion compared to those who did not (2580 ± 1086 Ω vs. 2409 ± 1427 Ω, p=0.8).

On further analysis of the 24-hour pH impedance study, there was no difference in baseline proximal impedance between patients with a positive symptom associated probability (SAP) for reflux and heartburn compared to those with a negative SAP (3040 ± 1020 Ω vs. 2935 ± 863 Ω, p=0.8). In contrast, baseline distal oesophageal impedance was significantly lower in SAP+ve patients than in SAP-ve patients (1302 ± 699 Ω vs. 2282 ± 1197 Ω, p=0.02).

5.3.2 In vitro assessment of proximal oesophageal integrity

5.3.2.1 Subjects

Of the 16 subjects recruited for the study, 15 had adequate paired biopsies allowing further analysis. The remaining subject was excluded due to one of the biopsies being too small to adequately cover the chamber aperture.

5.3.2.2 Baseline TER

Overall, the mean baseline TER in the distal oesophagus was 148.6 ± 82.7 Ω, and 179.0 ± 70.6 Ω in the proximal oesophagus. There was no significant difference between distal and proximal oesophageal baseline TER on paired analysis (p=0.24, figure 40).

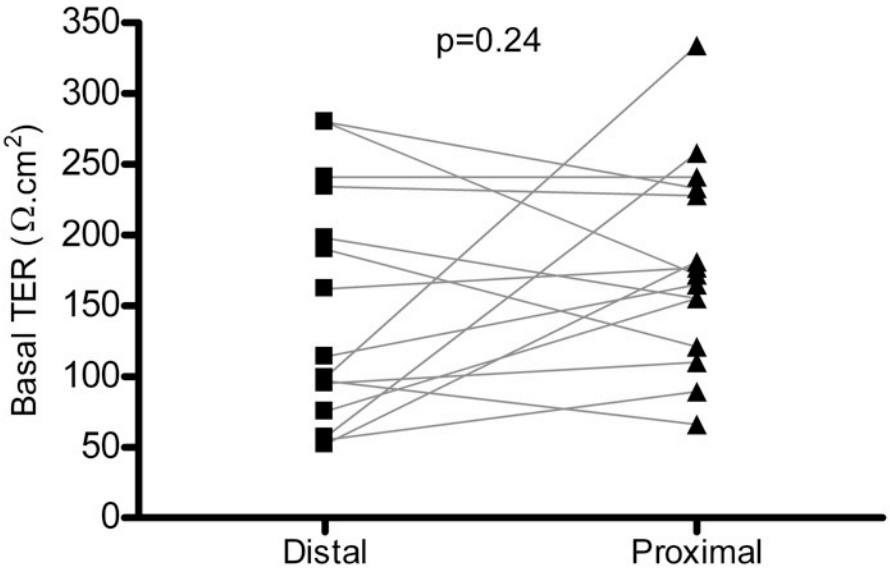


Figure 40: Baseline TER of biopsies from the distal and proximal oesophagus. The lines connect paired biopsies from the same patient.

5.3.2.3 Change in TER from baseline on exposure to acidic solution in biopsies from the proximal oesophagus

Overall, the percentage change in TER from baseline on exposure to the acidic solution in the distal oesophagus was −20.1 ± 17.0%. In the proximal oesophagus this value was −11.2 ± 13.7%. On paired analysis there was a non-significant trend towards a reduced susceptibility to TER change in the proximal oesophagus on acid exposure when compared to the distal oesophagus (p=0.19, figure 41).

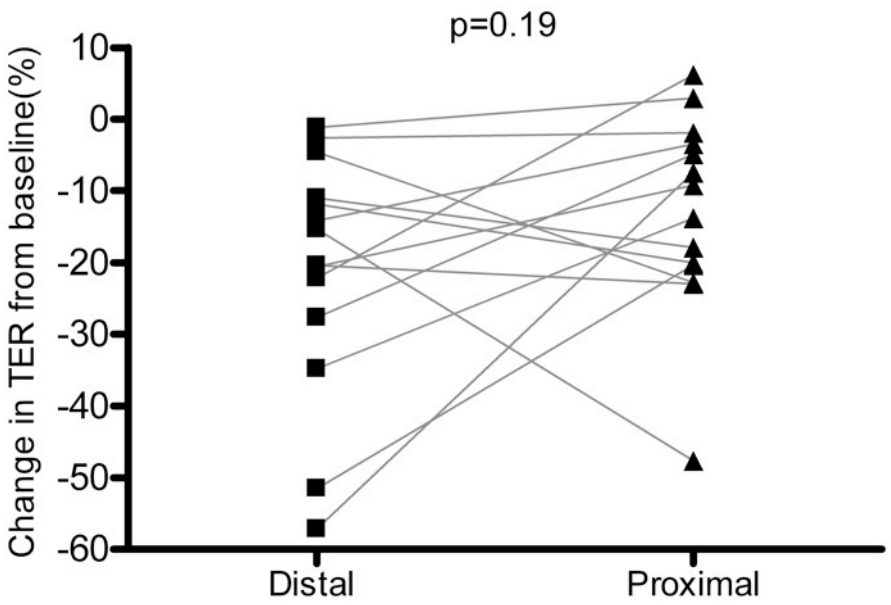


Figure 41: Change in TER from baseline on exposure to acidic solution. The lines connect paired biopsies from the same patient.

5.3.3 Histological studies: assessment of oesophageal mucosal nerve fibres

Overall, 8 proximal and 6 distal biopsies were suitable for immunohistochemical evaluation. These comprised of paired biopsies from 5 patients, and proximal unpaired biopsies from a further 3 patients, and unpaired distal biopsies from 1 further patient (total 9 patients used). Unpaired samples were used because the corresponding paired biopsy was inadequate for accurate orientation analysis. A total of 215 proximal, and 153 distal, 10 μm sections were examined. CGRP-IR fibres were identifiable in at least one section of all proximal biopsies, and in 5 of 6 distal biopsies. Fibres were identifiable in 55 of 215 proximal sections, and in 19 of 153 distal sections (p=0.002 for comparison, Fig 42A). Sections were otherwise similar in size and morphology.

Mucosal nerve fibres were seen to be strikingly more superficial in proximal oesophageal biopsies than in distal biopsies. Their morphology was also different:

proximal fibres were interspersed between flattened squamous epithelial cells with a laminar appearance, whilst distal fibres were interspersed between larger, rhomboid cells with a "pearl necklace" appearance (Fig 42C). To ensure neuronal staining, in both cases similar structures were labelled with the pan-neuronal neurofilament protein. The mean number of cells between the fibres and the luminal surface was 5.7 ± 4.1 in proximal biopsies and 22.2 ± 11.3 in distal biopsies ($p<0.0001$, figure 42B).

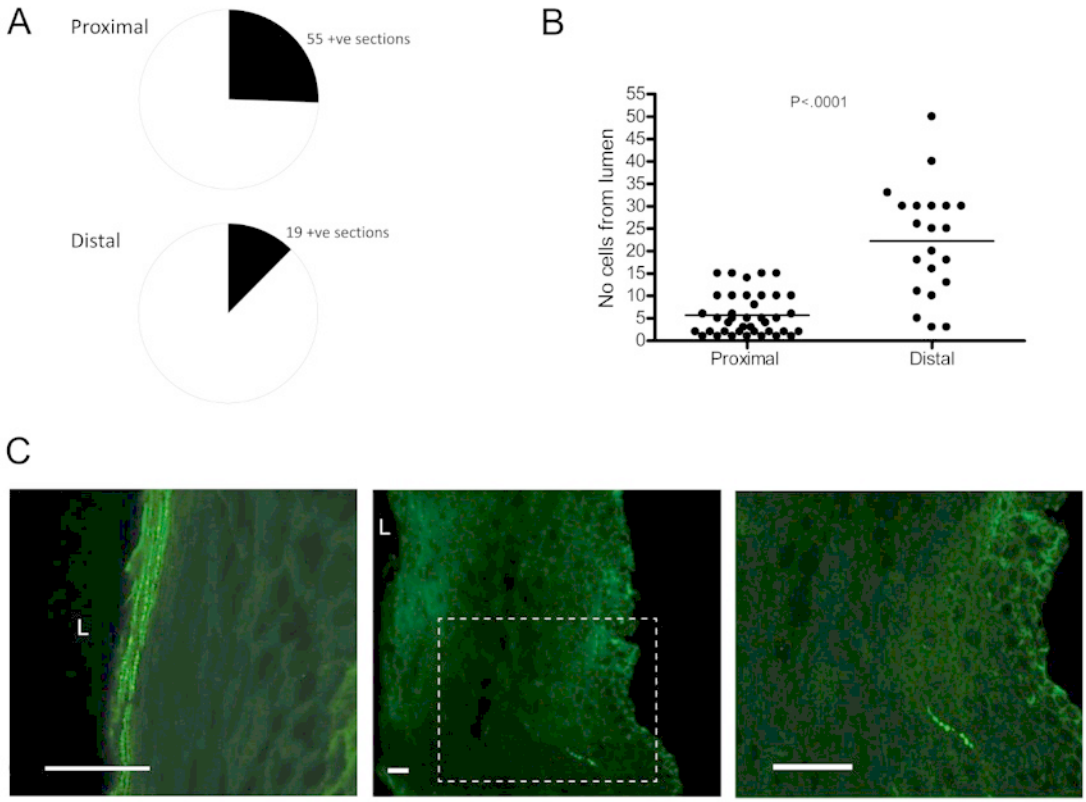


Figure 42: **A**: Proportion of sections in which CGRP-IR fibres were observed in proximal and distal oesophagus. **B:** Mean number of epithelial cells between CGRP-IR nerve fibres and the luminal surface in mucosal biopsies from the proximal and distal oesophagus. **C:** Representative examples of biopsies from the upper and lower oesophagus. The leftmost image (40x) is an example of a proximal oesophageal biopsy, with CGRP-IR fibres close to the luminal surface. The middle image (10x) is an example of the distal oesophagus, demonstrating a nerve fibre in the basal epithelium, shown at higher power (40x) in the rightmost image. L = lumen. Scale bar represents 50µm

5.4 Discussion

This chapter examined, *in vitro* and *in vivo*, the integrity of the proximal oesophageal mucosa, and its dynamic response to a direct or remote acid challenge in patients with heartburn symptoms. It also evaluated regional variability in mucosal sensory afferent nerves in the oesophagus.

We know already that these patients are more sensitive to reflux events that reach the proximal oesophagus compared to those restricted to the distal part.

The main results of the study showed:

1) A significantly *greater in vivo* baseline impedance in the proximal oesophagus compared to the distal.
2) A trend towards *higher* baseline oesophageal mucosal transepithelial electrical resistance (*in vitro*).
3) *Less* vulnerability of the proximal oesophageal mucosa to *in vitro* exposure to acid and bile.
4) In contrast with the relative robustness of the proximal oesophageal mucosal integrity, the density of mucosal peptidergic sensory nerve fibres was greater, and the location of the fibres was closer to the lumen in the proximal oesophagus when compared to the distal.
5) *In vivo* exposure of the distal oesophagus to acid causes a fall in impedance in the proximal, unexposed oesophagus, suggesting a spread in mucosal integrity changes.

There has been increasing interest in the role of the proximal oesophagus in symptom perception in gastro-oesophageal reflux disease. There is now substantial evidence from numerous studies indicating that the proximal

oesophagus is more sensitive to pain from mechanical and chemical stimuli than the distal oesophagus, and that this appears important in symptom perception in GORD[206-208, 213]. Oesophageal sensation is highly likely to have a multifactorial basis. Afferent innervation from chemo- and mechanosensitive fibres, spino-bulbar and cerebral processing of signals are all very important factors. Furthermore, the mucosal barrier function may have a significant pathophysiological role. In Chapter 3 it was demonstrated that the distal oesophageal mucosa is more vulnerable to acid exposure in patients with NERD compared to control subjects. These patients not only have microscopic mucosal changes in the distal oesophagus, (DIS) but they also exhibit similar histological changes in the more proximal part[164]. It was originally hypothesised that an enhanced perception of reflux events reaching the proximal oesophagus in GORD patients may be a phenomenon related to altered baseline and/or dynamic proximal mucosal integrity. The findings of this study suggest that this is not the case. The baseline proximal impedance is not lower in patients with NERD compared to healthy controls. In basal conditions the integrity of the proximal mucosa appears stronger than the distal (with significantly higher baseline impedance, and a trend towards a higher baseline TER). Additionally, the proximal oesophageal mucosal integrity, as measured by TER, does not appear more vulnerable to exposure to acidic solutions than the distal: indeed there is a trend towards less vulnerability in the proximal mucosa.

If an increased mucosal vulnerability to acid were a cause of the increased proximal sensitivity, one would expect a greater fall in TER during acidic exposure. It appears that the enhanced sensitivity of the proximal oesophagus is because of a different mechanism. It was then hypothesised that the proximal oesophageal mucosa might have a distinct quantity or distribution of sensory nerves.

Consequently biopsies from the distal and proximal oesophagus were examined for the presence of mucosal sensory afferent fibres. It was found that there was a greater density of sensory fibres in the proximal oesophageal mucosa. Furthermore, the location of these mucosal fibres was much closer to the oesophageal lumen in the proximal oesophagus when compared to the distal. To my knowledge, this is the first time that the distribution of human oesophageal mucosal sensory innervation has been investigated directly. The increased density and superficial distribution of the proximal mucosal afferent fibres may constitute the sensory component of a defensive mechanism against airway aspiration facilitated by proximal reflux events[313].

There are several features of the CGRP labelling that were seen which are important to note from a technical viewpoint. 1) The same labelling is seen with neurofilament and CGRP, confirming neuronal origin. 2) The superficial pattern of proximal labelling shown in figure 42C was not seen at all in distal oesophagus. 3) With variable focus the fibres could be followed back from the superficial layers to the deeper layers, indicating they are not disconnected from the parent axon, and both types of ending (deep and superficial) were seen in some sections of proximal oesophagus. 4) Many desquamating cell regions in the proximal oesophagus were CGRP negative. 5) The same CGRP antibody also labeled nerve fibres in human colon sections and whole-mounts (not shown). 6) All control images were negative.

In the presence of such a superficial distribution of sensory innervation, why is pain not felt more often when drinking acidic fruit juices or carbonated drinks? It is possible that activation of these afferents is not painful in normal conditions. Indeed, there are other examples of afferent activation without pain. It has been shown that acid irritation of gastric mucosa leads to local homeostatic reactions

and afferent nerves activation, but not neural activation in the spinal cord[314]. In contrast, in pathological situations, when these superficial nerves are exposed to refluxate there may be activation in the spinal cord and painful sensory perception. The *in vivo* provocative tests in this chapter may provide a potential reason as to why the fibres become sensitised in GORD. As described in previous experiments in healthy subjects, distal oesophageal acid perfusion not only provokes mucosal changes (drop in impedance and DIS) in the perfused area but also in the more proximal non-exposed oesophagus[161]. How these changes in proximal mucosal integrity contribute to proximal neural sensitisation is still unknown, but could include a more pronounced exposure of the superficial fibres and/or increased exposure and activation of fibres located slightly deeper in the mucosa. Furthermore, distal oesophageal acidification might also promote mucosal inflammatory changes along the whole length of the oesophagus.

Why are reflux events limited to the distal oesophagus less likely to be perceived as compared to those reaching the proximal oesophagus? Patients with NERD have impaired distal mucosal integrity. It has been hypothesised that distal DIS allows noxious components of the refluxate to access afferent endings that are located in the deep epithelium. So far, this hypothesis has not been experimentally demonstrated. The results presented in this chapter suggest that the relatively lower distal oesophageal sensitivity can be due to the differences in afferent mucosal innervation, whereby there are fewer fibres and deeper localisation in the distal oesophagus. It was interesting that neither the baseline impedance of the proximal oesophagus, nor the magnitude of change of proximal oesophageal impedance with distal acidification, was different between healthy controls, patients with functional heartburn, and patients with NERD. This is in contrast with the data from Chapter 4 showing that distal oesophageal impedance is normal

in functional heartburn but low in patients with non-erosive reflux disease. This suggests that oesophageal mucosal integrity may be of less relevance in the proximal compared to distal oesophagus.

It can be proposed that the spread of mucosal changes from a distal exposed area to a proximal non-exposed oesophagus may be important in modulating the perception of reflux episodes with high proximal extent. It has been observed that distal oesophageal exposure to weakly acidic solutions (containing pepsin and bile acids) can also produce spread of mucosal changes to the proximal oesophagus[161]. A similar type of distal oesophageal exposure occurs in patients "on" PPI therapy. It is possible, therefore, that the proximal spread of mucosal changes is part of a process that sensitises the proximal afferents to symptomatic perception of reflux: i.e. the painful perception of proximal reflux is dependent on distal oesophageal exposure to gastric contents (acid, or weakly acidic, depending on PPI therapy status). The mechanism underlying the mucosal spread of these changes is still unknown. A potential candidate mechanism can involve reflex connections with sympathetic pathways[315]. One can speculate that distal oesophageal acidification might trigger a sympathetic reflex that can, in turn, increase mucosal blood flow to the proximal oesophagus. The increased blood flow might modify mucosal ionic-liquid composition with decreased transepithelial resistance and increased exposure of superficial and deeper afferent nerve endings.

