

## Original Articles

# Detection and Clinical Significance of a Potential Mediator of Airway Remodelling in Preschool Wheezy Children

YY ZHANG, HZ XU, XE BAO, SX LI, YS WANG, Y CHEN, ZM CHEN

### Abstract

**Purpose:** To detect levels of matrix metalloproteinase (MMP)-9, tissue inhibitors of metalloproteinase (TIMP)-1 and transforming growth factor beta 1 (TGF- $\beta$ 1), and analysis the relationship among wheezing and airway remodelling-associated factors. **Methods:** The levels of MMP-9, TIMP-1 and TGF- $\beta$ 1 were detected by enzyme-linked immunosorbent assay. Samples of blood and nasopharyngeal aspirates were analysed for pathogens. Meanwhile, levels of immunoglobulin E (IgE) and percentage of eosinophils were measured. **Findings:** Serum levels of MMP-9 were much higher in wheezy children than in controls ( $p < 0.05$ ) while the level of TIMP-1 and TGF- $\beta$ 1 between the wheezy and control had no significant differences ( $p > 0.05$ ). No significant effects of recurrent wheezing, pathogen infection, IgE level or asthma high risk factors on serum level of MMP-9 were observed ( $p > 0.05$ ). **Conclusion:** We found that serum MMP-9 levels were higher in wheezy children, indicating a possibility for airway remodelling in wheezy children. However, recurrent wheezing, pathogen infection and hereditary backgrounds had no effect on the serum level of MMP-9.

### Key words

Airway remodelling; MMP-9; TGF- $\beta$ 1; TIMP-1; Wheezing

### Introduction

Asthma is a chronic inflammatory disease of the airway. The morbidity and mortality of asthma have significantly increased over the last 20 years. The mechanism of chronic asthma is complicated, which involving smooth-muscle dysfunction, chronic inflammation, airway structural changes, and collectively termed airway remodelling.<sup>1</sup>

Some showed that inflammation and airway remodelling occurred early in the natural history of bronchial asthma and were present even before asthma was diagnosed based on clinical symptoms.<sup>2,3</sup> Early intervention with inhaled corticosteroids can reduce airway inflammation, consequently retard the progression of airway remodelling and leading to an improved prognosis in asthma.<sup>4</sup> But steroids cannot reverse the established structural changes of airway remodelling.<sup>1</sup> Therefore early detection and intervention of airway remodelling are the key point for prevention and therapy for childhood asthma.

Wheezing in children is a complex problem, which can be classified into three wheezing phenotypes: transient early wheezing, non-atopic wheezing and atopic wheezing/asthma.<sup>5,6</sup> However, there is still a paucity of data to clarify that whether there is airway remodelling and what kind of correlation factors are existing in wheezy children before diagnosed as asthma. Many studies have indicated that matrix metalloproteinase (MMP)-9, transforming growth factor beta (TGF- $\beta$ ) and tissue inhibitors of metalloproteinase (TIMP)-1 played pivotal role in the development of airway remodelling.<sup>7-12</sup>

Department of Pulmonology, Children's Hospital, Zhejiang University School of Medicine, 57 Zhugan Xiang, Hangzhou 31003, China

YY ZHANG (張圓圓) Master of Medicine  
HZ XU (徐紅貞) Bachelor of Nursing  
XE BAO (鮑興兒) Master of Medicine  
SX LI (李淑嫻) Bachelor of Medicine  
YS WANG (王穎碩) Master of Medicine  
Y CHEN (陳穎) Bachelor of Medicine  
ZM CHEN (陳志敏) MD, PhD

Correspondence to: Prof. ZM CHEN

Received May 29, 2013

Here, we examined the levels of some airway remodelling-associated factors, such as MMP-9, TIMP-1 and TGF- $\beta$ 1 in young children with wheezing, and explored the effects of various conditions, such as different wheezing attacks, aetiological factors and hereditary backgrounds on the serum levels of MMP-9, TIMP-1 and TGF- $\beta$ 1.

