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Brief Communication

Limited Clinical Utility of Lipid-Laden Macrophage Index of Induced Sputum in Predicting Gastroesophageal Reflux-Related Cough

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ABSTRACT

Gastroesophageal reflux disease (GERD) is a common cause of chronic cough (CC). However, the diagnosis of GERD associated with CC based on 24-hour esophageal pH-monitoring or favorable response to empirical anti-reflux trials is invasive and time-consuming. Lipid-laden macrophages (LLMs) are supposed to be a biomarker for micro-aspiration of gastric content in the respiratory tract. This study was conducted to collect LLMs by the sputum induction technique and observe the relationship among the amount of LLMs, cough severity, parameters of 24-hour esophageal pH-monitoring and therapeutic response. The 24-hour esophageal pH-monitoring and sputum induction were performed on 57 patients with suspected GERD associated with CC. Thirty-four patients were followed up after empirical anti-reflux trials of 8 weeks to record the therapeutic response. Lipid-laden macrophage index (LLMI), a semiquantitative counting of LLMs, showed no significant correlation with the values of 24-hour esophageal pH monitoring at the proximal or remote electrode. No difference in LLMI or DeMeester score, as well as cough symptom association probability, were found between the responders and the non-responders. Reflux symptoms were more common in the responders (50%) compared to the non-responders (6%) ($P < 0.05$). Our study suggests that LLMI shows limited utility in clinically diagnosing GERD associated with CC as an underlying etiology or in predicting response to anti-reflux therapy. Anti-reflux therapy is more effective for CC patients with reflux symptoms than for those without.

Keywords: Cough; gastroesophageal reflux; macrophages; sputum; diagnosis

INTRODUCTION

Gastroesophageal reflux disease (GERD) associated with chronic cough (CC) is a common etiology of CC, especially in the European and US populations, accounting for 5%–40%.[1-3] The exact mechanism underlying GERD associated with CC remains unknown. Micro-aspiration is an alternative theory that has been suggested to play a role in GERD associated

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There are no financial or other issues that might lead to conflict of interest.

with CC, which supposes that gastric content induces the cough reflex by directly irritating the respiratory tract.[2,4] The 24-hour esophageal pH-monitoring test is recommended for patients with suspicious GERD associated with CC, which has been shown to be the most sensitive and specific diagnostic capability.[2,3] Unfortunately, 24-hour esophageal pH-monitoring has not been extensively used because its invasiveness and limited availability lead to difficult diagnosis.[3,5] Pulmonary macrophages that become laden with lipid are termed lipid-laden macrophages (LLMs).[6] The formation of LLMs are largely determined by the phagocytic ability of macrophages on lipid-containing material which is related to micro-aspiration.[7] Observing LLMs from sputum induction seems to be a non-invasive and more accessible methods to diagnose GERD associated with CC. Previous studies have found that LLMs in induced sputum are a marker for oropharyngeal reflux and possible gastric aspiration.[8] However, it is controversial whether LLMs in induced sputum would predict response to empirical anti-reflux treatment in patients with clinically suspicious GERD associated with CC. In this study, we aimed to explore the correlation among esophagus reflux, therapeutic response and lipid-laden macrophage index (LLMI), a semiquantitative counting of LLMs.

MATERIALS AND METHODS

Patients

Patients suspective of GERD associated with CC were recruited from the respiratory outpatient clinics of Guangzhou Institute of Respiratory Health from 2017 to 2019. In clinical practice, a validated, systematic, step-by-step diagnostic algorithm was used for determining the cause of CC, with uniform inclusion and exclusion criteria according to the cough guidelines proposed by the American College of Chest Physicians (ACCP) and Chinese Thoracic Society (CTS).[2,9]