In summary, this study suggests that the relative increased sensitivity of the proximal oesophagus in GORD is not primarily due to a baseline impaired mucosal integrity, but is associated with an increased density and more superficial location of proximal mucosal afferent nerves. It is thus far uncertain whether these anatomical differences are the sole mechanism for proximal sensitivity to reflux.

Our results confirm the findings from morphological studies that distal oesophageal exposure to gastric contents (acid and weakly acidic) can modulate proximal oesophageal mucosal integrity. This may also play an augmenting role in proximal sensitivity in GORD, perhaps by facilitating further activation of superficial and deep proximal afferents. This pathophysiological interaction is of potential therapeutic interest and deserves further study.

CHAPTER 6

Protection of human oesophageal mucosal integrity

CHAPTER 6: PROTECTION OF HUMAN OESOPHAGEAL MUCOSAL INTEGRITY

6.1 Introduction

The *in vitro* and *in vivo* studies presented in this thesis have suggested a role for an impaired oesophageal mucosal integrity in symptom pathogenesis of non-erosive reflux disease. They have shown that, in basal conditions, distal oesophageal impedance is lower in patients with non-erosive reflux disease than in control subjects or those with functional heartburn. Furthermore, the mucosa of patients with non-erosive reflux disease is more vulnerable to changes in transepithelial electrical resistance on exposure to refluxate-like solutions than control subjects, and appears to have a slow post-exposure recovery *in vivo*. Finally, those patients who have greater perception of an intra-oesophageal acid challenge appear to have a lower baseline impedance in the distal oesophagus. Given that it is proposed that such functional parameters are representative of impaired mucosal integrity, it may be desirable to treat these patients with a drug that protects the mucosa against the damaging effects of the refluxate, reducing its vulnerability and thereby decreasing perception of reflux events.

For some time, the possibility of using a locally acting topical therapy in GORD has been considered desirable. This approach has obvious benefits since it allows targeted therapy with a potential for limited systemic effects. There is a need for further treatments as a significant number of patients remain refractory to PPI therapy. Furthermore there have been recent warnings about the long-term safety of PPIs, particularly concerning an increased risk of intestinal infections.[5] A topical mucosal "protectant", as a similar concept to sunscreen in dermatology, may be

able to prevent the damaging effects of gastroesophageal reflux on oesophageal mucosal integrity.

A number of attempts to develop topical therapies for GORD have been considered. Perhaps one of the most well-known is sucralfate. Sucralfate is a salt of aluminium hydroxide and sucrose octasulphate. It is believed to have cytoprotective properties, and has been shown to have clinical therapeutic benefit in reflux disease and in healing of gastric ulcers without causing a meaningful change in gastric pH. An early study examined the protective effect of sucralfate against the damaging effects of experimental acid exposure of the gastric mucosa in anaesthetised rats[316]. Subsequently sucralfate was shown to be able to protect the oesophageal mucosa of the rabbit and cat against acid injury[317, 318]. Orlando *et al.* investigated its *in vitro* effect on rabbit oesophageal mucosa mounted in Ussing chambers[319]. Changes in electrical resistance on exposure to acidic solutions were measured. In untreated tissues, progressive fall in epithelial resistance was seen on acid exposure (as seen in the human tissue experiments of Chapter 3 in this thesis). Addition of sucralfate into the luminal bath reversed this fall in resistance (and increased luminal pH). A later experiment indicated that this property was due to a cytoprotective characteristic of the SO_4^{2-} ions contained in sucralfate (and sucrose octasulphate)[320].

In clinical application there is some evidence supporting the role of sucralfate in GORD. A single-blind comparison with cimetidine in 42 patients with reflux oesophagitis demonstrated improvement in 53% and complete macroscopic healing in 31% of the sucralfate group (similar to the cimetidine group)[263]. Similar findings were seen in a comparison study against ranitidine[264]. Sucralfate has not been comprehensively tested in those patients with refractory disease. A pilot study of 8 patients with reflux oesophagitis and symptoms not responding to high-

dose histamine receptor antagonists and prokinetics found that the addition of sucralfate for 8 weeks improved symptoms and endoscopic appearances[321]. Of course, the low patient numbers, uncontrolled and unblinded nature to this study should be considered when interpreting it. A scintigraphic imaging study demonstrated a significant limitation of sucralfate in offering oesophageal mucosal protection in the absence of erosion. This coating study revealed the failure of sucralfate to remain on the oesophageal wall after application in 70% of subjects[322].

Sodium alginate solutions (usually in combination with antacid) are frequently used in treatment of gastro-oesophageal reflux disease. In the presence of gastric acid, alginates precipitate, forming a gel that may confer benefit via its physical rather than chemical properties. Alginates are natural polysaccharide polymers isolated from brown seaweed. Chemically they are copolymers of α-L-guluronic and β-D-mannuronic acid residues connected by 1:4 glycosidic linkages. In an acidic environment alginic salts and alginic acids precipitate within minutes to form a viscous gel, and may have advantageous mechanical and adhesive properties.

The treatment of GORD with alginate-antacid combinations has been investigated in a number of studies. A randomised double-blind placebo trial of Gaviscon Advance (alginate, calcium carbonate and potassium bicarbonate) liquid in the treatment of patients with heartburn symptoms (with no endoscopic or physiological criteria) was published in 1999[266]. Ninety-eight patients were randomised to either 4 weeks of 10 ml Gaviscon Advance four times daily, or placebo. Physician and patient assessment found Gaviscon Advance to be superior to placebo in symptom control at 2 and 4 weeks. A further placebo-controlled crossover trial of Gaviscon tablets in 60 patients with meal-induced heartburn

found Gaviscon to be superior to placebo in symptom control (80% relief vs. 47%)[267]. There are theoretically advantageous properties of alginate-containing preparations over pure antacid. Despite these theoretical advantages, studies comparing alginate-antacid with pure antacids have been conflicting in describing differences between the two. Whilst some studies have suggested symptomatic benefit of alginate-antacids over antacid alone[323-325], others have found no differences between the two[259, 326].

What may be the mechanisms of the potential benefits of alginate solutions in patients with GORD? The gel that is formed when alginates encounter low pH or calcium is able to form a physical raft on top of the gastric juice. This floating capability is often enhanced by the inclusion of bicarbonate, which facilitates the production of CO_2 in the acid stomach environment, which is proposed to turn the raft into a foam that aids buoyancy[265]. This alginate gastric raft is able to reduce the number of acid reflux episodes in healthy volunteers[327, 328], with this property being considered as due to the viscous barrier surface tension reducing reflux through the gastro-oesophageal junction. A second important physical property of alginate rafts appears to be its ability to abolish or displace the post-prandial "acid pocket" in patients with symptomatic GORD[329].

It is possible that, in addition to the antacid and gastric mechanical properties of alginate-antacids, there may also be an oesophageal mucosal protective effect in gastro-oesophageal reflux disease. This is because they are thought to have additional bioadhesive properties to the oesophageal mucosa. Specific delivery and prolonged retention of a drug in the oesophagus is highly desirable in the treatment of GORD (and indeed other oesophageal disorders such as eosinophilic oesophagitis and hypersensitive oesophagus). The defensive properties of alginate

solutions are of interest in the treatment of GORD, where acid, pepsin and bile acid are all believed to be important in symptom pathogenesis.

The study presented in this chapter investigates the *in vitro* effects of an alginate-antacid solution (Gaviscon Advance) on human oesophageal mucosa when exposed to solutions containing acid, pepsin and bile acid.

It is hypothesised that human oesophageal mucosa integrity can be protected, and that mucosal vulnerability to acid exposure observed in NERD biopsies can be diminished with an alginate-based topical protectant solution.

The aim of the study is to test the *in vitro* feasibility of a topical protection of oesophageal mucosal integrity with an alginate solution.

6.2 Methods

6.2.1 Patients

Patients with typical symptoms of gastro-oesophageal reflux disease (heartburn and/or regurgitation) were recruited from the endoscopy department of the Royal London Hospital. Subjects with oesophageal erosions or Barrett's oesophagus were excluded from the study.

18 subjects were studied. Four were excluded due to having at least one inadequate biopsy according to the criteria described in Chapter 3. The remaining 14 subjects each had 3 biopsies that could be compared by application of one each of the "protectant solutions" (i.e alginate solution, viscous control and liquid control).

6.2.2 Endoscopy

Endoscopic procedures were performed under midazolam sedation or with pharyngeal local anaesthetic spray. Three oesophageal mucosal biopsies were taken (Radial Jaw 3 forceps, Boston Scientific, USA) from 3 cm above the squamo-columnar junction, and immediately placed in a pre-oxygenated Krebs-Henseleit buffer solution at pH 7.4. The biopsies were rapidly transported to the laboratory for Ussing Chamber study. All biopsies for the following studies were taken by the same endoscopist (Dr Woodland), using the same technique.

6.2.3 Materials

3 "mucosal protectant" solutions were used in this study, one test solution and two control solutions.

1) Alginate solution (Gaviscon advance, Reckitt Benckiser (UK) Ltd). Active ingredients sodium alginate 100 mg/ml, potassium bicarbonate 20 mg/ml, calcium carbonate 20 mg/ml. Viscosity 2470 cPs. Conductance 12.8 mS.
2) Viscous control. Hydrogenated glucose syrup, xanthan gum. Viscosity 3049 cPs. Conductance 1.9 mS.
3) Liquid control. Krebs-Henseleit solution at pH 7.4. No viscosity. Conductance 7.1 mS.

Viscosity of these solutions was determined by the mean of 3 measurements with a Brookfield LVDV-II+ viscometer. Conductance was measured using a Hanna EC215 Conductivity Meter.

6.2.4 Ussing chamber study

Biopsies were orientated and mounted in adapted Ussing chambers as described in Chapter 3. Biopsies were mounted in the Ussing chamber and equilibrated in Krebs-Henseleit buffer pH 7.4. After making a correction for fluid and circuit resistance, transmucosal potential difference was continuously monitored with Ag/AgCl electrodes. The basal transepithelial resistance (TER) was calculated according to Ohm's law from the voltage deflections induced by bipolar current pulses of 50μA, duration 200 ms every 6 seconds applied through platinum wires. All experiments were conducted in open-circuit conditions. The system was equilibrated at 37^0C until a stable TER baseline was established (typically 20 minutes).

After reaching a stable TER baseline, recording was paused and the chambers were removed from the apparatus and the two halves separated. This was done such that the "luminal" aspect of the biopsy was exposed. 200 μl of a "protectant

solution" was then applied to the exposed luminal aspect of the biopsy and left on the biopsy surface for 5 minutes (figure 43).

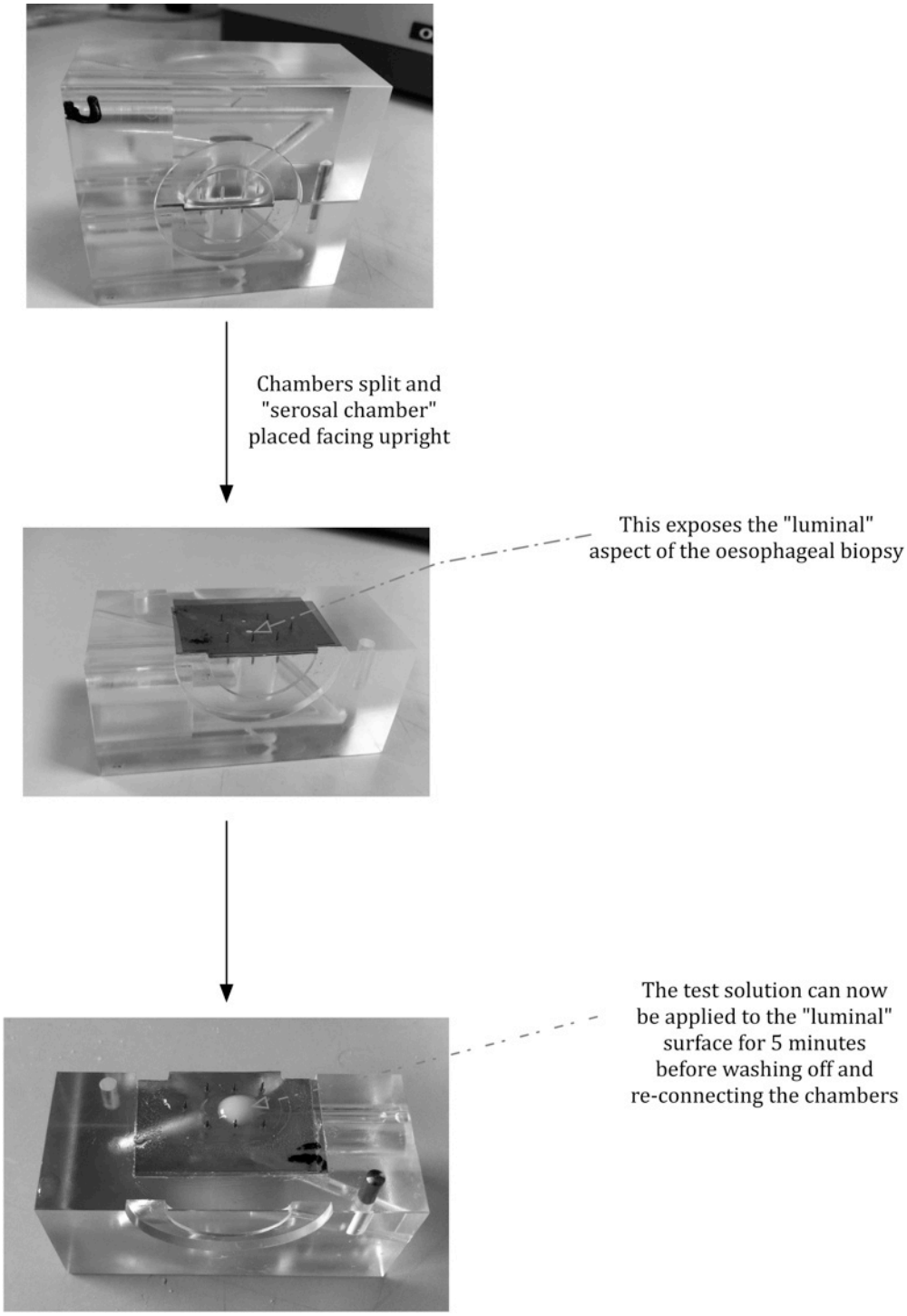


Figure 43: Technique for application of the mucosal protectant solution to human oesophageal biopsies in an Ussing chamber

For each patient, 3 biopsies were studied: i.e. each biopsy was exposed to either 1) Alginate solution; 2) Viscous control; 3) Liquid control. After 5 minutes the "protectant solution" was washed off with 5 ml Krebs-Henseleit pH 7.4 solution, and the chambers rejoined and filled with Krebs-Henseleit at pH 7.4. The biopsies were then allowed to re-equilibrate and wash in neutral solution for a further 15 minutes. For each biopsy the solution in the luminal chamber was then replaced with an acidic solution (Krebs-Henseleit at pH 2 + 1 mg/ml porcine pepsin + 1 mM taurodeoxycholic acid). This acidic exposure continued for 30 minutes, during which time TER was continuously measured (figure 44). Percentage change in TER from baseline at 30 minutes was calculated as described in Chapter 3.

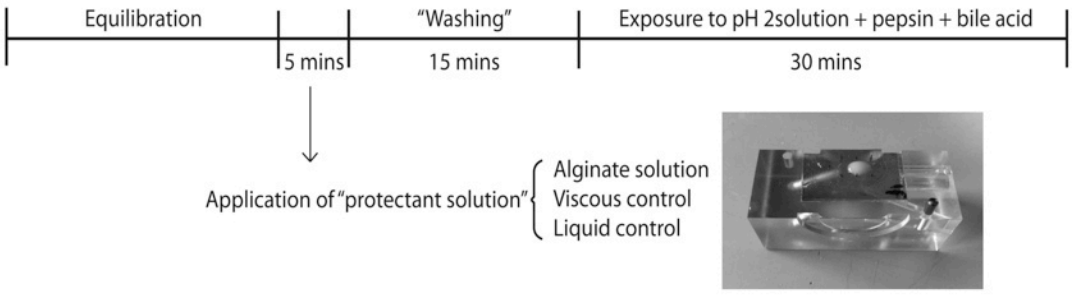


Figure 44: Study scheme for mucosal protectant experiment

6.2.5 Effect of antacid component of alginate solution

The viscous placebo solution does not contain antacid. It is possible that any protectant effect of the alginate solution is not due to the alginate, but rather due to the antacid component. To consider this possibility the experiment was repeated in biopsies from a further 6 subjects whereby the viscous control "protectant solution" was applied, this time containing 2.0 mmol calcium

carbonate (the same antacid at the same concentration as found in the alginate solution), and vigorously shaken before application. The protection experiments with this solution were conducted in the same way as detailed above.

6.2.6 Statistical methods

All data are expressed as mean ± standard deviation unless otherwise stated. Normality of distributions was assessed using a D'Agostino and Pearson omnibus normality test. Analysis of the change in TER that occurred on acid exposure in biopsies exposed to the 3 protectant solutions was done using repeated measures ANOVA with Bonferroni's multiple comparison test. Comparison between viscous control with and without antacid was done using an unpaired t test. Significance was declared at $p<0.05$.

6.3 Results

6.3.1 Ussing chamber experiments

Biopsies pretreated with liquid control solution did not appear to benefit from a protection effect, since there was mean change in TER of −21.1 ± 16.6%. This is very similar to the fall seen without topical treatment seen in the "unprotected" biopsies from patients when exposed to the acidic solution in Chapter 3 (-22.8 ± 11.9%).

