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Urine Club Cell 16-kDa Secretory Protein and Childhood Wheezing Illnesses After Lower Respiratory Tract Infections in Infancy

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Background: Infants with lower respiratory tract infections (LRTIs) are at an increased risk of developing childhood wheezing illnesses (including asthma), but it is not currently possible to predict those at risk for these long-term outcomes. The current objective was to examine whether urine levels of club cell 16-kDa secretory protein (CC16) at the time of an infant LRTI are associated with the development of childhood wheezing illnesses.
Methods: Prospective study of 133 previously healthy infants enrolled during a healthcare visit for a LRTI and followed longitudinally for childhood wheezing illnesses. Urine levels of CC16 at the time of enrollment were measured after validating a commercially available enzyme-linked immunosorbent assay kit for serum. The outcome of interest was parental report of subsequent childhood wheeze (defined as ≥1 episode of wheezing following the initial LRTI) at the 1-year follow-up visit. Logistic regression was used for the main analysis.
Results: The median (interquartile range) urine levels of CC16 (ng/mg of creatinine) at the time of an infant LRTI were 11.1 (7.7–20.1) for infants with subsequent childhood wheeze and 13.4 (8.3–61.1) for those without ($p=0.11$). In the main multivariate analysis using a logarithmic transformation of the urine levels of CC16, a twofold increase in urine levels of CC16 was associated with ~30% decreased odds (OR = 0.74 [95% confidence interval (CI) 0.56–0.98], $p=0.04$) of subsequent childhood wheeze after adjustment for potential confounders.
Conclusions: An inverse association was found between urine levels of CC16 at the time of an infant LRTI and the odds of subsequent childhood wheeze. Urine CC16 may be a useful biomarker of the development of childhood wheezing illnesses after LRTIs in infancy.

Introduction

Lower respiratory tract infections (LRTIs) are a major cause of morbidity and mortality in early childhood worldwide.[1,2] Bronchiolitis (the most common LRTI) is the number one cause of infant hospitalizations in the United States, and its inpatient-related costs have been estimated at ~$543 million per year.[2,3] In addition to their acute effects, LRTIs in infancy have also been associated with long-term outcomes, such as the development of childhood wheezing illnesses (including asthma).[4–7] This association is likely influenced by the specific infectious agent, the timing and/or severity of the infection, and certain host factors that may alter susceptibility to infection (e.g., family history of asthma),[7–9] but the precise underlying mechanisms remain largely unknown. Furthermore, although between 30% and 50% of infants with LRTIs subsequently develop childhood wheezing illnesses,[9–11] it is currently not possible to identify infants at risk for these long-term outcomes. Nor do we understand why some develop asthma and others do not.

Club cell 16-kDa secretory protein (CC16) (also known as CC10, uteroglobin, urine protein 1, or formerly as Clara cell secretory protein) is a candidate biomarker of lung injury produced mainly by the club cells (formerly known as Clara cells) of the distal bronchioles and, to a lesser extent, by the

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prostate, kidneys, and endometrium.[12–14] CC16 is secreted in large amounts into the bronchial epithelial lining fluid, and it is thought to have anti-inflammatory, immunoregulatory, and immunosuppressive effects.[12,13,15] In healthy subjects, small amounts of CC16 leak across the respiratory epithelium into the blood, likely through passive diffusion.[14] It is hypothesized that different lung disease mechanisms may result in increments or declines in serum levels of CC16.[13,14] For instance, respiratory disorders that increase the epithelial permeability (e.g., acute respiratory distress syndrome,[16] exposure to proinflammatory agents,[17] or severe chest trauma[18]) have been associated with higher serum levels of CC16. On the other hand, respiratory illnesses leading to small airway inflammation (e.g., asthma,[19–24] exposure to tobacco smoke,[25–27] or chronic obstructive pulmonary disease[25,27–29]) have been associated with lower serum levels of CC16.

Because CC16 in blood is entirely eliminated through glomerular filtration, serum and urine levels of CC16 are highly correlated.[30] As a result, there has been a recent interest in the utility of urine CC16 as a potentially useful, cost-effective, and noninvasive biomarker for respiratory disorders.[30,31]

The authors have previously demonstrated a severity-dependent relationship between infant LRTIs and the risk of developing asthma.[8] As CC16 may be a marker of both lung injury and immune response, the authors hypothesized that urine levels of CC16 at the time of an infant LRTI are associated with the development of childhood wheezing illnesses. To test this hypothesis, data obtained as part of the Tennessee Children's Respiratory Initiative (TCRI), a study of infants enrolled during an acute respiratory illness and followed longitudinally for the development of respiratory sequelae, were analyzed.