## Subjects and Methods

### Study Subjects

One hundred and twenty-seven young children aging from 1 month to 3 years (97 males, 30 females) hospitalized in our hospital from October 2008 to January 2009 were enrolled. The median age was  $10.9 \pm 7.7$  months old. All patients had clinical symptom of cough and wheezing, and had pulmonary signs of evident wheezing on admission. Children with tuberculosis of tracheobronchial lymph nodes, and respiratory tract foreign body were excluded. All wheezy children were inquired for individual history of wheezing and allergy, family allergy history in detail on admission. Thirty healthy children (16 males, 14 females, aging from 2 months to 3 years, the median age was  $12 \pm 5.5$  months old) were recruited through unrelated volunteer families. Healthy children had none of the following abnormalities: (1) parental history of atopic disease, such as asthma, eczema, allergic rhinitis; (2) high level of specific immunoglobulin (Ig) E antibodies against allergens and total serum IgE; (3) onset of wheezing; (4) increasing number of eosinophilia; (5) respiratory tract infection; or (6) evidence of another respiratory tract dysplasia. There were no significant differences in age, weight, and height between the groups. Research was carried out in compliance with the Helsinki Declaration. All of the data and materials in this manuscript were approved by the ethics committee of the Children Hospital of Zhejiang University School of Medicine. Approval number: 2010-GJ-012. The blood samples and nasopharyngeal aspirates were performed after the informed consents were signed by children's parents.

Patients were divided into groups with different criteria. According to the frequency of wheezing onset, they were divided into first wheezy group and recurrent group (wheezing  $\geq 2$ ). On the basis of pathogen detection, there were respiratory syncytial virus (RSV) positive group and RSV negative group, atypical pathogenic (including *Mycoplasma pneumoniae* (MP), *Chlamydia trachomatis* (CT) and *Chlamydia pneumoniae* (CP)) infection and non-atypical pathogenic infection group. Meanwhile, according

to the results of specific IgE antibodies against allergens and total serum IgE levels, they were divided into high IgE group (specific immunoglobulin E (SIgE)  $>0.35$  IU/ml or total immunoglobulin E (TIgE)  $>100$  IU/ml) and normal IgE group. Moreover, wheezy children were also divided into high risk group and low risk group for asthma, according to the GINA2006. The positive clinical index refers to the presence of a wheeze before the age of 3, and the presence of one major risk factor (parental history of asthma or eczema) or two of three minor risk factors (eosinophilia, wheezing without colds, and allergic rhinitis) the children with positive clinical index have been shown to predict the presence of asthma in later childhood<sup>13</sup> and therefore, they defined as high risk group.

### Measurement of Blood Sample of Subjects

Peripheral blood of wheezy children was obtained from each individual on admission. Peripheral blood of control children was obtained from each individual on health examination. Partial blood samples were kept at room temperature for 30 minutes, and centrifugated at 1000 r/min for 15 minutes. Then serum samples were separated and kept at  $-80^{\circ}\text{C}$  for determination of MMP-9, TIMP-1 and TGF- $\beta$ 1. The rest blood samples were detected for eosinophil counting, SIgE and TIgE determination, as well as IgM, IgG antibodies for MP, CP and CT. Meanwhile, the nasopharyngeal aspirates were also obtained on admission for detection of virus antigen and MP, CP, CT-Deoxyribonucleic acid (DNA). The subjects were enrolled with informed consent from their parents.

The percentage of eosinophil of peripheral blood was determined by blood analyser (Abbott cell-DYN 3700). The AllergyScreen-Test (Mediwiss Analytic, Moers, Germany) was performed for the semi-quantitative determination of circulating allergen-specific IgE in human serum. Specific IgE antibodies against allergens (dermatophagoides pteronyssinus, house dust, cat dander, dog dander, low ragweed, mulberry, cockroach, egg albumen, milk, fish, shrimp, crab, beef, limpet, mutton, mango, cashew nut, penicillium notatum, alternaria, aspergillus fumigatus, oak, elm, firmiana, surname, triangle leaf poplar) were measured in a range of 0.35-100 IU/ml. The results of the analysis were assigned to the Test Classes 0-6. Class 0: IgE  $<0.35$  IU/ml; Class 1: 0.35-0.69 IU/ml; Class 2: 0.7-3.49 IU/ml; Class 3: 3.5-17.49 IU/ml; Class 4: 17.5-49.9 IU/ml; Class 5: 50-100 IU/ml; Class 6:  $>100$  IU/ml. Class 1-6 means positive results. Total serum IgE levels

were measured using the same kit (on the membrane, a detection line is packed with a monoclonal anti-human IgE. This antibody reacts with the free human IgE in the serum of the patients. Total IgE  $\geq 100$  IU/ml means positive result).

