Diagnostic value of antineutrophil cytoplasmic antibodies in children with bronchiolitis obliterans

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Background: Diagnosis of childhood bronchiolitis obliterans (BO) is difficult owing to non-specific clinical presentations and limited investigational options. There is a lack in established serum biomarkers for BO. While the diagnostic value of antineutrophil cytoplasmic antibodies (ANCAs) has been discussed, little is known about this in BO. We aimed to investigate the serological profiles of ANCAs against myeloperoxidase (MPO-ANCA) and proteinase-3 (PR3-ANCA) in BO and acute pneumonia.

Methods: In this study, 42 BO children (BO group) and 43 with mild acute pneumonia (pneumonia group) were included, based on rigorous diagnostic criteria and additional constraints for minimizing selection bias. Serum MPO-ANCA and PR3-ANCA levels were measured on the first (baseline) and the last day of hospitalization (on discharge) by enzyme linked immunosorbent assay.

Results: Although the BO children had a longer hospital stay, the overall rate of positivity (≥180 AAU/mL) and median serum level of MPO-ANCA were higher in the BO group compared with the pneumonia group, either at baseline (69.1% vs. 9.3%, 292.00 vs. 104.75 AAU/mL, both P<0.001) or on discharge (61.9% vs. 9.3%, 310.50 vs. 95.42 AAU/mL). Similar was found for PR3-ANCA (38.1% vs. 4.7%, 106.66 vs. 54.56 AAU/mL at baseline; 35.7% vs. 2.3%, 97.98 vs. 57.23 AAU/mL on discharge, both P<0.001). There were a higher rate of dual-positivity and a lower rate of dual-negativity to both ANCAs in the BO group than those in the pneumonia group (all P<0.001).

Conclusions: Detection of MPO- and PR3-ANCA can help diagnosis of childhood BO.

Keywords: Bronchiolitis obliterans (BO); antineutrophil cytoplasmic antibodies (ANCAs); children

Submitted Apr 11, 2016. Accepted for publication May 09, 2016.

doi: 10.21037/jtd.2016.05.04

View this article at: http://dx.doi.org/10.21037/jtd.2016.05.04

Introduction

Bronchiolitis obliterans (BO), a chronic inflammatory process which involves the small airways and typically leads to progressive obliteration of the bronchioles (1), is noted among patients with severe infection, acute lung injury, autoimmune diseases, or in recipients of allogeneic transplantation (2,3). About 1% of children with infectious bronchiolitis may develop BO as a result of microbial respiratory infections (4-9). Unfortunately, BO in pediatric settings is often misdiagnosed as asthma, classical pneumonia or infectious bronchiolitis. This may be partly due to the non-specific clinical signs; moreover, current...
diagnostic tools show limitations in identifying BO. Briefly, chest radiography usually fails to reveal the lesions; high-resolution computed tomography (HRCT), with the greatest sensitivity and specificity (4,10) in imaging studies for BO, offers only circumstantial evidence of bronchiolar disease, whereas these evidence (such as typical mosaic pattern) share similarities to many other bronchiole-involving disorders (6,11,12); routine bronchoscopy is more useful for exclusive than for confirmatory diagnosis; pulmonary function tests are difficult to performed, and are merely for clinical reference in very young children aged below 5 (13); lung biopsy as the gold standard has a fairly low yield, as the specimens collected from patchy BO lesions are not always adequate (14). In addition, parents and children rarely prefer or comply to these invasive procedures. Repeated lung biopsy for follow-up in BO children can be thus more difficult. There is a dire need for a novel auxiliary approach which is diagnostically valuable and convenient in BO.

Antineutrophil cytoplasmic antibodies (ANCAs) are autoantibodies against components of neutrophils and monocytes (15). Two of these family members, the ANCAs against myeloperoxidase (MPO-ANCA) and proteinase-3 (PR3-ANCA), have been studied in primary vasculitis and autoimmune rheumatic diseases (16,17). In fact, recent research has linked ANCAs to a broad range of conditions, including autoimmune hepatopathy, Kawasaki disease, inflammatory bowel disease, and connective tissue diseases (18,19). In pulmonology, ANCAs have been reported detectable among patients with diffuse interstitial lung disease (20,21). In an uncontrolled study, we noted positive serum MPO- and PR3-ANCAs in 52.6% and 42.1%, respectively, of 19 patients with BO (22).

