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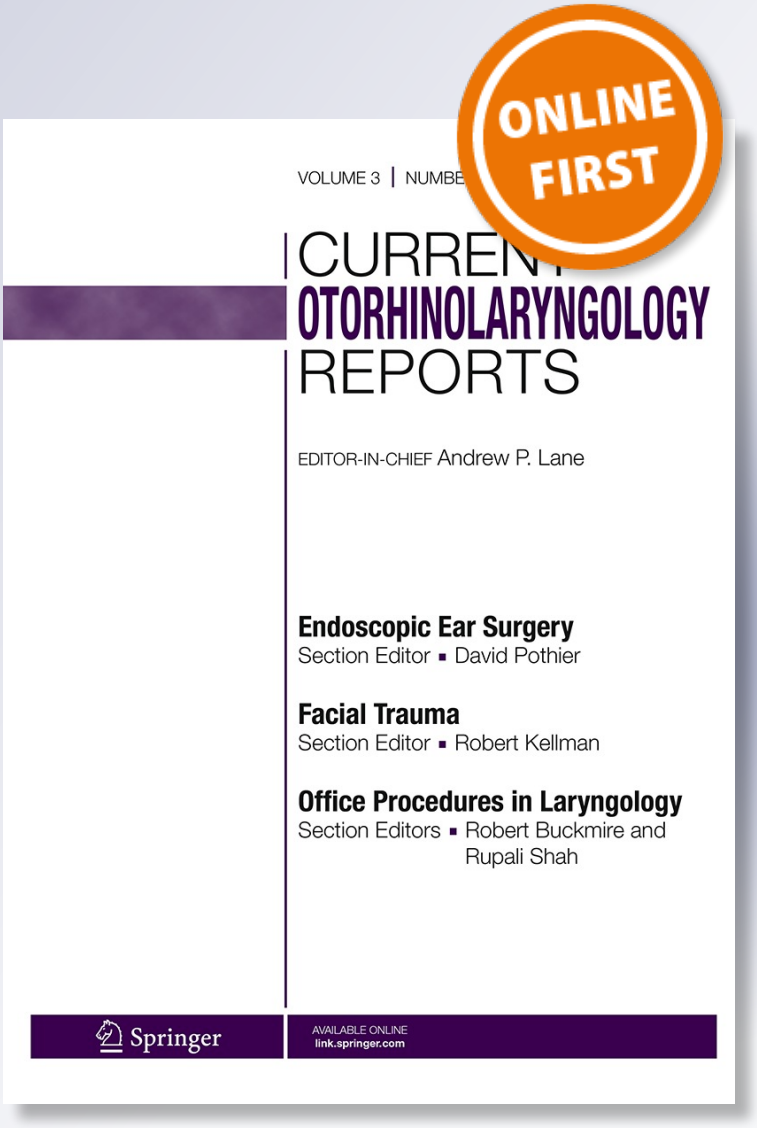

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LARYNGOPHARYNGEAL REFLUX (T. CARROLL, SECTION EDITOR)

The Role of Pepsin in LPR: Will It Change Our Diagnostic and Therapeutic Approach to the Disease?

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Abstract Laryngopharyngeal reflux (LPR) is the back flow of gastric contents into the laryngopharynx. It is estimated that this disease affects 20−40 % of the United States population and is commonly encountered by otolaryngologists. Patients with LPR present with symptoms due to chronic laryngeal irritation such as hoarseness and cough. Pepsin, a gastric enzyme, has been shown to be a specific and sensitive biomarker for LPR. Measurement of pepsin in patients with LPR symptoms holds promise as a reliable diagnostic test. Studies have shown that pepsin induces cell damage, inflammation, and neoplastic changes independently of gastric acid in an endocytosis-dependent manner. Thus, pepsin has been proposed as a novel therapeutic target, especially for patients experiencing refractory symptoms on currently available anti-reflux medications. Further research is needed to elucidate the exact role that pepsin plays in inflammatory and neoplastic diseases of the laryngopharynx and to develop pharmacologic agents targeting pepsin.

