Research Article

Identification of biomarkers for disease severity in nasopharyngeal secretions of infants with upper or lower respiratory tract viral infections

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Abstract

Lower respiratory tract infections (LRTIs) produced by viruses are the most frequent cause of morbidity and mortality in children younger than 5 years of age. The immune response triggered by viral infection can induce a strong inflammation in the airways and cytokines could be considered as biomarkers for disease severity as these molecules modulate the inflammatory response that defines the outcome of patients. Aiming to predict the severity of disease during respiratory tract infections, we conducted a 1-year follow-up observational study in infants who presented upper or lower respiratory tract infections caused by seasonal respiratory viruses. At the time of enrollment, nasopharyngeal swabs (NPS) were obtained from infants to measure mRNA expression and protein levels of IL-3, IL-8, IL-33, and thymic stromal lymphopoietin. While all cytokines significantly increased their protein levels in infants with upper and lower respiratory tract infections as compared to control infants, IL-33 and IL-8 showed a significant increase in respiratory syncytial virus (RSV)-infected patients with LRTI as compared to patients with upper respiratory tract infection. We also found higher viral loads of RSV-positive samples with a greater IL-8 response at the beginning of the symptoms. Data obtained in this study suggest that both IL-8 and IL-33 could be used as biomarkers for clinical severity for infants suffering from LRTIs caused by the RSV.

Keywords: cytokines, respiratory tract infection, RSV

Abbreviations: ELISA: enzyme-linked immunosorbent assay; LRTI: lower respiratory tract infection; NPS: nasopharyngeal swabs; RSV: respiratory syncytial virus; TSLP: thymic stromal lymphopoietin; URTI: upper respiratory tract infection

Introduction

Lower respiratory tract infections (LRTIs) in children younger than 5 years of age are considered a global public health problem that accounts for approximately 704 000 deaths annually and 60 million disability-adjusted life years worldwide [1]. Among them, bronchiolitis and pneumonia are the most common causes of children hospitalization, especially infants under 1 year of age [2]. LRTIs in children are also associated with long-term morbidity. There is abundant and strong evidence showing that infant hospitalized due to bronchiolitis can develop recurrent episodes of wheezing and asthma in the following years [3–6], which cause an overwhelming expense in health resources. In addition, up to a 13% of hospitalized children who suffered from at least one episode of pneumonia before adolescence will undergo a permanent reduction of lung capacity [7, 8]. These patients have shown an increased risk for chronic obstructive pulmonary disease in following years [9, 10]. Therefore, LRTI in children should be considered as a risk factor for long-term respiratory sequelae.

In children, LRTIs are mainly due to viruses, such as the respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, rhinovirus, adenovirus, and metapneumovirus [11, 12]. Although there are some exceptions, there are no specific therapies nor vaccines for respiratory infections caused...
by viruses; therefore, clinical efforts are focused on general support to reduce hospitalization rate [13]. The increasing demand of medical attention during winter outbreaks caused by respiratory viruses requires the development of improved strategies to reduce the viral burden. Early diagnosis of LRTIs could help physicians to consider a more accurate management of children suffering from viral infections. Besides, biomarkers to predict the severity and prognosis of LRTIs will contribute to decreasing the current high hospitalization requirements, and to identifying high-risk patients requiring a close clinical follow-up to avoid further health complications.

The exposure of the upper airway epithelium to a viral agent stimulates the production of several inflammatory mediators and the infiltration of immune cells into the airways [14]. This primary immune response against the virus might become exacerbated and sometimes lead to airway hyperresponsiveness, as well as a permanent dysregulation and susceptibility to developing recurrent wheezing and asthma [15–17].

Recent studies have shown that some cytokines produced in the respiratory tract of infants undergoing viral LRTIs may be useful to anticipate the severity of the acute illness and recurrent wheezing in the years following the infection [18–19]. Whether levels of these cytokines in nasopharyngeal secretions are also present in children with upper respiratory tract infections (URTIs) or whether they correlate with disease severity—or other long-term outcomes—has not been demonstrated yet. In the present study we aimed at identifying nasopharyngeal cytokines that could precisely predict disease severity in infants suffering from LRTIs. Here, we performed analyses to compare cytokines levels between infants suffering from LRTI and URTI caused by various viral infections. Our results allow us to propose that a panel of pro-inflammatory biomarkers might work as a good predicting tool for the acute phase severity of LRTIs and could guide the clinicians for support therapy and eventually help to distinguishing high-risk long-term morbidity infants.

