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Bronchoalveolar Lavage MMP-9 and TIMP-1 in Preschool Wheezers and Their Relationship to Persistent Wheeze

MICHEL D. S. ERLEWYN-LAJEUNESSE, LINDA P. HUNT, PETR POHUNEK, SARAH J. DOBSON, PUJA KOCHHAR, JANE A. WARNER, AND JOHN O. WARNER

Allergy and Inflammation Sciences [M.D.S.E.-L., S.J.D., P.K.], University of Southampton, Southampton SO16 6YD, United Kingdom; Clinical Sciences at South Bristol [L.P.H.], University of Bristol, Bristol BS8 1TH, United Kingdom; Pediatric Department [P.P.], University Hospital Motol, CZ-15006 Prague, Czech Republic; School of Biological Sciences [J.A.W.], University of Southampton, Southampton SO16 7PX, United Kingdom; Faculty of Medicine [J.O.W.], Imperial College, London W2 1PG, United Kingdom

ABSTRACT: Atopic preschool children are more likely to develop persistent wheezing, which could be a consequence of early airway remodeling. Protease–antiprotease balance between MMP-9 and its cognate inhibitor TIMP-1 may be involved in this process. Our hypothesis was that atopic wheezing preschool children would have an imbalance of MMP-9 to TIMP-1 in bronchoalveolar lavage (BAL). BAL from 52 preschool wheezers was compared with 14 controls without wheeze. A subgroup completed an International Study of Asthma and Allergy in Childhood symptom questionnaire 2 y later. Molar ratios of MMP-9/TIMP-1 were higher in wheezy children ($p < 0.001$; median 4.0%, range 0–8.7) than controls (0.6%, 0–1.8), and showed an excess of TIMP-1 in the airway. BAL TIMP-1 was raised in children with persistent wheezing ($p = 0.028$; 34.4 ng/mL, 9.1–93.1 compared with 10.6 ng/mL 6.1–18.6), as was serum levels of intercellular adhesion molecule-1 ($p = 0.027$). The absolute concentration of TIMP-1 in the airway, rather than its molar ratio with MMP-9, was associated with persistent wheezing. The processes involved with airway remodeling are complex but excess TIMP-1 may impede matrix protein turnover and thereby contribute to persistent changes in airway structure and wheezing. **(*Pediatr Res* 64: 194–199, 2008)**

Wheezing is a common respiratory symptom among preschool children and while most outgrow their disease by mid childhood, some continue to wheeze and develop asthma. Atopic preschool children are more likely to have persistent wheezing associated with progressively reducing pulmonary function (1). The cause of this deteriorating pulmonary function is not known, but airway remodeling is implicated in the disease process, and has been found in school-aged children with asthma (2–6). Thickening of the epithelial basement membrane (EBM) is an age related event detectable by 3 y of age in severe recurrent wheezers (7,8).

Although the inflammatory mechanisms underlying the remodeling process are not known, protease anti-protease balance is one factor that could lead to a disorder of matrix turnover (9). The matrixins are a family of proteases able to digest components of the extracellular matrix (ECM). Matrix metalloproteinase 9 (MMP-9) is the predominant matrixin in pulmonary tissue and is specifically inhibited by tissue inhibitor of metalloproteinase-1 (TIMP-1) in a 1:1 ratio (10). There are two opposing mechanisms by which MMP-9 and TIMP-1 could affect a remodeling process: First, an excess of active MMP-9 over TIMP-1 would allow unregulated destruction of the ECM leading to inflammation and wound repair (11). Second, an excess of TIMP-1 over MMP-9 would slow the turnover of the ECM, which may include thickening of the EBM.

Although, there is accumulating evidence of a role for MMP-9 and TIMP-1 in adult asthma (11–16), there are conflicting results in children, in an age group where structural remodeling may actually be occurring (17,18). The aim of this study was to investigate MMP-9 and TIMP-1 in bronchoalveolar lavage (BAL) from preschool wheezers. Our hypotheses were that those infants with atopy would have a different inflammatory process in the lower airways than nonatopic wheezers, and specifically that an imbalance of MMP-9 with its cognate inhibitor TIMP-1 would lead to persistence of symptoms.

