

RESEARCH LETTER

Nasopharyngeal CCL5 in infants with severe bronchiolitis and risk of recurrent wheezing: A multi-center prospective cohort study

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To the Editor:

Bronchiolitis is the leading cause of hospitalizations in US infants.¹ Additionally, small cohort studies indicate that 30%-40% of infants hospitalized for bronchiolitis (severe bronchiolitis) will subsequently develop recurrent wheezing and childhood asthma.¹ However, it remains unclear which infants with severe bronchiolitis will develop these chronic respiratory outcomes. Within the limited literature, a single-center analysis of 81 infants with respiratory syncytial virus (RSV) bronchiolitis demonstrated that upregulated expression of CCL5 (previously known as RANTES; a β -chemokine chemoattractant for inflammatory cells such as eosinophils) in nasal epithelium was associated with higher risk of developing childhood asthma.² However, despite the heterogeneity of bronchiolitis and subsequent risks of adverse respiratory outcomes,¹ we are not aware of another study that has investigated the role of airway CCL5 in other important populations (eg, infants with rhinovirus bronchiolitis). In the current study, we examined the relationship between nasopharyngeal CCL5 levels and risks of developing recurrent wheezing, according to

virus etiology, in a prospective cohort of infants with severe bronchiolitis.

Details of the study design, setting, participants, data collection, and analysis may be found in the Online Repository (Supplemental Methods). Briefly, this prospective cohort study, the 35th Multicenter Airway Research Collaboration (MARC-35)³ enrolled infants (age <12 months) at an initial episode of severe bronchiolitis at 17 sites across 14 US states (Table S1) during the 2011-2014 winter seasons. Bronchiolitis was defined according to the American Academy of Pediatrics guidelines—acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions.⁴ We excluded infants with previous enrollment during earlier bronchiolitis hospitalization, those who were transferred to a participating hospital >24 hours after the original hospitalization, those who were consented >24 hours after hospitalization, or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. All patients were treated at the discretion of the treating physician.

Using a standardized protocol, investigators collected nasopharyngeal samples within 24 hours of hospitalization. We tested the

*MARC-35 Investigators are present in Appendix.

samples for 17 respiratory viruses (eg, RSV, rhinovirus) using real-time PCR assays, and CCL5 levels using enzyme-linked immunosorbent assay. For the present analysis, to simplify data interpretation, we categorized CCL5 values into tertiles: low (<25.0 pg/mL), intermediate (25.0-74.0 pg/mL), and high (>74.0 pg/mL) CCL5. The primary outcome was recurrent wheezing by age 3 years. According to the 2007 NIH asthma guidelines,⁵ recurrent wheezing was defined as having at least 2 corticosteroid-requiring exacerbations in 6 months or having at least 4 wheezing episodes in 1 year that last at least 1 day and affect sleep. To examine heterogeneity of CCL5-outcome associations by type of viral infection, we constructed Cox proportional hazards model for each of 10 virus categories (solo RSV infection, solo rhinovirus infection, RSV-rhinovirus coinfection, and others).³ In the models, we adjusted for 11 potential confounders (age, sex, race/ethnicity, parental history of asthma, gestational age, season of birth, history of breathing problems, daycare attendance,

smoking exposure at home, use of positive pressure ventilation, and IgE sensitization at the enrollment) and accounted for patient clustering at the hospital level. To examine the robustness of the findings, we conducted a series of sensitivity analyses. First, to address potential dilutions of the specimens, we normalized the CCL5 level by using RNase P (host housekeeping gene) copy number, as we have previously described.⁶ Second, we performed a subgroup analysis excluding patients who had a breathing problem before the index hospitalization. Lastly, we modeled the outcome as a binary outcome by fitting a generalized linear mixed model for each of the 4 virology strata.

Of 921 infants in the longitudinal cohort, the median age was 3.2 months (IQR, 1.6-5.9 months) and 60% were male. Additionally, 44% were non-Hispanic white, 23% non-Hispanic black, and 30% Hispanics. All had detectable CCL5 at the index hospitalization for bronchiolitis, with the median concentration of 18.3 pg/

TABLE 1 Associations of nasopharyngeal CCL5 levels with the development of recurrent wheezing by age 3 years, according to virus groups

Virus groups and CCL5 levels	Model 1		Model 2		Model 3		Model 4	
	Cox proportional hazards model ^a		Sensitivity analysis using normalized CCL5 levels ^{ab}		Sensitivity analysis excluding patients who had a breathing problem episode before index hospitalization (n = 734) ^a		Sensitivity analysis modeling recurrent wheezing as binary variable ^c	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	OR (95% CI)	P-value
Sole RSV infection								
Low CCL5 (n = 197)	Reference	–	Reference	–	Reference	–	Reference	–
Intermediate CCL5 (n = 180)	1.57 (1.06-2.31)	.02	1.52 (1.03-2.23)	.04	1.83 (1.16-2.89)	.01	1.82 (1.12-2.94)	.02
High CCL5 (n = 164)	1.56 (1.04-2.32)	.03	1.50 (1.01-2.24)	.045	1.66 (1.03-2.66)	.04	1.70 (1.04-2.79)	.03
Sole rhinovirus infection								
Low CCL5 (n = 20)	Reference	–	Reference	–	Reference	–	Reference	–
Intermediate CCL5 (n = 15)	0.77 (0.21-2.84)	.69	0.62 (0.18-2.10)	.44	0.44 (0.13-1.52) ^d	.19	0.57 (0.09-3.80)	.56
High CCL5 (n = 17)	1.45 (0.45-4.61)	.53	2.50 (0.69-9.09)	.16	0.80 (0.27-2.37) ^d	.68	1.27 (0.21-7.52)	.79
RSV + rhinovirus coinfection								
Low CCL5 (n = 23)	Reference	–	Reference	–	Reference	–	Reference	–
Intermediate CCL5 (n = 39)	1.23 (0.39-3.84)	.72	1.56 (0.50-4.84)	.44	0.70 (0.18-2.72)	.61	1.35 (0.31-5.85)	.69
High CCL5 (n = 29)	0.55 (0.14-2.20)	.40	0.60 (0.16-2.25)	.45	0.23 (0.03-1.55)	.13	0.47 (0.08-2.80)	.41
Others								
Low CCL5 (n = 67)	Reference	–	Reference	–	Reference	–	Reference	–
Intermediate CCL5 (n = 72)	0.90 (0.50-1.61)	.72	1.13 (0.63-2.03)	.68	0.91 (0.43-1.90)	.80	0.95 (0.43-2.08)	.89
High CCL5 (n = 98)	0.66 (0.38-1.16)	.15	0.73 (0.41-1.32)	.30	0.61 (0.28-1.34)	.22	0.67 (0.32-1.41)	.29

CI, confidence interval; HR, hazard ratio; OR, odds ratio; RSV, respiratory syncytial virus.

Cox proportional hazards model that adjusts for weight at presentation (in addition to the covariates in the original model) is shown in Table S3.

^aCox proportional hazards model adjusting for patient-level confounders (age, sex, race/ethnicity, parental history of asthma, gestational age, season of birth, history of breathing problems, daycare attendance, smoking exposure at home, use of mechanical ventilation during the index hospitalization, and IgE sensitization [aeroallergen and/or food] at the enrollment) and accounting for potential patient clustering within the sites.

^bCCL5 levels normalized by RNase P copy number.

^cGeneralized linear mixed model adjusting for patient-level confounders (age, sex, race/ethnicity, parental history of asthma, gestational age, season of birth, history of breathing problems, daycare attendance, smoking exposure at home, use of mechanical ventilation during the index hospitalization, and IgE sensitization [aeroallergen and/or food]) and accounting for potential patient clustering within the sites.

^dUnadjusted Cox proportional hazards model because of the relatively small sample size.