**Materials and methods**

**Experimental design and study subjects**

This study was designed to evaluate the nasopharyngeal cytokine response during acute respiratory infections among infants and their clinical course for a 12-month follow-up period. Infants were enrolled exclusively for this purpose at the Hospital of the Red de Salud UC Christus from either, outpatient clinic, emergency room, hospitalized or hospitalized in intensive unit care. All participants were healthy infants consulting for acute respiratory symptoms that were categorized as suffering from an URTI or with lower airway respiratory infection (LRTI). Patients with URTI (n = 42) were mostly selected from outpatient clinic. Patients with LRTI (n = 43) were mostly selected from emergency room. During the same period, healthy infants (n = 11) who underwent a routine pediatric checkup with no respiratory symptoms were recruited as controls. For this study, all infants (patients and controls) were enrolled between May and September 2015 (fall and winter season in Chile, respectively). After children were discharged from the hospital, monitoring of all infants was conducted by telephone calls monthly for a 12-month period to complete a questionnaire of symptoms and illness events. Information obtained by telephone was double-checked with online medical records available of all subjects. The diagnosis performed by physician was made independently of the study team.

The inclusion criteria were: (i) age range: 3–24 months old, (ii) documented history of respiratory illness < 7 days, (iii) presence of clinical symptoms and signs compatible with upper or lower respiratory tract infection (e.g. rhinorrhea, nasal congestion, tachypnea, increased work of breathing, subcostal retractions, wheezing, crackles). The exclusion criteria were: (i) history of premature birth < 37 weeks, (ii) underlying disease (asthma, heart disease, cystic fibrosis, immunodeficiency, etc.), (iii) prior history of LRTI, (iv) previous chronic use of inhaled corticosteroids, and (v) patients who require immediate advance airway management or non-invasive ventilation. To avoid bias resulting from the cytokine response in severe patients, we decided to exclude them from the beginning of the study.

**Clinical definitions**

After the enrollment, patients were categorized as having either an upper or lower respiratory tract infection by the attending physician. When eligible for the study, a clinical member of the study was contacted, and the clinical definition was confirmed. URTI included at least one of the following conditions: rhinitis, pharyngitis, or acute otitis media. LRTI included either bronchiolitis or pneumonia. Family history and environmental exposure were registered as follows: (a) atopy was defined as having one or both parents with allergic rhinitis or atopic asthma diagnosed by a physician; (b) tobacco exposure was considered when any of the parents declared tobacco use in any period during the previous 6 months; (c) parents and caretakers were asked to report any respiratory infection, persistent respiratory symptoms in addition to wheezing episodes during follow-up; (d) all medication used during this period of time was also recorded and confirmed with online medical records. Recurrent wheezing was defined as having three episodes of wheezing diagnosed by a physician (unrelated to the study). Asthma was defined by a clinical diagnosis performed by a physician (unrelated to the study).

**Clinical samples**

NPS were collected using sterile flocked swabs, which were inserted into both nostrils to reach the posterior nasopharyngeal area in all participants. The swabs were transported in 1 ml of universal viral transport medium (BD, USA) and were processed and analyzed simultaneously by the Infectious Diseases and Molecular Virology Laboratory and by the Microbial Pathogenesis Laboratory at the Pontificia Universidad Católica de Chile. The Infectious Diseases and Molecular Virology Laboratory performed the virus identification using Molecular Panel PCR kit Luminex RPP Respiratory pathogens: A Influenza virus and subtyping H1, H3; B Influenza virus; Parainfluenza 1, 2, 3, 4; RSV (A and B), Adenovirus, Coronavirus NL63, 229E, OC43 and HKU1, Human Metapneumovirus, Rhinovirus/Enterovirus; Bocavirus, Chlamydothila pneumoniae, Legionella pneumophila, and Mycoplasma pneumoniae. The Microbial Pathogenesis Laboratory at the School of Biological Sciences at the Pontificia Universidad Católica de Chile performed the detection of protein levels and mRNA expression. Protein levels of cytokines were evaluated by commercial
enzyme-linked immunosorbent assay (ELISA) and a fraction of the sample was separated for extraction of RNA to detect cytokine transcript levels by RT-qPCR.

**Protein extract from nasopharyngeal samples**

A volume of 500 μl of sample was equally divided by adding 250 μl into two 1.5-ml Eppendorf tubes. Subsequently, both samples were centrifuged at 500 g for 10 min at 4°C and the supernatant from each sample was separated and stored at 4°C until its use. The protein extraction was performed according to a protocol previously described by González et al. [20]. Briefly, the pellet from one of the samples was used for protein extraction and the other sample was used for total RNA extraction. The pellet from the first sample was treated with 100 μl of RIPA Buffer (Pierce, Thermo Scientific #89900, USA) plus Protease inhibitor cocktail tablets (Roche #11836145001, USA), for 15 min at 4°C. The samples were mixed by vortex every 5 min. Then, samples were centrifuged at 500 g for 10 min at 4°C and the supernatant was collected and mixed with the supernatant obtained with the first centrifugation. Samples were tested by ELISA.