Methods and Materials

Study design and setting

The TCRI is a prospective cohort of term, predominantly non–low birth weight, previously healthy infants ($n=630$) enrolled during a healthcare visit for an acute respiratory illness at a single academic institution. The rationale and methods for the TCRI have been reported previously.[32] In brief, eligible infants were recruited at the time of an unscheduled clinic visit, emergency department visit, or hospitalization for an acute upper ($n=175$) or lower ($n=455$) respiratory tract infection during four winter respiratory viral seasons (from September to May of 2004 to 2008). Follow-up of participants to ascertain childhood wheezing illnesses through age 6 years is ongoing. Mothers provided consent for both their participation and their infants'. This study was approved by the Institutional Review Board of Vanderbilt University (Nashville, TN).

Study procedures

During study enrollment, research nurses administered an in-person research questionnaire to the mothers to collect information on the infant's past medical history and current respiratory health, sociodemographic characteristics, maternal history of asthma, and current exposure to secondhand smoking (SHS). They also obtained bag urine samples (mostly from hospitalized infants), which were maintained in repository for later analyses (see below). Through an initial structured medical chart review, information was gathered on each infant's acute illness visit (including current symptoms, physical examination, past medical history, and hospital course). Final diagnoses were obtained through a second medical chart review conducted after the visit discharge. A diagnosis of LRTI was based on both (1) a physician's diagnosis and (2) documentation of symptoms with duration ≤10 days (including any two of the following: cough, nasal congestion, rhinorrhea, wheezing, dyspnea, or fever) and requirement for an acute care clinic visit, an emergency department visit, or hospitalization (including 23-h stay). A panel of pediatricians reviewed cases that were not clearly identified to establish the final diagnosis. The severity of the LRTI was determined using the bronchiolitis severity score (BSS).[33] The BSS is a slightly modified version from the one by Tal *et al*.[34] It incorporates information obtained through the initial chart review, and ranges from 0 to 12, with higher scores indicating more severe illness (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/ped). Infants enrolled are followed up on a yearly basis up to the age of 6 years (either in person or by a phone interview) to determine childhood wheezing illnesses using the validated International Study of Asthma and Allergies in Children (ISAAC) questionnaire.[35,36]

Selection of participants

Urine levels of CC16 were measured in 133/455 infants with LRTIs, as described below. These 133 infants were selected among those infants with LRTIs with urine samples ($n=342$) based on the quantity of available urine, and were included in analyses (Supplementary Fig. S1).

Validation and measurement of urine CC16

As a first step, a commercially available enzyme-linked immunosorbent assay kit for serum CC16 (BioVendor, Chandler, NC) in urine was validated. The validation experiments included spike and recovery, multiple freeze–thaw cycles, and intra-assay controls on urine samples. This same kit has been previously validated for urine in another study.[30] Next, urine levels of CC16 (ng/mL) were measured according to the manufacturer's recommendations. 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; see Methods in the Supplementary Data).

Statistical analyses

The predictor of interest was the urine level of CC16 at the time of an infant LRTI, as measured with the assay described above. The outcome of interest was parental report of subsequent childhood wheeze (defined as ≥1 episode of wheezing following the initial LRTI) at the 1-year follow-up visit.

Descriptive statistics are presented as frequencies (percent) for categorical variables and median (interquartile

range [IQR]) for continuous variables. Comparisons between groups were conducted with Pearson's chi-square or Mann–Whitney *U*-tests, as appropriate.

For the main analysis, logistic regression was used to assess the unadjusted and adjusted association between urine levels of CC16 and subsequent childhood wheeze. The variables to be included in the multivariable models (infant's age, sex, and current exposure to SHS) were selected *a priori* based on published literature.[12,26,29,37] First-order interactions were tested for between urine levels of CC16 and each of the covariates mentioned above.

A sensitivity analysis was also conducted treating the values below the LOD as missing values and applying multiple imputation methods (see Methods in the Supplementary Data). These results were then compared to the main analysis that assigned values below the LOD a value of one-half the LOD.

