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Charcot-Leyden Crystal Concentration in Nasal Secretions Predicts Clinical Response to Glucocorticoids in Chronic Rhinosinusitis with Nasal Polyps

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Title: Charcot-Leyden Crystal Concentration in Nasal Secretions Predicts Clinical Response to Glucocorticoids in Chronic Rhinosinusitis with Nasal Polyps

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Short title: Nasal CLC predicts GC responses in CRSwNP.

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Keywords: Chronic rhinosinusitis with nasal polyps (CRSwNP); Charcot-Leyden crystals (CLCs); Receiver operating characteristic (ROC) curve; Corticosteroid resistance.
Capsule Summary

Charcot-Leyden Crystal concentration in nasal secretions may serve as a suitable non-invasive biomarker with predictive capability for response to GC therapy in CRSwNP patients, and possibly lead to better strategies for GC therapy in future.
To the Editor:

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a heterogeneous disease characterized by a defective immune barrier and massive inflammatory cell infiltration\textsuperscript{1,2}. Currently the most effective medical therapy in clinical practice is the use of glucocorticoids (GCs)\textsuperscript{3,4,5,E1}. However, many patients suffer from poor response to the therapy\textsuperscript{1,4,5}. Thus, developing biomarkers predicting GC response in CRSwNP patients would greatly improve the efficacy of GC and lighten the economic burden in clinical practice. Although several biomarkers associated with GC resistance have been discovered recently, most of these have been detected in polyp tissue samples\textsuperscript{6,7}. As the procedure for obtaining tissues often leads to unavoidable trauma of mucosa and increased risk of bleeding and infection, there is need for biomarkers that can be detected using noninvasive procedures.

Charcot-Leyden crystal (CLC) was first described in the 19\textsuperscript{th} century\textsuperscript{8}. It is a unique component of eosinophils and basophils and is regarded as a hallmark protein of eosinophilic inflammatory diseases. Previous studies have demonstrated the presence of CLC in sputum of patients diagnosed as allergic asthma or pulmonary ascariasis, and in the feces of patients with eosinophilic diseases of digestive system, such as ulcerative colitis and amebic Trichuris\textsuperscript{8}. In preliminary experiments we have successfully detected CLC in nasal secretions of CRSwNP patients, collected according to the noninvasive method of Watelet and colleagues\textsuperscript{9}. As rhinorrhea is one of the most common symptoms in these patients, and these secreted fluids can readily be collected using a noninvasive method, we have hypothesized that CLC in nasal secretions may serve as a predictive marker of GC response in CRSwNP.
patients. Thus, this study aimed to investigate the predictive capacity of CLC concentration in nasal secretions for GC response in CRSwNP patients.

This study was approved by the Ethics Committee of Beijing TongRen Hospital and all the participants provided written informed consent. Eighty-nine patients diagnosed as CRSwNP according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012\textsuperscript{E1} and who had no contraindications to GC were enrolled in the study. None of the patients had received any treatment with GC or immunomodulatory drugs within 4 weeks prior to enrollment. Patients with fungal sinusitis, allergic fungal rhinosinusitis, cystic fibrosis or primary ciliary dyskinesia were excluded from the study and all eligible patients were evaluated for comorbidity of allergic rhinitis, asthma and atopy. None of the enrolled patients were diagnosed with aspirin exacerbated respiratory disease (AERD). Clinical characteristics such as staging of CT and objective evaluation of olfactory function were determined as described previously\textsuperscript{E1-E6} (detailed criteria for each clinical characteristic are presented in Patients and samples section in the article’s Online Repository at www.jacionline.org). Bilateral nasal cavity secretions and polyp tissues were collected for comparison from each patient on admission to the hospital, as described previously\textsuperscript{9} (detailed information on collection of nasal secretions presented in Patients and samples section in the article’s Online Repository at www.jacionline.org). Following enrollment, all patients started a 2-week oral GC therapy (methylprednisolone, 24mg qm po), and at the end of this treatment period were divided into two groups according to nasal polyp size scoring (NPSS) system (see Table E1 in the article’s Online Repository at www.jacionline.org):
GC-responders group (n=48, patients whose change in NPSS was more than 1 point) and GC-non-responders group (n=41, patients whose change in NPSS was no more than 1 point).

