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RESEARCH ARTICLE

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Serum Clara cell protein and atopic phenotype in children up to 2 years of age

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Background: Low value of serum Clara cell protein (CC16) is associated with bronchial hyperreactivity in children.

Objective: To evaluate the serum CC16 in relation to atopy and previously manifested LRTD.

Methods: In the population of 163 healthy 5- to 24-month-old children, atopy was determined by Phadiatop-infant (serum-specific IgE≥0.35 kUA/L), serum CC16 by ELISA, while data on previously manifested low respiratory tract diseases (LRTD) were collected from the Health Care Center database.

Results: In atopic children, serum CC16 negatively correlated with age (r −.281, P=.041, n=53), while in nonatopic children, this correlation was positive (r .200, P=.036, n=110). Atopic ≥8-month-old children with previously manifested LRTD had lower level of CC16 (3.07 ng/mL) in relation to atopic children without LRTD at the same age (6.51 ng/mL), P=.029 (value of serum CC16≥4.8 ng/mL indicates atopic phenotype without LRTD 75% sensitivity, 87.5% specificity). In 8- to 24-month-old children with previously manifested pneumonia, serum CC16 was lower in atopic (2.9 ng/mL) in relation to nonatopic children (3.7 ng/mL), P=.029 (serum CC16 ≤3.4 ng/mL indicating atopy in the group of children with pneumonia, sensitivity 100%, and specificity 77%). Atopic 8- to 24-month-old children with previously manifested pneumonia had lower CC16 in relation to other atopic children in this age (P=.021) (for cutoff CC16≤3.4 ng/mL sensitivity 100%, specificity 77%), and also often chronic wheezing (atopic with pneumonia 83.3%, n=5/6 vs atopic without pneumonia 21.4%, n=3/14), P=.018.

Conclusion: Low serum CC16 is associated with previously expressed pneumonia and chronic wheezing in atopic children.

KEYWORDS
allergy, asthma, biomarkers, Clara cell protein

1 | INTRODUCTION

Atopy in children is defined as a certain pattern of immune response that causes damage of the lower respiratory tract during viral respiratory infections, exposure to aeroallergens, or air pollution, with persistent inflammation, aberrant reparation, hyperresponsiveness of the bronchial tree, and remodeling disorder of the airways, which all together represent the pathogenesis of asthma.[1-3] However, only a minority of atopic children will ever develop asthma, and it is still unclear what predisposes young children to have one allergic phenotype or another.[4,5]

Clara cell protein (CC16) is a small anti-inflammatory protein secreted by nonciliated bronchiolar Clara cells that protects the respiratory tract against oxidative stress and inflammation.[6] CC16 increases

the half-life of surfactant and reduces the level of eicosanoids by inhibition of phospholipase A2 (PLA2).[7] CC16 deficiency is associated with an increased susceptibility to lung injury due to environmental factors, with the development of inflammation in airways and appearance of bronchial hyperreactivity in children.[8-10]

The purpose of this study was to evaluate serum CC16 in population of symptom-free children up to 2 years of age in relation to atopy and previously manifested LRTD. Children with allergic asthma, and with the occurrence of asthma before the age of three, have a reduced lung function at early school age,[11] so it is considered that changes in the airways that occur during early childhood cause the expression of severe forms of asthma.[2,12] The first hypothesis of this study was that there is a negative correlation of serum CC16 with age in atopic children (possibility of lung development alteration), without correlation in nonatopic group. Second hypothesis was that children with previously manifested LRTD have lower level of serum CC16 in relation to children without LRTD, regardless of atopy,[10,13] and third, that children with previously manifested severe LRTD had lower serum CC16 level, because Clara cells appear to play a role in maintenance of airway epithelium after injury.[14]

2 | MATERIALS AND METHODS

This study is a part of population-based observational study of atopy and atopic phenotypes in the population of children between 5 and 24 months of age that has been conducted by the Public Health Institute of Kragujevac at the territory of Central Serbia. This study includes randomly chosen symptom-free children at a preventive pediatrics examination prior to vaccination/revaccination, with the written consent of parents. Atopy was determined by a qualitatively multitest Phadiatop-infant[15] (serum-specific IgE≥0.35 kUA/L) (Immunocap-100 system, Phadia AB; Thermo Fisher Scientific, Waltham, MA, USA). The study did not include children with data on pediatrics examinations due to any illnesses during a period up to 3 weeks before the study, children who received any systemic therapy, and children who had previously had a history of urinary tract disease. In the study group, there were no children with bronchopulmonary dysplasia. The sample size for determination of serum CC16 (n=163) was obtained according to the values taken from the study of Lagerkvist BJ.[6] The study group consisted of 53 atopic children (n=28 atopic children with LRTD and n=25 atopic children without LRTD) and control group of 110 nonatopic children (n=61 with LRTD and n=49 without LRTD). Serum CC16 was determined by human Clara cell protein enzyme-linked immunosorbent assay (BioVendor, Laboratorni medicina a.s Modrice, Czech Republic), sensitivity 0.02 ng/mL, assay range 2-100 ng/mL. During laboratory processing, samples were numbered, so that the person performing the assay did not have any personal data nor the data on clinical manifestations among children.

Data on previously manifested respiratory diseases were obtained using the questionnaire and the database of the Primary Health Care Center according to the International Classification of Diseases (Acute bronchial diseases: J21—bronchitis and J20—bronchiolitis; chronic bronchial disease: J44.9—morbus chronicus opstructivus non specificata and J45—asthma bronchiale; and J12-18—pneumoniae). Category "with LRTD" referred to children with previously manifested LRTD (average time elapsed since the last LRTD was 2 months), while category "without LRTD" referred to children without any data on previously manifested LRTD. Category "chronic wheezing" referred to children with previously manifested chronic bronchial diseases (J44.9 and J45).