Therefore we hypothesized that MPO- and PR3-ANCAs may be useful for facilitating diagnosis of BO. If with acceptable accuracy or specificity, serological tests could be clinically important in terms of patient convenience and non-invasiveness. Here we presented a preliminary study on their serological profiles in BO and whether they differ from the findings in bronchial pneumonia.

Methods

Study population

This was a prospective, observational study conducted between June 2009 and December 2013. During the study period, a total of 58 consecutive BO children (45 boys and 13 girls) treated in a tertiary teaching hospital in southern China, the First Affiliated Hospital of Guangzhou Medical University, were approached. The diagnostic criteria of BO were described elsewhere (23): (I) recurrent or persistent wheezing, dyspnea or coughing, and stridor lasting for ≥6 weeks after acute lower respiratory infection or acute lung injury, with no response to bronchodilators; (II) presence of clinical features inconsistent with the radiography findings, showing more severe symptoms in contrast to signs of hyperventilation on chest radiography; (III) mosaic perfusion pattern, bronchial wall thickening, bronchiectasis or atelectasis on chest HRCT; (IV) pulmonary function tests indicating obstructive ventilatory impairment; and (V) exclusion of other obstructive airway disorders that could lead to wheezing (such as asthma, primary ciliary dyskinesia, cystic fibrosis, foreign bodies, congenital abnormalities, tuberculosis), AIDS and other immune disorders (such as systemic lupus erythematosus and systemic vasculitis). For a rigorous confirmation of BO, in final analysis we included only those children who also fulfilled five of the following: (I) no response to regular inhaled corticosteroids and bronchodilators for ≥12 weeks; (II) negative bronchoscopic findings for airway malformations and tuberculosis; (III) retained exercise intolerance, and mosaic pattern on HRCT despite systemic corticosteroids therapy; (IV) absence of impaired renal function, such as microscopic hematuria or proteinuria; and (V) negative findings for antinuclear antibodies such as anti-Ro(SSA), anti-La(SSB), anti-Sm, or anti-dsDNA antibodies.

A contemporary cohort of 60 children (34 boys and 26 girls) with mild acute bronchial pneumonia was also approached as potential controls. The final inclusion was based on diagnostic criteria of mild acute bronchial pneumonia by Workgroup of Respiratory Diseases, Chinese Medical Association Society of Pediatrics (24), plus all of the following: (I) recurrent or persistent weekly; (II) no wheezing, no signs of lobar pneumonia, or severe pneumonia as manifested by hypoxemia and heart failure; (III) negative history for preterm birth, eczema, allergic rhinitis, scar formation, mechanical ventilation, and recurrent respiratory infections; (IV) no family history for allergies and keloidosis; (V) no medications with systemic corticosteroids during the present hospitalization.

For these subjects, we recorded data on their demographics, treatments, length of hospital stay, and serum MPO-ANCA and PR3-ANCA (see below).

Ethics statement

This study was approved by the Ethics Committee of First
Affiliated Hospital of Guangzhou Medical University (No. of Approval: GYFYY 2009-05-28). All experiments were performed in accordance with relevant guidelines and regulations of the Ethics Committee of First Affiliated Hospital of Guangzhou Medical University. Written informed consent was obtained from the legal guardians of all participants.

Measurement of serum MPO- and PR3-ANCAs

From each subject, 3 mL venous blood sample was collected on the first day (baseline) and the last day of hospitalization (on discharge). These samples were assigned unique identification numbers before transferring for measurement at the Central Laboratory of our institution. The technical team who completed the serological tests was blinded about patient information, including the diagnosis.

