

# Association between gastroesophageal reflux disease and vocal fold polyps

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## Abstract

The aim of this study is to explore the relationship between gastroesophageal reflux disease (GERD) and vocal fold polyps (VFPs).

This is a Case-Control study and was performed with the help of The Second Affiliated Hospital of Chongqing Medical University.

Twenty-seven patients with VFP and 20 controls without VFP were recruited between May and October 2018. All the subjects underwent a saliva pepsin test, completed the GerdQ questionnaire and 24-hour multichannel intraluminal impedance with pH (24-h MII-pH) monitoring. Twenty-five resected VFP specimens were examined with immunohistochemical (IHC) and double immunofluorescence (IF) staining.

The incidence of GERD in the VFP group was significantly higher than that in the control group ( $P = .003$ ). Patients with VFP had significantly higher GerdQ scores, pepsin concentrations, and pepsin-positive rates ( $P < .05$ ). Moreover, the number of proximal and upright reflux events was significantly higher in the VFP group ( $P < .05$ ). The pepsin concentration in saliva showed a significant positive correlation with the pepsin levels in tissues ( $r^2 = 0.50$ ,  $P = .011$ ). Pepsin and TGF- $\beta$ 1-positive cells were colocalized with CD45RO-positive cells. IHC staining showed that the majority of VFP patients had a positive expression of pepsin (20/25, 80%) and pepsin-positive cells were found in both the squamous epithelium and mesenchymal tissues. IHC staining of TGF- $\beta$ 1 in VFP revealed findings similar to those of pepsin staining.

GERD is an important risk factor for VFP. Pepsin may promote the aggregation of immune cells, increase the local cytokines, and promote inflammatory reaction, suggesting a potential new pathogenesis for VFP. The saliva pepsin test is a reliable method for GERD diagnosis.

**Abbreviations:** GERD = gastroesophageal reflux disease, IF = immunofluorescence, IHC = immunohistochemical, MII-pH = multichannel intraluminal impedance and PH, PBS = phosphate buffered saline, VFPs = vocal fold polyps.

**Keywords:** 24-hour multichannel intraluminal impedance and pH monitoring, gastroesophageal reflux, pepsin, vocal fold polyps

## 1. Introduction

Gastroesophageal reflux disease (GERD) is one of the most common chronic disorders of the digestive system. Acid reflux can not only damage the esophageal mucosa, but also reach the pharynx, larynx, nasal cavity, middle ear, and upper respiratory

tract and cause damage.<sup>[1,2]</sup> GERD is classified as reflux esophagitis if esophageal mucosal injury is present and non-erosive reflux disease if the esophageal mucosa is unaffected. Commonly used clinical tools for the diagnosis of GERD include gastroscopy, gastroesophageal reflux-related questionnaires such as the GerdQ scale, 24-hour pH-impedance monitoring, and the proton pump inhibitor diagnostic test. At present, 24-hour pH-impedance monitoring is the gold standard to diagnose GERD. However, this test is invasive and expensive and may be less sensitive than other tests used to diagnose GERD.<sup>[3]</sup> The measurement of pepsin in saliva is a new tool to detect GERD that is noninvasive and easy to perform. However, its sensitivity and specificity for diagnosing GERD require further study.<sup>[4,5]</sup> Pepsin is produced only in the stomach. When GERD occurs, pepsin and the contents of the stomach can reflux into the throat. Recent studies have found that pepsin also plays a pathogenic role in gastroesophageal reflux disease, and has a more obvious pathogenic effect on extraesophageal symptoms (such as pharyngeal discomfort, hoarseness, coughing, etc).<sup>[6,7]</sup>

Extraesophageal reflux disease is associated with many upper respiratory tract disorders such as chronic sinusitis, exudative otitis media, and other pharyngeal diseases, especially in children.<sup>[8-12]</sup> Jin et al found that pepsin can promote the immune response of tonsils, leading to increased expression of pre-inflammatory factors.<sup>[13]</sup>

Vocal fold polyps (VFPs) are some of the most common causes of hoarseness of voice, affecting patients' daily life and work.<sup>[14]</sup> Recent studies have found that laryngopharyngeal diseases such

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as VFP may be associated with GERD.<sup>[15]</sup> Wang et al found that pepsin expression in VFPs is significantly higher than that in normal vocal cord tissues, suggesting that pepsin reflux may be an important risk factor for VFPs.<sup>[16]</sup> It has been reported that many patients with VFPs have positive expression of pepsin in immunohistochemistry, suggesting that pepsin may be a risk factor for the disease.<sup>[17]</sup> Previous research showed positive immunohistochemical (IHC) staining of CD45RO and CD20 in VFPs, suggesting that the cellular immunity mediated by T cells and humoral immunity mediated by B cells are active in response to VFPs.<sup>[18]</sup> Previous studies also reported the expression of pepsin in laryngeal mucosa samples from patients with laryngeal reflux.<sup>[15]</sup> However, the relationship between GERD and VFP is still unclear. The purpose of this study was to explore the association between GERD and VFP and to understand the role of pepsin in the development of VFPs.

