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Methacholine and adenosine 5′-monophosphate challenges in children with post-infectious bronchiolitis obliterans

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ABSTRACT: Airway hyperresponsiveness (AHR) is a characteristic feature of asthma, but is also frequently demonstrated by children and adults with chronic obstructive lung diseases. AHR is usually measured by bronchial challenges using direct or indirect stimuli. The aim of this study was to compare these two types of bronchial challenge in children with post-infectious bronchiolitis obliterans (BO).

Methacholine and adenosine 5′-monophosphate (AMP) challenges were used as tools for the evaluation of AHR to direct and indirect stimuli, respectively, in children with post-infectious BO (n=28). These results were compared with those of asthmatic (n=30) and control children (n=25).

Altogether, twenty-two patients (78.6%) with post-infectious BO were hyperreactive to methacholine with a provocative concentration causing a 20% fall in forced expiratory volume in one second (PC_{20}) of <16 mg·mL^{-1}, but only six (21.4%) were hyperreactive to AMP with a PC_{20} of <200 mg·mL^{-1}. All patients with asthma responded positively to methacholine, and most (28, 93.3%) also responded positively to AMP. The majority of controls were insensitive to both challenges.

Airway hyperresponsiveness to methacholine is a frequent, but by no means universal, finding in children with post-infectious bronchiolitis obliterans, but is usually not accompanied by airway hyperresponsiveness to adenosine 5′-monophosphate. This finding suggests that airway hyperresponsiveness in patients with post-infectious bronchiolitis obliterans has characteristics that differ from those of asthmatic subjects.

KEYWORDS: Adenosine 5′-monophosphate, airway responsiveness, asthma, bronchial challenge test, methacholine, post-infectious bronchiolitis obliterans

Bronchiolitis obliterans (BO) refers to a clinical syndrome of chronic airflow obstruction associated with inflammatory changes in the small airways [1]. The increase in lung and bone marrow transplantation has led to a heightened interest in BO, as this is one of the important complications of those procedures [2]. However, BO occurs most commonly after an episode of respiratory infection in children [3]. Recently the prevalence of childhood post-infectious BO has been recognised to be greater than previously believed because high-resolution computed tomography (HRCT) is increasingly being utilised to investigate bronchial and small airway lesions [4, 5]. However, little is known about the functional consequences of post-infectious BO.

Airway hyperresponsiveness (AHR) is characterised by an exaggerated bronchoconstrictor response to provoking stimuli. AHR is usually measured by bronchial challenges using direct or indirect stimuli. Methacholine acts directly at the level of bronchial smooth muscle, whereas adenosine 5′-monophosphate (AMP) acts indirectly, causing primed mast cell degranulation and the release of pro-inflammatory mediators [6]. It has been shown that AHR to methacholine is present both in asthma and in chronic lung diseases, whereas AHR to AMP is present only in asthma [7, 8]. However, to the current authors' knowledge, these two types of bronchial challenge have never been compared in patients presenting with a diagnosis of post-infectious BO. It was hypothesised that children with post-infectious BO would exhibit AHR to methacholine, but not to AMP.

The purpose of the present study was to compare methacholine and AMP bronchial challenges in children with post-infectious BO; diagnoses were based on its typical clinical features and

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characteristic HRCT findings. Results were compared with those of a group of asthmatic children and a group of healthy controls who had experienced a lower respiratory infection but had normal HRCT findings.

METHODS

Altogether, 28 children with post-infectious BO were included in the study. They were selected from children who suffered from unresolved respiratory symptoms and/or signs (cough, dyspnoea, wheezing and crackles) after an episode of acute bronchiolitis or pneumonia. A diagnosis of BO was made in patients when they had a typical clinical history followed by chest HRCT findings that concurred with the diagnosis [9]. At the time of diagnosis, a median time of 11 months (range 3–29 months) had elapsed since the period of acute illness. Hospital records of these patients were reviewed. Pathogenic agents identified during the acute illness included *Mycoplasma pneumoniae* (n=13), adenovirus (n=4), measles virus (n=3), and respiratory syncytial virus (n=2), but no identification was available in six cases. HRCT features were expiratory air trapping (n=27), mosaic perfusion (n=23), bronchiectasis (n=18), bronchial wall thickening (n=8), and areas of atelectasis (n=4). No detectable clinical improvement after 2 weeks of treatment with inhaled β_2-agonist was requisite for the diagnosis of BO. Diagnoses of other chronic obstructive pulmonary diseases, such as cystic fibrosis, bronchopulmonary dysplasia, pulmonary tuberculosis, α_1-antitrypsin deficiency, and immunodeficiency were excluded on the basis of clinical, radiological and laboratory data. Medications used for treatment of diagnosed BO included antibiotics, oral or inhaled corticosteroids, and bronchodilators. However, none of the patients had received these medications within 1 month of bronchial challenges.