METHODS

Children under four were enrolled if they required a clinically indicated bronchoscopy with bronchoalveolar lavage for differential diagnostic purposes following recurrent or persistent wheeze with or without coughing. Atopy was defined as the presence of atopic dermatitis, or allergic rhinitis, or a raised serum eosinophil count or immunoglobulin E, or a positive skin prick test or radioallergosorbent test for a range of common food and aeroallergens. Subjects were recruited from Brussels, Paris, Prague, and Southampton between 1997 and 1999. Wheezers were excluded from the study if they had been treated with inhaled corticosteroids, cromoglycate, or antihistamines (including cough medicines) in the 2 wks preceding enrolment.

Controls were taken from a separate contemporaneous bronchoscopic study in children, who mostly had been bronchoscoped for stridor. As some wheezers also had stridor, controls with stridor and cough also had to have obvious structural airway abnormalities, such as airway malacia or external compression, which explained their symptoms.

The lavage and follow-up studies were fully approved by Southampton and South West Hampshire research ethics committee and by each institution's ethical review board. Informed consent from a parent or guardian was obtained before enrolment.

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Correspondence: M. Erlewyn-Lajeunesse, D. M., Paediatric Allergy and Immunology, Southampton University Hospital NHS Trust, Tremona Road, Southampton SO16 6YD, United Kingdom; e-mail: mich.lajeunesse@soton.ac.uk
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Abbreviations: BAL, bronchoalveolar lavage; **EBM,** epithelial basement membrane; **MMP-9,** matrix metalloproteinase 9; **MWU,** Mann-Whitney U test; **sICAM-1,** soluble intercellular adhesion molecule 1; **TIMP-1,** tissue inhibitor of metalloproteinase 1

Bronchoscopy and lavage. A transnasal flexible fiber-optic bronchoscopy and bronchoalveolar lavage, using a 3.5 mm pediatric scope, was performed using standard methods (19). The total lavage volume was between 2 and 3 mL/kg applied in three aliquots. The first aliquot was separated and termed bronchial wash, all subsequent aliquots were pooled and termed bronchoalveolar lavage (BAL). Only BAL was used in this analysis.

Both lavage and cell counts were prepared according to a standard method at each center's laboratory (20). Bronchial wash was sent for cultured using routine local laboratories and the significance of bacterial growth was a clinical decision based upon the opinion of the local investigator. Further analysis of BAL was performed centrally.

ELISA and RIA. ELISAs were performed according to the manufacturers' instructions. Markers were measured where the volume of BAL aliquots permitted. BAL was assayed for IL-8 (IL-8, R & D Systems Europe Ltd, UK), soluble ICAM-1 (R & D Systems Europe Ltd), MMP-9 (Amersham Pharmacia Biotech, UK) and TIMP-1 (Amersham Pharmacia Biotech). The limits of detection were 31.25 pg/mL, 10 ng/mL, 0.125 ng/mL, and 3.13 ng/mL respectively. Soluble ICAM-1 was also measured in serum. The MMP-9 ELISA was able to detect pro-MMP/TIMP complexes but not active MMP-9 when bound to TIMP-1. ECP was measured by radio-immunoassay (RIA) (Pharmacia & Upjohn Diagnostics AB; Uppsala, Sweden) in lavage and serum according to manufacturer's instructions.

Follow up. A subgroup of children recruited in Prague and Southampton were followed up by parental administered questionnaire for the International Study of Asthma and Allergy in Childhood (ISAAC) 23 mo after the BAL study closed (21). The questionnaire was translated and validated in Czech according to standard International Study of Asthma and Allergy in Childhood methodology (22).

Statistics. The lavage data were not normally distributed and this, together with the small number of measurements below the limit of detection, led us to use nonparametric analyses throughout. Multiple groups were compared using the Kruskal-Wallis test and pair wise comparisons using the procedure outlined by Siegel and Castellan (23) and adjusted for multiple comparisons unless otherwise stated. Spearman's rank correlation coefficients (r_S) were used between continuous variables.