### 1.1. Ethics statement

This study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University, and registered in the Chinese registry (registration number: ChiCTR1800015694).

## 2. Materials and methods

### 2.1. Study population and design

In this prospective study, 27 patients with hoarseness of voice who visited our hospital for the removal of VFPs (VFP group) and 20 healthy individuals without VFPs (control group) who underwent laryngoscopy examination between May and October 2018 were evaluated. The control group included 20 healthy individuals who were recruited via advertisement. The control group do not have other laryngeal pathologies. Among these 47 individuals, 2 were excluded because of intolerance to multi-channel intraluminal impedance with pH testing (MII-pH). Patients with a history of chronic disease, malignancy, previous esophageal/gastric surgery, and esophageal motor disorder were excluded. Additionally, patients taking antacid drugs or prokinetic agents a week before the test were excluded.

All the participants completed the GerdQ questionnaire comprising 6 questions (scores of 0–3 each), and a score  $\geq 8$  suggested GERD.<sup>[19,20]</sup> Afterward, the participants underwent 24-h MII-pH and a saliva pepsin test before VFP excision. After the surgery, the VFP specimens underwent detailed histological analysis including immunohistochemistry and double immunofluorescence (IF) staining.

### 2.2. Saliva pepsin test

Saliva was collected from the participants 1 hour after lunch during the 24-h ambulatory MII-pH monitoring period. The saliva was collected into tubes containing 0.5 mL of 0.01 M citric acid, refrigerated at 4°C, and analyzed with the Peptest lateral flow device (Chongqing Oumai Medical Equipment Co, Ltd) within 2 days.

The researcher performing this test was blinded. Using a 1 ml graduated pipette, approximately 1 ml of sample was transferred into a microcentrifuge tube. Then the sample was centrifuged for 5 minutes, and 80  $\mu$ l of the clear supernatant layer was collected. The 80  $\mu$ l sample was transferred to a tube and 240  $\mu$ l of migration buffer was added. A vortex mixer was used to mix the sample for 10 seconds. Finally, 80  $\mu$ l of the mixed sample was added to the well of the lateral flow device and left for 15 minutes. The lower

limit of detection for pepsin (as determined by the manufacturer) was set at 16 ng/mL. The test results were recorded as follows:

1. pepsin content  $< 16$  ng/ml was considered negative;
2. 16 to 75 ng/L was considered physiological reflux;
3. 75 to 125 ng/ml was weakly positive;
4. 125 to 200 ng/ml was positive; and
5. 200 ng/ml was a strong positive result indicating pathological reflux.<sup>[21]</sup>

### 2.3. 24-h MII-pH Monitoring

The study was performed with the Impedance-pH reflux monitoring system (Jinshan Science & Technology Group, Chongqing, China). The system contains a bifurcated catheter, a pH sensor, and 6 impedance sensors. The pH sensor was positioned 5 cm above the lower esophageal sphincter (determined with the pH set-up method). The 6 impedance sensors were located at 3, 5, 7, 9, 15, and 17 cm above the lower esophageal sphincter. All participants fasted for at least 8 hours, were off proton pump inhibitors for at least 7 days, and were encouraged to maintain their daily sleeping and eating habits. They were asked to record the timing of supine and upright positions, meals, and typical symptoms. The probe was placed for 24 hours and removed on the next day. The system recorded reflux data, which were analyzed with the system's software. A DeMeester score  $\geq 14.7$  suggested gastroesophageal reflux.<sup>[22]</sup>