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Introduction

Laryngopharyngeal reflux (LPR) is the back flow of gastric contents into the proximal aerodigestive tract or laryngopharynx. Other terms such as gastropharyngeal reflux (GPR) and esophagopharyngeal reflux (EPR) are synonymous with LPR and can be grouped into the comprehensive diagnosis of extraesophageal reflux (EER). LPR affects children and adults equally, and the clinical spectrum of this disease is extensive [1–3]. Unlike patients with gastroesophageal reflux (GER), which is limited to the esophagus, many LPR patients do not experience heartburn but present with symptoms due to chronic laryngeal irritation and inflammation such as chronic cough, throat clearing, post nasal drip, hoarseness or dysphonia, globus sensation, dysphagia, and dyspnea [1, 4] and significant evidence exists that chronic LPR contributes to life-threatening illness such as laryngeal cancer [5–7]. El-Serag et al. [8] showed the annual average rate of increase of reflux disease since 1976 was 4 %. In 2010, Koufman et al. [2, 3] estimated the prevalence of GERD and LPR in the United States by interviewing 656 randomly selected U.S. citizens. This study revealed that 40 % had reflux disease, 22 % having classic GERD and 18 % having LPR. It is estimated that LPR affects 20–60 % of the United States population [9, 10] and is present in up to 10 % of patients presenting to an otolaryngologist's office [11]. The economic burden of LPR is over 52 billion dollars per year which is 5.6 times more than the cost of GERD and 52 percent of that cost is attributed to the use of proton pump inhibitors (PPIs) [12•].

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The deleterious changes in the laryngopharynx observed in LPR develop following direct contact of the mucosa with the refluxed gastric contents consisting of acid as well as pepsin, bile, and pancreatic enzymes [13] and evidence supports a role for acid, pepsin and bile salts in LPR-related injury/disease [14, 15]. While many episodes of LPR are weakly or nonacid, pepsin is present in all refluxate [1, 16]. Pepsin is a gastric proteolytic enzyme made predominantly in the stomach [1, 16]. It is initially synthesized and secreted as the zymogen pepsinogen by chief cells in the gastric fundus and is subsequently cleaved in an autocatalytic fashion to produce the mature form of pepsin upon introduction to the acidic environment of the stomach lumen [16]. Pepsin is maximally active at pH 2 and continues to have activity up to pH 6.5. It retains stability up to pH 8 but is irreversibly inactivated at higher pH levels in which the secondary structure of the molecule is lost [1]. While the stomach and esophagus have internal defense mechanisms against pepsin such as mucus, peristalsis, and bicarbonate secretion, laryngeal tissues do not [17].

Diagnosis of LPR

Traditionally, diagnosis of LPR has relied on symptomatology, laryngoscopic identification of inflammatory changes, and invasive testing procedures such as pH monitoring [1, 18]. Laryngoscopic findings such as edema and erythema are often used to diagnose LPR [19]; however, studies showing high clinician-to-clinician variability [20] and findings in over 80 % of healthy controls [21] suggest that laryngoscopic diagnosis of LPR is highly subjective and nonspecific. The sensitivity and specificity of ambulatory pH monitoring as a means for diagnosing LPR have also been questioned; sensitivity of 24-h dual-pH probe (simultaneous esophageal and pharyngeal) monitoring has been shown to range from 50 to 80 % [11, 22].

Combined multichannel intraluminal impedance (MII) and pH monitoring (MII-pH) represented a major advance in LPR diagnostics by its ability to detect reflux events at weakly acidic or neutral pH [1]. MII-pH detects reflux events, irrespective of their pH, as changes in resistance to alternating current between two metal electrodes produced by the presence of bolus inside the esophageal or laryngeal lumen. Combined MII and manometry (MII-EM) provides simultaneous information on intraluminal pressure changes and bolus movement, whereas combined MII and pH (MII-pH) allows detection of reflux episodes and characterization of their pH. MII-pH provided several advantages over pH monitoring alone including differentiation between antegrade (i.e., swallow) and retrograde (i.e., reflux) bolus movements, characterization of reflux (i.e., gas, liquid, and solid), measurement of sequential reflux events while intraesophageal pH is <4.0, and detection of nonacidic reflux events [16].