Respiratory virus detection (including RSV, influenza virus A, B, parainfluenza virus 1, 2, 3, adenovirus) was performed by direct immunofluorescence using A Chemicon respirovirus assay reagent kit. DNA samples of MP, CP and CT from nasopharyngeal aspirates were detected by fluorescent quantitation polymerase chain reaction (PE5700, America), and reagent was provided by gene diagnosis centre of Daan Zhongshan university. Serum samples were detected for specific IgM and IgG antibody of CP by enzyme-linked immunosorbent assay (ELISA) (OuMeng; Bio Co., Beijing, China) according to the manufacturer's recommendations. Indirect ELISA kit (XiBei; Bio Co., Shanghai, China) was used to detect specific IgM and IgG antibody of MP and CT.

The absolute values of MMP-9, TIMP-1 and TGF- $\beta$ 1 levels in serum were determined by 2-site sandwich ELISA (Boster; Bio Co., Wuhan, China) according to the manufacturer's instructions with a serum dilution of 1:2. The intra-assay coefficient of variation was less than 10%, and the detection limitation for the assays was 156 pg/ml, 156 pg/ml and 15.6 pg/ml, respectively. The optical density was determined by using a microplate reader set to 450 nm (correction 540 nm).

## Statistical Analysis

Statistical analyses were performed using SPSS software (version 15.0). All data were expressed as median values (minimum value-maximal value). The comparisons were made by the Mann-Whitney U-test. Statistical significance was defined as  $p < 0.05$ .

## Results

Of the 127 wheezy children enrolled, 81 cases were first attack of wheezing, and 46 cases were recurrent wheezing. History of eczema was found in 51 cases, urticaria in 6 cases, drug allergy in 6 cases, and allergic rhinitis in 3 cases. Family history of allergy included parental asthma in 9 cases, parental allergic rhinitis in 14 cases, parental urticaria in 5 cases, sister asthma in 1 cases.

Of the 127 cases, the level of SIgE increased in 24 cases, TIgE increased in 41 cases, while both increased in 10 cases.

Of the children with SIgE level increased, 17 were found to be allergic to milk, 12 to mutton, 6 to beef, 2 to dermatophagoides pteronyssinus, 2 to cat dander, 1 to mould, 1 to house dust, 1 to dog dander, and some to multi-allergens. Using blood analyser, we found that there were 7 cases with blood eosinophilia above 4%.

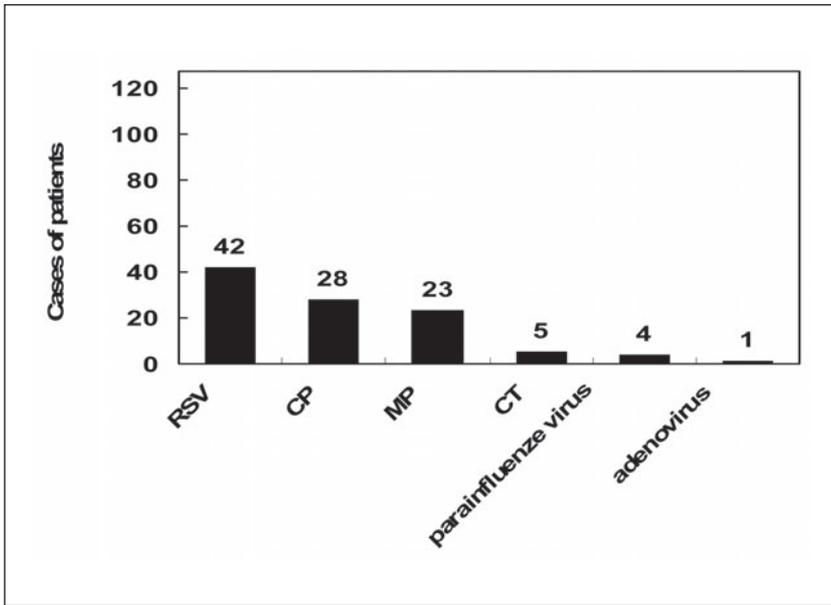
RSV infection were found in 42 cases, CP in 28 cases, MP in 23 cases, CT in 5 cases, parainfluenza virus in 4 cases, adenovirus infection in 1 case. And some of children had mixed infection with multi-pathogens (Figure 1).