### 2.4. Immunohistochemistry

Immunostaining was performed on paraffin sections of the specimens after deparaffinization and rehydration. The blocks were washed in phosphate buffered saline (PBS) and antigen retrieval was performed by putting the sections in citrate buffer (pH 6.0) and heating for 20 minutes. The sections were washed again in PBS and endogenous peroxidase was inactivated by covering the tissues with 3% hydrogen peroxide for 10 minutes. Then the tissues were sequentially stained with anti-pepsinogen A5 (PGA5; TA322389S, origene) and transforming growth factor (TGF)- $\beta$ 1 (TA313319, origene) at 37°C for 1 hour. A blank control without primary antibody was established. The sections were rinsed in PBS and a reaction enhancer was added at room temperature for 20 minutes. The sections were washed again in PBS and incubated at room temperature for 20 minutes with secondary antibody (PV-9001, ZSBG-Bio). After washing in PBS, 3,3'-diaminobenzidine was added to develop color at room temperature for 5 minutes. The sections were rinsed with water, and counterstained with hematoxylin. The proportion of positive cells in each section was scored on a 1 to 4 scale (1 =  $< 25\%$ , 2 = 25% to 50%, 3 = 51% to 75%, 4 =  $> 75\%$ ). Staining intensity was scored on a 0 to 3 scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe). Both of these scores were multiplied to obtain the final score: negative (–, 0–1), weakly positive (+, 2–4), moderately positive (++, 5–8), and strongly positive (+++, 9–12).

### 2.5. Double IF staining

The same procedures used for immunohistochemistry including deparaffinization, rehydration, and antigen retrieval were performed on the tissues. Normal goat serum was used to block the nonspecific antibody in PBS. Tissues were sequentially incubated with anti-PGA5 (1:25, TA322389S, origene) mixed with anti-CD20 (1:50, UM870002, origene), anti-PGA5 mixed

**Table 1****Patient characteristics.**

	VFP group (n=25)	Control group (n=20)	z	P value
Age (yr)	53.12±13.78	46.45±13.47	1.63	.111
Gender (male:female)	13:12	9:11	0.22	.641
Smoking	7	4	0.07	.786
BMI (kg/m <sup>2</sup> )	22.73±2.76	22.67±2.29	0.60	.552

BMI = body mass index, VFP = vocal fold polyps.

\*P&lt;.05 indicates significant difference.

with anti-CD45RO (1:75, TA807197S, origene), TGF-β1 (1:25, TA313319S, origene) mixed with anti-CD20, and TGF-β1 mixed with anti-CD45RO at 37°C for 2 hours. After rinsing, fluorescein isothiocyanate-conjugated goat anti-mouse IgG secondary antibody (1:50, KGAA25, KeyGEN) mixed with tetramethylrhodamine-conjugated goat anti-rabbit IgG secondary antibody (1:50, KGAA99, KeyGEN) was applied at 37°C for 1 hour. Counterstaining with 4',6-diamidino-2-phenylindole was performed and the samples were mounted with antifade solution. Finally, the tissues were examined under a fluorescence microscope (OLYMPUS, Tokyo, Japan).

**2.6. Statistical analysis**

Measurement data with normal distribution were expressed as mean ± standard deviation and data with non-normal distribution were expressed as median and range. Normally distributed continuous data were compared with the independent *t* test. Non-normally distributed continuous data were compared with the non-parametric test. Enumeration data and frequency were compared with the Chi-Squared test. Correlations between the non-normally distributed continuous variables were assessed using Spearman correlation analysis. All data were analyzed using SPSS version 22.0. A *P* value of <.05 was considered to be significant, with all tests reflecting 2-tailed comparisons.

**3. Results****3.1. Patient characteristics**

The 2 groups had no differences in terms of age, gender, smoking history, and body mass index. Comparisons of baseline

**Table 3****Prevalence and concentration of salivary pepsin in VFP group and control group.**

	VFP group (n=25)	Control group (n=20)	z	P value
Salivary pepsin concentration (ng/ml)	148.0 (16.0, 229.5)	16.0 (16.0, 101.8)	4.02	.045*
Pepsin (No./total No.)	60.0% (15/25)	30.0% (6/20)	-2.67	.008*

VFP = vocal fold polyps.

\*P&lt;.05 indicates significant difference.

characteristics between the VFP and control groups are summarized in Table 1.

**3.2. 24-h MII-pH monitoring and GerdQ**

Among the VFP patients, 52.0% (13/25) had a positive GerdQ score and the median value was 9 (7,11), while 15.0% (3/20) of patients in the control group had a positive score with a median value of 6.5 (5,7). Compared with the control group, positive GerdQ scores were significantly more frequent in the VFP group ( $\chi^2=6.64$ , *P*=.010) and the value of GerdQ was significant higher in this group (*z*=-3.33, *P*=.001) (Table 2).

The MII-pH positive rate was 48.0% (12/25) in the VFP group and 10.0% (2/20) in the control group. The esophageal acid reflux parameters were all higher in the VFP group, as well as the number of proximal reflux and upright reflux events (Table 2).

