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CONCISE REVIEW

Pepsin: biomarker, mediator, and therapeutic target for reflux and aspiration

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Extra-esophageal reflux is suspected to cause a wide range of clinical symptoms in the upper airways. Diagnosis and treatment has focused on acid, but realization of the role of nonacid reflux has resulted in research investigating the use of pepsin as a biomarker for gastric reflux and aspiration. Pepsin analysis can complement the use of questionnaires and office-based diagnosis and lessen the dependency on invasive and expensive diagnostic tests. Furthermore, pepsin as a first-line diagnostic biomarker has been shown to improve the accuracy of reflux diagnosis. In addition to its use as a diagnostic biomarker, pepsin has been shown to cause inflammation independent of the pH of the refluxate and thus despite acid suppression therapy. Research is ongoing to develop new therapies for airway reflux that specifically target pepsin.

Keywords: pepsin; reflux; airway; inflammation; carcinogenesis; therapeutics

Biomarker

The history of pepsin dates back to 1836, when it was discovered by Theodor Schwann.[1] In 1938, Herriott[2] studied the conversion of pepsinogen to pepsin, which is now considered to be the most aggressive proteolytic enzyme in gastric refluxate.[3] Pepsin has been identified as a biomarker of gastric reflux into the esophagus, airways, and lungs. Pepsin has been detected in saliva, sputum, and secretions from the trachea, lung, nose, sinus, middle ear, and exhaled breath condensate.

There are currently several diagnostic methods available to confirm or reject if reflux disease is responsible for a patient's symptoms. However, these tests are invasive, expensive, and have low sensitivity and specificity. For example, symptom questionnaires have 63% sensitivity and 67% specificity,[4] endoscopy has approximately 30% sensitivity for diagnosing reflux disease,[5] and pH-metry has sensitivity in the region of 60%.[6] None of these can be considered as first-line diagnostic tests. More recently, Peptest (RD Biomed Limited, UK), a noninvasive test based on lateral flow technology, was introduced to rapidly identify reflux in patients presenting with a range of reflux symptoms. Patients diagnosed with symptoms of gastro-esophageal reflux disease (GERD),[7,8] extra-esophageal reflux (EER), laryngopharyngeal reflux (LPR),[9] and various respiratory diseases[10] have been tested for reflux using Peptest to identify pepsin as a biomarker of reflux disease.

Pepsin is composed of a family of isoenzymes, with pepsin 3b being the most prominent. It is active over a wide pH range and is not irreversibly denatured until above pH 7.5.[11,12] This makes pepsin ideally suitable as a biomarker for detecting reflux in clinical samples and especially as a biomarker for detecting reflux above the upper esophageal sphincter for EER, LPR, and respiratory reflux (frequently referred to as airway reflux), which is considered more challenging to diagnose.

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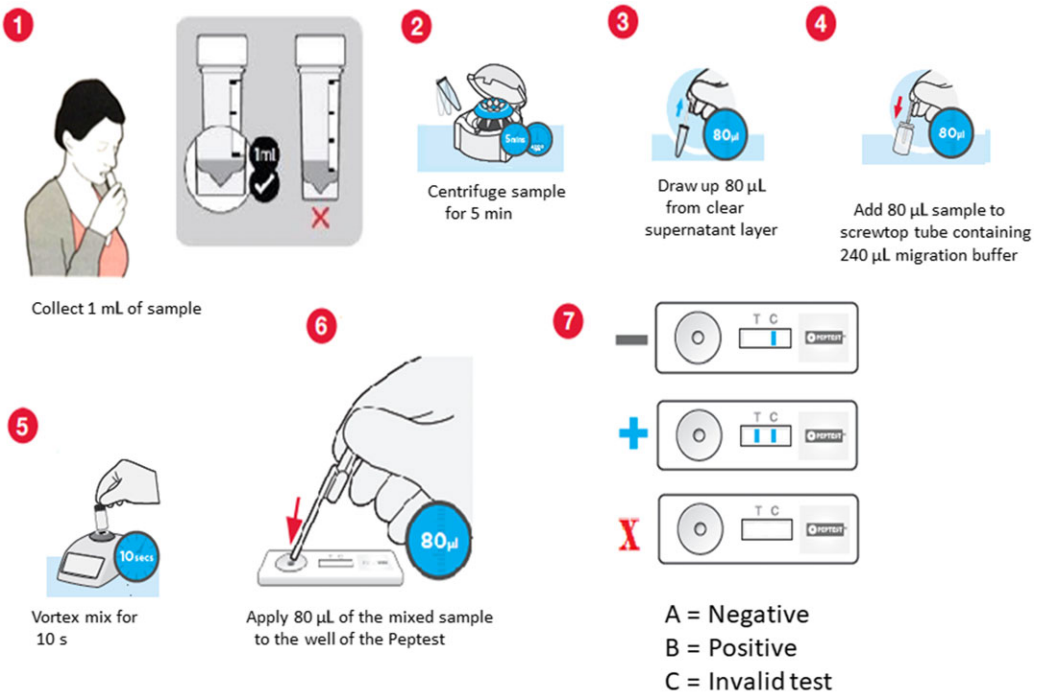