When biopsies were pre-treated with the viscous control solution, the acid-induced change in resistance was of −15.26 ± 13.8%, which was not significantly different to the liquid control results.

When biopsies were pre-treated with the alginate solution, acid failed to provoke a significant drop in resistance, with a mean change in TER of −2.7 ± 6.9% from baseline. This change was significantly smaller than was seen in both the viscous and liquid control biopsies (table 5 and figure 45).

Comparison	**Mean difference**	**P value**	**95% CI**
Alginate vs. Viscous Control	12.51	$P < 0.05$	2.170 to 22.85
Alginate vs. Liquid Control	18.35	$P < 0.001$	8.005 to 28.69
Viscous Control vs. Liquid Control	5.83	$P > 0.05$	-4.507 to 16.18

Table 5: Bonferroni's multiple comparison test of repeated measures ANOVA

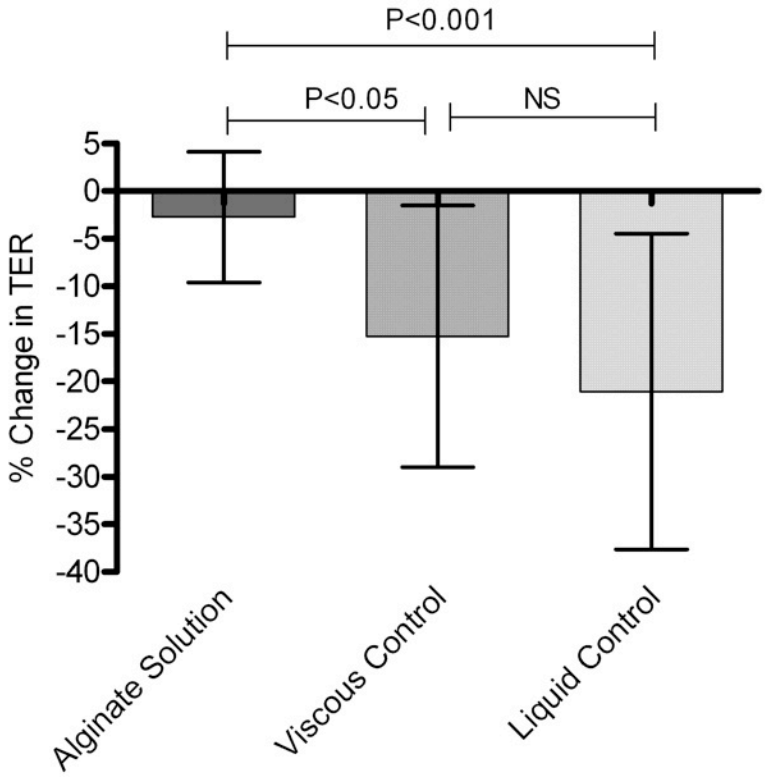


Figure 45: Comparison of change percentage change in TER compared to baseline on exposure of biopsy to pH2 solution containing pepsin and taurodeoxycholic acid after topical pre-treatment with alginate solution, viscous control and liquid control. NS = not significant

6.3.2 Assessment of contribution of antacid

The effect of addition of calcium carbonate 2.0 mmol to the viscous control was compared in 6 subjects. The mean change in TER from baseline in biopsies pre-treated with viscous control + antacid was −23.1 ± 9.1%, which was a similar change from baseline as seen in the standard viscous control (in fact numerically slightly greater change, but statistically insignificant at p=0.22, figure 46).

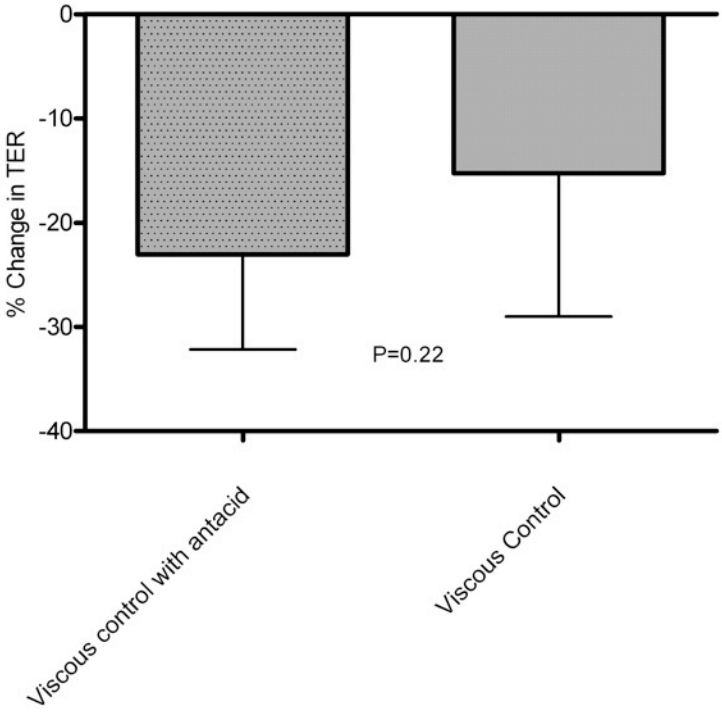


Figure 46: Comparison of percentage change in TER compared to baseline on exposure of biopsy to pH2 solution containing pepsin and taurodeoxycholic acid after topical pre-treatment with viscous control + 2.0 mmol calcium carbonate and viscous control alone

6.4 Discussion

If the mucosa of symptomatic patients is vulnerable to noxious effects of gastro-oesophageal refluxate, then a therapy that reduces the noxious exposure is desirable as it allows fast protection at the site of potential injury. This study investigated the potential of a topically applied alginate solution to reduce acid-induced integrity change in human oesophageal biopsies.

The study results were the following:

1) Topical pre-treatment with an alginate solution was able to significantly reduce *in vitro* change in TER caused by acid-pepsin-bile acid solutions.
2) This property appears to be independent of the viscosity of the solution, and independent of the presence of antacid.

As such, these *in vitro* results suggest that an alginate-containing solution may be able to reduce impact of refluxate on mucosal integrity. The *in vivo* implications of this require further investigation.

As previously described, attempts have previously been made to study the *in vitro* effects on mucosal electrical resistance of a topical treatment with sucralfate. Studies in cat, rabbit, and one study in human oesophageal mucosa (obtained from oesophagectomy specimens in patients with squamous cell carcinoma and exposed to prior radiotherapy) showed that sucralfate was able to attenuate the effect of acid exposure on mucosal electrical resistance[319, 320, 330]. This study is the first using oesophageal biopsies from human subjects without cancer or previous chemotherapy/radiotherapy.

In the study care was taken to control for the viscosity of the control solution as this could be a confounding factor if high viscosity was causing a physical barrier against the test solutions. To overcome this, a placebo solution of similar viscosity to the alginate solution (but lacking alginate or antacid) was used. The viscosity measured was actually very slightly higher in the viscous control than in the alginate solution (albeit in relative terms the results are very similar). This rejects the possibility that it is viscosity alone that protects the mucosa against access by the acid solution. Furthermore, in this study care was taken to be thorough in washing off "pre-treatment " solutions after applying them, both with a fast 5 ml pipette wash, then with a 15 minute period in neutral solution within the Ussing chamber before exposure to the acidic solution. If the alginate solution was an electrical insulator then application could have resulted in an artifactual high TER. However, the alginate solution was an excellent electrical conductor, with conductance that was in fact greater than seen in the control solutions.

It was possible that the protective effect of the alginate solution was due to its antacid component rather than the alginate itself. The addition of calcium carbonate antacid to the viscous control did not lead to an additional protective benefit over the standard viscous control. As such, it appears likely that the protective effect of the alginate solution is due to the alginate itself.

It is of interest to consider the mechanism by which alginate solutions may protect the oesophageal mucosa. Along with their mechanical properties at the gastro-oesophageal junction and on the acid-pocket, alginates have been found to demonstrate bioadhesive potential, a property determined primarily by polymer chain length and the presence of ionisable groups rather than e.g. the viscosity of the gel used[331]. Furthermore they appear to become adhesive on hydration (as occurs in the gastrointestinal tract). An *in vitro* porcine model of oesophageal

mucosal retention investigated the adhesiveness of fluorescein-labelled alginates. Alginates were applied to porcine oesophageal mucosa or cellular acetate, and washed continuously at 1 ml per minute with a series of solutions (including artificial and human saliva). With all of the alginates applied to the oesophagus there was approximately 20% mucosal retention after 30 minutes washing, suggesting a potential for bioadhesion. This appeared to be due to an alginate-mucosal interaction since there was no retention on a cellular acetate model[332]. A further study using a porcine *in vitro* model determined that the high molecular weight polymers exhibited better bioadhesion than low molecular weight polymers (with approximately 40% versus 20% retention at 20 minutes)[333]. It has also been shown that the nature of the vehicle used for the alginate preparation can influence bioadhesive properties. Suspensions containing a vehicle that required a low level of dilution to initiate swelling (such as glycerol) are more mucoretentive in *in vivo* studies[334]. When adhered to the oesophageal epithelium, the alginate solution may enable protection against refluxate damage. *In vitro* diffusion studies (using a dialysis membrane) have shown that the presence of alginates significantly reduces acid and pepsin diffusion across the membrane when compared to control[335]. The alginate-based formulation of Gaviscon Advance (Reckitt Benckiser (UK) Ltd) has been found, *in vitro*, to inhibit pepsin activity and to reduce pepsin and bile acid (taurocholate, glycocholate and deoxycholate) diffusion across an artificial membrane[336]. Since we know that mucosal integrity appears to be impaired by the presence of acid, pepsin and bile acid it would be possible that the impact Gaviscon has on diffusion and activity of these substances plays a role in the protective effect seen in this study. The bioadhesive properties appear to have enabled protection to be present after active and passive washing phases totalling more than 15 minutes.

Whilst these findings of our study are of interest, it is important to note that they are preliminary in nature. The *in vitro* testing environment of such a drug is obviously an artificial environment. The method of application for 5 minutes was not representative of normal conditions, as *in vivo* there is oesophageal peristalsis and saliva that will act to clear some of the drug. Although the solution was washed off before exposure to acid, during the application period no such washing occurred. There is also no indication of how these *in vitro* changes directly translate into the *in vivo* situation. However, these preliminary data serve as a platform to further study properties of topical protectant solutions (alginate-based or otherwise). It will be interesting to examine the *in vitro* effects of topical solutions in greater detail. For example, it is highly likely that acid exposure of the oesophageal epithelium causes activation of epithelial acid-sensitive receptors (such as TRPV1), with the release of inflammatory mediators[337, 338]. It is possible that release of such mediators can be evaluated (by sampling the "basal" chamber), and the effect of a topical protectant could be assessed. It may even be possible to "clamp" mucosal afferents and assess activation in response to acidification, which again could be assessed after topical protection. Furthermore, candidate acid/bile acid sensitive receptors could also be targeted with drugs added to the topical solution, and effect on integrity change or neurotransmitter released assessed *in vitro* before translation to the *in vivo* situation.

We know that PPIs are very effective therapies for GORD, and it would not be expected that a topical therapy could replace PPI as a treatment. However, as has previously been described, a significant number of patients remain refractory to PPI therapy, and as such there is an unmet need for improved therapy. The role of topical treatments would be expected to be either as an on-demand therapy in patients with mild disease, or as an add-on therapy in those with an incomplete

response to PPI or surgery. It is a particularly attractive strategy in PPI-refractory GORD since it could offer potentially protection against components of the refluxate other than strong acid (e.g. weak acid, bile) that are the cause of refractory reflux in some cases[70, 339]. Topical therapy could also serve as a potential therapy for those patients with acid-hypersensitive oesophagus, whereby they are sensitive to very short-lasting acid exposures (which can continue to occur on PPI therapy).

In summary, this study serves as a proof of concept that topical application of oesophageal mucosal protectants may have the ability to preserve mucosal integrity in the face of *in vitro* noxious exposures. Furthermore it presents a model that can be used in future assessment and development of topical mucosal solutions. Development of clinically effective topical solutions may meet an unmet need in PPI-refractory patients.

CHAPTER 7

General discussion

CHAPTER 7: GENERAL DISCUSSION

The prevalence of gastro-oesophageal reflux disease (GORD), Barrett's oesophagus and oesophageal adenocarcinoma is increasing rapidly. There is a clear link with obesity, which is also becoming an increasing public health problem. As such GORD is being increasingly encountered in primary and secondary healthcare. Refractory GORD remains a significant problem, and most patients who reach secondary care clinics have failed over the counter medications and then anti-secretory medications given in primary care. These patients present a challenge to the gastroenterologist assessing them. This means there is a need to better understand the pathophysiology of GORD and progress towards new treatment strategies.

Non-erosive disease perhaps presents the greatest challenge to clinicians, since it has less objective end points than erosive disease (in which mucosal healing can be evaluated) and it may have a poorer response to PPI therapy. The mechanisms of symptom perception are also less intuitive in non-erosive reflux disease, where there is an absence of obvious mucosal damage and inflammation. Nevertheless, recent years have highlighted the microscopic impairment of the oesophageal mucosa in non-erosive reflux disease, and this has prompted the hypothesis that an impaired mucosal integrity may play a role in disease pathogenesis and symptom perception. It has been suggested that this impaired integrity can be assessed not only morphologically (in the form of dilated intercellular spaces), but also functionally (in terms of *in vitro* transepithelial electrical resistance and *in vivo* oesophageal mucosal impedance). Dilated intercellular spaces are a static measure of integrity, and do not tell us about the response of the tissue to an aggressor challenge. Functional measures of integrity allow assessment of integrity

over time, and therefore can be used to assess the dynamic responses of the mucosa.

Throughout the thesis, the concepts of hypersensitivity and sensitisation are commented on, and their relationship in the context of this thesis deserve further discussion. In the opinion of many, the concept of hypersensitivity is a purely neural concept representing an abnormal neural response to a stimulus. The complex nature of the mucosal interface with the gastro-oesophageal refluxate has led the concept of hypersensitivity to be discussed in a broader sense through this thesis. Whilst the sensitivity to a stimulus can indeed be exaggerated by a purely abnormal neural response (at the peripheral afferent level, or at the central spinal or cerebral level), other variables in the locality of the mucosa will affect the strength of the neural response to a given stimulus, and thus are also described in terms of hypersensitivity. This means that, in this context, there are times when the oesophageal mucosa is hypersensitive to reflux due to an abnormal barrier function, not a neural abnormality. In such a case there may be increased activation of normal neurones due to increased access of noxious stimuli to it (because of an impaired mucosal integrity).

In contrast, chapter 5 describes the concept of an "increased sensitivity" of the proximal oesophagus compared to the distal oesophagus. In this context it is not intended to reflect an abnormal process, but rather a differential distribution of "normal" afferent nerves that may result in the proximal oesophageal mucosa being more sensitive to a given stimulus than the distal mucosa.

There is also the possibility that, in reality, the story is even more complex. As yet we are unsure of the mechanisms that result in changes to the permeability of the mucosa. It may be that the same mechanisms that contribute to permeability

changes can also cause neuronal sensitisation (such as may happen in the case of an inflammatory reaction to the stimulus) and thus cause hypersensitivity both by increasing access or noxious stimuli to the afferent nerve and by causing an abnormal response of the nerve itself. We do not know whether such processes may also be involved in the proximal oesophagus in NERD, but if so this could accentuate the already marked differences of the normal proximal mucosa compared to the distal.

At the end of Chapter 1, remaining questions were identified, and these shall now be addressed in the light of the findings of the studies presented in this thesis.

How does human oesophageal mucosa compare with animal oesophageal mucosa previously used in experimental work?

Our *in vitro* and *in vivo* experiments have provided novel data that can be compared to animal studies. Except for a few studies addressing baseline human mucosal characteristics, previous *in vitro* studies of functional oesophageal integrity have been assessed in animals (predominantly rabbits and rats).

An immediately noticeable finding is that the baseline TER in the human mucosa is much lower than that seen in other animals. In Ussing chamber studies of rabbit oesophageal mucosa, Farré *et al.* found baseline TER values in the range of approximately 1500 to 2500 $\Omega.cm^2$ [153]. The group of Tobey *et al.* also measured baseline rabbit oesophageal mucosal TER to be approximately 2000 $\Omega.cm^2$ [340]. The data from studies presented in this thesis demonstrate a much lower TER baseline, in the range of 68 to 285 $\Omega.cm^2$. A part of the reason for this discrepancy between rabbit and human baseline TER may be due to size of the tissue sample. In the aforementioned studies rabbit oesophagus was cut in sections and mounted in

chambers with an aperture of 0.3 to 1.2 cm^2. In the human studies an aperture of 0.017 cm^2 was used. There is considerably more "edge effect" at smaller aperture sizes: i.e. there is inevitably damage at the edge of the biopsy due to pressure from the apposing halves of the chamber, and for a smaller aperture the circumference where this damage occurs is a higher overall proportion of the tissue being studied. To investigate this further we have also mounted mucosal sections from human oesophagectomy specimens in chambers with a 0.5 cm^2 aperture, and found the baseline to be around 300 to 400 $\Omega.cm^2$. This suggests that edge effect can have a significant impact, but nevertheless the basal TER is considerably lower than is seen in rabbits. Corroborating with our data are two studies from other groups who have examined basal, static, TER in human oesophageal mucosal biopsies. Jovov *et. al.* in the USA have published values between approximately 70 to 300 $\Omega.cm^2$ [133], and Weijenborg *et al.* in the Netherlands have found values between approximately 70 to 125 $\Omega.cm^2$ [294]. Overall this suggests an inherent difference in the baseline integrity characteristics of rabbit and human oesophageal mucosa.