The advent of MII-pH monitoring led to significant advances in understanding of LPR disease (LPRD) and crucial differences between its pathophysiology and that of GER disease (GERD). Tamhankar et al. [23] demonstrated that PPI therapy decreases acidity of the refluxed fluid but not the frequency or duration of events. In another study, 35 % (70/200) of patients who were on PPI therapy continued to have symptoms associated with nonacidic reflux [24]. Tutuian et al. [25] demonstrated that proximal reflux episodes were significantly associated with symptoms irrespective of pH [26] and that in 26 % of 50 patients with chronic cough despite treatment with twice-daily dosing of PPIs, cough was associated with nonacidic reflux. In that same study, six patients underwent laparoscopic Nissen fundoplication, a surgical procedure for treatment of reflux, who subsequently became asymptomatic and were able to discontinue PPI therapy. In another study by Iqbal et al. [27], 85 % of study patients had relief of EER symptoms after fundoplication, the majority of whom had previously failed PPI therapy.

By demonstrating the inefficacy of acid-targeting therapeutics for LPR, association of symptoms with nonacid reflux, and symptom alleviation upon interventions that abrogate reflux of all gastric contents, the data garnered through MII-pH technology brought about a shift in perception of LPR as a primarily acid-mediated disease, to one significantly mediated by nonacid components of reflux for which typical GERD treatment would not suffice. While MII-pH monitoring has proved to be an invaluable tool for LPR research, its invasiveness and cost [16] prevent its use as a mainstay in diagnosis. MII-pH studies highlighting the significance of nonacid reflux in LPR, however, have served to stimulate research in nonacid components of reflux. Recent studies demonstrating pepsin as a sensitive and specific biomarker for LPR and mediator of laryngeal inflammation implicate it as an attractive minimally or noninvasive diagnostic and potential therapeutic target for LPRD [16, 28].

Pepsin: Biomarker of Reflux

As mentioned previously, pepsin is present in all refluxate in contrast to other gastric components such as acid or bile salts which may or may not be present. Pepsin analysis, like MII, is able to detect reflux irrespective of pH and can be used to monitor LPR in patents on PPIs. Unlike MII, pepsin analysis is minimally invasive as it does not require use of transnasal catheters, and it can be performed on samples of saliva and sputum which are easily obtainable

[29]. Opposed to MII monitoring, pepsin analysis can also provide direct detection of refluxate at sites of airway damage potentially attributable to EER, and to date has demonstrated the presence of pepsin in the laryngopharynx, trachea, lung, sinus, middle ear, combined sputum and saliva, and exhaled breath condensate [29–33].

Although pepsin assay alone does not indicate a causal relationship to damage, the presence of pepsin in the laryngopharynx does indicate reflux [29–33]. As aforementioned, pepsin is primarily synthesized by gastric chief cells in the form of pepsinogen A and subsequently cleaved upon exposure to the acidic environment of the stomach, thereby generating the mature pepsin enzyme. While serum pepsinogen may be present in the airway tissues of patients with gastritis and pepsinogen has been reported to be produced by metaplastic tissue of the esophagus, production of mature pepsin protein has only been reported in the stomach [34–38]. Real-time (RT)-polymerase chain reaction (PCR) and Western blot analysis has been used to confirm that the laryngopharynx does not produce pepsinogen A or pepsin in LPR patients or healthy controls [6, 39]. Thus, pepsin in the laryngopharynx is a specific marker for LPR [6, 16].

A range of laboratory methods exist to detect pepsin in biopsy, lavage, aspirate, saliva or sputum specimens including enzymatic assays and antibody-based assays such as immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), and Western blot and a clinical test for pepsin is also now commercially available. Many studies have demonstrated that the measurement of pepsin by these methods in patients with LPR holds promise as a diagnostic test. It has been demonstrated to be both a specific and sensitive marker of reflux.

In a study by our group [28], pepsin was detected in laryngeal epithelia from 26/27 confirmed LPR patents but was not detected in any of the 19 control subjects without LPR ($p < 0.001$). Pepsin analysis was performed via Western blot of homogenized laryngeal biopsies. Western blot is capable of discriminating between pepsin at 35 kDa and pepsinogen at 43 kDa, and the anti-human pepsin antibody utilized was shown to have high pepsin-to-pepsinogen specificity and high sensitivity, with a limit of detection of <0.05 ng of pepsin [28].