**Measurement of cytokines by ELISA in nasopharyngeal samples**

Cytokine levels in NPS were determined by commercial ELISA (PeproTech, USA). The following cytokines were evaluated: IL-3 (#900-K13), IL-8 (#900-M18), IL-33 (#900-M398), and thymic stromal lymphopoietin (TSLP) (#900-K334). The protocol was performed according to manufacturer’s instructions. Briefly, treated nasopharyngeal swabs were incubated in an ELISA plate previously activated with specific capture antibody anti-IL-3, anti-IL-8, anti-IL-33, and anti-TSLP provided by the kit. Then, the plate was blocked and incubated with detection antibodies. The limit of detection for the cytokines measured ranged from 8 to 4000 pg/ml.

**RNA isolation from nasopharyngeal samples**

RNA from nasopharyngeal swabs was purified using TRIzol LS reagent (Invitrogen, #10296010, USA), following the manufacturer’s instructions. Total RNA was isolated using the pellet obtained from 250 μl of clinical samples and 1 ml of TRIzol LS reagent. Samples were stored at –80°C.

**Quantitative real-time RT-PCR assay**

The relative expression of the selected cytokines was obtained from 2 μg of total RNA in clinical samples of infected patients by comparing their cycle threshold (Ct) with samples from healthy patients (control). The IL-3, IL-8, IL-33, and TSLP cytokines were quantified using the commercial kit TaqMan(R) Gene Expression Assays (Applied Biosystems): human IL-33 (#Hs00369211_m1), human IL-3 (#Hs00174117_m1), human IL-8 (#Hs00174103_m1), and human TSLP (#Hs01572933_m1). The normalization was performed using the TaqMan probe of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #Hs99999905_m1, Applied Biosystems) as reference gene, using the following formula: ΔCt = ΔCt(target gene) – ΔCt(GAPDH). The fold-change for the samples was defined as the relative expression compared with the corresponding control and was calculated as follows: 2^(-ΔΔCt), where ΔΔCt = ΔCt(patients) – ΔCt(healthy). Reactions were carried out using the One-Step RNA-to-Ct master mix reagent (Applied Biosystems, #4392938), and the program consisted of 15 min at 48°C, followed by 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. All samples were analyzed at least by duplicate in an Applied Biosystems Step-One Plus thermocycler (Thermo Fisher Scientific, USA).

**Viral load determination**

RSV-positive samples with high (n = 21) and low (n = 9) levels of mRNA (high: >10 of fold increase and low: <10 of fold increase) or protein (high: >300 pg/ml and low: <100 pg/ml) of IL-8 were used to determine viral loads. From 1.5 μg of extracted RNA, cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, #17889). The real-time PCR was performed using 200 ng of cDNA to quantify the RSV N gene using a standard curve with known concentrations (2.5 × 10^9 to 2.5 × 10^5 copies/μl) of the pET15b plasmid containing the N-RSV gene. For the PCR reaction, 2× SYBR green master mix (Bio-Rad, #172-5270), 10 μM of forward, and reverse primers for the N gene were used. The program consisted of 30 s at 95°C, 40 cycles of 5 s at 95°C, and 30 s at 60°C. A melting curve was incorporated following 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C. All samples were analyzed at least by duplicate in an Applied Biosystems Step-One Plus thermocycler (Thermo Fisher Scientific, USA). A positive result was defined when the Ct value of gen N was ≤35. This information was not released to the clinicians; therefore, it did not influence clinical decisions. The Ct values obtained were interpolated to the curve and analyzed in Graphpad 9.0 software. Copies/μl results were normalized for each 200 ng of cDNA.

**Statistical analyses**

Continuous data were presented as median and interquartile range. Categorical data were expressed by percentages. Bivariate analysis included Student-t for independent samples and ANOVA test followed by GT2 de Hochberg or Games-Howell post hoc tests depending on the results of Bartlett test of homogeneity of variances. In addition, non-parametric Mann–Whitney, Kruskal–Wallis, χ², and Fisher exact tests were applied. Multivariate analysis using logistic regression after multiple imputation by chained equation was applied. We built 20 imputed data sets after log transformation of continuous variables. Because the large number of variables relative to sample size, variables were selected by Bayesian Information Criteria using strategy similar to Chen 2013 [21]. Statistical analysis was performed using GraphPad Prism 6 software, IBM SPSS 17.0, and R packages MASS, mice, and miceadvs. P-value < 0.05 was taken as statistically significant.