Data for the clinical characteristics for each patient were expressed as the medians and interquartile ranges (IQR) except for binary variables and age (expressed as the mean and standard deviation) (see Table E2 in the article’s Online Repository at www.jacionline.org).

Concentration of CLC in the nasal secretions was analyzed by Enzyme-Linked Immunosorbent Assay (ELISA). Statistical analysis was performed by GraphPad Prism6 and receiver operating characteristic (ROC) curves were drawn using SPSS 24.0 (IBM Corp, Armonk, NY) (details presented in the Methods section in this article’s Online Repository at www.jacionline.org). Statistical significance was considered at p value of less than 0.05.

The two groups were not significantly different in terms of gender, age, smoking history, nasal obstruction, rhinorrhea, facial pain or subjective olfactory disorder. However, patients in GC-responders group had higher rates of comorbid allergic rhinitis (p=0.025, OR=3.600, 95%CI=1.184-10.94) and atopy (p=0.020, OR=2.824, 95%CI=1.190-6.699), than patients in GC-non-responders group. Similarly, patients in GC-responders group showed significantly greater severity of olfactory disorder (p=0.035), higher E/M (total ethmoid sinus scores/total maxillary sinus scores) ratios of Lund-Mackay scoreE6 (p<0.001), and higher percentage of peripheral blood eosinophils (p=0.016), than patients in GC-non-responders group. There was no significant difference in percentage of neutrophils between the two groups (p=0.172). Histological evaluation of polyp tissue revealed that patients in GC-responders group had significantly higher numbers of infiltrating eosinophils (p<0.001).
and lower numbers of infiltrating neutrophils ($p<0.001$) compared with patients in GC-non-responders group.

Analysis of the average concentration of CLC in nasal secretions showed that this was significantly higher (105.3ng/mL; IQR: 51.08-153.5ng/mL) in patients in the GC-responders group than in patients in the GC-non-responders group (0.6460ng/mL; IQR: 0-29.34ng/mL); $p<0.001$) (see Table E2 in this article’s Online Repository at www.jacionline.org). Four patients in the GC-responders had much higher CLC concentrations (outliers, marked red in Figure 1B; with three being 1000ng/mL and one 651.8ng/mL). Despite excluding these data, the difference between the median=90.02ng/mL (IQR: 50.18-136.3ng/mL) value for the GC-responders group was still found to be significantly higher than for GC-non-responders group ($p<0.001$).

To determine the specific factors associated with polyp recurrence, binary logistic regression model method of forward Wald mode was employed for further comparison between the groups. All the clinical characteristics; including CLC concentration in nasal secretions, percentages of eosinophils, neutrophils, lymphocytes and plasma cells in polyp tissues, percentage of eosinophils in peripheral blood, Lund-Mackay Score E/M ratio, objective olfactory function and comorbid rates of allergic rhinitis and atopy; which had been found to be significantly different by preliminary analyses were introduced as variables in the binary logistic regression model method. The multivariate analysis revealed that, only CLC concentration in nasal secretions ($p<0.001$, OR=1.043, 95%CI=1.020-1.066) and percentage of eosinophils in polyp tissues ($p<0.001$, OR=1.056, 95%CI=1.025-1.087) showed the
potential for predicting GC-response in CRSwNP patients (see Table E3 in this article’s Online Repository at www.jacionline.org). ROC curves for these two parameters (shown in Figure 1A) demonstrated the corresponding area under curve (AUC) to be 0.897 and 0.855 for CLC concentration in nasal secretions and the percentage of eosinophils in polyp tissues, respectively. According to the Youden’s index (Table E4), the optimal cut-off value for CLC concentration in nasal secretions to predict GC response was 30.065ng/mL (Youden’s index=67.6%, with a sensitivity of 89.6% and a specificity of 78.0%; Figure 1B), and the optimal cut-off value for percentage of eosinophils in polyp tissues to predict GC response was 41.50% (Youden’s index=66.6%, with a sensitivity of 81.3% and a specificity of 85.4%; Figure 1C).