In 2012, Yuksel et al. [40] reported the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of a salivary pepsin test in patients with GERD compared to controls. Both sensitivity and specificity of a rapid lateral flow test (Peptest^{TM}) was reported to be 87 %. Saliva was collected from 58 patients with GERD and 51 healthy controls. Twelve percent (6/51) of the controls and 22 % (13/58) of the patents with GERD tested positive for pepsin. There was a step-wise increase in pepsin positive samples among the GERD patients: GERD symptoms only (24 %), abnormal pH monitoring (43 %) and esophagitis (55 %) ($p < 0.001$). For patients with objective GERD (abnormal pH and/or esophagitis), salivary pepsin showed a PPV of 85 % and a NPV of 68 %, and it was concluded that a positive salivary pepsin test may obviate the need for more invasive and expensive diagnostic testing. With regards to the low prevalence of salivary pepsin in patients with GERD, it was noted that for pepsin to be present in saliva, refluxate must reach the oropharynx which is not common in GERD or could be due to the timing of saliva collection [40].

More recently, Hayat et al. [41••] tested 100 asymptomatic controls and 111 patients with heartburn with salivary pepsin testing and MII-pH monitoring. One-third of asymptomatic patients had pepsin in saliva but at a low concentration. Patients with GERD had a higher prevalence and concentration of pepsin in saliva. A pepsin concentration greater than 16 ng/ml in saliva, had a sensitivity of 78.6 % and a specificity of 64.9 % for the diagnosis of reflux-related symptoms. A sample with a high pepsin concentration (>210 ng/ml) had a sensitivity of 98.2 % [41••].

A study by Wassenaar et al. [42] investigated pepsin detection in patients with clinical LPR before and after fundoplication with the intent of determining (1) the incidence of pepsin detection in patients with clinical LPR, (2) the concordance between pepsin detection in laryngeal biopsy specimens and sputum, (3) the ability of antireflux surgery to eradicate pepsin from the upper airway, and (4) the relationship of the presence of pepsin, pre or postoperatively, to the presence of symptoms of LPR. Hoarseness was the most common primary presenting LPR symptom followed by cough and globus sensation. Pepsin was detected in laryngeal mucosa in eight of nine patients preoperatively. In the five patients who had both biopsy and sputum samples available, four had a correlation between pepsin detection in sputum and biopsy. Postoperatively, pepsin was only detected in one patient. Postoperatively, six patients had good symptom improvement, two had mild improvement, and one had no improvement. The one patient without preoperative pepsin remained negative postoperatively and was the only one who did not have normalization of reflux measured by pH monitoring and reported no response to treatment of the primary LPR symptom. Authors concluded that the measurement of pepsin in patients with GERD and LPR symptoms improves the accuracy of diagnosis of LPR. Pepsin is frequently detected in the airway (by biopsy or sputum) in patients with documented GERD and signs and symptoms or LPR, and there is good concordance between pepsin detection in biopsy and sputum samples. Furthermore, antireflux surgery was successful in eradicating pepsin from the upper airway and preoperative pepsin detection

seems to predict clinical improvement of LPR after antireflux surgery [42].

While additional, larger studies are needed, the studies to date demonstrate that pepsin is a sensitive and specific marker for reflux. Measurement of pepsin in patients with LPR holds promise as a reliable diagnostic test that is less invasive and less expensive than current methods.

Pepsin: Mediator of Cell Damage

It is currently unknown what specific reflux-related mechanisms lead to laryngopharyngeal reflux disease (LPRD) signs and symptoms [43]. Acidity of the reflux alone may cause tissue damage at the level of the upper airway [44]. However, as mentioned previously, studies using combined MII-pH monitoring have shown that many episodes of gastric reflux are nonacidic and that weakly and nonacidic gastric reflux is associated with persistent symptoms in as many as 20 percent of acid-suppressed patients [24]. Thus, the role of nonacidic refluxate components warrants consideration in the pathogenesis of LPRD.