The inclusion criteria were as follows: 1) age > 18 years; 2) cough as the sole or predominant symptom lasting for at least 8 weeks, with normal chest X-rays; 3) no evidence for cough variant asthma and eosinophilic bronchitis: normal spirometry (forced expiratory volume in one second [FEV1] % predicted >80% with FEV1/forced vital capacity [FVC] ≥ 0.7), negative bronchial provocation test, sputum eosinophils < 2.5% and normal FeNO (FeNO < 25 ppb); and 4) no evidence for upper airway cough syndrome (UACS): absence of UACS-relevant symptoms and abnormal examinations. Moreover, the exclusion criteria were as follows: 1) current smokers or people who quitted smoking for less than 2 years; 2) use of angiotensin-converting enzyme inhibitors or nasal medications (oily nose drops); and 3) patients who had a history of chronic foreign aspiration and lipid storage disease, or had ever received nutritional supplementation with lipid emulsions.

Recipients received a fully detailed assessment of etiology, including dual-channel esophageal pH-monitoring test and sputum induction. Patients were assigned to receive anti-reflux therapy including antacids and prokinetic agents. The proton pump inhibitors and prokinetic agents used in the study were esomeprazole and domperidone, respectively. Esomeprazole (20 mg bid) and domperidone (10 mg tid) were prescribed for 8 weeks. The efficacy of anti-reflux therapy was recorded after 8-week follow-up. Patients were divided into 2 groups according to the treatment response, and the improvement was assessed by cough symptom score. The patients with more than 50% improvement in cough symptom after anti-reflux therapy were considered the responder group, while the others were classified

the non-responder group. All subjects signed the consent form. The study was approved by the Institutional Review Board (IRB) of the First Affiliated Hospital of Guangzhou Medical University (No. IRB 201911).

The 24-hour dual-channel esophageal pH-monitoring test

All patients underwent double-channel 24-hour esophageal pH-monitoring in accordance with previous studies.[4,8] None of the subjects had received anti-reflux treatment before. Reflux episodes were defined as a decrease in esophageal pH below 4 for longer than 5 seconds, followed by an increase in pH for a minimum of 4.5, thus avoiding oscillating phenomena. This enabled us to detect the total acid exposure time (seconds), percentage of total time (%), mean acid clearance time (seconds), number of reflux episodes, time of the longest reflux episode (seconds) and number of reflux episodes over 5 minutes. Deemester score and cough symptom association probability (SAP) were able to be detected which reflected the severity of reflux and relationship between reflux and cough respectively.

Induced sputum and staining of LLMs

Induced sputum was obtained from all the recipients as previously described.[8,9,10] The sputum was induced before the initiation of anti-reflux treatment with hypertonic saline using an ultrasonic nebulizer. The induction was carried out with an aerosol of 3% saline for 3 sessions of 7 minutes each. After each inhalation, the patient was asked to cough and expectorate sputum into a universal container. The induction was stopped if patients had obvious respiratory symptoms. All the subjects underwent the procedure safely. The sputum was separated from saliva and put on the slide. After air-drying and neutral formaldehyde fixation, the slides were counterstained with hematoxylin and Oil-Red-O for scoring and LLMI calculation.[6]

Measurement of LLMI

We used the LLMI to quantify lipid accumulation in sputum macrophages as previously proposed.[8,9] The amount of intracellular lipid in each macrophage was evaluated using a grading system for semi-quantitating[10]: Grade 0, absence of intracellular lipid droplets; Grade 1, one or a few lipid droplets; Grade 2, many distinct droplets; Grade 3, many confluent droplets with visible nucleus; and Grade 4, many confluent intracellular droplets completely opacifying the cytoplasm and obscuring the nucleus (**Fig. 1**). LLMI was the sum of the scores for 100 consecutive macrophages, which ranges from 0 to 400. The LLMI was determined by the mean of values assessed independently by 2 observers.

Statistical analysis

Correlations between LLMI and esophageal pH-monitoring parameters were analyzed by Pearson's correlation. Difference in LLMI and pH-monitoring parameters were compared between the positive and negative response groups by the Mann-Whitney test. Categorical variables are presented as percentages, and their comparisons were made using the χ^2 or Fisher's exact test. Differences with a $P < 0.05$ were considered statistically significant.