When considering the effects of acidic solutions on dynamic changes of TER in animal and humans there is less data available to compare. The best comparator is the study by Farré *et al.* investigating the effect of acidic solutions containing pepsin and bile acid on TER of rabbit oesophageal mucosa in Ussing chambers[153]. In this study, the concentrations of bile acids used were mostly high (2 to 5 mmol/l), but a concentration of 0.5 mmol/l was also used. It can be seen that, when the mucosa was exposed to pH 2 solution with pepsin and 0.5 mmol/l taurodeoxycholic acid, there was a mean change in TER of −17%. On exposure to a similar solution with a 2 mmol\l bile acid concentration there was a mean change of -58%. The overall mean change in TER seen in our studies using 1 mmol/l

taurodeoxycholic acid was −14.4%, i.e. slightly less than that seen in the rabbit mucosa when exposed to the lower concentration of bile acid. This suggests that, although ionically less "tight" at baseline, the human mucosa may be less vulnerable to integrity changes on exposure to refluxate-like solutions. At baseline, since the TER is formed almost entirely from characteristics of paracellular ion diffusion, it suggests that this pathway is more ionically permeable in humans than in rabbits. Differences in resistance in this pathway are likely to be due to differences in the tight junction-apical membrane morphology and/or function between species, but this is as yet untested. Changes in TER on exposure to reflux is likely to involve more complicated, dynamic mechanisms, and these can be discussed when considering the *in vitro* and *in vivo* response of human oesophageal mucosa to acid.

An important question is whether the previous studies in animal oesophageal mucosa can be used to understand human disease. There are several qualitative similarities with humans that suggest they can. For example, the formation of DIS in response to acid is similar, and the functional changes in impedance and TER are also similar. As such, mechanistically, they appear similar to humans. The main differences are in the quantitative results of baseline TER values and response to acid. Of course, perhaps the greatest benefit of studying human tissue rather than animal is the possibility of clinicopathological correlation.

How does the normal human oesophageal mucosa respond when it is exposed to reflux (experimentally and in vivo)?

It is known that patients with non-erosive reflux disease, but not healthy controls, display dilated intercellular spaces (DIS) in the oesophageal mucosal basal epithelium, and that the healthy oesophagus develops DIS when exposed to acid.

The development of DIS is an "all or nothing" phenomenon, and its measurement has inherent difficulties (such as a difficulty to perform truly "random" measurements, and large intra-individual variability of spaces), meaning that interpretation of dynamic changes and objective comparison within closely matched groups (e.g. between patients with non-erosive reflux disease) is not feasible. As such the studies presented in this thesis used measures of functional integrity to assess dynamic changes on exposure to acidic solutions. These *in vitro* and *in vivo* experiments reveal that, on exposure to acidic solutions, the human oesophageal mucosa responds with an impairment of integrity. *In vitro*, this was measured in terms of a reduction in TER from baseline during the exposure. This was tested on exposure to acidic and weakly acidic solutions (representative of refluxate composition both in "off" and "on" PPI conditions). It can be seen that, in all subjects, the exposure to the acidic solution (pH 2 + pepsin + bile acid) results in an overall greater mean reduction in TER compared to the weakly acidic solution (pH 5 + pepsin + bile acid) (-14.4 ± 15.3% vs. -1.6 ± 11.0%), suggesting that this change in integrity is indeed pH dependent.

The *in vivo* studies used intraluminal mucosal impedance as a surrogate marker of mucosal integrity. In the study presented in Chapter 4, we can see that perfusion of a neutral (pH 6.7) solution did not cause any reduction in impedance after a 10 minute exposure. This corroborates findings from Farré *at al.* during their morphological studies of the healthy human oesophagus, where they found that *in vivo* neutral perfusion did not cause any change in intercellular space diameter[161]. In contrast, perfusion of acidic (pH 1) solution causes a profound fall in impedance. For all subjects the mean change in distal oesophageal impedance from baseline 5 minutes after cessation of the acidic perfusion was -1215 Ω (a change of −51%). It was very apparent that this change in impedance was not a brief, transient

phenomenon, but was long-lasting. At 90 minutes post acid perfusion the mean baseline was still only 73% of baseline.

The reason for the acid-induced changes in human oesophageal mucosal integrity are as yet unknown, but are of interest to future understanding and management of GORD. The most widely held belief is that the mechanism underlying such changes in integrity are related initially to acid-induced epithelial junctional barrier damage. The theory proposed by Orlando *et al.* is that acid is able to directly damage this junction, leading initially to increased ionic permeability, then further disruption as water follows chloride ions into the epithelium and swells the intercellular spaces (and so increasing permeability further)[291]. This group has published a study indicating that there is cleavage of e-cadherin (an important component of the epithelial adherens junction) in the mucosa of patients with GORD (including erosive disease)[133]. This was associated with a reduction in basal TER in corresponding biopsies studied in Ussing chambers. This model is an intuitive one, but the relationship is not necessarily causal. It is entirely possible that the disruption in integrity occurs via a more indirect pathway. It has been noted that the squamous epithelium of the oesophagus has numerous receptors in quite superficial locations (such as the acid-sensitive receptor TRPV1)[337]. Acid may be able to directly stimulate such receptors on the superficial epithelium of the oesophagus, resulting in release of several inflammatory mediators such as platelet activating factor (PAF)[338]. These in turn could mediate inflammatory disruption of the epithelial integrity, such as is seen in eosinophilic oesophagitis (where DIS is also seen[341]). Such inflammatory disruption could lead to peripheral sensitisation by increasing permeability to noxious components of the refluxate and by sensitising mucosal sensory afferent nerves. Our studies cannot support one or the other of these hypotheses, and this can be a focus of work in the future.

The other phenomenon we have observed in our *in vivo* studies is the "spread" of integrity change from the distal to proximal oesophagus on distal acid perfusion (i.e. perfusion of the distal oesophagus was able to cause a change in impedance in not only the distal, perfused oesophagus, but also in the proximal, unexposed oesophagus). This phenomenon was seen previously, again using morphological means, by Farré *et al.*[161]. Acid perfusion of the distal oesophagus of healthy volunteers (without pre-existing DIS) was able to provoke DIS formation in the proximal oesophagus. Correspondingly, patients with non-erosive reflux disease have DIS in the distal and (less often acid exposed) proximal oesophageal mucosa. Perhaps these observations support the more "indirect" theory of integrity impairment. Mechanisms other than direct acid exposure are able to affect oesophageal mucosal integrity. Furthermore, it appears that mucosal integrity does not begin and end at DIS. The *in vivo* and *in vitro* studies in this thesis have been performed in patients, who in many cases, would be presumed to have pre-existing DIS (i.e. those with refractory GORD in the *in vitro* study, and especially those with GORD not currently taking PPI in the *in vivo* study). Despite this likely pre-existing DIS in both proximal and distal oesophagus, acid exposure is able to cause further disruption in integrity in both the distal and proximal oesophagus. It would suggest that cell barrier function is not at its most impaired when DIS is present and can be further damaged perhaps by further acid damage to intercellular junctions and/or activation of epithelial receptors (whose availability to refluxate may be enhanced by the presence of DIS).

Is the oesophageal mucosa different or more vulnerable to reflux in different disease phenotypes?

A very interesting and potentially important finding of the *in vitro* and *in vivo* studies presented in this thesis has been the difference in response of the oesophageal mucosa between patients with different disease phenotypes. The *in vitro* study in Chapter 3 compared mucosal biopsies from patients with refractory reflux symptoms and control patients with no upper gastrointestinal symptoms. It found that biopsies from symptomatic patients had a more dramatic reduction in TER than controls when exposed to the same refluxate-like solution. This was true for the acidic (pH 2 + pepsin + bile acid) solution, but perhaps even more interestingly it was also true for the weakly acidic (pH 5 + pepsin + bile acid) solution. This solution was representative of the "on" PPI condition. Biopsies from the control subjects did not respond to exposure to this solution with a fall in TER. In contrast, the symptomatic patients responded with a statistically significant mean reduction in TER. It has been documented that some subjects who are refractory to PPI therapy are symptomatic to weakly acidic (i.e. pH >4) reflux episodes[339]. This study raises the intriguing possibility that a reason for refractory GORD in some cases may be a distinct vulnerability of the oesophageal mucosa to weakly acidic reflux events.

The *in vivo* study in Chapter 4 compared mucosal integrity characteristics (as measured by impedance) of symptomatic patients with non-erosive reflux disease and functional heartburn. It can be seen that, at baseline, the distal oesophageal impedance is significantly lower in patients with non-erosive reflux disease (with increased 24-hour acid exposure) than in patients with functional heartburn (with physiological acid exposure). This lower impedance does indeed appear to be related (at least in part) to chronic acid exposure since the baseline impedance

correlates significantly with the 24-hour acid exposure time. Whilst the baseline impedance characteristics and findings of the *in vitro* study in Chapter 3 offer insight into vulnerability to acid damage, the acid perfusion study in Chapter 4 investigates the recovery capacity of the mucosa after acid injury. Using impedance as a surrogate marker, it can be seen that not only is there a long-lasting impairment of distal oesophageal mucosal integrity after acid perfusion, but there is an intra-individual variability in the rate of recovery of the mucosa after exposure. The rate of impedance recovery after cessation of acid perfusion was significantly slower in subjects with non-erosive reflux disease than was seen in patients with functional heartburn. Bringing together the findings from Chapters 3 and 4 it could be suggested that there is a mucosal phenotype seen in patients with non-erosive reflux disease of impaired baseline integrity (at least as measured by impedance), more dramatic impairment of integrity when exposed to reflux events, and a slower recovery of this integrity after the reflux event has passed. It could be hypothesised that the occurrence of regular reflux episodes with resulting damaged mucosal integrity and the slow recovery of the integrity is able to perpetuate the low baseline integrity seen in patients with non-erosive reflux disease (which is likely to reflect a situation of vulnerability to reflux perception). On treatment with PPI, the extent of integrity change during reflux is likely reduced, and the baseline impedance is seen to increase in treated subjects[156]. However, there remain a subset of patients whose mucosa remains vulnerable to weakly acidic reflux, and these patients may remain refractory to PPI.

What is the relationship between human oesophageal sensitivity to acid (heartburn perception) and oesophageal mucosal integrity?

An intuitive step from investigating the mucosal changes described above is to consider the relationship between oesophageal integrity and acid sensitivity. There is circumstantial evidence for a relationship between integrity and symptom perception. In morphological terms it has been shown that DIS is present in patients with GORD, but not in controls[163]. Furthermore, the DIS resolves on successful treatment of GORD[166], except in the circumstance when symptoms persist on PPI treatment, in which case DIS can be seen to persist[167]. In functional terms, this thesis has shown that impedance is lower in patients with non-erosive reflux disease than in those with functional heartburn, and others have shown impedance increases with successful PPI therapy[156]. Clinically, it has been shown that a reflux event is more likely to be perceived if it was recently preceded by a prior reflux event[210, 213], which in the context of our findings may be explained by the presence of a transiently more impaired mucosal integrity due to a previous "acid exposure burden". The study in Chapter 4 also attempted to address this question more objectively. Not all subjects are able to perceive an oesophageal acid perfusion as heartburn, even those with GORD (an observation that has led to a reduction in the use of the acid sensitivity test in clinical practice). The study showed that subjects who perceived the acid perfusion as heartburn had a significantly lower baseline distal oesophageal impedance than those who did not. It could be argued that this is because those with a lower baseline impedance were mostly represented by the non-erosive reflux disease phenotype (rather than functional heartburn), and as such were more likely to have acid sensitivity. Countering this suggestion was the finding that even when only those with functional heartburn were included in the analysis, there was still a significantly

lower baseline impedance in acid perceivers. Supporting the hypothesis is a recent abstract (presented at UEGW 2012) by a group from Amsterdam, who have found that *in vivo* sensitivity to acid perfusion was negatively correlated to *in vitro* basal integrity markers (transmucosal fluorescein flux)[294].

The findings discussed above suggest strongly that mucosal integrity plays an important role in pathogenesis and symptom perception in GORD. It may be that the variability in mucosal vulnerability between subjects may play a part in explaining why a similar acid reflux burden can result in a wide variability of symptom severity. Of course, however, mucosal integrity is only a part of the process leading to symptomatic reflux disease. As well as the integrity changes, there will also be factors such as activity of mucosal nociceptors and afferent nerve fibres, contribution of other properties of the refluxate (e.g. volume and gas causing oesophageal distension), and spinal and cerebral processing of sensory information that influence perception. The findings of the studies in Chapter 5 are a useful platform for further discussion of this point.

Is the regional difference in oesophageal sensitivity observed in humans due to distinct oesophageal mucosal characteristics?

It has been shown in multiple studies that reflux events reaching the proximal oesophagus are more likely to be perceived than those only reaching the distal oesophagus[211-214]. It has also been suggested that the proximal oesophagus is more sensitive to acid perfusion than is the distal oesophagus. Given the findings summarised in the paragraphs above, it was hypothesised that part of the reason for this relative hypersensitivity may be related to a more vulnerable mucosal integrity in the proximal oesophagus compared to the distal. This may have been expressed as a more impaired integrity at baseline (as measured by baseline *in*

vitro TER or *in vivo* impedance), or a more dramatic change in TER on exposure to refluxate-like solutions. The findings in Chapter 5 did not support this hypothesis. The baseline impedance was higher in the proximal compared to distal oesophagus, and there was also a trend towards a higher basal TER in the proximal mucosal biopsies. The change in TER of proximal biopsies was not greater on exposure to acidic solutions, and in fact trended towards a smaller effect compared to distal biopsies. These findings demonstrate the multifaceted nature of oesophageal nociception, involving several factors in addition to mucosal integrity. In fact, it appears logical that there may be different factors involved in proximal versus distal reflux nociception. The distal oesophagus, even in healthy individuals, is subject to dozens of acid reflux episodes every day. It is required to be relatively resistant to reflux perception. On the other hand, the proximal oesophagus is usually required to be very sensitive (at least at a subliminal level) since the presence of a reflux event at the proximal oesophagus threatens aspiration into the airways, and hence must trigger reflex activity (such as upper oesophageal sphincter closure and peristaltic bolus clearance) in defence[308]. The lack of difference in integrity between proximal and distal oesophageal mucosa led us to investigate other characteristics of the oesophageal mucosa, in the form of afferent mucosal innervation. Striking differences were found in the sensory afferent innervation of the distal and proximal oesophagus, with the nerve fibres being more numerous and in a position much closer to the luminal surface in the proximal mucosa. This afferent neuronal distribution is ideally located to perform the fast, defensive actions required, usually in the absence of pain. The deeper epithelial location of the distal oesophageal afferent nerves perhaps reveal insight into why mucosal integrity is important in this region. An intact mucosal barrier may be able to mostly “shield” these afferent neurones (and their corresponding

nociceptive receptors) from noxious components of the refluxate. As the integrity of this barrier is impaired, access to these nociceptive neurones is increased, and as DIS develops perhaps the available surface area of these nociceptors increases, further sensitising the mucosa.

In the proximal oesophagus, as mentioned, acidic solutions (such as those in drinks) do not usually cause heartburn symptoms. So what sensitises these superficial neurones to cause more perception in reflux disease? Mucosal integrity, or at least the mechanisms underlying the changes in mucosal integrity may have an important role. In particular, the "spread" of mucosal changes from distal to proximal oesophagus may be important in modulating the perception of the proximal oesophagus. Whether this is via an inflammatory "field change" in the oesophagus, via changes in local blood supply, or via an alternative mechanism is unknown. The result may be a sensitisation of afferent neurones and a recruitment of more available nociceptors via disruption of the barrier integrity. This paradigm would suggest that, indeed, distal acidification via gastro-oesophageal reflux is required to sensitise the proximal oesophagus to painful perception of proximal reflux. More studies are required to investigate this interesting possibility.

Can the human oesophageal mucosa integrity be protected with a topical agent?

The thesis has indicated that mucosal integrity has an important role in the pathophysiology of GORD. Although PPIs are an excellent treatment for GORD, there is still a large unmet need of 20-30% of disease sufferers with PPI-refractory symptoms. For these patients an alternative strategy that protects not just against strong acid, but other components of the refluxate and lower acid concentrations is warranted. A topical therapy that can protect the mucosa against these components and that can reduce the damaging effects on mucosal integrity would

be an attractive add-on treatment. The potential of an alginate-antacid solution to offer *in vitro* protection against the changes in mucosal integrity seen in oesophageal mucosal biopsies on exposure to acidic solutions (as seen in Chapter 3) was investigated. Such compounds have potential to act as a mucosal protectant due to the bioadhesive properties of the alginate component[335], and the potential defence against pepsin and bile acid diffusion[336]. Indeed, it was found that topical pre-treatment with an alginate compound was able to diminish the changes in integrity seen when the distal oesophageal biopsy was exposed to an acidic solution containing pepsin and bile acid. Control experiments suggest that this protective property was independent of the viscosity of the alginate solution, or the presence of concomitant antacid. It is appreciated that clinical experience informs us that such alginate-antacids are not efficacious as monotherapy in all but the mildest GORD. However, the role of such agents is likely to be greatest as add-on treatments to PPI. Furthermore, by examining the role of alginates on mucosal integrity, we may be able to target treatment to groups where we believe the most benefit could be found such as those with refractory GORD due to perception of weakly acidic reflux. It is even possible that alginates could be used in cases of proximal oesophageal sensitivity to reflux, both by protecting the proximal oesophageal mucosa directly, but also possibly by preventing proximal sensitisation via protection of the distal oesophagus. The model presented in this thesis can be used and adapted to evaluate new topical treatments *in vitro* with an aim to establishing the compounds with the best protection and best adhesion. Further alterations to such compounds, and addition of drugs for local delivery can be proposed and tested as our knowledge of the mechanisms of oesophageal mucosal integrity changes grows.

