SUMMARY

Background
Gastro-oesophageal reflux disease (GERD) is one of the commonest diseases of Western populations, affecting 20 to 30% of adults. GERD is multifaceted and the classical oesophageal symptoms such as heartburn and regurgitation often overlap with atypical symptoms that impact upon the respiratory system and airways. This is referred to as extra-oesophageal reflux disease (EERD), or laryngopharyngeal reflux (LPR), which manifests as chronic cough, laryngitis, hoarseness, voice disorders and asthma.

Aim
The ‘Reflux and its consequences’ conference was held in Hull in 2010 and brought together a multidisciplinary group of experts all with a common interest in the many manifestations of reflux disease to present recent research and clinical progress in GERD and EERD. In particular new techniques for diagnosing reflux were showcased at the conference.

Methods
Both clinical and non-clinical key opinion leaders were invited to write a review on key areas presented at the ‘Reflux and its consequences’ conference for inclusion in this supplement.

Results and conclusion
Eleven chapters contained in this supplement reflected the sessions of the conference and included discussion of the nature of the refluxate (acid, pepsin, bile acids and non-acid reflux); mechanisms of tissue damage and protection in the oesophagus, laryngopharynx and airways. Clinical conditions with a reflux aetiology including asthma, chronic cough, airway disease, LPR, and paediatric EERD were reviewed. In addition methods for diagnosis of reflux disease and treatment strategies, especially with reference to non-acid reflux, were considered.
Review article: nature and properties of gastro-oesophageal and extra-oesophageal refluxate

J. P. Pearson & S. Parikh

INTRODUCTION

Gastric juice moving in a retrograde fashion out through the cardiac (lower oesophageal) sphincter and up the oesophagus is a normal physiological event. Based on measurements using 24 h ambulatory oesophageal impedance-pH monitoring of a healthy population of 72 adults, the median number of reflux events was 44 with a 75th percentile of 58 events. Interestingly, only just over half of these events were acidic (pH < 4.0) the rest being weakly acidic.1 In our own impedance studies, we have taken 58 as the normal level of reflux events to compare normals to lung transplant patients.2 It is clear that reflux occurs in the normal population; however, the number of events may be affected by having an impedance catheter in place.

The main aggressive agents in gastric juice are acid, pepsin, bile salts, bacteria (particularly in patients on proton pump inhibitor (PPI) therapy) and pancreatic proteolytic enzymes. With acid, the pH is likely to be below 4 for a significant time in the oesophagus, where the protective mechanisms against acid are the strongest outside of the stomach. These include peristalsis, carbonic anhydrase production of bicarbonate, heat shock protein expression, a surface layer of dead cells which

SUMMARY

Gastric juice contains many damaging agents against which the stomach has effective defences including a mucus bilayer which generates an unstirred layer which supports surface neutralisation of acid and forms a diffusion barrier to pepsin. However, once gastric contents leave the stomach and enter the oesophagus and the upper airways the protective mechanisms are much reduced. The major aggressors in gastric refluxate are acid, pepsin and bacteria. In addition, gastric refluxate will contain, but not always, duodenal factors such as bile acids and pancreatic enzymes. Acid in the majority of reflux events will remain at a damaging level, i.e. below pH 4.0 for a significant amount of time in the oesophagus. Bile acids have been demonstrated to be damaging in many experimental models; however, great care needs to be taken when interpreting these results because the concentrations and the form of the bile acids used do not always reflect the in vivo situation. Pepsin is an acidic protease but has the potential to damage extra-gastric tissues at pHs up to 6.0 and will not be irreversibly inhibited until pH 7.5 or above. Trypsin, if it passes through the stomach at pH 4.0 or above or rapidly though low pH of 2 or less, will retain activity and can go on to cause damage. With the increase in the use of proton pump inhibitors to treat gastro-oesophageal reflux the elevation of gastric pH has allowed bacterial overgrowth of the stomach. Consequently, a reflux event can lead to the establishing of bacterial colonies outside of the stomach, notably into the airways and lungs.
can be shed off to protect the viable cells from acid exposure and salivary mucus and bicarbonate.3

In terms of acid in the oesophagus, proton pump inhibitors will reduce the level and time of acid exposure in gastro-oesophageal reflux disease (GERD) patients; however, acid exposure will still tend to be above normal.4 It is important to point out here that although PPI therapy will increase the pH and reduce the volume of gastric juice with the pH of refluxate being mainly between 5 and 7 that is weakly acidic.5 It will not directly affect pepsin secretion and critically it will not prevent reflux.6 Therefore on PPIs gastric juice will still contain the other damaging components.

MAJOR AGGRESSORS IN GASTRIC JUICE WITH POTENTIAL TO DAMAGE EXTRA-OESOPHAGEAL TISSUES

Bile acids
Bile acids can be found in gastric juice and are present as a result of reflux from the duodenum through the pylorus into the stomach (duodenogastric reflux). Bile acids have been measured in fasting gastric juice using an enzymatic method based on a 3α hydroxydehydrogenase, which acts on the hydroxyl group of the bile acid steroid ring and in the process releasing reduced NAD. The NADH is then reacted with nitrotetrazolium blue to produce formazan catalysed by diaphorase. The resulting blue colour development can be quantified at 540 nm. This assay can be made more sensitive by using thio-NAD\n in the presence of excess NADH, which results in enzyme cycling and the rate of formation of thio-NADH can be determined directly by a change in absorbance at 405 nm, with a claimed lower detection limit of 1 μmol/L. Using the above methods fasting gastric bile acid levels ranged from 0 to 150 μmol/L, median 0 μmol/L (n = 20) and 0–410 μmol/L, median 100 μmol/L (n = 14) in controls and oesophagitis patients respectively.7 In our studies, using the enzymatic method, in fasting gastric juice of patients undergoing routine upper GI endoscopy the values ranged from 10 to 10 000 μmol/L with a median of 55 μmol/L and only seven of the 60 samples having bile acid levels above 500 μmol/L (see Table 1). It is likely that gastric juice bile acid concentrations increase in the postprandial period. This can be implied from the increases in bile acid concentrations in oesophageal aspirates increasing from a mean of 6 to 12 μmol/L and 10 to 60 μmol/L from fasting to postprandial in controls and GERD patients respectively.8 Therefore reflux after a meal is potentially more damaging than in the fasting state. Based on the levels of bile acids present in gastric juice and the normal serum range 0–10 μmol/L. Along with the manufacturers claim for the enzymatic assay of a detection limit of 1 μmol/L, in our hands 2 μmol/L and the findings of Klokkenburg et al.9 that ‘outcomes lower than 5 μmol/L may not be reliable’. Bile acid levels in the oesophagus would be measurable and present at potentially damaging levels in the oesophagus but once the refluxate has left the oesophagus and entered the pharynx/larynx and has been diluted by upper airway and salivary secretions, in many cases bile acids will be below detectable levels. In particular detection is unlikely if the refluxate is aspirated into the lungs and collected by bronchoalveolar lavage which involves a 100–200 times dilution with saline. We would recommend that more sensitive quantitation methods are used for extra-oesophageal refluxate, e.g. HPLC which can determine levels of individual bile acids with a limit of detection of 0.07–0.6 μmol/L and quantification in the range of 0.2–1.8 μmol/L.10 However, the most sensitive detection system available is tandem mass spectrometry with a quantitation limit of 0.1 μmol/L and with pre-extraction using a C18SPE column eluted with methanol the limit can be improved to 1 nmol/L.

Bile acids have been demonstrated in many studies to be damaging to oesophageal and extra-oesophageal tissues. In a perfused rabbit oesophagus model, bile acids at concentrations up to 5 mmol/L have been shown to damage the mucosa particularly at low pH with the taurine conjugated bile acids, taurodeoxycholate and taurocholate causing increased permeability to hydrogen ions and mucosal damage, while un-conjugated ones did not.11, 12 Bile salts have the potential to damage tissues as far away from the stomach as the lungs. Exposure of type II pneumocytes in culture to chenodeoxycholate in μmol/L concentrations caused increases in cell membrane permeability and decreased cell viability.13 In our laboratory, we have demonstrated that lithocholate (the free acid) concentrations above 10 μmol/L cause significant cell death of primary bronchial epithelial cells in culture. Further recent studies by Farre et al.,14 using rabbit oesophageal mucosa set up in Ussing chambers have confirmed that the conjugated bile acid sodium salts glycocholate and taurodeoxycholate at concentrations between 0.5 and 5 mmol/L, at pH 2.0 damaged the mucosa as seen by decreased electrical resistance. Interestingly, they further demonstrated that these conjugated bile acids and un-conjugated deoxycholate caused damage at higher pHs of 5.0 and 7.4 when used at the higher
mmol/L concentrations, 2 and above in the presence of 1 mg/mL pepsin. From the above studies, bile acids are damaging in the mmol/L range; however, there is conflicting data as to the levels of bile acids reaching the extra-gastric tissues.

In addition, in experimental assessments of bile acid damage, the form of bile acids in vivo needs to be carefully considered. Bile acids exist in several forms, as free acids, bile salts with sodium and potassium, conjugated with glycine or taurine and conjugated bile salts with sodium or potassium. Bile salts are synthesised in the liver from cholesterol. The two primary bile acids are cholic acid and chenodeoxycholic acid, having three and two hydroxyl groups present on the steroid rings respectively. In the colon, cholic acid and chenodeoxycholic acid are converted to the secondary bile acids deoxycholic acid and lithocholic acid by bacteria, with two and one hydroxyl groups present on the steroid rings respectively. Consequently lithocholate with only one hydroxyl group is the most lipid soluble and potentially the most damaging to cells. In the liver, bile acids are conjugated to glycine or taurine. Any free bile acids and conjugated bile acids are converted to sodium or potassium salts in the alkaline conditions of hepatic bile. The question therefore is what is the distribution of bile acids in bile and in different regions of the GI lumen and in what form are they present?

In human, bile only a trace of free bile acids are present and glycine conjugates make up around 75% and taurine conjugates around 25% (Table 2).\textsuperscript{10} Bile acids detected and quantitated in aspirated duodenal fluid were conjugated, with the free bile acid, cholic acid being below the level of detection (0.07–0.6 μmol/L). Conjugated bile acids were present in the following percentages: glycodeoxycholate 48%, glycochenodeoxycholate 20%, glycocholate 15%, taurochenodeoxycholate 8% and taurocholate 6%. In gastric aspirates again conjugates predominate with no free cholic acid detectable. The conjugated bile acids were as follows: glycodeoxycholate 48%, glycochenodeoxycholate 39%, glycol-cholate 35%, taurochenodeoxycholate 4% and taurocholate 6%.\textsuperscript{10} In human oesophageal aspirates, again no free bile acids have been

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<th>TBA (μM)</th>
<th>GJ sample</th>
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<td>n.d.</td>
<td>60</td>
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n.d., not detectable (detection limit 10 μM); TBA, total bile acids.

Mean: 55 (10–10 010) μM. Bile acid concentration was measured using the 3α-hydroxysteroid dehydrogenase assay.
found with 60% glycocholate, 16% glycodeloxycholate and 15%, glycochenodeoxycholate the remaining ~10% being made up of taurine conjugates and glycolithocholate.\(^8\) It appears from the above studies that free bile acids should not be used in experimental models of reflux. The last question relating to bile acids is their damaging potential. Do they have a direct detergent effect on the cell membrane or do they need to enter the cell? Based on the pK\(a\) values in Table 2, the glycine conjugates, which make up 70%, would be uncharged at pHs below 4, the values occurring in refluxate. Glycine conjugates would therefore be hydrophobic and lipid membrane soluble. They could then accumulate inside the cell, as once inside they would ionise, become hydrophilic at pH 7.4 and be trapped inside the cell. As taurine conjugates have pK\(a\) values below pH 2, only a small fraction will be protonated (uncharged) at pHs between 2 and above. So these are unlikely to accumulate inside cells as a result of a reflux event. Another point to consider is that at very low pH, bile acids will precipitate and therefore cease to be damaging. It is also possible that some charged bile acids may be actively taken up by upper GI tract mucosal cells, e.g. in the oesophagus by scavenging receptors.

A situation where un-conjugated bile acids may become important is in patients on acid suppression therapy, where bacterial overgrowth occurs in the stomach and these bacteria could deconjugate the bile acids.\(^16\) Then bile acids with pK\(a\) values around 5 could damage mucosa by entering cells at pHs in the weakly acid reflux range above pH 5.

The direct detergent effect of bile acids requires a hydrophobic and a hydrophilic region and is affected by solubility and pK\(a\), as detergent behaviour is altered by ionisation state. Therefore taurine conjugates would be expected to have a topical detergent effect across the whole pH range of refluxate. Taurine conjugates are so called acid resistant detergents.

### Pepsins

Pepsins are by definition acidic proteases and have maximal activity against a protein substrate such as haemoglobin between pH 1.9 and 3.6.\(^23\) This wide range is because human gastric juice contains eight different pepsins. The major pepsin is pepsin 3, making up 70.3 ± 2.6% of the total.\(^24\) Pepsin 3 has a pH optimum between 2.4 and 2.8 against haemoglobin. It is important to note that human pepsin 3 retains between 5% and 10% of its activity against purified protein at pH 5.0 and 10% or more at pH 4.0 and retains measurable activity up to pH 6.0. In addition, pepsins ability to damage tissue also extends up to pH 6.0 as seen in the pig larynx model.\(^3\) The ability to degrade and damage mucosal tissue up to pH 6.0 is important when considering reflux from the stomach into the upper airways and lungs as reflux events in the weakly acidic range, pH 5.0 and above have been widely reported.\(^14\) Consequently, pepsin must be considered a potential damaging agent up to pH 6.0. A further consideration is that pepsin could be taken

<table>
<thead>
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<th>Table 2</th>
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<tr>
<td>CA</td>
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<tr>
<td>DCA</td>
<td>48–241 (\times) 10(^3)</td>
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<td>GCA</td>
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<td><strong>Taurine conjugated</strong></td>
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<td>TCA</td>
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<td>TCDCA</td>
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CA, cholic acid; DCA, deoxycholic acid; CDCA, chenodeoxycholic acid; GCA, glyco-cholic acid; GDCA, glyco-deoxycholic acid; GCDCA, glyco-chenodeoxycholic acid; TCA, tauro-cholic acid; TDCA, tauro-deoxycholic acid; TCDCA, tauro-chenodeoxycholic acid; RT, room temperature.

The solubilities are in water ~pH 5.0 at RT. The data in this table are taken from or calculated from figures in references 15 and 17–22.
up by the mucosal cells of the aerodigestive tract and cause intracellular damage in regions of low pH inside the cell, e.g. lysosomes. Although pepsin is inactive above pH 6.0, it is still un-denatured. In gastric juice, pepsin retains its native structure up to pH 7.5 and possibly higher. Therefore, pepsin present in a refluxate could bind to the mucosa, remains native but inactive after neutralisation of the refluxate and be reactivated by a subsequent reflux event below pH 6.0 or by the passage over the mucosa of an acidic drink, e.g. cola pH 2.5–2.8. The presence of pepsin in the refluxate makes it a potential biomarker of reflux as well as a factor in the aetiology of tissue damage. Unlike the measurement of bile acids, there is a sensitive pepsin ELISA available with a lower quantitation limit of 1 ng/mL.

Pancreatic proteolytic enzymes

Pancreatic exocrine secretions contain several proteolytic enzymes including trypsin and chymotrypsin. Trypsin has been the most studied. It is secreted as trypsino-gen and activated by an intestinal enzyme, enteropeptidase. The activation involves removal of a 6 amino acid aspartate rich peptide. This is unlike the conversion of pepsinogen to pepsin which is auto-catalytic and pH dependent. Trypsin has a pH range of activity between 6 and 10 with optima around pH 8. For trypsin to have a major role in damage to the aerodigestive tract it must pass through the stomach from the duodenum without being inactivated and it must reach the oesophagus and above at pHs within its activity range. Rat models demonstrate the damaging potential to the oesophagus of trypsin. In models where duodenal contents are introduced into the oesophagus by means of an oesophagogastrodudenostomy, where the duodenum is joined to the stomach above the pylorus and to the oesophagus. That is a situation where duodenal contents do not pass through an intact stomach. This gastroduodenal reflux model induced oesophageal erosions and ulcer formation within 8 weeks of surgery and the use of trypsin inhibitors reduced the damage. Also in humans with distal gastrectomy reconstructed with an anastomosis between the remaining stomach and the duodenum, trypsin activity was elevated in oesophageal washings, pH range 5.2–7.2 average 6.3, in patients with severe oesophagitis. However, in both these situations duodenal juice has not passed through a normal intact stomach. When it has passed through an intact stomach, only seven of 365 oesophageal aspirated contained active trypsin and the level of trypsin activity was only above 20 μg/mL when the pH was above 4.6. In our studies, trypsin activity in human gastric juice correlated with the pH of the juice, i.e. the higher the pH the higher the trypsin level. In further experiments, trypsin was stable when exposed to pepsin at pH 4.0 for 6 h but destroyed by incubation with pepsin at pH 2.2 for 4 h. Consequently, for trypsin to be a major source of damage to tissues above the stomach, it must pass through the stomach at a pH above 2.2, a situation present in patients on PPI therapy.

Bacteria/bacterial products

We have shown in experiments with human primary lung epithelial cells that human gastric juice at 1/100 to 1/1000 dilution with media (therefore pH will be 7.4) could cause almost 100% cell death and dialysis and filtration reduced the level of cell death. Recently Mertens et al. using primary bronchial cells from one patient have exposed them to diluted gastric juice collected from 11 patients on or off PPI therapy having routine endoscopy. Gastric juice from patients on PPI therapy produced a significantly higher Il-8 response than patients off PPI and that the pH of gastric juice as collected significantly correlated with Il-8 production. Filtration of the gastric juice significantly reduced the Il-8 response. These results imply a role for constituents in gastric juice of patients on PPI therapy, that cause significant inflammatory responses, that are removed at least in part by filtration. As on PPI therapy the gastric pH would be sufficiently high to permit some bacterial overgrowth in the stomach, candidates for these constituents are bacteria and bacterial products such as endotoxins.

CONCLUSIONS

In refluxate:

(i) Acid is damaging to the oesophagus but once outside the oesophagus it will be rapidly neutralised.

(ii) Pepsin can damage all extra-gastric tissues at pHs up to 6.

(iii) Bile acids are potentially damaging but most models do not use the in vivo form of bile acids. The levels of bile acids outside of the stomach and oesophagus are difficult to quantitate accurately.

(iv) Trypsin could cause damage if it retains activity after passing through the stomach.

(v) Bacteria and bacterial products can cause tissue damage if they have survived exposure to gastric juice.

Acid can be controlled by PPI therapy all the other damaging factors remain potentially damaging on PPI therapy and may have their damaging ability enhanced by the increase in the pH of gastric juice.
REFERENCES


INTRODUCTION
Gastro-oesophageal reflux disease (GERD), by definition, is a clinical disorder whose signs and symptoms result from the contact of tissue with components of the refluxate. The most common symptom of GERD is ‘heartburn’, a reflection of acid damage to the oesophageal epithelium.1 Heartburn is a substernal burning discomfort, often worse after meals and on reclining and temporarily relieved by antacids. Based on estimates that ~20% of adults have heartburn at least weekly, GERD is one of the most common conditions in Americans. It is also costly because of procedures used in diagnosis and in prescriptions for acid-inhibiting medication used in treatment. Moreover, heartburn occurs at night as well as day, and so interferes with sleep and work, impairing both productivity and quality of life.2 Though rarely a cause of death, 1/3rd of GERD subjects with heartburn have on upper endoscopy erosive oesophagitis, and this may progress to strictures and/or Barrett’s oesophagus, the latter a risk factor for oesophageal adenocarcinoma. The remaining two-thirds with heartburn from GERD have normal-appearing oesophageal mucosa on endoscopy and so are said to have non-erosive reflux disease (NERD).1 The pathogenesis of GERD is accepted as arising from prolonged contact of oesophageal epithelium with refluxed gastric hydrochloric acid (HCl), and the accuracy of these concepts supported by the success of therapy with proton pump inhibitors (PPIs), both in controlling heartburn and healing erosions.3 However, given the presence of physiologic reflux and the fact that healthy subjects experience no heartburn during oesophageal acid perfusion (Bernstein test),4 it is evident that not all acid contact with the oesophageal epithelium is damaging, and this a testament in part to defences within the epithelium proper. Indeed, as the oesophageal epithelium lacks a well-defined mucus coat and surface protection against injury is in part the result of the antireflux mechanisms that limit the frequency of reflux, in part the result of the luminal clearance mechanisms that limit the duration of acid contact with the oesophageal epithelium and in part due to ‘tissue resistance’ which provides protection during contact of acid with epithelium. When acid contact with the epithelium exceeds a critical level – which varies and depends on level of acidity, duration of contact, and epithelial integrity – damage occurs that early on is recognised by the symptom of heartburn and development of dilated intercellular spaces within the epithelium. Both heartburn and dilated intercellular spaces reflect a breach in the barrier function of oesophageal epithelium. The location of the breach and how it promotes both the symptom of heartburn and the progression of non-erosive reflux disease to erosive oesophagitis are described as is the rationale for the development of two aberrant means of repair – stricture and Barrett’s oesophagus.