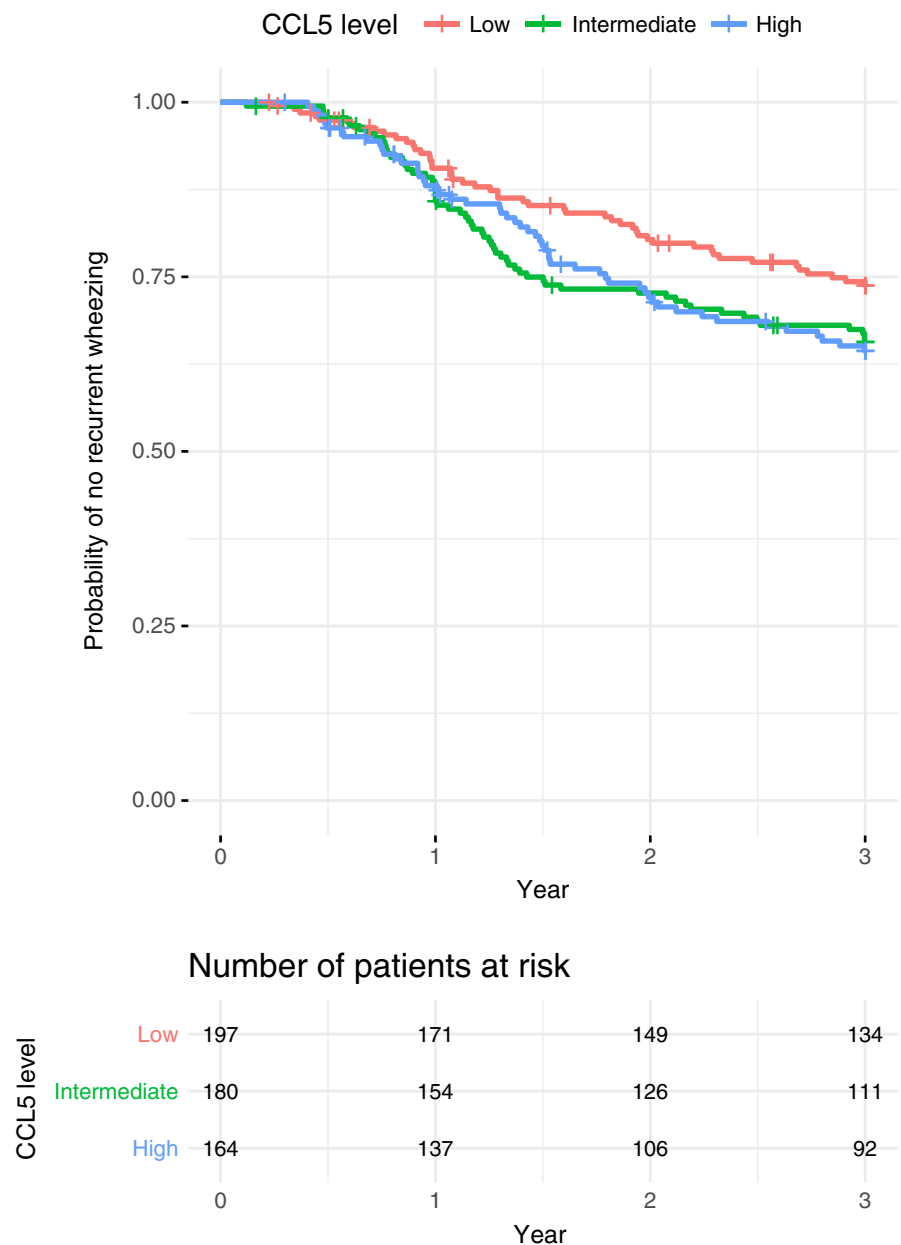
Bold results are statistically significant.

mL (IQR, 42.0-103.9 pg/mL). Overall, 32% developed recurrent wheezing by age 3 years. Between CCL5 strata, most patient characteristics did not differ, while infants with low CCL5 levels were younger and more likely to have RSV infection (both $P < 0.05$; Table S2). There was a significant interaction between CCL5 levels and virus category with regard to risk of recurrent wheezing ($P_{\text{interaction}} = .01$), indicating that CCL5-outcome associations differ by virus. Indeed, among infants with solo RSV infection, compared to those with low CCL5 levels, the risk of recurrent wheezing was significantly higher in those with intermediate and high CCL5 levels (25%, 33%, and 33%, respectively) with corresponding adjusted HR of 1.57 (95% CI, 1.06-2.31; $P = .02$) and 1.56 (95% CI, 1.04-2.32; $P = .03$; Table 1 and Figure 1). In contrast, among the 3 other virus categories, CCL5 levels were not significantly

associated with risks of recurrent wheezing (all $P \geq .15$; Table 1 and Figure S1). This heterogeneity persisted across the sensitivity analyses with the use of normalized CCL5 levels, exclusion of patients with prior breathing problems, and use of generalized linear mixed models (Table 1) (Figure S2).

The observed association between CCL5 levels and risks of recurrent wheezing in RSV bronchiolitis is concordant with earlier reports. In a single-center study of 30 Korean infants with RSV infection, Chung et al⁷ reported associations between higher nasal CCL5 levels and higher risks of recurrent wheezing during the 1-year follow-up period. In another single-center analysis of 81 infants with RSV bronchiolitis, Bacharier et al² reported associations between elevated CCL5 mRNA expression and higher risks of childhood asthma by age 6 years. Similarly, another single-

FIGURE 1 Proportion of patients with solo respiratory syncytial virus (RSV) infection who remained recurrent wheezing-free over a 36-month period. Among infants with solo RSV infection, the risks of developing recurrent wheezing were significantly higher in those with intermediate CCL5 (adjusted HR, 1.57; 95% CI, 1.06-2.31; $P = .02$) and those with high CCL5 (adjusted HR, 1.56; 95% CI, 1.04-2.32; $P = .03$), compared to infants with low CCL5 levels. The number at risk indicates the number of patients at risk for developing recurrent wheeze at each age. The numbers decrease with age as patients who develop the outcome are censored in the survival analysis. As requested, we have added this information to the Figure Legend section



center study of 320 Chinese infants with RSV bronchiolitis also demonstrated that a polymorphism in the *CCL5* gene is associated with higher serum *CCL5* levels and subsequent risks of recurrent wheezing by age 3 years.⁸ Our current multicenter study of 921 infants with severe bronchiolitis corroborates these earlier reports on RSV bronchiolitis, and extends them by demonstrating the lack of association in infants with other virus infections (eg, rhinovirus bronchiolitis).

Exploring the underlying mechanism of these findings is beyond the scope of the current analysis. However, the association in solo RSV infection may be causal—ie, severe RSV infection promotes exuberant *CCL5*-induced airway inflammation thereby contributing to recurrent wheezing. Alternatively, the expression of *CCL5* during RSV infection is genetically determined,⁸ and hence elevated levels may be simply a marker of infants who are predisposed to develop recurrent wheezing. Interestingly, the *CCL5*-recurrent wheezing association was not observed in the other virus groups. The lack of significant association was also found in the RSV-rhinovirus coinfection group, while it is difficult to speculate whether rhinovirus infection modifies RSV-*CCL5*-recurrent wheezing associations given the limited sample size. Regardless, our findings lend additional support to the emerging concept that bronchiolitis is a heterogeneous condition with different pathobiological mechanisms and risks of adverse respiratory outcomes.¹

Our study has potential limitations, such as parental report of wheezing outcome and the absence of a “control” group. Additionally, our observations were based on nasopharyngeal *CCL5* levels, while bronchiolitis also involves lower airways. However, the literature has shown significant correlations between upper and lower airway inflammatory mediators, including *CCL5*.⁹ The use of upper airway specimens is also unavoidable as lower airway sampling is overly invasive in young infants. Next, the current analysis investigated the relationship of airway *CCL5* with risks of recurrent wheezing rather than incident asthma. However, the presence of recurrent wheezing carries a 5.5-fold increased risk of developing asthma by age 6 years.¹⁰ Finally, while the study cohort comprised a racially/ethnically diverse US sample, we must cautiously generalize our inferences beyond infants with severe bronchiolitis. Still, our data remain directly relevant for 130 000 hospitalized US infants each year, of which 30%-40% will develop asthma.¹

In sum, this pre-planned analysis in a multicenter cohort of 921 infants with severe bronchiolitis demonstrated that the relationship of nasopharyngeal *CCL5* levels with risk of recurrent wheezing differs by infecting virus. Specifically, higher *CCL5* levels were associated with 50% increased risk of recurrent wheezing only in infants with solo RSV bronchiolitis. Although causal inference remains premature, our observations—in conjunction with prior studies—advance research into the complex interplay between respiratory virus infection and host immunity in early infancy, and their integrated contribution to lung health in childhood.