A group of 30 patients with asthma was also recruited. These patients had a history of asthma symptoms (cough, wheezing and dyspnoea) extending over the previous year, which had been controlled by an as-needed bronchodilator either with or without anti-inflammatory agents. They were diagnosed as having asthma based on a methacholine (provocative concentration causing a 20% fall in forced expiratory volume in one second (FEV_1) PC_{20} of <16 mg·mL^{-1} or airway reversibility (an increase in FEV_1 of $>12\%$ after bronchodilator administration). Patients with a history of near-fatal asthma or major exacerbations necessitating the use of systemic corticosteroids were excluded.

A control group of 25 children with a history of low respiratory tract infection was included for purposes of comparison. Control cases were identified by reviewing the hospital records of children who had been admitted to the authors' hospital due to acute bronchiolitis or pneumonia. Letters were sent to their parents requesting cooperation. Subjects with no current respiratory symptoms and normal HRCT findings were selected. Median duration between hospitalisation for lower respiratory infection and inclusion in the study was 18 months (range 6–49 months). Infectious agents at the time of hospitalisation included *Mycoplasma pneumoniae* (n=10), pneumococcus (n=4), *Haemophilus influenzae* (n=3), respiratory syncytial virus (n=2), and adenovirus (n=2). No identification was available in four cases.

Blood samples were obtained from all study subjects for the determination of total eosinophil counts, and skin-prick testing was performed on all subjects to assess atopy. Atopy was defined as the presence of at least one positive reaction (>3 mm weal diameter) to a battery of 12 common airborne allergens. To be eligible for the study, subjects had to be capable of performing pulmonary function tests in a reproducible way (*i.e.* a coefficient of variation of FEV_1 in three consecutive flow–volume curves of $<5\%$) and were required to have an FEV_1 of $\geqslant 60\%$ of the predicted value [10]. Both methacholine and AMP challenges were performed at the same time of the day with an interval of 3–14 days. All studied subjects were asked to stop using antihistamine, inhaled bronchodilators and other medications for 48 h and inhaled corticosteroids for 7 days before the tests. None of the subjects had exhibited any symptoms of an upper respiratory infection in the month preceding the tests.

Methacholine inhalation tests were carried out using a modification of the method described by CHAI *et al.* [11], and the AMP challenge test was a modification of the method of the European Respiratory Society [12]. Fresh solutions of methacholine and AMP were prepared in buffered saline solution at concentrations ranging from 0.075–50 mg·mL^{-1} for methacholine and from 3.125–400 mg·mL^{-1} for AMP. Lung function was measured using a computerised spirometer (Microspiro-HI 298; Chest, Tokyo, Japan), and the largest value of triplicate FEV_1 on each occasion was used for analysis. A Rosenthal-French dosimeter (Laboratory for Applied Immunology, Baltimore, MD, USA), triggered by a solenoid valve set to remain open for 0.6 s, was used to generate an aerosol from a DeVilbiss 646 nebuliser (DeVilbiss Health Care, Somerset, PA, USA), with pressurised air at 137.89 kPa (20 lb·in^{-2}). Each subject inhaled five inspiratory capacity breaths of buffered saline solution and increasing concentrations of methacholine or AMP, respectively, at 5-min intervals. This gave an output of 0.009 ± 0.0014 mL (mean $\pm$ SD) per inhalation. FEV_1 was measured 90 s after inhalation at each concentration level. The procedure was terminated when the FEV_1 decreased by $>20\%$ of its post-saline value or when the highest methacholine (50 mg·mL^{-1}) or AMP (400 mg·mL^{-1}) concentrations were reached. The percentage decline of FEV_1 from the post-saline value was plotted against the log concentrations of the inhaled methacholine or AMP. Methacholine PC_{20} and AMP PC_{20} were calculated by interpolating between two adjacent data points if the FEV_1 decreased by $>20\%$.

Parents gave written informed consent for their children to participate in the study. The study protocol was approved by the hospital's Ethics Committee.