RESULTS

Subject characteristics and controls. Fifty-two wheezers were recruited to the study and compared with 14 controls recruited over the same time period. Study groups and their clinical details are shown in Table 1. No wheezers or controls had received oral or inhaled Corticosteroids in the 2 wk before bronchoscopy. One nonatopic wheezer had a transfusion of human normal immunoglobulin 3 wks before bronchoscopy for a history of recurrent otitis media and hypogammaglobulinemia of subclass IgG2. Serum and bronchoalveolar lavage findings are shown in Table 2. Total nucleated cell counts (TNCC) and serum soluble intercellular adhesion molecule 1 (sICAM-1) were significantly different between groups yet pair wise comparison failed to show significant differences between controls, atopic and nonatopic wheezers ($0.05 < p < 0.10$ and $p > 0.05$, respectively).

MMP-9 and TIMP-1. MMP-9, but not TIMP-1 concentrations, in BAL were significantly different between wheezers and controls. The median MMP-9 concentration for all wheezers was 3.0 ng/mL (range <0.1–55.7, $n = 36$) compared with controls 0.8 ng/mL (<0.1–4.8 ng/mL, $n = 11$, $p = 0.002$). When wheezers were divided by atopy (Table 2, Fig. 1 panel *A*), MMP-9 concentrations remained significantly different between groups ($p = 0.005$), with significant pair wise differences between nonatopics and controls (unadjusted $p < 0.05$), and atopics and controls (unadjusted $p < 0.05$), but not between atopics and nonatopic wheezers. These pair wise differences were not significant when we adjusted for three multiple comparisons ($p > 0.1$). Among wheezers, BAL neutrophil proportions correlated with MMP-9 ($r_S = 0.7$, $p < 0.001$, Fig. 1 panel *B*). TIMP-1 was not significantly different between all wheezers and controls ($p = 0.194$, median 18.4 ng/mL, 1.2–205, $n = 36$), and remained so when categorized as atopic or nonatopic ($p = 0.341$, Table 2, Fig. 1 panel *C*). Unlike MMP-9, TIMP-1 did not correlate to any particular cell type.

Ratios of MMP-9 to TIMP-1 were calculated using the molar weights of the two proteins and expressed as a percentage. Molar ratios were significantly higher in all wheezers (4.0%, 0–8.7%, $n = 35$, this excluding a single outlier of 201%) than controls ($p < 0.001$). This difference between groups remained when wheezers were considered as atopic and nonatopic ($p < 0.001$, Table 2, Fig. 1 panel *D*). Molar ratios for nonatopic wheezers were significantly different from controls ($p < 0.05$). Atopic wheezers were also significantly different from controls ($p < 0.05$) but this was borderline when adjusted for three comparisons ($0.05 < p < 0.10$).

TIMP-1 was in molar excess in the airway of both wheezers and controls. Only one wheezy child had an excess of MMP-9 with molar ratio of 201%. This child was atopic, with bacteria present on BAL culture and a neutrophilia of 54% on BAL cytology suggesting active infection.

Table 1. *Clinical details*

Presenting complaint	Control Stridor*	Wheeze with or without cough		
		All wheezers	Nonatopic wheezers	Atopic wheezers
N	14	52	27	25
Age	1.7 (0.2–5.1)†	1.1 (0.5–3.8)	0.9 (0.5–3.8)	1.1 (0.5–3.5)
Males	7 (50%)	31 (60%)	15 (56%)	16 (64%)
Stridor	12 (86 %)*	3 (6%)	2 (7%)	1 (4%)
Airway malacia‡	7 (50%)	2 (4%)	1 (4%)	1 (4%)
Cough	3 (21%)	28 (54%)	14 (52%)	14 (56%)
Positive cultures	0/7	15/43 (35%)	11/22 (50%)	4/21 (19%)
Antibiotics§	NA	11 (21 %)	7 (26%)	4 (16%)
Inhaled corticosteroids§	NA	12 (23%)	9 (33%)	3 (12%)

Wheezing subjects are shown in total, and the atopic subgroup as a separate column. Age is shown as median (range) in years.