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We thank the MARC-35 study hospitals and research personnel for their ongoing dedication to bronchiolitis and asthma research (see Table S1 in the Online Repository). We also thank Ashley F. Sullivan, MS, MPH and Courtney Tierney, MPH (Massachusetts General Hospital, Boston, MA) and Alkis Togias, MD (National Institute of Allergy and Infectious Diseases) for their contributions to the study.

CONFLICT OF INTERESTS

Dr. Piedra has provided bronchiolitis-related consultation for Gilead, Novavax, and Regeneron. Dr. Spergel has provided consultation for Dannone, DBV Technologies, Pfizer, and Regeneron. The other authors have no financial relationships relevant to this article to disclose.

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REFERENCES

- Hasegawa K, Mansbach JM, Camargo Jr CA. Infectious pathogens and bronchiolitis outcomes. *Expert Rev Anti Infect Ther*. 2014;12:817-828.
- Bacharier LB, Cohen R, Schweiger T, et al. Determinants of asthma after severe respiratory syncytial virus bronchiolitis. *J Allergy Clin Immunol*. 2012;130:e3.
- Hasegawa K, Mansbach JM, Ajami NJ, et al. Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalized for bronchiolitis. *Eur Respir J*. 2016;48:1329-1339.
- Ralston SL, Lieberthal AS, Meissner HC, et al. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. *Pediatrics*. 2014;134:e1474-e1502.
- US Department of Health and Human Services. *National Asthma Education and Prevention Program: Expert Panel Report 3 (EPR 3): Guidelines for the Diagnosis and Management of Asthma (NIH Publication 08-4051)*. Bethesda, MD: National Institutes of Health; 2007.
- Piedra FA, Mei M, Avadhanula V, et al. The interdependencies of viral load, the innate immune response, and clinical outcome in children presenting to the emergency department with respiratory syncytial virus-associated bronchiolitis. *PLoS One*. 2017;12:e0172953.
- Chung HL, Kim SG. RANTES may be predictive of later recurrent wheezing after respiratory syncytial virus bronchiolitis in infants. *Ann Allergy Asthma Immunol*. 2002;88:463-467.
- Tian M, Liu F, Wen GY, Shi SY, Chen RH, Zhao DY. Effect of variation in RANTES promoter on serum RANTES levels and risk of recurrent wheezing after RSV bronchiolitis in children from Han, Southern China. *Eur J Pediatr*. 2009;168:963-967.
- McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ, Walsh GM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. *Am J Respir Cell Mol Biol*. 2008;39:560-568.
- 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(4 Pt 1):1403-1406.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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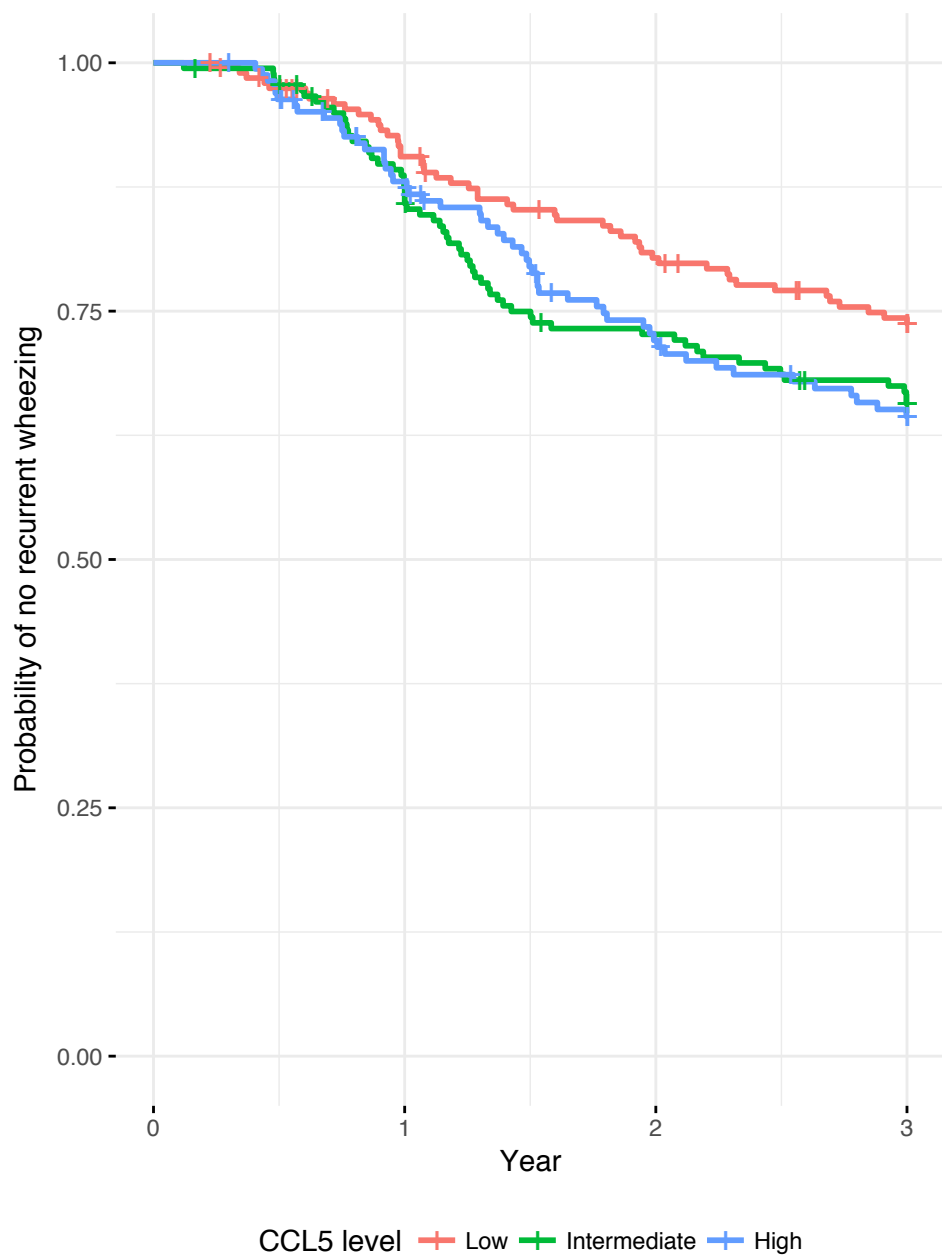
APPENDIX

MARC-35 Investigators (PubMed Collaborators): The following individuals were MARC-35 site investigators and are Collaborators on this paper.

