U-EPX levels and wheezing in infants and young children with and without RSV bronchiolitis

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Summary
An association between severe infant bronchiolitis due to respiratory syncytial virus (RSV) and subsequent wheezing is well documented. High levels of urinary eosinophil protein X (U-EPX) have been related to active disease in asthmatic children. The aim of this study was to analyse whether RSV bronchiolitis leads to an increase in U-EPX levels and whether wheezing is more common in children with high U-EPX values.

Seventeen infants requiring in-ward care for verified RSV lower respiratory tract infection were followed and compared with age-matched controls. A reference group without a history of RSV bronchiolitis was also included.

At inclusion at mean age 3.3 months and at follow-up at mean age 32.9 months, U-EPX levels were comparable in the RSV group. However, at follow-up at mean age 6.4 months, the RSV group had significantly increased levels of U-EPX compared with inclusion (median 167.8; range 46.2–470.7 vs. 122.8; 43.7–266.0 \(\mu\text{g/mmol creatinine}; P=0.023\)) and also significantly increased compared with the 6-month-old controls (167.8 vs. 93.0; 19.0–204.0 \(\mu\text{g/mmol creatinine}; P=0.0095\)). RSV infected subjects that experienced wheezing had significantly higher U-EPX values both at inclusion and at age 32.9 months than those who did not. Also, in the reference group (mean age 18.4 months), the children who had wheezed during the preceding year had higher U-EPX levels than those who had not wheezed.

In conclusion, RSV bronchiolitis severe enough to require in-ward care produces a significant, but transient increase in U-EPX. Furthermore, a high U-EPX at baseline appears to be associated with an increased risk of future wheezing.

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KEYWORDS
Asthma; Bronchiolitis; EPX; Infant; Respiratory syncytial virus; Wheezing

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**Introduction**

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infections (LRTI) among infants. Wheezing following RSV LRTI is a well-documented phenomenon.\(^1\) The Tucson study found that obstructive respiratory symptoms following RSV in early childhood tend to diminish gradually with age until, by the age of 13 years, they no longer show any significant increase in prevalence.\(^2\) Even so, a tendency towards reversible obstruction was documented.\(^2\) The severity of the original disease and the time of disease onset might influence the long-term effects, as Sigurs et al. noted a remaining increase in the risk of obstructive symptoms at 13 years of age in patients who had been admitted to hospital for severe RSV bronchiolitis as infants.\(^3\)

Th2 lymphocytes, mast cells and eosinophil granulocytes are crucial components of the asthmatic inflammation. Upon activation, eosinophil granulocytes degranulate and release highly reactive proteins, including Major Basic Protein (MBP), Eosinophil Cationic Protein (ECP) and Eosinophil Protein X (EPX). ECP has been used as a marker of disease and eosinophil activity in asthma, as well as a method for assessing the response to glucocorticoid treatment.\(^4\)\(^–\)\(^6\) ECP can be measured in serum or nasopharyngeal secretions.\(^7\) Urinary (U) EPX is an alternative non-invasive eosinophil marker in children with asthmatic symptoms.\(^5\)\(^–\)\(^6\)\(^,\)\(^8\)

The aim of this study was to investigate whether RSV bronchiolitis leads to an increase in U-EPX levels compared with healthy, age-matched controls. We also wanted to see whether there was a relationship between the levels of U-EPX and the presence of post-infection wheezing.

**Subjects and methods**

### Subjects

Seventeen children requiring hospitalization for RSV bronchiolitis at the Department of Pediatrics, Landspitali University Hospital, Reykjavik, Iceland, were included. RSV infection was diagnosed by direct (IMAGEN, DakoCytomation) and indirect (Biotrin, respiratory viral panel) immunofluorescent staining of nasopharyngeal aspirates and viral culture performed at the Department of Virology, Landspitali University Hospital, Reykjavik. Samples of nasopharyngeal secretions were obtained by passing a polyethylene catheter into the nasopharynx, followed by the application of gentle suction and rinsing into collection traps containing 2.5 mL of saline.