Figure 1. Schematic procedure of Peptest from sample collection to sample analysis.

For a diagnostic test to be considered as a first-line test, it needs to be simple to use, noninvasive with high patient compliance, give rapid results, and be cost-effective. Peptest has been clinically validated throughout the UK and Europe and has been used to determine the presence of pepsin in, for example, saliva samples provided by patients at specific time points. Samples are collected in the morning upon waking and before eating and cleaning teeth, postprandial 60 min after finishing a meal, or 15 min after experiencing symptoms. All samples are stored in a refrigerator before being analyzed for the presence of pepsin using Peptest. Sample collection tubes are centrifuged at 4000 rpm for 5 min; 80 µL from the surface layer of the centrifuged sample is then transferred to a microtube containing 240 µL of migration buffer and the sample mixed with a vortex mixer for 10 s. Afterward, 80 µL of the mixed sample is transferred to the circular well of a lateral flow device (LFD; Fig. 1). The LFD contains two unique antipepsin human monoclonal antibodies, one to capture and another to detect pepsin. The intensity of the pepsin test line within the window of the LFD is measured using a Peptest cube reader and automatically converted into the concentration of pepsin (ng/mL) present. Preliminary data are being collected from an ongoing study.

There is growing evidence that reflux into the airways is responsible for the etiology and the exacerbation of a range of respiratory conditions, including idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, chronic cough, cystic fibrosis, and chronic rhinosinusitis. Some of our groups have published extensively on the ability of pepsin to function as a biomarker of reflux in the upper airways, but data are still evolving on the association between the presence of pepsin and combined multichannel intraluminal impedance pH-metry (pH-MII) data. Pepsin has excellent positive and negative predictive values as a biomarker to detect reflux, and is known to decrease markedly after fundoplication.[13] One prior study has evaluated the connections between pH-MII, salivary pepsin, and nasopharyngeal reflux testing and shown that pepsin and pH-MII appear to correlate well, while nasopharyngeal reflux probe data did not seem to be as effective.[14]

Our group has designed a study to directly evaluate the ability of pH-MII to detect abnormal pharyngeal refluxate exposure and correlations

with salivary pepsin presence in patients with LPR and asymptomatic volunteers. Patients are being recruited for participation in this study as part of the normal clinical care in our tertiary Laryngology and Professional Voice clinic for complaints of chronic cough, dysphonia, globus sensation, throat irritation/clearing, and throat pain. Patients all receive standard 24-h pH-MII testing using the Sandhill ComforTec LPR probe system (Diversatek Healthcare, Milwaukee, WI), which includes a pharyngeal impedance array that straddles the pharyngeal pH detector. In addition, five daily sputum specimens are collected for pepsin analysis during the study period: upon placement of the probe, 30 min following each meal, and upon awakening. An additional nasal lavage is collected at the time of pH-MII probe placement for pepsin analysis. Sputum pepsin levels are being assessed via standard ELISA assay using our proprietary pepsin antibody, and confirmed by western blot. We define elevated reflux exposure as any pharyngeal pH drop below 4.0, or greater than 40 proximal impedance events. Our study to date has included 26 symptomatic patients and 13 controls. Our preliminary data support the potential for salivary pepsin to serve as a noninvasive screening modality for elevated pharyngeal reflux exposure and suspected LPR. Sputum pepsin presence appears to correlate well with some pH-MII parameters that are elevated in GERD patients, but further patient recruitment is required to define specific correlations between patient symptoms, pharyngeal pH-MII data, and salivary pepsin presence.