**Results**

**Clinical variables**

A total of 98 infants were enrolled during two consecutive years for this study as follows: URTI = 42, LRTI = 45, and controls = 11. The study was explained in detail to the parents of all the subjects and they signed an informed consent. Out of the 45 patients with LRTI, 4 presented pneumonia and 41 presented bronchiolitis. Twenty-six infants were admitted to the hospital due to respiratory distress and two for having apnea as a first symptom. All 28 admitted patients stayed for a median of 4 days (range: 1–15 days) and were able to drop off oxygen 1 day previous discharge. No
further complications were reported during 1 month after discharge from hospital. Out of the 42 URTI patients, only one progressed to develop bronchiolitis and was admitted to the hospital due to respiratory distress and stayed for 6 days. Although patients that presented with URTI and LRTI were more frequently exposed to kerosene than controls, other clinical characteristics were similar between groups (Table 1). Virus identification after diagnosis resulted as follow: RSV-positive \( n = 41 \), hMPV-positive \( n = 2 \), ADV-positive \( n = 1 \), FLU-positive \( n = 3 \), and PIV-positive \( n = 3 \) and negative for all viruses \( n = 37 \). All control infants \( n = 11 \) tested negative for viruses. Detection of any virus was more common in patients who presented with LRTI than those who presented with URTI (Table 1).

Almost all the subjects were followed for a 12-month period by means of phone calls. However, five patients were lost at 6 months and 11 patients were lost up at 12 months. During the follow-up period there was no report of hospital admission. All control infants completed the 12 months of following up. There were no significant differences between symptoms, wheezing episodes, inhaled corticosteroid requirement, or presence of recurrent wheezing at 6 or 12 months of following up, except for common cold at 6 months that presented more frequently in patients with URTI. After 12 months of follow-up, asthma was more common in LRTI compared to when compared to URTI patients (Table 2).

### Table 1: Clinical characteristics of patients

| Clinical characteristic | Controls \( n = 11 \) | URTI \( n = 42 \) | LRTI \( n = 45 \) | Total \( n = 98 \) | \( P \)-value URTI vs LRTI (\( \chi^2 \) test) |
|-------------------------|------------------------|-----------------|-----------------|----------------|----------------------------------|
| Age (range) months      | 4.0 m (1–6.3)          | 8.0 m (0.3–19.9) | 5.0 m (0.3–20) | 52/46         | 0.012                            |
| Gender: male/female     | 5/6                    | 23/19           | 24/21           |               | ns                               |
| Tobacoo exposure        | 5 (45%)                | 17 (40%)        | 18 (40%)        | 40 (41%)      | ns                               |
| Parental asthma         | 1 (9%)                 | 12 (29%)        | 10 (22%)        | 23 (23%)      | ns                               |
| Kerosene exposure       | 2 (18%)                | 28 (67%)        | 24 (53%)        | 54 (55%)      | ns                               |
| Days of illness (>3)    | 0 (0%)                 | 26 (62%)        | 39 (87%)        | 65 (66%)      | <0.012                           |
| Virus identification    | 0 (0%)                 | 19 (45%)        | 33 (73%)        | 52 (53%)      | 0.010                            |
| RSV identification      | 0 (0%)                 | 12 (29%)        | 29 (64%)        | 41 (42%)      | 0.0001                           |

ns: non-significant.

### Table 2: Clinical outcomes

| Clinical outcomes       | Controls \( n = 11 \) | URTI \( n = 42 \) | LRTI \( n = 45 \) | Total \( n = 98 \) | \( P \)-value URTI vs LRTI (\( \chi^2 \) test) |
|-------------------------|------------------------|-----------------|-----------------|----------------|----------------------------------|
| Cough at 1 month        | 2 (18%)                | 33 (79%)       | 36 (80%)        | 71 (72%)      | ns                               |
| Cough at 6 months       | 1 (9%)                 | 13 (31%)       | 16 (36%)        | 30 (31%)      | ns                               |
| Common cold at 1 month  | 1 (9%)                 | 7 (17%)        | 3 (7%)          | 11 (11%)      | ns                               |
| Common cold at 6 months | 4 (36%)                | 27 (64%)       | 16 (36%)        | 56 (57%)      | 0.0074                           |
| Common cold at 12 months| 6 (55%)                | 31 (74%)       | 30 (67%)        | 79 (81%)      | ns                               |
| Wheezing at 6 months    | 2 (18%)                | 16 (38%)       | 16 (36%)        | 34 (35%)      | ns                               |
| Wheezing at 12 months   | 3 (27%)                | 18 (43%)       | 22 (49%)        | 43 (44%)      | ns                               |
| Recurrent wheezing at 6 months | 0 (0%) | 2 (5%) | 4 (9%) | 6 (6%) | ns |
| Recurrent wheezing at 12 months | 0 (0%) | 2 (5%) | 3 (7%) | 5 (5%) | ns |
| IC use at 6 months      | 0 (0%)                 | 7 (17%)        | 5 (11%)         | 12 (12%)      | ns                               |
| IC use at 12 months     | 0 (0%)                 | 5 (12%)        | 7 (16%)         | 12 (12%)      | ns                               |
| Asthma at 12 months     | 0 (0%)                 | 0 (0%)         | 4 (9%)          | 4 (4%)        | 0.047                            |