Recent studies have demonstrated that pepsin plays a role in mucosal damage and inflammation during nonacidic reflux [5, 45–47]. At a neutral pH, pepsin is enzymatically inactive but stable (below pH 8.0) and is taken up by laryngeal and hypopharyngeal cells by receptor-mediated endocytosis. Once taken up, pepsin is retained in intracellular vesicles of low pH where it is presumed to be reactivated [45]. Additional studies analyzing cellular morphology, mitochondria function, and the expression of stress response genes in laryngeal specimens and cultured hypopharyngeal cells treated with pepsin confirmed that the endocytosed pepsin causes mitochondrial damage and changes the expression of several genes implicated in stress and toxicity [46]. Thus, pepsin may contribute to the signs and symptoms associated with weakly and nonacidic LPR [5, 46]. Our group [47] further investigated the potential of pepsin to contribute to mucosal damage and demonstrated that endocytosed nonacidic pepsin induces a proinflammatory cytokine gene expression profile in hypopharyngeal cells in vitro similar to that which contributes to disease severity in GERD patients.

Chronic stimulation of inflammatory molecular signaling pathways is a well-established contributor to tumorigenesis [48]. Many clinical studies have shown a high prevalence of LPR in patients with laryngeal cancer [49, 50]; however, many of these studies lack appropriate controls and are confounded by the fact that the majority of patients with laryngeal carcinoma have significant tobacco and alcohol histories. Wight et al. [51] reported in 2003 a high frequency of reflux in laryngeal cancer patients who were nonsmokers and nondrinkers. In 2011, Tae et al. [50] compared the frequency and severity of LPR by dual-probe pH monitoring in patients with laryngeal cancer and a control population: LPR patients with chronic cough and globus sensation. The prevalence and severity of LPR was significantly higher in cancer patients than the controls. Furthermore, Cekin et al. [52] reported the prevalence of LPR in new patients presenting with a laryngeal lesion was 69.8 % and there was a significant relationship between LPR positivity and presence of a malignant/premalignant laryngeal lesion ($p = 0.03$). Additional studies examined the direct effect of various individual components of refluxate including acid, pepsin and bile salts on laryngeal cells. These demonstrated a significant role for pepsin and bile acids in carcinogenesis, in a dose-dependent manner with greater toxicity at a lower pH [53]. Using an in vivo hamster buccal pouch model of squamous cell carcinoma (SCC), our group found a significant increase in tumor volume in hamster cheek pouches exposed to pepsin (0.1 mg/ml; pH 2) plus a known carcinogen—7, 1 dimethylbenzanthracene (DMBA)—compared to exposure to DMBA alone, suggesting that pepsin in acid reflux does promote tumorigenesis [54]. In addition, our group has reported that exposure of hypopharyngeal FaDu cells and normal human primary epithelial cells to pepsin (0.1 mg/ml; pH 7) causes a significant change in the expression of 27 genes implicated in carcinogenesis and of 22 microRNAs specifically known to be altered in head and neck SCC [6]. Pepsin increased proliferation in both FaDu SCC cells and normal laryngeal primary epithelial cells by significantly increasing the percentage of cells in S phase in a time- and dose-dependent manner. These data demonstrate that pepsin, present in laryngopharyngeal cancer specimens but not in normal laryngeal control cells, may initiate epithelial cell dysregulation promoting laryngopharyngeal cancer [17]. More recently, our group demonstrated that hypopharyngeal FaDu cells chronically exposed to nonacidic pepsin demonstrated increased cell migration and colony-forming ability relative to control cells indicating that chronic pepsin exposure acts as a promoter of tumorigenesis and metastasis of airway epithelium [7]. In 2015, Sereg-Bahar et al. [55••] conducted a study comparing the pH, the level of bile acids, the total pepsin, and its enzymatic activity in saliva in patients with T1 laryngeal cancer to a healthy control group. The cancer patients had higher levels of total pepsin and bile acids compared to the healthy patients suggesting that LPR has a role in the development of laryngeal carcinoma [51].

Combined, these studies demonstrate that pepsin induces cell damage, inflammation, and neoplastic changes independently of acid in an endocytosis-dependent manner.

Pepsin: Therapeutic Target for Treatment of LPR

The treatment of LPR is aimed at eliminating symptoms, managing complications and maintaining symptom remission [1]. Treatment ranges from conservative measures like lifestyle modifications and dietary changes to pharmacologic acid suppression to antireflux surgical procedures [43].