SUMMARY
The oesophageal epithelium is exposed to refluxed gastric acid on a daily basis; yet most subjects have no symptoms or signs of tissue damage. Protection against injury is in part the result of the antireflux mechanisms that limit the frequency of reflux, in part the result of the luminal clearance mechanisms that limit the duration of acid contact with the oesophageal epithelium and in part due to ‘tissue resistance’ which provides protection during contact of acid with epithelium. When acid contact with the epithelium exceeds a critical level – which varies and depends on level of acidity, duration of contact, and epithelial integrity – damage occurs that early on is recognised by the symptom of heartburn and development of dilated intercellular spaces within the epithelium. Both heartburn and dilated intercellular spaces reflect a breach in the barrier function of oesophageal epithelium. The location of the breach and how it promotes both the symptom of heartburn and the progression of non-erosive reflux disease to erosive oesophagitis are described as is the rationale for the development of two aberrant means of repair – stricture and Barrett’s oesophagus.
zone for buffering luminal acid, one of its most critical defences against acid back diffusion is its barrier function.5

PROTECTION
Barrier function against acid entry into the epithelium resides in both the apical membranes and apical junctional complexes (AJCs) of surface cells in stratum corneum. The apical cell membranes and integral membrane proteins representing cation channels are designed to prevent acid diffusion directly into the cell cytosol while the AJCs are designed to prevent acid diffusion into the intercellular space.5 This is evident experimentally by showing that neither intracellular pH of surface cells nor transepithelial electrical resistance ($R_T$) of rabbit oesophageal epithelium are materially altered when tissues are exposed luminally to HCl at pHs as low as 2.0.6, 7 The reason for this is that the apical membranes are composed of a hydrophobic lipid bilayer and the cation channels are pH-sensitive, i.e. when luminal pH falls to 3.5, channel conformation changes in a way that precludes cations, including $H^+$, from moving from lumen to cell cytosol.8 Additionally, $H^+$ diffusion into the intercellular space is limited by the AJC, the latter comprised of three structures: tight junctions, adherens junctions and desmosomes (Figure 1). The tight junctions and adherens junctions have bridging proteins (claudins/occludin and e-cadherin respectively) that encircle the cell to create directly a diffusion barrier to ion movement while desmosomes which are spot welds have bridging proteins (desmoglein/desmocollin) that impede ion diffusion by maintaining close apposition of the lateral cell membranes.9, 10 By limiting the rate of $H^+$ diffusion, these structures defend against reduction in cytosolic or intercellular pH as the latter compartments have a readily replenishable supply of buffering substances, especially bicarbonate ions generated by carbonic anhydrases or derived by diffusion from blood.11, 12

TISSUE DAMAGE
Experimentally, when luminal acidity in contact with rabbit oesophageal epithelium is reduced to a pH <2.0, a break in the epithelial barrier emerges – the latter shown by a steady decline in $R_T$.7, 12 Furthermore, the decline in $R_T$ is attributed to a breakdown in the junctional defence leading to an increase in paracellular permeability (Figure 2). The change in junctional defence has been documented by circuit analysis, fluorescein and mannitol flux, and development of dilated intercellular spaces (DIS) in oesophageal epithelium on transmission electron microscopy.13–16 DIS emerge because the $H^+$-induced increase in paracellular permeability enables the accompanying anion, $Cl^-$, to diffuse into the intercellular space, and to do so in sufficient quantities to create an osmotic force for water movement.17 This increase in water movement into the intercellular space separates the lateral cell membranes yielding DIS. Just as important as identifying that acid produces an increase in paracellular permeability, is the means by which it does it. Thus, it was shown that the change in AJC that causes the increase in paracellular permeability was the primary point of attack by acid rather than an indirect consequence of cell acidification. This was determined by monitoring intracellular pH within surface cells residing within intact oesophageal epithelium using pH microelectrodes.6 When the epithelium is exposed to pH 1.6 to

Figure 1 | This illustrates the major contributors to the barrier function of oesophageal epithelium. Note the apical membrane and tripartite intercellular apical junctional complex comprised of the tight junction, adherens junction and desmosome.
record the decline in $R_T$, it is noted that intracellular pH declines and stabilises at pH 6.5. If intracellular pH, however, is lowered to pH 6.5 by serosal exposure to HCl, pH 3.0, there is no decline in $R_T$. Hence, the break in the barrier, i.e. decline in $R_T$ at luminal pH 1.6 is the cause of the decline in intracellular pH and not the effect of a decline in intracellular pH; and this consistent with the conclusion that luminal acid directly attacks and damages the AJC leading to an increase in paracellular permeability. Further supporting this conclusion is the observation that luminal acid-induced cell necrosis in oesophageal epithelium can be prevented with a cell-impermeant buffer on the serosal side of the tissue (as replacement for bathing solution bicarbonate) and that this protection by extracellular buffer occurs without preventing the luminal acid-induced increase in paracellular permeability, i.e. reduction in $R_T$.

The reason for focusing on the acid-induced increase in paracellular permeability is that this occurrence threatens the viability of oesophageal epithelium by enabling luminal acid to more freely diffuse into and acidify the intercellular space. Moreover, acidification of the intercellular space allows by extension direct acidification of the squamous cell basolateral membrane (Figure 2). Acidification of this membrane is of concern because it contains a basolateral Na-independent, $\text{Cl}^-/\text{HCO}_3^-$ exchanger capable of transferring a low extracellular pH into a low intracellular pH by moving extracellular $\text{Cl}^-$ into the cell in exchange for intracellular $\text{HCO}_3^-$ out of the cell.\[7, 18\] [Note: As the $\text{HCO}_3^-$ removed from the cell is derived from carbonic acid ($\text{H}_2\text{CO}_3$), loss of cytosolic $\text{HCO}_3^-$ is the equivalent of cytosolic gain of $\text{H}^+$.] In effect, intracellular acidification is readily translated into intracellular acidification, the latter the proximate cause for cell oedema and necrosis in acid-exposed oesophageal epithelium. That acidification of the intercellular compartment leads to cell oedema and necrosis and this via activation of basolateral membrane $\text{Cl}^-/\text{HCO}_3^-$ exchange has also been tested and supported by serosal exposure of oesophageal epithelium to acid, pH 2.0.\[7, 12\] Notably, the acid-induced cell oedema and necrosis could be effectively prevented by serosal pre-treatment of the epithelium with a disulfonic stilbene derivative, a compound known to block $\text{Cl}^-/\text{HCO}_3^-$ exchange. These data are all consistent with the following sequence: Luminal acid directly attacks and damages the AJC which results in an increase in paracellular permeability. The increase in paracellular permeability promotes cell necrosis, first by acidifying the intercellular space which in turn acidifies the cell cytosol. The cell cytosol is acidified because of the ability of $\text{H}^+$ and $\text{Cl}^-$ to traverse the basolateral cell membrane via a membrane Na-independent, $\text{Cl}^-/\text{HCO}_3^-$ exchanger. The mechanism by which cell acidification is translated into cell oedema has been investigated and shown to be due to the inactivation of $\text{K}^+$ channels and sodium pump and simultaneous activation of a $\text{NaKCl}$ cotransporter.\[7, 19–21\] The $\text{NaKCl}$ cotransporter loads the cell with ions to create an osmotic force for excess water movement into the cytosol, producing cell oedema. That this is the case was shown by the ability to prevent cell oedema by pre-treatment of cells with bumetanide, a compound known to inhibit the $\text{NaKCl}$ cotransporter.\[20\] The proximate cause of cell death remains somewhat elusive but is likely mediated by the acid-induced elevation in intracellular $\text{Ca}^{2+}$, the latter known to activate such destructive enzymes as phospholipases, ribonuclease-
es and proteases as well as to impair cell respiration in some cell types via activation of the mitochondrial permeability transition.\(^2\)\(^2\)\(^3\)

Notably, the phenomena described above for acid-exposed and acid-damaged rabbit oesophageal epithelium have also been identified in acid-exposed and reflux-damaged human oesophageal epithelium. For instance, it has been observed that: (i) perfusion of the oesophagus with acid precipitates heartburn and lowers the electrical potential difference in NERD;\(^2\)\(^4\) (ii) perfusion of the oesophagus with acid results in development of DIS;\(^2\)\(^5\) (iii) oesophageal biopsies from GERD have lower \(R_\Delta\) and higher fluorescein fluxes than healthy oesophageal epithelium;\(^2\)\(^6\) (iv) oesophageal biopsies from GERD, both erosive and non-erosive forms, have DIS;\(^1\)\(^4\) and (v) DIS in GERD resolves, along with heartburn, upon treatment with PPIs.\(^2\)\(^7\) Taken together, these observations establish that heartburn develops in GERD when an increase in paracellular permeability – evident by acid-induced lowering of PD and development of DIS – enables luminal acid sufficient access to trigger the firing of nociceptors within the oesophageal epithelium.\(^2\)\(^8\) Moreover, because human, like rabbit, squamous epithelial cells have a basolateral Na-independent, \(\text{Cl}^-/\text{HCO}_3^-\) exchanger,\(^2\)\(^9\)\(^3\)\(^0\) it is clear that acidification of the intercellular space will result in the lowering of intracellular pH, thereby risking cell injury and necrosis.

Cell damage at the microscopic level can progress to macroscopic damage and erosions because of the increasing size of the defect in the epithelial barrier. However, this can be repaired through one of two mechanisms: restitution or regeneration.\(^3\)\(^1\)--\(^3\)\(^5\) Regeneration, which requires cell replication, is slow, occurring over days to weeks depending on the size of the defect. This is because it requires DNA and protein synthesis by progenitor cells residing within the stratum germinativum. Restitution, however, can restore barrier function relatively fast, over minutes to hours, because it does not require cell replication but instead uses cell migration to close a barrier defect. In the case of restitution, viable cells adjacent to areas of necrosis migrate over a scaffold or basement membrane to re-establish epithelial continuity. Mediating these reparative processes, whether regeneration or restitution, are a variety of growth factors, but for these to be effective requires an environment of neutral or near neutral pH. This is because restitution and regeneration are exquisitely acid-sensitive, with the former being inhibited at pH 6.5 and abolished at pH 3.\(^1\)\(^5\) This suggests that for wound repair to be effective that luminal acidity needs to be controlled through the use of acid-inhibiting drugs. This particularly the case in oesophagus which lacks a well-defined pre-epithelial defence comprised of mucus and bicarbonate-rich unstirred water layer for surface buffering of \(\text{H}^+\). In effect, when the rate of cell necrosis exceeds the rate of squamous repair, the consequence is erosive oesophagitis. Ultimately if squamous repair falters, two aberrant forms of repair emerge: stricture and Barrett’s oesophagus.\(^1\) A stricture aids repair in GERD by creation of a fibrotic barrier to acid reflux while Barrett’s oesophagus, representing a lining of specialised columnar epithelium in oesophagus, aids repair by providing a more acid-resistant epithelium.\(^3\)\(^6\) The latter is the case because specialised columnar epithelium can secrete mucus and bicarbonate and has a junctional barrier dominated by claudin-18, the latter conferring greater resistance to acid penetration into the intercellular space.\(^3\)\(^7\)--\(^3\)\(^0\)

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Review article: uptake of pepsin at pH 7 - in non-acid reflux - causes inflammatory, and perhaps even neoplastic, changes in the laryngopharynx

N. Johnston

INTRODUCTION

Laryngopharyngeal reflux (LPR) contributes to voice disorders, otolaryngological inflammatory disorders, and is associated with upper airway neoplasia. Treatment is currently focussed on increasing the pH of the refluxate as it was thought that the refluxate would not cause injury at higher pH. However, many patients with reflux-attributed laryngeal injury/disease have persistent symptoms despite maximal acid suppression therapy. Recent studies using combined multichannel intraluminal impedance (MII) with pH monitoring showed a positive symptom association with non- and weakly acidic reflux and an association between non-/weakly acidic reflux and refractory symptoms on proton pump inhibitor (PPI) therapy. Thus, the role of acid alone in the development of reflux related laryngeal pathology is being questioned and studies examining the effects of the other components of the refluxate are needed. Crucially, our data supports a role for pepsin in reflux-attributed laryngeal injury/disease, independent of the pH of the refluxate.

There is substantial evidence in the literature demonstrating a significant association between reflux of gastric

SUMMARY

We describe herein how pepsin causes laryngeal epithelial cell damage at pH 7, and thus in non-acidic refluxate. Our data may help explain why some patients have refractory symptoms on maximal proton pump inhibitor therapy, and help explain the reported symptom association with non-acidic reflux events. We report mitochondrial and Golgi damage in laryngeal epithelial cells exposed to pepsin at pH 7. Cell toxicity was also demonstrated using the MTT cytotoxicity assay. Pepsin at pH 7 significantly alters the expression levels of multiple genes implicated in stress and toxicity. We also report that pepsin (0.1 mg/mL, pH 7) induces a pro-inflammatory cytokine gene expression profile in hypopharyngeal FaDu epithelial cells in vitro similar to that which contributes to disease in gastro-oesophageal reflux patients. Moreover, using a Human Cancer PathwayFinder SuperArray, we have shown that pepsin (0.1 mg/mL, pH 7) significantly alters the expression of 27 genes implicated in carcinogenesis. Collectively, these data suggest a mechanistic link between exposure to pepsin, even in non-acidic refluxate, and cellular changes that lead to laryngopharyngeal disease including cancer.

In this context, our unexpected finding that pepsin is taken up by human laryngeal epithelial cells by receptor-mediated endocytosis is highly relevant. Pepsin has been previously assumed to cause damage by its proteolytic activity alone, but our discovery that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis opens the door to a new mechanism for cell damage, and downstream, the development of new therapies for reflux disease – receptor antagonists and/or pepsin inhibitors.
contents into the laryngopharynx (LPR) and laryngeal inflammatory diseases, voice disorders and even neoplastic diseases of the laryngopharynx. It has been estimated that up to 50% of patients with laryngeal and voice disorders have significant symptoms of LPR. However, the exact role of LPR in injury and disease remains controversial. Several factors complicate this area of research.

First, while there is general agreement that PPIs are effective in treating gastro-oesophageal reflux disease (GERD), their efficacy for the treatment of LPR remains in doubt. Because many patients with reflux-attributed laryngeal symptoms and endoscopic signs do not respond to acid suppression therapy as well, or at all, compared with patients with GERD, some believe that LPR cannot be the cause of their symptoms and injury. It has been suggested that the laryngeal mucosa is more sensitive to the damaging effects of gastric refluxate than the oesophagus and thus these patients require higher doses and a longer trial of PPIs. In 2006, Vaezi et al. reported a prospective multicentre, randomised study which evaluated the efficacy of PPI’s in treating LPR. They found no difference in LPR response to PPI or placebo. However, it has been suggested that these data may be inconclusive because the inclusion criteria could have produced a dilution effect. Of the 145 subjects included, most were marginal cases with minimally troubling symptoms based on their LPR–health-related quality of life assessment and absence of pharyngeal acid reflux on pH monitoring. More recently, Reichel et al. reported that patients with symptoms and endoscopic signs of LPR showed a statistically significant improvement in both symptoms and physical findings on esomeprazole vs. placebo for 12 weeks. A substantial placebo effect was noted at 6 weeks; however, this was no longer evident at 12 weeks.

Second, there are many nonspecific symptoms and findings of LPR. This has resulted in an over-diagnosis of LPR, and subsequently an inappropriate use of PPIs in patients exhibiting similar symptoms and findings which are unrelated to LPR. As a result, this has likely increased the number of patients included in studies investigating the efficacy of PPI therapy, who do not actually have LPR.

Third, combined MII with pH monitoring (MII-pH), has been introduced to our field relatively recently as a method of measuring and supporting with the diagnosis of LPR especially in identifying those patients (around 20%) who do have a reflux/symptom relationship. It should be noted that the majority of MII-pH studies (approximately 80%) have a negative symptom association with non-acid or weakly acidic reflux and extra-oesophageal symptoms. However, a significant association between non- and weakly acidic reflux and persistent symptoms on PPI therapy has been shown in approximately 20% of patients. Patients with signs and symptoms associated with non-acidic and weakly acidic reflux would likely have a negative pH test and would not benefit from PPI therapy. Diagnosis and treatment have focused on the acidity of the refluxate because it was thought that the other components of the refluxate would not be injurious at higher pH. However, it is now known that certain bile acids are injurious at higher pH, and our data support a role for pepsin in reflux-attributed laryngeal injury and disease, independent of the pH of the refluxate. Given: (i) the multiple reports of refractory reflux-attributed laryngeal symptoms and endoscopic findings on maximal PPI therapy; (ii) that studies using MII-pH reveal a positive symptom association with non- and weakly acidic reflux events; and (iii) we now know that pepsin and bile acids are injurious at higher pH, the role of acid alone in reflux-attributed signs and symptoms has to be questioned and subsequently the efficacy of acid suppression therapy for treating such.

The objective of our ongoing studies are to: (i) elucidate pepsin as a causal agent involved in early events in carcinoma of the laryngopharynx; (ii) isolate and identify the receptor with which pepsin interacts on the surface of human laryngeal epithelial cells; and (iii) further delineate the effects of receptor-mediated uptake of pepsin on the biochemistry and biology of laryngeal epithelium. Our long-term goal is to develop more effective, better targeted, therapeutics for patients with reflux disease, specifically for that large population that have persistent symptoms despite maximal acid suppression therapy. The potential protective effect of irreversible inhibitors of pepsin activity is currently being investigated. Following identification of the receptor with which pepsin interacts, antagonists will be developed and tested using in vitro and in vivo models, to determine whether they prevent pepsin uptake and injury.

**DIAGNOSIS OF LPR**

For the diagnosis of LPR, most physicians rely on a combination of the patients’ symptoms, laryngeal findings and reflux testing results. Ambulatory 24 h double-probe (simultaneous oesophageal and pharyngeal), pH monitoring and impedance testing are the most widely applied. There are several disadvantages to
using double-probe pH monitoring. This technique cannot detect non-acid reflux events, which are now known to be associated with laryngeal symptoms and endoscopic findings.\textsuperscript{15–17, 31} Furthermore, calculations of the sensitivity of dual-probe pH monitoring for the detection of LPR range from 50% to 80%.\textsuperscript{4} MII was introduced to our field more recently as a method of measuring and supporting with the diagnosis of LPR. The MII system measures changes in electrical conductivity of intraluminal content as a bolus more through the oesophagus and into the laryngopharynx. In Alternating Current circuits the resistance to electrical current flow is called impedance. MII permits not only identification of liquid, gaseous, or mixed intra-oesophageal/intra-pharyngeal materials, but also the direction of their travel. Furthermore, MII technology in conjunction with a pH sensor allows discrimination of acid (pH < 4.0) from weakly acidic (pH 4.0–6.5) and non-acidic (pH 7 and above) reflux.

**TREATMENT OF LPR**

Treatment of LPR depends on the type and severity of symptoms and signs and is usually empirical. Patients with LPR are typically prescribed PPIs, such as Nexium, to control the acidity of the refluxate. PPIs inhibit the H\textsuperscript{+}/K\textsuperscript{+} ATPase enzyme that catalyses acid secretion in parietal cells in the stomach and thus are potent gastric acid suppressing agents. However, PPI therapy appears to have limited ability to protect patients from reflux-attributed symptoms and injury. In fact, it has been suggested that 25–50% patients have refractory symptoms on maximal PPI therapy. These patients can be subdivided into three groups: (i) Patients with symptom association with breakthrough acid reflux: This patient population may benefit from an increase in dose of their PPI or an H\textsubscript{2}-receptor antagonist at bedtime. (ii) Patients who have symptom association with non-acidic reflux events: These patients would likely have a negative pH test and would not benefit from PPI therapy. Surgery is one of the few options for these patients. Several studies in the literature report resolution of reflux-attributed voice disorders and laryngeal symptoms and endoscopic findings after fundoplication.\textsuperscript{32} (iii) Patients who have no symptom association: Reflux is unlikely to be the cause of symptoms and injury in this population and thus other causes should be investigated. Combined MII with pH monitoring (MII-pH) is now being used to correlate symptoms with reflux events to help identify potentially PPI-responsive acid reflux patients, who should be distinguished from both non-acid reflux and nonreflux laryngitis patients unlikely to respond to acid suppression therapy.

Using MII-pH monitoring, Tamhankar \textit{et al.}\textsuperscript{17} showed that PPI therapy decreases the H\textsuperscript{+} ion concentration in the refluxed fluid, but does not significantly affect the frequency or duration of reflux events. Kawamura \textit{et al.}\textsuperscript{31} reported on a comparison of GER patterns in 10 healthy volunteers and 10 patients suspected of having reflux-attributed laryngitis. Using a bifurcated MII-pH reflux catheter, the investigators found that gastric reflux with weak acidity (above pH 4.0), is more common in patients with reflux-attributed laryngitis compared with healthy controls. Oelschlager \textit{et al.}\textsuperscript{33} demonstrated that the majority of reflux episodes into the pharynx are in fact non-acidic. More recently, Sharma \textit{et al.}\textsuperscript{15} reported that 70/200 (35%) patients on at least twice daily PPI had a positive symptom index for non-acidic reflux. Tutui\textit{an et al.}\textsuperscript{16} also recently reported that reflux episodes extending proximally are significantly associated with symptoms irrespective of the pH of the refluxate. Here, we present a hypothetical paradigm to explain these observations.

**ROLE OF PEPSIN IN INFLAMMATORY DISEASE OF THE LARYNGOPHARYNX**

Pepsin is a proteolytic enzyme produced only in the stomach, initially secreted in zymogen form as pepsinogen by gastric chief cells. Hydrochloric acid in the stomach causes the pepsinogen to unfold and cleave itself in an autocatalytic fashion, generating pepsin – the active form. Pepsin is maximally active at pH 2.0, but can cause tissue damage above this pH, with complete inactivation not occurring until pH 6.5.\textsuperscript{11, 34, 35} While pepsin is inactive at pH 6.5, it remains stable until pH 8.0 and thus can be reactivated when the pH is reduced. Pepsin is not irreversibly inactivated until pH 8.0.\textsuperscript{34, 35} Thus, even if the pepsin which we have detected in, for example, laryngeal epithelia is inactive\textsuperscript{21, 22} (mean pH of the laryngopharynx is 6.8) it would be stable and thus could sit inactive/dormant in the laryngopharynx and have the potential to become reactivated by a decrease in pH. Using a specific and sensitive antibody against human pepsin, we have demonstrated the presence of pepsin in laryngeal epithelial biopsy specimens taken from patients with reflux-attributed laryngeal disease; not detected in normal control subjects.\textsuperscript{11, 21, 22} In these studies, we also report a significant association between the presence of pepsin and depletion of laryngeal protective proteins; carbonic anhydrase isoenzyme III (CAIII) and squamous epithelial stress protein Sep70. Using an established
porcine in vitro model, we have demonstrated that exposure of laryngeal mucosa to pepsin, though not to low pH alone, causes depletion of CAIII and Sep70 protein levels. These findings suggest that the pepsin present in the laryngeal epithelia of patients with reflux-attributed laryngeal disease is likely to be the causal factor for the observed depletion of CAIII and Sep70 proteins in these same patients.

We have recently documented co-localisation of pepsin with clathrin in laryngeal epithelial cells, a widely accepted marker of the receptor-mediated pathway. This supports our previous findings of co-localisation of pepsin with transferrin, another marker of the receptor-mediated pathway. Together, these immuno-electron microscopy data strongly suggest that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis. However, molecules taken up by fluid phase endocytosis can also rarely be detected in clathrin coated pits. Thus, it was necessary to confirm real receptor-type behaviour. We performed competitive binding experiments with unlabelled ligand (pepsin) in the cold to ascertain whether binding is saturable and can be competed for, characteristics of receptor-mediated uptake. Using pepsin labelled with tetramethyl rhodamine isothiocyanate (TRITC) we documented uptake of pepsin by laryngeal epithelial cells and its presence inside the cell after incubation at 37 °C for 5–10 min. In competitive binding experiments, where cells were exposed to an excess of free/unlabelled pepsin at 4 °C prior to incubation with pepsin-TRITC, pepsin-TRITC was not detected inside the cells even after 30 min at 37 °C. If pepsin-TRITC was being taken up by general fluid-phase endocytosis, prior incubation with an excess of unlabelled pepsin at 4 °C would not have significantly affected uptake. One would have expected to see uptake of pepsin-TRITC at the same rate as before – in intracellular vesicles after 5–10 min at 37 °C. However, in the case of specific receptor-mediated uptake, the high concentration of unlabelled ligand (pepsin) saturated the receptors at 4 °C and was taken up when warmed to 37 °C. Only once receptors recycle to the cell surface, will you see labelled pepsin (pepsin-TRITC) inside the cells. These competitive binding experiments confirm that uptake of pepsin is saturable and thus unequivocally receptor-mediated. This is further supported by our finding that pepsin remains on the cell surface in the presence of wortmannin, an inhibitor of receptor-mediated endocytosis, but is detected inside intracellular vesicles in the presence of DMA, an inhibitor of fluid phase but not receptor-mediated endocytosis.