**3.2.1. Prevalence of positive pepsin and pepsin concentration in saliva.** In total, 60.0% (15/25) of VFP patients had a positive saliva pepsin test result, which was significantly higher than the percentage in the control group (30.0%) (6/20). The median pepsin concentration in the VFP group was 148.0 (16.0, 229.5) ng/ml, with the highest pepsin concentration of 438.0 ng/ml in the VFP group. In contrast, the median pepsin concentration in the control group was 16.0 (16.0, 101.8) ng/ml and the highest pepsin concentration was 170 ng/ml (Table 3).

**3.2.2. Prevalence of GERD.** All subjects in this study completed 3 tests (GerdQ scale, saliva pepsin test, and 24-hour pH monitoring). The patients with positive results in at least 2 tests were deemed to have GERD. The incidence of GERD in the VFP group was 52.0% (13/25), which was significantly higher than

**Table 2****Results of 24-h MII-pH monitoring and GerdQ in the VFP group and control group.**

	VFP group (n=25)	Control group (n=20)	z	P value
GerdQ score	9.00 (7.00, 11.00)	6.50 (5.00, 7.00)	-3.33	.001*
Total reflux time (min)	22.00 (7.50, 90.00)	3.05 (1.60, 22.25)	-2.99	.003*
Time of pH<4 (%)	1.60 (0.60, 6.45)	0.25 (0.10, 1.63)	-2.90	.004*
No. of acid reflux events	33.00 (10.00, 69.00)	9.00 (5.00, 26.25)	-2.58	.010*
No. of long reflux events>5 min	0.00 (0.00, 4.00)	0.00 (0.00, 0.75)	-2.07	.038*
DeMeester score	12.60 (2.85, 25.85)	1.60 (0.80, 6.93)	-2.99	.003*
No. of proximal reflux	8.00 (2.50, 14.00)	3.50 (0.00, 7.50)	-2.34	.019*
No. of upright reflux events	26.00 (12.50, 37.00)	9.50 (3.25, 28.00)	-2.00	.045*
No. of supine reflux events	4.00 (0.00, 10.50)	10.00 (2.00, 24.75)	-1.95	.051
Positive pH monitoring test, % (No./total No.)	48.0 (12/25)	10.0 (2/20)	7.49	.006*
Positive GerdQ. % (No./total No.)	52.0 (13/25)	15.0 (3/20)	6.64	.010*

24-h MII-PH = 24-hour multichannel intraluminal impedance with pH, VFP = vocal fold polyps.

\*P&lt;.05 indicates significant difference.



**Table 4**  
The prevalence of GERD in VFP group and control group.

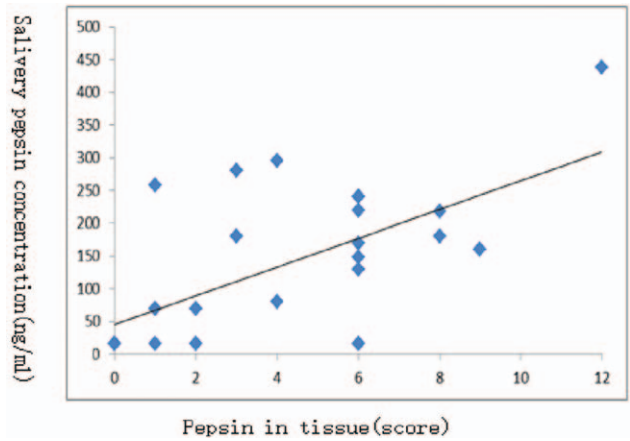
	GERD		Total	$\chi^2$	P value
	Present	Absent			
VFP group (n=25)	13	12	25	8.82	.003*
Control group (n=20)	2	18	20		
Total	15	30	45		

GERD = gastroesophageal reflux disease, VFP = vocal fold polyps.  
\*  $P < .05$  indicates significant difference.

that in the control group [10% (2/20) ( $\chi^2=8.82$ ,  $P=.003$ )] (Table 4).