In regard to the effect on GC, NPSS of GC-responders and GC-non-responders patients demonstrated no difference before GC treatment \( (p=0.968) \), but significant differences in NPSS of post-GC treatment \( (p<0.001) \) and change in NPSS pre- and post-GC therapy \( (p<0.001) \). Analysis of the CLC concentrations in 29 patients (12 of whom were GC-responders and 17 GC-non-responders) for whom samples were available, indicated that CLC was detectable in both subgroups pre- and post-GC treatment and that GC treatment significantly decreased CLC concentration in the GC-responders group \( (p=0.002) \), but not in the GC-non-responders group \( (p=0.211) \) (Figure 1D and 1E).

Additional analysis of the result for CLC concentrations indicated that patients with CLC concentrations lower than 30.065ng/mL \( (n=37) \) showed significantly greater differences in responses to GC therapy compared with the patients with CLC concentrations higher than
the cut-off value (n=52) (p<0.001) (Figure 2A). Although the NPSS was not significantly different between the two groups before GC therapy (p>0.999), this was significantly decreased after GC therapy in patients with higher CLC concentrations compared to patients with lower CLC concentrations (p<0.001) (Figure 2B-C). Moreover, patients with higher levels of CLC concentrations in nasal secretions displayed more severe infiltration of eosinophils in both polyp tissues (p<0.001) and peripheral blood than patients with lower CLC concentrations (p=0.014) (Figure 2D-E); while infiltration of neutrophils was not significantly different in either polyp tissues (p=0.302) or peripheral blood (p=0.071) of patients between the two groups (Figure 2F-G). In contrast, CLC concentrations in nasal secretions were not significantly associated with distribution of gender, age, smoking history, comorbid asthma rhinitis or atopy (see Figures E1A-F in this article’s Online Repository at www.jacionline.org). Similarly, any of the four subjective symptoms (see Figures E2A-D in this article’s Online Repository at www.jacionline.org), objective olfactory test, Lund-Mackay score of paranasal sinuses or E/M ratios (see Figure E3A-C in this article’s Online Repository at www.jacionline.org) were also not significantly associated with CLC concentrations in the CRSwNP patients.

In this study we investigated the predictive significance of CLC concentration in nasal secretions for GC response of CRSwNP, and found that this noninvasive sampling method had a similar predictive capacity as the percentage of eosinophils in polyp tissues. CLC concentrations in nasal secretions were associated with GC responses and that GC therapy significantly decreased the CLC concentrations. Nevertheless, all the other parameters;
including gender, age, smoking history, comorbid asthma, comorbid allergic rhinitis, comorbid atopy, severity of symptoms, or staging of CT scan; had little impact on the CLC concentration in the nasal secretions. Also, CLC concentrations in nasal secretions were correlated with percentage of eosinophils in tissue polyps (Spearman correlation model, \( p<0.001 \) \( R=0.5 \)). The possibility that nasal secretions are as valuable as polyp tissues for predicting GC response in CRSwNP patients is particularly important as the collection of nasal secretions is much easier, safer and more tolerable than collection of polyp tissues, and the risk of trauma and hemorrhage are lower. Additionally, the use of nasal secretions is an efficient and accurate method of both detecting and counting various inflammatory cells.

In the case of the four GC-responders patients with much higher nasal CLC concentrations (outliers) than the rest of the group (marked red in Figure 2B), further analysis demonstrated that in these patients the clinical indices of percentage of eosinophils in polyp tissues, comorbidity of asthma, allergic rhinitis or percentages of inflammatory cells in tissue polyps were not significantly different compared to the other patients in the group.

Considering the efficacy and accuracy of GC therapy, it is important to predict GC response of patients prior to administration of GC therapy for long periods. Furthermore predicting the response to GC therapy in CRSwNP patients is particularly important, because GC insensitivity is often observed in a large number of this group of patients. In this regard, our finding that 46.1% (41/89) CRSwNP patients were insensitive to GC therapy is in accordance with the findings of a more recent study by Hong and colleagues\(^6\), which reported 44.2% (23/52) of CRSwNP patients to be non-responsive to GC therapy, and demonstrates
that CLC concentration in nasal secretions is likely to be a suitable biomarker for predicting GC response for CRSwNP patients.