CHAPTER 8

Future directions

CHAPTER 8: FUTURE DIRECTIONS

The *in vitro* effects of acid exposure on the integrity of human oesophageal mucosa are intriguing and are of clinical relevance. The molecular mechanisms of these changes are subject to discussion, but with little in the way of supporting experimental data. In an initial study of this we wish to evaluate the inflammatory response of the mucosa and its temporal relationship to changes in integrity (as measured by TER). We would aim to sample the "basal" bathing solution of the Ussing chamber at time intervals and perform ELISA for inflammatory mediators such as IL-8. We propose to plot the release of inflammatory mediators against changes in TER, with an aim of establishing whether any inflammatory response precedes or follows integrity change in the mucosa. This may guide us closer to understanding the mechanism of integrity impairment in response to acid.

The mucosal behaviour in response to *in vivo* acid perfusion is of interest to us. We see that the oesophageal mucosa of different individuals can handle acid in different ways. In general those with GORD appear to have a lower baseline impedance and slower post-acid impedance recovery than those with normal acid exposure on 24-hour study, but there is some significant overlap. Indeed, some patients with functional heartburn have mucosal behaviour that is very similar to what is seen in patients with non-erosive reflux disease. The diagnosis of functional heartburn is very much dependent on results of reflux monitoring. There is some discussion as to whether a 24-hour reflux study is sufficient for diagnosis of functional heartburn. Some subjects will inevitably react to the presence of a recording catheter by modifying their behaviour: perhaps eating less freely, exercising less, or sleeping less well. Prolonging the study may allow better

acclimatisation, and it has been seen that a 48, 72 or even 96 hour study can increase the pickup of true reflux disease. We propose that mucosal behaviour may help identify those subjects who initially are identified as having functional heartburn on a 24-hour study, but who would be revealed as having pathological gastro-oesophageal reflux disease on a prolonged study. We intend to investigate this possibility with a study using *in vivo* acid challenge and prolonged pH monitoring in patients with heartburn.

We have identified a difference in mucosal afferent innervation between the distal and proximal oesophagus. We intend, in collaboration with Professor Blackshaw at our institution, to investigate this further. First we wish to fully delineate these nerves into their spinal and vagal components. Second we wish to investigate (with a combination of immunohistochemistry and RNA analysis) the relative proximal and distal distribution of candidate nociceptive receptors including TRPV1, TRPV4, TRPA1, and ASIC3.

We wish to objectively measure proximal and distal sensitivity to thermal and chemical stimuli in healthy volunteers and patients, and relate this to mucosal integrity, and nerve and receptor distribution as obtained from mucosal biopsies. With this we hope to build a more comprehensive picture of the factors involved in oesophageal nociception.

Finally, we also wish to investigate further the potential of using a mucosal protectant in reflux disease. In the shorter term we intend to expand on our *in vitro* studies, and perform *in vivo* studies of the effect on mucosal integrity using impedance. We would like to investigate the effect on pain sensitivity to oesophageal acid perfusion, and relate this to any effects on integrity. We are also very interested in the effect prior treatment with a topical agent may have on post-acid exposure impedance recovery, and on proximal changes in integrity after

distal acid perfusion. In the medium term future we would like to better understand the duration of the protective effect, and the effect on multiple acid exposures. In the longer term future we would like to use our *in vitro* and *in vivo* studies to investigate for better adhesive-protective formulations, and to attempt to combine the topical solution with drugs, the targets being determined by our studies on nociceptor distribution in the oesophagus (for example, a topical TRPV1 antagonist).

Overall, the research involved in this PhD thesis aimed to build on our knowledge of oesophageal mucosal integrity and nociception in gastro-oesophageal reflux disease, and to increase our potential to develop new effective treatments for GORD.

REFERENCES

1. Toghanian S, Wahlqvist P, Johnson DA, et al. The burden of disrupting gastro-oesophageal reflux disease: a database study in US and European cohorts. Clin Drug Investig 2010;30:167-78.
2. Palmer ED. The hiatus hernia-esophagitis-esophageal stricture complex. Twenty-year prospective study. Am J Med 1968;44:566-79.
3. Sifrim D, Zerbib F. Diagnosis and management of patients with reflux symptoms refractory to proton pump inhibitors. Gut 2012;61:1340-54.
4. Woodland P, Sifrim D. The refluxate: The impact of its magnitude, composition and distribution. Best Pract Res Clin Gastroenterol 2010;24:861-71.
5. Moayyedi P, Leontiadis GI. The risks of PPI therapy. Nature Publishing Group. Volume 9: Nature Publishing Group, 2012:132-139.
6. Vakil N, van Zanten SV, Kahrilas P, et al. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. Am J Gastroenterol 2006;101:1900-20; quiz 1943.
7. Dent J, Brun J, Fendrick AM, et al. An evidence-based appraisal of reflux disease management--the Genval Workshop Report. Gut. Volume 44 Suppl 2, 1999:S1-16.
8. Dent J. Symptom evaluation in reflux disease: workshop background, processes, terminology, recommendations, and discussion outputs. Gut. Volume 53, 2004:iv1-iv24.
9. Sontag SJ, Hirschowitz BI, Holt S, et al. Two doses of omeprazole versus placebo in symptomatic erosive esophagitis: the U.S. Multicenter Study. Gastroenterology 1992;102:109-18.
10. Lind T, Havelund T, Carlsson R, et al. Heartburn without oesophagitis: efficacy of omeprazole therapy and features determining therapeutic response. Scand J Gastroenterol 1997;32:974-9.
11. Inadomi JM, McIntyre L, Bernard L, et al. Step-down from multiple- to single-dose proton pump inhibitors (PPIs): a prospective study of patients with heartburn or acid regurgitation completely relieved with PPIs. Am J Gastroenterol 2003;98:1940-4.
12. Klauser AG, Schindlbeck NE, Muller-Lissner SA. Symptoms in gastro-oesophageal reflux disease. Lancet 1990;335:205-8.

13. Dent J, Vakil N, Jones R, et al. Accuracy of the diagnosis of GORD by questionnaire, physicians and a trial of proton pump inhibitor treatment: the Diamond Study. Gut 2010;59:714-21.
14. Johnsson F, Joelsson B, Gudmundsson K, et al. Symptoms and endoscopic findings in the diagnosis of gastroesophageal reflux disease. Scand J Gastroenterol 1987;22:714-8.
15. Winters C, Jr., Spurling TJ, Chobanian SJ, et al. Barrett's esophagus. A prevalent, occult complication of gastroesophageal reflux disease. Gastroenterology 1987;92:118-24.
16. Dakkak M, Hoare RC, Maslin SC, et al. Oesophagitis is as important as oesophageal stricture diameter in determining dysphagia. Gut 1993;34:152-5.
17. Zhang X, Geboes K, Depoortere I, et al. Effect of repeated cycles of acute esophagitis and healing on esophageal peristalsis, tone, and length. Am J Physiol Gastrointest Liver Physiol 2005;288:G1339-46.
18. Irwin RS, Curley FJ, French CL. Chronic cough. The spectrum and frequency of causes, key components of the diagnostic evaluation, and outcome of specific therapy. Am Rev Respir Dis 1990;141:640-7.
19. Shaker R, Milbrath M, Ren J, et al. Esophagopharyngeal distribution of refluxed gastric acid in patients with reflux laryngitis. Gastroenterology 1995;109:1575-82.
20. Sontag SJ, O'Connell S, Khandelwal S, et al. Most asthmatics have gastroesophageal reflux with or without bronchodilator therapy. Gastroenterology 1990;99:613-20.
21. Lundell LR, Dent J, Bennett JR, et al. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. Gut 1999;45:172-80.
22. El-Serag HB. Epidemiology of non-erosive reflux disease. Digestion 2008;78 Suppl 1:6-10.
23. Guidelines--Rome III Diagnostic Criteria for Functional Gastrointestinal Disorders. J Gastrointestin Liver Dis 2006;15:307-12.
24. Dent J, El-Serag HB, Wallander MA, et al. Epidemiology of gastro-oesophageal reflux disease: a systematic review. Gut 2005;54:710-7.

25. Ruigomez A, Garcia Rodriguez LA, Wallander MA, et al. Natural history of gastro-oesophageal reflux disease diagnosed in general practice. Aliment Pharmacol Ther 2004;20:751-60.
26. Yuen E, Romney M, Toner RW, et al. Prevalence, knowledge and care patterns for gastro-oesophageal reflux disease in United States minority populations. Aliment Pharmacol Ther 2010;32:645-54.
27. Wong BC, Kinoshita Y. Systematic review on epidemiology of gastroesophageal reflux disease in Asia. Clin Gastroenterol Hepatol 2006;4:398-407.
28. Trudgill NJ, Kapur KC, Riley SA. Familial clustering of reflux symptoms. Am J Gastroenterol 1999;94:1172-8.
29. Mohammed I, Cherkas LF, Riley SA, et al. Genetic influences in gastro-oesophageal reflux disease: a twin study. Gut 2003;52:1085-9.
30. Lembo A, Zaman M, Jones M, et al. Influence of genetics on irritable bowel syndrome, gastro-oesophageal reflux and dyspepsia: a twin study. Aliment Pharmacol Ther 2007;25:1343-50.
31. Locke GR, 3rd, Talley NJ, Fett SL, et al. Risk factors associated with symptoms of gastroesophageal reflux. Am J Med 1999;106:642-9.
32. Carre IJ, Johnston BT, Thomas PS, et al. Familial hiatal hernia in a large five generation family confirming true autosomal dominant inheritance. Gut 1999;45:649-52.
33. Asling B, Jirholt J, Hammond P, et al. Collagen type III alpha I is a gastro-oesophageal reflux disease susceptibility gene and a male risk factor for hiatus hernia. Gut 2009;58:1063-9.
34. Jirholt J, Asling B, Hammond P, et al. 4-aminobutyrate aminotransferase (ABAT): genetic and pharmacological evidence for an involvement in gastro esophageal reflux disease. PLoS One 2011;6:e19095.
35. Drovdlic CM, Goddard KA, Chak A, et al. Demographic and phenotypic features of 70 families segregating Barrett's oesophagus and oesophageal adenocarcinoma. J Med Genet 2003;40:651-6.
36. Sun X, Elston R, Barnholtz-Sloan J, et al. A segregation analysis of Barrett's esophagus and associated adenocarcinomas. Cancer Epidemiol Biomarkers Prev 2010;19:666-74.

37. Su Z, Gay LJ, Strange A, et al. Common variants at the MHC locus and at chromosome 16q24.1 predispose to Barrett's esophagus. Nat Genet 2012;44:1131-6.
38. Locke GR, 3rd, Talley NJ, Fett SL, et al. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. Gastroenterology 1997;112:1448-56.
39. Isolauri J, Laippala P. Prevalence of symptoms suggestive of gastro-oesophageal reflux disease in an adult population. Ann Med 1995;27:67-70.
40. Kotzan J, Wade W, Yu HH. Assessing NSAID prescription use as a predisposing factor for gastroesophageal reflux disease in a Medicaid population. Pharm Res 2001;18:1367-72.
41. El-Serag HB, Petersen NJ, Carter J, et al. Gastroesophageal reflux among different racial groups in the United States. Gastroenterology 2004;126:1692-9.
42. Jacobson BC, Somers SC, Fuchs CS, et al. Body-mass index and symptoms of gastroesophageal reflux in women. N. Engl. J. Med. Volume 354, 2006:2340-2348.
43. Terry P, Lagergren J, Wolk A, et al. Reflux-inducing dietary factors and risk of adenocarcinoma of the esophagus and gastric cardia. Nutr Cancer 2000;38:186-91.
44. Diaz-Rubio M, Moreno-Elola-Olaso C, Rey E, et al. Symptoms of gastro-oesophageal reflux: prevalence, severity, duration and associated factors in a Spanish population. Aliment Pharmacol Ther 2004;19:95-105.
45. Savarino E, Pohl D, Zentilin P, et al. Functional heartburn has more in common with functional dyspepsia than with non-erosive reflux disease. Gut 2009;58:1185-91.
46. Hu WH, Wong WM, Lam CL, et al. Anxiety but not depression determines health care-seeking behaviour in Chinese patients with dyspepsia and irritable bowel syndrome: a population-based study. Aliment Pharmacol Ther 2002;16:2081-8.
47. Nojkov B, Rubenstein JH, Adlis SA, et al. The influence of co-morbid IBS and psychological distress on outcomes and quality of life following PPI therapy in patients with gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2008;27:473-82.

48. Johnson LF, Demeester TR. Twenty-four-hour pH monitoring of the distal esophagus. A quantitative measure of gastroesophageal reflux. Am J Gastroenterol 1974;62:325-32.
49. Hila A, Bouali H, Xue S, et al. Postprandial stomach contents have multiple acid layers. J Clin Gastroenterol 2006;40:612-7.
50. Clarke AT, Wirz AA, Seenan JP, et al. Paradox of gastric cardia: it becomes more acidic following meals while the rest of stomach becomes less acidic. Gut 2009;58:904-9.
51. Vo L, Simonian HP, Doma S, et al. The effect of rabeprazole on regional gastric acidity and the postprandial cardia/gastro-oesophageal junction acid layer in normal subjects: a randomized, double-blind, placebo-controlled study. Aliment Pharmacol Ther 2005;21:1321-30.
52. Rohof WO, Bennink RJ, de Ruigh AA, et al. Effect of azithromycin on acid reflux, hiatus hernia and proximal acid pocket in the postprandial period. Gut 2012.
53. Smith JL, Opekun AR, Larkai E, et al. Sensitivity of the esophageal mucosa to pH in gastroesophageal reflux disease. Gastroenterology 1989;96:683-9.
54. Tuttle SG, Rufin F, Bettarello A. The physiology of heartburn. Ann Intern Med 1961;55:292-300.
55. Sifrim D, Holloway R, Silny J, et al. Acid, nonacid, and gas reflux in patients with gastroesophageal reflux disease during ambulatory 24-hour pH-impedance recordings. Gastroenterology 2001;120:1588-98.
56. Zerbib F, Roman S, Ropert A, et al. Esophageal pH-impedance monitoring and symptom analysis in GERD: a study in patients off and on therapy. Am J Gastroenterol 2006;101:1956-63.
57. Emerenziani S, Sifrim D, Habib FI, et al. Presence of gas in the refluxate enhances reflux perception in non-erosive patients with physiological acid exposure of the oesophagus. Gut 2008;57:443-7.
58. Goldberg HI, Dodds WJ, Gee S, et al. Role of acid and pepsin in acute experimental esophagitis. Gastroenterology 1969;56:223-30.
59. DeMeester TR, Wang CI, Wernly JA, et al. Technique, indications, and clinical use of 24 hour esophageal pH monitoring. J Thorac Cardiovasc Surg 1980;79:656-70.
60. Robertson D, Aldersley M, Shepherd H, et al. Patterns of acid reflux in complicated oesophagitis. Gut 1987;28:1484-8.

61. Savarino E, Tutuian R, Zentilin P, et al. Characteristics of reflux episodes and symptom association in patients with erosive esophagitis and nonerosive reflux disease: study using combined impedance-pH off therapy. Am J Gastroenterol 2010;105:1053-61.
62. Bechi P, Pucciani F, Baldini F, et al. Long-term ambulatory enterogastric reflux monitoring. Validation of a new fiberoptic technique. Dig Dis Sci 1993;38:1297-306.
63. Pace F, Sangaletti O, Pallotta S, et al. Biliary reflux and non-acid reflux are two distinct phenomena: a comparison between 24-hour multichannel intraesophageal impedance and bilirubin monitoring. Scand J Gastroenterol 2007;42:1031-9.
64. Fiorucci S, Distrutti E, Di Matteo F, et al. Circadian variations in gastric acid and pepsin secretion and intragastric bile acid in patients with reflux esophagitis and in healthy controls. Am J Gastroenterol 1995;90:270-6.
65. Miller LJ, Malagelada JR, Longstreth GF, et al. Dysfunctions of the stomach with gastric ulceration. Dig Dis Sci 1980;25:857-64.
66. Schindlbeck NE, Heinrich C, Stellaard F, et al. Healthy controls have as much bile reflux as gastric ulcer patients. Gut 1987;28:1577-83.
67. Theisen J, Nehra D, Citron D, et al. Suppression of gastric acid secretion in patients with gastroesophageal reflux disease results in gastric bacterial overgrowth and deconjugation of bile acids. J Gastrointest Surg 2000;4:50-4.
68. Marshall RE, Anggiansah A, Owen WA, et al. The relationship between acid and bile reflux and symptoms in gastro-oesophageal reflux disease. Gut 1997;40:182-7.
69. Koek GH, Tack J, Sifrim D, et al. The role of acid and duodenal gastroesophageal reflux in symptomatic GERD. Am J Gastroenterol 2001;96:2033-40.
70. Tack J, Koek G, Demedts I, et al. Gastroesophageal reflux disease poorly responsive to single-dose proton pump inhibitors in patients without Barrett's esophagus: acid reflux, bile reflux, or both? Am J Gastroenterol 2004;99:981-8.
71. Karamanolis G, Vanuytsel T, Sifrim D, et al. Yield of 24-hour esophageal pH and bilitec monitoring in patients with persisting symptoms on PPI therapy. Dig Dis Sci 2008;53:2387-93.