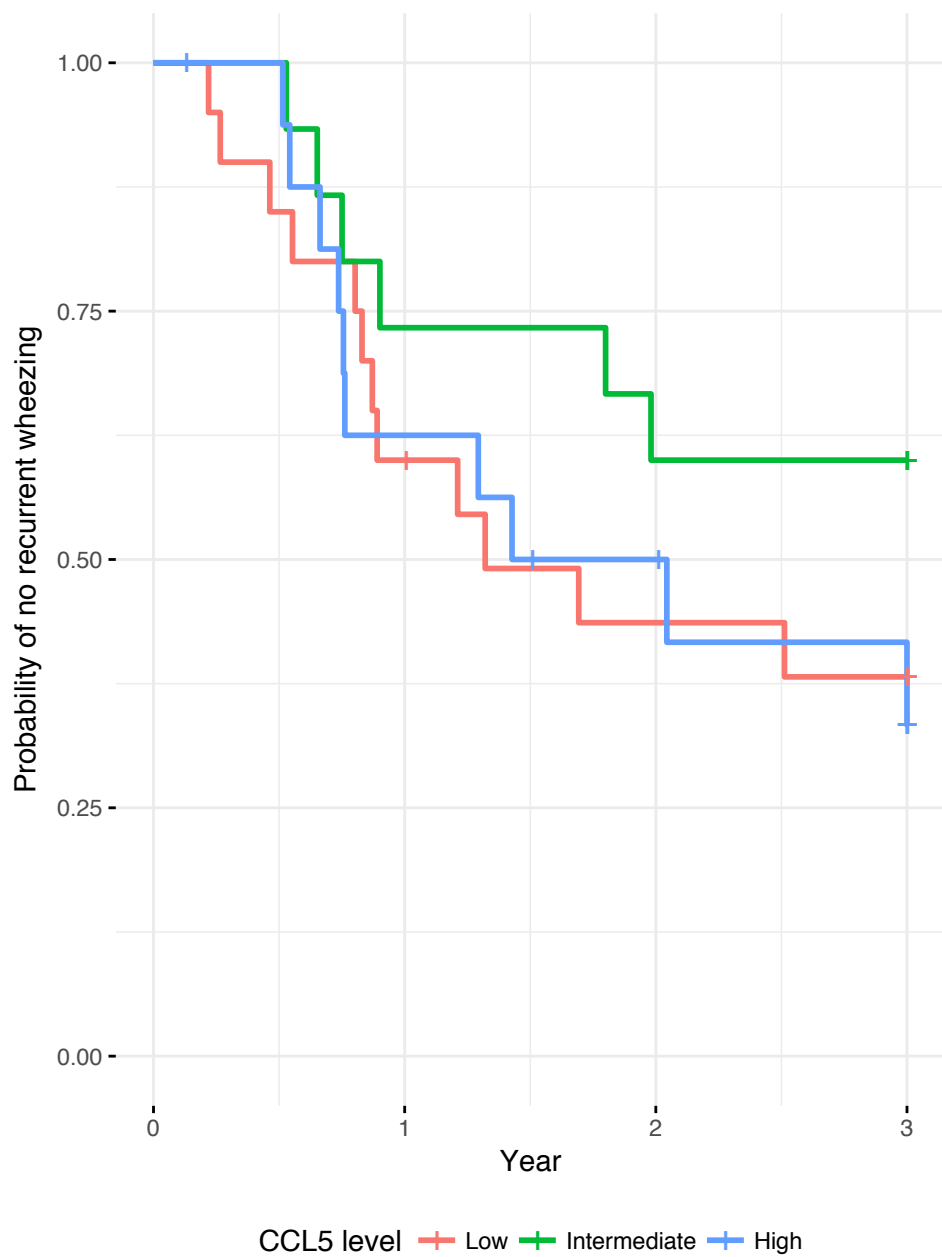
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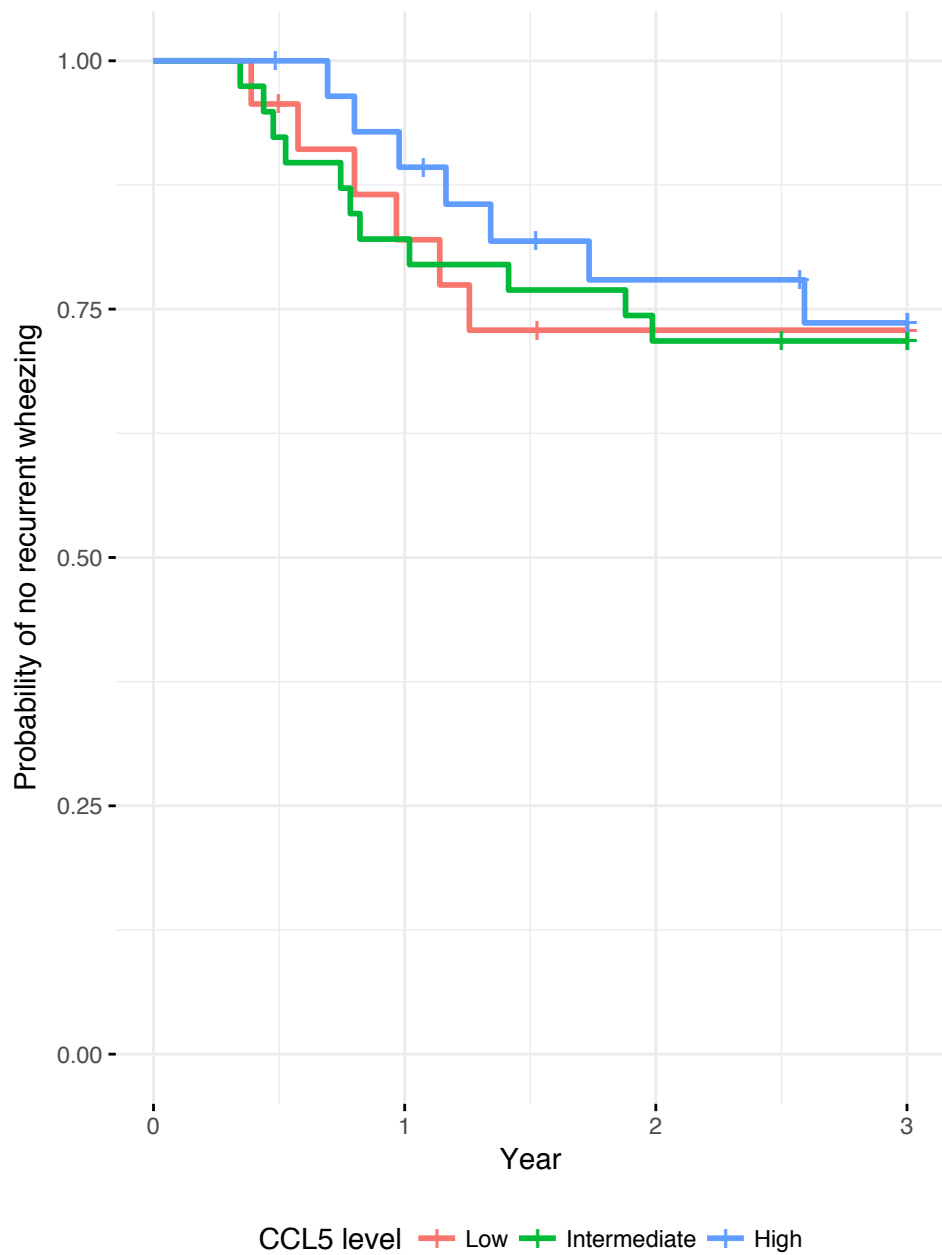
A) Solo RSV infection