ns: non-significant.

### URTI and LRTI patients presented low cytokines mRNA expression in nasopharyngeal swabs

The mRNA levels of il-3, il-33, il-8, and tslp in URTI and LRTI patients were compared to controls, showing variable changes. IL-8 mRNA level showed a significant increase in patients with LRTI as compared to controls, while IL-3 and IL-33 mRNA expression showed no significant difference among the groups evaluated. TSLP mRNA expression presented a significant decrease in URTI and LRTI patients compared to controls (Fig. 1).

### Protein levels of cytokines in nasopharyngeal swabs varied according to the clinical diagnosis

Cytokine levels were measured in NPS from URTI and LRTI patients using ELISA technique. IL-3, IL-8, IL-33 showed a significant increase in both URTI and LRTI patients as compared to controls. TSLP showed a significant increase only in URTI. Remarkably, levels of IL-33 and IL-8 in URTI and LRTI had a 100-fold increase as compared to control subjects. The results also showed that IL-3 and TSLP levels were higher in patients with URTI than in patients with LRTI, while IL-8 levels were higher in LRTI patients as compared to subjects presenting an URTI (Fig. 2). Additional analyzes for all cytokines were performed in infants with LRTIs and those with more than 3 days of illness. Analyses consisted of comparing the response of subjects who showed cytokine levels below...
100 pg/ml (weak response) and those who had a cytokine response above 300 pg/ml, a stronger response. Data showed a difference in the response (strong 78% vs. weak 54%, χ² test P = 0.046; Table 3) of these participants, mainly in IL-33 levels, where there is a higher percentage of subjects with the strong response (IL-33 levels >300 pg/ml) versus weaker responses. Based on these observations, it can be suggested that higher concentrations of IL-33 associate with an increased response and disease severity for the patient. Finally, Table 4 shows the results with URTI and LRTI infants younger and older than 6 months, where it was observed that patients older to 6 months presented a strong response of the IL-33, IL-3, and TSLP levels in NPS, as compared to infants younger than 6 months of age (IL-33: 59% vs. 33%, χ² test P = 0.049; IL-8: 54% vs. 23%, χ² test P = 0.027; TSLP: 29% vs. 19%, χ² test P = 0.0006; Table 4).

High levels of IL-8 and IL-33 are detected in nasopharyngeal swabs of RSV patients with LRTI

Cytokine levels were evaluated from RSV-positive (n = 41) and RSV-negative (n = 43). IL-3, IL-8, and IL-33 showed a significant increase in their levels in RSV-positive patients as compared to controls (Fig. 3). IL-3 levels did not show significant differences between RSV-positive and RSV-negative patients, while IL-8 levels were significantly increased in RSV-positive patients as compared to RSV-negative patients. IL-33 levels were also significantly increased in RSV-positive, and RSV-negative patients as compared to healthy controls (Fig. 3) and also a significant increase of IL-33 is observed in RSV-positive patients as compared to RSV-negative patients. TSLP levels did not show a significant difference among RSV-positive patients and controls, but a significant increase is observed in RSV-negative patients as compared to controls. Importantly, comparison of cytokine levels in RSV-positive patients with URTI and LRTI showed that IL-8 and IL-33 was significantly increased in patients with LRTI and compared to URTI (Fig. 4).