72. Gasiorowska A, Navarro-Rodriguez T, Wendel C, et al. Comparison of the degree of duodenogastroesophageal reflux and acid reflux between patients who failed to respond and those who were successfully treated with a proton pump inhibitor once daily. Am J Gastroenterol 2009;104:2005-13.
73. Moffat RC, Berkas EM. Bile esophagitis. Arch Surg 1965;91:963-6.
74. Harmon JW, Johnson LF, Maydonovitch CL. Effects of acid and bile salts on the rabbit esophageal mucosa. Dig Dis Sci 1981;26:65-72.
75. Nehra D, Howell P, Pye JK, et al. Assessment of combined bile acid and pH profiles using an automated sampling device in gastro-oesophageal reflux disease. Br J Surg 1998;85:134-7.
76. Gotley DC, Appleton GV, Cooper MJ. Bile acids and trypsin are unimportant in alkaline esophageal reflux. J Clin Gastroenterol 1992;14:2-7.
77. Buttar NS, Wang KK, Leontovich O, et al. Chemoprevention of esophageal adenocarcinoma by COX-2 inhibitors in an animal model of Barrett's esophagus. Gastroenterology 2002;122:1101-12.
78. Sarosi G, Brown G, Jaiswal K, et al. Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus. Dis Esophagus 2008;21:43-50.
79. Fein M, Peters JH, Chandrasoma P, et al. Duodenoesophageal reflux induces esophageal adenocarcinoma without exogenous carcinogen. J Gastrointest Surg 1998;2:260-8.
80. Nehra D, Howell P, Williams CP, et al. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. Gut 1999;44:598-602.
81. Huo X, Juergens S, Zhang X, et al. Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-kappaB activation in benign Barrett's epithelial cells. Am J Physiol Gastrointest Liver Physiol 2011;301:G278-86.
82. Lillemoe KD, Johnson LF, Harmon JW. Role of the components of the gastroduodenal contents in experimental acid esophagitis. Surgery 1982;92:276-84.
83. Nagahama K, Yamato M, Nishio H, et al. Essential role of pepsin in pathogenesis of acid reflux esophagitis in rats. Dig Dis Sci 2006;51:303-9.
84. McCallum RW, Berkowitz DM, Lerner E. Gastric emptying in patients with gastroesophageal reflux. Gastroenterology 1981;80:285-91.

85. Shay SS, Eggli D, McDonald C, et al. Gastric emptying of solid food in patients with gastroesophageal reflux. Gastroenterology 1987;92:459-65.
86. Stacher G, Lenglinger J, Bergmann H, et al. Gastric emptying: a contributory factor in gastro-oesophageal reflux activity? Gut 2000;47:661-6.
87. Schwizer W, Hinder RA, DeMeester TR. Does delayed gastric emptying contribute to gastroesophageal reflux disease? Am J Surg 1989;157:74-81.
88. Penagini R, Hebbard G, Horowitz M, et al. Motor function of the proximal stomach and visceral perception in gastro-oesophageal reflux disease. Gut 1998;42:251-7.
89. Vu MK, Straathof JW, v d Schaar PJ, et al. Motor and sensory function of the proximal stomach in reflux disease and after laparoscopic Nissen fundoplication. Am J Gastroenterol 1999;94:1481-9.
90. Richter JE, Wu WC, Johns DN, et al. Esophageal manometry in 95 healthy adult volunteers. Variability of pressures with age and frequency of "abnormal" contractions. Dig Dis Sci 1987;32:583-92.
91. Schoeman MN, Tippett MD, Akkermans LM, et al. Mechanisms of gastroesophageal reflux in ambulant healthy human subjects. Gastroenterology 1995;108:83-91.
92. Mittal RK, Rochester DF, McCallum RW. Sphincteric action of the diaphragm during a relaxed lower esophageal sphincter in humans. Am J Physiol 1989;256:G139-44.
93. Mittal RK, Fisher MJ. Electrical and mechanical inhibition of the crural diaphragm during transient relaxation of the lower esophageal sphincter. Gastroenterology 1990;99:1265-8.
94. Mittal RK. Hiatal hernia and gastroesophageal reflux: another attempt to resolve the controversy. Gastroenterology 1993;105:941-3.
95. Kaul B, Petersen H, Myrvold HE, et al. Hiatus hernia in gastroesophageal reflux disease. Scand J Gastroenterol 1986;21:31-4.
96. Mittal RK, Holloway RH, Penagini R, et al. Transient lower esophageal sphincter relaxation. Gastroenterology 1995;109:601-10.
97. Dent J, Dodds WJ, Friedman RH, et al. Mechanism of gastroesophageal reflux in recumbent asymptomatic human subjects. J Clin Invest 1980;65:256-67.
98. Dodds WJ, Dent J, Hogan WJ, et al. Mechanisms of gastroesophageal reflux in patients with reflux esophagitis. N Engl J Med 1982;307:1547-52.

99. Dent J, Holloway RH, Toouli J, et al. Mechanisms of lower oesophageal sphincter incompetence in patients with symptomatic gastrooesophageal reflux. Gut 1988;29:1020-8.
100. Mittal RK, McCallum RW. Characteristics and frequency of transient relaxations of the lower esophageal sphincter in patients with reflux esophagitis. Gastroenterology 1988;95:593-9.
101. Holloway RH, Kocyan P, Dent J. Provocation of transient lower esophageal sphincter relaxations by meals in patients with symptomatic gastroesophageal reflux. Dig Dis Sci 1991;36:1034-9.
102. Mittal RK, McCallum RW. Characteristics of transient lower esophageal sphincter relaxation in humans. Am J Physiol 1987;252:G636-41.
103. Martin CJ, Patrikios J, Dent J. Abolition of gas reflux and transient lower esophageal sphincter relaxation by vagal blockade in the dog. Gastroenterology 1986;91:890-6.
104. Holloway RH, Wyman JB, Dent J. Failure of transient lower oesophageal sphincter relaxation in response to gastric distension in patients with achalasia: evidence for neural mediation of transient lower oesophageal sphincter relaxations. Gut 1989;30:762-7.
105. Anggiansah A, Taylor G, Marshall RE, et al. Oesophageal motor responses to gastro-oesophageal reflux in healthy controls and reflux patients. Gut 1997;41:600-5.
106. Dodds WJ, Kahrilas PJ, Dent J, et al. Analysis of Spontaneous Gastroesophageal Reflux and Esophageal Acid Clearance in Patients with Reflux Esophagitis. Neurogastroenterology & Motility 1990;2:79-89.
107. Fornari F, Blondeau K, Durand L, et al. Relevance of mild ineffective oesophageal motility (IOM) and potential pharmacological reversibility of severe IOM in patients with gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2007;26:1345-54.
108. Kahrilas PJ, Dodds WJ, Hogan WJ, et al. Esophageal peristaltic dysfunction in peptic esophagitis. Gastroenterology 1986;91:897-904.
109. Barham CP, Gotley DC, Mills A, et al. Oesophageal acid clearance in patients with severe reflux oesophagitis. Br J Surg 1995;82:333-7.
110. Singh P, Adamopoulos A, Taylor RH, et al. Oesophageal motor function before and after healing of oesophagitis. Gut 1992;33:1590-6.

111. Timmer R, Breumelhof R, Nadorp JH, et al. Oesophageal motility and gastro-oesophageal reflux before and after healing of reflux oesophagitis. A study using 24 hour ambulatory pH and pressure monitoring. Gut 1994;35:1519-22.
112. Helm JF, Dodds WJ, Pelc LR, et al. Effect of esophageal emptying and saliva on clearance of acid from the esophagus. N Engl J Med 1984;310:284-8.
113. Dutta SK, Matossian HB, Meirowitz RF, et al. Modulation of salivary secretion by acid infusion in the distal esophagus in humans. Gastroenterology 1992;103:1833-41.
114. Dutta SK, Agrawal K, Mahmoud MA. Modulation of salivation and heartburn in response to the site of acid infusion in the human oesophagus. Aliment Pharmacol Ther 2010;32:795-800.
115. Williams SE, Turnberg LA. Demonstration of a pH gradient across mucus adherent to rabbit gastric mucosa: evidence for a 'mucus-bicarbonate' barrier. Gut 1981;22:94-6.
116. Guillem P, Billeret V, Buisine MP, et al. Mucin gene expression and cell differentiation in human normal, premalignant and malignant esophagus. Int J Cancer 2000;88:856-61.
117. Quigley EM, Turnberg LA. pH of the microclimate lining human gastric and duodenal mucosa in vivo. Studies in control subjects and in duodenal ulcer patients. Gastroenterology 1987;92:1876-84.
118. Hopwood D, Coghill G, Sanders DS. Human oesophageal submucosal glands. Their detection mucin, enzyme and secretory protein content. Histochemistry 1986;86:107-12.
119. Brown CM, Snowdon CF, Slee B, et al. Neural influences on human esophageal and salivary alkali secretion. Dig Dis Sci 1995;40:1642-50.
120. Brown CM, Snowdon CF, Slee B, et al. Effect of topical oesophageal acidification on human salivary and oesophageal alkali secretion. Gut 1995;36:649-53.
121. Orlando RC, Lacy ER, Tobey NA, et al. Barriers to paracellular permeability in rabbit esophageal epithelium. Gastroenterology 1992;102:910-23.
122. Tobey NA, Argote CM, Vanegas XC, et al. Electrical parameters and ion species for active transport in human esophageal stratified squamous epithelium and Barrett's specialized columnar epithelium. Am J Physiol Gastrointest Liver Physiol 2007;293:G264-70.

123. Angelow S, Yu AS. Claudins and paracellular transport: an update. Curr Opin Nephrol Hypertens 2007;16:459-64.
124. Inai T, Kobayashi J, Shibata Y. Claudin-1 contributes to the epithelial barrier function in MDCK cells. Eur J Cell Biol 1999;78:849-55.
125. Yu AS, Enck AH, Lencer WI, et al. Claudin-8 expression in Madin-Darby canine kidney cells augments the paracellular barrier to cation permeation. J Biol Chem 2003;278:17350-9.
126. McCarthy KM, Francis SA, McCormack JM, et al. Inducible expression of claudin-1-myc but not occludin-VSV-G results in aberrant tight junction strand formation in MDCK cells. J Cell Sci 2000;113 Pt 19:3387-98.
127. Wen H, Watry DD, Marcondes MC, et al. Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5. Mol Cell Biol 2004;24:8408-17.
128. Jeansonne B, Lu Q, Goodenough DA, et al. Claudin-8 interacts with multi-PDZ domain protein 1 (MUPP1) and reduces paracellular conductance in epithelial cells. Cell Mol Biol (Noisy-le-grand) 2003;49:13-21.
129. Asaoka D, Miwa H, Hirai S, et al. Altered localization and expression of tight-junction proteins in a rat model with chronic acid reflux esophagitis. J Gastroenterol 2005;40:781-90.
130. Furuse M, Hirase T, Itoh M, et al. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 1993;123:1777-88.
131. Stevenson BR, Siliciano JD, Mooseker MS, et al. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. J Cell Biol 1986;103:755-66.
132. Shen L. Tight junctions on the move: molecular mechanisms for epithelial barrier regulation. Ann. N. Y. Acad. Sci. Volume 1258, 2012:9-18.
133. Jovov B, Que J, Tobey NA, et al. Role of E-cadherin in the pathogenesis of gastroesophageal reflux disease. Am J Gastroenterol 2011;106:1039-47.
134. Perez-Moreno M, Fuchs E. Catenins: keeping cells from getting their signals crossed. Dev Cell 2006;11:601-12.
135. Capaldo CT, Macara IG. Depletion of E-cadherin disrupts establishment but not maintenance of cell junctions in Madin-Darby canine kidney epithelial cells. Mol Biol Cell 2007;18:189-200.

136. Christie KN, Thomson C, Xue L, et al. Carbonic anhydrase isoenzymes I, II, III, and IV are present in human esophageal epithelium. J Histochem Cytochem 1997;45:35-40.
137. Tobey NA, Reddy SP, Khalbuss WE, et al. Na(+)-dependent and -independent Cl-/HCO3- exchangers in cultured rabbit esophageal epithelial cells. Gastroenterology 1993;104:185-95.
138. Tobey NA, Koves G, Orlando RC. Human esophageal epithelial cells possess an Na+/H+ exchanger for H+ extrusion. Am J Gastroenterol 1998;93:2075-81.
139. Donne A. Recherches sur quelques unes des proprietes chimiques des secretions, et sur les courants electriques qui existent dans les corps organises. Ann Chim Phys 1834;57:398-416.
140. Rehm WS. Evidence that the major portion of the gastric potential originates between the submucosa and mucosa. Am J Physiol 1946;147:69-77.
141. Rovelstad RA, Owen CA, Jr., Magath TB. Factors influencing the continuous recording of in situ pH of gastric and duodenal contents. Gastroenterology 1952;20:609-24.
142. Helm WJ, Schlegel JF, Code CF, et al. Identification of the Gastroesophageal Mucosal Junction by Transmucosal Potential in Healthy Subjects and Patients with Hiatal Hernia. Gastroenterology 1965;48:25-35.
143. Davenport HW, Warner HA, Code CF. Functional Significance of Gastric Mucosal Barrier to Sodium. Gastroenterology 1964;47:142-52.
144. Beck IT, Hernandez NA. Transmural potential difference in patients with hiatus hernia and oesophageal ulcer. Gut 1969;10:469-76.
145. Khamis B, Kennedy C, Finucane J, et al. Transmucosal potential difference; diagnostic value in gastro-oseophageal reflux. Gut 1978;19:396-8.
146. Orlando RC, Powell DW, Bryson JC, et al. Esophageal potential difference measurements in esophageal disease. Gastroenterology 1982;83:1026-32.
147. Orlando RC, Bryson JC, Powell DW. Mechanisms of H+ injury in rabbit esophageal epithelium. Am J Physiol 1984;246:G718-24.
148. Tobey NA, Hosseini SS, Argote CM, et al. Dilated intercellular spaces and shunt permeability in nonerosive acid-damaged esophageal epithelium. Am J Gastroenterol 2004;99:13-22.

149. Fromter E, Diamond J. Route of passive ion permeation in epithelia. Nat New Biol 1972;235:9-13.
150. Martinez SD, Malagon IB, Garewal HS, et al. Non-erosive reflux disease (NERD)--acid reflux and symptom patterns. Aliment Pharmacol Ther 2003;17:537-45.
151. Siddiqui A, Rodriguez-Stanley S, Zubaidi S, et al. Esophageal visceral sensitivity to bile salts in patients with functional heartburn and in healthy control subjects. Dig Dis Sci 2005;50:81-5.
152. Vaezi MF, Richter JE. Contribution of acid and duodenogastro-oesophageal reflux to oesophageal mucosal injury and symptoms in partial gastrectomy patients [see comment]. Gut 1997;41:297-302.
153. Farre R, van Malenstein H, De Vos R, et al. Short exposure of oesophageal mucosa to bile acids, both in acidic and weakly acidic conditions, can impair mucosal integrity and provoke dilated intercellular spaces. Gut 2008;57:1366-74.
154. Shay S, Tutuian R, Sifrim D, et al. Twenty-four hour ambulatory simultaneous impedance and pH monitoring: a multicenter report of normal values from 60 healthy volunteers. Am J Gastroenterol 2004;99:1037-43.
155. Sifrim D, Zhang X, Emerenziani S. Patients with severe esophagitis and Barrett's esophagus have a distinct low intraluminal impedance. A screening test? Gut 2004;53(Suppl VI):A111.
156. Kessing BF, Bredenoord AJ, Weijenborg PW, et al. Esophageal acid exposure decreases intraluminal baseline impedance levels. Am J Gastroenterol 2011;106:2093-7.
157. Farre R, Blondeau K, Clement D, et al. Evaluation of oesophageal mucosa integrity by the intraluminal impedance technique. Gut 2011;60:885-92.
158. Zhong C, Duan L, Wang K, et al. Esophageal intraluminal baseline impedance is associated with severity of acid reflux and epithelial structural abnormalities in patients with gastroesophageal reflux disease. J Gastroenterol, 2012.
159. Orlando RC, Powell DW, Carney CN. Pathophysiology of acute acid injury in rabbit esophageal epithelium. J Clin Invest 1981;68:286-93.