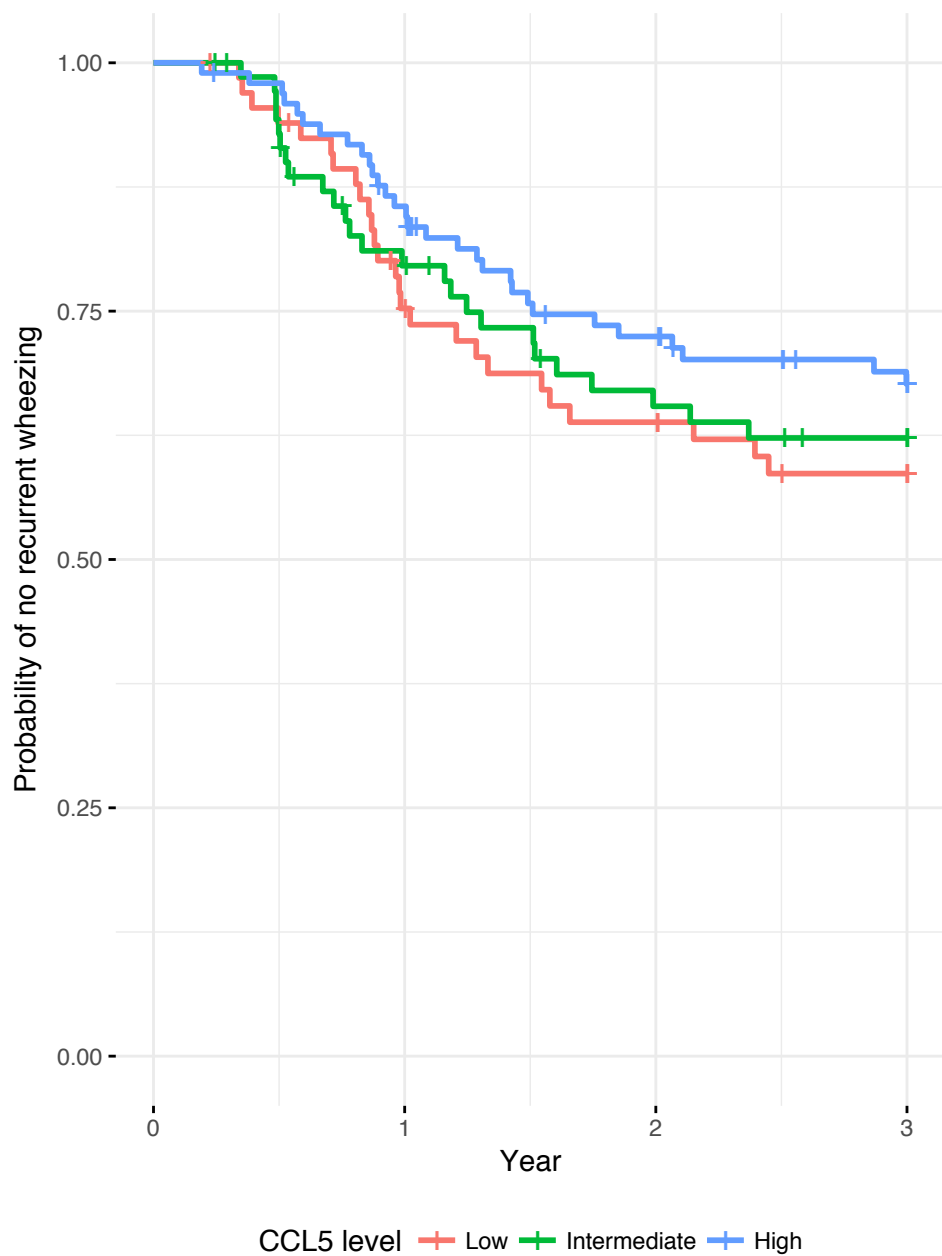
B) Solo rhinovirus infection



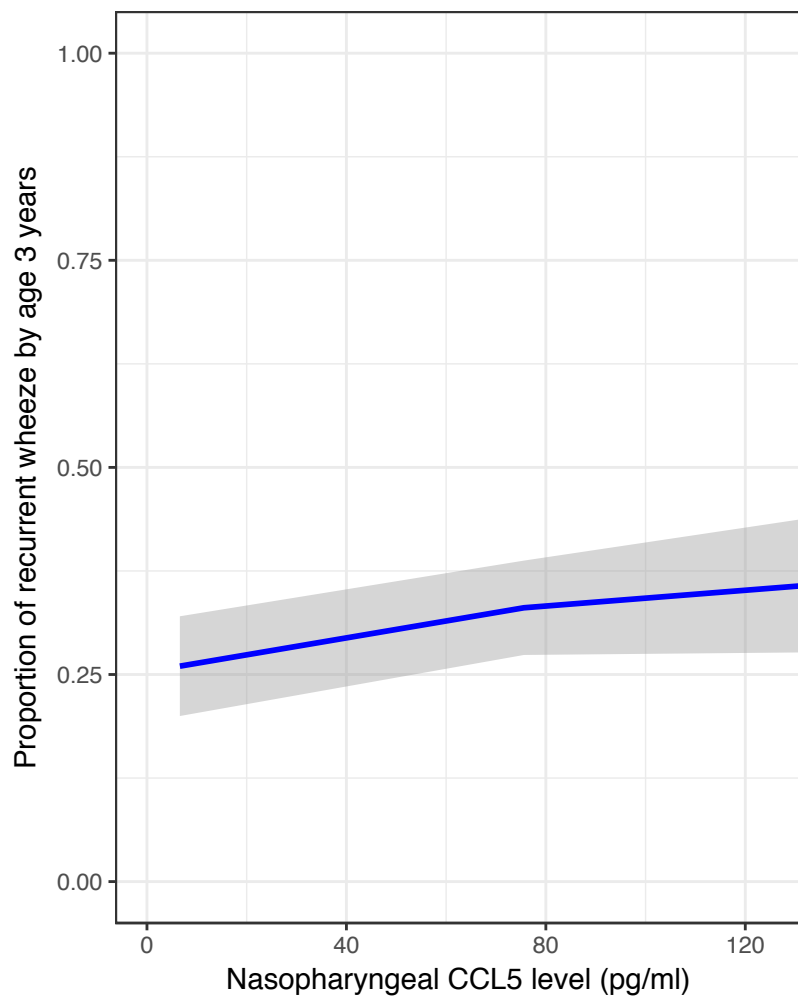
C) RSV and rhinovirus coinfection



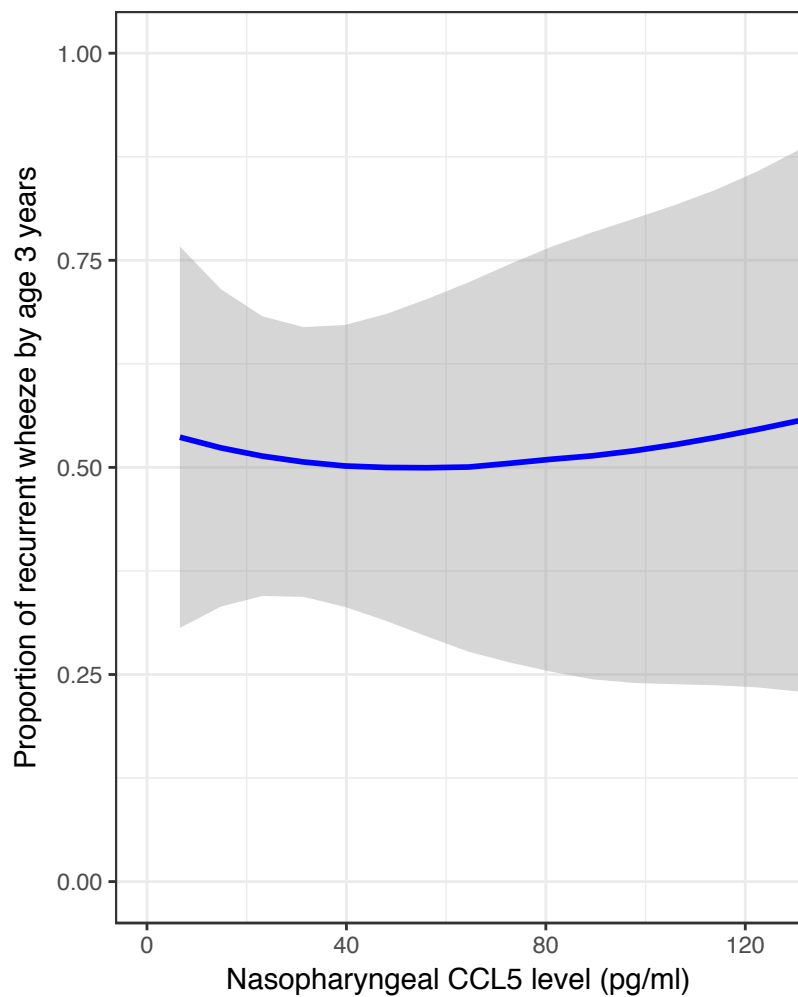
D) Other virus group



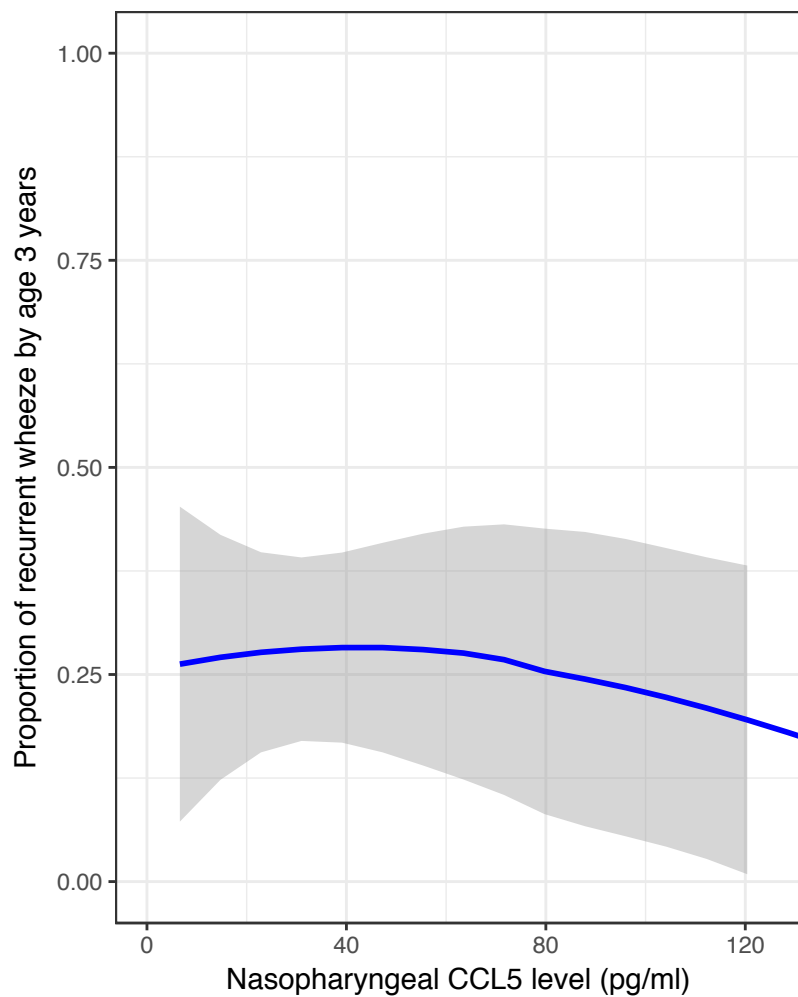
A. Sole RSV infection



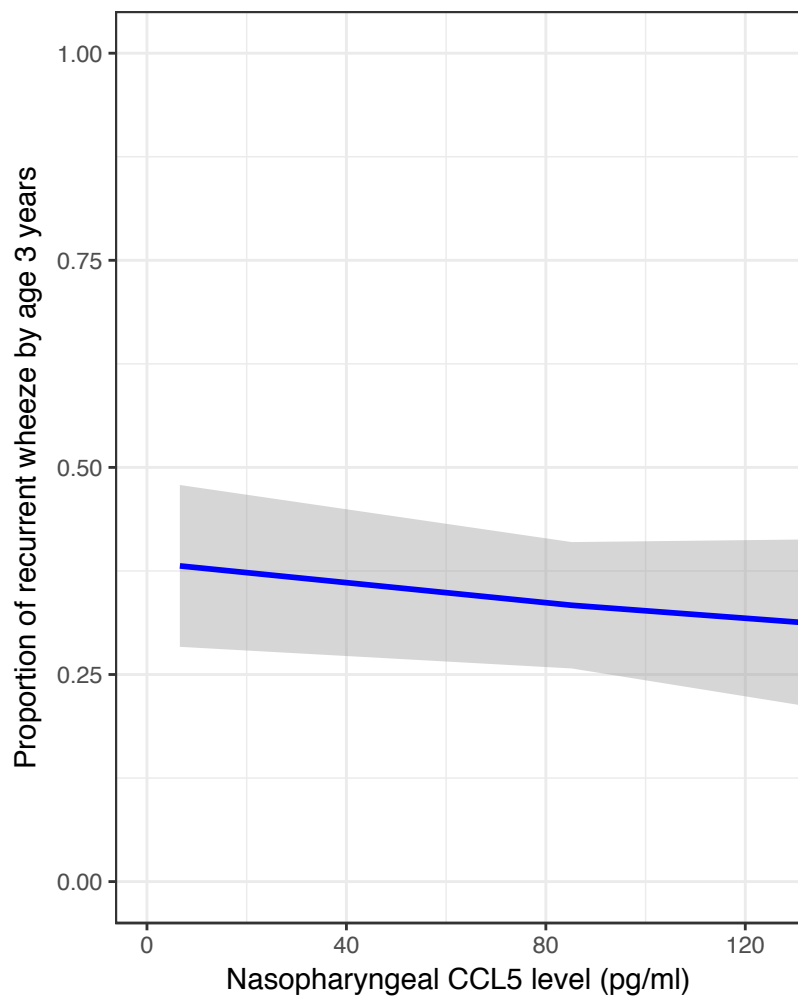
B. Sole rhinovirus infection



C. RSV + rhinovirus coinfection



D. Others



ONLINE REPOSITORY

Nasopharyngeal CCL5 in infants with severe bronchiolitis and risk of recurrent wheezing: A multicenter prospective cohort study

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SUPPLEMENTAL METHODS

Study Design, Setting, and Participants

This is a pre-planned analysis of the data from a multicenter prospective cohort study of infants (age <1 year) hospitalized with bronchiolitis (severe bronchiolitis). This study, called the 35th Multicenter Airway Research Collaboration (MARC-35)(1-6), was coordinated by the Emergency Medicine Network (EMNet)(2), a collaboration of 245 participating hospitals.

Using a standardized protocol, the investigators at 17 sites across 14 U.S. states (**Table S1**) enrolled infants hospitalized with an attending physician diagnosis of bronchiolitis during three consecutive bronchiolitis seasons from November 1 to April 30 (2011-2014). Bronchiolitis was defined by the American Academy of Pediatrics guidelines – acute respiratory illness with some combination of rhinitis, cough, tachypnea, wheezing, crackles, and retractions (7). We excluded infants with previous enrollment during earlier bronchiolitis hospitalization, those who were transferred to a participating hospital >24 hours after the original hospitalization, those who were consented >24 hours after hospitalization, or those with known heart-lung disease, immunodeficiency, immunosuppression, or gestational age <32 weeks. All patients were treated at the discretion of the treating physician.

Of 1016 infants initially enrolled into the MARC-35 cohort, 921 (91%) completed the run-in procedure (contact at 1-week after hospital discharge and 3-weeks after hospitalization) and comprised the longitudinal (analytic) cohort. The institutional review board at each of the participating hospitals approved the study. Written informed consent was obtained from the parent or guardian.

Data Collection

Investigators conducted a structured interview that assessed patients' demographic characteristics, medical, family, and environmental history, and details of the acute illness. Emergency department and hospital chart reviews provided further clinical data, such as medical management and disposition. All data were reviewed at the EMNet Coordinating Center at Massachusetts General Hospital (Boston, MA), and site investigators were queried about missing data and discrepancies identified by manual data checks.

