Supplementary Data

Methods

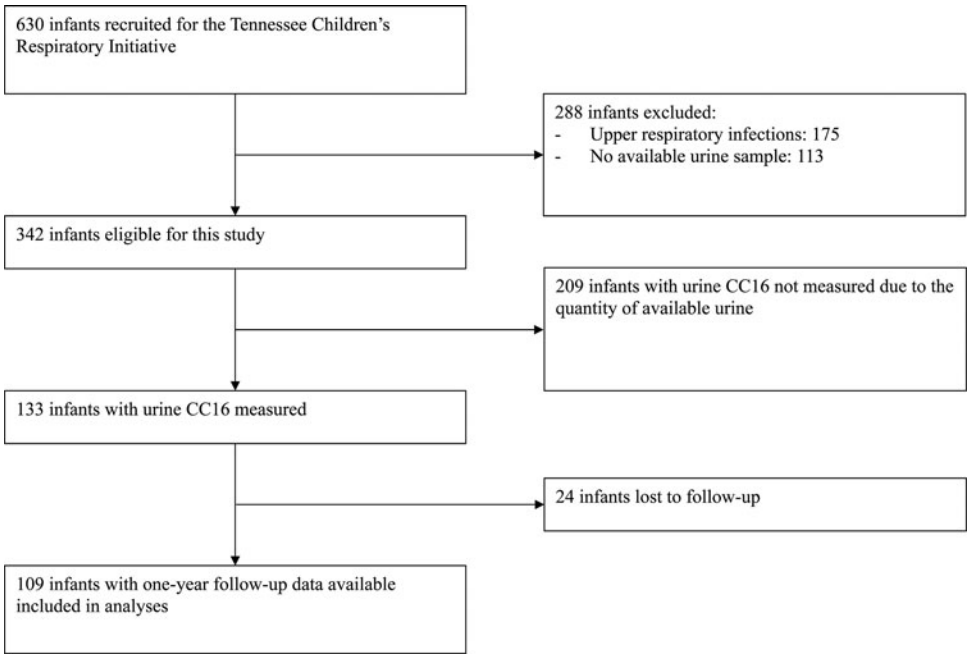
Validation and measurement of urine CC16

As a first step, a commercially available enzyme-linked immunosorbent assay (ELISA) kit was validated for serum club cell 16-kDa secretory protein (CC16; BioVendor, Chandler, NC) in urine. The validation experiments included spike and recovery, multiple freeze–thaw cycles, and intra-assay controls on urine samples. In the spike and recovery experiment, a healthy infant control sample was spiked with 15.6, 31.2, 62.5, 125, 250, and 500 IU/mL concentrations and then measured as unknowns against the standard curve made up in the supplied diluent according to the manufacturer's recommendations. The spike and recovery experiment was successful and showed that the sample urines did not interfere with the analyte detection.

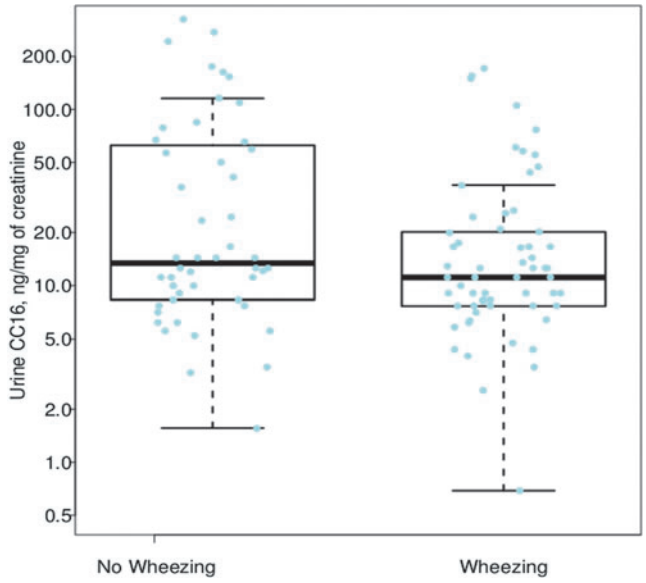
For the freeze–thaw cycles, samples from control and sick infants were spiked with a known amount of CC16 and then aliquoted into five microcentrifuge tubes and frozen. These samples were then subjected to one to five freeze–thaw cycles and measured as unknowns against the ELISA standard curves made up in the kits' supplied diluent according to the manufacturer's recommendations. There was no evidence of degradation with increasing freeze–thaw cycles. Intra- and inter- assay controls were also performed. These were 7.7% and 12.5% for the quality control high samples, and 3.7% and 6.5% for the quality control low samples. Urine levels of CC16 (ng/mL) were then measured according to the manufacturer's recommendations. In particular, the kit uses a polyclonal antibody precoated on a microtiter plate. The standard curve is made by diluting a standard solution to the following concentrations: 0, 2, 5, 10, 20, 40, and 100 ng/mL. The standard solutions and samples are diluted with dilution buffer and pipetted into the wells, and the biomarker is bound to the immobilized antibody. After any unbound substances are washed away, biotin-labeled CC16 antibody is added. The plate is washed once again and Streptavidin-HRP is added. Following a wash to remove any unbound antibody-enzyme reagent, a color developing substrate is added. Intensity of color develops in proportion to the amount of the marker that is bound in the initial step. After the color development is stopped, the intensity of color is read at a specific wavelength on a spectrophotometer, and the concentration of the samples is determined by converting the optical density to concentration employing the standard curve. All samples were tested in duplicate. The values below the instrument's limit of detection (LOD; 2 ng/mL) were assigned a value one-half the LOD (i.e., 1 ng/mL). Finally, urine creatinine (mg/mL) was determined by a chemical assay based on Jaffe's reaction (Enzo Life Sciences, Farmingdale, NY). Urine levels of CC16 (ng/mL) were corrected to account for urine concentration by dividing them by urine creatinine values (mg/mL).