RESULTS

Subject characteristics

A total of 57 subjects with suspicions of GERD associated with CC were enrolled. All of them received standard anti-reflux therapy. Thirty-four patients were followed up successfully.

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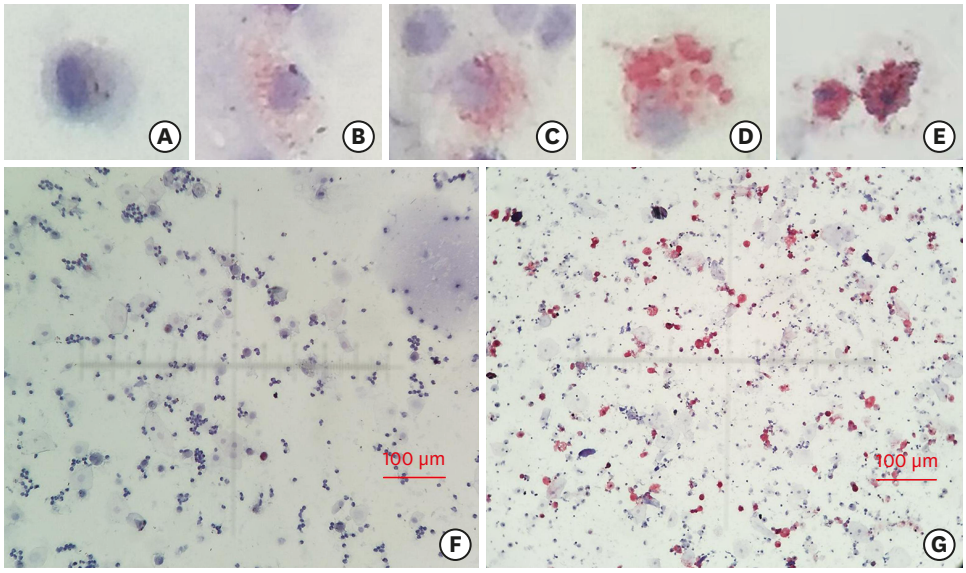

Fig. 1. Determination of LLMI. Cytoplasmic lipid droplets was detected by staining with Oil-Red-O. Sputum macrophages were graded by semiquantitating the amount of intracellular lipid. LLMI was the sum of the scores for 100 macrophages. (A) Grade 0: no lipid droplets. (B) Grade 1: one or a few lipid droplets. (C) Grade 2: many distinct droplets. (D) Grade 3: many droplets with visible nucleus. (E) Grade 4: many droplets completely covering the cytoplasm and obscuring the nucleus. (F) A sample of low LLMI. (G) A sample of high LLMI (bar = 100 μm).
LLMI, lipid-laden macrophage index.

They were classified into the positive (n = 18) and negative response groups (n = 16). Patients' baseline characteristics are presented in **Table 1**. There were no significant differences in age and sex distribution between the 2 groups.

Correlation between values of 24-hour pH monitoring and LLMI

LLMI showed no significant correlation with Deemester score ($P = 0.767$) or SAP ($P = 0.480$). No correlation was observed between LLMI and esophageal pH-monitoring data at the proximal channel (all $P > 0.05$), and neither was there any significant correlation between LLMI and data at the distant probe (all $P > 0.05$). The P values are presented in **Table 2**.

Difference between the positive and negative response group

No significant differences in LLMI, DeMeester score or SAP were observed between the 2 groups (all $P > 0.05$) (**Fig. 2**). Also, there was no significant difference in the data of 24-hour dual channel PH monitoring between the 2 groups (**Table 1**). Compared to the positive response group which included 9 patients with reflux symptoms (regurgitation and/or heartburn) as the initial symptom, only 1 patient in the negative response group suffered from reflux symptoms as the initial symptom ($P < 0.01$) (**Table 1**). Furthermore, regurgitation (50%) was found to have significantly higher sensitivity compared to heartburn (16.7%) in predicting response to treatment ($\chi^2 = 4.17$, $P < 0.05$). No significant differences were detected in specificity between regurgitation (93.75%) and heartburn (100%) ($P > 0.05$). From the current data, regurgitation showed better predictive value in the response to anti-reflux treatment than heartburn.