**3.2.3. Correlation of pepsin in tissue with pepsin in saliva.**

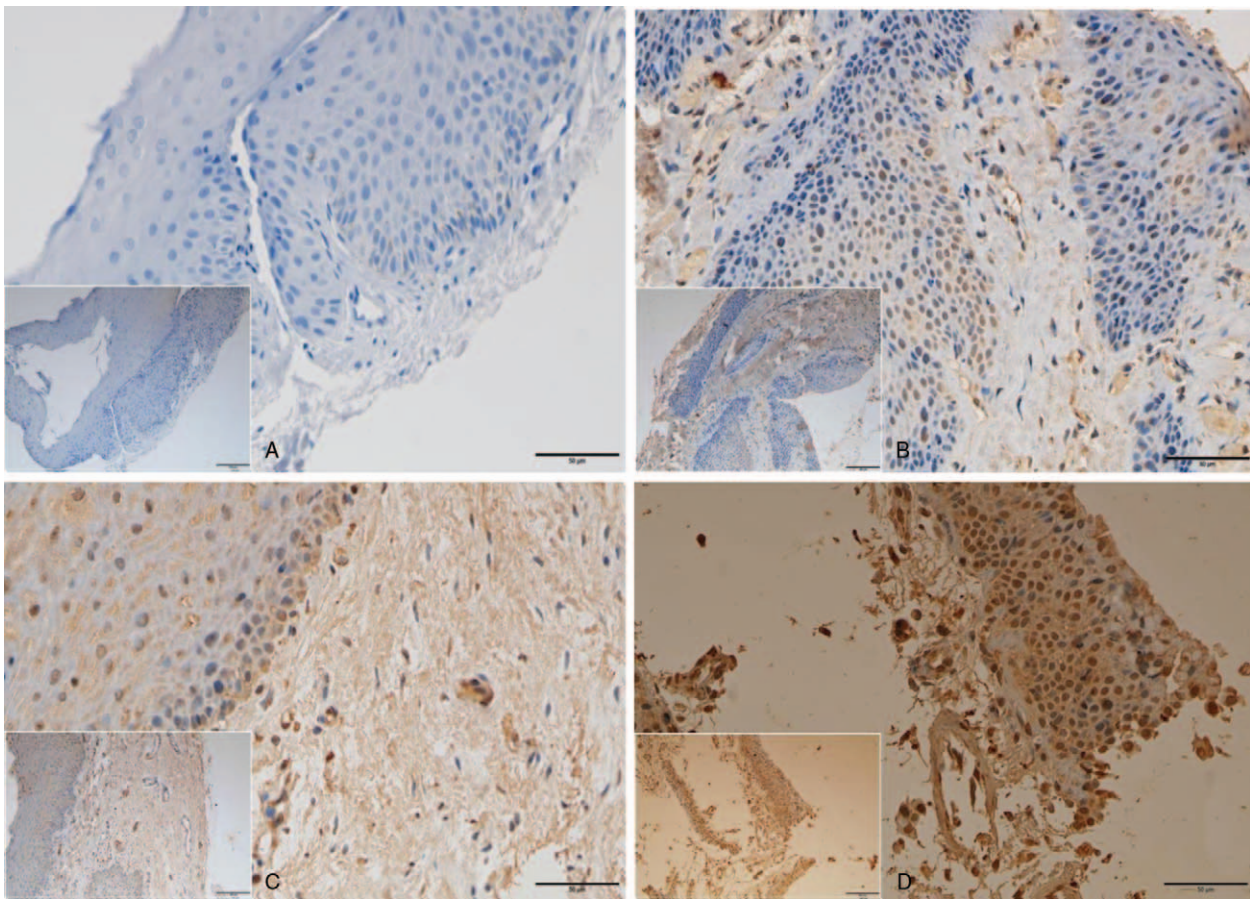
Pepsin-positive cells were detected in the VFP squamous epithelium and mesenchymal tissues. Positive staining for pepsin was mainly found in the cytoplasm and cell nucleus. Stomach tissues were used as the positive control for pepsin detection, and PBS instead of pepsin antibody was used as a negative control. IHC staining for pepsin was negative (Fig. 1A) in 5 patients (20%), weakly positive (Fig. 1B) in 9 patients (36%), moderately positive (Fig. 1C) in 9 patients (36%), and strongly positive (Fig. 1D) in 2 patients (8%). The salivary pepsin concentration