While PPI therapy is a mainstay in the treatment of GERD, its efficacy for the treatment of LPRD remains doubtful [43]. In clinical practice, it is believed that patients with reflux laryngitis require higher doses and longer trials of PPIs than those with typical GERD given the assumption that the upper airway is more sensitive to acid reflux than the esophagus [56–58]. However, placebo-controlled trials have previously failed to demonstrate any therapeutic benefit of PPIs [59–64]. Some benefit of PPI therapy in LPR patients has been reported, for example Reichel et al. [65] and Lam et al. [66] performed randomized, double-blind, placebo-controlled trials with PPIs (esomeprazole 20 mg twice daily for 3 months and rabeprazole 20 mg twice daily for 3 months, respectively) with both studies showing significant improvement in symptoms and Reichel et al. found improvement in laryngeal exam. However, upon closer review of these two studies, Vaezi [67] argued that the actual significant improvement was for heartburn symptoms, not chronic throat symptoms. Other studies have shown that the proportion of patients with improvement in laryngeal symptoms with PPI therapy is higher in GERD patients than in those without GERD [68–70]. Given the paucity of data supporting a beneficial effect of reflux treatment on the extraesophageal symptoms [22, 64], the American Gastroenterological Association guidelines for GERD recommended against the use of acid-suppression therapy for acute treatment of patients with potential extraesophageal reflux syndromes (laryngitis, asthma) in the absence of typical GERD symptoms [71]. Despite such advisement, treatment for LPR continues to frequently involve empiric therapy with PPIs and monitoring of symptoms [18]. Given the potential risks of prolonged PPI therapy, its associated cost, and the high percentage of LPR patients for which PPI therapy has been shown ineffective by MII-pH monitoring, an alternative treatment for LPR is desirable [72].

Given pepsin's role in nonacidic LPR, it has been proposed as a novel therapeutic target, especially for patients experiencing refractory symptoms on PPIs [5, 6, 45–47]. Approximately $26 billion/year is currently being spent on PPIs for the treatment of LPR, despite their poor efficacy for this patient population [12]. We and others have discussed the promise of irreversible inhibitors of peptic activity and/or receptor antagonists as potential new therapeutics for LPRD [46, 73•]. We believe that EER is much more dependent on pepsin-mediated damage in the laryngeal and airway mucosa than with acid. In this regard, we are currently leading an international drug discovery program to develop a drug which specifically targets pepsin. There are two mechanisms by which one can target pepsin: (1) irreversible inactivate the enzyme to prevent it from becoming reactivated inside intracellular compartments of lower pH and (2) via a receptor antagonist to prevent pepsin being taken up by receptor mediated endocytosis. It should be noted that pepstatin, a commercially available potent inhibitor of pepsin, has poor water soluble characteristics and poor pharmacokinetic properties. Thus, preclinical evaluations of new pepsin inhibitor compounds to document bioavailability and efficacy are underway.

Conclusion

Laryngopharyngeal reflux is a common disease encountered by otolaryngologists. The approach to its diagnosis and management remains controversial but advances in the research investigating the role of pepsin in LPR have led to proposals in novel diagnostic methods and therapeutic targets. Studies to date, have revealed that pepsin is a specific and sensitive biomarker for LPR, and its measurement in patients with LPR symptoms holds promise as a potential reliable method of diagnosis that is both less invasive and less expensive than current controversial diagnostic methods. In addition, pepsin has been shown to be a mediator of cell damage independently of gastric acid in an endocytosis-dependent manner and without effect from bile salts. For this reason, pepsin has been proposed as a novel therapeutic target for the treatment of LPR, especially in patients experiencing refractory symptoms on PPIs. Ongoing studies aim to elucidate the exact role that pepsin plays in inflammatory and neoplastic diseases of the laryngopharynx as well as to develop pharmacologic agents aimed at targeting pepsin, specifically agents that are irreversible inhibitors of pepsin activity and/or pepsin receptor antagonists.

Compliance with Ethics Guidelines

Conflict of Interest Drs. Johnston and Samuels have a patent pending on Novel anti-pepsin therapeutic. Dr. Luebke has no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- •• Of major importance

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