DISCUSSION

Main mechanisms of gastroesophageal reflux associated with CC are the esophagobronchial vagal reflex and micro-aspiration.[1,5] In this study, we investigated the role of micro-aspiration

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Table 1. The baseline characteristics of patients

Characteristics	Total	Positive	Negative	*P* value (positive *vs.* negative)
Numbers	57	18	16	NA
Age (yr)	42.0 ± 13.5	42.9 ± 12.8	39.7 ± 13.1	0.927
Sex (male/female)	24/33	8/10	10/6	0.292
With reflux symptoms	10	9	1	**0.008**
Without reflux symptoms	47	9	15	
LLMI	22.5 (6.5–45.5)	25 (6.5–46.5)	22 (5.75–48.5)	0.998
Cough severity (VAS score)				
Before treatment	5.5 ± 1.2	5.4 ± 1.1	5.6 ± 1.5	0.825
After treatment	3.2 ± 2.4	1.2 ± 0.8	5.4 ± 1.4	**0.001**
24-hour dual channel PH monitoring				
DeMeester	6.5 (2.8–11.3)	7.7 (3.1–23.9)	9.5 (4.7–11.3)	0.328
SAP	0 (0–85.5)	39 (0–87.8)	45 (0–98)	0.597
Proximal channel (acid exposure)				
Total acid exposure time (sec)	18 (0–198)	27 (0–465)	15 (0–186)	0.578
Percentage of total time (%)	0 (0–3)	0 (0–6)	0 (0–0.3)	0.530
Mean acid clearance time (sec)	9 (0–27)	16.5 (0–41.8)	8.5 (0–24.3)	0.699
Number of reflux episodes	1 (0–6)	1.5 (0–7.5)	2 (0–7)	0.973
The longest reflux episode (sec)	10 (0–69)	22.5 (0–193.5)	10 (0–85.8)	0.530
Remote channel (acid exposure)				
Total acid exposure time (sec)	858 (303–2,187)	1,464 (259.5–5,193)	1,581 (618–2,325)	0.328
Percentage of total time (%)	1.1 (0.4–2.9)	1.9 (0.3–6.5)	2.3 (0.8–2.9)	0.328
Mean acid clearance time (sec)	28 (14–40.5)	30 (12–49)	32 (15.3–45.8)	0.856
Number of reflux episodes	40 (18.0–57.6)	41.5 (15.5–112.8)	43 (29–66.3)	0.827
Time of the longest reflux episode (sec)	186 (60–348)	318 (40–450)	213 (111–358.5)	0.408
Number of reflux episodes over 5 min	0 (0–1.1)	1.1 (0–2.2)	0 (0–0.8)	0.408

Data are presented as mean ± standard deviation or median (interquartile range).
SAP, symptom association probability; LLMI, lipid-laden macrophage index; VAS, visual analogue scale; SAP, symptom association probability; NA, not applicable.
P values < 0.05 are in bold.

Table 2. Correlations between LLMI and 24-hour esophageal pH-monitoring parameters

	LLMI (n = 57)	
	r	*P*
DeMeester score	−0.040	0.767
Cough SAP (%)	0.096	0.480
Proximal channel (acid exposure*)		
Total acid exposure time (sec)	−0.133	0.326
Percentage of total time (%)	−0.211	0.116
Mean acid clearance time (sec)	−0.084	0.536
Number of reflux episodes	−0.133	0.240
Time of the longest reflux episode (sec)	−0.110	0.415
Remote channel (acid exposure*)		
Total acid exposure time (sec)	−0.102	0.449
Percentage of total time (%)	−0.107	0.427
Mean acid clearance time (sec)	−0.240	0.720
Number of reflux episodes	0.011	0.935
Time of the longest reflux episode (sec)	−0.089	0.508
Number of reflux episodes over 5 min	0.070	0.604