Relationship between the production of cytokines at the beginning of the study and clinical outcome of patients in a follow-up year

Cytokine levels were analyzed to estimate the relative risk to develop a more severe clinical course during illness, or to develop a clinically relevant outcome during follow-up after primary illness. A multivariate regression logistic model built to evaluate a more severe clinical course using LRTI as outcome resulted in an increased risk for IL-8 (OR = 1.85; CI 1.11–3.09; P-value 0.02) and also an increased risk for virus identification (OR = 3.21; CI 1.10–9.34; P-value 0.03). In other words, a difference of 2.7 (Euler’s number) in IL-8 levels increase 1.85 the risk for developing LRTI and 3.2 the risk to identify a virus during the illness.
Higher levels of IL-8 associate with higher levels of viral load of RSV

Viral loads were determined from cDNA using a standard curve with known concentrations of a plasmid containing the N-RSV gene. This assay was performed in a total of 23 positive samples for RSV, in which 14 samples showed high levels of the chemokine IL-8 and nine samples showed low levels of IL-8 by qPCR (Fig. 1) and by ELISA (Figs 3 and 4). These results indicated that a higher viral load was present in samples with greater IL-8 response. These data indicate a significant difference between patients with higher IL-8 expression as compared to those with low expression of this cytokine (Fig. 5). Higher viral loads in the samples associated with an exacerbated IL-8 response in children with LRTI positive for RSV.

Table 3: High cytokines levels versus clinical characteristics and outcome

| Clinical characteristics and outcome | Patients | P-value ($\chi^2$ test) |
|-------------------------------------|---------|------------------------|
| Low IL-33 levels < 100 pg/ml ($n = 22$) | 12 (54%) | 22 (78%) | 0.046 |
| Days of illness (>3) | | | |
| Low IL-8 levels < 100 pg/ml ($n = 24$) | 20 (83%) | 14 (74%) | ns |
| Days of illness (>3) | | | |
| Low IL-3 levels < 50 pg/ml ($n = 40$) | 32 (80%) | 21 (70%) | ns |
| Days of illness (>3) | | | |
| Low TSLP levels < 30 pg/ml ($n = 32$) | 26 (81%) | 29 (69%) | ns |
| Days of illness (>3) | | | |

ns: non-significant.
The aim of this work was to evaluate whether a pro-inflammatory cytokines panel could detect disease severity in infants with respiratory tract infections. We observed that IL-3, IL-8, and IL-33, and TSLP were significantly increased in nasopharyngeal swabs of infants with respiratory tract infections as compared to controls. Furthermore, in this study we corroborated that IL-8 levels are significantly increased in children with LRTI as compared to URTI. The multivariate regression logistic model also supports the use of IL-8, a severity marker for the development of LRTI. In RSV-positive patients, we also observed higher levels of IL-33 in patients with LRTI as compared to RSV-negative patients. Furthermore, we detected elevated levels of IL-33 is associated with >3 days of illness, as compared to children with low levels of this cytokine in NPS. These results provide evidence that detection of IL-8 and IL-33 in NPS in addition to microbiological diagnosis could provide additional information about the severity of the respiratory tract infection.

Based on the properties reported for these cytokines, this panel was designed to understand why some infants with a respiratory virus infection develops a severe clinical course and other resolve as an upper respiratory infection. IL-8 has been frequently reported as a biomarker for viral infection in infants specifically those among different studies, explicitly in infants with RSV bronchiolitis [22–25]. In these works, it has been shown an important influx of neutrophils to the airways and a prolonged survival of these cells that could explained by the IL-8 effects. This current study was able to show a significant increase both in IL-8 protein values (Fig. 2) and also a higher mRNA expression of IL-8 (Fig. 1) in NPS from infants with LRTI compared to in infants with URTI infants. Tabarani

### Table 4: Cytokines levels in patients under and over 6 months of age

| Patients                  | <6 months (age) | >6 months (age) | P-value (χ² test) |
|---------------------------|-----------------|-----------------|------------------|
| High IL-33 levels (>300 pg/ml) | 10 (33%)        | 16 (59%)        | <0.049           |
| High IL-8 levels (>300 pg/ml) | 12 (23%)        | 14 (54%)        | ns               |
| High IL-3 levels (>100 pg/ml)   | 4 (11%)         | 10 (33%)        | <0.027           |
| High TSLP levels (>60 pg/ml)     | 11 (29%)        | 26 (19%)        | <0.0006          |

ns: non-significant.