* Two control children did not have stridor: one had unexplained tachypnoea, the other a normal bronchoscopy following persistent changes to the right middle lobe. Both had negative BAL cultures, normal serum IgE, and no clinical response to $\beta 2$ agonist inhalers.

† Only one child was older than 4 yrs in the control group.

‡ Functional obstruction of the airway, such as laryngo or bronchomalacia.

§ Medication given in the 6 wk before bronchoscopy.

Table 2. *Serum and bronchoalveolar lavage*

	Control	Nonatopic	Atopic	*p*
Total nucleated cells	1.52	2.75	3.73	0.004
$\times 10^5$/mL	0–4.99	0.14–200.1	0.53–184.8	
	14	25	24	
Cellular viability (%)	82	72	71	0.077
	61–95	35–100	38–100	
	12	24	24	
Epithelial cells (%)	2	1	4	0.224
	0–5	0–19	0–24	
	12	24	22	
Macrophages (%)	90	92	85	0.171
	74–98	5–99	26–97	
	14	25	24	
Neutrophils (%)	3	2	5	0.648
	0–14	0–87	0–54	
	14	25	24	
Lymphocytes (%)	7	4	9	0.439
	1–22	0–26	0–31	
	14	25	24	
Eosinophils (%)	0	0	0	0.122
	0–0.7	0–2.0	0–27.6	
	14	25	24	
Interleukin 8	46	46	66	0.910
(pg/mL)	<31–234	<31–580	<31–4085	
	7	22	21	
Eosinophil cationic	2.2	2.4	2.2	0.608
protein (μg/L)	<2.0–5.4	<2.0–151.8	<2.0–90.3	
	8	24	21	
Serum ECP (μg/L)	NA	24	28	0.690*
		3–72	3–125	
		12	17	
sICAM-1 (ng/mL)	81	77	76	0.880
	42–186	24–206	27–230	
	12	23	22	
Serum sICAM-1	NA	333	409	0.025*
(ng/mL)		243–755	313–636	
		22	21	
MMP-9 (ng/mL)	0.8	3.7	2.2	0.005
	<0.1–4.8	0.8–54.6	<0.1–55.7	
	11	19	17	
TIMP-1 (ng/mL)	27	18	21	0.341
	15–87	4–108	1–206	
	11	19	17	
MMP-9/TIMP-1	0.6	5.1	3.0	< 0.001
molar ratio (%)	0–1.8	1.1–87.5	0–201.4	
	11	19	17	

The table shows medians with ranges and *n*. Samples were from BAL unless stated. Where lower limits shows a < figure values were below the limit of detection for this assay. *p* values are calculated from three groups Kruskal Wallis tests except.

* Mann-Whitney U test was employed.