Pepsin is thought to cause damage by its proteolytic activity alone, digesting the structures that maintain cohesion between cells. Our discovery that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis is a novel scientific finding which could also have important clinical implications. If pepsin taken up by the cell was merely targeted to lysosomes for degradation, a role for pepsin in reflux-attributed injury would seem unlikely. However, we have shown that pepsin can be detected in late endosomes 6 h following a 20 min exposure, revealing that it is not merely targeted to lysosomes for degradation. Our preliminary investigations also suggest that intracellular pepsin is intact. When cultured FaDu cells are incubated with pepsin-TRITC (10 ng/mL) at 4 °C for 1 h and then warmed to 37 °C, a single band is detected at 35 kDa (corresponding to the correct molecular weight of pepsin) by sodium dodecyl sulphate – polymerase gel electrophoresis (SDS–PAGE). A polypeptide band was not detected when cells were incubated at 4 °C. At 4 °C, endocytosis is stopped and thus any pepsin present remains on the cell surface. When the cells are warmed to 37 °C, pepsin is taken up by the cell by receptor-mediated endocytosis and can be detected in intracellular vesicles. Detection of a single band at 35 kDa by SDS–PAGE when pepsin is inside the cell, suggests that intracellular pepsin is intact. We intend to isolate intracellular organelles via differential centrifugation and analyse the intracellular pepsin by SDS–PAGE to confirm that it is intact.

Interestingly, the proteolytic activity of pepsin is not essential for receptor-mediated uptake, as inactive pepsin is taken up by receptor-mediated endocytosis. Receptors and their ligands are typically sorted in late endosomes or the TRG. Using antibodies against Rab-9 (a marker of late endosomes) and TRG-46 (a marker of the TRG) we have confirmed the presence of pepsin in these intracellular compartments (Johnston et al., in press). As our SDS–PAGE data suggest that intracellular pepsin is intact, it is possible that it could become reactivated in either of these intracellular compartments, which are approximately pH 5. Pepsin, even when inactive, remains stable below pH 8. Thus, pepsin below pH 8 taken up by the cell is stable and thus has the potential to become reactivated by a subsequent decrease in pH as in late endosomes or the TRG. It should be noted, while pepsin is maximally active at pH 2.0, it has 40% of its maximum activity at pH 5. We intend to both reversibly and irreversibly inhibited pepsin in an indirect approach to investigate whether inactive pepsin (pepsin at pH 7) taken up by receptor-mediated endocytosis...
causes damage by becoming reactivated inside the cell. If pepsin does become reactivated intracellularly in vivo, a reversible, but not an irreversible, inhibitor of peptic activity would be expected to prevent pepsin from becoming reactivated inside the cell and subsequently causing damage. Alternatively, it may be that activation of the cell surface receptor by pepsin results in a cell signalling cascade ultimately having a negative effect on normal cell function. The process of signal transduction, whereby binding of a ligand to its receptor initiates a signalling cascade, is often dysregulated in disease. It is unlikely that there is a specific cell surface receptor for pepsin, but perhaps it is more plausible that pepsin somehow exploits another receptor on laryngeal epithelial cells. One would presume that a receptor antagonist would be required to prevent peptic injury by this mechanism. Our long-term goal is to elucidate this novel mechanism for peptic injury and to test pepsin inhibitors and receptor antagonists using in vitro and in vivo models.

To test our hypothesis that inactive pepsin can be taken up by laryngeal epithelial cells and cause intracellular damage, perhaps by becoming reactivated inside the cell in late endosomes or the TRG (compartments of lower pH) or by initiating a cell signalling event following interaction with a cell surface receptor, we exposed cultured epithelial cells to pepsin (0.1 mg/mL human pepsin 3b) at pH 7, for either 1 or 12 h at 37 °C, washed three times briefly and examined by transmission electron microscopy. 20 The cells remained viable following a 1- and 12-h incubation with pepsin at neutral pH. Cell and nuclear membranes were intact. However, both mitochondria and Golgi were clearly damaged. Mitochondria were swollen and the cristae degraded in cells exposed to pepsin (0.1 mg/mL) at pH 7 for 1 h at 37 °C. Further mitochondrial damage was evident in cells exposed to pepsin for 12 h. Golgi were also swollen in cells exposed to pepsin for 12 h. Control cells, which were incubated for the same time period, in the absence of pepsin, showed no signs of mitochondrial or Golgi damage. The mitochondrial damage we observed in human FaDu epithelial cells exposed to pepsin (0.1 mg/mL) at pH 7 is probably an early indicator of necrosis and supports our hypothesis that pepsin can cause injury to laryngeal epithelial cells in non- and weakly acidic refluxate. There is no doubt that pepsin will be more injurious to the laryngeal epithelium in acidic refluxate. However, our data reveals that it could also cause damage in non-acidic refluxate. In support of a pepsin effect on mitochondria, initially observed by transmission electron microscopy, we also report cell toxicity measured by a MTT cell toxicity colorimetric assay kit (Sigma-Aldrich Corp., St Louis, MO, USA). The key component of this kit is 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide or MTT, which can be used to measure mitochondrial activity in living cells. A decrease in absorbance, compared with control, is indicative of damage. We exposed cultured FaDu cells to pH 7 or 5.5 ± pepsin (0.1 mg/mL) for 1 h at 37 °C. Data from three biological replicates, read in triplicate, were analysed using one-way analysis of variance. Importantly, a significant increase in toxicity was detected following exposure to pepsin at pH 7 compared with pH 7 control (P < 0.01). This finding supports our electron microscopy data showing mitochondrial damage by pepsin at neutral pH.

Mitochondria are known to play a central role in cell metabolism, and damage – and subsequent dysfunction – in mitochondria is an important factor in a wide range of human diseases. 38 While seemingly unrelated, there is a common thread between the different diseases associated with mitochondrial damage: cellular damage causing oxidative stress and the accumulation of reactive oxygen species. These oxidants then damage mitochondrial DNA, resulting in mitochondrial dysfunction and death. 39 There is evidence that CAIII protects against oxidative damage 40, 41 that has been shown to occur experimentally from reflux. 42, 43 We have shown that CAIII expression levels are depleted in patients with LPR and that laryngeal CAIII levels are depleted following exposure to pepsin in vitro.11, 22 The possible link between reflux-attributed laryngeal injury/disease, depleted levels of protective CAIII by pepsin, and the mitochondrial damage observed following exposure to pepsin, warrants further investigation. Perhaps depletion of laryngeal CAIII by LPR of pepsin results in the accumulation of reactive oxygen species and subsequent mitochondrial damage.

In addition to depletion of CAIII, we have also reported that patients with LPR have depleted levels of laryngeal Sep70, compared with normal control subjects. Furthermore, both CAIII and Sep70 proteins are depleted following exposure to pepsin, but not low pH alone, in vitro.11, 21, 22 More recently, we found that patients with LPR have depleted levels of MUC 2, 3 and 5ac mRNA, and that pepsin prevents production of these mucins in vitro. 44 However, given that pepsin is a proteolytic enzyme, it is likely that pepsin would have a more global effect, rather than cause damage by depleting the expression of a select few genes/proteins. To this end, a
Human Stress and Toxicity PathwayFinder PCR Array (SABiosciences, Frederick, Maryland, USA) was used to examine the effect of pepsin, at neutral pH, on the expression of 84 genes whose expression levels is indicative of stress and toxicity. Cultured FaDu cells were incubated with complete growth media ± pepsin (0.1 mg/mL) for either 1 or 12 h at 37 °C, washed and processed for real-time RT–PCR. Data from three biological replicates were analysed using the RT² Profiler PCR Array Data Analysis software – student’s t-test. Our data indicates that pepsin significantly alters the expression levels of multiple genes implicated in stress and toxicity.20 The expression levels of seven genes, implicated in stress and toxicity, were significantly upregulated following 1 h incubation with pepsin (0.1 mg/mL, pH 7). A time response was observed: the expression levels of 25/84 of these genes were significantly altered following a 12 h incubation with pepsin. A long exposure time was used in these initial experiments to see if an effect could be observed. We anticipated that we would only see an effect by exposure to pepsin at neutral pH after a long time period, compared with pepsin at acidic pH where one would expect to see an effect relatively quickly. The morphological changes we observed would have been missed by simply examining gross morphology (for example, H&E stained sections examined by light microscopy) and require detailed examination of the intracellular structures (using transmission electron microscopy). While damage clearly occurs, the cells do remain viable and thus potentially able to recover from a single insult. It is likely that permanent injury and symptoms would result from multiple uncontrolled reflux events, as is thought to occur in LPR patients. Time course, repeated exposure and pulse chase experiments will now be performed. However, compared with controls, pepsin is clearly injurious to laryngeal epithelial cells at neutral pH. A SuperArray for inflammatory cytokines and receptors was also used to investigate whether pepsin, at pH 7, elicits an inflammatory response.45 This is important as the consequence of reflux damage and the cause of symptoms is de facto chronic inflammation. The expression of a number of inflammatory cytokines and receptors was altered in human hypopharyngeal epithelial cells following overnight treatment with pepsin at neutral pH >1.5-fold change in gene expression was detected for CCL20, CCL26, IL8, IL1F10, IL1A, IL5, BCL6, CCR6 and CXCL14 (P < 0.05). These pro-inflammatory cytokines and receptors are known to be involved in inflammation of the oesophageal epithelium in response to reflux and contribute to the pathophysiology of reflux oesophagitis.45 These data indicate that refluxed pepsin may contribute to laryngeal inflammation associated with non-acidic gastric reflux including that experienced by patients despite maximal acid suppression therapy.

ROLE OF PEPSIN IN CANCER OF THE LARYNGOPHARYNX

Laryngeal carcinoma accounts for about 1% of all newly diagnosed cancers in the US. Approximately, 11 000 new cases are diagnosed every year and about 4300 deaths per year are attributed to laryngeal carcinoma. Despite a decrease in the number of people who smoke in the US, the incidence of laryngeal cancer actually appears to be rising. Unfortunately, the prognosis remains poor and the mortality rate high, with a 5-year survival rate of 40%.46–50 Tobacco and alcohol are well-known established risk factors. Other risk factors include human papilloma virus, radiation exposure, occupational exposure and LPR.4 The latter remains controversial and requires further investigation, especially as it has become one of the most common chronic diseases of adults in the US. For many reasons, it is very difficult to prove that reflux is a causal agent in the development of laryngeal cancer. Many clinical studies have shown a high prevalence of LPR in patients with laryngeal cancer4, 50; however, these studies are confounded by the fact that the majority of patients with laryngeal cancer have a significant smoking and alcohol history, and many lack appropriate controls. Another difficulty is the lack of uniformity in establishing the diagnosis of GERD and LPR in the literature.

While it seems logical that chronic laryngeal inflammation could lead to a neoplastic lesion, it remains unclear whether reflux laryngitis is a precursor to laryngeal cancer. It is hoped that research in cell biology of reflux may eventually lead to an answer to this age-old question, since population and other clinical studies have too many confounding variables. Gabriel and Jones31 were among the first to present evidence suggesting this possibility. Many others have also suggested an association.4, 7, 52–55 To further explore the association between LPR and laryngeal cancer, several investigators have examined the direct effect of the individual components of gastric refluxate – mainly acid, pepsin and bile acids – on laryngeal cancer, several investigators have examined the direct effect of the individual components of gastric refluxate – mainly acid, pepsin and bile acids – on laryngeal cell and molecular biology and pathology.4, 56, 57 These studies demonstrated a significant role for pepsin and bile acids in carcinogenesis, in a dose-dependent manner with greater toxicity at lower pH. Interestingly, several clinical studies evaluating patients with prior gastrectomy suggest that the components of non-acidic reflux promote the development of laryngeal...
We report that exposure of hypopharyngeal epithelial cells to pepsin (0.1 mg/mL, pH 7) causes a significant change in the expression of 27 genes implicated in carcinogenesis (Johnston N, unpublished data). Analysis of these genes strongly suggests that pepsin exposure causes an increase in cell proliferation and thus may contribute to oncogenic transformation by aberrant cell growth. This was investigated further using propidium iodide staining and flow cytometry. Pepsin was indeed found to significantly increase the percentage of cells in S phase in a dose-dependent manner. Growth curve data are consistent with pepsin causing an increase in cell proliferation and thus support our flow cytometry data.

CONCLUSION AND FUTURE DIRECTIONS

Our data strongly suggest that pepsin may be responsible for laryngeal symptoms and injury associated with non-and weakly acidic reflux and help explain why many patients have refractory symptoms on maximal acid suppression therapy. Moreover, our preliminary data demonstrate that pepsin may even initiate neoplastic changes which could result in the development of laryngopharyngeal cancer. We are currently exposing human hypopharyngeal and laryngeal epithelial cells to human pepsin at pH 7 in time-course and dose-response experiments. The effect of pepsin on cell viability and cytotoxicity will be measured using the Vybrant Cell Metabolic Assay. An accurate measurement of cell proliferation will be obtained using the more superior Click-iT Edu Proliferation Assay. The Cell Clonogenic Survival Assay will be used to test the capability of adherent cells to survive and replicate following exposure to pepsin and an Anoikis Assay will be used to measure anchorage-independent growth and monitor anoikis propelled cell death. Finally, using microarray technology, the expression of 113 gene indicators of the 15 different signal transduction pathways involved in oncogenesis will be examined to explore the possible molecular mechanisms by which pepsin dysregulates hypopharyngeal and laryngeal epithelial cells. We are also testing pepsin inhibitors in our in vitro models to see if they prevent peptic injury. Once we have identified the receptor with which pepsin interacts, we will also design and synthesise receptor antagonists to test in our in vitro models. If our in vitro studies demonstrate that pepsin inhibitors and/or receptor antagonists prevent pepsin uptake/injury, an in vivo model will be used to investigate the clinical usefulness of such pharmacological agents.

REFERENCES


Review article: effects of pepsin and alginate in an animal model of squamous cell carcinoma

J. Allen*, S. P. Tinling†, N. Johnston‡ & P. Belafsky*

INTRODUCTION
Head and neck squamous cell carcinoma (HNSCC) represents 5–8% of malignancies worldwide, with the larynx the most common site, contributing >11 000 new cases annually in the US.1–4 The primary risk factors associated with development of these malignancies are tobacco and alcohol abuse.1–9 More than 95% of laryngeal cancers are associated with tobacco use.2–5 Other proposed aetiological agents in pharyngolaryngeal cancers include genetic predisposition, human papillomavirus, inhaled irritants particularly occupational exposure and dietary factors.1, 2, 5 Gastro-oesophageal reflux (GER), laryngopharyngeal reflux (LPR) and more recently duodenogastro-oesophageal reflux have also been suggested as causal agents in pharyngolaryngeal carcinogenesis.1–3, 5–10 Despite a documented higher prevalence of GER and LPR in patients with pharyngolaryngeal carcinoma,1, 3, 5, 6, 8 it has been difficult to ascertain (i) whether reflux is ‘associated’ with tumour formation rather than ‘causal’, and (ii) what agent in the reflux enhances tumorigenesis, because population based studies are confounded by the high prevalence of smokers and drinkers in this patient population. It is well known that smoking and alcohol promote reflux by modifying lower oesophageal sphincter function, and the effects of both agents combined are synergistic, both on reflux and

SUMMARY
Gastro-oesophageal and laryngopharyngeal reflux disease have been linked to laryngopharyngeal carcinoma; however, human studies are often confounded by the effects of smoking and alcohol. The hamster buccal pouch animal model of mucosal carcinogenesis allows testing of isolated interventions to assess the influence of each on cancer progression. The purpose of this study was to determine the effect of human pepsin on tumour growth in an animal model, and to determine whether topical alginate would modify tumour growth. Sixty male Syrian hamsters were divided into two treatment groups. Forty hamsters received application of known carcinogen 7,12-dimethylbenzanthracene (DMBA) to both cheek pouches. In addition purified activated human pepsin was applied to the left cheek pouch. Twenty hamsters received application of human pepsin and DMBA to both cheek pouches, with the left cheek pouch receiving topical alginate solution prior to pepsin application. Tumour volume was compared between sides, with the paired t-test. The results showed a statistically significant increase observed in tumour volume in the hamsters receiving combined DMBA and human pepsin, compared with those receiving DMBA alone (P < 0.0001). A statistically significant reduction in tumour volume was observed in hamsters that received alginate prior to DMBA and human pepsin application, compared with hamsters painted with DMBA and human pepsin alone (P < 0.0001).
on tumour growth.7 Trying to tease out how risk factors aside from smoking and alcohol contribute to HNSCC development is difficult, as the population of patients who are lifelong nonsmokers and nondrinkers, who have developed squamous cell carcinoma of the head and neck, is small.

Animal models offer the opportunity to study the effects of potential carcinogens or interventions, in isolation from confounding factors. The hamster buccal cheek pouch (HBP) model is an established model of human mucosal squamous cell carcinoma.11 Developed in 1954 by John Salley, it has been accurately characterised and extensively utilised as a reliable model of sequential carcinogenesis.11, 12 A progression from hyperplasia, to dysplasia and then to invasive carcinoma can be achieved, which enables controlled assessment of the effects of risk factors and various interventions.11, 13 Adams et al.14 used the HBP model to assess the effect of acid and porcine pepsin on tumour growth in the pouch. Results suggested that this ‘gastric mix’ enhanced tumour growth.14 The purpose of this investigation was to evaluate the effect of acidified human pepsin on tumour proliferation and determine whether inhibition of pepsin activity by an alginate ‘bandage’ would counteract this effect.

MATERIALS AND METHODS
This study was conducted using sixty 4-week-old male golden Syrian hamsters (Mesocricetus auratus) obtained from Charles River Laboratories (Wilmington, MA). The animals were reared on a 12 h light/dark cycle, with the temperature maintained between 20 and 24 °C and given Rodent Laboratory Chow No. 5001 (Ralston Purina Co., St Louis, MO, USA) and distilled water ad libitum. The study was performed in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Welfare Act (7 U.S.C et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee of University of California, Davis.

Isolation of Pepsin 3b
Human pepsin was derived from gastric aspirates of crude gastric juice collected from patients undergoing transnasal oesophagoscopy for clinical indications. Approximately 5–10 mL of gastric juice was aspirated from patients at the conclusion of their scheduled procedure. Crude gastric juice samples were pooled and pepsin 3b isolated by anion exchange chromatography. Informed consent was obtained from all patients, and collection was approved by Institutional Review Board.

Crude gastric juice was initially filtered through 113 V, 320 mm diameter Whatman filter paper (Fisher Scientific, Pittsburgh, PA, USA) to remove mucoid and particulate matter. The filtrate was then dialysed against 50 mM sodium acetate buffer, pH4.1 at 4 °C. Partial extraction of pepsins was performed using a diethyl amino ethyl (DEAE) cellulose column (DE52; Whatman Inc., Clifton, NJ, USA) equilibrated with cold dialysis buffer. Negatively charged proteins, including pepsin 3b, were eluted from the DE52 column with salt (sodium acetate/1 M NaCl). Fractions containing protein (determined by measuring OD @ 280 nm) were pooled an dialysed against 50 mM sodium acetate at 4 °C to remove all salt prior to performing the second anion exchange with a Pharmacia Mono Q 5/50 column (GE Healthcare Ltd, Piscataway, NJ, USA) developed with a gradient of 0.15–0.3 mol/L NaCl in 50 mmol/L sodium acetate, pH 4.1.15 Isolated pepsin 3b was stored at −20 °C in 50% glycerol.

Alginate solution. Liquid alginate preparation (Gaviscon Advance; Reckitt Benckiser, Hull, UK) containing 100 mg sodium alginate/20 mg potassium bicarbonate per millilitre was used in all animals.

Exposure protocol
The sixty male hamsters were housed in groups of four to five hamsters per cage and were checked three times...
weekly, and weighed monthly. The Institutional Veterinarian reviewed the hamsters to ensure tumour growth did not cause undue stress or discomfort. The hamsters were divided into two treatment groups.

**Group 1.** Forty hamsters right and left cheek pouches were dosed three times per week for 14 weeks with 50 μL of 0.5% solution of 7,12-dimethylbenz[a]anthracene (DMBA), a known carcinogen, dissolved in heavy mineral oil. DMBA was applied with a micropipette to ensure a uniform volume per application. The animal was restrained with a neck pinch and the pipette tip was placed in approximately the same location within the cheek pouch for each application. The location was one centimetre from the lateral edge of the pouch and opposite the last molar. In addition, in a similar manner, the left cheek pouch received 20 μL of human pepsin (0.1 mg/mL) activated by hydrochloric acid at pH 2.0.

**Group 2.** Using the same method, 20 hamster’s right cheek pouches were coated three times per week with liquid alginate (400 μL) for 4 weeks. Ten minutes after alginate application, both right and left cheek pouches were administered 10 μL human pepsin (0.1 mg/mL) activated by hydrochloric acid at pH 2.0. In the same hamsters, during weeks 5–14, 200 μL of liquid alginate was applied thrice weekly to the right cheek pouch. After waiting 10 min, DMBA (50 μL of 0.5% solution dissolved in mineral oil) was applied to both right and left cheek pouches.

At week 15, all hamsters were euthanised by CO₂ asphyxiation. Cheek pouches were everted and examined with an Olympus operating microscope (Olympus America Inc., Centre Valley, PA, USA). All lesions were measured and counted. Lesions were measured to the nearest mm with a flexible ruler by an examiner blind to exposure status (Figure 1). A composite score was formed for each cheek pouch by totalling the combined numbers and diameters of all lesions noted. Data were coded, recorded, and analysed in SPSS 17.0 for the Macintosh (Chicago, IL, USA).

Specimens were obtained from cheek pouches for histological examination, and for real-time polymerase chain reaction (RT-PCR) analysis of gene microarrays. Specimens for histology were placed in tagged permeable bags and preserved in a solution of 2% glutaraldehyde and 2% paraformaldehyde mixed with phosphate buffered saline. Specimens for real-time PCR analysis were placed in 1.5 mL Sarstedt microcentrifuge tubes (Newton, NC, USA), snap frozen in liquid nitrogen, then stored at −80 °C prior to analysis.

**Histological processing**

After fixation the specimens were rinsed in two changes of saline and then dehydrated in a graded series of acetone, and embedded in epoxy-araldite resin. Each block was bisected along a plane which bisected the tumour and adjacent normal tissue using a 0.04 mm thick diamond wafering blade. Slices were then mounted and 1 micron sections were cut, mounted on slides and stained with toluidine blue and basic fuchsin. Samples for histological microscopy were chosen to correspond with the average sample size for each experimental group.

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**Figure 4** | Histological micrograph (4× magnification) of squamous cell carcinoma from hamster buccal cheek pouch treated with 7,12-dimethylbenzanthracene and pepsin, demonstrating keratin pearls and fungiform growth pattern.