Statistical analyses

A sensitivity analysis was conducted, treating the values below the LOD as missing values and applying multiple imputation methods. Multiple imputation was performed using the ''aregImpute'' algorithm and the ''fit.mult.impute,'' as implemented in the Hmisc package in R with regression type matching. Fifteen imputation data sets were used in this analysis. All variables used in the logistic regression model were included in the imputation models. The imputation methods curtailed to the range of observed urine levels of CC16 (ng/mg of creatinine). These results were then compared to our main analysis that assigned values below the LOD a value of one-half the LOD.



SUPPLEMENTARY FIG. S1. Flow diagram of infants in the Tennessee Children's Respiratory Initiative included in this study.



SUPPLEMENTARY FIG. S2. Box plots of urine levels of club cell 16-kDa secretory protein according to subsequent childhood wheeze. The box plots display the median (bold line), the interquartile range (box hinges), and the minimum and maximum value of the data (upper and lower whiskers).

Supplementary Table S1. Bronchiolitis Severity Score

Respiratory rate per minute	*Flaring or retractions*[a]	*Pulse oxygen saturation on room air*	*Wheezing*[a]	*Score assigned to each column*
<30	None	>94	None	0
31–45	Mild	90–94	End expiratory, audible only by stethoscope	1
46–60	Moderate	85–89	Full expiratory, audible only by stethoscope	2
>60	Severe	<85	Audible without stethoscope[b] or markedly decreased air exchange on auscultation	3
				Total score

The score assigned to each column was obtained from reviewing the child's medical record at the enrollment visit. The total score was obtained adding the individual values from the last row.

[a]A score of 1.5 is assigned if information is not otherwise specified.

[b]This included maternal report.

Supplementary Table S2. Comparison of Infants with LRTIs That Were and Were Not Included in Analyses*

	*Included (*n = *133)*	*Not included (*n = *322)*
Age at enrollment (weeks)	11.0 (6.0–17.0)	10.0 (6.0–23.0)
Female sex	41 (31%)	159 (49%)*
Race/ethnicity		
Black	23 (17%)	58 (18%)
White	85 (64%)	192 (60%)
Hispanic	17 (13%)	36 (11%)
Other	8 (6%)	35 (11%)
Gestational age (weeks)	39 (38–39)	39 (38–40)
Birth weight (g)	3,345 (3,090–3,579)	3,274 (2,948–3,629)
Current exposure to SHS	29 (22%)	114 (36%)*
Maternal asthma	21 (16%)	61 (19%)
Insurance type		
Private	54 (41%)	88 (27%)*
Medicaid	71 (53%)	211 (66%)
None	8 (6%)	22 (7%)
Bronchiolitis severity score	7.0 (4.5–9.0)	6.5 (4.0–8.5)*
LRTI visit type		
Unscheduled outpatient visit	3 (2%)	39 (12%)*
Emergency department visit	21 (16%)	36 (11%)
Hospitalization	109 (82%)	247 (77%)

Data are presented as the number (%) for binary variables or median (interquartile range) for continuous variables. Percentage calculated for children with complete data.

*$p < 0.05$ for the comparisons between groups. Results from Pearson's chi-square tests or Mann–Whitney *U*-tests, as appropriate.

LRTI, lower respiratory tract infection; SHS, second-hand smoke.

Supplementary Table S3. Comparison of Infants with LRTIs With and Without 2-Year Follow-Up Data*

	*Without 2-year follow-up data (*n = *24)*	*With 2-year follow-up data (*n = *109)*
Age at enrollment (weeks)	11.5 (5.8–16.2)	10.0 (6.0–17.0)
Female sex	11 (46%)	30 (28%)
Race/ethnicity		
Black	2 (8%)	22 (20%)*
White	14 (58%)	76 (70%)
Hispanic	7 (29%)	10 (9%)
Other	1 (4%)	1 (1%)
Gestational age (weeks)	39 (38–39)	39 (38–39)
Birth weight (g)	3,345 (3,118–3,530)	3,374 (3,090–3,629)
Current exposure to SHS	6 (25%)	23 (21%)
Maternal asthma	8 (33%)	13 (12%)*
Insurance type		
Private	2 (8%)	52 (48%)*
Medicaid	21 (88%)	50 (46%)
None	1 (4%)	7 (6%)
Bronchiolitis severity score	7.8 (4.8–8.6)	7.0 (4.5–9.0)
LRTI visit type		
Unscheduled outpatient visit	1 (4%)	2 (2%)
Emergency department visit	1 (4%)	20 (18%)
Hospitalization	22 (92%)	87 (80%)

Data are presented as the number (%) for binary variables or median (interquartile range) for continuous variables. Percentage calculated for children with complete data.

*$p < 0.05$ for the comparisons between groups. Results from Pearson's chi-square tests or Mann–Whitney *U*-tests, as appropriate.

Supplementary Table S4. Analysis of Urine CC16 at the Time of an Infant LRTI and Subsequent Childhood Wheeze Using Multiple Imputation for CC16 Values Below the LOD

	OR [95% CI][a]	p-*Value*
Unadjusted		
Urine CC16 (ng/mg of creatinine)	0.70 [0.58–0.86]	<0.001
Adjusted[b]		
Urine CC16 (ng/mg of creatinine)	0.66 [0.48–0.90]	0.008

[a]OR [95% CI] for a twofold increase in urine levels of CC16.

[b]Logistic regression model adjusted for infant's age, sex, and current exposure to second-hand smoke.

CC16, club cell 16-kDa secretory protein; LOD, limit of detection.