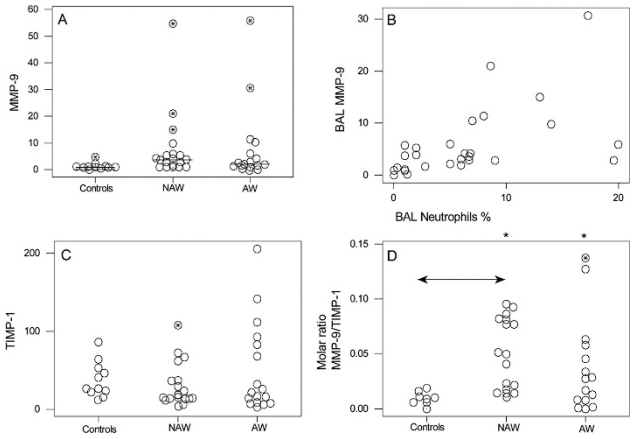

Figure 1. MMP-9 and TIMP-1 in BAL of preschool wheezers. (*A*) Plot of MMP-9 (ng/mL) by group. There was an overall difference between groups of $p = 0.005$, but no pair wise differences reached significance when adjusted for multiple comparisons. (*B*) Scatter plot of BAL neutrophil proportion and MMP-9 concentrations in wheezers. BAL neutrophils correlated to MMP-9 concentrations (r_S 0.7, $p < 0.001$). Two values with MMP-9 >50 ng/mL were excluded from this graph to aid visual comparison. These values were included in the statistical analysis. (*C*) Plot of TIMP-1 (ng/mL) by group. There were no significant differences overall or between groups. (*D*) Plot of molar ratios of MMP-9/TIMP-1. Outliers ratio >0.2 are excluded to aid visual comparison and are indicated as *. They were included in the statistical analysis. Overall, difference between groups was $p < 0.001$ with pair wise comparison between Controls and NAW of $p < 0.05$ indicated by arrowed line.

The effect of positive bacterial culture. Positive bacterial cultures were found in 50% (11/22) of nonatopic wheezers and 19% (4/21) of atopics (χ^2 $p = 0.070$, Table 1). These were common respiratory pathogens including *Haemophilus influenza*, Streptococcus *pneumonia, Moraxella catarrhalis,* and *Staphylococcus aureus*. Only one subject, whose BAL was positive on culture, was taking oral antibiotics at the time of the bronchoscopy. The effect of bacteria on our results was examined where culture results were available. Cellular viability was reduced by positive cultures ($p = 0.05$ compare negative culture wheezers) but there was no effect on neutrophil proportion or IL-8. MMP-9 was different between wheezers with positive cultures ($p = 0.015$; 4.8 ng/mL, 1.1–55.7, $n = 10$), negative cultures (2.2 ng/mL, 0–30.6, $n = 21$), and controls (0 ng/mL, 0–4.8, $n = 5$). TIMP-1 was reduced in wheezers with positive cultures ($p < 0.05$; 9.3 ng/mL, 1.2–72.5) compared with those with negative cultures (29.3 ng/mL, 8.1–205.6). As a result, molar ratios were higher in those with positive cultures ($p < 0.05$; 11.5%, 2.2–201.4) than negative cultures (1.8%, 0–9.5).

Follow-up study. Twenty-five wheezers (25/52, 48%) in two of the original centers were followed up by questionnaire. Twenty families responded (20/25, 80%) with replies from 12 atopic and eight nonatopic wheezers. There were no differences in clinical characteristics between those who did and those who did not respond to the survey. The average age of the respondents was 4.3 y (range 2.6–6.9) and the mean time between bronchoscopy and follow up was 2.6 y (range 2.0–3.6).

Fifteen (15/20, 75%) had wheezed in the last year and these persistent wheezers were younger than those who had outgrown their symptoms (Mann-Whitney U (MWU) test, $p = 0.043$). Persistent respiratory symptoms are shown in Table 3. There was no relationship between persistence of wheeze and age at bronchoscopy (MWU, $p = 0.394$). All atopics (11/12, 92%) had persistent symptoms except for one child, whose parents reported that their child had never been wheezy. Four nonatopic wheezers had outgrown their symptoms at follow up. None had developed clinical signs of atopy since bronchoscopy. By comparison, three of four nonatopics with persistent wheezing had developed either allergic rhinitis or atopic dermatitis since the time of bronchoscopy. The lone

Table 3. *Respiratory symptoms in persistent wheezers*

	Response (%)
Wheezed at anytime in the past	19 (95)
Diagnosis of asthma ever	14 (70)
Wheezed in the last 12 mo (persistent)	15 (75)
Attacks of wheezing in the last 12 mo	
None	5 (25)
1 to 3	7 (35)
4 to 12	8 (40)
Sleep disturbance due to wheezing	
Never woken	8 (40)
Less than once per week	4 (20)
Once or more nights per wk	3 (15)
Speech limiting severity in last 12 mo	2 (10)
Exercise induced symptoms in last 12 mo	10 (50)
Nocturnal cough in last 12 mo	10 (50)

The table shows the prevalence of reported symptoms amongst the 20 families who responded to the follow-up questionnaire survey. Persistent wheezers are defined as those who had wheezed in the last 12 months.

persistent nonatopic wheezer had a raised serum ECP at time of bronchoscopy at 6 mo old but still had no clinical atopic features 2.5 y later.