**Figure 5** | Histological photomicrograph (20× magnification) of squamous cell carcinoma from hamster buccal pouch treated with 7,12-dimethylbenzanthracene and pepsin, showing keratin pearls and disordered architecture.
Calibrated digital images were captured using a PixelFly digital camera (The Cooke Corp., Auburn Hills, MD, USA) attached to an Olympus BX60 microscope (Olympus America In., Center Valley, PA, USA).

Cancer PathwayFinder SuperArray
RNA was isolated as directed by the SuperArray RT\(^2\)-qPCR-Isolation Kit (SuperArray, Frederick, MD, USA) and the concentration and quality were assessed by UV spectroscopy and agarose gel electrophoresis. RNA was reverse transcribed using the SuperArray RT\(^2\) First Strand Kit. FaDu cell cDNA was diluted in RT2 SYBR Green/Fluorescein qPCR Master Mix and aliquoted into a 96-well RT\(^2\) Profiler PCR Array (Human Cancer PathwayFinder PCR Array; SuperArray). Real-time RT-PCR was performed in an iCycleriQ Multicolor Real-Time PCR Detection System (Bio-Rad Life Science, Hercules, CA, USA). RT-PCR conditions were as follows: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, immediately followed by a melt curve of 95 °C for 1 min, 65 °C for 2 min and sixty 10 s cycles of 0.5 °C increase. Three biological replicates for each condition were performed.

Data analysis
Tumour composite scores were compared between cheek pouches using the paired-samples \(t\)-test. For RT-PCR analysis, three biological replicates were performed. RT-PCR samples exhibiting more than one peak within the melt curve were excluded from analysis. The means of the housekeeping genes for each replicate was subtracted from the gene value to normalize the data. Data analysis was performed using the SuperArray online interface (http://www.SuperArray.com/pcrarraydataanalysis.php).

RESULTS

Group 1: Effect of pepsin
The right cheek pouch receiving DMBA alone, produced an average composite score of 9.7 (±7.2) mm. The left cheek pouch receiving DMBA plus pepsin, produced an average composite score of 15.1 (±7.9) mm (\(P < 0.0001\), Figure 3). The average number of lesions in the right cheek pouch was 6.6 (±4.7), and in the left cheek pouch was 8.75 (±4.3), \(P < 0.002\). Histological examination of lesions demonstrated invasive squamous cell carcinoma in both right and left cheek pouches (Figures 4 and 5).

Group 2: Effect of alginate vs. pepsin
The right cheek pouch receiving alginate, pepsin and DMBA, produced an average composite score of 0.225 (±0.6) mm. The left cheek pouch receiving only pepsin and DMBA, produced an average composite score of 2.4 (±2.5) mm (\(P < 0.001\)). The average number of lesion in the right cheek pouch was 0.25 (±0.6), and in the left cheek pouch was 1.7 (±1.9), \(P < 0.001\).

Histological findings
Histological examination of specimens from both right and left cheek pouches showed moderately differentiated squamous cell carcinoma (Figures 4 and 5).

Real-time polymerase chain reaction
Real-time polymerase chain reaction analyses demonstrated upregulation and downregulation of several genes involved in cell cycle regulation, formation of metastases and cytokine production in pepsin- vs. nonpepsin-treated samples (Table 3). The small number of samples (\(n = 6\)) precluded multivariate statistical analysis of differences, therefore only side to side comparisons are made.

DISCUSSION
Laryngopharyngeal reflux has been implicated in multiple disorders and diseases of the laryngopharynx including chronic laryngitis, vocal process granuloma, chronic cough, dysphonia, globus and laryngopharyngeal carcinoma.\(^4, 7, 9, 16, 17\) The proposed aetiology of the latter is via a pathway of chronic reflux-mediated irritation and inflammation that leads eventually to dysplastic change, culminating in formation of carcinoma.\(^17, 18\) Association of chronic inflammation with dysplasia and development of malignancy is seen in other organs, for example, oesophageal reflux leading to Barrett’s metaplasia and adenocarcinoma.\(^2, 9, 18–20\) As suggested by Koufman and Burke,\(^19\) the aetiology of laryngopharyngeal cancer is likely to be multifactorial, a result of a complex interplay of genetics, environment and spontaneous events. So far the literature is divided on the role that GER and LPR plays in development of carcinoma.\(^1–10, 16, 18–20\) In a well-summarised review, Qadeer \textit{et al.} propose three possible scenarios. First, that GER and LPR are associated with laryngeal cancer, because of smoking and alcohol related refluxogenic effects, but not causal alone. Secondly, that GER or LPR are independent risk factors for development of malignancy, or finally that GER or LPR may be a co-factor in development of cancer, enhancing the effects of other carcinogens such as smoking or alcohol.\(^1, 16, 21\) Human studies in support of these scenarios are limited by either the confounding effects of smoking and alcohol in the majority of patients, or small numbers of participants, and therefore diminished statistical
power, inherent in studying nonsmoker nondrinker patients with laryngopharyngeal carcinoma.3, 5–8, 16, 19, 20

Animal models
The hamster buccal cheek pouch model is an established model of human mucosal carcinoma. Tumours are histologically and morphologically similar to human SCC, and can be induced reliably with topical application of a known carcinogen (DMBA).11, 13 The pouch can be easily accessed and examined without anaesthetising the animals. Limitations of the HBP model are that the human oral cavity does not have such a pouch, and that HBP tumours may contain genetic mutations dissimilar to typical human oral cavity tumours.13 The HBP has been used in a single study aimed at assessing the effect of GER on tumour growth. Adams et al.14 utilised this model in a study where hydrochloric acid and porcine pepsin were applied following DMBA application, to elucidate whether tumorigenesis was enhanced. An increased number of tumours were seen in animals treated with ‘gastric content mix’14. Alternative animal work regarding reflux effects on cancer promotion has been undertaken in Wistar rats exposed to a mix of HCl, pepsin and sodium nitrate, and to duodenogastro-oesophageal reflux.21, 22 Del Negro et al.22 did not find dysplastic or carcinomatous change in rats treated over a 6-month period. Ling et al.21 likewise could not demonstrate carcinoma formation in rats with duodenal content-induced laryngitis, but did note an increased Ki67 proliferation index. Both studies had relatively short exposure periods (up to 30 weeks), and neither used a promoter, which may explain the lack of lesions seen within the study period. In humans, it may take years of exposure and inflammation to induce malignant degeneration. In this study using an animal model, application of acidified human pepsin in addition to a known carcinogen increased the tumour volume evident in the cheek pouch.

Pepsin-mediated injury
Refluxate is a complex mix of substances including hydrochloric acid, pepsin, bicarbonate, mucus, prostaglandins, food, intrinsic factor and refluxed duodenal content such as trypsin and bile acids.17, 23, 24 Acid secreted by the gastric epithelium has long been considered the most injurious element of the refluxate, and the proton pumps responsible for acid secretion have been therapeutic targets. Although we can now reduce acid secretion to near zero, the same cannot be said of symptoms. This suggests that acid is not the only injurious substance present. Other factors contributing to tissue damage include pepsin, bile acids and trypsin.2, 4, 7, 10, 18, 21–25 Recent work has examined the role of pepsin in oesophageal and laryngeal injury.17, 23, 24, 26–31 Pepsin is the major enzyme in gastric juice and may reach concentrations of 1 mg/mL in the stomach. Pepsin is activated by acid and is most potent in a low pH environment, but can retain proteolytic effect up to pH 6.5, and is not irreversibly inactivated until >pH 8.23, 26 Some authors now support pepsin as the main aetiological factor in oesophageal and laryngeal reflux damage.6, 17, 23, 24, 26, 28 Pepsin can adhere to the laryngeal mucosa, or be absorbed into pharyngeal secretions. It may be inactive at that time, as the typical pH of the pharyngolarynx is 6.8; however, later exposure to low pH, as happens with a reflux episode, can reivate sequestered pepsin, promoting inflammation and cell damage.17, 26–28 The laryngeal mucosa actively endocytoses pepsin, and the pepsin may remain viable within the cell cytosol, or be transported to the Golgi apparatus and late endosomes. These organelles maintain low pH and reactivation of pepsin may occur within these structures and result in significant cell damage.17, 27 Pepsin may induce gene activation for inflammatory cytokines in human hypopharyngeal cells, and alter the production of protective mucus in these cells.17, 28 Notable depletion of protective proteins such as carbonic anhydrase isoenzyme III (CAIII) and squamous epithelial stress protein Sep70 can be found in

Table 3 | Cancer SuperArray gene expression profiles

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold change in expression</th>
<th>P-value (left vs. right)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APAF-1</td>
<td>−2.01</td>
<td>0.016</td>
</tr>
<tr>
<td>Bcl-XL</td>
<td>+1.51</td>
<td>0.048</td>
</tr>
<tr>
<td>IL-8</td>
<td>+1.69</td>
<td>0.057</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>+2.57</td>
<td>0.17</td>
</tr>
<tr>
<td>Telomerase reverse transcriptase</td>
<td>+1.99</td>
<td>0.076</td>
</tr>
<tr>
<td>Synuclein – gamma</td>
<td>+1.62</td>
<td>0.016</td>
</tr>
<tr>
<td>Col 18A1</td>
<td>+1.51</td>
<td>0.033</td>
</tr>
<tr>
<td>Metastasis associate 2</td>
<td>+1.73</td>
<td>0.025</td>
</tr>
<tr>
<td>Pinin, desmosome associated protein</td>
<td>+1.62</td>
<td>0.042</td>
</tr>
<tr>
<td>Inteigin /5</td>
<td>+1.31</td>
<td>0.046</td>
</tr>
</tbody>
</table>

APAF-1, apoptotic peptidase activating factor; Bcl-XL, B-cell lymphoma Extra Large; IL-8, interleukin 8; TIMP-1, tissue inhibitors of metalloproteinases 1; Col 18A1, collagen 18A1.
pepsin exposed laryngeal tissue, such as seen in patients with LPR.26, 30-33 These findings strongly implicate pepsin as a key mediator in reflux-related tissue damage, and suggest a pathway through which pepsin/reflux injury may inhibit the cell’s ability to cope with mutagenic insults.

**Gene expression changes**

The analysis of differential gene expression in pepsin-treated vs. nontreated samples in this study demonstrates more than 1.5-fold changes in gene expression in several cell cycle genes. Apoptosis regulators APAF-1 (apoptosis peptidase activating factor 1) and Bcl-XL (B-cell lymphoma Extra Large) have been previously demonstrated to be down- and upregulated respectively, by pepsin action.34, 35 In samples from our study, APAF-1 expression was decreased more than twofold, and Bcl-XL expression increased by more than 1.5-fold in cheek pouches treated with pepsin compared with the nontreated side. This alteration in APAF-1/Bcl-XL expression reduces caspase-9 activation, a crucial cell signal that commits a cell to apoptosis. In a study by Huang et al.,35 more than 40% of laryngeal squamous cell carcinomas (11/27) exhibited decreased APAF-1 expression compared with paraneoplastic laryngeal tissues. Telomerase expression is implicated in cell immortalisation that is associated with transformation to a malignant phenotype. Cheek pouch tissue exposed to pepsin demonstrated almost a twofold increase in expression of telomerase reverse transcriptase supporting the development of immortality in the carcinoma cells. Tumour development is dependent on vascular support. Interleukin 8 (IL-8) is a chemotactic factor released by inflammatory cells that has a potent angiogenic effect. Johnston et al.17 demonstrated marked upregulation of IL-8 in human hyperplagyngeal cells treated with non-acid pepsin. Our hamster tissue exposed to pepsin showed a differential expression of IL-8 of 1.69-fold compared with nonpepsin exposed tissue. Tissue inhibitors of metalloproteinases (TIMPs) are a family of proteins that regulate proliferation and apoptosis of cells, and regulate angiogenic and inflammatory responses. Increased expression of TIMP family members correlates with malignancy in human cancers, hyperproliferation of keratinocytes, malignant conversion of dysplastic cells and grade of laryngeal cancer.36, 37 Our PCR analysis of pepsin-exposed tissue demonstrated 2.57-fold increase in TIMP-1 expression. Together this epigenetic shift in expression of RNA suggests pepsin-mediated effects that may potentially enhance malignant transformation in the setting of inflammation. This requires further work with larger sample sizes in human laryngeal cancer specimens.

**Alginate bandage protection**

Proton pump inhibitors are efficacious acid suppressors. They do not exert any effect on pepsin secretion, nor do they reduce reflux volume or duration.26 Reflux may still occur while taking PPI, but it will be non-acid. An alternative approach to control of reflux symptoms is to use physical barriers. The use of alginates derived from seaweeds, has been investigated in this role. Alginates are polysaccharide copolymers that form a mesh-like gel structure which can act as a biological sieve.23 The properties of the gel can be altered by changing the relative proportions of guluronic acid and mannuronic acid in the mix. This adjusts the cross-linking and changes the pore sizes within the gel mesh, critically adjusting the permeability of the substance.23, 25 Tang et al.25 demonstrated that adhered alginate gel significantly reduced both proton (acid) diffusion and pepsin diffusion in a dose-dependent fashion. Strugala et al.23 showed reduced pepsin diffusion in alginate, by up to 82% compared with controls. Furthermore the alginate used in their study significantly retarded diffusion of bile acids as well. They simulated repeated reflux events by using multiple 5 mL aliquot exposures of pepsin and bile acids. Even after 10 exposures, alginate gel absorbed 50% of pepsin in the sample.23 Alginate gel will coat mucosal surfaces and can stay adherent for up to 60 min without being washed off by saliva flow.25 Thus alginate gel may act like gastric mucus, forming a physical barrier to diffusion of acid and pepsin, and preventing contact with the cell surface and subsequent damage.23, 25 In our study, addition of alginate gel suspension to the hamster cheek pouch receiving DMBA and human pepsin, significantly reduced the number and size of carcinoma lesions formed (P < 0.001).

**LIMITATIONS**

While animal models provide an experimental environment which may be controlled to a large degree, inherent limitations exist in extrapolating animal findings to humans. In this study we chose to limit hamster numbers by using each hamster as its own control, and expose the left and right cheek pouches differently. Although the hamster pouch is capacious and largely used for storage of food by the animal, it is possible for the hamster to transfer food particles from one pouch to the other. This may result in cross-contamination of test substances. If this has occurred we would expect our
results to be biased towards finding no difference between the two sides. Our results, therefore, may underestimate the true effect of pepsin and alginate. Because of limitations on animal numbers and comfort, we were unable to paint the HBP of Group 2 animals with DMBA for a full 14 weeks. This resulted in smaller lesions overall produced in both cheek pouches but still allowed comparison of right and left pouches in the same animals. Unfortunately it limits comparison between Group 1 and Group 2 as the length of exposure to carcinogen was different. We utilised a carcinogenic substance, DMBA, in this model to grow squamous carcinoma. The additive effect of pepsin combined with DMBA increased the tumour volume produced. This supports pepsin as a co-factor in carcinogenesis, but does not suggest that pepsin alone is sufficient for tumour initiation. As previously mentioned, it is likely that pharyngolaryngeal cancer has a multifactorial aetiology, and our results support reflux as a potential independent co-factor in this process. In humans, reflux may act synergistically with other initiators or promoters such as smoking, alcohol, genetic mutations, human papilloma virus or dietary factors.

CONCLUSIONS

The study data support a role for pepsin in promoting carcinogenesis in an animal model of squamous cell carcinoma. Alginate suspension provided protection from pepsin-enhanced tumour growth. These findings support previous work that links GER and LPR with pharyngeal and laryngeal cancer.

REFERENCES


INTRODUCTION
Gastro-oesophageal reflux disease (GERD) is a condition that develops when reflux from the stomach into the oesophagus causes troublesome symptoms and/or mucosal damage. GERD is a very common disorder; up to 40% of adults in the US report having symptoms of heartburn, regurgitation, throat clearing or cough regularly.

Many patients who seek medical care because of this condition are labelled as GERD patients and empirically treated with proton pump inhibitors (PPI). However, many patients have persistent symptoms despite acid-suppressive therapy. Recent observations have estimated that continued symptoms may occur in 40–50% of these patients and can be attributed to a variety of causes.\(^1\)

Several studies have shown that non-acid reflux episodes can also cause symptoms of GERD. As PPIs do not reduce the number of reflux episodes but only change the acidity, non-acid reflux episodes can persist as the cause of symptoms.\(^2\)\(^,\)\(^3\) Another possible reason for treatment failure is an erroneous diagnosis of GERD. Several disorders can be misinterpreted as GERD, and treatment with a PPI is unlikely to resolve the symptoms in these patients. Moreover, a potential cause of treat-
ment failure is insufficient inhibition of gastric acid secretion by the PPI therapy. This may occur either because of limited effectiveness of the PPI itself or because patients are not compliant to the therapy.

Advances in technology to evaluate oesophageal function have clarified the role of non-acid reflux in the pathogenesis of persistent oesophageal symptoms. We will review the clinical importance of non-acid reflux, including presentation, diagnostic testing and proposed therapy.

Definitions
Gastro-oesophageal reflux describes the retrograde flow of gastric content into the oesophagus. It occurs even in healthy individuals and may be regarded as ‘physiologic’ as long as it does not induce oesophageal mucosal abnormalities or troublesome symptoms.

For a number of years, clinicians and investigators have considered oesophageal pH monitoring to be the gold standard for identifying gastro-oesophageal reflux (GER) episodes. Acid reflux is detected by pH monitoring as a rapid decline in oesophageal pH from above to below 4.0. Non-acid reflux episodes are defined by detection of reflux of gastric contents with a pH > 4.0 (i.e. above the threshold used by conventional pH monitoring to identify acid reflux).

Strictly speaking, acid reflux is defined as reflux episodes with a nadir pH < 4; weakly acidic as reflux episodes with a nadir pH between 4 and 7, and weakly alkaline as reflux episodes with a nadir pH above 7. We prefer separating GER episodes into acid and non-acid using the conventional cut-off value of 4.0. This is also a level of acidity at which oesophageal mucosal injury does not occur.

For many years, GERD was diagnosed by the presence of oesophageal erosions (erosive oesophagitis). Recent studies have documented that up to 70% of reflux patients have typical reflux symptoms (i.e. heartburn and/or regurgitation) in the absence of endoscopically visible oesophageal mucosal injuries, making non-erosive reflux disease (NERD) the more common form of presentation. When including oesophageal symptom association into the definition, NERD patients can be further subclassified into two distinct groups:

(i) Patients with an abnormal acid exposure time (AET).

(ii) Patients with normal AET and positive symptom association (‘hypersensitive oesophagus’).

We believe that patients with ‘Functional heartburn’ (i.e. endoscopically normal oesophagus, normal distal oesophageal acid exposure and negative symptom association between heartburn and GER episodes) should not be included in the NERD group given that there is no evidence correlating symptoms to reflux. They usually have a poorer response to acid suppressive therapy and are more likely to have accompanying psychopathology.

Detection of non-acid reflux episodes
Conventional pH monitoring measures acid reflux by detecting decreases in distal oesophageal pH. However, changes in oesophageal pH are an imperfect measure of gastro-oesophageal reflux, particularly in the setting of acid suppression therapy. This method does not allow assessment of non-acid reflux, which may occur when the gastric contents are buffered (in the postprandial period), in patients with atrophic gastritis, or during pharmacological acid suppression. Furthermore, symptoms related to reflux will not always reflect the acidity of the refluxate but may be attributed to other factors such as the volume of the refluxate, the distensibility of the oesophagus and its sensitivity to sudden presence of liquid or gas/liquid reflux.

As a result, there have been several efforts to more accurately quantify reflux of gastric contents (and the association with symptoms) using techniques that do not depend solely upon changes in pH. One of these methods involves detection of bilirubin concentrations in oesophageal refluxate, a technique that can help detect reflux of duodenal contents. In addition, a variety of radiographic methods to detect GER have been described.

More recently, combined multichannel intraluminal impedance and pH testing (MII–pH) monitoring has permitted a detailed understanding of the relationship between symptoms and all types of GER.4 The technique is based upon detection of changes in resistance to electrical currents and thus does not rely upon changes in pH. Liquids refluxing from the stomach into the oesophagus are detected by MII–pH monitoring as decreases in the electrical resistance (i.e. impedance) to alternating current progressing over time from distal to proximal (i.e. retrograde bolus movement). The information provided by the pH electrode evaluates the acidity of the refluxate and is then used to simply classify reflux episodes into acid or non-acid types. Impedance-detected reflux episodes associated with a decline in pH from above to below 4.0 are considered acid, whereas impedance-detected reflux episodes during which the pH remains above 4.0 are considered to be non-acid.5, 6
This method has been shown to be sensitive to assess the number and type of reflux episodes as well as the relationship between symptoms and reflux episodes at all pH levels (both acid and non-acid). Although the use of this technique is increasing, it is not regularly available at all GI practices, and referral to a specialised diagnostic centre may be needed.

MII–pH catheter characteristics
Ambulatory MII–pH can be performed with different catheters that incorporate a varying number of impedance-measuring segments and pH electrodes in different configurations. A typical catheter has a pH electrode positioned 5 cm above the manometrically determined lower oesophageal sphincter (LOS; as in conventional pH testing), with the possibility of placing additional pH sensors in the stomach or the proximal oesophagus. Six or more impedance-measuring segments in the catheter (each composed of two metal ring electrodes, usually spaced 2 cm apart) detect impedance changes along variable lengths of the oesophagus.

Principles of MII–pH monitoring
Intra-oesophageal impedance is measured across a series of closely spaced electrodes within the oesophageal lumen (thus the term ‘multichannel intraluminal impedance’ MII). When surrounded by air, there is almost no current flow between the electrodes and therefore the impedance is very high. Any other material present within the oesophagus (saliva, swallowed material, and gastric reflux) will cause changes of the electric conductivity directly related with ionic concentration. With this technique it is possible to identify movement of intra-oesophageal material in either an antegrade or retrograde direction. In addition, the changes in the impedance enables detailed characterisation of reflux episodes, including composition (air, liquid or mixed), proximal extent, velocity and clearance time.

Thus, during combined MII–pH monitoring, impedance detects the reflux episode (retrograde bolus movement), and pH determines its acidity (acid or non-acid). This technique also provides information on:
- the number of acid and non-acid reflux episodes;
- number of liquid, gas and mixed reflux episodes;
- proximal extent of reflux episodes;
- bolus contact time (i.e. percentage of time bolus was present at 5 cm above the LOS; and
- acid contact time (i.e. percentage of time pH < 4.0 at 5 cm above the LOS).

Acid and non-acid reflux detected by MII–pH monitoring (Figure 6). Impedance changes in six measuring segments spanning the oesophagus (Z1–Z6) and pH changes from a single sensor in the distal oesophagus (above) and gastric fundus (below) are shown; the dotted line represents a pH of 4.0.