As shown in Figure 2A, the levels of MMP-9 in wheezy group were significantly increased when compared to the level of the controls ( $p = 0.000 < 0.05$ ). However, there was no significant difference of the levels of TIMP-1 and TGF- $\beta$ 1 in serum between wheezy group and controls ( $p > 0.05$ , respectively) (shown in Figures 2B-C).

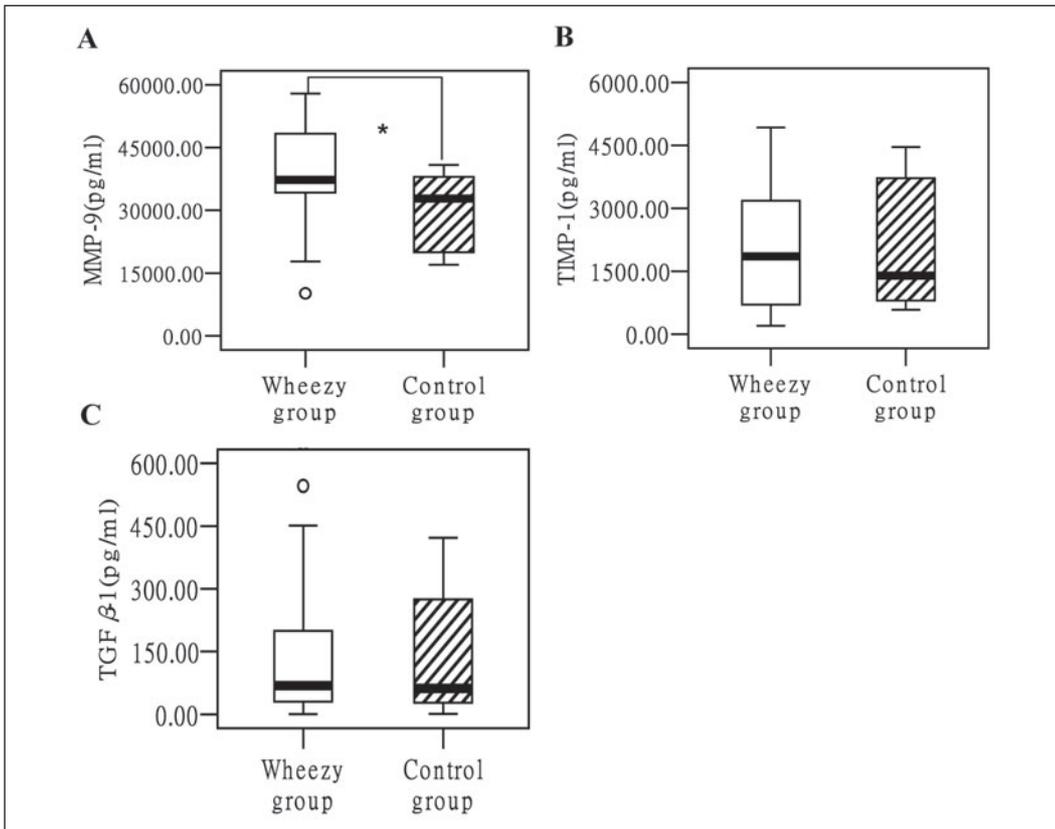
Considering only MMP-9 levels was different between the wheezy children with the controls, we then analysed the effects of various factors as recurrent wheezing attacks, aetiological factors and hereditary backgrounds on serum MMP-9 levels. As shown in Figure 3A, there were no statistically significant differences between first wheezy group and recurrent wheezy group ( $p > 0.05$ ). Since Viral respiratory, especially RSV and atypical pathogens infections are universal in the first few years of life, and can cause wheezing.<sup>14</sup> We next detected the level of MMP-9 in RSV/atypical pathogen infection group and non-RSV/atypical pathogen infection group. However, there was no significant differences between these two groups ( $p > 0.05$ ) (Figures 3B-C). IgE rising and high risk factors are concerned as attack of asthma. We were wondering whether the asthmatic high risk factors and IgE would affect the levels of airway remodelling-associated mediators. As shown in Figures 3D-E, Neither the asthmatic high risk factors nor IgE level had direct effect or correlation effect on the levels of MMP-9 ( $p > 0.05$ ).