Inflammatory markers in BAL and serum were compared with light of current symptoms. BAL TIMP-1 and serum sICAM-1 were related to persistent wheezing. No other serum and BAL markers showed a similar relationship. Figure 2 shows how the concentration of TIMP-1 in BAL was significantly higher among persistent wheezers (median 34.4 ng/mL, range 9.1–93.1; MWU, $p = 0.028$) compared with those who had outgrown their symptoms (10.6 ng/mL, 6.1–18.6). Serum sICAM-1 was also raised among persistent wheezers ($p = 0.027$). The median serum concentration was 477 ng/mL (243–588) among persistent wheezers compared with 269 ng/mL (252–337) among transient wheezers (Fig. 2). Some nonatopic persistent wheezers had raised levels of TIMP-1 and serum sICAM-1 before the onset of atopic disease (Fig. 2).

DISCUSSION

Our study suggests that it is not the imbalance of MMP-9 with TIMP-1 that is related to persistent wheezing in the preschool years, but rather the absolute values of BAL TIMP-1 and serum sICAM-1. An acute TIMP-1 response has been observed in adult studies of allergic airway inflammation (24–27), and has also correlated to airway wall thickening (15). In children, TIMP-1 was increased in upper airway secretions of severe respiratory syncytial virus bronchiolitis and we speculate that TIMP-1 may be involved in inflammation leading to post bronchiolitic wheezing (28). Our results are very different from that seen in chronic lung disease of prematurity, where TIMP-1 is reduced early in the disease process (29). We propose that TIMP-1 excess may impair matrix turnover, leading to thickening of the EBM and airway remodeling, but may also have an immunomodulatory role that may well be crucial to its mode of action (10). The association of serum sICAM-1 with persistent wheezing indicates airway inflammation. Infant serum sICAM-1 predicted the onset of wheezing in the second year of life (30). Together our results suggest that different inflammatory processes separate persistent from transient wheezers early in life. The implication is that inflammation is associated with structural airway changes leading to persistence of wheezing.

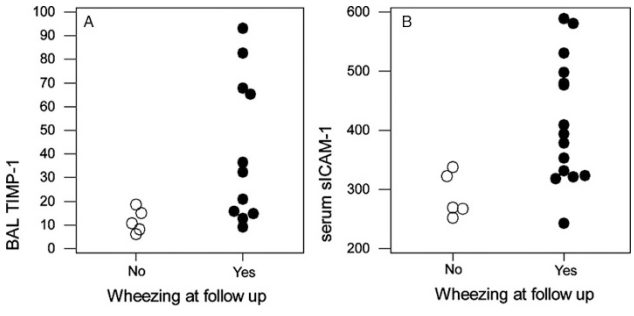

Figure 2. The relationship of TIMP-1 and serum sICAM-1 at bronchoscopy to persistence of wheeze. Panels A and B show plots of BAL TIMP-1 and serum sICAM-1 against persistence of wheeze: ○ no wheeze at follow up; ● persistent wheezers at time of bronchoscopy.

MMP-9 was increased in both atopic and nonatopic wheezers and we consider this to be a marker for acute inflammation, as adult studies show that MMP-9 increases during exacerbations of asthma (24,26,27,31,32). In our study TIMP-1 excess was the rule as MMP-9 only exceeded TIMP-1 molar concentrations in one child, who clearly had a lower respiratory tract infection; yet raised MMP-9 was also seen in those with positive bacterial culture of BAL. Whether this is infection or not is unclear as neutrophil proportions and IL-8 in were not increased by the presence of bacteria in the lavage. Bacteria are frequently isolated from BAL in preschool wheezers and their presence is cited as being from upper respiratory tract contamination of the bronchoscope (33–37). TIMP-1 was reduced in these children, like adults with pneumonia (38), suggesting that bacteria are proinflammatory; however, other markers of inflammation like IL-8 were not increased in this group. If high TIMP-1 is associated with persistent wheezing, then the lower TIMP-1 concentrations in children with positive bacterial cultures may be a marker of protection in keeping with the hygiene hypothesis, where bacterial infection reduces the subsequent risk of allergic disease.