Epidemiology
The epidemiology of clinically relevant non-acid reflux is poorly understood. In one literature review, only 60% of patients treated for erosive oesophagitis and only about 40% of patients treated for suspected NERD had a complete symptom response with PPIs. The challenge in these PPI failures, or partial responders (i.e. patients with persistent symptoms on acid suppressive therapy) is to separate them from patients with other non-GERD causes of persistent symptoms accounting for the incomplete symptom response.

In our multicentre study using combined MII–pH monitoring, we found 37% of 168 patients with persistent symptoms on a PPI twice daily had symptoms associated with non-acid reflux, and an additional 11% had symptoms associated with continued acid reflux. Similar results were described in a European multicentre study in which 32% of 60 patients with persistent symptoms on PPI therapy had symptoms associated with non-acid reflux. Regurgitation and cough were most frequently associated with non-acid reflux. A second study from our laboratory found that 42% of 200 patients with persistent symptoms evaluated on a PPI twice daily had a positive symptom association with reflux, 35% non-acid only. See Figure 7.
Clinical manifestations
Non-acid reflux episodes do not always cause symptoms. Thus, there are patients who may have asymptomatic non-acid reflux. PPIs are potent gastric acid suppressants but do not affect structural and motility abnormalities at the gastro-oesophageal junction responsible for GERD [i.e. hiatal hernias, decreased LOS pressure, transient LOS relaxations (TLOSRs)]. Therefore, PPIs do not decrease reflux; they simply change the acidity of the refluxate.5, 6

The data noted above with combined MII–pH monitoring have clarified the extent to which symptoms can be attributed to non-acid reflux. However, the exact mechanism by which non-acid reflux episodes produce symptoms remains uncertain. One possibility is that abrupt distention of the lower oesophagus stimulates mechanoreceptors in the oesophagus.

Extra-oesophageal symptoms. It has been suggested that so-called extra-oesophageal GERD symptoms such as cough and throat clearing can be attributed to non-acid reflux. One group described the relationship between non-acid reflux and cough in 22 patients.7 About 30% of cough episodes were temporally associated with reflux. In approximately one-half of these patients, cough preceded reflux while in the remaining patients reflux preceded cough (acid 32% and non-acid 17%).

We observed similar findings in a group of 50 patients with chronic cough who underwent combined MII–pH monitoring while on acid suppressive therapy with a PPI twice daily with or without an H2 – receptor antagonist at bedtime.8 There was an association between cough and non-acid reflux (i.e. a positive symptom index) in 13 patients (26%). These patients tended to be younger and were more likely to be male compared with those who had non-acid reflux that was not associated with cough.

In our most recent study of 250 patients with persistent symptoms despite twice daily PPI therapy, about 20% of patients with cough or throat clearing had a positive symptom association with non-acid reflux. This was, of course, a lower association than occurred for heartburn or regurgitation; both being around 50%.

Diagnosis
In patients with ongoing symptoms despite acid suppression therapy and normal endoscopy, it is desirable to perform reflux monitoring on medication (twice daily PPI therapy given 30 min before breakfast and dinner). It has been our practice to perform combined MII–pH monitoring in these patients.9

MII–pH represents a good option in this setting because it detects both acid and non-acid reflux.

Table 4 | Reflux reduction therapies

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgical (fundoplication)</td>
<td></td>
</tr>
<tr>
<td>Endoscopic: is there an effective one?</td>
<td></td>
</tr>
<tr>
<td>Medical</td>
<td></td>
</tr>
<tr>
<td>Decrease TLOSRs (baclofen and related compounds)</td>
<td></td>
</tr>
<tr>
<td>Increase LESP (bethanechol)</td>
<td></td>
</tr>
<tr>
<td>Improve gastric emptying (metoclopramide, domperidone)</td>
<td></td>
</tr>
<tr>
<td>Decrease reflux (alginic acid prn)</td>
<td></td>
</tr>
<tr>
<td>Decrease visceral sensation (imipramine and related compounds)</td>
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</tbody>
</table>

TLOSRs, transient lower oesophageal sphincter relaxations; LESP, lower esophageal sphincter pressure.

Table 5 | Potential reflux inhibitors decrease transient lower oesophageal sphincter relaxations

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Description</th>
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<tbody>
<tr>
<td>Baclofen (Arbaclofen/Xenoport)</td>
<td>GABA agonist</td>
</tr>
<tr>
<td></td>
<td>CNS side effects</td>
</tr>
<tr>
<td>AZD3355 (AstraZeneca)</td>
<td>Effects peripheral GABA receptors (possibly less CNS effects)</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>ADX10059 (Addex)</td>
<td>Decrease activation of m-glutamate receptor 5 (mGluR5)</td>
</tr>
<tr>
<td></td>
<td>Less CNS effects</td>
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CNS, central nervous system; GABA, gamma-amino butyric acid.
MII–pH parameters used are primarily the number of reflux episodes and the symptom association analysis. The clinical importance of an abnormal number of non-acid reflux episodes is controversial. Association between reflux (acid or non-acid) measured by MII–pH and symptoms is reported with the use of either the symptom index (SI) or symptom association probability (SAP).

We favour using the symptom index (SI) representing the number of symptoms associated with reflux divided by the total number of symptoms recorded by a patient during the monitoring period expressed in percent. Patients in whom at least one-half of the symptoms are associated with reflux (i.e. SI ≥ 50%) are considered to have symptomatic gastro-oesophageal reflux (i.e. positive test). By contrast, patients in whom less than one-half of symptoms are associated with reflux (i.e. SI < 50%) are considered to have a negative study, i.e. persistent symptoms on PPI therapy due to causes other than reflux.

A separate SI is calculated for each of the patient’s symptoms. In our 250 patient study on ‘PPI failures’ analysis of abnormal number of GER episodes, in addition to the SI, resulted in separation into patient groups shown in Figure 8. Surprisingly, the largest positive diagnosis group was identified as a ‘sensitive oesophagus’. See Figure 8.

With this in mind, when MII–pH monitoring is performed in patients with ongoing symptoms despite acid suppressive therapy, three clinical scenarios are possible:

(i) Patients with positive association between acid reflux and symptoms, which will require further acid suppression; i.e. increasing the dose or changing to a different medication or timing of dosing.

(ii) Patients with adequate acid control but ongoing non-acid reflux with a positive reflux–symptom association will require treatment beyond acid suppression.

(iii) Patients with no relationship between reflux and symptoms should prompt evaluation for non-reflux causes of symptoms.

Treatment

Treatment approaches for non-acid reflux are evolving and depend on both symptom severity and individual patient characteristics. Conservative treatment may involve lifestyle modifications such as avoiding late and large meals, staying upright for at least 3 h after a meal, weight loss, smoking cessation and limiting alcohol intake and sleeping with the head of the bed elevated. However, the efficacy of these measures to reduce non-acid reflux has not been formally investigated.

In attempts to reduce the number of reflux episodes different strategies have been proposed, classified as reflux reduction therapies (Table 4).

There have been few studies of specific medical therapy. A report of 10 patients found that a preparation of sodium alginate and potassium bicarbonate (Gaviscon advance) given postprandially decreased the number of acid reflux episodes and distal oesophageal acid exposure 5 cm above the LOS.

Other medical therapies include baclofen, a gamma-amino butyric acid (GABA)-B agonist which will decrease postprandial reflux of all types, both acid and non-acid.11, 12 This agent will decrease TLOSRs through its action as a GABA-B agonist. However, it is not well tolerated by all patients, with adverse events including drowsiness, nausea and lowering of seizure threshold.

A few studies have used baclofen as a treatment for non-acid reflux. A small placebo controlled study reported improvement in symptoms and postprandial reflux with regular dosing of baclofen.13 Studies with reflux inhibitors similar to baclofen are being widely pursued. (Table 5).

Surgery (fundoplication) is indicated when the above measures fail, or as a treatment option. This suggestion assumes that the patient has no contraindications to surgery and that a skilled laparoscopic surgeon with experience in fundoplication is available. Patients need to be counselled on the limited data supporting this approach as lack of response to a PPI has traditionally been a negative predictor for response to laparoscopic fundoplication.
Fundoplication has the potential to reduce non-acid reflux by strengthening anatomic antireflux mechanisms. We evaluated the response to antireflux surgery in 19 patients undergoing laparoscopic Nissen fundoplication following combined impedance-pH monitoring while on a PPI twice daily. Prior to surgery, 18 of the 19 patients had a positive symptom index for GERD symptoms and reflux on acid suppressive therapy. At a mean follow-up of 14 months, 16 of 17 patients (94%) were asymptomatic or markedly improved. Persistent symptoms were noticed in one patient with a negative symptom index during the preoperative impedance-pH testing and in a second patient in whom hoarseness recurred 9 months after the operation: a third patient was lost to follow-up.

**RECOMMENDATIONS**

Treatment approaches for non-acid reflux are evolving. Our approach is shown in Figure 9 based mainly upon clinical observations (Grade 2C). We suggest a trial of the lifestyle modifications described above and medical therapy with baclofen 5 to 20 mg before meals plus alginic acid prn. In patients with continued, bothersome symptoms in whom the diagnosis is secure based upon combined MII–pH monitoring, we suggest laparoscopic Nissen fundoplication.

![Figure 9](image_url)  
**Figure 9** Flow diagram – gastro-oesophageal reflux disease approach.

**REFERENCES**


Review article: identifying the causes of reflux events and symptoms – new approaches

M. Fox

INTRODUCTION

The aim of investigation in patients with suspected gastro-oesophageal reflux disease (GERD) is to provide clinically relevant measurements of underlying pathology that explain the cause and guide effective management of mucosal disease and symptoms. Routine physiological measurement of gastrointestinal function rarely meets these ideals, especially in patients with reflux symptoms persisting during acid-suppressive therapy.

In the past, endoscopy-negative reflux disease (ENRD) was considered a mild disease, with increasing grades of erosive reflux disease (ERD) and Barrett’s columnar lined oesophagus indicating increasing severity of disease. This concept focuses on injury to the oesophageal mucosa and pH-monitoring was developed to assess what degree of acid exposure was related to mucosal disease. The introduction of proton pump inhibitor (PPI) medication that effectively inhibits gastric acid production and heals ERD has shifted attention to oesophageal symptoms. On this basis ENRD is no longer considered a mild disease because affected patients often have subjectively severe and/or atypical symptoms such as cough, and their response to acid suppression is often incomplete. The need to explain the causes of these ‘treatment resistant’ problems led to the introduction and widespread adoption of combined pH-impedance monitoring. This tech-

SUMMARY

Gastro-oesophageal reflux disease (GERD) is present if the passage of gastric contents back into the oesophagus causes either mucosal disease or symptoms. The aim of clinical investigation in patients with suspected GERD is not only to establish the diagnosis, but also to identify underlying pathology and guide specific management. Unfortunately, standard endoscopy and physiological measurement of oesophageal function by manometry and ambulatory pH measurement rarely meet these ideals. The need to improve clinical management of patients, especially those with endoscopy negative disease and symptoms persisting during acid-suppressive therapy has re-focused attention on the pathophysiology of disease. This review summarises new approaches and new technologies that have been introduced for the investigation of GERD. These include high-resolution endoscopy, detection of dilated inter-cellular spaces on histology, combined pH-impedance studies, prolonged wireless pH monitoring, detection of aerosolised acid in the pharynx, detection of pepsin in expectorated saliva, measurement of gastro-oesophageal distensibility and monitoring of gastro-oesophageal function after a meal by high-resolution manometry. The potential role of these advances to improve clinical practice is considered. Throughout, emphasis is given to the need to identify underlying causes of reflux events and symptoms and how the findings of investigation could be used to guide rational and effective treatment.
nique demonstrated that acid-suppression does not suppress reflux events and that persistent symptoms in this group are often related to weakly acid reflux (liquid or gas). The need to treat these patients has, in turn, re-focused attention on the underlying pathophysiology of GERD.

**GERD pathophysiology**

Reflex of air (‘belching’) occurs most commonly during transient lower oesophageal sphincter relaxations (TLOSRs) triggered by gastric distension. Acid secretions and other gastric contents may also pass into the oesophagus during such events. GERD patients do not necessarily have more TLOSRs than healthy controls. Rather, structural changes and instability increase the distensibility of the oesophago-gastric junction (OGJ) and the likelihood of reflux during TLOSRs and at other times (e.g. on straining). It is likely that the same changes allow greater volumes of gastric contents to pass the reflux barrier and to extend further into the proximal oesophagus. Once reflux has occurred, ineffective motility and clearance are important also because exposure to acid and other noxious substances in refluxate (e.g. pepsin, bile salts) increase the risk of mucosal disease and other complications. Whether reflux then triggers patient symptoms depends on a dynamic interaction between the severity of the underlying event and sensitivity to that event (Figure 10).

**Endoscopy and biopsy**

Endoscopy and biopsy is performed routinely in patients with reflux symptoms that persist during acid suppression to exclude other conditions, such as eosinophilic oesophagitis, that respond to other therapies. It would be convenient if a definitive diagnosis of GERD could be made at the same procedure; however, the majority of patients have ENRD on standard white light illumination. Recent studies have shown that high-resolution endoscopy with narrow band imaging reveals micro-erosions and other subtle abnormalities at the OGJ in ENRD (Figure 11). Biopsies reveal dilated intercellular spaces on electron microscopy in the majority of ENRD patients that could facilitate activation of nociceptive visceral afferent nerve receptors (Figure 12). Moreover, dilated intercellular spaces can be induced by perfusion of acid (even at pH > 4) and resolve on acid suppressive therapy. Such changes could be sensitive diagnostic markers of GERD; however, specialist equipment and histology are required and the utility of these findings in clinical practice has not been determined.

**Recent developments in oesophageal pH and pH-impedance studies.** Gastro-oesophageal reflux disease is present when the passage of gastric contents into the oesophagus damages the mucosa or causes symptoms. Thus, the analysis of reflux studies should provide not only a direct assessment of disease severity (e.g. acid exposure, number of reflux events, proximal extent) but also an assessment of whether reflux events are the cause of symptoms (reflux–symptom association). It has been proposed that the likelihood of a reflux event causing a symptom provides an assessment of visceral sensitivity. This concept is supported by pH-impedance studies in which ENRD patients have less acid reflux but are more likely to report symptoms in response to acid or weakly acid reflux than ERD patients (Figure 13). Similarly, preliminary results from prolonged wireless pH monitoring (Bravo System, Given, Yoqneam, Israel) show that although the overall symptom burden increases with oesophageal acid exposure, the response to acid suppression treatment is inversely correlated to symptom index. This suggests that, independent of acid exposure,
patients with a high symptom index (i.e. visceral hypersensitivity) are more likely to experience persistent symptoms on treatment than those with a low symptom index. These observations may explain the paradox that patients with severe oesophageal acid exposure and mucosal disease often respond well to acid suppression, whereas patients with ENRD and functional heartburn with mildly elevated or normal acid exposure but a high symptom index often continue to report symptoms on treatment. It should be noted that the high day-to-day variability of symptoms make such an analysis less secure for 24 h than more prolonged pH monitoring and that the most appropriate reporting of reflux–symptom association data for this purpose has not been established.

**Laryngopharyngeal reflux studies.** Epidemiological studies report an association between GERD and extraoesophageal disease; however, a large, well-designed clinical trial failed to find a benefit of high-dose PPI treatment and highlighted the difficulty of establishing a link between acid reflux and laryngopharyngeal symptoms. Affected patients may not have elevated oesophageal or pharyngeal acid exposure or a definite reflux–symptom association on pH ± impedance studies. This may be because the pharynx and larynx are exquisitely sensitive even to small amounts of even weakly acidic reflux, especially in the presence of pepsin and other noxious gastric contents. Moreover, once damaged, affected structures heal only slowly and local irritation may cause symptoms independent of reflux events. Impedance is not sensitive in the pharynx and standard pH studies detect very few reflux events. Recently a minimally invasive device for detection of oropharyngeal acid reflux (Restech; Respiratory Technology Corp., San Diego, CA) was introduced that is well-tolerated and sensitive even to tiny droplets of acid (Figure 14). Preliminary findings suggest that this increases diagnostic yield of reflux as a cause of laryngopharyngeal symptoms.

An alternative approach is the detection of pepsin in expectorated saliva [Pepsin Lateral Flow Device (Peptest); RD Biomed Limited, Hull, UK], a proteolytic enzyme that remains active even in weakly acid environs, adheres to and damages laryngopharyngeal structures. Pepsin is not sensitive in the pharynx and standard pH studies detect very few reflux events. Recently a minimally invasive device for detection of oropharyngeal acid reflux (Restech; Respiratory Technology Corp., San Diego, CA) was introduced that is well-tolerated and sensitive even to tiny droplets of acid (Figure 14). Preliminary findings suggest that this increases diagnostic yield of reflux as a cause of laryngopharyngeal symptoms.

**Figure 11** | Compared with control (a), narrow band imaging revealed that a significantly higher proportion of patients with endoscopy-negative reflux disease had increased number (b), dilatation (c), tortuosity (d) of intrapapillary capillary loops, microerosions (e) and increased vascularity at the squamocolumnar junction. Reproduced with permission from Sharma et al.²³
produced only in the stomach and its presence in the throat can only be as a consequence of reflux. Measurement is simple and non-invasive using a near patient test (Figure 15) and its detection may be of particular value to assess whether reflux is causing intermittent symptoms that do not occur every day or chronic, persistent

**Figure 12** | Photomicrographs of oesophageal mucosa, obtained using transmission electron microscopy (TEM) of the suprabasal layer (original magnification, ×3500). (a) and (c) Patients with endoscopy-negative reflux disease and erosive oesophagitis, respectively, before treatment. (b) and (d) Resolution of DIS after high-dose acid suppression treatment. Reproduced with permission from Calabrese et al.\(^{14}\)

**Figure 13** | The majority of symptoms in all patients with reflux symptoms not taking acid suppressive medications are related to acid reflux events. In endoscopy-negative reflux disease (ENRD) patients, especially the subgroup with physiological levels of acid reflux (i.e. functional heartburn), the proportion of symptoms related to weakly acid reflux is higher than that in erosive reflux disease (ERD) patients. In addition, the presence of gas in the refluxate significantly enhances the probability of reflux perception. This finding is likely to reflect visceral hypersensitivity to oesophageal stimulation and to explain why ENRD patients respond less well to therapy. Derived from data reported by Emerenziani et al.\(^{16}\)

**Figure 14** | Restech pharyngeal pH catheter in position lateral to the uvula in the oropharynx, a position that causes minimal discomfort to the subject. The light emitting diode aids placement. The design can detect liquid or aerosolised acid, prevents artefact because of drying out of the electrode or loss of sensitivity caused by tissue contact; all properties that facilitate accurate pH monitoring in the oropharynx. Reproduced with permission from Sun et al.\(^{18}\)
symptoms (e.g. sore throat) that are not associated with individual reflux events and symptoms.

**High-resolution manometry and Endo-FLIP.** Although endoscopy and pH ± impedance studies that detect mucosal disease or reflux events are required to diagnose GERD, these techniques do not provide a direct assessment of underlying pathology. By contrast, oesophageal manometry can assess the pressure and length of the OGJ, detects TLOSRs and the occurrence of reflux events. High-resolution manometry (HRM) with one pressure sensor per centimetre from the pharynx to the stomach is a recent advance. HRM data are presented in real time as a ‘spatiotemporal’ plot, a compact, visually intuitive display of gastro-oesophageal function. Not only does this increase the diagnostic sensitivity to peristaltic dysfunction and allow a detailed assessment of the structural integrity and function of the reflux barrier in GERD, but also HRM facilitates the extraction of meaningful information from physiological challenges (e.g. test meal) that frequently trigger reflux and symptoms (Figure 16). In addition, manometry can identify rumination, a behavioural disorder in which subconscious contraction of the abdominal wall forces gastric contents back into the oesophagus producing symptoms that frequently present as ‘treatment resistant’ GERD. By identifying the underlying mecha-
nism of reflux events, HRM has the potential to direct specific medical and surgical treatment.

An important property of the reflux barrier that is not assessed by manometry is distensibility (i.e. the ease with which the OGJ is opened). The endoscopic functional luminal imaging probe (Endo-FLIP system; Crospon Medical Devices, Galway, Ireland) uses Impedance Planimetry to measure the cross-sectional area (CSA) at multiple points across the OGJ at various distension pressures. This technique has been validated and recent studies have confirmed that OGJ distensibility is greater in GERD patients than healthy individuals and reduced after fundoplication (Figure 17). Endo-FLIP has not, as yet, been compared with the results of ambulatory oesophageal pH studies that provide a definitive diagnosis of GERD; however, it is likely to identify patients in whom increased numbers of reflux events are resulting from mechanical disruption of the reflux barrier and this could help select patients who would benefit from anti-reflux surgery.

CONCLUSION

The success of scientific medicine is based on the identification and treatment of the pathophysiological basis of disease. In the past, investigation of GERD has been limited to confirming the diagnosis and the treatment of GERD has been empiric acid suppression or surgery. New approaches and new technologies are now available to assess every aspect of GERD pathophysiology, including motor and sensory dysfunction. This information could play an important role in directing future clinical management to address the individual causes of patient symptoms and disease.

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Review article: reflux and asthma – mechanisms of interaction and asthma outcomes

S. M. Harding

INTRODUCTION

Asthma is a heterogeneous clinical syndrome characterised by nonspecific airway hyperresponsiveness and inflammation.1 Asthma is highly prevalent and affects approximately 300 million people worldwide.2 The triggers and/or precipitants of asthma are numerous and include viruses, allergens, occupational exposures, hormones, drugs, exercise, stress, smoking, as well as co-morbidities such as gastro-oesophageal reflux, rhinosinusitis and obesity.3 These triggers and/or precipitants differ among individuals.

Reflux is a trigger and/or a co-morbid disorder in approximately 75% of asthmatics.4 A systematic review in adult asthmatics notes reflux symptom prevalence to be 59%, abnormal oesophageal acid contact times present in 51%, hiatal hernia present in 51% and endoscopic evidence of oesophagitis in 37%.5 In adults, 41% of asthmatics had reflux-associated asthma symptoms.6 In asthma patients who do not have reflux symptoms, 61% had abnormal oesophageal acid contact times consistent with the diagnosis of gastro-oesophageal reflux disease.7

SUMMARY

Asthma is a heterogeneous clinical syndrome characterised by nonspecific airway hyperresponsiveness and inflammation. Reflux is a trigger and/or co-morbid disorder in approximately 75% of asthmatics. Mechanisms exist between the oesophagus and the lung, including common embryonic foregut origins and vagal innervation. Oesophageal acid results in bronchoconstriction that can be ablated with vagotomy. Autonomic dysfunction with heightened vagal tone and local axonal reflexes are also active. Oesophageal acid potentiates the bronchoconstrictive effect of other asthma triggers. Microaspiration induces bronchoconstriction and alters the immune response to a Th-2 response in animal models. All of these mechanisms can induce airway inflammation including neuroinflammatory mediators, airway eosinophilia, neutrophilia and macrophage activation. Cytokines levels, including IL-5, IL-6 and IL-8, are also increased. Proton pump inhibitor treatment lowers exhaled breath condensate 8-isoprostan levels.