The serum levels of MPO- and PR3-ANCAs were measured by using MPO and PR3 ELISA kits (ZEUS Scientific, Branchburg, New Jersey, USA), respectively, according to the manufacturer’s instructions. Briefly, 100 μL of 1:21 diluted serum sample was added into reaction wells, followed by 30 min incubation at 25 degree Celsius (°C). After five washes, 100 μL of antibody-enzyme conjugate was added, and the plate was incubated for another 30 min at 25 °C. After three washes, 100 μL of substrate was added into each well and, following 15-min incubation at 25 °C, 50 μL of stop solution was added. Finally, the optical density at 450 nm (OD) for each well was measured by using a microplate reader (ThermoFisher Scientific, Shanghai, China). The levels of MPO- and PR3-ANCAs were calculated based on the formula: Test Specimen ANCA (AAU/mL) = Test Specimen OD × Calibrator Unit Value/Calibrator OD. For either MPO-ANCA or PR3-ANCA, the cut-off value for positive test was ≥180 AAU/mL, according to manufacturer’s recommendation.

Statistical analysis

Data were analyzed with the Statistical Package for the Social Sciences version 13.0 (SPSS Inc., Chicago, IL, USA). Kolmogorov-Smirnov test was used for evaluating the data normality. Normally distributed values were presented as mean ± standard deviation (SD), non-normally distributed values as median and interquartile range (IQR), and the range of reference values as 95% confidence intervals (95% CI). Normally distributed data were compared by using an independent t-test, otherwise by using a rank-sum test for independent samples. Categorical data were expressed with rate or proportion, and analyzed with Chi-squared test or Fisher’s exact test where appropriate. A P value <0.05 was considered significant.

Results

General demographics and clinical characteristics of the study population

There were 42 BO children (BO group) and 43 with mild acute bronchial pneumonia (Pneumonia group) included in the final analysis, while the remaining 33 were excluded because of incompatibility with the inclusion criteria or additional constraints. The general demographics and clinical characteristics of children in both groups are shown in Table 1. According to the timing of sample collection, the median interval between two serum tests in either group was the median length of hospital stay.

For the treatments during hospitalization, the both groups received antibiotics, expectorants, and salbutamol. In addition, the BO children received empirical small-dose azithromycin (3 to 5 mg/kg/d) and oral prednisone (1.5 to 2.0 mg/kg/d) or IV methylprednisolone equivalent.

Serological profiles of MPO-ANCA in bronchiolitis obliterans versus mild acute pneumonia

There were more children in the BO group with positive findings for serum MPO-ANCA at baseline [29 (69.1%) vs. 4 (9.3%), χ²=75.662, P<0.001] and on discharge [26 (61.9%) vs. 4 (9.3%), χ²=61.339, P<0.001], respectively, compared with the pneumonia group (Table 2, Figure 1A,B). At baseline, the median level of serum MPO-ANCA in BO children was 292.00 vs. 104.75 AAU/mL in children with pneumonia (Z=3.586, P<0.001); these figures were 310.50 vs. 95.42 AAU/mL on discharge (Z=3.498, P<0.001).

Within-group comparisons did not show statistical differences in the overall rate of positivity and serum level of MPO-ANCA between baseline and on discharge either in BO or in the pneumonia group (both P>0.05).

Serological profiles of PR3-ANCA in bronchiolitis obliterans versus mild acute pneumonia

Similarly, there were more BO children with positive findings for serum PR3-ANCA at baseline [16 (38.1%) vs. 2 (4.7%), χ²=32.262, P<0.001] and on discharge [15
(35.7%) vs. 1 (2.3%), \( \chi^2 = 37.557, P<0.001 \), respectively, compared with the pneumonia group (Table 3, Figure 1C,D). At baseline, the median level of serum PR3-ANCA in BO children was 106.66 vs. 54.56 AAU/mL in those with pneumonia (Z=3.982, P<0.001); these figures were 97.98 vs. 57.23 AAU/mL on discharge (Z=2.888, P<0.001). Again, within-group comparisons did not show statistical differences in the overall rate of positivity and serum level of PR3-ANCA between baseline and on discharge either in BO or in the pneumonia group (both P>0.05).