The urine levels of CC16 were transformed to a logarithmic (log2) scale for regression modeling analyses, and results were backtransformed for ease of interpretation. All statistical analyses were performed using R v3.1.0.[38] Statistical significance was defined as a $p < 0.05$.

Results

The baseline characteristics of the 133 infants with LRTIs included in the analyses are presented in Table 1. The majority of these infants were younger than 6 months of age, male, white, and enrolled at the time of a hospitalization. Most of them had a relatively high BSS, no history of maternal asthma or current exposure to SHS, and government health insurance for low-income families (Medicaid). There were no significant associations between the urine levels of CC16 at the time of an infant LRTI and any of the baseline characteristics, including BSS ($p > 0.05$).

Table 1. Baseline Characteristics of Infants with LRTIs (N = 133)

Age at enrollment (weeks)	11.0 (6.0–17.0)
Female sex	41 (31%)
Race/ethnicity	
Black	23 (17%)
White	85 (64%)
Hispanic	17 (13%)
Other	8 (6%)
Gestational age (weeks)	39 (38–39)
Birth weight (g)	3,345 (3,090–3,579)
Current exposure to SHS	29 (22%)
Maternal asthma	21 (16%)
Insurance type	
Private	54 (41%)
Medicaid	71 (53%)
None	8 (6%)
BSS	7.0 (4.5–9.0)
LRTI visit type	
Unscheduled outpatient visit	3 (2%)
Emergency department visit	21 (16%)
Hospitalization	109 (82%)
Urine CC16 (ng/mL)	1.0 (1.0–5.8)
Urine CC16 (ng/mg of creatinine)	12.5 (7.7–35.7)

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

LRTI, lower respiratory tract infection; SHS, second-hand smoking; BSS, bronchiolitis severity score; CC16, club cell 16-kDa secretory protein.

Compared with infants with LRTIs included in analyses, those with LRTIs not included were more likely to be female and to have been enrolled at the time of an unscheduled outpatient visit. They were also more likely to have a history of current exposure to SHS, no insurance, and a lower BSS. There were no other significant differences between infants with LRTIs included and not included in analyses (see Supplementary Table S2).

Of the 133 infants with LRTIs, 109 (~82%) had 1-year follow-up data (Supplementary Fig. S1). Of these, 61 (~56%) had parental report of subsequent childhood wheeze (with 35 [~57%] of them reporting ≥4 episodes of wheezing since the enrollment visit). The median (IQR) age for follow-up was 1.1 (1.0–1.9) years in those with subsequent childhood wheeze, and 1.1 (1.0–2.0) in those without ($p = 0.6$). The median (IQR) urine levels of CC16 (ng/mg of creatinine) at the time of an infant LRTI were 11.1 (7.7–20.1) for infants with subsequent childhood wheeze and 13.4 (8.3–61.1) for those without ($p = 0.11$; Supplementary Fig. S2). Infants with LRTIs without 2-year follow-up data were more likely to be Hispanic, to have a history of maternal asthma, and to have Medicaid insurance. There were no other significant differences between infants with LRTIs with and without 2-year follow-up data (see Supplementary Table S3).

In the main unadjusted analysis using the logarithmic transformation of the urine levels of CC16, a significant inverse association of the urine levels of CC16 at the time of an infant LRTI with the risk of subsequent childhood wheeze was found (unadjusted OR = 0.79 [95% CI 0.63–0.99], $p = 0.0497$). The adjustment for infant's age, sex, and current exposure to SHS did not weaken the association. In the main adjusted analysis, a twofold increase in urine levels of CC16 (ng/mg of creatinine) at the time of an infant LRTI was associated with ~30% decreased odds (adjusted OR = 0.74 [95% CI 0.56–0.98], $p = 0.04$) of subsequent childhood wheeze (Fig. 1). No significant first-order interactions between urine levels of CC16 and the *a priori* selected covariates were detected ($p > 0.05$ for each interaction term). Consistent results were obtained when analyzing urine CC16 without the creatinine correction (ng/mL; Table 2).

There were 75 (56%) samples with urine CC16 values below the LOD. A sensitivity analysis was conducted by treating the values below the LOD as missing data and applying multiple imputations methods, obtaining similar results when compared to the main analysis. In both the unadjusted and adjusted models using multiple imputations, the urine levels of CC16 were inversely associated with the risk of subsequent childhood wheeze (see Supplementary Table S4).