Reflux treatment improves asthma outcomes in selected asthmatics with reflux symptoms, with placebo-controlled trials showing improved peak expiratory flow rates and asthma quality of life, although these findings are modest. Asthmatics without reflux symptoms do not show asthma improvement with PPI therapy. Reflux therapy-responsive asthma may represent a distinct asthma phenotype. Future research is needed to identify biomarkers or clinical variables that identify this phenotype.
The importance of reflux as a trigger of asthma is recognised. In the 5 to 10 years follow-up of the European Community Respiratory Health Survey of 16 191 participants from five northern European countries, nocturnal reflux symptoms were an independent risk factor for asthma onset. Reflux is recognised as a significant risk factor for recurrent asthma exacerbations as well as a key factor in difficult-to-treat asthma.

While such observations cannot address causality, asthma patients with reflux treatment-responsive asthma may represent a distinct asthma phenotype. Currently, there is no validated test or biomarker that identifies these asthmatics. Furthermore, in individual asthmatics, reflux may be present; however, reflux may not be a trigger or exacerbating factor for their asthma. Thus, placebo-controlled trials may not show significant impact across the entire group of asthmatics with reflux. Medical reflux therapy primarily targets gastric acid secretion and not reflux itself, so reflux still occurs. Although investigators note that certain phenotypic asthma or oesophageal variables may predict asthma response to reflux therapy, much work needs to be done in this area.

To further understand this asthma phenotype, predisposing factors for reflux development in asthmatics will be reviewed. Furthermore, three questions will be asked: (i) What are the potential mechanisms of interaction between the oesophagus and the lung? (ii) Can these mechanisms initiate airway inflammation? (iii) Does reflux therapy improve asthma outcomes?

Predisposing factors for reflux development in asthmatics

There are many potential predisposing factors for reflux development in asthmatics. Asthmatics have autonomic dysregulation with heightened vagal tone. During asthma exacerbations or in episodes of increased work of breathing, the lower oesophageal sphincter (LES) barrier may be overcome because of marked increases in negative intrathoracic pressure. Obesity and hiatal hernia are risk factors for reflux development and both of these conditions are prevalent in asthmatics.

Asthma medications can promote reflux. Intravenous theophylline and aminophylline increase gastric acid secretion and decrease LES pressure. Oral slow-release theophylline increases reflux symptoms by 170% and upright oesophageal acid contact times by 24%. This effect is more pronounced in asthmatics with therapeutic serum theophylline levels. Inhaled beta-2 adrenergic receptor agonists, such as albuterol, cause a dose-dependent reduction in LES pressure and alter oesophageal contraction amplitude in the oesophageal body. Thus, inhaled beta-2 agonists, especially in sequential doses used during acute asthma exacerbations, alter oesophageal motor function and predispose to reflux development. There are no data currently available examining oesophageal effects of inhaled long-acting beta agonists. Oral corticosteroids (prednisone 60 mg per day for 7 days) increases oesophageal acid contact times at both the distal \( (P < 0.002) \) and proximal \( (P < 0.007) \) pH probes in asthmatics with minimal reflux symptoms and stable asthma. In a longitudinal cohort study utilising the UK General Practice Research Database of 9712 asthmatics, the use of oral corticosteroids for more than 3 months was a risk factor for reflux development (odds ratio 4.5; 95% confidence intervals 1.0–19.5).

Furthermore, airflow obstruction induces transient LES relaxations, the principle mechanism of individual reflux episodes. Lifestyle factors are also important. In 261 asthmatics and 218 controls, 50% of the asthmatics had reflux symptoms that awakened them from sleep and 33% of them noted asthma symptoms. Sixty percent of these asthmatics ate right before bedtime, which was related to reflux symptoms during sleep. These reflux predisposing factors may be more important during acute asthma exacerbations and in asthmatics with difficult-to-treat asthma.

Potential mechanisms of interaction between the oesophagus and the lung

There are many potential mechanisms of interaction between the oesophagus and the lung, including sharing common embryonic foregut origins and vagal innervation. The lower oesophageal sphincter may be a respiratory-derived structure based on transcription factor expression experiments. The crural diaphragm, which is considered the extrinsic sphincter of the antireflux barrier, has sensory and motor innervation by way of the vagus nerve. Transient LES relaxations are vagally mediated where there is simultaneous relaxation of the LES and the crural diaphragm.

A vagal bronchoconstrictive reflex is present. In a dog model, oesophageal acid increased respiratory resistance which was abolished with vagotomy. In asthmatics and normal controls, oesophageal acid decreased peak expiratory flow rates and increased airway resistance, even without evidence of proximal oesophageal acid – a marker of microaspiration. Atropine pre-treatment partially ablated this bronchoconstrictive response. In 136 subjects referred for oesophageal testing, oesophageal acid infusion decreased heart rate, \( \text{FEV}_1 \), and oxygen saturation.
A positive Bernstein test was not required for this response and atropine pre-treatment ablated this response. Rosztóczy et al. examined the presence of an oesophago-bronchial reflex (EBR) in 43 consecutive asthmatics and 20 subjects with chronic cough without asthma. The EBR was assessed by oesophageal acid perfusion and methacholine challenge tests. Subjects who had a positive EBR were more likely to have a positive Bernstein test and more acid reflux in the supine position. However, the unanswered question remains – could the presence of an EBR be a biomarker identifying reflux-treatment responsive asthma? The vagal reflex does not end in the pons. Central sensitisation occurs that alters vagal reflex output. These reflexes respond to oesophageal acid and distention of the oesophagus. Vagal input is also active in microaspiration-induced bronchoconstriction as vagotomy abolishes the effect.

Furthermore, asthmatics with reflux have autonomic dysregulation with heightened vagal tone. Local axonal reflexes are also active such that nitric oxide-containing neurons in the oesophageal myenteric plexus project axons directly to the trachea. In animal models, oesophageal acid induces the release of tachykinins and substance P, resulting in airway oedema. Neurokinin receptors are found in human airways, and astmatic airways have increased expression of these receptors. Stimulation of these receptors results in bronchoconstriction and vascular and pro-inflammatory effects. In asthmatics with reflux, a positive correlation exists between oesophageal acid exposure, and substance P and neurokinin A levels in induced sputum.

Heightened bronchial reactivity may also play a role in reflux-triggered asthma. For instance, in 105 consecutive asthmatics, a correlation was noted between the provocative dose of methacholine required to reduce the FEV\textsubscript{1} by 25% and the number of reflux episodes \((r = 0.56; \ P = 0.05)\). This relationship was stronger in asthmatics with reflux \((r = 0.98; \ P = 0.001)\). Heightened bronchial reactivity from reflux episodes also occurs during sleep. Monitoring oesophageal pH and respiratory resistance during sleep, reflux episodes were associated with higher respiratory resistance compared with baseline. Moreover, there was a correlation noted between respiratory resistance and reflux duration. Oesophageal acid also potentiates the bronchoconstrictive effect of methacholine and voluntary isocapnic hyperventilation of dry air. This effect was also abolished with atropine pre-treatment. Microaspiration causes bronchoconstriction and increases respiratory resistance. Further, microaspiration shifts the immune response to a Th-2 response in a murine asthma model.

**Induction of airway inflammation by oesophageal reflux**

These mechanisms induce airway inflammation. Vagally induced bronchoconstriction is associated with airway eosinophilia, which is blocked with atropine pretreatment. Airway neutrophilia correlates with oesophageal acid contact times in humans. Exhaled breath condensate 8-isoprostane levels were higher in asthmatics with reflux. Proton pump inhibitor therapy lowered 8-isoprostane levels. In animal models, acid in the stomach elevated IL-5, IL-6, and IL-8 levels and increased neutrophil numbers in bronchoalveolar lavage fluid. Macrophage activation and matrix metalloproteinase-9 expression was enhanced with gastric fluid. These data support the hypothesis that oesophageal contents can impact bronchomotor tone and airway inflammation.

**Asthma outcomes with reflux therapy**

Reflux therapy improves asthma outcomes in selected asthmatics. Littner et al. noted in a multicentre, double-blind, placebo-controlled trial in 207 moderate to severe asthmatics using lansoprazole 30 mg BID for 24 weeks, that symptoms did not improve (primary outcome); however, quality of life improved. Fewer patients had asthma exacerbations or required prednisone. Kiljander et al. noted in 767 moderate to severe persistent asthmatics that esomeprazole 40 mg twice daily for 16 weeks improved peak expiratory flow rates in asthmatics with both reflux symptoms and nocturnal asthma symptoms, as well as in asthmatics taking long-acting beta agonists. Asthmatics without reflux symptoms did not have improved outcomes. This finding is verified by the American Lung Association’s Asthma Clinical Research Centers which performed a placebo-controlled, randomised trial in 412 inadequately controlled asthmatics without reflux symptoms. Even in subjects with abnormal oesophageal acid contact times, esomeprazole 40 mg twice a day for 24 weeks did not improve asthma control, pulmonary function, asthma quality of life or bronchial reactivity. It is noteworthy that asthmatics without reflux symptoms with oesophageal pH evidence of proximal reflux reported worse asthma and health-related quality of life. More recently, Kiljander et al. reported results in 828 moderate to severe asthmatics with symptomatic reflux randomised to receive placebo or esomeprazole 40 mg daily, or 40 mg twice daily for 26 weeks. Both treatment doses improved FEV\textsubscript{1} and
asthma quality of life compared with placebo at treatment end; however, these improvements were minor. A Cochrane System database review noted that subgroups of asthmatics may benefit from medical reflux therapy.

Carefully controlled surgical trials examining fundoplication on asthma outcomes are lacking. In a review of 24 trials evaluating 417 asthmatics, fundoplication improved asthma symptoms in 79%, asthma medication use in 88% and pulmonary function in 27%. In a controlled trial of 62 asthmatics with reflux at the 2 year follow-up, asthma symptom scores improved in 43% of the asthmatics undergoing fundoplication compared with less than 10% in the medically treated group (ranitidine 150 mg three times daily) and the placebo group \( P = 0.0009 \).

CONCLUSIONS

In conclusion, oesophageal contents can alter lung function and impact airway inflammation. Reflux treatment improves asthma outcomes in selected asthmatics. Previous asthma outcome studies identified potential predictors of asthma response. These include the presence of regurgitation more than once a week, abnormal amounts of acid in the proximal oesophagus, higher oesophageal acid contact times, difficult to control asthma, non-allergic asthma and nocturnal asthma. Currently there is insufficient data as to whether other variables, including reflux-associated asthma symptoms, Bernstein-positive subjects, or asthmatics with heightened EBR, higher levels of exhaled breath condensate (EBC) 8-isoprostane levels or lower EBC pH can predict asthma response.

Other potential identifiers that need study include salivary, sputum or bronchoalveolar lavage markers of reflux, including pepsin or bile acid. Cytokine profiles or neuroinflammatory biomarkers may also be important. Comprehensive clinical phenotypic characterisation and comprehensive bio-specimen analyses of asthmatics who respond to reflux therapy compared with those who do not, needs to be carried out. Potentially, patients with reflux therapy-responsive asthma may represent a distinct asthma phenotype. Further research is needed to identify the phenotypic characteristics and biomarkers that identify this phenotype.

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INTRODUCTION

Numerous reports in the 1980s and 1990s examined the profile of patients presenting with chronic cough, which is arbitrarily defined as cough lasting greater than 8 weeks. These studies identified three common causes for chronic cough: asthma, postnasal drip syndrome (PNDS), more latterly called the Upper Airways Cough Syndrome and gastro-oesophageal reflux disease (GERD). The paradigm was applied around the world in clinics treating patients with cough. However, some patients steadfastly refused to be pigeonholed into one of these diagnostic categories and were labelled idiopathic cough.

In the Hull Cough Clinic, we adopted a similar paradigm and developed a probabilistic based paradigm of therapeutic trials aimed at discovering the underlying cause of the cough. It became increasingly apparent, however, that none of the diagnostic categories claimed in the original studies were well supported by the clinical observations in individual patient groups. Repeated attempts via histological examination, or the detection of inflammatory markers and mediators were unsuccessful in differentiating objectively these diagnostic categories. The asthmatic cough was strikingly different from classic atopic asthma, with an onset of middle age often without obvious atopy and sometimes even without bronchoconstriction. Here the term cough variant asthma was coined to describe patients with chronic cough and bronchial hyperresponsiveness but without overt bronchoconstriction. Sputum eosinophilia was present. In a further variation even bronchial hyperresponsiveness was dropped from the classification and chronic cough with sputum eosinophilia described. This form of airway TH2 response was termed eosinophilic bronchitis, which in the eyes of enthusiasts became a separate condition. The ‘disease’ of postnasal drip syndrome has never found much credence in Europe.

SUMMARY

Chronic cough was previously thought to be attributed to three main aetiologies: asthma, reflux and postnasal drip. There is little to differentiate these three different diagnoses in the patients with chronic cough in terms of histology, cytokine profile or clinical history. In fact, the uniform clinical history exhibited by patients with chronic cough points to a single aetiology – airway reflux. In support of this, a questionnaire has been developed which identifies key elements within the clinical history which is answered positively by the overwhelming majority of patients presenting to a cough clinic. A new technology, including airway pH and exhaled breath condensate pepsin levels provide supporting evidence for the hypothesis that chronic cough is really a single diagnosis which has been termed the cough hypersensitivity syndrome. The syndrome is not necessarily precipitated by acid reflux and therefore therapy is directed more at motility and inhibition of the hypersensitivity components of the syndrome. The application of these diagnostic methodologies to other airway diseases such as severe asthma and recurrent exacerbations of COPD has revealed that many of the features of the cough hypersensitivity syndrome are also present in these conditions.

A. H. Morice

Review article: reflux in cough and airway disease

A. H. Morice

Hull York Medical School, University of Hull, Cardiovascular & Respiratory Studies, Castle Hill Hospital, Castle Road, Cottingham, East Yorkshire HU16 5JQ, UK.

Correspondence to:
Prof. A. H. Morice, Hull York Medical School, University of Hull, Cardiovascular & Respiratory Studies, Castle Hill Hospital, Castle Road, Cottingham, East Yorkshire HU16 5JQ, UK.
E-mail: a.h.morice@hull.ac.uk
chiefly a diagnosis found in the Americas. As far back as the 1800s, an English surgeon reported that on his visit to the United States he had come across a new condition – PNDS. In the ACCP guidelines on chronic cough, it is defined by response to therapy with first generation antihistamines. There are no objective tests to describe the syndrome. Reliance on therapeutic response to define a particular condition is at best risky and open to the problems associated with the use of old fashioned drugs developed with a multitude of activities. More recently the drug most frequently used in the treatment of PNDS in the Americas – dextromethorphan has been shown to be an antagonist of the capsaicin cough receptor, TRPV1. Thus, while there is no doubt that patients with chronic cough can present with predominantly nasal and upper airway symptoms the response to a therapeutic agent cannot be said to define a particular syndrome, and PNDS is really a symptom which is a facet of a larger overall pathological process.

The final diagnosis within the 20th Century triad of chronic cough was reflux disease. Unfortunately, the term GERD had been commandeered by the gastroenterologists to describe the dyspeptic symptoms of heartburn and indigestion. Here acid is the predominant player in the evolution of the condition. When close associations were sought between chronic cough and GERD, while there were correlations they were insufficient to prove causality. This was because whilst acid is undoubtedly the major pathogenic factor in heartburn, non-acid reflux is the major precipitant of the symptoms associated with airway reflux. Before this was realised, patients would be sent for conventional 24 h oesophageal pH assessment which clearly delineates heartburn-associated GERD. The report would read that there was no evidence of reflux. In fact, the true report should have read there was no evidence of liquid acid reflux.

It was thus by applying an unsatisfactory series of existing paradigms to the diagnosis of chronic cough that

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HULL COUGH HYPERSENSITIVITY QUESTIONNAIRE

Name: ______________________________
D.O.B:________________________ UN:___________
DATE OF TEST:________________________

Please circle the most appropriate response for each question

<table>
<thead>
<tr>
<th>Within the last MONTH, how did the following problems affect you?</th>
<th>0 = no problem and 5 = severe/frequent problem</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoarseness or a problem with your voice</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Clearing your throat</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>The feeling of something dripping down the back of your nose or throat</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Retching or vomiting when you cough</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Cough on first lying down or bending over</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Chest tightness or wheeze when coughing</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>A tickle in your throat, or a lump in your throat</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Cough with eating (during or soon after meals)</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Cough with certain foods</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Cough when you get out of bed in the morning</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Cough brought on by singing or speaking (for example, on the telephone)</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>Coughing more when awake rather than asleep</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>A strange taste in your mouth</td>
<td>0 1 2 3 4 5</td>
</tr>
</tbody>
</table>

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Figure 18 | Hull cough hypersensitivity questionnaire.
three separate ‘diseases’ were invented to describe the syndrome and those patients not fitting into these boxes were then assigned as idiopathic. In truth, the overwhelming majority of patients with chronic cough present with a remarkably uniform history, elements of which clearly and undoubtedly point to the origin in reflux disease.\(^{16}\)

**Cough hypersensitivity syndrome**

Patients with chronic cough give a remarkably uniform clinical history. This story has two components. Firstly, the precipitants of airway reflux, which is probably gaseous and mildly acidic in nature, give striking clues to the clinician. For example, patients not infrequently complain of cough after meals. This postprandial paroxysm of coughing typically occurs 10 min after the meal.\(^{16}\) This of course is the peak time for lower oesophageal sphincter opening,\(^{17}\) allowing the air swallowed during eating to be belched up. There is no heartburn as the food has neutralised gastric acid.

The second component of the syndrome is the consequence of the reflux, which leads to hypersensitivity of the irritant receptors on upper airway nerves. These nociceptors are mainly of the transient receptor potential (TRP) family.\(^{18}\) While there are twenty-seven different TRP receptors described fortunately those of interest within the cough sphere are temperature receptors and, in particular, TRPV1, TRPA1 and TRPM8. These temperature receptors are promiscuous in binding to a wide range of environmental stimuli and provide the defence against the inhalation of noxious physical and chemical stimuli. In chronic cough (and probably in acute viral respiratory tract infection cough), these receptors are up-regulated and increased in number.\(^{19, 20}\) Thus, the patient with a chronic cough will complain of paroxysms induced by exposure to noxious chemical stimuli, including smoke (acreolin), or a change in atmospheric temperature (TRPV1 and TRPA1). These responses have previously been dismissed as nonspecific whereas in fact they represent specific adaption with increased nociception in response to the challenge of airway reflux.

**Figure 19** Demonstration of grossly abnormal episodes of airway pH in patient with severe asthma. The trace shows repeated falls in pH indicating episodes of gaseous acid reflux into the pharynx.
The diagnosis of reflux cough
There is no readily available objective test which indicates the presence of airway reflux. The clinical history, however, has been codified into a fourteen point questionnaire, which has recently been validated (Figure 18). Each question independently tests for the cough hypersensitivity syndrome and the upper limit of normal is 13 out of 70. Perhaps the greatest utility of the questionnaire is that it directs the questioning into aspects of the history which are unequivocally reflux associated with components of the non-acid reflux nature, such as the dysmotility associated with vomiting and retching and the repetitive throat clearances.

New technologies for the diagnosis of airway reflux
Recently combined multichannel intraluminal impedance and pH testing (MII-pH) has been used to determine the role of reflux in patients presenting with chronic cough.

Multichannel intraluminal impedance-pH is extensively described by Arevelo, Sharma and Castell in their paper on Symptomatic Non-acid Reflux: The New Frontier in GERD in this supplement.

Two more recent modalities of examination of airway reflux have been developed. Firstly, the sampling of pharyngeal airway pH maybe undertaken by the Restech probe, which has been specifically designed to sit at the back of the pharynx and sample gaseous pH. Thus, the gaseous belch responsible for airway reflux can be detected. However, many of these belches are of a neutral or mildly acid nature. None the less valuable information can be obtained proving airway reflux which had previously been misdiagnosed as other airway conditions. For example, in Figure 19 grossly abnormal episodes of airway pH are demonstrated in a female with severe ‘asthma’ who had multiple ITU admissions before curative fundoplication. The second modality is the estimation of airway pepsin using exhaled breath condensate. Previous methods for collecting exhaled breath condensate relied on laboratory based technologies. A recent development has been a device for the patient to take home and collect samples during attacks. Here the presence of pepsin within the exhaled breath is indicative of a reflux during coughing, asthma or acute exacerbations of COPD.

Airway reflux in other respiratory conditions
Many other respiratory conditions are misdiagnosed because airway reflux is not widely recognised. In asthma, a TH2 response maybe precipitated by the aspiration of airway reflux. This maybe a TH1 response with wheezing and neutrophils but often a TH2, eosinophilic led response occurs which mimics classic asthma. Late onset asthma, i.e. asthma presenting in the 40s and 50s is often described as intrinsic asthma, because it was clearly differentiated by the early physicians from extrinsic allergic asthma. However, patients with this form of asthma, which is often resistant to conventional treatment, answer highly to the cough hypersensitivity questionnaire and fundoplication can lead to significant improvements.

In numeric terms, viral infections are clearly the most common cause of exacerbations of COPD, but in those patients who are ‘frequent flyers’, i.e. those patients with multiple admissions in a short period of time an alternative aetiology must be evident. Classic GERD is highly associated with COPD exacerbations. We have recently surveyed our frequent flyers at Castle Hill Hospital and find that a highly abnormal score on the cough hypersensitivity questionnaire is the norm. We believe that these patients represent COPD patients with additional airway reflux. Finally, those patients with bronchiectasis, cystic fibrosis, lung transplantation rejection and fibrosing alveolitis also respond highly to questionnaires and associations with GERD have been reported. So, airway reflux may not only be responsible for chronic cough but is a precipitating factor in a spectrum of respiratory disease, which was previously thought to be idiopathic in nature.

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Review article: laryngopharyngeal reflux – the ear, nose and throat patient

M. G. Watson

INTRODUCTION
Laryngopharyngeal reflux (LPR) is now a common diagnosis in UK ENT practice, although acceptance of the disease continues to attract a degree of controversy. The term was first used by Koufman in 1991.1 It differs from classical gastro-oesophageal reflux disease (GERD) in several respects. Gastro-oesophageal reflux (GER) produces classical symptoms of heartburn and regurgitation, whereas LPR may present with a spectrum of throat symptoms.

GER is characterised by abnormal acid clearance times in the oesophagus (<5% is physiological). This leads to oesophagitis, which can be detected easily at upper gastrointestinal endoscopy. In LPR, oesophageal acid clearance is often normal, with brief ‘flashes’ of reflux reaching to the upper oesophageal sphincter and beyond, into the laryngopharynx. These reflux episodes may consist of mainly gas carrying an aerosol of gastric contents, rather than a large volume liquid bolus.2 The upper aerodigestive tract is much more sensitive to the presence of refluxate than the oesophagus.