It should be noted that pepsin may only be transiently present in saliva as a result of the transient nature of reflux and intermittent influence of swallowing, both of which will vary with food intake. To that end, optimization of the timing and method of acquisition of salivary samples to yield the greatest balance of sensitivity and specificity is essential if pepsin is to be used clinically as a marker of disease. Although saliva samples are typically collected at least 1 h after meals to avoid detection of postprandial reflux events, there have not been any large-scale studies to determine the optimal timing or method of sample collection, and hence the optimal timing for collection of samples is still unclear. Larger studies with more frequent collection of samples may allow determination of the ideal time for sample collection, which might in turn facilitate the use of salivary pepsin as a more sensitive and specific tool for diagnosing airway disease.

While salivary pepsin does represent evidence of reflux events and tracheal or bronchoalveolar lavage (BAL) pepsin is clear evidence of aspiration, measurement of pepsin in secretions may not adequately reflect its uptake via receptor-mediated endocytosis in laryngeal and lung epithelial cells. As such, intracellular pepsin or tissue-bound pepsin may be more indicative of pepsin-mediated disease than salivary or tracheal/BAL pepsin. Other factors, such as compromise of mucosal defense mechanisms of the tissues in question, may predispose to increased endocytosis of pepsin, setting the stage for its intracellular activation and downstream development of an inflammatory response. Although the invasiveness of sample collection would likely preclude the use of pepsin within tissues as a diagnostic marker, the precise role of pepsin in LPR and other reflux-mediated airway diseases may be elucidated by future studies examining both factors, which might contribute to increased receptor-mediated endocytosis of pepsin, and the relationship between pepsin in salivary secretions and pepsin in laryngeal tissues.

Mediator

Proton pump inhibitors (PPIs) are the gold standard therapy for GERD. However, the efficacy of PPIs for the treatment of airway reflux, including LPR, is poor.[15] The airway is believed to be more sensitive to gastric refluxate than the esophagus; thus, patients with reflux laryngitis are given higher doses and longer trials of PPIs compared to patients with typical GERD.[16–18] Despite higher doses and longer trials of PPIs, most placebo-controlled trials revealed little to no therapeutic benefit[19–24] and it has been highlighted that the two studies which did suggest some therapeutic benefit of PPIs for the treatment of LPR[25,26] actually showed improvement in heartburn symptoms, not chronic throat symptoms.[23] Given the lack of data supporting a beneficial effect of PPIs for EER symptoms, it has been recommended against their use for proximal airway reflux in the absence of classic GERD symptoms.[27] However, PPIs are still frequently prescribed for LPR.[28] Given the potential risks of prolonged PPI use, the financial cost of such, and their poor efficacy for the treatment of LPR, an alternative treatment for LPR is needed.[3,29]

Combined multichannel intraluminal impedance and pH monitoring has been used to demonstrate the poor efficacy of PPIs for the treatment of LPR,[30] symptom association with nonacid reflux, and symptom alleviation following surgery to prevent reflux of all gastric contents.[31] The data obtained using this technology changed the perception that LPR and airway reflux are acid-mediated diseases, but instead primarily mediated by nonacid reflux events for which PPI therapy is not sufficient.[3]

Studies analyzing cell morphology, mitochondrial function, and the expression of stress response genes in laryngeal specimens and cultured hypopharyngeal epithelial cells treated with pepsin revealed that endocytosed nonacid pepsin causes toxicity.[32,33] The consequence of reflux damage and cause of symptoms is chronic mucosal inflammation. In that regard, pepsin, independent of acid, induces expression of a cytokine profile in hypopharyngeal cells similar to that in reflux esophagitis and which are known to contribute to the pathophysiology of GERD.[34] Thus, pepsin may contribute to symptoms and endoscopic findings associated with nonacid reflux and despite PPI therapy.