160. Carney CN, Orlando RC, Powell DW, et al. Morphologic alterations in early acid-induced epithelial injury of the rabbit esophagus. Lab Invest 1981;45:198-208.
161. Farre R, Fornari F, Blondeau K, et al. Acid and weakly acidic solutions impair mucosal integrity of distal exposed and proximal non-exposed human oesophagus. Gut 2010;59:164-9.
162. Barlow WJ, Orlando RC. The pathogenesis of heartburn in nonerosive reflux disease: a unifying hypothesis. Gastroenterology 2005;128:771-8.
163. Tobey NA, Carson JL, Alkiek RA, et al. Dilated intercellular spaces: a morphological feature of acid reflux--damaged human esophageal epithelium. Gastroenterology 1996;111:1200-5.
164. Caviglia R, Ribolsi M, Gentile M, et al. Dilated intercellular spaces and acid reflux at the distal and proximal oesophagus in patients with non-erosive gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2007;25:629-36.
165. Caviglia R, Ribolsi M, Maggiano N, et al. Dilated intercellular spaces of esophageal epithelium in nonerosive reflux disease patients with physiological esophageal acid exposure. Am J Gastroenterol 2005;100:543-8.
166. Calabrese C, Bortolotti M, Fabbri A, et al. Reversibility of GERD ultrastructural alterations and relief of symptoms after omeprazole treatment. Am J Gastroenterol 2005;100:537-42.
167. Vela MF, Craft BM, Sharma N, et al. Refractory heartburn: comparison of intercellular space diameter in documented GERD vs. functional heartburn. Am J Gastroenterol 2011;106:844-50.
168. Sifrim D, Holloway R, Silny J, et al. Composition of the postprandial refluxate in patients with gastroesophageal reflux disease. Am J Gastroenterol 2001;96:647-55.
169. Johnston BT, McFarland RJ, Collins JS, et al. Symptom index as a marker of gastro-oesophageal reflux disease. Br J Surg 1992;79:1054-5.
170. Wiener GJ, Richter JE, Copper JB, et al. The symptom index: a clinically important parameter of ambulatory 24-hour esophageal pH monitoring. Am J Gastroenterol 1988;83:358-61.

171. Aanen MC, Bredenoord AJ, Numans ME, et al. Reproducibility of symptom association analysis in ambulatory reflux monitoring. Am J Gastroenterol 2008;103:2200-8.
172. Knowles CH, Aziz Q. Basic and clinical aspects of gastrointestinal pain. Pain 2009;141:191-209.
173. Berthoud HR, Blackshaw LA, Brookes SJ, et al. Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. Neurogastroenterol Motil 2004;16 Suppl 1:28-33.
174. Blackshaw LA, Gebhart GF. The pharmacology of gastrointestinal nociceptive pathways. Curr Opin Pharmacol 2002;2:642-9.
175. Yu S, Undem BJ, Kollarik M. Vagal afferent nerves with nociceptive properties in guinea-pig oesophagus. J Physiol 2005;563:831-42.
176. Kollarik M, Ru F, Brozmanova M. Vagal afferent nerves with the properties of nociceptors. Auton Neurosci 2010;153:12-20.
177. Reeh PW, Kress M. Molecular physiology of proton transduction in nociceptors. Curr Opin Pharmacol 2001;1:45-51.
178. Sugiura T, Dang K, Lamb K, et al. Acid-sensing properties in rat gastric sensory neurons from normal and ulcerated stomach. J Neurosci 2005;25:2617-27.
179. Holzer P. Acid-sensitive ion channels in gastrointestinal function. Curr Opin Pharmacol 2003;3:618-25.
180. Page AJ, Brierley SM, Martin CM, et al. Different contributions of ASIC channels 1a, 2, and 3 in gastrointestinal mechanosensory function. Gut 2005;54:1408-15.
181. Price MP, McIlwrath SL, Xie J, et al. The DRASIC cation channel contributes to the detection of cutaneous touch and acid stimuli in mice. Neuron 2001;32:1071-83.
182. Ramsey IS, Delling M, Clapham DE. An introduction to TRP channels. Annu Rev Physiol 2006;68:619-47.
183. Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci 2001;24:487-517.
184. Yiangou Y, Facer P, Dyer NH, et al. Vanilloid receptor 1 immunoreactivity in inflamed human bowel. Lancet 2001;357:1338-9.
185. Ward SM, Bayguinov J, Won KJ, et al. Distribution of the vanilloid receptor (VR1) in the gastrointestinal tract. J Comp Neurol 2003;465:121-35.

186. Amadesi S, Nie J, Vergnolle N, et al. Protease-activated receptor 2 sensitizes the capsaicin receptor transient receptor potential vanilloid receptor 1 to induce hyperalgesia. J Neurosci 2004;24:4300-12.
187. Vaezi MF, Richter JE. Role of acid and duodenogastroesophageal reflux in gastroesophageal reflux disease. Gastroenterology 1996;111:1192-9.
188. Burnstock G, Wood JN. Purinergic receptors: their role in nociception and primary afferent neurotransmission. Curr Opin Neurobiol 1996;6:526-32.
189. Page AJ, O'Donnell TA, Blackshaw LA. P2X purinoceptor-induced sensitization of ferret vagal mechanoreceptors in oesophageal inflammation. J Physiol 2000;523 Pt 2:403-11.
190. Grundy D, Al-Chaer ED, Aziz Q, et al. Fundamentals of neurogastroenterology: basic science. Gastroenterology 2006;130:1391-411.
191. Kirkup AJ, Brunsden AM, Grundy D. Receptors and transmission in the brain-gut axis: potential for novel therapies. I. Receptors on visceral afferents. Am J Physiol Gastrointest Liver Physiol 2001;280:G787-94.
192. Al-Chaer ED, Westlund KN, Willis WD. Sensitization of postsynaptic dorsal column neuronal responses by colon inflammation. Neuroreport 1997;8:3267-73.
193. Sarkar S, Thompson DG, Woolf CJ, et al. Patients with chest pain and occult gastroesophageal reflux demonstrate visceral pain hypersensitivity which may be partially responsive to acid suppression. Am J Gastroenterol 2004;99:1998-2006.
194. Sarkar S, Aziz Q, Woolf CJ, et al. Contribution of central sensitisation to the development of non-cardiac chest pain. Lancet 2000;356:1154-9.
195. Sarkar S, Hobson AR, Hughes A, et al. The prostaglandin E2 receptor-1 (EP-1) mediates acid-induced visceral pain hypersensitivity in humans. Gastroenterology 2003;124:18-25.
196. Willert RP, Woolf CJ, Hobson AR, et al. The development and maintenance of human visceral pain hypersensitivity is dependent on the N-methyl-D-aspartate receptor. Gastroenterology 2004;126:683-92.
197. Organization TG. A gallup survey on heartburn across America. Princeton, NJ: The Gallup Organization, 1988.

198. Fass R, Naliboff BD, Fass SS, et al. The effect of auditory stress on perception of intraesophageal acid in patients with gastroesophageal reflux disease. Gastroenterology 2008;134:696-705.
199. Farre R, De Vos R, Geboes K, et al. Critical role of stress in increased oesophageal mucosa permeability and dilated intercellular spaces. Gut 2007;56:1191-7.
200. Cao J, Papadopoulou N, Kempuraj D, et al. Human mast cells express corticotropin-releasing hormone (CRH) receptors and CRH leads to selective secretion of vascular endothelial growth factor. J Immunol 2005;174:7665-75.
201. Christensen J. The oesophagus. In: Christensen J, Wingate DL, eds. A Guide to Gastrointestinal Motility. London: Wright PSG, 1983:75-100.
202. Andrew BL. The nervous control of the cervical oesophagus of the rat during swallowing. J Physiol 1956;134:729-40.
203. Neuhuber WL, Kressel M, Stark A, et al. Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde DiI and DiA tracing: focus on myenteric ganglia. J Auton Nerv Syst 1998;70:92-102.
204. Rodrigo J, Hernandez CJ, Vidal MA, et al. Vegetative innervation of the esophagus. III. Intraepithelial endings. Acta Anat (Basel) 1975;92:242-58.
205. Barthelemy P, Sabeur G, Jammes Y. Reflex relationships between the cervical esophagus and the respiratory system in cats. Neurosci Lett 1996;217:81-4.
206. Patel RS, Rao SS. Biomechanical and sensory parameters of the human esophagus at four levels. Am J Physiol 1998;275:G187-91.
207. Krarup AL, Olesen SS, Funch-Jensen P, et al. Proximal and distal esophageal sensitivity is decreased in patients with Barrett's esophagus. World J Gastroenterol 2011;17:514-21.
208. Thoua NM, Khoo D, Kalantzis C, et al. Acid-related oesophageal sensitivity, not dysmotility, differentiates subgroups of patients with non-erosive reflux disease. Aliment Pharmacol Ther 2008;27:396-403.
209. Weusten BL, Smout AJ. Analysis of 24-h oesophageal pH and pressure recordings. Eur J Gastroenterol Hepatol 1995;7:1147-51.
210. Bredenoord AJ, Weusten BL, Curvers WL, et al. Determinants of perception of heartburn and regurgitation. Gut 2006;55:313-8.

211. Cicala M, Emerenziani S, Caviglia R, et al. Intra-oesophageal distribution and perception of acid reflux in patients with non-erosive gastro-oesophageal reflux disease. Aliment Pharmacol Ther 2003;18:605-13.
212. Tutuian R, Vela MF, Hill EG, et al. Characteristics of symptomatic reflux episodes on Acid suppressive therapy. Am J Gastroenterol 2008;103:1090-6.
213. Zerbib F, Duriez A, Roman S, et al. Determinants of gastro-oesophageal reflux perception in patients with persistent symptoms despite proton pump inhibitors. Gut 2008;57:156-60.
214. Emerenziani S, Ribolsi M, Sifrim D, et al. Regional oesophageal sensitivity to acid and weakly acidic reflux in patients with non-erosive reflux disease. Neurogastroenterol Motil 2009;21:253-8.
215. Menges M, Muller M, Zeitz M. Increased acid and bile reflux in Barrett's esophagus compared to reflux esophagitis, and effect of proton pump inhibitor therapy. Am J Gastroenterol 2001;96:331-7.
216. Vaezi MF, Singh S, Richter JE. Role of acid and duodenogastric reflux in esophageal mucosal injury: a review of animal and human studies. Gastroenterology 1995;108:1897-907.
217. Lagergren J, Bergstrom R, Lindgren A, et al. Symptomatic gastroesophageal reflux as a risk factor for esophageal adenocarcinoma. N Engl J Med 1999;340:825-31.
218. Caldwell MT, Lawlor P, Byrne PJ, et al. Ambulatory oesophageal bile reflux monitoring in Barrett's oesophagus. Br J Surg 1995;82:657-60.
219. Miwa K, Sahara H, Segawa M, et al. Reflux of duodenal or gastro-duodenal contents induces esophageal carcinoma in rats. Int J Cancer 1996;67:269-74.
220. Castell DO. Diet and the lower esophageal sphincter. Am J Clin Nutr 1975;28:1296-8.
221. McArthur K, Hogan D, Isenberg JI. Relative stimulatory effects of commonly ingested beverages on gastric acid secretion in humans. Gastroenterology 1982;83:199-203.
222. Pehl C, Pfeiffer A, Wendl B, et al. The effect of decaffeination of coffee on gastro-oesophageal reflux in patients with reflux disease. Aliment Pharmacol Ther 1997;11:483-6.

223. Wright LE, Castell DO. The adverse effect of chocolate on lower esophageal sphincter pressure. Am J Dig Dis 1975;20:703-7.
224. Becker DJ, Sinclair J, Castell DO, et al. A comparison of high and low fat meals on postprandial esophageal acid exposure. Am J Gastroenterol 1989;84:782-6.
225. Hampel H, Abraham NS, El-Serag HB. Meta-analysis: obesity and the risk for gastroesophageal reflux disease and its complications. Ann Intern Med 2005;143:199-211.
226. Perez AR, Moncure AC, Rattner DW. Obesity adversely affects the outcome of antireflux operations. Surg Endosc 2001;15:986-9.
227. Scarpellini E, Blondeau K, Vos R, et al. M1826 the role of body mass index and waist circumference in determining reflux pattern and proximal extent of reflux events. Gastroenterology 2009;135(5, Suppl. 1):A426.
228. Chow WH, Blot WJ, Vaughan TL, et al. Body mass index and risk of adenocarcinomas of the esophagus and gastric cardia. J Natl Cancer Inst 1998;90:150-5.
229. Pehl C, Pfeiffer A, Wendl B, et al. Different effects of white and red wine on lower esophageal sphincter pressure and gastroesophageal reflux. Scand J Gastroenterol 1998;33:118-22.
230. Kubo A, Levin TR, Block G, et al. Alcohol types and sociodemographic characteristics as risk factors for Barrett's esophagus. Gastroenterology 2009;136:806-15.
231. Anderson LA, Cantwell MM, Watson RG, et al. The association between alcohol and reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma. Gastroenterology 2009;136:799-805.
232. Pandeya N, Williams G, Green AC, et al. Alcohol consumption and the risks of adenocarcinoma and squamous cell carcinoma of the esophagus. Gastroenterology 2009;136:1215-24, e1-2.
233. Hila A, Castell DO. Nighttime reflux is primarily an early event. J Clin Gastroenterol 2005;39:579-83.
234. Stanciu C, Bennett JR. Effects of posture on gastro-oesophageal reflux. Digestion 1977;15:104-9.
235. Harvey RF, Gordon PC, Hadley N, et al. Effects of sleeping with the bed-head raised and of ranitidine in patients with severe peptic oesophagitis. Lancet 1987;2:1200-3.

236. Kapur KC, Trudgill NJ, Riley SA. Mechanisms of gastro-oesophageal reflux in the lateral decubitus positions. Neurogastroenterol Motil 1998;10:517-22.
237. Khoury RM, Camacho-Lobato L, Katz PO, et al. Influence of spontaneous sleep positions on nighttime recumbent reflux in patients with gastroesophageal reflux disease. Am J Gastroenterol 1999;94:2069-73.
238. van Herwaarden MA, Katzka DA, Smout AJ, et al. Effect of different recumbent positions on postprandial gastroesophageal reflux in normal subjects. Am J Gastroenterol 2000;95:2731-6.
239. Ness-Jensen E, Lindam A, Lagergren J, et al. Decreased gastroesophageal reflux symptoms after tobacco smoking cessation in a prospective population-based cohort study: the HUNT study. UEGW. Amsterdam, 2012.
240. Bell NJ, Hunt RH. Role of gastric acid suppression in the treatment of gastro-oesophageal reflux disease. Gut 1992;33:118-24.
241. Jones DB, Howden CW, Burget DW, et al. Acid suppression in duodenal ulcer: a meta-analysis to define optimal dosing with antisecretory drugs. Gut 1987;28:1120-7.
242. Bell NJ, Burget D, Howden CW, et al. Appropriate acid suppression for the management of gastro-oesophageal reflux disease. Digestion 1992;51 Suppl 1:59-67.
243. DeVault KR, Castell DO. Guidelines for the diagnosis and treatment of gastroesophageal reflux disease. Practice Parameters Committee of the American College of Gastroenterology. Arch Intern Med 1995;155:2165-73.
244. Fackler WK, Ours TM, Vaezi MF, et al. Long-term effect of H2RA therapy on nocturnal gastric acid breakthrough. Gastroenterology 2002;122:625-32.
245. Robinson M. Review article: current perspectives on hypergastrinaemia and enterochromaffin-like-cell hyperplasia. Aliment Pharmacol Ther 1999;13 Suppl 5:5-10.
246. Massoomi F, Savage J, Destache CJ. Omeprazole: a comprehensive review. Pharmacotherapy 1993;13:46-59.
247. Lew EA. Review article: pharmacokinetic concerns in the selection of anti-ulcer therapy. Aliment Pharmacol Ther 1999;13 Suppl 5:11-6.
248. Wolfe MM, Sachs G. Acid suppression: optimizing therapy for gastroduodenal ulcer healing, gastroesophageal reflux disease, and stress-related erosive syndrome. Gastroenterology 2000;118:S9-31.

249. Miner P, Jr., Katz PO, Chen Y, et al. Gastric acid control with esomeprazole, lansoprazole, omeprazole, pantoprazole, and rabeprazole: a five-way crossover study. Am J Gastroenterol 2003;98:2616-20.
250. Vakily M, Zhang W, Wu J, et al. Pharmacokinetics and pharmacodynamics of a known active PPI with a novel Dual Delayed Release technology, dexlansoprazole MR: a combined analysis of randomized controlled clinical trials. Curr Med Res Opin 2009;25:627-38.
251. Havelund T, Laursen LS, Skoubo-Kristensen E, et al. Omeprazole and ranitidine in treatment of reflux oesophagitis: double blind comparative trial. British medical journal (Clinical research ed.). Volume 296: BMJ Group, 1988:89.
252. Castell DO, Richter JE, Robinson M, et al. Efficacy and safety of lansoprazole in the treatment of erosive reflux esophagitis. The Lansoprazole Group. Am J Gastroenterol 1996;91:1749-57.
253. Castell DO, Kahrilas PJ, Richter JE, et al. Esomeprazole (40 mg) compared with lansoprazole (30 mg) in the treatment of erosive esophagitis. Am J Gastroenterol 2002;97:575-83.
254. Richter JE, Kahrilas PJ, Johanson J, et al. Efficacy and safety of esomeprazole compared with omeprazole in GERD patients with erosive esophagitis: a randomized controlled trial. Am J Gastroenterol 2001;96:656-65.
255. Dekkers CP, Beker JA, Thjodleifsson B, et al. Double-blind comparison [correction of Double-blind, placebo-controlled comparison] of rabeprazole 20 mg vs. omeprazole 20 mg in the treatment of erosive or ulcerative gastro-oesophageal reflux disease. The European Rabeprazole Study Group. Aliment Pharmacol Ther 1999;13:49-57.
256. Mossner J, Holscher AH, Herz R, et al. A double-blind study of pantoprazole and omeprazole in the treatment of reflux oesophagitis: a multicentre trial. Aliment Pharmacol Ther 1995;9:321-6.
257. Croxtall JD, Scott LJ. Dexlansoprazole modified release: in erosive oesophagitis and non-erosive reflux disease. Drugs 2010;70:1593-601.
258. Metz DC, Howden CW, Perez MC, et al. Clinical trial: dexlansoprazole MR, a proton pump inhibitor with dual delayed-release technology, effectively controls symptoms and prevents relapse in patients with healed erosive oesophagitis. Aliment Pharmacol Ther 2009;29:742-54.