Discussion

Infants with LRTIs have substantially higher odds of subsequently developing childhood wheezing illnesses (including asthma) when compared with the general population,[4–11] but it is not currently possible to identify those at risk for these long-term outcomes. This lack of prognostic

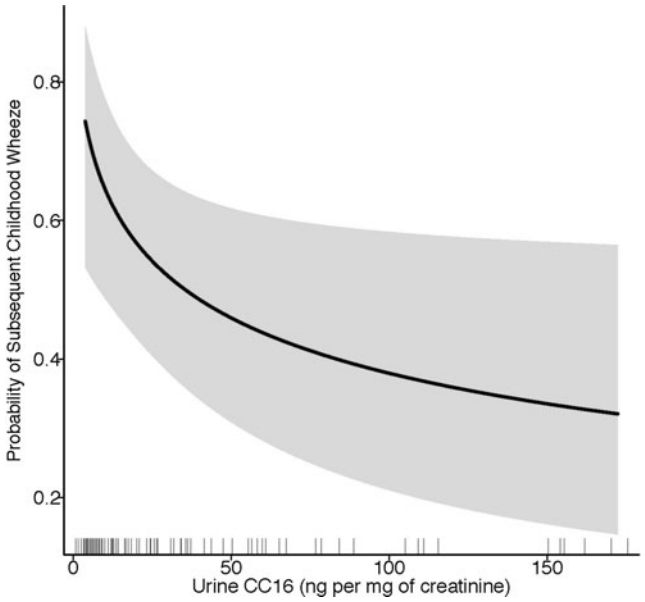

FIG. 1. Predicted probability of subsequent childhood wheeze by urine CC16 at the time of an infant LRTI. The solid black line represents the predicted probability values, and the shaded gray bands represent the lower and upper 95% confidence intervals. These estimates were obtained from a logistic regression model adjusted for infant's age, sex, and current exposure to second-hand smoking. CC16, club cell 16-kDa secretory protein; LRTI, lower respiratory tract infection.

data has led to substantial uncertainty among caregivers and healthcare providers as to which patients would benefit from preventive strategies, education, early intervention, or referral to a specialist. Thus, for both clinical and research purposes, noninvasive biomarkers of the development of childhood wheezing illnesses following LRTIs are needed.[9] Urine biomarkers offer several advantages compared with serum samples, as they simplify collection, reduce costs, and may decrease participant attrition.[30] In addition, biomarkers can shed light on the mechanisms of disease pathogenesis. In the present study, an inverse association was found between urine levels of CC16 at the time of an infant LRTI and the odds of subsequent childhood wheeze.

The precise function of CC16 is unknown, although it appears to protect the respiratory tract against epithelial injury, inflammation, and oxidative stress.[12,14] CC16 modulates the expression and/or activity of inflammatory proteins such as interferon γ, tumor necrosis factor α, and phospholipase A_2.[12,39–41] Phospholipase A_2 is involved in the arachidonic acid cascade, and indirectly controls the availability of prostaglandins and leukotrienes.[42] CC16 may also attenuate mucous secretion and suppress Th2 T-cell differentiation.[43,44] Following acute viral infections, transgenic mice deficient in CC16 develop a more severe lung inflammation with increased Th2 cytokine levels, airway reactivity, and mucous production, as well as prolonged viral persistence.[45,46] Reconstitution of CC16 in mice is able to reverse these altered phenotypes.[46] The CC16 gene maps to a genetic region (11q12–13) that has been linked to asthma and atopy in some studies.[47,48] The A38G polymorphism in the promoter region of this gene decreases the amount of expressed CC16 and has been found to be associated with wheezing disorders in children and adults.[19,21,22,49,50] Taken together, these findings support the concept that CC16 can have an important role in the pathogenesis of these diseases.

Table 2. Analysis of Urine CC16 at the Time of an Infant LRTI and Subsequent Childhood Wheeze Without Creatinine Correction

	OR [95% CI][a]	p-*Value*
Unadjusted		
Urine CC16 (ng/mL)	0.85 [0.68–1.07]	0.2
Adjusted[b]		
Urine CC16 (ng/mL)	0.76 [0.58–0.99]	0.04

[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 SHS.