**Table 1** General demographics and clinical characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BO (n=42)</th>
<th>Pneumonia (n=43)</th>
<th>Z/( \chi^2 ) value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months)</td>
<td>21.00</td>
<td>60.00</td>
<td>-4.345</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IQR</td>
<td>26</td>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>22.23–32.84</td>
<td>44.48–62.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Girls</td>
<td>35 (83.3)</td>
<td>26 (60.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys</td>
<td>7 (16.7)</td>
<td>17 (39.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median duration of symptoms (months)</td>
<td>3.50</td>
<td>0.25</td>
<td>-7.702</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IQR</td>
<td>9.13</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>0.24–0.35</td>
<td>4.79–11.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median length of hospital stay (days)</td>
<td>25.50</td>
<td>7.00</td>
<td>-7.421</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>IQR</td>
<td>13.00</td>
<td>5.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>95% CI</td>
<td>24.26–32.55</td>
<td>7.41–10.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Clinical symptoms and signs during hospitalization, n (%)

- Fever: 33 (78.6) vs. 41 (95.4), Z=11.317, P<0.001*
- Cough: 41 (97.6) vs. 43 (100.0), Z=0.497*
- Persistent wheezing: 42 (100.0) vs. 0, Z=0.001*
- Shortness of breath: 36 (85.7) vs. 0, Z=0.001*
- Exercise intolerance or limited activities: 42 (100.0) vs. 0, Z=0.001*
- Acropachy: 12 (28.6) vs. 0, Z=0.014*
- Three depressions signs: 24 (57.1) vs. 0, Z=0.001*
- Transcutaneous oxygen saturation <85%: 25 (59.5) vs. 0, Z=0.001*

Medical history, n (%)

- Premature birth (<37 weeks): 4 (9.5) vs. 0, Z=0.002*
- Low birth weight (<2.5 kg): 3 (7.1) vs. 0, Z=0.014*
- Mechanical ventilation: 18 (42.9) vs. 0, Z=0.001*
- Severe pneumonia: 33 (78.6) vs. 0, Z=0.001*

BO, bronchiolitis obliterans; IQR, interquartile range; 95% CI, 95% confidence interval. *, Chi-square test; †, Fisher exact probability test was used instead of the Chi-square test, owing to the zero value in the pneumonia group.

Single versus dual positivity or negativity to the antineutrophil cytoplasmic antibodies (ANCAs) in bronchiolitis obliterans versus mild acute pneumonia

In the BO group, single-positivity to MPO-ANCA alone...
Table 2 Serological profiles of MPO-ANCA in the bronchiolitis obliterans (n=42) and pneumonia (n=43) groups*

<table>
<thead>
<tr>
<th>MPO-ANCA</th>
<th>Baseline</th>
<th>On discharge</th>
<th>χ²/Z value</th>
<th>P value</th>
<th>Baseline</th>
<th>On discharge</th>
<th>χ²/Z value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive findings, n (%)</td>
<td>29 (69.1)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>4 (9.3)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>75.662</td>
<td>&lt;0.001</td>
<td>26 (61.9)&lt;sup&gt;†&lt;/sup&gt;</td>
<td>4 (9.3)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>61.339</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Serum level (AAU/mL)</td>
<td>292.00&lt;sup&gt;†&lt;/sup&gt;</td>
<td>104.75&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>3.586</td>
<td>&lt;0.001</td>
<td>310.50&lt;sup&gt;†&lt;/sup&gt;</td>
<td>95.42&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>3.498</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Median IQR</td>
<td>514.26</td>
<td>62.11</td>
<td>348.85</td>
<td>49.80</td>
<td>348.85</td>
<td>49.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BO, bronchiolitis obliterans; MPO, myeloperoxidase; ANCA, antineutrophil cytoplasmic antibody; MPO-ANCA, antineutrophil cytoplasmic antibody against myeloperoxidase. *, non-parametric tests for two independent samples were used owing to the non-normality in data distribution (P<0.05); †, P>0.05 for the within-group comparison between baseline and on discharge in the children with BO; ‡, P>0.05 for the within-group comparison between baseline and on discharge in the children with mild acute pneumonia.