LLMI, lipid-laden macrophage index; SAP, symptom association probability.
*Acid exposure is defined as episode of a fall in pH to < 4.0 at the esophageal electrode. Acid clearance time was defined as the time from the moment the esophageal pH dropped below 4 until it recovered to a value of 4.0 or until a new reflux episode started. All data were analyzed by spearman correlation.

in patients with suspected GERD associated with CC by measuring LLMs in sputum. The mechanism for the formation of LLMs is largely determined by the phagocytic ability of macrophages on lipid-containing material. Micro-aspiration, in which refluxate reaches the airways through the proximal esophagus and the larynx, is another main source of lipid-

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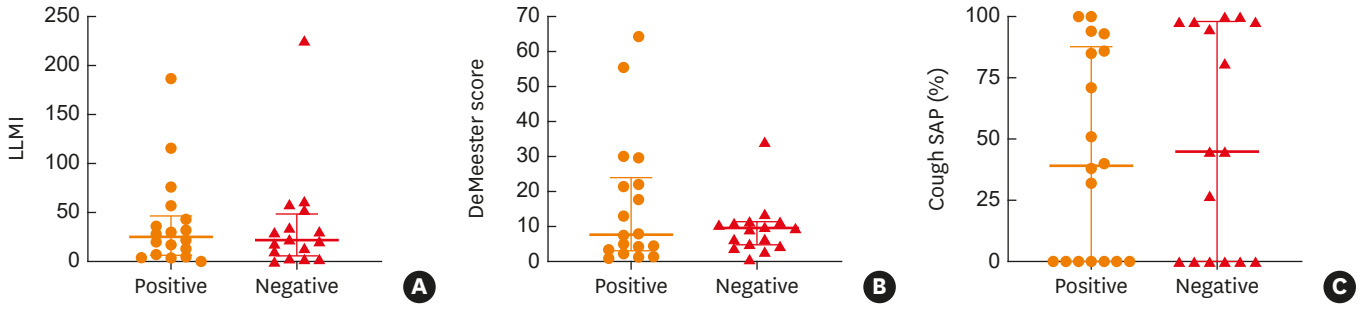

Fig. 2. Comparison of LLMI, DeMeester score, and cough SAP between positive and negative response groups. (A) No difference of LLMI in sputum was found between 2 group. (B) No difference of DeMeester score was observed between 2 group. (C) No difference of SAP was found between 2 group. All $P > 0.05$.
LLMI, lipid-laden macrophage index; SAP, symptom association probability.

containing material which is common in GERD.[9] The lipid-containing material can also come from external stimuli like air pollution, e-cigarettes and fat content diet.[11,12] Hence, irrespective of the type of reflux (acidity or non-acidity), the aspiration of lipid-containing reflux content into the airways contributes to the formation of LLMs.

The diagnosis of GERD associated with CC is based on CC associated with gastroesophageal reflux symptoms or reflux evidence proved by 24-hour esophageal pH-monitoring as well as favorable response to anti-reflux empirical trials.[2,3,5] However, esophageal pH-monitoring is time-consuming, invasive and limitedly available. Empirical trials also cost time and may result in unnecessary use of medication. LLMI is a simple index acquired from bronchoalveolar lavage fluid (BALF) or induced sputum.[4,8,10,13] Many previous studies used bronchoalveolar lavage (BAL) to obtain LLMI.[4,13] However, compared to BAL, induced sputum is non-invasive and more acceptable for patients. It would be meaningful if LLMI of induced sputum could serve as a routine predictor for response to anti-reflux treatment. However, in the current study, no correlation was found between LLMI and pH monitoring parameters. No difference in LLMI was observed between the positive and negative respond groups.