In vitro studies suggest that bile can cause laryngeal inflammation at both acid and nonacid pH. However, others have suggested that "there is no evidence that the same mechanism occurs in the human larynx."[35] *In vitro* exposure to bile acids causes blebbing of the cell membrane,[36] but to our knowledge this has never been reported in laryngeal mucosa from patients with LPR. The concentrations of bile salts and acids used in previous studies were high, ranging from 5 to 50 mM. Bile salt concentration in the human duodenum ranges from 10 to 22 mM.[37] The physiological bile acid/salt content in gastric refluxate reaching the laryngopharynx is expected to be in the micromolar range.[38] To our knowledge, this bile salt/acid concentration has not been shown to cause laryngeal damage. However, there is no clear evidence quantifying the concentration of each bile acid in the laryngopharynx. It should also be noted that the unconjugated bile acids that cause damage at higher pH, consistent with the environment of the laryngopharynx, are rarely found in gastric refluxate.[38,39] Further research is needed to clarify the role of refluxed bile in airway inflammatory disease.

Over 900,000 adults receive invasive mechanical ventilation (MV) in the United States each year.[40,41] Though MV is an essential, life-saving therapy for critically ill patients, it also places them at risk of morbid complications termed ventilator-associated complications (VACs).[42] VACs are heath care–acquired conditions that are considered preventable. They include acute lung injury, ventilator-associated pneumonia, atelectasis, and pulmonary edema. Acquisition of VACs confers heightened risk of poor outcomes, including death and prolonged MV. Aspiration has been proposed by many as a key factor in the development and worsening of numerous VACs (Fig. 2).[43,44] Aspiration of both gastric contents (gastric aspiration) and of oropharyngeal secretions (salivary aspiration) has been described. The relative contribution of either type of aspiration on the development of VACs is unknown, because recognizing and distinguishing between these different types of aspiration remains challenging. Clinicians need to detect aspiration early so that interventions targeted toward gastric aspiration, oropharyngeal aspiration, or both can be used to prevent further aspiration events and ameliorate poor outcomes. Salivary amylase, which is not normally found in the lungs, has recently been proposed as a biomarker for oropharyngeal aspiration. A recent study reported that BAL amylase is not only associated with risk factors for aspiration, but may also be useful as an early screening tool to guide management of patients suspected of aspiration.[45] We recently investigated the prevalence of gastric aspiration in a cohort of pediatric patients with chronic respiratory symptoms and in patients with tracheostomies by assessing the presence of pepsin in BAL specimens.[46] Pepsin-positive BAL specimens were identified in 25 patients who underwent bronchoscopy (74%) and 22 patients with tracheostomy (71%). All specimens from controls ($n = 11$) were negative for pepsin.

Role of pepsin in aero-digestive cancers

In addition to a role for LPR in laryngeal inflammatory disease, many have also shown an association between LPR and laryngeal cancer and suggest that LPR is a significant risk factor; however, causality has never been proven. It is accepted that chronic reflux into the esophagus causes inflammation—reflux esophagitis. Chronic reflux esophagitis can cause a metaplastic change in the cells that line