The age and gender characteristics of the infants are shown in Table 1. Median weight at birth was 3400 g (range 1665–4600 g) and the median length was 50 cm (range 43–55 cm). A follow-up of the children was made after 3 and 30 months. At the follow-up after 30 months, parents were asked to fill out a semi-structured questionnaire relating to wheezing during the preceding 12-month period. If wheezing had occurred, the number of times was noted. Parents were also questioned about the presence of atopic disease (atopic dermatitis, asthma, allergic rhinoconjunctivitis or food allergy) among themselves and siblings (Table 1).

Two separate groups of age-matched, healthy controls of 3 and 6 months (n = 12 and 13, respectively) were randomly recruited from the Centre for Child Health Services in Reykjavik and investigated correspondingly (Table 1). In addition, a reference group of 25 children without a history of RSV bronchiolitis, mean age 18.4 months, was randomly recruited from the Centre for Child Health Services.

| Table 1: Age, gender characteristics, and presence of atopic disease among parents and siblings in the study groups. |
|---|---|---|---|---|
| | Inclusion Age six months | Age 33 months | Non-bronchiolitis reference group |
| | RSV group | Controls | RSV group | Controls |
| N | 17 | 12 | 17 | 13 | 17 | 25 |
| Mean age, months | 3.3 | 3.3 | 6.4 | 6 | 32.9 | 18.4 |
| Male, % | 53 | 67 | 53 | 46 | 53 | 68 |
| Female, % | 47 | 33 | 47 | 54 | 47 | 32 |
| Atopic disease in parents and siblings, % | 82 | 91 | 82 | 82 | 82 | 80 |
Health Services (Table 1). Also, these parents were asked to fill out a semi-structured questionnaire relating to wheezing during the preceding 12-month period. If wheezing had occurred, the number of times was noted. Parents were also questioned about the presence of atopic disease among themselves and siblings.

U-EPX measurements and white blood cell counts

U-EPX levels were determined in all children at all time points. In the RSV group the first U-EPX samples were obtained during the RSV infection within 24 h from admission. Urine samples were collected and stored at $-20$ °C until measurements were performed. Due to the diurnal variation in U-EPX, samples were taken between 9 and 12 a.m. U-EPX levels were measured with a radioimmunoassay with a detection limit of approximately 3 μg/L (Pharmacia Diagnostics, Uppsala, Sweden) and related to urinary creatinine concentration (Jaffé’s reaction, HiCo Creatinine, Boehringer Mannheim GmbH, Germany) to compensate for urinary water dilution. Venous blood was obtained, and analysed the same day. The white blood cell and differential counts were analysed using Cell-Dyn 4000 (USA System, Santa Clara, CA, USA).

Skin prick test

Thirty months after inclusion, at mean age 32.9 months, all children in the RSV group were examined with a skin prick test. The SPT included the following allergens: *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, grass pollen, dog, cat, cow’s milk (raw and boiled), egg white, peanuts, wheat, fish (cod) and soy. The extracts of the ALK Soluprick® kit (ALK, Hørsholm, Denmark) were used. Skin prick test was also performed in the 18-month-old children recruited from the Centre for Child Health Services.

Statistical analysis

Statistical analyses were conducted using the JMP® software (SAS Institute Inc., Cary, NC, USA). Since most of the U-EPX samples did not fit a normal distribution, non-parametric tests were used. To compare U-EPX samples among the RSV-infected subjects with those of the age-matched controls and among wheezers and non-wheezers, Wilcoxon’s two-sample test was used. For an evaluation of the U-EPX samples among RSV-infected subjects at different times, Wilcoxon’s matched-pairs test was performed. The statistical data are presented as the median and range. A $P$-value $<0.05$ was regarded as statistically significant.

Ethics

The project was approved by the Icelandic National Ethics Committee. The participating parents signed statements of informed consent.