Refluxate contains both hydrochloric acid and enzymes, notably pepsin. Activated pepsin is probably the causative agent for mucosal inflammation in LPR, rather than acid alone.3 In an animal model, it has been shown that reflux episodes lasting for 30 s three times per week are sufficient to cause significant mucosal damage. This is in stark contrast to GERD, where the oesophagus can withstand the presence of refluxate for up to 5% of each 24 h period.

While LPR and GERD may exist independently, evidence is growing that as severity increases, it is more likely that features of both will be present.4 This article will describe current practice in diagnosis and treatment of LPR patients in a UK ENT out-patient clinic.

Diagnosis
In UK practice, most patients are diagnosed following a thorough clinical assessment. This begins with taking a careful case history. LPR symptoms are listed in Table 6. A patient completed questionnaire to quantify symptoms is an important part of the initial work-up, to aid in diagnosis and to set a baseline, which can be used later to assess response to treatment. The Reflux Symptom Index (RSI), described by Belafsky et al.,5 is the most commonly used tool.

An ENT examination is then performed, with particular attention being paid to the larynx and pharynx. Indirect laryngoscopy with a mirror is not adequate in this setting: rigid or flexible laryngoscopy is essential, preferably using a digital system with video recording and still image capture. Features of LPR on examination are listed.
in Table 7. These may be quantified using the Reflux Finding Score (RFS), for the same purposes as the RSI described above.

Most UK patients do not undergo further investigations at this stage. Any further tests are reserved for those who do not respond well to treatment, or in whom the diagnosis is unclear. LPR investigations are listed in Table 8.

Barium swallow is no longer routinely used to diagnose reflux disease, although it still has an important role if pharyngeal pouch is suspected. The Bernstein acid perfusion test is mainly of historical interest, but has been used recently in research into reflux-related cough. Upper gastrointestinal endoscopy is valuable in the diagnosis of GERD, but is often normal in LPR. Oesophageal manometry may not be directly helpful in the diagnosis of LPR, but it is necessary to determine the position of upper and lower oesophageal sphincters to guide the correct placement and spacing of electrodes for pH monitoring, and is very useful in the assessment of oesophageal dysmotility, which may be contributing to symptoms.

The 24 h dual channel pH monitoring, with electrodes in the distal oesophagus and at or just above the upper sphincter, has been the mainstay for diagnosis of LPR. Single channel pH recordings can diagnose GERD, but may miss LPR. Some authors have considered that any episodes of reflux reaching the pharynx are abnormal, although others feel that ≥4 episodes in 24 h are needed to make the diagnosis. More recently, multichannel intraluminal impedance measurement has been added to pH monitoring (MII-pH) and may improve diagnostic accuracy, particularly for weakly acidic or non-acidic reflux events, although again there is variation in the interpretation of normative data. Both techniques are invasive (involving transnasal insertion of catheter), expensive to perform, and are poorly tolerated by some patients. Protocols which require the patient to come off their antireflux medication for a period prior to the test can result in a worsening of symptoms. For patients where surgical intervention for LPR is being considered, physiological confirmation of the condition by pH monitoring or MII-pH is normally required.

A non-invasive test that gives rapid results would be very helpful in patient management, rather than waiting for the results of pH monitoring or MII-pH. One non-invasive procedure which has recently become available (Peptest, RD Biomed Limited, Hull, UK) uses a pepsin immunoassay applied within a lateral flow device for sampling pharyngeal secretions: if pepsin is detected, then the patient probably suffers from LPR. Another minimally invasive procedure involves monitoring airway pH in the pharynx to detect aerosolised reflux (Restech, Respiratory Technology Corp., San Diego, CA, USA). In ventilated patients, measurement of pH in exhaled breath condensate may be valuable.

One study has found that in patients with cough related to LPR, which did not respond to standard medication, that some patients were found to be positive for Helicobacter pylori, and improved following eradication of the organism.

It must be borne in mind that although LPR is a possible cause of laryngopharyngeal inflammation, it is by no means the only cause. Other causes of laryngeal inflammation are listed in Table 9.

**Table 7** | Laryngopharyngeal reflux examination findings

(i) Erythema/hyperaemia, 3 point inflammation (both arytenoids and base of epiglottis)
(ii) Posterior commissure hypertrophy
(iii) Granuloma
(iv) Pseudosulcus
(v) Vocal cord oedema
(vi) Thick intralaryngeal mucus
(vii) Ventricular obliteration
(viii) Extra laryngeal signs: red mucosa in nasopharynx, lingual tonsil
(ix) Hypertrophy

**Table 6** | Laryngopharyngeal reflux symptoms

(i) Dysphonia
(ii) Swallowing difficulty (‘pseudodysphagia’) 
(iii) Globus/feeling of lump in throat (FLIT)
(iv) Throat clearing/tickle in throat
(v) Cough/choking
(vi) Thick mucus in throat ‘Post Nasal Drip’ or ‘Catarrh’
(vii) Laryngospasm/cough syncope
(viii) Sore throat
(ix) Hypertrophy
advice sheet is normally given to the patient, along with an explanation of the condition and how to manage it. The main principles include avoiding fatty foods, fizzy drinks (especially cola) and fruit juices. Food should not be taken late in the evening. Smoking should be avoided, and alcohol (particularly spirits, white and rose wine) will aggravate the condition. Patients prone to supine reflux will benefit from using extra pillows or propping up the head of the bed, and sleeping on the left side rather than the right may reduce the tendency to reflux. Vigorous exercise such as jogging or circuit training may exacerbate symptoms. Obesity and tight clothing will also make matters worse.

A reflux advice session from a trained therapist may improve compliance, and voice therapy for those patients with hoarseness can improve outcomes.

Liquid alginate preparations have been shown to be effective in treatment of LPR symptoms, either alone or in combination with proton pump inhibitors (PPI). They have the advantage of being a nonsystemic medication. If used as sole treatment, they should be given after each meal and last thing at night: nothing should be taken by mouth after the nocturnal dose.

H2 receptor antagonists have largely been superseded by PPIs, which are more effective at blocking gastric acid secretion, although they may have a role in those patients who suffer from nocturnal acid breakthrough despite twice daily PPI.

Proton pump inhibitor drugs currently available on the UK market are shown in Table 10, along with their equivalent doses. Omeprazole and lansoprazole are available as generic preparations: rabeprazole, esomeprazole and pantoprazole are still proprietary drugs. All PPIs act for 12–14 h, so in LPR they need to be taken twice daily to give full 24 h protection given the sensitivity of the upper aerodigestive tract to refluxate. A period of 2–3 months is necessary to establish benefit from the medication. Although there has been controversy regarding the use of PPIs in LPR, more recent research has shown evidence of benefit. If patients do not respond to the first drug chosen, or if they suffer unacceptable side effects, it is worth trying another drug as some patients respond better to one PPI than another. Most patients require treatment for approximately 6 months, and then should be gradually weaned from the drug to minimise the possibility of PPI rebound. Figure 21 illustrates a PPI dosage reduction schedule based on my own experience in using rabeprazole.

Proton pump inhibitor drugs are at best an indirect treatment for LPR, helping to reduce the activation of pepsin. At present there are no drugs which directly oppose the action of pepsin. Prokinetic agents such as domperidone or metoclopramide can be helpful in GERD, especially when there is oesophageal dysmotility, but they are usually unhelpful in LPR. Newer agents that protect against transient lower oesophageal sphincter relaxation are in development.

### Table 8 | Laryngopharyngeal reflux investigations

- (i) Usually none: clinical diagnosis
- (ii) Barium swallow
- (iii) Acid perfusion test (Bernstein)
- (iv) Oesophagoscopy
- (v) Manometry
- (vi) Oesophageal pH monitoring
- (vii) Oesophageal impedance (MCII-pH)
- (viii) Pepsin testing (Peptest)
- (ix) Airway pH monitoring (Restech)
- (x) Exhaled breath condensate pH monitoring

### Table 9 | Causes of laryngeal inflammation

- (i) Reflux
- (ii) Smoking/inhaled irritants
- (iii) Alcohol
- (iv) Allergy
- (v) Virus infections
- (vi) Voice abuse

![LPR treatment algorithm](image-url)
including GABA<sub>B</sub> agonists, and metatropic glutamate receptor 5 antagonists.

In the longer term, most patients can manage their condition by dietary and lifestyle modification, supplemented by a liquid alginate at night. Some patients also find it helpful to have an ‘as required’ PPI available.

A small number of patients either fail to respond to medical treatment or require long-term high-dose PPI to control their symptoms. If it is confirmed following investigation that LPR is present after 24 h dual channel pH monitoring or MCII-pH, then laparoscopic fundoplication has been found to be effective.

**CONCLUSION**

Laryngopharyngeal reflux is a commonly diagnosed condition in UK ENT clinics. Most patients are treated on the basis of a clinical diagnosis, with invasive investigations being reserved for patients where the diagnosis is uncertain or treatment proves difficult. New less invasive techniques (e.g. Peptest, Restech) may be of greater value in UK practice. The majority of cases respond to dietary and lifestyle modification, and medical treatment, with only a small proportion going on to receive antireflux surgery.

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INTRODUCTION

Gastro-oesophageal reflux disease (GERD) in children is present when reflux of gastric contents is the cause of troublesome symptoms and/or complications.\(^1\) The main areas implicated in extra-oesophageal reflux relate to the pulmonary and ENT systems and the oral cavity (dental erosions).\(^2\) Gastro-oesophageal reflux (GER) is common in asymptomatic children and especially in infants and therefore the presence of GER may be coincidental and not be contributing to any lung or ENT symptoms or disease that is occurring.

Many children with neurological or neuromuscular disorders have accompanying GERD and extra-oesophageal reflux disease. In such cases, dysfunctional swallowing may occur in isolation or in addition to GER and be associated with recurrent pulmonary aspiration.

Gastro-oesophageal reflux associated pulmonary disease

Respiratory symptoms occurring in association with GER could be the result of recurrent micro-aspirations but importantly, similar symptoms have been noted to occur without the actual penetration of gastric contents into the airway; a finding which could be related to direct acid stimulation of the lower oesophagus.\(^3\) Respiratory disease therefore may be: causally related to GER due to micro-aspiration, causally related to GER due to stimulation of the vagal reflex via pharyngeal or laryngeal vagal receptors or causally unrelated to GER (coincidental). The extra work of breathing associated with respiratory disease (e.g. asthma or cystic fibrosis) and treatments used may make GER more likely. Determining whether GER and the subsequent aspiration of gas-

SUMMARY

In children, respiratory and ENT disorders are associated with extra-oesophageal reflux. These include asthma, recurrent pneumonia, cough, apnoea, sinusitis, otitis media, laryngomalacia, recurrent croup and recurrent respiratory papillomatosis. The traditional tests of barium swallow, 24 h pH probing have limited ability to proving causality between the gastro-oesophageal reflux and the extra-oesophageal symptoms. Multichannel oesophageal impedance measurements have now been studied in children and when combined with pH monitoring will like become the gold standard investigation. It is also important but difficult to determine if the refluxate is entering the lung and while it has recently been shown that the lipid laden macrophage index obtained from bronchoalveolar lavage (BAL) lacks specificity detecting pepsin in BAL is both sensitive and specific for pulmonary aspiration.

Children with GER who have failed medical therapy are commonly referred for antireflux surgery. There is a paucity of well-controlled prospective data on the efficacy of fundoplication in children. The long-term results of antireflux surgery are mixed. Those children with isolated lower oesophageal sphincter incompetence can be expected to do well while those with dysmotility, such as neurologically impaired children, and those with reflux-related respiratory disease have a high rate of symptom recurrence.
Asthma

In a recent systematic review including 19 studies, Thakkar et al. determined that GERD was present on average in 23% (range 19–80%) of children with asthma. When only the five case–control studies were included, the odds ratio of having GER in asthma was 5.5. However, in a systematic review for the Cochrane Collaboration, Gibson et al. concluded that while antireflux treatment helps GER symptoms, there is little evidence that it improves asthma symptoms.

More recently, Sopo et al. specifically reviewed whether proton pump inhibitor (PPI) treatment improved asthma symptoms in children with asthma and GERD. They found four studies of which three reported benefit; however, the only double blind RCT showed no benefit.

Recurrence pneumonia

Owayed et al. extensively investigated 238 children with recurrent pneumonia. In children with a known major condition (e.g. neurological handicap or congenital heart disease), recurrent pulmonary aspiration was the likely diagnosis in almost half the children. In otherwise healthy children with recurrent pneumonia, recurrent pulmonary aspiration was second in frequency to asthma. Children with recurrent pulmonary aspiration also suffer more frequent symptomatic lower respiratory viral infections.

Chronic cough

In children with known underlying chronic lung disease, chronic cough has been reported to occur with GERD. Farrell et al. while investigating children with GERD found that those with chronic cough were more likely to have pepsin detectable in bronchoalveolar lavage (BAL) samples.

Two recent studies using strict criteria to define GER; (i) reflux index (i.e. the percentage of time that the distal oesophagus is exposed to a pH < 4) of ≥4% on pH metry or (ii) oesophageal biopsy showing reflux oesophagitis and the resolution of the cough with medical antireflux treatment. In both studies, GERD was found to be an unusual cause of chronic cough identified in less than 5% of cases. Five paediatric studies looking at the effectiveness of GERD treatment in children with cough were identified in a Cochrane review. While improvements in GERD symptoms were noted, there was insufficient evidence to make definitive conclusions as to whether GERD therapy is beneficial in chronic cough. Given the above data showed a relation between chronic cough and GERD in only a minority of patients, studies looking at the use of GERD treatment in this subgroup of children with chronic cough would be very worthwhile.

Apnoea and acute life-threatening episodes.

The association between GER and apnoea of prematurity (AOP) has been intensively studied but remains controversial. Several recent studies using multiple intraluminal impedance which detects both acid and non-acid GER found that GER episodes were equally likely before as after an apnoeic event. However, Corvaglia et al., in a well-designed study, used simultaneous and synchronised polysomnography and pH-impedance monitoring to detect and characterise apnoea, breathing movements, airflow, heart rate and O2 saturation while recording both acid and non-acid reflux events. This study confirmed that GER can trigger apnoea with the frequency of apnoea in the 30 s after GER being greater than in the 30 s before GER events. Overall the higher the number of GER triggered events that an infant suffered the higher the number of apnoeas observed. It should be noted that there is pathophysiological evidence for apnoea causing GER episodes too.

Results of studies reporting the prevalence of GER in infants presenting with ALTE are conflicting. Three...
small studies reported no increased frequency of GER in those with ALTE compared with controls, while a fourth and larger study with 62 infants suggested a much larger prevalence GER in those with ALTE (42% vs. 8%).

**ENT disease**

Extra-oesophageal reflux has been implicated in ENT conditions such as; sinusitis, otitis media, congenital stridor (laryngomalacia) and recurrent croup.

There has been steadily increasing awareness of the importance of GER moving further up into the larynx and pharynx (laryngopharyngeal reflux) in the pathogenesis and management of a variety of conditions involving the paediatric airway, in addition to other pathologies common in paediatric ENT. The interaction of reflux and the work of the paediatric airway surgeon can be broadly considered in three main groups:

(i) In certain conditions, it is possible that reflux is in part, or possibly, entirely responsible for the condition. For instance in laryngomalacia reflux is very commonly identified but it remains unclear as to whether the reflux is a cause or consequence of the condition.

(ii) There are some pathologies where there is a clearly identified cause but reflux disease may well worsen the condition. An important example of this is recurrent respiratory papillomatosis (RRP), caused by the Human Papilloma virus, which frequently requires multiple endoscopic procedures over protracted periods of time. There are a small number of uncontrolled studies which suggest that reflux could have a role to play in the severity of RRP.

(iii) Some airway procedures, particularly reconstructive procedures for airway stenosis may be compromised by failure to recognise and appropriately control gastro-oesophageal reflux.

There is, unfortunately, a paucity of good evidence on which to base treatment decisions regarding the role of GER in the paediatric airway and a significant amount of our knowledge base is based on expert opinion. This is an area where there is a clear requirement for further research into the effects of reflux.

One reason for the lack of robust evidence linking GER as the cause of respiratory or ENT disorders relates to our limited ability to confirm by conclusive testing that a child who has GER has related extra-oesophageal symptoms due to the GER.

**Investigations**

When faced with a child with respiratory and/or ENT symptoms and the need to either diagnose or exclude GER or extra-oesophageal reflux as the cause the clinician needs to; (i) ascertain if there is evidence of dysfunctional swallowing and direct aspiration of food/fluid with swallowing; (ii) determine if GER is occurring; (iii) determine if the contents of gastro-oesophageal reflux is entering the lung and based on these data, assess the likeliness that the symptoms are indeed related to the GER events. This can be a direct relationship, e.g. due to triggering of vagal receptors in the oesophagus, larynx or pharynx, but also a delayed effect as might be present in recurrent pneumonias. In the first group, an attempt can be made to prove a statistical association between GER and symptoms, while an association in the latter group can only be derived from indirect evidence. Additional problems with diagnosis result from a lack of standards for detecting both pulmonary aspiration and even the presence of pathological GER.

**Investigations of dysfunctional swallowing**

It is important to remember that recurrent small volume aspiration (micro-aspiration) may result from dysfunctional swallowing, sometimes referred to as ‘aspiration from above’. This is particularly important in the pre-term infant, neurologically handicapped child and babies with structural airway disorders (repaired oesophageal atresia or tracheoesophageal fistula, laryngeal cleft or cleft palate). Investigations for aspiration with dysfunctional swallowing include; history with observation of feeding, barium swallow, videofluoroscopic swallow study (VFSS), fibre-optic endoscopic evaluation of swallowing (FEES), bronchoscopy e.g. for laryngeal cleft, radionuclide salivagram and a trial of treatment (e.g. withholding oral feeds).

Investigations to determine if GER is present include:

(i) Upper gastro-intestinal contrast studies (useful for detecting anatomical defects, can not discriminate between physiological and pathological reflux).

(ii) 24 h oesophageal pH monitoring (used by many, but lacks sensitivity for detecting GER episodes as it does not detect weakly acidic GER events, which have been shown to be the majority of GER events in infants).

(iii) Gastric scintigraphy (‘milk scan’, lacks standardisation and has not been proven to be helpful in the diagnosis of GERD).

(iv) Oesophageal endoscopy and biopsy (only helpful when oesophagitis is considered, the absence, however, of oesophagitis in no way means that GER can be excluded as a cause of extra-oesophageal symptoms).
(v) Trial of treatment (e.g. proton pump inhibitors, often used in adults, but no results have been reported in children).

More recently multichannel intraluminal (oesophageal) impedance (MII) measurement has been shown to be a better way to detect both acid and non-acidic reflux. Multichannel intraluminal (oesophageal) impedance is a relatively new technique for evaluating oesophageal function and GER in children. As refluxate is a better conductor than an empty oesophagus, while air does not conduct electrical current, it is possible to use changes in impedance at multiple levels of the oesophagus to allow detection of both acid and non-acid reflux. In MII testing, no normal values are available for most of the paediatric age range, with the exception of tube fed premature infants. Strenuous efforts have been made and are ongoing to establish reference values for other age groups. Most recently, data from the German Pediatric Impedance Group (G-PIG) on 700 children presenting with symptoms suggestive of GER, on which multiple intraluminal impedance (MII)-pH recordings were performed, were presented. Thirty-seven percent of these children showed abnormal MII and pH-study, 18% showed only pathologic pH measurements and 45% had an abnormal MII-Recording only. In this population, extra-oesophageal symptoms of GER were seen more often in younger children and were more often related to a normal pH- but abnormal MII study. This shows in this largest systematically standardised data collection of MII-pH in children worldwide that a large amount of patients with GER-disease would not have been recognised by pH-measurement alone. Although these data are more than helpful in interpreting MII studies, it is unlikely that truly normative data will ever become available because of ethical considerations preventing the study of healthy children with invasive techniques.

Interobserver studies on MII-pH are well under way (including 10 international centres with four in Germany) which will help define reproducibility and validity of the use of this technology in clinical routine. There is still a certain amount of subjectivity in the interpretation of the data and the results of these studies will shed light on the differences and whether they influence clinical recommendations and research results. The validation of the automated analysis software implemented in the recording systems has been a high priority task in the past. The data analysis is now complete and first results of the expert scored database against the software, as well as improved software versions based on the findings are expected in the near future. A validated analysis software may be the last step for MII-pH from bench to bedside.

Using symptom association scores to establish a relation between symptoms and GER events would theoretically provide convincing evidence of causality and bypass the problem of unavailable healthy controls. However, several difficulties arise, one of which is the fact that the available means of establishing a temporal association over a standard 24 h period or any other time interval have been developed in adults with heartburn as a presenting symptom. In paediatrics, only adolescents present with heartburn and the younger the child, the less specific presenting symptoms are. It was recently shown that symptom association probability (SAP) scores as used in adults cannot simply be used for these nonspecific symptoms. SAP in infants can further be optimised, when the minimal number of symptoms is taken into account. In addition, different time epochs should be used for different symptoms in children. Furthermore, novel statistical means are being developed for the analysis of tracings with low baselines. Finally, and clinically most important, it was shown that PPI therapy is equally effective in infants as placebo but has more side effects. Physiologically, this is not remarkable, as gastric juice in these infants is very effectively buffered by frequent milk feedings. However, it is very common in paediatric wards to start infants on PPI therapy if they show signs of gastro-oesophageal reflux disease.

Tests to determine whether GER is associated with pulmonary aspiration

Upper gastrointestinal contrast studies may show gross reflux and occasionally the radiologist can observe the refluxate being aspirated. Gastro-oesophageal radionuclide scanning involves feeding the child with milk mixed with radiolabelled technetium. Subsequent serial scintigraphy to detect and locate tracer activity is performed for several hours after the feed. GER is identified when a column of radioactivity reappears in the oesophagus and aspiration has occurred when the radioactivity appears within the airways or lungs. Bronchoscopy and bronchoalveolar lavage (BAL) can be used to diagnose aspiration of stomach contents using the detection of staining for lipid using Oil Red within alveolar macrophages (Lipid Laden Macrophage Index, LLMI). To improve the specificity of this test researchers have attempted to determine if the lipids accumulated in macrophages are exogenous (rather than endogenous metabolic products of lipid membranes). Elidemir et al. using milk protein...
immunocytochemistry reported in a murine model that after a single aspiration of human milk a large number of alveolar macrophages displayed positive staining for alpha lactalbumin and beta lactoglobulin whereas none of the negative controls display immunoreactivity. This test needs further development.

The protein pepsin is produced in the stomach and should not be detectable in BAL fluid. The detection of pepsin in BAL fluid may be both a specific and sensitive method of diagnosing GER-related pulmonary aspiration. However, BAL sampling is an invasive procedure in children requiring anaesthesia. Induced sputum is a potentially less invasive method to obtain lower respiratory samples from older children. Unfortunately, a very recent study suggests that pepsin is detectable from both salivary and sputum samples of children with no respiratory or gastrointestinal symptoms suggesting that this is unlikely to be beneficial as a test. Likewise, Koksal et al. did not find that the LLMI in induced sputum to be useful in diagnosing pulmonary symptoms secondary to GER.