## Discussion

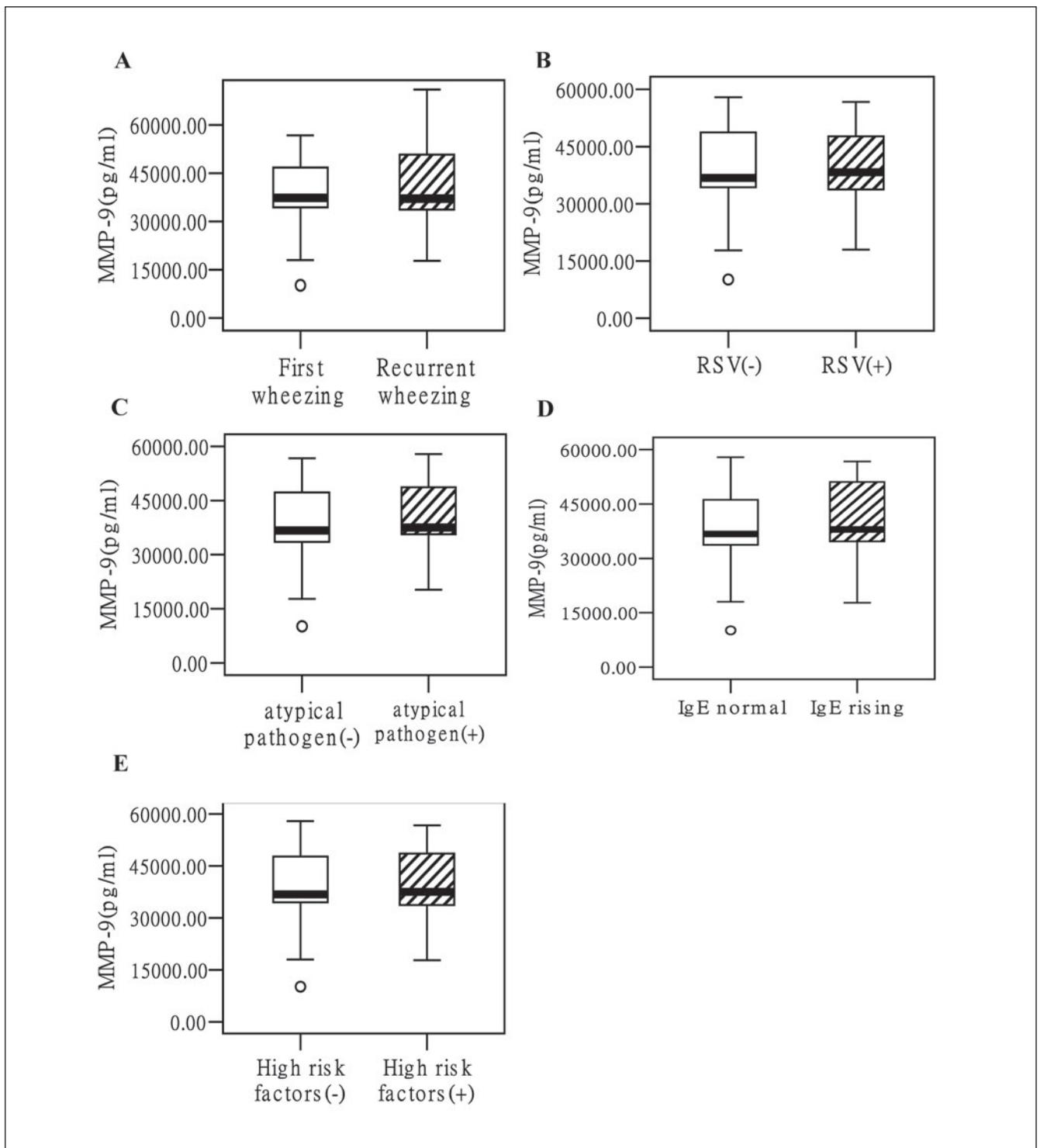
Airway remodelling in asthma was first described in 1922 by Hubert and Koessler in cases of fatal asthma.<sup>15</sup> It refers to structural changes, including loss of epithelial integrity, thickening of basement membrane, subepithelial fibrosis, goblet cell and submucosal gland enlargement, increased smooth muscle mass, decreased cartilage integrity, and increased airway vascularity.<sup>16</sup> The main reason of airway remodelling is excessive deposition of extracellular matrix (ECM). Many studies in asthma have