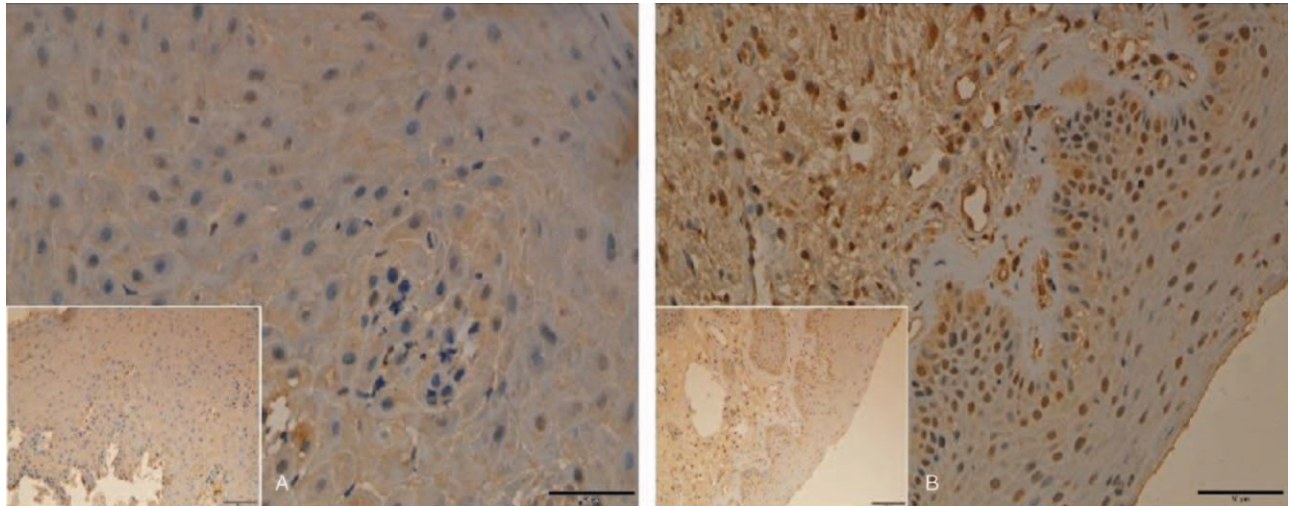
**Figure 2.** Correlation between the pepsin levels in the tissues and salivary pepsin concentration.

had a significant positive correlation with pepsin levels in the tissues ( $r^2=0.50$ ,  $P=.011$ ) (Fig. 2).

**3.2.4. TGF- $\beta$ 1 expression in the VFPs.** Immunostaining for TGF- $\beta$ 1 was detected in VFP specimens, both in the epithelium



**Figure 1.** Pepsin immunohistochemical staining ( $\times 400$ ) in the vocal fold polyps: (A) Negative pepsin (–) in both cytoplasm and nucleus seen as blue color. (B) Weakly positive pepsin (+) staining seen as diffuse cytoplasmic brown granules of weak staining. (C) Moderately positive pepsin (++) staining seen as diffuse cytoplasmic and some nuclear brown granules of moderate staining. (D) Strongly positive pepsin (+++) staining seen as diffuse cytoplasmic and nuclear brown granules of strong staining.



**Figure 3.** TGF- $\beta$ 1 immunohistochemical staining ( $\times 400$ ) in the vocal fold polyps: TGF- $\beta$ 1-positive cells were found in the squamous epithelium and mesenchymal tissue (A and B).

and mesenchymal tissues (Fig. 3). Positive results were detected in similar regions as pepsin staining, suggesting a possible relationship between pepsin exposure and inflammation.

**3.2.5. Pepsin and TGF- $\beta$ 1 were detected in CD45RO-positive cells in VFps.** Double IF staining for CD45RO was performed to characterize the pepsin-positive cells and TGF- $\beta$ 1-positive cells. CD45RO is the differentiation antigen for memory T lymphocytes and CD45RO-positive cells were observed in the VFps (Fig. 4) colocalized with pepsin and TGF- $\beta$ 1-positive cells (Fig. 4A, 4B). In contrast, we found no colocalization of pepsin and CD20, nor that of TGF- $\beta$ 1 and CD20.

#### 4. Discussion

This study found that, compared with the control group, individuals in the VFP group had a significantly higher incidence of GERD as well as higher GerdQ scores, saliva pepsin concentrations, positive rates, DeMeester scores, and proximal reflux events, suggesting that GERD is closely related to the occurrence of VFps. GERD could be an important risk factor for the formation of VFps. A previous study showed that pepsin in throat epithelial cells could lead to the lack of 2 kinds of protective proteins (carbonic anhydrase isoenzyme III and Sep 70), resulting in damage to the laryngeal mucosa.<sup>[23]</sup> Additionally, increased vascular permeability, degeneration, hypoxia, local edema, and fibrosis may contribute to the development of VFps.<sup>[24]</sup> Changes in the microcosmic, macroscopic, and immune expression of many individuals with VFps have been attributed to other factors such as traumatic voice behavior, smoking, and apoptosis.<sup>[25]</sup> Nonetheless, previous studies have shown that the formation of VFps in many patients is related to GERD.<sup>[26]</sup>