Nasopharyngeal aspirate samples were collected by trained site investigators using the standardized protocol that was utilized in a previous multicenter cohort study of children with bronchiolitis (8, 9). All sites used the same collection equipment (Medline Industries, Mundelein, IL) and collected the samples within 24 hours of hospitalization. No sampling-related adverse events were reported. The nasopharyngeal sample was added to transport medium, immediately placed on ice and then stored at -80°C . The transport media was Iscove media with 15% glycerol – a robust matrix without causing inhibition or interference with any of the methods used for virus quantitative polymerase chain reaction (PCR), cytokine/chemokine, and other biomarker testing (4, 10-15). Frozen samples were shipped in batches on dry ice to Baylor College of Medicine, where they were tested for 17 respiratory viruses (e.g., respiratory syncytial virus [RSV], rhinovirus) by using real-time PCR assays (8, 9, 16). In addition, to test the hypothesis that an elevated level of CCL5 in the upper airway is associated with a higher risk of developing recurrent wheezing, we also measured CCL5 levels by using enzyme-linked immunosorbent assay (ELISA) (17), as detailed below.

Primary exposure – Nasopharyngeal CCL5

Nasopharyngeal CCL5 concentration was quantified by using Quantikine Human

CCL5/RANTES Immunoassay (R&D Systems, Minneapolis, MN) – a quantitative solid phase sandwich ELISA. The minimum detectable concentration of CCL-5 is 6.6 pg/ml, with an intra-assay coefficient of variations of <3% and inter-assay coefficient of variations of <7%. The assay was performed according to the manufacturer’s instructions (18).

IgE Measurement

Serum specific IgE (sIgE) was measured at the enrollment using two different assays (ImmunoCAP sIgE and ImmunoCAP ISAC) at the Phadia Immunology Reference Laboratory (Portage, MI, USA). The sIgE allergen assays conducted were milk, egg white, peanut, cashew nut, and walnut; a positive test was defined as ≥ 0.35 kU/L. The ImmunoCAP ISAC microarray immunoassay measures IgE antibodies to 112 components from 51 allergen sources including foods (e.g., eggs, cow’s milk, fish, shellfish, tree nuts, peanuts, soybeans, wheat, and some fruits) and aeroallergens (e.g., grasses, weeds, trees, animals [cat, dog, horse, and mouse], molds, dust mites, and cockroach). A positive result was defined as ≥ 0.30 ISU-E (ISAC Standardized Units); ISU-E provides an indication of IgE levels and is standardized to ImmunoCAP sIgE units.