**Figure 1** Aetiologic types of 127 preschool wheezy children in our hospital. One hundred and twenty-seven preschool wheezy children hospitalised in our hospital from October 2008 to January 2009 were enrolled. The samples of blood and nasopharyngeal aspirates from these children were detected for different pathogen.



**Figure 2** The serum levels of airway remodelling-associated mediator in wheezy group and control group. The absolute values of MMP-9, TIMP-1 and TGFβ-1 levels in serum were determined by ELISA assays. Panels A-C represent MMP-9, TIMP, TGFβ-1, respectively. (Panel A) MMP-9 levels were higher in wheezy group than controls (\*p<0.05). Data represented as median.



**Figure 3** The serum levels of MMP-9 in different groups. The levels of MMP-9 in serum were determined by ELISA assays. Panels A-E represent the effects of various factors such as the frequency of wheezing onset, RSV and atypical pathogens infections, IgE rising and asthmatic high risk factors on serum MMP-9 levels, respectively. Data represented as median.

indicated that cytokines, chemokines (such as MMPs), and growth factors (such as TGF- $\beta$ ) released from inflammatory and structural cells in the airway play a pivotal role in the development of remodelling.<sup>7-12</sup> TGF- $\beta$ 1 is a pleiotropic cytokine which plays important roles on the proliferation, differentiation and ECM metabolism of airway structural cell.<sup>17,18</sup> MMPs, a family of zinc- and calcium-dependent enzymes, are responsible for the degradation of ECM. Among MMPs family, MMP-9 is mostly studied and relevant to asthma. As a counterbalance, TIMP-1 inhibits the enzymatic activity of MMP-9 by stoichiometric 1:1 binding. The increasing of MMP-9 over TIMP-1 has been found to be associated with airway wall inflammation; but the decreasing of MMP-9 over TIMP-1 has been found to be associated with airway wall thickening, as well as chronic airflow obstruction. The balance between MMP-9 and TIMP-1 is considered a major theory to explain the progression of asthmatic airway remodelling.<sup>19,20</sup> The importance of airway remodelling as an early and consistent component of childhood asthma has been emphasized in recent studies.<sup>11</sup> Nevertheless, researches about whether there are airway remodelling in young children with wheezing before diagnosed as asthma are still lacking. In our study, we collected 127 wheezy children and 30 healthy controls, and analysed the differences of levels of MMP-9, TIMP-1 and TGF- $\beta$ 1 between wheezy children and normal controls. To our interest, we found that serum levels of MMP-9 were significantly increased in wheezy group compared with the controls, indicating that MMP-9 maybe a high risk factor to cause airway remodelling in wheezy children. We did not observed the significant differences in the levels of TIMP-1 between wheezy children and controls, so the molar ratios of MMP-9/TIMP-1 were not to be analysed. Concerning the increasing of MMP-9 in wheezy children, regular follow-up and initiate early intervention probably should be carried out in these wheezy children. However, Saglani et al<sup>21</sup> reported that epithelial reticular basement membrane thickening and the eosinophilic inflammation characteristic of asthma in older children and adults are not present in wheezy infants. Further investigation to determine the relationship between wheezy infants and airway remodelling should be carried out in the future.

Wheezing is usually associated with a number of factors. Early-life viral infection causes acute illness and can be associated with an increased risk for subsequent development of asthma/recurrent wheezing. The most commonly detected viruses are RSV.<sup>22</sup> Our study had shown a 33% RSV infection in wheezy children, which occupied

89% in the occurrence of viral infection. Recent studies<sup>23,24</sup> have emphasized potential associations with other pathogens for developing wheezing and asthma, especially with atypical bacterial pathogens *Mycoplasma pneumoniae* and *Chlamydothila pneumoniae*. In this article, of the 28 children with CP infection, 7 cases were combined with RSV, 5 cases with MP, 4 cases with MP and RSV.