259. Graham DY, Patterson DJ. Double-blind comparison of liquid antacid and placebo in the treatment of symptomatic reflux esophagitis. Dig Dis Sci 1983;28:559-63.
260. Weberg R, Berstad A. Symptomatic effect of a low-dose antacid regimen in reflux oesophagitis. Scand J Gastroenterol 1989;24:401-6.
261. Grove O, Bekker C, Jeppe-Hansen MG, et al. Ranitidine and high-dose antacid in reflux oesophagitis. A randomized, placebo-controlled trial. Scand J Gastroenterol 1985;20:457-61.
262. Szabo S. The mode of action of sucralfate: the 1 x 1 x 1 mechanism of action. Scand J Gastroenterol Suppl 1991;185:7-12.
263. Hameeteman W, von d Boomgaard DM, Dekker W, et al. Sucralfate versus cimetidine in reflux esophagitis. A single-blind multicenter study. J. Clin. Gastroenterol. Volume 9, 1987:390-394.
264. Simon B, Mueller P. Comparison of the effect of sucralfate and ranitidine in reflux esophagitis. Am. J. Med. Volume 83, 1987:43-47.
265. Johnson FA, Craig DQM, Mercer AD, et al. The effects of alginate molecular structure and formulation variables on the physical characteristics of alginate raft systems. International Journal of Pharmaceutics. Volume 159, 1997:35-42.
266. Chatfield S. A comparison of the efficacy of the alginate preparation, Gaviscon Advance, with placebo in the treatment of gastro-oesophageal reflux disease. Curr Med Res Opin 1999;15:152-9.
267. Lanza FL, Smith V, Page-Castell JA, et al. Effectiveness of foaming antacid in relieving induced heartburn. South Med J 1986;79:327-30.
268. Goves J, Oldring JK, Kerr D, et al. First line treatment with omeprazole provides an effective and superior alternative strategy in the management of dyspepsia compared to antacid/alginate liquid: a multicentre study in general practice. Aliment Pharmacol Ther 1998;12:147-57.
269. Mann SG, Cottrell J, Murakami A, et al. Prevention of heartburn relapse by low-dose famotidine: a test meal model for duration of symptom control. Aliment Pharmacol Ther 1997;11:121-7.
270. Bright-Asare P, El-Bassoussi M. Cimetidine, metoclopramide, or placebo in the treatment of symptomatic gastroesophageal reflux. J Clin Gastroenterol 1980;2:149-56.

271. McCallum RW, Ippoliti AF, Cooney C, et al. A controlled trial of metoclopramide in symptomatic gastroesophageal reflux. N Engl J Med 1977;296:354-7.
272. Ramirez B, Richter JE. Review article: promotility drugs in the treatment of gastro-oesophageal reflux disease. Aliment Pharmacol Ther 1993;7:5-20.
273. Valenzuela J, Defillipi C, Gurtierrez F. Effect of domperidone on patients with symptomatic gastroesophageal reflux. Dig Dis Sci 1980;25:716A.
274. Masci E, Testoni PA, Passaretti S, et al. Comparison of ranitidine, domperidone maleate and ranitidine + domperidone maleate in the short-term treatment of reflux oesophagitis. Drugs Exp Clin Res 1985;11:687-92.
275. Nissen R. [A simple operation for control of reflux esophagitis]. Schweiz Med Wochenschr 1956;86:590-2.
276. Spechler SJ. Comparison of medical and surgical therapy for complicated gastroesophageal reflux disease in veterans. The Department of Veterans Affairs Gastroesophageal Reflux Disease Study Group. N Engl J Med 1992;326:786-92.
277. Spechler SJ, Lee E, Ahnen D, et al. Long-term outcome of medical and surgical therapies for gastroesophageal reflux disease: follow-up of a randomized controlled trial. JAMA 2001;285:2331-8.
278. Carlson MA, Frantzides CT. Complications and results of primary minimally invasive antireflux procedures: a review of 10,735 reported cases. J Am Coll Surg 2001;193:428-39.
279. American Gastroenterological Association (2008, May 19). Nearly 40 Percent Of GERD Patients Taking PPIs Experience Recurring Symptoms. ScienceDaily. .
280. El-Serag H, Becher A, Jones R. Systematic review: persistent reflux symptoms on proton pump inhibitor therapy in primary care and community studies. Aliment Pharmacol Ther 2010;32:720-37.
281. Campos GM, Peters JH, DeMeester TR, et al. Multivariate analysis of factors predicting outcome after laparoscopic Nissen fundoplication. J Gastrointest Surg 1999;3:292-300.
282. Khajanchee YS, Hong D, Hansen PD, et al. Outcomes of antireflux surgery in patients with normal preoperative 24-hour pH test results. Am J Surg 2004;187:599-603.

283. Bytzer P, van Zanten SV, Mattsson H, et al. Partial symptom-response to proton pump inhibitors in patients with non-erosive reflux disease or reflux oesophagitis - a post hoc analysis of 5796 patients. Aliment Pharmacol Ther 2012;36:635-43.
284. Weijenborg PW, Cremonini F, Smout AJ, et al. PPI therapy is equally effective in well-defined non-erosive reflux disease and in reflux esophagitis: a meta-analysis. Neurogastroenterol Motil 2012;24:747-57, e350.
285. Gerson LB, Huff FJ, Hila A, et al. Arbaclofen placarbil decreases postprandial reflux in patients with gastroesophageal reflux disease. Am J Gastroenterol 2010;105:1266-75.
286. Boeckxstaens GE, Beaumont H, Mertens V, et al. Effects of lesogaberan on reflux and lower esophageal sphincter function in patients with gastroesophageal reflux disease. Gastroenterology 2010;139:409-17.
287. Sifrim D, Castell D, Dent J, et al. Gastro-oesophageal reflux monitoring: review and consensus report on detection and definitions of acid, non-acid, and gas reflux. Gut 2004;53:1024-31.
288. Souza RF. The pathogenesis of gastroesophageal reflux disease. Gastroenterol Hepatol (N Y) 2012;8:263-5.
289. Rieder F, Cheng L, Harnett KM, et al. Gastroesophageal reflux disease-associated esophagitis induces endogenous cytokine production leading to motor abnormalities. Gastroenterology 2007;132:154-65.
290. Tobey NA, Argote CM, Awayda MS, et al. Effect of luminal acidity on the apical cation channel in rabbit esophageal epithelium. Am J Physiol Gastrointest Liver Physiol 2007;292:G796-805.
291. Tobey NA, Gambling TM, Vanegas XC, et al. Physicochemical basis for dilated intercellular spaces in non-erosive acid-damaged rabbit esophageal epithelium. Dis Esophagus 2008;21:757-64.
292. Fass R. Erosive esophagitis and nonerosive reflux disease (NERD): comparison of epidemiologic, physiologic, and therapeutic characteristics. J Clin Gastroenterol 2007;41:131-7.
293. Orlando RC. Pathophysiology of gastroesophageal reflux disease: esophageal epithelial resistance. In: Castell DO, Richter JE, eds. The esophagus. 4th ed. Philadelphia: Lippincott Williams & Wilkins, 2004:421-33.

294. Weijenborg PW, Smout A, Bredenoord AJ. Hypersensitivity to acid in patients with non-erosive reflux disease is associated with impaired oesophageal muocsal integrity. Gut 2012;61 (suppl 3):A82.
295. Altomare A, Ma J, Guarino MP, et al. Platelet-activating factor and distinct chemokines are elevated in mucosal biopsies of erosive compared with non-erosive reflux disease patients and controls. Neurogastroenterol Motil 2012;24:943-e463.
296. Orlando RC, Paterson WG, Harnett KM, et al. Esophageal disease: updated information on inflammation. Ann N Y Acad Sci 2011;1232:369-75.
297. Knowles CH, Aziz Q. Visceral hypersensitivity in non-erosive reflux disease. Gut 2008;57:674-83.
298. Vela MF, Camacho-Lobato L, Srinivasan R, et al. Simultaneous intraesophageal impedance and pH measurement of acid and nonacid gastroesophageal reflux: effect of omeprazole. Gastroenterology 2001;120:1599-606.
299. Rohof WO, Bennink RJ, Boeckxstaens G. Study on the Mechanisms Underlying PPI Resistance in GERD Patients. Gastroenterology 2012;142:S-203.
300. Zerbib F, Belhocine K, Simon M, et al. Clinical, but not oesophageal pH-impedance, profiles predict response to proton pump inhibitors in gastro-oesophageal reflux disease. Gut 2012;61:501-6.
301. Savarino E, Marabotto E, Zentilin P, et al. The added value of impedance-pH monitoring to Rome III criteria in distinguishing functional heartburn from non-erosive reflux disease. Dig Liver Dis 2011;43:542-7.
302. Bernstein LM, Baker LA. A clinical test for esophagitis. Gastroenterology 1958;34:760-81.
303. Weusten BL, Roelofs JM, Akkermans LM, et al. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. Gastroenterology 1994;107:1741-5.
304. Tanaka S, Chu S, Hirokawa M, et al. Direct measurement of acid permeation into rat oesophagus. Gut 2003;52:775-83.
305. Weijenborg PW, Rohof WO, Akkermans LM, et al. Esophageal Electrical Tissue Impedance Spectroscopy Can Detect Esophageal Permeability Changes in Gastroesophageal Reflux Disease During Upper Endoscopy Gastroenterology 2012;142:S-166.

306. King AL, Anggiansah A, Anggiansah R, et al. Acid perfusion test: a useful test for evaluating esophageal acid sensitivity. Dig Dis Sci 2005;50:1611-5.
307. Richter JE, Pandolfino JE, Vela MF, et al. Utilization of wireless pH monitoring technologies: a summary of the proceedings from the Esophageal Diagnostic Working Group. Dis Esophagus 2012.
308. Shaker R, Hogan WJ. Reflex-mediated enhancement of airway protective mechanisms. Am J Med 2000;108 Suppl 4a:8S-14S.
309. Torrico S, Kern M, Aslam M, et al. Upper esophageal sphincter function during gastroesophageal reflux events revisited. Am J Physiol Gastrointest Liver Physiol 2000;279:G262-7.
310. Neuhuber WL. Sensory vagal innervation of the rat esophagus and cardia: a light and electron microscopic anterograde tracing study. J Auton Nerv Syst 1987;20:243-55.
311. Dutsch M, Eichhorn U, Worl J, et al. Vagal and spinal afferent innervation of the rat esophagus: a combined retrograde tracing and immunocytochemical study with special emphasis on calcium-binding proteins. J Comp Neurol 1998;398:289-307.
312. Wank M, Neuhuber WL. Local differences in vagal afferent innervation of the rat esophagus are reflected by neurochemical differences at the level of the sensory ganglia and by different brainstem projections. J Comp Neurol 2001;435:41-59.
313. Shaker R, Lang IM. Reflex mediated airway protective mechanisms against retrograde aspiration. Am J Med 1997;103:64S-73S.
314. Schuligoi R, Herzeg G, Wachter C, et al. Differential expression of c-fos messenger RNA in the rat spinal cord after mucosal and serosal irritation of the stomach. Neuroscience 1996;72:535-44.
315. Sengupta JN. Electrophysiological recording from neurons controlling sensory and motor functions of the esophagus. Am J Med 2001;111 Suppl 8A:169S-173S.
316. Harrington SJ, Schlegel JF, Code CF. The protective effect of sucralfate on the gastric mucosa of rats. J Clin Gastroenterol 1981;3:129-34.
317. Schweitzer EJ, Bass BL, Johnson LF, et al. Sucralfate prevents experimental peptic esophagitis in rabbits. Gastroenterology 1985;88:611-9.

318. Katz PO, Geisinger KR, Hassan M, et al. Acid-induced esophagitis in cats is prevented by sucralfate but not synthetic prostaglandin E. Dig Dis Sci 1988;33:217-24.
319. Orlando RC, Turjman NA, Tobey NA, et al. Mucosal protection by sucralfate and its components in acid-exposed rabbit esophagus. Gastroenterology 1987;93:352-61.
320. Orlando RC, Tobey NA. Why does sucralfate improve healing in reflux esophagitis? The role of sucrose octasulfate. Scand J Gastroenterol Suppl 1990;173:17-21.
321. Ros E, Pujol A, Bordas JM, et al. Efficacy of sucralfate in refractory reflux esophagitis. Results of a pilot study. Scand J Gastroenterol Suppl 1989;156:49-55.
322. Taal BG, Vales Olmos RA, Boot H, et al. Assessment of sucralfate coating by sequential scintigraphic imaging in radiation-induced esophageal lesions. Gastrointest Endosc 1995;41:109-14.
323. Stanciu C, Bennett JR. Alginate-antacid in the reduction of gastro-oesophageal reflux. Lancet 1974;1:109-11.
324. Chevrel B. A comparative crossover study on the treatment of heartburn and epigastric pain: Liquid Gaviscon and a magnesium--aluminium antacid gel. J Int Med Res 1980;8:300-2.
325. Grossman AE, A.P. K, Rhodes JB, et al. Reflux esophagitis. A comparison of old and new medical management. J Kans Med Soc 1973;74:423-4.
326. McHardy G. A multicentric, randomized clinical trial of Gaviscon in reflux esophagitis. South Med J 1978;71 Suppl 1:16-21.
327. Castell DO, Dalton CB, Becker D, et al. Alginic acid decreases postprandial upright gastroesophageal reflux. Comparison with equal-strength antacid. Dig Dis Sci 1992;37:589-93.
328. Dettmar PW, Hampson FC, Taubel J, et al. The suppression of gastro-oesophageal reflux by alginates. Int J Clin Pract 2007;61:1654-62.
329. Kwiatek MA, Roman S, Fareeduddin A, et al. An alginate-antacid formulation (Gaviscon Double Action Liquid) can eliminate or displace the postprandial 'acid pocket' in symptomatic GERD patients. Aliment Pharmacol Ther 2011;34:59-66.
330. Orlando RC, Tobey NA, Schreiner VJ, et al. Cytoprotection by sucralfate: role of sulfate ions. Scand J Gastroenterol Suppl 1987;140:23-7.

331. Smart JD, Kellaway IW, Worthington HE. An in-vitro investigation of mucosa-adhesive materials for use in controlled drug delivery. J Pharm Pharmacol 1984;36:295-9.
332. Batchelor HK, Banning D, Dettmar PW, et al. An in vitro mucosal model for prediction of the bioadhesion of alginate solutions to the oesophagus. Int J Pharm 2002;238:123-32.
333. Smart JD, Riley RG, Tsibouklis J, et al. The retention of 14C-labelled poly(acrylic acids) on gastric and oesophageal mucosa: an in vitro study. Eur J Pharm Sci 2003;20:83-90.
334. Richardson JC, Dettmar PW, Hampson FC, et al. Oesophageal bioadhesion of sodium alginate suspensions: particle swelling and mucosal retention. Eur J Pharm Sci 2004;23:49-56.
335. Tang M, Dettmar P, Batchelor H. Bioadhesive oesophageal bandages: protection against acid and pepsin injury. Int J Pharm 2005;292:169-77.
336. Strugala V, Avis J, Jolliffe IG, et al. The role of an alginate suspension on pepsin and bile acids - key aggressors in the gastric refluxate. Does this have implications for the treatment of gastro-oesophageal reflux disease? J Pharm Pharmacol 2009;61:1021-8.
337. Cheng L, de la Monte S, Ma J, et al. HCl-activated neural and epithelial vanilloid receptors (TRPV1) in cat esophageal mucosa. Am J Physiol Gastrointest Liver Physiol 2009;297:G135-43.
338. Ma J, Harnett KM, Behar J, et al. Signaling in TRPV1-induced platelet activating factor (PAF) in human esophageal epithelial cells. Am J Physiol Gastrointest Liver Physiol 2010;298:G233-40.
339. Sifrim D. Acid, weakly acidic and non-acid gastro-oesophageal reflux: differences, prevalence and clinical relevance. Eur J Gastroenterol Hepatol 2004;16:823-30.
340. Tobey NA, Djukic Z, Brighton LE, et al. Lateral cell membranes and shunt resistance in rabbit esophageal epithelium. Dig Dis Sci 2010;55:1856-65.
341. Mueller S, Aigner T, Neureiter D, et al. Eosinophil infiltration and degranulation in oesophageal mucosa from adult patients with eosinophilic oesophagitis: a retrospective and comparative study on pathological biopsy. J Clin Pathol 2006;59:1175-80.