### Figure 3: Cytokines levels in nasopharyngeal samples from RSV+ and RSV(−) patients, as compared to healthy controls.

Protein levels of cytokines in NPS were evaluated by ELISA in RSV+ (N = 41), Flu+ (N = 3), MPV (N = 2), PIV (N = 3), ADV (N = 1), and virus− (N = 31) infants and control infants (N = 11). The concentrations of IL3, IL8, IL33, and TSLP are expressed in pg/ml and interquartile values are shown as a horizontal bar and square. Statistical analysis was performed using a non-parametric ANOVA test with a post Kruskal–Wallis test ****P < 0.0001, **P < 0.01 and *P = 0.050 respect to RSV+. A Mann–Whitney test was performed to compare values between two groups and P-values of significant differences are indicated above each graph.
et al. reported similar results in patients with moderate to severe RSV bronchiolitis, showing a direct correlation between IL-8 values and illness severity. In fact, their nasopharyngeal IL-8 values in RSV-positive infants were similar to our results despite of virus identification. Although IL-8 levels for RSV-positive infants in our study were 50 times higher as compared to other respiratory viruses and compared to infants with RSV-negative (Fig. 3). Likewise, Diaz et al. showed that nasopharyngeal IL-8 levels in infants with viral bronchiolitis were directly associated with disease severity (such as increase of oxygen requirements) in the regression analysis [25]. Our findings regarding IL-8 mRNA expression and IL-8 protein levels in patients with LRTI corroborates the importance during RSV LRTIs and suggest its potential as biomarker for RSV severity illness. We also found higher viral loads of RSV-positive samples with higher levels of IL-8 expression at the beginning of the symptoms. Remarkably, 90% of these samples were children with bronchiolitis during the initial diagnosis, and 67% of these were hospitalized. This suggests a positive correlation between viral load, IL-8 production, and the severity of the disease in children with LRTI positive for RSV. Several studies agree with these data [26, 27] and indicated that this correlation can be found in children under 5 years old, especially if the infection is for the first time. On the contrary, other studies showed that viral loads were inversely proportional to disease severity and clinical outcomes [28]. Considering these observations, further studies are necessary to conclusively define an association between high RSV viral load and exacerbated response of il-8. However, here, for the first time, the expression of IL-8 was correlated with RSV

**Figure 4:** Cytokines levels in nasopharyngeal swabs according to clinical diagnosis of URTI and LRTI, as compared to controls in RSV+ patients. Protein levels of cytokines in NPS were evaluated by ELISA in infants with URTI (N = 12) and LRTI (N = 29) with an RSV+ diagnosis. The concentrations of IL-3, IL-8, IL-33, and TSLP are expressed in pg/ml. Median and interquartile values are shown as a horizontal bar and square. Statistical analysis was performed using a Mann–Whitney test to compare values between two groups and P-values of significant differences are indicated above each graph.

**Figure 5:** Higher viral loads of RSV in samples with greater IL8 response. Linear regression analysis and N-Ct interpolation for viral load determination in RSV-positive samples with high (n = 21) and low (n = 9) levels of IL8 mRNA or protein. This was determined by real-time PCR with a standard curve with known concentrations (2.5 × 10^9 to 2.5 × 10^3 copies/μl) of the pET15b plasmid containing the N-RSV gene. The Y axis, in logarithmic scale (log10), showing a median and interquartile range. Statistical analysis was performed using a Mann–Whitney test to compare values between two groups and P-values of significant differences are indicated above each graph.
viral loads and disease severity. This is an important factor that could be highly useful for directing future clinical studies.

We have previously reported a good correlation between IL-3 levels from the lower airways of RSV bronchiolitis infants with the number of episodes of wheezing over a 3-year period of following up [29]. Lu et al. have also reported that IL-3 could be used as a predictive biomarker for asthma for children of years old (OR 3.22). Although this study has the weakness of linking a viral bronchiolitis episode during infancy with IL-3 levels obtained in 1–5 years later [30]. This new data showing an increased IL-3 response in both upper and lower respiratory tract infections (Fig. 2), it was unable to predict recurrent wheezing or asthma afterwards in any of all logistic models applied. Intriguing finding of our study was the fact that infants presented more frequently a stronger IL-3 response when they were older than 6 months of age. These results should lead us to interpret the IL-3 data with caution and to give relevance the age of the subject that could be responsible for the difference in the results. Thus, IL-3 may have a role as a modulator of the cellular response, but more information is required to understand its predictive potential.