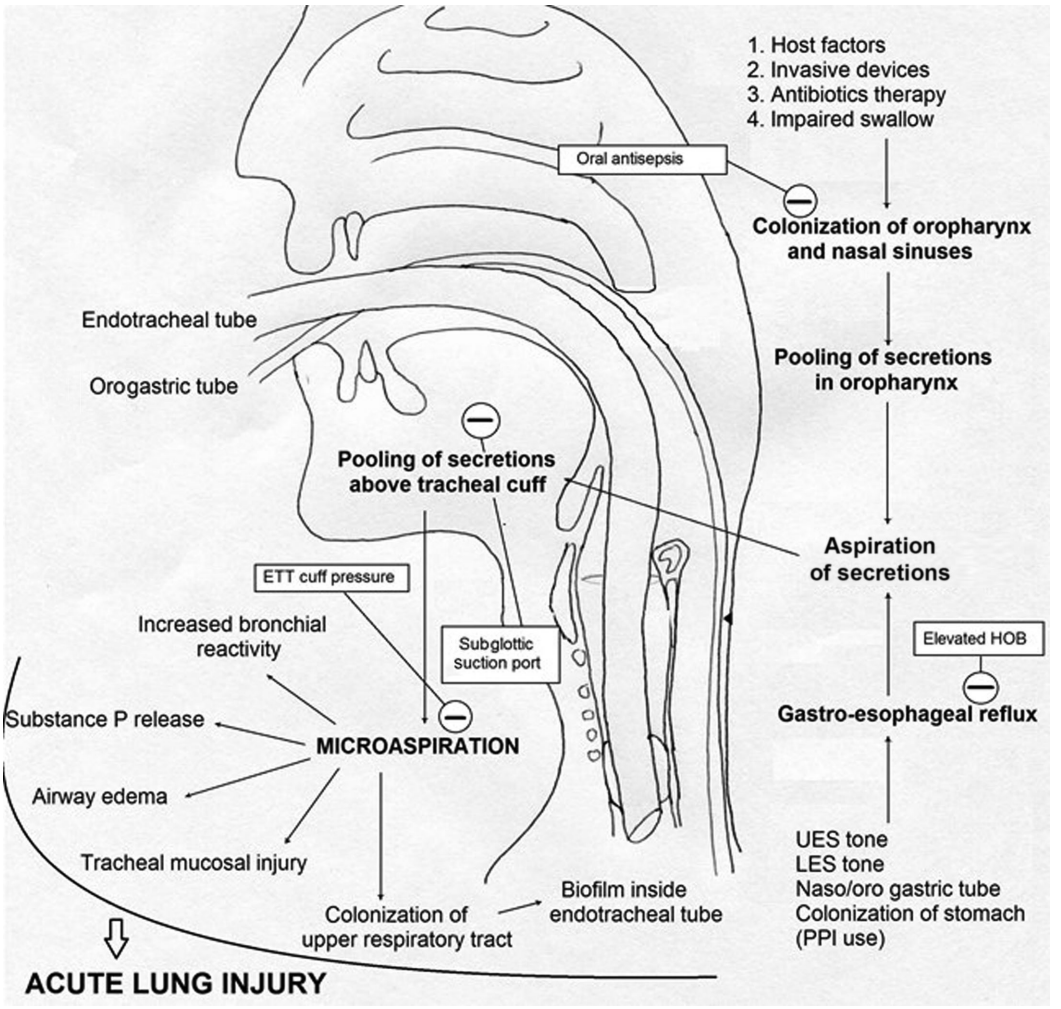

Figure 2. **Framework of aspiration in patients with endotracheal tubes receiving mechanical ventilation.**

the lower esophagus—Barrett's esophagus—and patients with Barrett's esophagus have an increased risk of esophageal adenocarcinoma.[47] Chronic inflammation in reflux esophagitis is known to cause DNA damage, hyperproliferation, and inhibit apoptosis.[48] The laryngopharynx is known to be more susceptible to damage by gastric refluxate than the esophagus, perhaps because it lacks intrinsic defense mechanisms present in the esophagus to protect against damage by gastric refluxate: peristalsis, saliva, and bicarbonate production. Thus, it is reasonable to assume that chronic uncontrolled LPR could not only cause inflammatory but perhaps also neoplastic pathologies in the laryngopharynx, as it is known to in the esophagus. Our group has shown that pepsin is often detectable in the larynges of cancer patients, but absent in patients without clinical signs of reflux or inflammatory and neoplastic disease.[49] *In vitro* studies demonstrate that pepsin: (1) induces a dose- and time-dependent promotion of proliferation in both normal laryngeal and transformed hypopharyngeal epithelial cultures,[49] (2) causes gene and microRNA expression changes consistent with promotion of neoplasia, (3) causes resistance to apoptosis,[49,50] and (4) increases colony forming ability in nonacid pepsin–treated cells relative to control cells.[50] An *in vivo* hamster study demonstrated an increase in tumor volume in animals exposed to active pepsin.[39] Taken together, these data strongly suggest that chronic pepsin exposure will promote tumorigenesis, highlighting a role

for LPR of pepsin in the carcinogenesis of the laryngopharynx.