Primary Outcome – Recurrent Wheezing by Age 3 years

Recurrent wheezing was defined in the 2007 NIH asthma guidelines as having at least two corticosteroid-requiring exacerbations in six months or having at least four wheezing episodes in one year that last at least one day and affect sleep (19). Among children with this clinical history and a positive modified Asthma Index, the NIH guidelines recommend that clinicians begin inhaled corticosteroids for children age 0-4 years to reduce impairment and risk of exacerbations (20, 21).

After the index hospitalization for bronchiolitis (i.e., enrollment), trained interviewers began interviewing parents/legal guardians by telephone at 1 and 3 weeks after hospital discharge, then at 6-month intervals (i.e., at ages 6, 12, 18, 24, 30, and 36 months). For infants aged >6 months at the index hospitalization, the 6-month intervals commenced at age 12 months. At each follow-up interview, data were collected about the child's prescribed medication (inhaled corticosteroids or montelukast, to help prevent or treat breathing problems) and breathing problem episodes since study enrolment or last follow-up interview. Of 921 children enrolled in the analytic cohort, 805 (87%) completed the age 3-year interview.

Statistical Analyses

To simplify data presentation and interpretation, we categorized the nasopharyngeal CCL5 values into tertiles: low (<25.0 pg/ml), intermediate (25.0-74.0 pg/ml), and high CCL5 (>74.0 pg/ml) levels. We compared the patient characteristics, clinical presentation, virology, nasopharyngeal microbiota profiles, and laboratory data, according to the CCL5 status, by using Wilcoxon-Mann-Whitney test or chi-square test as appropriate.

Next, we modeled the time-to-event (i.e., the development of recurrent wheezing) by fitting adjusted Cox proportional hazards models. The time-to-event for each participant was defined as the period from the date of birth to when the outcome occurred within the 36-month follow-up period. Patients who did not have an outcome were censored at their last follow-up interview or time of withdrawal. In the multivariable model, we first adjusted for patient-level characteristics (age, sex, race/ethnicity, parental history of asthma, gestational age, season of birth, history of breathing problems, daycare attendance, smoking exposure at home, acute severity defined by the use of positive pressure ventilation [continuous positive airway pressure

and/or intubation] during the index hospitalization, IgE sensitization [aeroallergen and/or food] at the enrollment, and respiratory viruses detected by PCR) and accounted for patient clustering at the hospital-level by including the sites as a random-effect. Based on *a priori* knowledge (17, 22), we first tested for interactions between CCL5 status and virology. As the model indicated a statistically significant CCL5–virology interaction ($P_{\text{interaction}}=0.01$), we then stratified the analysis by virology (solo RSV infection, solo rhinovirus infection, RSV/rhinovirus coinfection, and others). The evaluation of scaled Schoenfeld residuals was not statistically significant for each of the covariates in the model, indicating no violation of the proportionality of hazards assumption.

To examine the robustness of the findings, we conducted sensitivity analyses. First, to address potential dilutions of the specimens, we normalized the CCL5 level by using RNase P (host housekeeping gene) copy number, as we have previously described (10). Second, we performed a subgroup analysis excluding patients who had a breathing problem before the index hospitalization. Lastly, we modeled the outcome as a binary outcome by fitting a generalized linear mixed model for each of the four virology strata. Analyses used R version 3.3. All P-values were two-tailed, with $P<0.05$ considered statistically significant.

SUPPLEMENTAL REFERENCES

1. Mansbach JM, Hasegawa K, Henke DM, Ajami NJ, Petrosino JF, Shaw CA, et al. Respiratory syncytial virus and rhinovirus severe bronchiolitis are associated with distinct nasopharyngeal microbiota. *J Allergy Clin Immunol*. 2016;137(6):1909-13 e4.
2. Hasegawa K, Mansbach JM, Ajami NJ, Espinola JA, Henke DM, Petrosino JF, et al. Association of nasopharyngeal microbiota profiles with bronchiolitis severity in infants hospitalized for bronchiolitis. *Eur Respir J*. 2016;48:1329-39.
3. Hasegawa K, Mansbach JM, Ajami NJ, Petrosino JF, Freishtat RJ, Teach SJ, et al. Serum cathelicidin, nasopharyngeal microbiota, and disease severity in infants hospitalized with bronchiolitis. *J Allergy Clin Immunol*. 2017;139(4):1383-6.
4. Hasegawa K, Mansbach JM, Ajami NJ, Petrosino JF, Freishtat RJ, Teach SJ, et al. The relationship between nasopharyngeal CCL5 and microbiota on disease severity among infants with bronchiolitis. *Allergy*. 2017;[Epub ahead of print].
5. Stewart CJ, Mansbach JM, Wong MC, Ajami NJ, Petrosino JF, Camargo Jr CA, et al. Associations of nasopharyngeal metabolome and microbiome with severity among infants with bronchiolitis: A multi-omic analysis. *Am J Respir Crit Care Med*. 2017;196(7):882-91.
6. Mansbach JM, Hasegawa K, Ajami NJ, Petrosino JF, Piedra PA, Tierney CN, et al. Serum LL-37 level is associated with severity of bronchiolitis and viral etiology. *Clin Infect Dis*. 2017.
7. Ralston SL, Lieberthal AS, Meissner HC, Alverson BK, Baley JE, Gadomski AM, et al. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. *Pediatrics*. 2014;134(5):e1474-502.

8. Hasegawa K, Jartti T, Mansbach JM, Laham FR, Jewell AM, Espinola JA, et al. Respiratory syncytial virus genomic load and disease severity among children hospitalized with bronchiolitis: Multicenter cohort studies in the US and Finland. *The Journal of infectious diseases*. 2015;211(10):1550-9.
9. Mansbach JM, Piedra PA, Teach SJ, Sullivan AF, Forgey T, Clark S, et al. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. *Arch Pediatr Adolesc Med*. 2012;166(8):700-6.
10. Piedra FA, Mei M, Avadhanula V, Mehta R, Aideyan L, Garofalo RP, et al. The interdependencies of viral load, the innate immune response, and clinical outcome in children presenting to the emergency department with respiratory syncytial virus-associated bronchiolitis. *PLoS One*. 2017;12(3):e0172953.
11. Bennett BL, Garofalo RP, Cron SG, Hosakote YM, Atmar RL, Macias CG, et al. Immunopathogenesis of respiratory syncytial virus bronchiolitis. *The Journal of infectious diseases*. 2007;195(10):1532-40.
12. Laham FR, Trott AA, Bennett BL, Kozinetz CA, Jewell AM, Garofalo RP, et al. LDH concentration in nasal-wash fluid as a biochemical predictor of bronchiolitis severity. *Pediatrics*. 2010;125(2):e225-33.
13. Mian AI, Laham FR, Cruz AT, Garg H, Macias CG, Caviness AC, et al. Nitric oxide metabolites as biomarkers for influenza-like acute respiratory infections presenting to the emergency room. *Open Respir Med J*. 2012;6:127-34.
14. Mehta R, Scheffler M, Tapia L, Aideyan L, Patel KD, Jewell AM, et al. Lactate dehydrogenase and caspase activity in nasopharyngeal secretions are predictors of bronchiolitis severity. *Influenza and other respiratory viruses*. 2014;8(6):617-25.

15. Nicholson EG, Schlegel C, Garofalo RP, Mehta R, Scheffler M, Mei M, et al. Robust Cytokine and Chemokine Response in Nasopharyngeal Secretions: Association With Decreased Severity in Children With Physician Diagnosed Bronchiolitis. *The Journal of infectious diseases*. 2016;214(4):649-55.
16. Beckham JD, Cadena A, Lin J, Piedra PA, Glezen WP, Greenberg SB, et al. Respiratory viral infections in patients with chronic, obstructive pulmonary disease. *The Journal of infection*. 2005;50(4):322-30.
17. Tian M, Liu F, Wen GY, Shi SY, Chen RH, Zhao DY. Effect of variation in RANTES promoter on serum RANTES levels and risk of recurrent wheezing after RSV bronchiolitis in children from Han, Southern China. *European journal of pediatrics*. 2009;168(8):963-7.
18. R&D Systems. Human CCL5/RANTES Quantikine ELISA Kit [cited 2016 September 15]. Available from: https://www.rndsystems.com/products/human-ccl5-rantes-quantikine-elisa-kit_drn00b_-_product-details.
19. US Department of Health and Human Services. National Asthma Education and Prevention Program: Expert Panel Report 3 (EPR 3): Guidelines for the Diagnosis and Management of Asthma (NIH Publication 08-4051). Bethesda, MD, National Institutes of Health, 2007. 2007.
20. Bisgaard H, Hermansen MN, Loland L, Halkjaer LB, Buchvald F. Intermittent inhaled corticosteroids in infants with episodic wheezing. *N Engl J Med*. 2006;354(19):1998-2005.
21. Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szeffler SJ, et al. Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N Engl J Med*. 2006;354(19):1985-97.