Results

U-EPX in the RSV group compared with controls

At the time of inclusion, U-EPX levels were comparable in the RSV group and the control group (Fig. 1). At the follow-up after 3 months, at mean age 6.4 months, the RSV group exhibited significantly higher U-EPX values in relation to the inclusion values (median 167.8; range 46.2–470.7 vs. 122.8; 43.7–266.0 μg/mmol creatinine; $P = 0.023$) (Fig. 1). U-EPX in the RSV group was also significantly higher compared with age-matched 6-month-old controls (167.8; 46.2–470.7 vs. 93.0; 19.0–204.0 μg/mmol creatinine; $P = 0.0095$) (Fig. 1). There were no significant differences in U-EPX between the two 3- and 6-month-old control groups. Thirty months after inclusion, at mean age 32.9 months, the U-EPX levels in the RSV group were again comparable to the values at inclusion (NS).

Wheezing

At the follow-up visit 30 months after the bronchiolitis wheezing during the preceding 12 months was evaluated in the RSV group. Sixty-five per cent
(11/17) had experienced wheezing. Median value (range) for the number of episodes of wheezing (for children who had experienced wheezing) was 4 (1–12).

In the group of 18-month-old children without a history of RSV bronchiolitis 48% (12/25) had experienced wheezing during the preceding 12 months, median value for number of wheezing episodes was 1 (1–5).

**U-EPX and wheezing**

In the RSV group, a relationship was seen between wheezing and U-EPX values at inclusion and at the follow-up after 30 months. Subjects who had experienced wheezing during the 12 months before the follow-up had significantly higher U-EPX values than non-wheezers, both at inclusion (165.7; 43.7–266.0 vs. 76.9; 58.6–137.4 µg/mmol creatinine; \( P = 0.039 \)) (Fig. 2) and 30 months later (141.9; 63.0–264.0 vs. 59.9; 55.3–262.5 µg/mmol creatinine; \( P = 0.050 \)). However, at the follow-up 3 months after inclusion, there was no difference in U-EPX levels between future wheezers and non-wheezers (167.8; 96.5–470.7 vs. 168.6; 46.2–208.3 µg/mmol creatinine; \( P = 0.51 \)).

Also, the children in the group of 18-month-old children without a history of RSV bronchiolitis who had wheezed during the preceding year had higher U-EPX levels than those who had not wheezed (138.5; 87.0–340.0 vs. 103.0; 19.0–197.0 µg/mmol creatinine; \( P = 0.030 \)).

**Skin prick test**

No children in the RSV group had a positive SPT at the last visit, nor were any positive SPTs found in the group of 18-month-old children from the Centre for Child Health Services.

**White blood cell count**

Three months after RSV infection eosinophils had a median value (range) of 0.4 \((0.1–0.7) \times 10^9/L\) vs. 0.25 \((0.1–0.8) \times 10^9/L\) at inclusion, NS. However, this 3-month follow-up value was significantly higher than the 30-month follow-up value, 0.4 \((0.1–0.7) \times 10^9/L\) vs. 0.2 \((0–0.7) \times 10^9/L\), \( P = 0.049 \). There were no statistically significant differences in total white blood cell counts or monocyte counts.

**Discussion**

Compared with the inclusion values and compared with the controls, a significant rise in U-EPX was seen in the RSV group at the follow-up 3 months after inclusion. This finding probably reflects an ongoing eosinophil activation as a result of the RSV infection. However, the increase was transient and 30 months later the values were comparable to those at inclusion. Thus, the RSV infection does not seem to have induced a persistent eosinophil activation and the clinical significance of the transient increase in U-EPX levels is therefore uncertain. Regarding the eosinophil counts in peripheral blood, the difference was not statistically significant between the values at inclusion and at the follow-up 3 months later, but significantly higher at that follow-up compared with the follow-up at age 32.9 months. Largely, the changes in U-EPX and eosinophils followed the same pattern.

Many studies have reported an association between RSV disease and childhood wheezing.\(^1\)–\(^3\) In this limited material, at the follow-up 30 months after inclusion 65% of the children in the RSV group had experienced wheezing during the previous 12 months. This might, at least partly, represent an
airway hyperresponsiveness induced by the RSV infection.