Therapeutic target

Johnston *et al.* discovered that pepsin is taken up by laryngeal epithelial cells by receptor-mediated endocytosis.[32,51] Receptors and their ligands are typically sorted in late endosomes and the transreticular Golgi (TRG), in which the pH is approximately 5. Pepsin has approximately 40% of its maximal activity at this pH.[12,52] Thus, when inactive pepsin is taken up by laryngeal epithelial cells, it could potentially be reactivated in the lower pH microenvironment of the intracellular compartments, subsequently causing intracellular damage. Using immuno-electron microscopy, colocalization of pepsin with Rab-9 and TRG-46—markers of late endosomes and the TRG, respectively—has been documented.[33] These findings reveal an entirely novel mechanism for pepsin-induced cellular injury in nonacid reflux and could offer an explanation as to why most patients with reflux-attributed laryngeal injury and disease have symptoms while on PPI therapy. Thus, while pepsin is known to play a role in GERD at low pH due to its proteolytic activity, the activity/stability data and discovery of pepsin uptake and intracellular pepsin reveal a role for pepsin in EER, where the acidity of the gastric refluxate may not be as clinically relevant. In support of this, it has been shown by electron microcopy and MTT cytotoxicity assay that pepsin causes laryngeal cell damage at pH 7 and it has thus been speculated that pepsin may contribute to symptoms and endoscopic findings despite PPI therapy. The consequence of reflux damage and cause of symptoms is chronic mucosal inflammation. In this regard, pepsin, independent of acid, induced expression of a cytokine profile in hypopharyngeal cells similar to that in reflux esophagitis and which is known to contribute to the pathophysiology of GERD.[34] These data indicate that refluxed pepsin, even in nonacid and weak acid reflux, likely causes mucosal inflammation in the laryngopharynx, which PPIs will not address—perhaps explaining reflux-attributed symptoms and findings refractory to PPI. Receptor-mediated uptake of nonacid pepsin, as in nonacid LPR, and the subsequent inflammatory and neoplastic changes that occur,[34,39,49,50] will not be prevented by PPIs since pepsin appears to either become reactivated in the lower pH microenvironment of the TRG and late endosomes, or either initiates or dysregulates a cell signaling cascade following activation of the cell surface receptor.[33]

Given pepsin's role in nonacid LPR, it has been proposed as a novel therapeutic target, especially for patients experiencing refractory symptoms on PPIs.[3,33] Approximately $26 billion/year is currently spent on PPIs for the treatment of LPR alone, despite their poor efficacy for this patient population.[53] The promise of irreversible inhibitors and/or receptor antagonists as potential new therapeutics for LPR has been discussed in the literature.[3,33] There are two mechanisms by which one can target pepsin: (1) irreversibly inactivate the enzyme to prevent it from becoming reactivated inside the TRG and late endosomes, and (2) via a receptor antagonist to prevent receptor-mediated uptake of pepsin. It should be noted that while the commercially available inhibitor of pepsin, Pepstatin A, is a potent inhibitor, it does have poor water-soluble characteristics and poor pharmacokinetic properties. One of our groups is performing preclinical evaluations of new pepsin inhibitor compounds to document bioavailability and efficacy.

Summary

Pepsin appears to be a sensitive and specific biomarker for reflux and aspiration, but larger studies with more frequent collection of samples are needed to determine the ideal time for sample collection to yield the greatest sensitivity and specificity before it can be used in routine clinical practice. In light of the poor efficacy of PPIs for airway reflux and role of nonacid pepsin in laryngeal inflammatory and neoplastic disease, drug discovery studies are underway to develop a new therapeutic for airway reflux which specifically targets pepsin. Receptor-mediated uptake of nonacid pepsin, as in nonacid EER reflux events, will not be prevented by acid suppression therapy since pepsin appears to either become reactivated inside intracellular compartments with lower pH or initiate/dysregulate a cell signaling cascade following activation of the cell surface receptor. A receptor antagonist or irreversible inhibitor is needed to prevent this.

Competing interests

P.W.D. is a Founder Director of RD Biomed Limited, the company that developed Peptest. N.J. is preparing a new composition-of-matter patent for

a therapeutic for airway reflux that specifically targets pepsin. J.M.B. has a consulting arrangement with Diversatek.

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