Several studies have recently indicated different biomarkers, which might be associated with decreased response to GC in CRSwNP patients. For example, increased neutrophilia in nasal polyps, increased serum amyloid A (SAA), an apolipoprotein associated with high-density lipoprotein, upregulation of Mucin 4 and down-regulation of Mucin 1, E7, and IL-25 have been associated with GC insensitivity. However, all these biomarkers have been investigated in polyp tissues; thus, making the use of CLC concentration in nasal secretions a suitable easy and faster to employ alternative for predicting GC response in CRSwNP patients.

A major limitation of the present study is that it was conducted in Asian patients whose response to steroids is poor due to a higher prevalence of non-eosinophilic nasal polyps. Therefore, the finding for a predictive capability of nasal CLC secretion for nasal polyp response to GC is not generalizable to the western patients and needs to be investigated further in this population. Nevertheless, the determination of specific secreted proteins in nasal secretions may serve as reliable noninvasive biomarkers in the future to predict the response of CRSwNP to GC.

In summary, our study has demonstrated that CLC concentration in nasal secretions is a suitable noninvasive biomarker that could predict GC response for CRSwNP patients. Routine use of this biomarker in clinical practice may lead to better strategies for GC therapy in the future.
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Ph.D. #1,2, Chengshuo Wang, M.D., Ph.D. #1,2
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2 Beijing Key Laboratory of Nasal Diseases, Beijing Institute of Otolaryngology, Beijing 100005, China
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management of chronic rhinosinusitis with nasal polyposis. Eur Arch Otorhinolaryngol. 2017; 274(10):3559-3566


Figure 1: CLC concentration in nasal secretions and predictive value. (A) The area under curve (AUC) for CLC concentration in nasal secretions was 0.897 (OR=1.043; 95% CI: 1.020-1.066) while the AUC for percentage of eosinophils in polyp tissues was 0.855 (OR=1.056; 95%CI: 1.025-1.087). (B) Distribution of CLC concentration in nasal secretions in GC-responders group (n=48, four outliers are marked in red) and GC-non-responders group (n=41; p<0.001). The optimal cut-off value determined by Youden’s index was 30.065ng/mL. (C) Distributions of percentage of eosinophils in polyp tissues (p<0.001) in GC-responders group (n=48) and GC-non-responders group (n=41). The optimal cut-off value determined by Youden’s index was 41.50%. (D) Change in nasal CLC concentration before and after GC therapy in GC-responders group (n=12) and (E) GC-non-responders group (n=17).

Figure 2: Differences in clinical characteristics between patients with nasal CLC concentration lower (n=37) or higher than the cut-off value (n=52); (A) Number of CG-Responders (black columns)/Non-responders (gray columns); (B) NPSS post-GC therapy; (C) Change in NPSS pre- and post-GC therapy. (D) % eosinophils in polyp tissues; (E) % eosinophils in peripheral blood; (F) % neutrophils in polyp tissues; (G) % neutrophils in peripheral blood.
**TABLES**

**Table E1.** Nasal Polyp Size Scoring (NPSS) System

<table>
<thead>
<tr>
<th>Polyp Score</th>
<th>Polyp Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No polyps</td>
</tr>
<tr>
<td>1</td>
<td>Small polyps in the middle meatus not reaching below the inferior border of the middle concha</td>
</tr>
<tr>
<td>2</td>
<td>Polyps reaching below the lower border of the middle turbinate</td>
</tr>
<tr>
<td>3</td>
<td>Large polyps reaching the lower border of the attachment of inferior turbinate or polyps medial to the middle concha</td>
</tr>
<tr>
<td>4</td>
<td>Large polyps causing almost complete congestion/obstruction of the inferior meatus</td>
</tr>
</tbody>
</table>
Table E2. Demographic and Clinical Characteristics of GC-Responders and GC-non-Responders