We compared the U-EPX values in the children who subsequently wheezed in the RSV group with those that did not. We found significantly higher U-EPX inclusion values in the wheezing children. This relationship was not apparent at the U-EPX peak 3 months after RSV infection, but was again significant at the follow-up after 30 months. This is consistent with the early results of Koller et al. and by Reijonen et al., which indicated a possible predictive role for S-ECP levels in the development of persistent wheezing. In this context it is also of interest to recollect that Ehlenfield et al. found that eosinophilia at the time of RSV bronchiolitis generally predicted the development of wheezing persisting into later childhood. Also, Øymar et al. found a normal or high eosinophil count in infants with severe viral wheeze to predict subsequent recurrent wheezing, while U-EPX was not a predictive factor for recurrent wheezing in their study. U-EPX appears to have a baseline level, which is individual for each child, as the values at inclusion and at the follow-up 30 months later were comparable. The wheezing children had significantly higher EPX at inclusion as well as at the follow-up 30 months later compared with those who did not wheeze. Thus, in this study wheezing seemed to correlate to baseline U-EPX levels. This may explain why an association was not seen between U-EPX values and wheezing 3 months after RSV infection when the RSV infection probably had induced a more generalized eosinophil activation. The finding of an association between baseline U-EPX and wheezing may be consistent with a hypothesis that RSV infection induces wheezing in the genetically predisposed infant. Since it seems to be an increased baseline U-EPX that shows relationship to wheezing, perhaps that level is of greater clinical interest than the transient increase following infection.

The transient elevation of U-EPX at the follow-up after 3 months seemed to be related to the RSV infection since it was not seen in the group of age-matched controls. In contrast, higher U-EPX levels in children with wheezing were seen both in the RSV group and in the randomly recruited group of children aged 18 months. In the RSV group significantly higher U-EPX values were seen already at inclusion. Therefore, high U-EPX values may indicate children prone to wheeze, possible identifying future asthmatic children. This could be interpreted to indicate that the relation between high U-EPX values and inclination to wheezing was independent of any risk of wheezing caused by the RSV infection.

Although atopic diseases were common among parents and siblings in this study, no child in either the RSV group or in the group of randomly recruited 18-month-old children exhibited a positive SPT. We were surprised at this finding and we do not know if this was due to chance or if positive SPTs just had not yet developed at this young age. Whether RSV bronchiolitis contributes to the subsequent development of allergic sensitization is the subject of debate. The Tucson study did not reveal any correlation between LRTI and positive SPTs, or differences in the mean total IgE between LRTI subgroups. Stein et al. concluded that RSV infection was not correlated to the development of atopic asthma or allergic sensitization. This can be compared with the results of the Sigurs study, which indicated that severe RSV infection in infancy was an independent risk factor for the development of asthma, as well as allergic sensitization to aeroallergens at the age of 13 years. The subjects in the Tucson and Sigurs studies differed substantially in terms of age at disease onset and severity of illness. The children in the Sigurs study had severe bronchiolitis while those in the Tucson study were less severely ill. In the present study the children were hospitalized but not seriously ill. It is possible that the severity of the initial disease may be important for the development of asthma and/or allergy after RSV infection. Furthermore, the age at disease onset could influence disease outcome. The children in the Sigurs long-term study were younger (mean age 4 months) than the subjects in the Tucson study ( < 3 years). Mean age in the present study was 3.3 months.

A limitation of the present study is the relatively small number of cases and controls. Furthermore, ideally the same controls should have been followed from the beginning of the study instead of having two cross-sectional control groups. A longitudinally followed control group could also have been used as control group at the follow-up of the RSV group after 30 months. Although we were able to perform comparisons within the group of randomly recruited children aged 18 months, those children could not be used as a proper control group for the 30-month follow-up of the RSV group.

In conclusion, RSV bronchiolitis severe enough to require in-ward care produces a significant yet transient increase in U-EPX. Furthermore, subjects in the RSV group who had experienced wheezing during the year preceding the follow-up 30 months after the bronchiolitis had significantly higher U-EPX values both at inclusion and at that follow-up. Thus, a high U-EPX at baseline appears to increase the risk of future wheezing.
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