Figure 1 Serum levels of MPO-ANCA and PR3-ANCA in individual subjects of the BO and pneumonia groups. The serum levels are converted into natural logarithms to facilitate graphic presentation. (A,C) The BO group (n=42); (B,D) the pneumonia group (n=43). Red dashed line: the natural logarithm (5.2) of cut-off value (180 AAU/mL). BO, bronchiolitis obliterans; MPO-ANCA, antineutrophil cytoplasmic antibody against myeloperoxidase; PR3-ANCA, antineutrophil cytoplasmic antibody against proteinase-3; Adm, day of admission (baseline); Dis, day of discharge (on discharge).
A remarkably higher rate of dual-positivity to MPO- and PR3-ANCAs (16 (38.1%) vs. 1 (2.3%), $\chi^2=40.500$, $P<0.001$) and a lower rate of dual-negativity to both ANCAs (13 (31.0%) vs. 38 (88.4%), $\chi^2=57.473$, $P<0.001$) in the BO group compared with the pneumonia group. On discharge, 15 children (35.7%) in the BO group were dual-positive to both ANCAs, compared to none in the pneumonia group (Table 4); and the rate of dual-negativity to both ANCAs remained to be lower in the BO group [16 (38.1%) vs. 38 (88.4%), $\chi^2=53.625$, $P<0.001$].

**Discussion**

Pathologically confirmed BO cases were first reported in 1901 (25). In 1973, 52 more cases of BO were described by Gosink (26). With the advances in diagnostics, increasing patients with BO have been identified over the past decades. Unfortunately, the diagnosis of childhood BO remains difficult owing to various technical setbacks (4,10,11,14), or hampered by the invasiveness and poor patient compliance. In a study by Colom (27), the diagnosis of BO in 120 children ineligible for lung biopsy due to young age, airflow limitation or other reasons relied alternatively on clinical scoring and a 10-year follow-up. Serological tests of biomarkers could thus be clinically useful as a convenient approach for diagnosis. However, such biomarkers in BO are yet to be established.

Recently, the clinical value of ANCAs has been discussed in diseases ranging from glomerulonephritis, vasculitis to
ulcerative colitis (16-19,22,28). Studies on ANCs were driven by the putative endothelial damage by massive release of oxygen free radicals and proteolytic enzymes owing to ANCA-induced neutrophil activation under inflammatory conditions, although the exact mechanism remains to be clarified (29,30). Following this logic, ANCs may also be involved in chronic lung diseases [such as interstitial lung disease (20,21)] where airway neutrophilia is frequently present and considered to correlate with disease severity (31). Studies have shown high neutrophil counts in the bronchoalveolar lavage fluid (BALF) of patients with BO after lung transplantation, but not in those without BO, which might be explained by long-term neutrophil colonization in the lungs (32). Among measles patients who subsequently developed BO, there was also an increase in BALF neutrophils compared with controls (7).

In 2012, we noted a high positive rate of ANCs among 19 BO children with a history of severe pulmonary infection, although that cohort was small and did not include any control group (22). Unlike in adults, childhood BO is related more to severe, recurrent respiratory infections (6,7,33,34). Serum ANCs may also be associated with various microbial infections (4-9). Given these, we recruited children with mild acute pneumonia as controls. Our selection criteria for the pneumonia group were rigorously designed to exclude asthma, allergies, scarring and other conditions that might complicate our results.

The present study showed significantly higher positive rates and serum levels of MPO- and PR3-ANCAs in the BO group compared with the pneumonia group, indicating an association between ANCs and BO. Our study was not designed to elucidate on the mechanism underlying this observation. However, based on findings for ANCA in other diseases (35,36), we speculated that ANCs in these BO patients might have activated neutrophils to release large amounts of oxygen free radicals and proteolytic enzymes, which in turn caused epithelial detachment and necrosis in the small airways, and resulted in abnormal tissue repair, submucosal fibrosis, hyalinization, and collagen deposition. Subsequently, the fibrosis and scarring could lead to narrowing, distortion and occlusion of the bronchiolar lumen.