IL-33 has been described as an ‘alarming’ cytokine, because its release occurs spontaneously when the respiratory epithelium has been damaged [31]. IL-33 is a mediator of the epithelium innate response after infection, given it induces a Th2 response [31]. Saravia et al. have found elevated levels for IL-33 in nasal aspirates from infants hospitalized for RSV bronchiolitis, which went down to undetectable levels after 4 weeks of the infection [32]. A recent study has also related high nasopharyngeal levels of IL-33 with bronchiolitis severity [33]. Our results showed that nasopharyngeal IL-33 levels were higher in RSV-infected infants compared to control (Fig. 3). In contrast to IL-8, IL-33 levels also increased in URTI due to other viruses (Fig. 3). This suggest that IL-33 released from the respiratory epithelium could be responding to different virus infection, not only RSV. IL-33 levels were not different between infants with URTI and LRTI as we expected (Fig. 2), but the level of this cytokine was higher in RSV-positive patients with LRTI as compared to RSV-positive patients with URTI (Fig. 4). This could be explained by the fact that IL-33 main role should occur in the upper respiratory epithelium, where virus induces the initial innate response either in the beginning of the infection in the upper airways and/or as the infection progress to the lower airways. An additional finding of this study was to find a strong IL-33 response in higher proportion of infants older than 6 months of age in order to consider age as an additional factor in the innate response. We hypothesized that IL-33 response will depend on many variables that need to be evaluated for considering its use as a predictive biomarker in LRTIs, such as atopy in predisposed infants or asthma in adults [34].

TSLP has been identified as an important cytokine in allergic asthma development [35] and also has been proposed as one of the mediators involved in the upregulation of inflammatory response during RSV infection [35]. García-García et al. have increased TSLP levels in nasal aspirates in infant with longer hospitalization or those who required intensive care unit stay [35]. This led them to hypothesize that a polarized Th2 response would be the explanation for a complicated clinical course and eventually the shift to explain recurrent wheezing or asthma development. We were able to corroborate higher TSLP levels in infants with URTI and LRTI as compared with healthy infants (Fig. 2). These results were according with the hypothesis about the relevant role for the upper airway epithelium in the production of this cytokine. TSLP mRNA expression was found severely decreased in our results (Fig. 1) suggesting a downregulation mechanism at the time either upper or lower respiratory infection were clinically relevant. Lee et al. was able to demonstrate that TSLP response in airway epithelial cells is the key cytokine involved in the Th2 response and after RSV infection on the onset of recurrent wheezing or asthma [36].

Our idea in the future is that diagnostic kits can include simultaneously the detection of the virus causing the infection as well as markers (cytokines) that can be predict disease severity for the patient. Such information would allow better and faster management of patients, based on the detection of high levels of, e.g., IL-8 or IL-33, or the sum of both when a patient has RSV. To detect both the virus and the severity marker, it is extremely important that during the processing of the sample the virus proteins are recovered from the infected cells (in cell lysates) and not only in the supernatant (where the cytokines or severity markers would be found). In conclusion, for a better diagnosis that includes virus and disease severity by immunodiagnostic techniques, it is necessary to have a sample that includes both cell lysates and supernatants.

The limitations of our study are the short follow-up period to evaluate the outcome of patients (1 year after of infection) and that the clinical symptoms and clinical definitions were kept simple aiming to facilitate the data registration by phone. The disadvantage of this is it could have led to oversight additional clinical information relevant for the outcome. Last, our data should be interpreted cautiously due to the large variability on the results and the predominant detection of RSV over other respiratory viruses and the reduced number of control infants available to compare the results of children with respiratory tract infections, which could bias the results described in this study.

Conclusions

In summary, here we describe that IL-3, IL-8, IL-33, TSLP are elevated in nasopharyngeal secretions of children with respiratory tract infections caused by respiratory viruses, being IL-8 and IL-33 significantly increased in RSV-positive patients with LRTI patients. These results suggest that these cytokines could be used as severity markers for respiratory tract infections caused by viruses in children, which could be included as part of the parameters tested as part of diagnosis panels for viral identification. Additionally, it is important to carry out more studies that correlate high levels of IL-8, viral load, and severity in children with RSV infection.

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Conflict of interest
The authors declare the following conflict of interest: The patent for severity markers title ‘Use of il-3, il-33, and il-12p40 for characterization of the respiratory infections by syncytial respiratory virus’ was request in several countries.

Author contributions
P.J.B., Y.V., and S.M.B. conceived and planned the experiments. Y.V. and I.A.G. carried out the experiments. P.J.B., A.A.B., A.M.C., and M.F. contributed to sample collection and preparation. P.J.B., Y.V., I.A.G., O.P., C.A.R., A.M.K., and S.M.B. contributed to the interpretation of the results. P.J.B., Y.V., and S.M.B. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Data availability
Data available on request. The data underlying this article will be shared on reasonable request to the corresponding author.

Ethical Approval
Institutional Review Board statement: Informed consent was obtained for all infant’s parents who participated in this study, which was approved by the Scientific Ethics Committee (CEC-MEDUC) from the Pontificia Universidad Católica de Chile (Approval number 13-294).

Informed consent statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Permission to reproduce: Not applicable.

That the animal research adheres to the ARRIVE guidelines (https://arriveguidelines.org/arrive-guidelines): Not applicable.

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