Statistical analysis

Data are presented as mean $\pm$ SD. PC_{20} values were log transformed before statistical analysis. FEV_1 values were expressed as percentages of predicted based on data from the local population [13]. Subjects were considered to have AHR to methacholine or AMP if they showed a decline in FEV_1 of 20% or more at a concentration of methacholine <16 mg·mL^{-1} [14] or AMP <200 mg·mL^{-1} [15] (*i.e.* a methacholine PC_{20} <16 mg·mL^{-1} or an AMP PC_{20} <200 mg·mL^{-1}). Censored values of 100 mg·mL^{-1} for methacholine and 800 mg·mL^{-1} for AMP were awarded for those who did not

show an FEV1 fall of <20% after inhaling the maximal concentration of methacholine (50 mg·mL^{-1}) or AMP (400 mg·mL^{-1}). Screening of data for differences among the three groups was performed using ANOVA test. When differences were significant, each pairing was examined using the unpaired t-test. Differences in frequencies between two groups were analysed using the Chi-squared test. Correlations between variables were calculated using Pearson's correlation test. A p-value of $\leqslant 0.05$ was taken to be significant.

RESULTS

The clinical characteristics and spirometric values of the three study groups are shown in table 1. There was no significant difference in terms of age or sex ratio between the three groups. The prevalence of atopy and blood eosinophil counts were significantly higher in the asthma group than in the other two groups. Mean baseline FEV1 values before methacholine and AMP challenges were significantly lower in the BO group than in the asthma or control group, and the difference between the latter two groups was not significant. No significant difference was observed between the mean baseline FEV1 values before the two challenges within any of the three groups. When the staging system for bronchiolitis obliterans syndrome (BOS) after lung transplantation [16] was applied in the current study's BO patients, two patients were classified as BOS 0, 10 as BOS 0-p (a potential BOS stage), 13 as BOS 1, and three as BOS 2.

Methacholine challenge test results are shown in figure 1. It can be seen that three subjects in the BO group and 20 subjects in the control group failed to respond to the highest concentration (50 mg·mL^{-1}) of methacholine. All 30 subjects in the asthma group, 22 subjects (78.6%) in the BO group, and three subjects (12%) in the control group had a PC_{20} <16 mg·mL^{-1}, the cut-off point for AHR. The frequencies of AHR to methacholine in the three groups differed significantly (Chi-squared test, p=0.00). There was considerable overlap between the methacholine PC_{20} results of the asthma and BO groups, and mean values were not significantly different for hyperreactive subjects with post-infectious BO (n=22, geometric mean (range of 1 SD): 2.18 mg·mL^{-1} (0.68–6.70)) and those with asthma (n=30, 1.27 mg·mL^{-1} (0.33–4.87), p=0.14).

AMP challenge test results are shown in figure 2. All but two subjects in the asthma group, eight in the BO group,

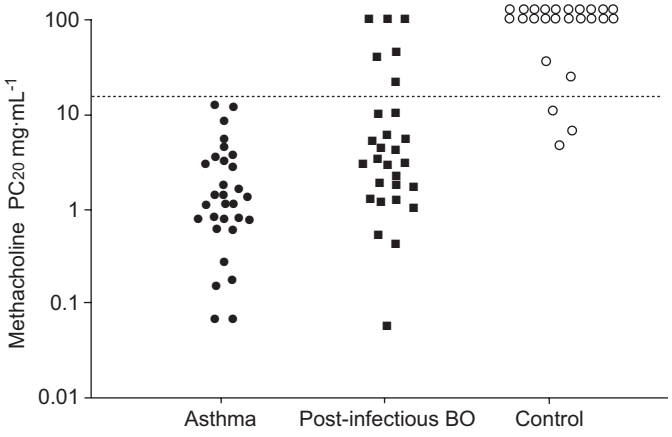

FIGURE 1. Distribution of methacholine provocative concentration causing a 20% fall in forced expiratory volume in one second (PC_{20}) in children with asthma, in children with post-infectious bronchiolitis obliterans (BO), and in controls. The dotted line represents the PC_{20} limit of 16 mg·mL^{-1}.