22. Bacharier LB, Cohen R, Schweiger T, Yin-Declue H, Christie C, Zheng J, et al.
Determinants of asthma after severe respiratory syncytial virus bronchiolitis. *J Allergy Clin Immunol.* 2012;130(1):91-100 e3.

Table S1. Principal investigators at the 17 participating sites in MARC-35

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Table S2. Patient characteristics of infants hospitalized for bronchiolitis by nasopharyngeal CCL5 level

Variables	Low CCL5 level <25 pg/ml n=307	Intermediate CCL5 level 25-74 pg/ml n=306	High CCL5 level >74 pg/ml n=308	P-value
Patient characteristics				
Age (mo), median, (IQR)	2.9 (1.4-5.4)	2.8 (1.5-5.3)	4.0 (2.3-7.0)	<0.001
Male sex	189 (62)	182 (59)	183 (59)	0.83
Race/ethnicity				0.25
Non-Hispanic white	141 (46)	130 (43)	130 (42)	
Non-Hispanic black	66 (22)	66 (22)	78 (25)	
Hispanic	83 (27)	102 (33)	90 (29)	
Other	17 (6)	8 (3)	10 (3)	
Parental history of asthma	105 (34)	98 (32)	102 (33)	0.86
Maternal smoking during pregnancy	53 (18)	32 (11)	41 (14)	0.08
Prematurity (32-37 weeks)	48 (16)	61 (20)	62 (21)	0.27
Birth weight				0.22
<3.0 pounds (<1.3 kg)	1 (<1)	2 (1)	0 (0)	
3.0-4.9 pounds (1.4-2.2 kg)	15 (5)	23 (8)	18 (6)	
5.0-6.9 pounds (2.3-3.1 kg)	97 (32)	106 (35)	108 (35)	
≥7.0 pounds (≥3.2 kg)	193 (63)	175 (57)	178 (58)	
Season of birth				0.053
Spring (March-May)	33 (11)	28 (9)	51 (17)	
Summer (June-August)	65 (21)	54 (18)	65 (21)	
Fall (September-November)	126 (41)	126 (41)	109 (35)	
Winter (December-February)	83 (27)	98 (32)	83 (27)	
Previous breathing problems before the index hospitalization*	63 (21)	54 (18)	70 (23)	0.29
History of eczema	39 (13)	47 (15)	51 (17)	0.39
Ever attended daycare	69 (23)	65 (21)	77 (25)	0.53
Smoking exposure at home	53 (17)	39 (13)	46 (15)	0.29
Corticosteroid use before the index hospitalization	47 (15)	40 (13)	51 (17)	0.47
Duration of breathing problem prior to the hospitalization, < 24 hours	21 (7)	15 (4.9)	16 (5.2)	0.53
Clinical course at the index hospitalization				
Weight at presentation (kg), median (IQR)	5.7 (4.5-7.5)	5.8 (4.6-7.3)	6.8 (5.3-8.2)	<0.001
Received corticosteroids during pre-hospitalization visit	27 (9)	25 (8)	31 (10)	0.72
Use of mechanical ventilation (CPAP and/or intubation)	17 (6)	19 (6)	13 (4)	0.54
Laboratory data at the index hospitalization				
Nasopharyngeal CCL5 (pg/ml),	6.6	41.9	160.8	<0.001

median (IQR)	(6.6-18.3)	(31.6-55.2)	(103.8-324.4)	
Blood eosinophils $\geq 4\%$	30 (11)	29 (11)	23 (9)	0.57
Serum LL-37 (ng/ml), median (IQR)‡	46 (34-60)	46 (35-62)	46 (32-62)	0.70
Serum 25-hydroxyvitamin D (ng/ml), median (IQR)	25 (17-32)	27 (18-33)	27 (20-34)	0.03
Aeroallergen sensitization†	2 (1)	9 (3)	4 (1)	0.07
Food sensitization†	54 (18)	57 (19)	60 (20)	0.83
Virology at the index hospitalization				
Sole RSV infection	197 (64)	180 (59)	164 (53)	0.02
Sole rhinovirus infection	20 (7)	15 (5)	17 (6)	0.68
RSV + rhinovirus coinfection	23 (8)	39 (13)	29 (9)	0.09
Others	67 (22)	72 (24)	98 (32)	0.01
Nasopharyngeal microbiota profile at the index hospitalization§				0.34
<i>Haemophilus</i> -dominant profile	57 (19)	58 (19)	61 (20)	
<i>Moraxella</i> -dominant profile	72 (24)	68 (22)	59 (19)	
<i>Streptococcus</i> -dominant profile	77 (26)	98 (32)	83 (27)	
Mixed profile	95 (32)	81 (27)	103 (34)	
Recurrent wheezing				
Recurrent wheezing by age 3 years	93 (30)	102 (33)	101 (33)	0.69

Data are no. (%) of infants unless otherwise indicated. Percentages may not equal 100 because of rounding. Proportions are column-wise proportions.

Abbreviations: CPAP, continuous positive airway pressure; IQR, interquartile range; RSV, respiratory syncytial virus

* Defined as a child having cough that wakes him/her at night and/or causes emesis, or when the child has wheezing or shortness of breath without cough

† Defined by having one or more positive values for allergen-specific IgE at the enrollment

‡ The concentration of serum LL-37, the main active form of human cathelicidin.

§ These four nasopharyngeal microbiota profiles were derived by applying an unsupervised clustering approach to the 16S rRNA gene sequence data.

Table S3. Sensitivity analysis of associations between nasopharyngeal CCL5 levels with development of recurrent wheezing by age 3 years, according to virus groups

Virus groups and CCL5 levels	Sensitivity analysis	
	Cox proportional hazards model	
	<i>HR (95% CI)</i>	<i>P-value</i>
Sole RSV infection		
Low CCL5	Reference	-
Intermediate CCL5	1.58 (1.07-2.33)	0.02
High CCL5	1.54 (1.03-2.30)	0.04
Sole rhinovirus infection		
Low CCL5	Reference	-
Intermediate CCL5	0.77 (0.18-3.19)	0.71
High CCL5	1.45 (0.44-4.71)	0.54
RSV + rhinovirus coinfection		
Low CCL5	Reference	-
Intermediate CCL5	1.06 (0.33-3.42)	0.93
High CCL5	0.54 (0.14-2.12)	0.37
Others		
Low CCL5	Reference	-
Intermediate CCL5	0.91 (0.51-1.64)	0.76
High CCL5	0.67 (0.38-1.17)	0.16

Bold results are statistically significant

Cox proportional hazards model adjusting for patient-level confounders (age, sex, race/ethnicity, parental history of asthma, gestational age, season of birth, history of breathing problems, daycare attendance, smoking exposure at home, weight at presentation, use of mechanical ventilation during the index hospitalization, and IgE sensitization [aeroallergen and/or food] at the enrollment) and accounting for potential patient clustering within the sites.

Figure Legends

Figure S1. Proportion of patients who remained recurrent wheezing-free over a 36-month period according to virus categories at index hospitalization for severe bronchiolitis

A) Solo RSV infection (n=541). **B)** Solo rhinovirus infection (n=52). **C)** RSV and rhinovirus coinfection (n=91). **D)** Other virus group (n=237). Corresponding hazards ratio are shown in **Table 1**.

Figure S2. Association of nasopharyngeal CCL5 levels and risk of recurrent wheezing

The fitted lines represent locally weighted scatterplot smoothed (loess) curve, with 95% confidence intervals. **A)** Solo RSV infection (n=541). **B)** Solo rhinovirus infection (n=52). **C)** RSV and rhinovirus coinfection (n=91). **D)** Other virus group (n=237).