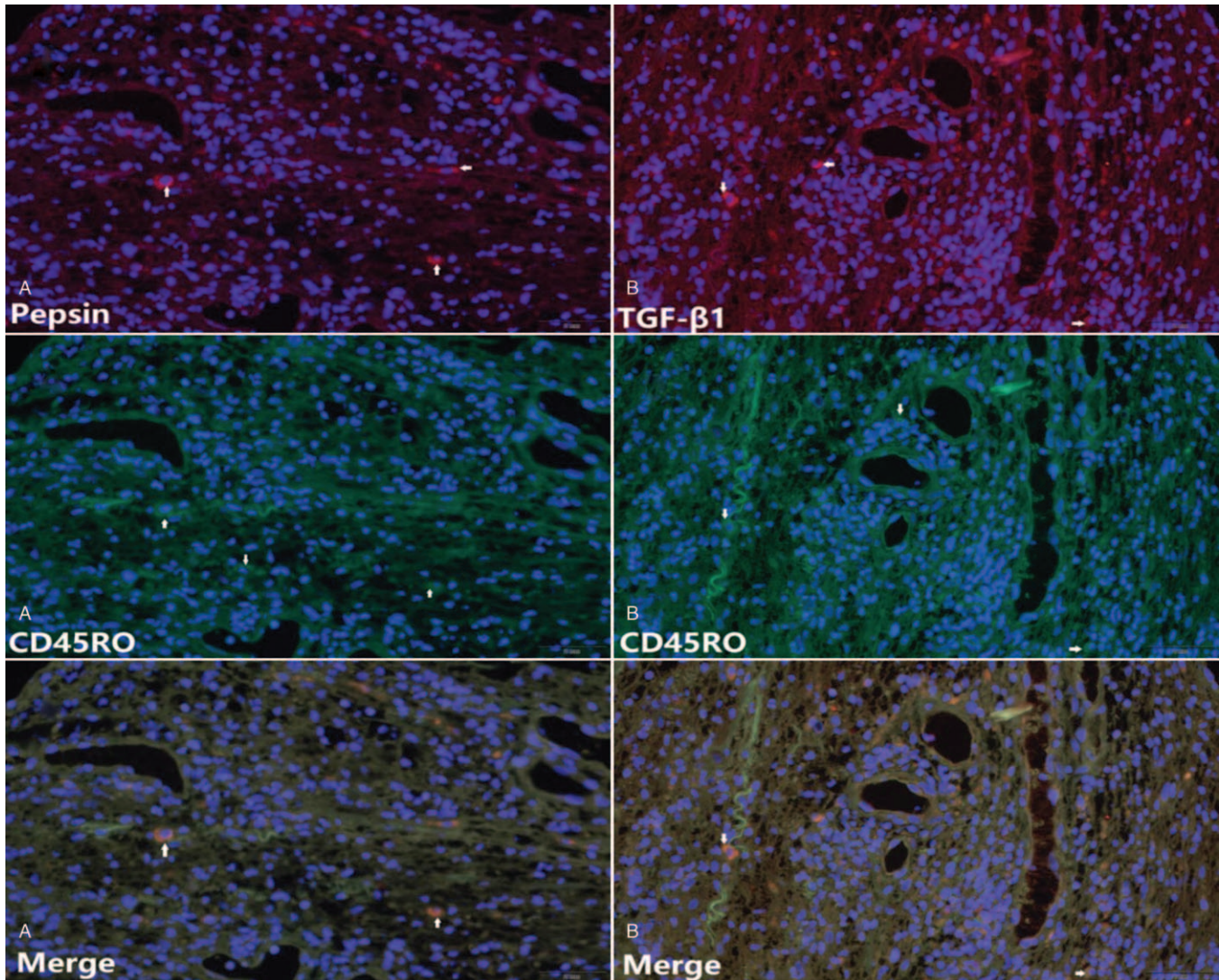
In this study, the incidence of gastroesophageal reflux in the VFP group was significantly higher than that in the control group (Table 3). Both acid reflux and non-acid reflux occur in GERD patients, and non-acid reflux is suspected to be an important factor in laryngeal inflammation.<sup>[27]</sup> The GerdQ score reflects the subjective symptoms of patients. In this study, the GerdQ scores of patients with VFps were significantly higher than those of the

control group, indicating that some patients with VFps had more obvious reflux symptoms. The concentration of salivary pepsin in the VFP group was also significantly higher than that in the control group. Therefore, the high level of pepsin in saliva suggested the occurrence of reflux, and the reflux in patients with VFps was more serious than that in the control group.