It would be important to note that environmental factors (such as silica dusts), bacterial or viral infections, drugs and genetic susceptibility may interact to affect the positive findings for ANCs (37). In the present study with rigorous inclusion criteria to minimize confounding factors, MPO-ANCA was detected in >60%, and PR3-ANCA in >35%, respectively, of the BO children. These positive findings persisted over a median duration of 25.50 days, and were mostly noted in children who were dual-positive to MPO- and PR3-ANCA (38.1% at baseline, 35.7% on discharge). In contrast, the pneumonia group showed much lower positive rates of MPO-ANCA (<9.5%) or PR3-ANCA (<5.0%) either at baseline or on discharge (a shorter median of 7.00 days later); and according to our observation, the positivity was more likely to be single-positivity to MPO- or PR3-ANCA alone, and much less frequently to be dual-positivity to the both (Table 4). The serum ANCs levels in these single-positive children (n=4 at baseline and n=5 on discharge) (Table 4) were considerably low, noted to be near although modestly above the cut-off value (180 AAU/mL). In addition, the majority of ANCA single-positive pneumonic children differed in patient identification between the baseline and on discharge (Figure 1B,D). These observations suggested two probable reasons: acute infections might also contribute to transient positivity of ANCs or, the single-positive findings in pneumonic children may arise from systematic error of the testing in our study. We expect that future studies could be elaborated to further minimize the confounding effect of acute infections and improve the diagnostic potential of serum ANCs in BO.

A positive correlation between ANCs and disease activity has been reported in primary vasculitis, where serum ANCA levels decreased or diminished in patients who responded to treatment but increased in those who relapsed (38,39). Therefore these authors proposed using ANCs as a biomarker for diagnosing ANCA-associated vasculitis, evaluating disease severity, and predicting prognosis. In the present study, the serum MPO- and PR3-ANCA levels were high in BO children at baseline, and remained so over a longer duration of hospital stay. Interestingly, ANCs as autoimmune antibodies are mainly IgG with a half-life of ~21 days. Thus, studies with longer period to follow up the serum ANCs in these BO children should be of interest, and in fact, our ongoing study is now looking at this until the end of 2016 (≥3 years after the first measurement). Since ANCA production might be activated by MPO and PR3 antigens on the surface of neutrophils that colonize the pulmonary tissues as a result of small airway obstruction, recurrent inflammation and infections, we speculated that even after short-term anti-inflammatory and antimicrobial treatments, the serum ANCA levels would remain elevated.
until the airway neutrophilic infiltration is substantially improved. Compared with acute infections like simple pneumonia, the airway neutrophilic infiltration can be much more severe in BO, and hence probably the longer duration and greater amplitude of elevated serum MPO- and PR3-ANCAs as found in the present study.

We should acknowledge inadequate basic research on serum ANCAs in BO, the small sample size in our study, and the presence of ANCAs in many other disorders, may prevent us from recommending ANCA as a specific biomarker of BO. Nonetheless, the present study may be an early attempt to shed light on serum ANCA profiles in BO. When interpreted in the context of clinical signs, medical history and imaging findings, serum ANCA tests may become a convenient auxiliary approach for diagnosis of BO. In particular, dual-positivity to the two ANCAs seemed to offer specificity in determining BO. For single-positivity to ANCA, we speculated that using a higher cut-off value (220 AAU/mL) instead of 180 AAU/mL, would also yield higher specificity in the diagnosis (Figure 1), although this needs further clarification in larger studies.

There were several limitations in the present study. Firstly, none case of BO in this study was diagnosed based on open lung biopsy. This could potentially result in a selection bias. We attempted to circumvent this bias with rigorous constraints in patient inclusion. At the time of writing, these BO patients are still in follow-up. Secondly, the mean age and the gender distribution were not comparable between the with BO and pneumonia groups. While this mis-match is to be controlled in future studies, to the best of our knowledge, there is little evidence in the literature suggesting patient age and gender may interfere with serum ANCA levels. Thirdly, a healthy control group was not present because this was an observational study based on clinical practice, and that serum ANCA tests in healthy subjects were not part of a regular health checkup. Fourthly, follow-up data on MPO- and PR3-ANCAs in the BO children were not available, because the follow-up is ongoing until the end of 2016. Future studies are therefore needed to address these limitations, validate our findings, and further clarify the clinical value of ANCAs in BO.

Acknowledgements

The authors are grateful to Ms. Mei Jiang for her generous assistance in medical statistics, and to Kangni Chen who edited the format of the text. Special gratitude goes to the technical team at Central Laboratory, First Affiliated Hospital of Guangzhou Medical University, for their work in serological tests.

Funding: Guangdong Natural Science Foundation (Project No. S2012010008784); Guangdong Provincial Science and Technology Project (Project No. 2014A020212356); Guangzhou Municipal Science and Technology Innovation Project (Project No. 201504281719217).

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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