Falsey et al<sup>25,26</sup> showed infection with RSV contributed heavily to the repeated chronic inflammation and airway remodelling. Other study also demonstrated that MP infection elicited an inflammatory milieu in the lungs that skewed the immune response toward the Th2-type, thus exacerbating the pathophysiological changes associated with asthma.<sup>27</sup> In order to approach the effects of pathogens and different wheezing attacks on the levels of airway remodelling-associated mediators, wheezy children were respectively divided into RSV positive group and RSV negative group, atypical pathogenic infection group and atypical pathogenic un-infection group, first wheezy group and recurrent wheezy group. And then levels of MMP-9 between these groups were compared. Nevertheless, we did not observed any differences of serum level of MMP-9, which confirm that recurrent wheezing and pathogens infection are not the precipitating factors for airway remodelling. Further studies are needed in this issue.

Since most cases of asthma begin during the first years of life, identification of young children at high risk of developing the disease is an important public health priority. Castro-Rodriguez et al<sup>28</sup> concluded for the prediction of asthma: a stringent index included frequent wheezing during the first 3 year of life and either one major risk factor (parental history of asthma or eczema) or two of three minor risk factors (eosinophilia, wheezing without colds, and allergic rhinitis); a loose index required any wheezing during the first 3 year of life plus the same combination of risk factors described previously. And their study also found that 59% of children with a positive loose index and 76% of those with a positive stringent index had active asthma in at least one survey during the school years. Asthma is a chronic inflammatory disease of the airway, in which IgE plays a pivotal role by involving in type I allergy. Elevated serum IgE are is associated with asthma and wheezing during childhood, and associated with the severity of asthma, further correlates with airway remodelling.<sup>29,30</sup> To understand the relationship between children with asthmatic risk factors and airway remodelling-associated mediators, we divided children into high risk group and low risk group, or high IgE group and normal IgE group,

then analysed the levels of MMP-9. However, there were no statistically significant differences among above-mentioned groups, suggesting there is no relationship between asthmatic risk factors and airway remodelling-associated mediators.

In conclusion, we demonstrated much higher levels of serum airway remodelling-associated factor MMP-9 in young wheezy children. However, recurrent wheezing, RSV infection, atypical pathogenic infection, IgE rising or asthma high risk factors had no effect on the serum level of MMP-9. Intimate follow-up needs to be carried out for wheezy children, especially for children with higher MMP-9.

## Acknowledgements

We sincerely thank Yufeng Liang for his excellent statistical assistance. We are grateful respiratory staff for collecting samples of blood and nasopharyngeal aspirates.

Contributors: All authors contributed to design, analysis, interpretation and writing of the manuscript.

Funding: This work was supported by grants from the Zhejiang Province Natural Science Foundation of China (LQ13H100002), the Zhejiang Medicine & Health Research Fund (No. 2013KYB152), and Scientific Research Fund of Zhejiang Provincial Education Department (Y201328580).