<table>
<thead>
<tr>
<th></th>
<th>GC-Responder (48)</th>
<th>GC-non-Responders (41)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male/Female)</td>
<td>29/19</td>
<td>28/13</td>
<td>0.510</td>
</tr>
<tr>
<td>Age (Mean±SD)</td>
<td>44.69 ± 11.52</td>
<td>44.63 ± 11.66</td>
<td>0.983</td>
</tr>
<tr>
<td>Asthma (Y/N)</td>
<td>18/30</td>
<td>16/25</td>
<td>1</td>
</tr>
<tr>
<td>Allergic Rhinitis (Y/N)</td>
<td>16/32</td>
<td>5/36</td>
<td>0.025</td>
</tr>
<tr>
<td>Atopy (Y/N)</td>
<td>32/16</td>
<td>17/24</td>
<td>0.020</td>
</tr>
<tr>
<td>Smoking History (Y/N)</td>
<td>9/39</td>
<td>7/34</td>
<td>1</td>
</tr>
<tr>
<td>Nasal Obstruction (Median; IQR)</td>
<td>7.0; 6.0-8.0</td>
<td>7.0; 6.0-8.0</td>
<td>0.895</td>
</tr>
<tr>
<td>Rhinorrhea (Median; IQR)</td>
<td>6.0; 4.3-6.8</td>
<td>5.0; 3.5-6.5</td>
<td>0.101</td>
</tr>
<tr>
<td>Facial Pain (Median; IQR)</td>
<td>0; 0-2.8</td>
<td>0; 0-2.0</td>
<td>0.514</td>
</tr>
<tr>
<td>Subjective Olfactory Disorder (Median; IQR)</td>
<td>6.0; 4.0-10.0</td>
<td>4.0; 0-8.5</td>
<td>0.080</td>
</tr>
<tr>
<td>Objective Olfactory Function (Median; IQR)</td>
<td>5.0; 3.0-5.0</td>
<td>3.0; 1.0-5.0</td>
<td>0.035</td>
</tr>
<tr>
<td>Lund-Mackay Score (Median; IQR)</td>
<td>19.5; 14.3-22.0</td>
<td>17.0; 14.0-20.0</td>
<td>0.154</td>
</tr>
<tr>
<td>Lund-Mackay Score E/M Ratio (Median; IQR)</td>
<td>2.6; 2.0-3.5</td>
<td>2.0; 1.9-2.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nasal Polyp Size Score (Pre-GC Therapy) (Median; IQR)</td>
<td>5.0; 4.0-6.0</td>
<td>5.0; 4.0-6.0</td>
<td>0.767</td>
</tr>
<tr>
<td>Nasal Polyp Size Score (Post-GC Therapy)</td>
<td>2.0; 2.0-3.0</td>
<td>5.0; 4.0-5.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Measure</td>
<td>Median (IQR)</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>-----------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Nasal Polyp Size Score (Post Minus Pre)</td>
<td>-2.0; -3.0 - -2.0</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Eosinophils% (Tissues)</td>
<td>60.75; 10.52; 0-37.17</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Neutrophils% (Tissues)</td>
<td>0; 0-1.255</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes% (Tissues)</td>
<td>21.50; 48.70; 31.96-65.85</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Plasma Cells% (Tissues)</td>
<td>14.95; 21.30; 9.65-40.15</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Eosinophils% (Peripheral Blood)</td>
<td>5.950; 4.500; 1.650-7.900</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Neutrophils% (Peripheral Blood)</td>
<td>53.45; 56.00; 49.45-63.05</td>
<td>0.172</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (Nasal Secretions)</td>
<td>1.0; 0-2.0</td>
<td>0.032</td>
<td></td>
</tr>
<tr>
<td>CLC Concentration (Nasal Secretions)</td>
<td>105.3; 0.6460; 0-29.34</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>
Table E3. Binary Logistic Regression for Potential Markers Associated with GC Response