Unfortunately, in a particular child, it is often impossible to prove that aspiration is taking place or that GER is the cause of the respiratory symptoms and a trial of anti-GER treatment may be warranted.

Medical treatment of gastro-oesophageal reflux pulmonary disease
Treatment of the respiratory features of extra-oesophageal reflux logically calls for treatment of the underlying GER. This involves pharmacological management using thickened feeds, altering gastric pH, and prokinetic agents and while such treatments likely improve symptoms of GER, it is more difficult to prove improvement in respiratory symptoms.

Management of extra-oesophageal reflux in children - a surgical perspective
In paediatric practice, GER is considered to be pathological if associated with symptoms or complications which include respiratory and ENT disease, oesophagitis or failure to thrive (because of caloric loss through vomiting). A high proportion of children with pathological GER have an underlying neurological impairment and in this group, reflux is part of a much wider ‘pan-gut’ motility disorder. The standard surgical approach to patients with GER disease who have failed medical therapy is fundoplication. Fundoplication, one of the three most commonly performed major operations in childhood, is at least temporarily effective in patients whose underlying problem is primary lower oesophageal sphincter incompetence.

Much has been written about antireflux surgery in childhood. The vast majority of publications have been retrospective analyses with the potential for observer bias and often without objective outcome measures. Nevertheless, some authors report very impressive, albeit subjective, medium to long-term results of fundoplication in both neurologically normal and neurologically impaired children. Minimally invasive antireflux surgery has become increasingly popular leading some

Figure 22 | Gastrojejunostomy.

Figure 23 | Surgical jejunostomy.
to speculate that the threshold to intervene surgically may have reduced following the introduction of the laparoscope, and also effective prokinetics being taken off the market. On the other hand, some authors are more sanguine about the results of surgical intervention. In a prospective randomised trial of fundoplication (vs. medical therapy) in adults, almost two-thirds of patients were using antireflux medication at long-term post-operative follow-up, while Goyal reported symptom relapse in 60% of neurologically impaired children following Nissen fundoplication. Unsurprisingly, redo Nissen fundoplication also has a high failure rate. Some paediatric surgeons are therefore re-thinking their surgical strategy towards the management of GER with one centre reporting a 50% reduction in Nissen fundoplication in recent years. Unfortunately there is a paucity of randomised controlled trials assessing the efficacy of antireflux surgery in childhood evidenced by the fact that in two recent Cochrane reviews on the subject, the authors were unable to recruit a single appropriate article. One randomised controlled trial compared the metabolic impact of laparoscopic and open fundoplication in children. However, clinical outcomes were not reported.

Evidence for the efficacy of fundoplication in reflux-related respiratory disease is mixed. In adults, post-operative improvement in laryngopharyngeal reflux symptoms (hoarseness, globus, cough and pharyngitis) has been reported by Westcott et al. A review of antireflux surgery in asthmatics found that surgery may improve asthma symptoms but not pulmonary function and Wetscher noted improvement in respiratory symptoms in the majority of adult patients following either complete or partial wrap fundoplication. Furthermore, following lung transplantation, reflux-related bronchiolitis obliterans syndrome, a major cause of morbidity and mortality, may improve following antireflux surgery. However, none of these observations has been based on randomised controlled trials. Demeester reported that adults with GER and respiratory symptoms were more likely to respond well to fundoplication if they had normal oesophageal motility. In paediatric practice, a high proportion of patients with concomitant GER and respiratory disease have oesophageal dysmotility such as those with a history of oesophageal atresia or neurological impairment. Recurrence of respiratory symptoms following antireflux surgery is therefore common. Smith et al. reported recurrent pneumonia in 40%, while Cheung noted that the incidence of pneumonia was unchanged following fundoplication. These poor results are due either to ongoing aspiration of oropharyngeal secretions (common in neurologically impaired children or those with previous oesophageal atresia) or because GER has recurred. While some authors report low rates of recurrent GER, others report rates as high as 60% particularly in neurologically impaired patients.

It is no surprise therefore that alternative surgical strategies, such as feeding jejunostomy or oesophagojejunostomy, have been sought. Placement of a gastrojejunostomy tube has the advantage of allowing gastric decompression as well as establishing jejunal feeding (Figure 22). However, difficulties maintaining tube placement often renders these tubes impractical for long-term use. A more permanent solution is a surgical jejunostomy (Figure 23). While this is a relatively simple procedure to perform, complications are reported to occur in over a third of cases. With either type of jejunal feeding device, the patient is restricted to continuous feeding over many hours, and aspiration of gastric secretions may still occur. Oesophagogastric disconnection with oesophagojejunal anastomosis (Figure 24) is a major procedure, and is used almost exclusively in severely neurologically impaired patients. The advantages include establishment of bolus gastrostomy feeding (the stomach having been disconnected from the oesophagus) and less pooling of oropharyngeal secretions in the oesophagus. While reported series are small, some encouraging results have been published. However, the complication rate in

**Figure 24** | Oesophagogastric disconnection with Oesophagojejunostomy anastomosis.
these complex patients is high ranging from 30 to 43%, 61, 62

Otolaryngologists have been adopting surgical strategies to prevent aspiration. Botulinum toxin A injection into the salivary glands reduces saliva production in children with neurological disorders, 63 but its effect on chronic aspiration has not been thoroughly studied. Tracheotomy may fail to control aspiration leading some otolaryngologists to recommend laryngotracheal separation in intractable cases. 64

CONCLUSION

Respiratory and ENT disorders are associated with extraoesophageal reflux. These include asthma, recurrent pneumonia, cough, apnoea, sinusitis, otitis media, laryngomalacia, recurrent croup and recurrent respiratory papillomatosis. Proving causality between the gastrooesophageal reflux and extra-oesophageal symptoms has proven more difficult.

Multichannel oesophageal impedance measurements have now been studied in children and when combined with pH monitoring are becoming the recommended standard of investigation.

Children with extra-oesophageal GER who have failed medical therapy are commonly referred for antireflux surgery. There is a paucity of well-controlled prospective data on the efficacy of fundoplication in these children. As long as these data are unavailable, only children with life-threatening symptoms should be considered for surgical treatment.

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INTRODUCTION

Gastro-oesophageal reflux disease (GERD) is defined as chronic symptoms or mucosal damage caused by the abnormal reflux of gastric contents into the oesophagus. Symptoms and oesophageal injury are traditionally considered to be caused by acid and the proteolytic enzyme pepsin. The greater the frequency and amount of acid exposure, the more severe the degree of oesophagitis. The majority of patients with symptoms of GERD, however, do not have endoscopic evidence of oesophagitis and up to 60% of patients with non-erosive reflux disease (NERD) have normal oesophageal acid exposure on ambulatory pH testing. These patients are less likely to improve with proton pump inhibitors (PPIs) and the clinician must think beyond the use of antacids to optimise treatment outcomes.

The mainstay of therapy for GERD has focused on enhancing the safety and efficacy of antacids. Proton pump inhibitors (PPIs) are the most effective medications available to increase intragastric pH. Newer, faster acting drugs with longer half-lives are continuously being developed. Even with these potent antacids, however, a significant percentage of persons with symptoms of GERD fail to improve with PPI therapy. In this group of patients, refractory symptoms may be the result of non-acid reflux (NAR). Treatment of NAR ranges from simple reassurance to antireflux surgery. Current treatment algorithms include a combination of dietary and lifestyle modifications, antacid medication, inhibition of transient lower oesophageal sphincter relaxations, and/or enhancement of the antireflux barrier. Because of the nonspecific nature of patient symptoms and the frequently elusive diagnosis, it is essential to exhaust conservative treatment options before resorting to surgical intervention. The purpose of this manuscript is to review the contemporary nonsurgical management of NAR.

SUMMARY

A significant percentage of people with gastro-oesophageal reflux disease (GERD) fail to improve with proton pump inhibitor (PPI) therapy. In this group of patients, refractory symptoms may be the result of non-acid reflux (NAR). Treatment of NAR ranges from simple reassurance to antireflux surgery. Current treatment algorithms include a combination of dietary and lifestyle modifications, antacid medication, inhibition of transient lower oesophageal sphincter relaxations, and/or enhancement of the antireflux barrier. Because of the nonspecific nature of patient symptoms and the frequently elusive diagnosis, it is essential to exhaust conservative treatment options before resorting to surgical intervention. The purpose of this manuscript is to review the contemporary nonsurgical management of NAR.

Diagnosis of NAR

Ambulatory pH testing has been available for over 30 years. Both hard-wired and wireless telemetry pH sensors are available to quantify the frequency and duration of oesophageal acidification. These devices, however, are not capable of measuring NAR. Combined multi-
channel intraluminal impedance and pH monitoring (MII-pH) is a relatively new diagnostic tool that has the ability to detect reflux episodes independent of pH. Although fluoroscopy can identify NAR, MII-pH monitoring is the only available test capable of quantifying the frequency and duration of NAR episodes.\textsuperscript{11, 12} When a patient fails to improve despite adequate acid suppression, several options exist for the subsequent evaluation. First, the clinician should consider alternative causes for the patient’s symptoms. Cough, chest tightness and pain, postnasal drip, throat clearing, globus and hoarseness can all be caused by entities other than reflux. Comprehensive upper aerodigestive tract endoscopy should be considered to rule out infectious, neoplastic and allergic disease responsible for the persistent symptoms. If alternative causes cannot be identified and endoscopy is unremarkable, MII-pH monitoring may be considered. The test may be performed on or off medication. Advantage of keeping the patient on PPIs is that if the study reveals the presence of NAR only, increasing the dose of PPIs will have no additional benefit.\textsuperscript{9, 13} If the study reveals persistent acid reflux, however, increasing the dosage of acid suppressive medication, changing the PPI, or adding a histamine-2 receptor antagonist may be beneficial. The advantage of performing a pH study off medication is that a baseline level of acid and NAR can be obtained. Hard-wired ambulatory pH and impedance testing, however, is painful, unsightly, causes dysphagia and reduces reflux-provoking behaviour.\textsuperscript{14} This significantly limits the reliability of hard-wired catheter-based testing. Because of this shortcoming, a wireless reflux monitoring system has been developed (Bravo pH System, Given Imaging, Ltd., Yoqneam, Israel). The Bravo pH monitoring system utilises a wireless capsule that is atraumatically placed in the oesophagus. Patients with the wireless system are typically unaware of the capsule. Instead of going home with a tube coming out of their nose, the only thing that they have to be concerned with is a cell phone-sized receiver attached to their belt or purse. Because the monitoring is painless, patients do not miss a day of work and continue to partake in their normal daily activities. This is thought to enhance the reliability of the diagnostic test. The major disadvantage of the Bravo system is its inability to detect NAR. The test should therefore be reserved for evaluating patients off PPIs and other antacid medications.

The role of lifestyle modifications in the treatment of NAR
There are little data to support the use of diet and lifestyle modifications as a solitary treatment of NAR. The majority of patients with NAR, however, do not have erosive oesophagitis, intestinal metaplasia or dysplasia. NAR, therefore, is frequently a symptom driven, quality-of-life disease and it is appropriate to recommend dietary and lifestyle modifications as primary or complementary treatment. Table 11 lists the dietary and lifestyle modifications recommended by our centre.

The role of proton pump inhibitors in NAR
The success of acid suppression in the management of patients with GERD is reflected in the dramatic reduction of elective surgery for peptic ulcer disease. Mucosa healing with PPIs is usually achieved within 8 weeks of treatment in the majority (>90%) of patients with oesophagitis.\textsuperscript{8, 10} In addition to elevating gastric pH, PPIs may exert an effect on patients with NAR by reducing the volume of postcibal gastric contents. Gastric volume as determined by magnetic resonance imaging is reduced by 12% after 7 days of twice-daily 40 mg esomeprazole.\textsuperscript{15} The effect of PPI on gastric volume reduction may last 75 min after a meal.\textsuperscript{15} There is a suspected relationship between gastric volume and the frequency of transient lower oesophageal sphincter relaxation associated reflux events.\textsuperscript{16, 17} A reduction in gastric volume should reduce both acid and NAR. The effect of PPIs on gastric volume, however, may be negated by the effect of the drugs on gastric emptying. A systematic review evaluating the effects of PPIs on gastric emptying suggests that the drugs delay gastric emptying of solid food presumably through a reduction in intragastric peptic digestion.\textsuperscript{18}

<table>
<thead>
<tr>
<th>Table 11</th>
<th>Dietary and lifestyle modifications</th>
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<tr>
<td><strong>Recommended dietary and lifestyle modifications</strong></td>
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<tr>
<td>Smoking cessation</td>
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<tr>
<td>Elimination of alcohol consumption</td>
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<td>Weight loss with a low-fat, low-carbohydrate diet</td>
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<tr>
<td>Elevate the head of bed during sleeping</td>
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<tr>
<td>Stress reduction</td>
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<tr>
<td>Exercise</td>
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<tr>
<td>Avoid spicy and acidic foods</td>
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<tr>
<td>Avoid carbonated beverages</td>
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<tr>
<td>Reduce caffeine consumption</td>
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<tr>
<td>Avoid peppermint and spearmint</td>
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<tr>
<td>Avoid chocolate</td>
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<td>Avoid the consumption of large meals</td>
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<tr>
<td>Avoid eating and lying down for 3 h</td>
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Thus, the influence of PPIs on gastric volume is multifactorial, and the overall effect of the drugs on the frequency of reflux events is negligible. In fact, symptoms of reflux may persist in nearly 50% of patients with NERD who are treated with once-daily PPI.

Transient lower oesophageal sphincter relaxation and reflux ‘inhibitors’

The lower oesophageal sphincter (LES) plays a crucial role in maintaining the mechanical barrier necessary for prevention of gastric reflux. Reflux mainly occurs during prolonged relaxations of the LES not related to swallowing. These relaxations are known as transient lower oesophageal sphincter relaxations (TLESRs) and are thought to play a pivotal role in the mechanism of reflux in both healthy subjects and GERD patients. Pharmacological inhibition of TLESRs is a promising approach in an attempt to reduce NAR.

Baclofen is a γ-Aminobutyric acid B (GABA_B) receptor agonist primarily used to treat spasticity and hiccups. Lehmann et al. demonstrated that activation of the GABA(B) receptor inhibits TLESRs in dogs. Shortly after, Lidums et al., assessed the effect of 40 mg baclofen given 90 min before a meal on postprandial gastrooesophageal reflux and TLESRs in 20 healthy volunteers and reported that baclofen significantly inhibited gastrooesophageal reflux by TLSER inhibition. Using MII-pH, Vela et al. compared acid and NAR after placebo and baclofen 40 mg in nine healthy volunteers and nine heartburn patients and found that Baclofen reduces postprandial acid and NAR and their associated symptoms. Ciccaglione et al. studied the effect of acute (1 day) and chronic (4 weeks) administration of baclofen 10 mg four times daily in GERD patients and normal controls. Baclofen reduced 24 h reflux frequency and duration and increased gastric pH in both GERD patients and controls. When given for 1 month to reflux patients, baclofen reduced both reflux frequency and duration, increased intragastric pH and improved symptom scores. Koek et al. investigated the effect of baclofen in sixteen patients with persistent non-acid duodenal reflux during PPI therapy. Baclofen 5 mg three times daily was increased by 5 mg every fourth day until a maintenance dose of 20 mg three times daily was reached. Baclofen improved duodenal reflux and associated symptoms that persisted during PPI therapy alone.

Potential side effects of baclofen are not inconsequential and include constipation or diarrhoea, nausea, dizziness, drowsiness, urinary difficulty, headache and muscle weakness. The side effect profile of the medication has a significant effect on patient compliance. New GABA(B) agonists with fewer side effects are under development and preliminary trials are promising for use as add-on therapy to PPIs. In a recent multicentre, randomised, double-blind, crossover study comparing an investigational prodrug of the active R isomer of baclofen vs. placebo, Arbaclofen placarbil decreased reflux and associated symptoms with good tolerability. The safety, efficacy and advantages of this new drug remains to be established in phase IIb and III trials.

Prokinetics

Prokinetics or gastroprokinetics exert their influence by increasing acetylcholine concentration by antagonising the D2 receptor which inhibits acetylcholine release and by inhibiting acetylcholinesterase which metabolises acetylcholine. Higher acetylcholine levels increase gastrointestinal peristalsis and stimulate gastrointestinal motility. Vaezi et al., in a double-blind placebo controlled crossover study, evaluated the efficacy of Cisapride in postgastrectomy patients with duodenogastro-oesophageal reflux. Cisapride significantly reduced duodenogastrooesophageal reflux and improved symptom scores. Miyamoto et al. offered the prokinetic mosapride 5 mg three-times daily to patients failing therapy with once-daily PPI. The addition of the prokinetic improved symptom scores in persons dissatisfied with PPI monotherapy.

In a similar study without a placebo arm, Fujiyama et al. evaluated the effect of mosapride 15 mg daily in PPI-resistant NERD patients. The addition of mosapride to omeprazole significantly improved reflux symptoms. A recent Cochrane review evaluating the benefit of prokinetics, however, concluded that there is
insufficient evidence to conclude that prokinetic therapy is superior to placebo in the treatment of reflux oesophagitis. The questionable benefit of prokinetics, in addition to the drugs’ propensity to increase the QT interval and cause cardiac arrhythmia and death, has significantly limited the use of these medications by most clinicians.

Alginates
Alginates have been used for years for the over-the-counter treatment of reflux disease. Sodium alginate has a unique mechanism of action and can form a mechanical antireflux barrier that may be beneficial in the treatment of acid and NAR. In the presence of gastric acid, the alginate precipitates and forms a gel. The alginate is combined with sodium or potassium bicarbonate (Gaviscon Advance, Reckitt Benckiser Healthcare Ltd, Hull, UK). When in contact with gastric acid, the bicarbonate is converted into carbon dioxide which becomes trapped within the gel precipitate, converting it into foam which floats on the surface of the gastric contents, similar to a raft on water (Figure 25). Raft formation occurs rapidly, often within a few seconds of dosing; hence alginate-containing antacids are comparable with traditional antacids for speed of onset of relief. Chattfield et al. in a randomised, double-blind multicentre study reported that a raft-forming alginate suspension was superior to placebo for treatment of GERD. The role of a liquid alginate suspension in protecting the oesophagus from damage by pepsin and bile acids was recently investigated using a series of in vitro models. The authors reported that the formulation reduced pepsin activity and was able to limit both pepsin and bile acids from the refluxate. The value of liquid alginate in the management of extra-oesophageal reflux was recently assessed in an open-label pilot study. Significant improvement was noticed in symptom scores and clinical findings with the alginate suspension.

CONCLUSIONS
Recent advances in our ability to measure NAR by means of MII-pH monitoring has improved our understanding and treatment of this aspect of reflux disease. PPIs have a limited role in the management of the non-acid component of reflux, although patients may welcome the relief they get from reduction of their acid reflux. Currently available therapeutic options include diet and lifestyle modifications, inhibition of TLESRs and/or enhancement of the antireflux barrier.

REFERENCES
ACKNOWLEDGEMENTS

Declaration of personal interests:

Guest editors:

Peter W. Dettmar has served as a consultant to Reckitt Benckiser Healthcare (UK) Ltd and owns ordinary shares in Reckitt Benckiser and GW Pharmaceuticals.

Robert C. Heading has received remuneration as a speaker and advisory board member from Nycomed (formerly ALTANA Pharma) and from Reckitt Benckiser. He owns ordinary shares in Novartis, Procter & Gamble and Reckitt Benckiser.

Donald O. Castell has served as a speaker, consultant or advisory board member for Xenoporo, Addex, NorGINE, SANDHILL SCIENTIFIC and Pfizer. He has received research funding from Pfizer, Xenoporo and Addex.

Authors:

Jeffrey Pearson has served as a speaker at a symposium organised by Reckitt Benckiser Healthcare (UK) Ltd.

Shruti Parikh has no personal interests to declare.

Roy C. Orlando has served as consultant and has research support from AstraZeneca. He also has research support from Takeda Pharmaceuticals and recent research support from Procter & Gamble.

Nikki Johnston has no personal interests to declare. Jaqueline Allen has no personal interests to declare. Steven P. Tinling has no personal interests to declare.

Peter C. Belafsky has served as a consultant for Medtronic Inc., Olympus Inc., Nestle, Inc. and Primal Pictures, and has received research funding from Coapt Inc., Nestle, Inc. and Arthrocure Inc. He is on the Clinical Advisory Board for Covidien. He owns a patent for the swallowing expansion device (ID).

Donald O. Castell has served as a speaker, consultant or advisory board member for Xenoporo, Addex, NorGINE, SANDHILL SCIENTIFIC and Pfizer. He has received research funding from Pfizer, Xenoporo and Addex.

Lubin F. Arevalo has no personal interests to declare.

Neeraj Sharma has no personal interests to declare.
Mark Fox has served as a speaker, a consultant and an advisory board member for Movetis, and has received research funding from Reckitt Benckiser, AstraZeneca, Given Imaging and Nestle.

Susan M. Harding has served as a consultant to Caremark/CVS under a contract with the University of Alabama at Birmingham (Jul 1, 2008–Dec 31, 2009) and receives research funding from the National Institutes of Health/National Heart Blood and Lung Institute (R02 HL 075614-5 9/23/08–5/31/12).

Alyn H. Morice has received consulting fees from Boehringer Ingelheim, Pfizer, GlaxoSmithKline, and Proctor & Gamble, lecture fees from Boehringer Ingelhei and AstraZeneca, and grant support from Proctor & Gamble.

Mark Watson has served as a speaker and advisory board member for Reckitt Benckiser, and has received donations to a Departmental Trust Fund from Reckitt Benckiser.

Michael D. Shields has received honoraria for speaking at educational meetings and received hospitality allowing attendance at International conferences from the following pharmaceutical companies: GlaxoSmithKline, AstraZeneca, Novartis, Altana, 3M, Merck Sharp & Dohme and Alk Abello.

Michiel van Wijk has no personal interests to declare.

William McCallion has no personal interests to declare.

Tobias Wenzl has served as a speaker, a consultant or an advisory board member for Sandhill Scientific, Tecnomatix Germany, and Reckitt Benckiser, and has received research funding from AstraZeneca and Sandhill Scientific.

Petros Karkos has no personal interests to declare.

Declaration of funding interests: This supplement has been commissioned and funded by Reckitt Benckiser Healthcare (UK) Ltd. The authors have received an honorarium from Reckitt Benckiser Healthcare (UK) Ltd for writing their articles. Reckitt Benckiser Healthcare (UK) Ltd did not have editorial control over the content of the articles prior to publication. The articles have been reviewed by three guest editors who each received an honorarium from Reckitt Benckiser Healthcare (UK) Ltd.

Guest editors declaration: The three Guest Editors Peter W. Dettmar, Robert C. Heading and Donald O. Castell have individually reviewed each paper submitted for inclusion in the supplement entitled “Review article: Reflux and its consequences – the laryngeal, pulmonary and oesophageal manifestations”. All papers have been fully approved by all three Guest Editors and no ethical problems with any contributions were detected.