Two other studies have measured MMP-9 and TIMP-1 in asthmatic children. Both have methodological differences that make it difficult to compare with our results. Tang *et al.* found that MMP-9 and TIMP-1 were raised in alveolar macrophages from asthmatic children (17). This study used immunohistochemistry to identify MMP-9 and TIMP-1 in alveolar macrophage vacuoles. The MMP-9/TIMP-1 molar ratios observed were much greater than our study with controls reaching 100% and wheezers 76%. However, it is not clear whether observed MMP-9 and TIMP-1 were due to cellular synthesis, or were the result of ingestion of airway material. Both scenarios may be radically different from that found in the epithelial lining fluid and BAL. Doherty *et al.* used techniques similar to ours, but in older children with stable asthma, pretreated with ICS (18). They observed reduced MMP-9 in asthmatics, and TIMP-1 concentrations one tenth of those described here. These results contradict ours and are probably related to differences in the patient groups under consideration, as they were not in an acute phase of their disease.

Limitations and ethical considerations of this study. Pediatric research that involves invasive procedures is both practically and ethically difficult. We chose to study wheezy children who required bronchoscopy for clinical indications. Despite the growing safety record of pediatric bronchoscopy, it is still rarely indicated for preschool wheeze (39). The study required four large European centers recruiting over 3 y to enroll just over 50 subjects. In consequence, the children were a mixed group whose indications for bronchoscopy included severe or atypical symptoms and failure of standard therapies. The severity of their symptoms makes extrapolation of the results to the general population of infant wheezers difficult.

Finding appropriate controls for research requiring invasive procedures in children is also extremely difficult. The control cases in this study are believed to represent as near to normal physiology as is possible within ethical and practical constraints. Widely accepted normal data has been taken from bronchoscopy findings in similar children (40–42).

We did not expect to evaluate lavage bacteria in infant wheeze, and our study was limited by the absence of central or standardized culture mechanisms. Cell counts were performed using a standardized method but were not validated between centers, and this probably accounts for the lack of further differences between groups. Results were missing for many of the children enrolled in this study, which also introduced bias and reduced sample size in an already small study. We would recommend that future studies perform a full evaluation for infectious agents in lavage specimens from infant wheezers.

The questionnaire follow up was limited by its size. Most children had persistent wheeze and were almost exclusively atopic, which reduced our ability to compare between transient and persistent disease. Transient wheezers were older at follow up than those whose symptoms persisted, but there was no relationship between age at bronchoscopy, when the samples were taken, and persistent wheezing. It is unfortunate that the numbers were too small to look for other potential sources of bias and our findings should be interpreted with this in mind.

In conclusion, this small study of BAL findings in preschool wheezers has shown that MMP-9 is raised in proportion of wheezy children, but that in general TIMP-1 outweighs MMP-9 in both normal and wheezy airways. The absolute concentration of TIMP-1 may be related to airway remodeling in some atopic children as persistent wheezers had raised BAL TIMP-1 and serum sICAM-1 on initial evaluation. The profibrotic activity of TIMP-1 may be responsible for EBM thickening leading to inflammation, which would also upregulate sICAM-1 expression. Thus, we propose that recurrent insults to the airway epithelium, which occur more frequently in atopic than nonatopic infants, result in a process that induces airway remodeling at a very early stage in disease evolution, and this process is more likely to lead to persistent disease. However, the role of MMP-9, TIMP-1 and their molar ratio in prediction of airway remodeling remains impossible to interpret with accuracy, as they remain surrogate markers of the remodeling process. Until biopsy studies are able to provide a basis for their validity, both in adults and in infancy, their significance can remain only speculative.

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