Most[19–24] but not all[51] studies in children or adults have found an inverse association between serum levels of CC16 and prevalent asthma or its related phenotypes. In a recent cross-sectional study of 203 adult elite athletes, low serum levels of CC16 were also associated with a history of frequent upper respiratory tract infections.[52] To date, only a few pediatric studies have been conducted in this topic, all of which used serum levels of CC16 and had a cross-sectional design. In a study of 100 Australian children aged 0–18 years, asthmatics had lower serum levels of CC16 when compared with nonasthmatics.[19] Similar results were obtained in a study of 51 children aged 0–14 years in Greece.[20] Two other larger studies in Chinese children found lower serum levels of CC16 in children with childhood wheezing illnesses, although these findings were mostly present in children who were homozygous for the A38G polymorphism.[21,22] In a recent large prospective study of children in three different countries, low serum levels of CC16 at 4–6 years of age were associated with decreased lung function by 16 years of age, although the outcome of asthma was not assessed.[29] To the authors' knowledge, this is the first study to examine the relationship between urine CC16 and the development of childhood wheezing illnesses.

The mechanisms behind an inverse association between serum or urine levels of CC16 and childhood wheezing illnesses found in this and other studies can only be speculated on. In both LRTIs and childhood wheezing illnesses (including asthma), there can be substantial small airway inflammation with resultant damage of the club cells of the distal airways. This can lead to a decrease production of CC16 and, thus, lower amounts of this biomarker leaking across the respiratory epithelium into the blood as long as the epithelial permeability remains intact.[27] In other respiratory diseases where the epithelial permeability is severely affected (such as acute respiratory distress syndrome), a positive correlation between serum or urine levels of CC16 and these conditions may occur, as has been previously shown.[16]

The present study has considerable strengths, including a prospective design, the use of a commercially available test in urine samples, and an analytical approach accounting for potential confounders. Several limitations to the findings are also recognized. First, there were significant differences in

certain baseline characteristics between infants included and not included in analyses and those with and without follow-up data, which may suggest a selection bias. However, some of these differences can be explained by the study design, as urine samples were more likely available for hospitalized infants. Second, as is the case for any observational study, there could be residual confounding by measured or unmeasured variables. The variables to be included in the multivariable models (age, sex, and current exposure to SHS) were selected *a priori* based on the published literature.[12,26,37] Due to the relatively small sample size, more variables in the same multivariable models could not be included, but additional analysis replacing current exposure to SHS with other potential confounders (such as maternal asthma or BSS) did not change the results (data not shown). Urine levels of CC16 could be also affected by several other factors (e.g., post-renal excretion of CC16, diurnal variation, and physical activity).[13,30,53] However, it is unknown if these factors are truly important in children, as the limited data available are in adults.[13,14] Furthermore, urine levels of CC16 have been highly correlated to serum levels (which are less likely to be affected by some of these factors) in other studies using a similar instrument.[14,29,30] In the present study, similar results were obtained when analyzing urine CC16 with and without the creatinine correction, but there could still be residual confounding by the renal function and/or hydration status at the time of the urine sample. Third, there were a large number of values below the LOD. It was not possible to evaluate if this differs from other studies examining urine levels of CC16, as there are only a limited number of publications on this field and this information is rarely included in the results. To overcome this limitation, a sensitivity analysis was conducted using multiple imputation methods, obtaining similar results to those of the main analysis (which further supports the conclusions). Fourth, due to limited power, sensitivity or specificity analyses could not be conducted to evaluate the utility of urine CC16 for risk prediction assessment. Fifth, urine levels of CC16 could be a predictor of asthma independent of LRTIs. Sixth, longitudinal measurements of urine CC16 were not available. Lastly, although the results were significant, the overall strength of the association was relatively weak. Thus, it is unlikely that urine CC16 can be used as a standalone biomarker. Nonetheless, the data raise the possibility of using urine CC16 in combination with other clinical risk factors to select research participants at high risk of developing childhood wheezing illnesses following LRTIs and/or to understand better the mechanisms of disease development.

In summary, it was found that urine levels of CC16 at the time of an infant LRTI are associated with subsequent childhood wheeze. The results of the present study suggest that urine CC16 may be a useful, readily accepted, noninvasive biomarker of the development of childhood wheezing illnesses following infant LRTIs. Larger studies are needed to confirm the utility of urine CC16 as a predictive tool of childhood wheezing illnesses following LRTIs, establish standard values, and validate these findings in other populations.

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Author Disclosure Statement

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