<table>
<thead>
<tr>
<th>Markers</th>
<th>Odd Ratio</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLC Concentration (Nasal Secretions)</td>
<td>1.043</td>
<td>1.020-1.066</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eosinophils% (Tissue)</td>
<td>1.056</td>
<td>1.025-1.087</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
Table E4. Determination of the Optimal Cut-off value for Each Marker

<table>
<thead>
<tr>
<th>Cut-off value</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Youden’s Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>28.425</td>
<td>0.896</td>
<td>0.732</td>
<td>0.628</td>
</tr>
<tr>
<td>29.340</td>
<td>0.896</td>
<td>0.756</td>
<td>0.652</td>
</tr>
<tr>
<td><strong>30.065</strong></td>
<td><strong>0.896</strong></td>
<td><strong>0.780</strong></td>
<td><strong>0.676</strong></td>
</tr>
<tr>
<td>32.188</td>
<td>0.875</td>
<td>0.780</td>
<td>0.655</td>
</tr>
<tr>
<td>35.292</td>
<td>0.854</td>
<td>0.780</td>
<td>0.635</td>
</tr>
<tr>
<td>38.870</td>
<td>0.833</td>
<td>0.805</td>
<td>0.638</td>
</tr>
<tr>
<td>40.500</td>
<td>0.813</td>
<td>0.829</td>
<td>0.642</td>
</tr>
<tr>
<td><strong>41.500</strong></td>
<td><strong>0.813</strong></td>
<td><strong>0.854</strong></td>
<td><strong>0.666</strong></td>
</tr>
<tr>
<td>43.000</td>
<td>0.792</td>
<td>0.854</td>
<td>0.645</td>
</tr>
<tr>
<td>44.500</td>
<td>0.771</td>
<td>0.854</td>
<td>0.624</td>
</tr>
</tbody>
</table>
The diagram shows a comparison of Eosinophil% between two groups: High CLC Concentration and Low CLC Concentration. The data points indicate a significant difference, with a p-value of less than 0.001.
Online Repository

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**Conflict of interest:** The authors declare no conflict of interest.

**Keywords:** Chronic rhinosinusitis with nasal polyps (CRSwNP); Charcot-Leyden crystals (CLCs); Receiver operating characteristic (ROC) curve; Corticosteroid resistance.
MATERIALS AND METHODS

Patients and samples

Eighty-nine patients diagnosed as CRSwNP according to the European Position Paper on Rhinosinusitis and Nasal Polyps (EPOS) 2012\textsuperscript{E1}, and scheduled to undergo functional endoscopic sinus surgery (FESS) in Beijing TongRen Hospital, were enrolled to this study. Patients who had contraindications for glucocorticoids or had used glucocorticoids or immunomodulatory drugs within 4 weeks before the study were excluded. Patients with aspirin exacerbated respiratory disease (AERD), fungal sinusitis, allergic fungal rhinosinusitis, cystic fibrosis or primary ciliary dyskinesia were also excluded. Allergic rhinitis was diagnosed according to the Allergic rhinitis and its impact on asthma (ARIA) 2008\textsuperscript{E2} criteria. Asthma was diagnosed as defined by Global Initiative For Asthma (GINA) 2014\textsuperscript{E3}. Atopy was evaluated by measurement of serum specific IgE (Phadia, Uppsala, Sweden, cut-off value: 0.35kUa/L). This study was approved by the ethics committee of Beijing TongRen Hospital and all the participants provided written informed consent prior to entry to the study.

All eligible patients were asked to self-evaluate their symptoms according to 0-10 Visual Analogue Scale (VAS)\textsuperscript{E4}. In addition, all the participants underwent CT scans of paranasal sinuses and the outcomes of CT scan were evaluated by Lund-Mackay Staging system\textsuperscript{E5, E6}. Objective olfactory test was performed as described by Haruna S, et al\textsuperscript{E7}. Endoscopic examinations were conducted to
investigate the polyp size of each side of the nasal cavity. The endoscopic images were evaluated and scored independently by two otolaryngologists who were blinded to the design of the trial. As a result of the examination, all the patients had scored at least 4 in total before the GC therapy was undertaken. The data of peripheral blood were obtained from a routine blood test.