## Declaration of Interest

None

## References

- Jenkins HA, Cool C, Szeffler SJ, Covar R, Brugman S, Gelfand EW, et al. Histopathology of severe childhood asthma: a case series. *Chest* 2003;124:32-41.
- Bush A. How early do airway inflammation and remodeling occur? *Allergol Int* 2008;57:11-9.
- Pohunek P, Warner JO, Turzíkóvá J, Kudmann J, Roche WR. Markers of eosinophilic inflammation and tissue re-modelling in children before clinically diagnosed bronchial asthma. *Pediatr Allergy Immunol* 2005;16:43-51.
- Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999;104:356-63.
- Castro-Rodriguez JA, Garcia-Marcos L. Wheezing and Asthma in childhood: an epidemiology approach. *Allergol Immunopathol (Madr)* 2008;36:280-90.
- Martinez FD. Development of wheezing disorders and asthma in preschool children. *Pediatrics* 2002;109:362-7.
- Fedorov LA, Wilson SJ, Davies DE, Holgate ST. Epithelial stress and structural remodelling in childhood asthma. *Thorax* 2005;60:389-94.
- Chetta A, Zanini A, Foresi A, D'Ippolito R, Tipa A, Castagnaro A, et al. Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma. *Clin Exp Allergy* 2005;35:1437-42.
- Avdalovic MV, Putney LF, Schelegle ES, Miller L, Usachenko JL, Tyler NK, et al. Vascular remodeling is airway generation-specific in a primate model of chronic asthma. *Am J Respir Crit Care Med* 2006;174:1069-76.
- Zou H, Nie XH, Zhang Y, Hu M, Zhang YA. Effect of basic fibroblast growth factor on the proliferation, migration and phenotypic modulation of airway smooth muscle cells. *Chin Med J (Engl)* 2008;121:424-9.
- Moynihan BJ, Tolloczko B, El Bassam S, Ferraro P, Michoud MC, Martin JG, et al. IFN-gamma, IL-4 and IL-13 modulate responsiveness of human airway smooth muscle cells to IL-13. *Respir Res* 2008;9:84.
- Borowski A, Kuepper M, Horn U, Knüpfer U, Zissel G, Höhne K, et al. Interleukin-13 acts as an apoptotic effector on lung epithelial cells and induces pro-fibrotic gene expression in lung fibroblasts. *Clin Exp Allergy* 2008;38:619-28.
- O'Byrne P, Bateman ED, Bousquet J, Clark T, Paggiario P, Ohta K, et al. Global Strategy for Asthma Management and Prevention 2006. Available from URL: <http://www.ginasthma.org>.
- Gern JE. Rhinovirus and the initiation of asthma. *Curr Opin Allergy Clin Immunol* 2009;9:73-8.
- Redington AE, Howarth PH. Airway wall remodelling in asthma. *Thorax*. 1997;52:310-2.
- Bergeron C, Al-Ramli W, Hamid Q. Remodeling in asthma. *Proc Am Thorac Soc* 2009;6:301-5.
- Bossé Y, Rola-Pleszczynski M. Controversy surrounding the increased expression of TGF beta 1 in asthma. *Respir Res* 2007;24:66.
- Doherty T, Broide D. Cytokines and growth factors in airway remodeling in asthma. *Curr Opin Immunol* 2007;19:676-80.
- Ohbayashi H, Shimokata K. Matrix metalloproteinase-9 and airway remodeling in asthma. *Curr Drug Targets Inflamm Allergy* 2005;4:177-81.
- Matsumoto H, Niimi A, Takemura M, Ueda T, Minakuchi M, Tabuena R, et al. Relationship of airway wall thickening to an imbalance between matrix metalloproteinase-9 and its inhibitor in asthma. *Thorax* 2005;60:277.
- Sagiani S, Malmström K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. *Am J Respir Crit Care Med* 2005;171:722-7.
- Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev* 2010;23:74-98.
- Daian CM, Wolff AH, Bielory L. The role of atypical organisms in asthma. *Allergy Asthma Proc* 2000;21:107-11.
- Johnston SL, Martin RJ. Chlamydia pneumoniae and Mycoplasma pneumoniae: a role in asthma pathogenesis? *Am J Respir Crit Care Med* 2005;172:1078-89.

25. Falsey AR, Formica MA, Hennessey PA, Criddle MM, Sullender WM, Walsh EE. Detection of respiratory syncytial virus in adults with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;173:639-43.
26. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med* 2005;352:1749-59.
27. Bakshi CS, Malik M, Carrico PM, Sellati TJ. T-bet deficiency facilitates airway colonization by *Mycoplasma pulmonis* in a murine model of asthma. *J Immunol* 2006;177:1786-95.
28. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. *Am J Respir Crit Care Med* 2000;162:1403-6.
29. Sherrill DL, Stein R, Halonen M, Holberg CJ, Wright A, Martinez FD. Total serum IgE and its association with asthma symptoms and allergic sensitization among children. *J Allergy Clin Immunol* 1999;104:28-36.
30. Wickman M, Lilja G, Soderstrom L, van Hage-Hamsten M, Ahlstedt S. Quantitative analysis of IgE antibodies to food and inhalant allergens in 4-year-old children reflects their likelihood of allergic disease. *Allergy* 2005;60:650-7.