Our experiments showed that patients with VFps had higher salivary pepsin concentrations and pepsin-positive rates. Previous studies have found that pepsin is inactive when the pH value is between 6 and 8, but the structure is stable. When the pH value is below 6, pepsin can be reactivated.<sup>[28]</sup> Therefore, pepsin can remain stable on the surface of the throat (pH $\approx$ 6.8) at body temperature. When extra-esophageal reflux reaches the laryngeal area, pepsin can be transferred into laryngeal cells via receptor-mediated endocytosis.<sup>[23,29]</sup> Samuels and Johnston et al found that inactive pepsin can be reactivated and damage cells in 2 ways. For example, when acidic reflux happens, the pH of the environment decreases to the appropriate range for pepsin activation. Moreover, the Golgi apparatus and lysosomes have a lower pH, hence the pepsin endocytosed by laryngeal and hypopharyngeal epithelial cells can be reactivated and cause intracellular damage.<sup>[23]</sup> We also found that 80% of the VFP samples showed positive pepsin staining. Moreover, the positive rate of pepsin in VFP tissues was higher than the positive rate for 24-h MII-pH monitoring and salivary pepsin. In this study, we found that the pepsin concentration in saliva positively correlated with the pepsin level in VFP tissues. Jiang et al reported that pepsin IHC staining of laryngeal mucosa is a sensitive and specific method for the diagnosis of laryngeal reflux in clinical practice.<sup>[15]</sup> However, biopsy of the laryngeal mucosa is an invasive procedure. Alternatively, a salivary pepsin test can be used as a reliable method for the diagnosis of extraesophageal reflux.

As shown in Figure 4, pepsin was co-located with CD45RO as well as TGF- $\beta$ 1 and CD45RO. The present study is the first of its kind to show that pepsin staining correlates with the expression of inflammatory factors and is co-localized with CD45RO, suggesting that pepsin may lead to the activation of memory T lymphocytes and mediate immune responses, indicating a





**Figure 4.** Double immunofluorescence staining ( $\times 400$ ) in the vocal fold polyps: (A) Immunofluorescence double labeling for pepsin (red) and CD45RO (green) shows cytoplasmic staining. (B) Immunofluorescence double labeling for TGF- $\beta 1$  (red) and CD45RO (green) shows cytoplasmic staining.

potential new pathogenesis for VFP. CD45RO cells can secrete a variety of factors (IFN $\gamma$ , TNF $\alpha$ , IL-4, IL-6).<sup>[30]</sup> When the throat and vocal cord mucosa is stimulated by pathogenic agents via antigen cell processing, T helper cells are activated, releasing a variety of related cytokines and causing inflammatory cell aggregation, proliferation, and activation. Local inflammation that persists for too long can eventually lead to tissue damage. Studies have reported that the expression of many inflammatory cytokines and receptors such as CCL26, IL8, IL1F10, IL1A, IL5, BCL6, CCR6, and CXCL14 genes in human pharyngeal epithelial cells after overnight exposure to pepsin (under neutral pH conditions) was more than 1.5 times that of the control group.<sup>[31]</sup> This suggests that not only acid reflux, but also pepsin in non-acid reflux can cause pharyngeal inflammation. TGF- $\beta 1$  is one of the known mediators of inflammation.<sup>[32–34]</sup> As shown in Figures 2 and 3, TGF- $\beta 1$  was found in regions similar to those stained by pepsin in VFPs, suggesting that pepsin staining was associated with the expression of inflammatory factors. These findings suggest that pepsin may promote the aggregation of

immune cells and lead to the increase of local cytokines in patients with VFP.

There are some limitations to the current study. First, the sample size was small. Second, it was a single center study. Future multicenter trials with larger sample sizes are required to validate the findings of this study. Third, due to ethical reasons, we could not obtain vocal cord tissues from normal subjects in the control group. Therefore, we could not examine whether normal physiological reflux can also cause positive staining of the vocal cords with pepsin. In addition, the cytokines in the VFPs were not directly examined in this study, hence the direct effect of pepsin on the cytokines in VFPs remains unknown. Further experiments are needed to explore the mechanism in detail.

## 5. Conclusions

There is a close association between GERD and VFP. In this study, we demonstrated that pepsin may promote the aggregation of immune cells, leading to the increase of local cytokines and

thereby promoting the development of an inflammatory response, which may possibly explain the link between GERD and VFP. Moreover, the salivary pepsin test can be a reliable alternative to pepsin IHC staining for the diagnosis of extraesophageal reflux.

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## Author contributions

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**Resources:** Xia Li.

**Supervision:** ZhaoXia Yang.

**Validation:** ZhaoXia Yang.

**Writing – original draft:** Yazhu Zou.

**Writing – review & editing:** ZhaoXia Yang.

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