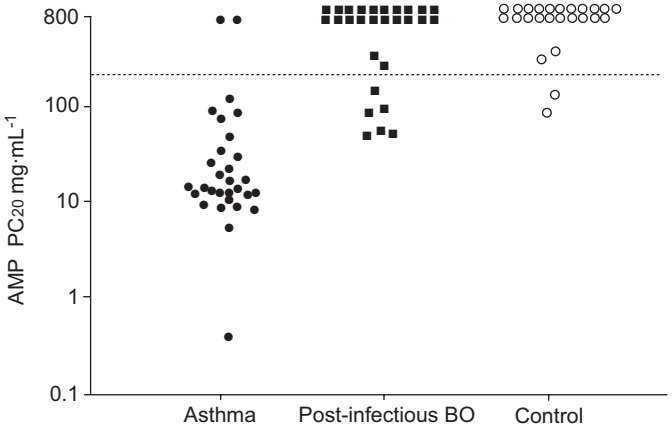

FIGURE 2. Distribution of adenosine 5′-monophosphate (AMP) provocative concentration causing a 20% fall in forced expiratory volume in one second (PC_{20}) in children with asthma, in children with post-infectious bronchiolitis obliterans (BO), and in controls. The dotted line represents the PC_{20} limit of 200 mg·mL^{-1}.

TABLE 1 Clinical characteristics and spirometric values of the three study groups

	Asthma	Post-infectious bronchiolitis obliterans	Control
Patients n (M/F)	30 (17/13)	28 (16/12)	25 (12/13)
Age yrs	9.5±1.8	8.2±1.2	9.3±2.9
Atopy n (%)	23 (76.7)¶	13 (46.4)$^{+}$	11 (44.0)
Blood eosinophils μL^{-1}	487±332¶	248±223^{+}	252±132
FEV1$^{\#}$ methacholine challenge	91.2±19.9	77.6±21.1$^{¶,+}$	95.9±15.2
FEV1$^{\#}$ AMP challenge	92.5±16.1	78.0±18.9$^{¶,+}$	95.7±14.1

Data are presented as mean±SD, unless otherwise stated. M: male; F: female; FEV1: forced expiratory volume in one second; AMP: adenosine 5′-monophosphate. Atopy was defined as the presence of at least one positive reaction (>3 mm weal diameter) to a battery of 12 common airborne allergens. $^{\#}$: pre-test baseline values (% predicted); ¶: p<0.05 *versus* control group; $^{+}$: p<0.05 *versus* asthma group.

and four in the control group responded to concentrations up to 400 mg·mL^{-1}. The frequency of AHR to AMP (PC_{20} <200 mg·mL^{-1}) in the asthma group (28/30, 93.3%) was significantly higher than that (6/28, 21.4%) in the BO group, which was not significantly different from that (2/25, 8%) of the control group. Of subjects hyperreactive to AMP, those with asthma (n=28) had a significantly lower AMP PC_{20} (16.58 mg·mL^{-1} (5.69–48.33)) than those with post-infectious BO (n=6, 77.41 mg·mL^{-1} (50.61–118.41), p=0.00).

In order to examine the effect of baseline lung function on response to methacholine or AMP, the relationship between PC_{20} and initial FEV_1 was calculated. No correlation for either methacholine PC_{20} or AMP PC_{20} was found with baseline FEV_1 either in the asthma group (r=0.13, p=0.51, and r=-0.05, p=0.78, respectively) or in the BO group (r=0.12, p=0.55, and r=-0.22, p=0.27, respectively). The frequency of AHR to methacholine was not statistically different for subjects with a low FEV_1 (<80% predicted) (14/16, 87.5%) and those with a normal FEV_1 (⩾80% predicted) (8/12, 66.7%, p=0.35) in the BO group (data not shown).

There was an inverse correlation between AMP PC_{20} and blood eosinophil counts in the asthma group (r=-0.39, p=0.03) but not in the BO group (r=-0.18, p=0.42). No correlation was found between methacholine PC_{20} and blood eosinophil counts in either the asthma group (r=-0.04, p=0.84) or the BO group (r=-0.12, p=0.58) (data not shown).

Individual values for the two challenges and the correlations between them in the asthma and BO groups are shown in figure 3. Of the 22 subjects with post-infectious BO who had AHR to methacholine, only five (22.7%) showed AHR to AMP, whereas the combination of AHR to methacholine and AMP was found in 28 asthma patients (93.3%). Moreover, methacholine PC_{20} and AMP PC_{20} correlated significantly in the asthma group (r=0.51, p=0.00), but not in the BO group (r=0.29, p=0.13). There were no differences in the clinical picture, atopic status, or airway pathogens at the time of antecedent respiratory infections among children with post-infectious BO who responded to both methacholine and AMP (n=5), those who responded to methacholine but not to AMP (n=17), those who responded to neither methacholine nor AMP (n=5), and one who responded only to AMP.