We initially hypothesized that LLMI somehow correlates to pH monitoring parameters, especially at the proximal probe. However, no correlation between LLMI and pH monitoring parameters suggests that LLMI might not be a significant biomarker for acid gastroesophageal reflux. Previous studies showed controversial results about the relationship between reflux and LLMI.[4,9,13,14] Parameswaran[9] reported higher LLMI of sputum in subjects with oropharyngeal reflux than in those without. In contrast, similar to our study, Köksa *et al.*[10] and Rosen *et al.*[14] found that the LLMI of BALF is not an indicator of pulmonary symptoms secondary to gastroesophageal reflux in adults or children. These results indicate that micro-aspiration into the lungs might be absent even in patients who suffer from gastroesophageal reflux. A plausible explanation for this may be that although some GERD patients associated with CC have an abnormal anti-reflux barrier, they could have functional epiglottis and the cough reflex which protects their lungs from reflux content.[15,16]

Patients with CC who had a positive response to anti-reflux therapy were considered to have GERD associated with CC, while patients who did not respond well were considered to have non- or refractory GERD associated with CC.[17] No difference in LLMI levels in either group implies that LLMI may have insignificant correlation with therapeutic effects in our patients. Since gastroesophageal reflux content could induce formation of LLMs, the negative results imply that micro-aspiration seems to be a less likely cause of GERD associated with CC.

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Pepsin is another biomarker uniquely produced in the stomach, and the presence of pepsin in the lungs suggests the transfer of gastric content to the airway, a consequence of reflux and micro-aspiration.[15,18] Similarly, Decalmer *et al.*[15] showed that patients with GERD patients associated with CC had levels of pepsin in BAL similar to healthy control subjects. Grabowski *et al.*[19] found no difference in pepsin levels in sputum between patients with CC and the control group. It seems that micro-aspiration may not be the main mechanism for GERD-associated CC. A previous study revealed that repeated esophageal acid stimulation of guinea pigs can induce airway neurogenic inflammation and neurochemical alterations in the neural pathways of the vagus-mediated esophageal bronchial reflex.[20,21] Another study demonstrated that the dorsal vagal complex is involved in the esophagobronchial reflex and modulates neurogenic airway inflammation induced by gastroesophageal reflux.[22] It is indicated that CC might be triggered by the esophagobronchial reflex encompassing peripheral and central pathways. Taken together, the esophagobronchial reflex is more likely to be involved in GERD-associated CC.[23,24]

Both acid and non-acid refluxes can be the reason for gastroesophageal reflux cough. In accordance with a previous study,[3] there is no significant benefit of receiving anti-acid therapy for patients with rare or no heartburn that was provoked by acid reflux. Compared to the non-responder group, the responder group contains more patients with regurgitation and/or heartburn, which indicates that CC patients with reflux symptoms are more sensitive to anti-reflux therapy than those without. Furthermore, our result indicated that regurgitation was more predictive of therapeutic response than heartburn in suspected GERD associated with CC. Anti-reflux therapy might be helpful in managing patients with GERD associated with CC. The diagnostic value of various reflux symptoms requires further investigations with larger samples.

There are some limitations that need to be considered. First, multichannel intraluminal impedance pH monitoring, which can detect both acidic reflux and nonacidic refluxes, was not used in this study. This means that the number of detected refluxes might be less than the actual one, which may contribute to the negative results of the relation between LLMI and reflux monitoring. Secondly, this study failed to record the therapeutic responses of all patients. Including more patients in both groups will be better for analyzing the characteristics of 2 populations. Thirdly, changes in LLMI were unknown in sputum of those who compared after anti-reflux therapy, which warrants further studies. Finally, as an observational study, placebo effect is an inevitable limitation. Hence, a placebo control group to minimize this effect should be considered in future experiments.

In conclusion, this study suggests that LLMI shows limited utility in clinically diagnosing GERD associated with CC as an underlying etiology or in predicting response to anti-reflux therapy. Anti-reflux therapy might be more helpful for CC patients with reflux symptoms than those without.

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