On admission to the hospital, bilateral nasal cavity secretions from each patient were collected by the method described by Watelet et al. Each sample was diluted with 0.5mL 0.9% natural saline (NS) and kept at room temperature for 1 hour, following which the samples were centrifuged into a Falcon tube and stored at 4°C until further measurements. Polyp tissues (3×3 mm for each participant) were also obtained right after the nasal secretions were collected. The patients received a 2-week therapy of oral GC (methylprednisolone, 24mg qm po), and at the end of treatment were divided into GC-responders group (n=48) and GC-non-responders group (n=41), based on the change in polyp size scores. Patients who failed to have a reduction of more than 1 polyp score, based on nasal polyp size scoring system after GC therapy, were defined as GC-non-responders.

Histological evaluation

Histological criteria were determined by Hematoxylin and eosin (H&E) staining. Tissue samples were dehydrated, embedded in paraffin and then cut at 5 µm thicknesses, using a Leica RM2235 cryostat (Leica Microsystems, Bannockburn, IL, USA). All sections were examined by optical microscopy at a magnification of
×400, and the absolute number and percentage of eosinophils, neutrophils, plasma cells and lymphocytes were calculated. For each section, the absolute number and percentage of each cell type were recorded as the mean data of three non-overlapping regions, and the final evaluation for each patient’s tissue sample was recorded as the mean data of five sections. The counts of inflammatory cells were performed by two pathologists who were blinded to the study design.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

The detection and estimation of CLC concentration in nasal secretions were performed using commercial ELISA kits (Cloud-Clone Corp, Wuhan, China). The detection range of this kit was 0.312-20 ng/mL CLC. Prior to assay, all samples of the nasal secretions were diluted 50-fold with 0.9% NS, and the assay was conducted strictly according to the manufacturer’s instructions.

**Statistical analysis**

All data were expressed as the medians and interquartile ranges (IQR), except for binary variables and age, which were expressed as the mean and standard deviation. The statistical analysis was performed using GraphPad Prism6. All parametric variants were analyzed by Student’s *t*-tests and the nonparametric variants by Mann-Whitney U tests. The binary variables were analyzed by Fisher’s tests and Chi-square tests and binary logistic regression was used to find the potential predictive markers for GC response. Receiver operating characteristic (ROC) curves were generated to determine the optimal cut-off point, using SPSS version 24.0 (IBM
Corp, Armonk, NY). The predictive ability of the CLC concentration in nasal secretion for GC sensitivity was determined by the area under the ROC curve (AUC) and the optimal cut-off point was determined by Youden’s Index. Values of \( p<0.05 \) were considered statistically significant. All data were analyzed using two-tailed tests.
REFERENCES


FIGURE LEGENDS

Figure E1: Effect of gender (black columns for male and gray columns for female) (A), age (B), comorbid asthma (C), comorbid allergic rhinitis (D), comorbid atopy (E), and smoking history (F) on nasal CLC concentration lower or higher than the cut-off value (black columns for yes and gray columns for no in Figure E1C-F).

Figure E2: Severity and incidence of the chronic rhinosinusitis symptoms in patients with nasal CLC concentration lower or higher than the cut-off value; (A) nasal obstruction, (B) rhinorrhea, (C) facial pain, and (D) subjective olfactory disorder.

Figure E3: Difference of patients with nasal CLC concentration lower or higher than the cut-off value in (A) objective olfactory test, (B) Lund-Mackay score for paranasal sinuses on CT scan and (C) ratios of Lund-Mackay scores of ethmoid sinus/Lund Mackay scores of the maxillary sinus.
A scatter plot showing years old on the y-axis and CLC concentration on the x-axis. The plot is split into two groups: High CLC Concentration and Low CLC Concentration. The p-value is given as 0.739.
A bar chart showing the comparison between High CLC Concentration and Low CLC Concentration. The chart indicates that the number for High CLC Concentration is significantly higher than for Low CLC Concentration, with a p-value of 0.077.
$p = 0.781$
A box plot showing visual analogue scale scores for High CLC Concentration and Low CLC Concentration. The p-value is 0.389.