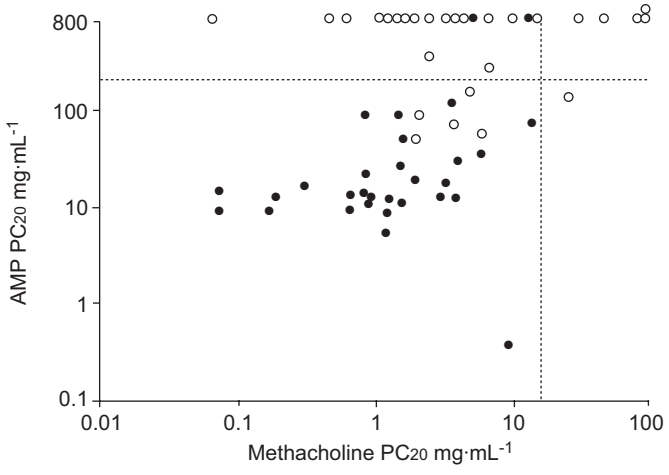

FIGURE 3. Scatter plots of adenosine 5′-monophosphate (AMP) provocative concentration causing a 20% fall in forced expiratory volume in one second (PC_{20}) against methacholine PC_{20}. ●: subjects with asthma; ○: subjects with post-infectious bronchiolitis obliterans. The horizontal dotted line represents the PC_{20} limit of 200 mg·mL^{-1} for AMP and the vertical line the PC_{20} limit of 16 mg·mL^{-1} for methacholine.

DISCUSSION

The current study is the first to compare methacholine and AMP challenge test results in children with post-infectious BO. The majority of these children were hyperresponsive to methacholine, but not to AMP, which was in contrast with the finding in patients with asthma, most of who responded positively to both challenges.

Post-infectious BO is an unusual consequence of epithelial injury of the lower respiratory tract, but may be more common than previously thought. A definitive diagnosis of BO was traditionally made with histopathological confirmation *via* lung biopsy. However, this procedure is invasive and does not always confirm the diagnosis because of the patchy distribution of airway involvement. During the last decade, HRCT of the thorax for patients with respiratory diseases has enhanced the ability of clinicians to identify BO noninvasively [5, 9]. Adequate evidence indicates that a diagnosis of BO can be made based on typical clinical features and characteristic HRCT findings [17]. All patients in the BO group showed a mosaic perfusion and/or bronchiectasis on HRCT. Other chronic obstructive pulmonary diseases were excluded with appropriate measures. The diagnosis of post-infectious BO in the present study is therefore considered to be reasonably valid. In fact, 12 mildly affected patients, judged from the normal pulmonary function test results (FEV_1⩾80%), were included. However, most of them (10/12, 83.3%) showed a decline in small airway flow function, as measured by forced expiratory flow between 25 and 75% of vital capacity ($FEF_{25–75\%}$; ⩽75% predicted). Were it not for the use of HRCT with a high index of suspicion, this group would have been considered as having a nonspecific sequela of a previous respiratory infection rather than having BO. A control group was selected that consisted of children with previous admission for lower airway infections but with complete clinical and radiological resolution, in order to preclude assigning a role for these infections in causing AHR.

The occurrence of AHR to methacholine is well documented in post-transplant BO [18, 19]. A positive methacholine challenge test after lung transplantation has been proposed as an early marker of BO [18]. Little is known about AHR in post-infectious BO. Two studies [7, 8] have shown that children with asthma and those with chronic lung diseases (cystic fibrosis, ciliary dyskinesia and BO) have AHR to methacholine, but only those with asthma are hyperreactive to AMP. However, because of the small number of cases involved and the undefined aetiology of BO, the results are inconclusive concerning response to methacholine and AMP in post-infectious BO.

In the present study, the majority of patients with post-infectious BO were hyperreactive to methacholine, but only a minor proportion of these patients were hyperreactive to AMP.

However, all patients with asthma responded positively to methacholine, and most also responded positively to AMP. A methacholine PC_{20} of 16 $mg \cdot mL^{-1}$ was chosen as the AHR cut-off. This may appear high, but is considered clinically relevant, since ''borderline'' AHR (PC_{20} 4–16 $mg \cdot mL^{-1}$), defined according to the American Thoracic Society guidelines [14], was demonstrated by a considerable proportion (5/30, 16.7%) of asthma patients. A cut-off point of 200 $mg \cdot mL^{-1}$ was chosen for AMP PC_{20}, because this allowed asthma patients and healthy controls to be discriminated in previous studies [15, 20]. In fact, all but two asthmatic patients in the present study responded positively to AMP <200 $mg \cdot mL^{-1}$, which suggests that this concentration represents an appropriate upper limit for the evaluation of AHR. Of those subjects hyperreactive to methacholine, AHR to AMP was present in only five (22.7%) of 22 patients with post-infectious BO, compared with 28 (93.3%) of 30 asthmatic subjects. The lower frequency of positive responses to methacholine or AMP in the BO group, at least as compared with the asthmatic group, cannot be accounted for by the effects of previous medications, as none of the BO patients had received anti-inflammatory agents, such as corticosteroids or macrolide antibiotics, within 1 month of the challenges. The current study's results indicate that AHR to methacholine in patients with post-infectious BO is usually not accompanied by AHR to AMP, whereas most asthmatic subjects showed positive responses to methacholine and AMP. This response implicates different inflammatory processes besides the structural changes that occur in post-infectious BO, because AHR to AMP may differ in its pathophysiological pathway from that to methacholine [21].

The mechanisms underlying AHR to methacholine in the BO group are not clear. It should be considered that other causes of AHR, such as asthma, might have been overlooked or misattributed to BO. However, this is believed to be unlikely, because the clinical response to bronchodilators in the BO group was minimal, and mosaic perfusion on HRCT, which was present in most cases of the BO group, is rarely or never seen in uncomplicated asthma [22, 23]. The mean baseline lung function was lower in the BO group, despite the exclusion of subjects with severe airflow limitation (FEV_1 <60% predicted) prior to challenge. This may account for the high frequency of AHR to methacholine observed in the BO group, since the degree of AHR may be related to some extent to the degree of resting airway obstruction as measured by FEV_1 [24]. However, no correlation was found between methacholine PC_{20} and baseline FEV_1, and AHR to methacholine was shown by a high proportion (8/12, 66.7%) of patients with a normal FEV_1 ($\geq$80% predicted), which suggests that a low baseline FEV_1 is not a major factor of AHR to methacholine. Of the other possible factors, peripheral airway wall thickening, which does not affect baseline airway calibre, may enhance airway responsiveness, as would a loss of elastic recoil because of the decreased load against which the muscle contracts [25].

The inflammatory mediator release originating from airway mast cells plays an integral role in the response to inhaled AMP [6]. Clinical studies in asthma patients have shown that AHR to AMP reflects an underlying eosinophilic or atopic airway inflammation more accurately than AHR to methacholine [21, 26]. This hypothesis is supported by the current authors' observation of a positive correlation between blood eosinophil counts and AMP PC_{20}, but not between blood eosinophil counts and methacholine PC_{20} in the asthma group. The poor response to AMP in patients with post-infectious BO was matched by the absence of an increased blood eosinophil count. No information is available about any cellular or mediator changes in the airways of patients with post-infectious BO. The results of this study may suggest that mast cells are not increased either in number or activation status in this disease.

The discussion so far has assumed that airway responsiveness to methacholine or AMP is accurately reflected by the measurement of FEV_1. FEV_1 measures air flow at high- and mid-lung volumes. Other respiratory indices, such as $FEF_{25–75\%}$, are considered to be more sensitive indicators of obstruction in small airways [27], which are the physiological sites of airflow limitation in BO. However, $FEF_{25–75\%}$ is more variable and less reproducible than FEV_1. In addition, as full vital capacity may not be delivered in a forced expiratory manoeuvre in the presence of severe airway obstruction, $FEF_{25–75\%}$ may underestimate the degree of airway obstruction [28]. Thus, the use of this index in assessing AHR may be misleading. The relative contribution of the proximal conducting airway and the distal small airways to AHR has been studied in patients with suspected post-transplant BO [29]. This study concluded that AHR, as measured by FEV_1, arises from involvement of the small airways. However, whether this is the case for post-infectious BO needs to be determined by a future study.

In conclusion, airway hyperresponsiveness to methacholine is a frequent, but by no means universal, finding in children with post-infectious bronchiolitis obliterans, but is usually not accompanied by airway hyperresponsiveness to adenosine 5′-monophosphate. This suggests that airway hyperresponsiveness in patients with post-infectious bronchiolitis obliterans has different characteristics to those occurring in asthmatic subjects.

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