The software SPSS for Windows was used for statistical analysis, and G*Power 3.1.9.2 was used for determination of sample and effect size. The difference in the frequency of independent variables in relation to a dependent variable was determined by χ^2 test and Fisher's exact test, as well as contingency table 3×2. Correlation between continuous variables was determined with Spearman's test (for power 90%, eliminating type II error, atopic children n=53, minimal effect size was medium ρ=0.4 (critical t=2.0, δ=3.30), and for nonatopic children n=110, minimal effect size was small ρ=0.3 (critical t=1.98, δ=3.27)). To compare the concentration of the serum CC16 among atopic and nonatopic children and among children with different respiratory diseases, nonparametric tests Mann-Whitney and Kruskal-Wallis were used (for power 90%, eliminating type II error, min ARE distribution, N1=53 atopic, N2=110 nonatopic, minimal effect size was d=0.58, and for analysis with minimal simple size—for 8- to 24-month-old atopic children with pneumonia, N1=6 vs 8- to 24-month-old nonatopic children with pneumonia, N2=13, effect size for difference in serum CC16 was large d=1.84; critical t=2.13, δ=3.48). ROC curve was used to obtain a decision marker value of CC16 and age for LRTD, atopy and previously manifested pneumonia. Univariate analysis of variance was used to determine the effect of the age and pneumonia to low serum levels of CC16 in atopic children. We considered P value less than .05 as statistically significant in all analyses. Values of the serum CC16 concentration were shown as median, range, interquartile range, and number of cases.

2.1 | Ethical principles

The study was conducted in accordance with the ethical standards of the Helsinki Declaration. Taking biological material from children (serum) was conducted in a medical institution under the supervision of a pediatrician and with the written consent of parents. The study was approved by the Ethics Committee of the Public Health Institute in Kragujevac (No 01-5163/2010).

3 | RESULTS

Statistical parameters of serum CC16 in symptom-free children up to 2 years of age (n=163) were the following: median 4.3 mg/mL, range 16.1, minimum 1.6, maximum 17.7. The analysis of correlation between serum CC16 and age (Figure 1) shows that in atopic children, serum CC16 decreases with age (Sp ρ: −0.281, P=.041, n=53), while in nonatopic children increases (Sp ρ: 0.200, P=.036, n=110). In 5- to 7-month-old group of children, there was no difference in serum

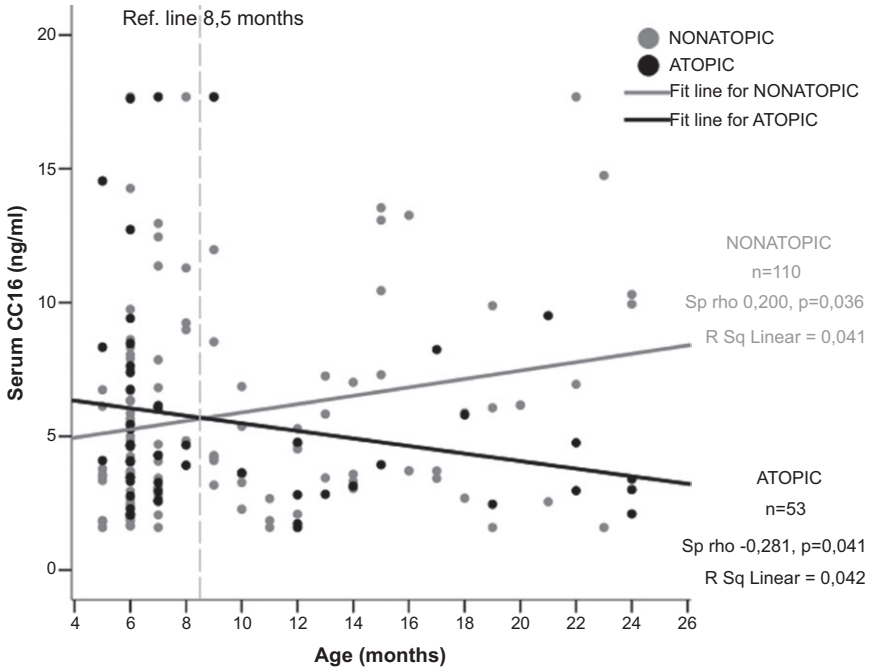


FIGURE 1 Correlation of serum level CC16 with age in atopic and nonatopic children

CC16 concentration between atopic and nonatopic children (Md=4.6, n=33 vs Md=3.9 ng/mL, n=58, *P*=.223), but in children between 8 and 24 months of age, atopic children had lower serum CC16 values (Md=3.5 ng/mL, range 16.1, interquartile range 1.95, n=20) in relation to the nonatopic children (Md=5.3 ng/mL, range 16.1, interquartile range 6.4, n=52,) at the same age (*P*=.036).

3.1 | Age, LRTD, and CC16

There were no statistically significant differences between atopic and nonatopic children with LRTD in the median age at the study time (10 months, n=28 vs 10 months, n=61; *P*=.929), number of LRTD (2× vs 2×; *P*=.253), as well as in median age of first LRTD (6 vs 6 months; *P*=.676), or in the median time that had elapsed since the last diagnosis of LRTD (2 vs 2 months; *P*=.753). Analysis of children's age and data on LRTD showed that children with LRTD (n=89) were older in relation to children without LRTD (n=74) (10 vs 6 months, *P*<.001; for cutoff ≥7.5-month sensitivity 62%, specificity 77%; area 0.742 CI95% 0.666-0.818), in atopic group (10 vs 6 months, *P*=.001) and in nonatopic group (10 vs 6 months, *P*<.001).

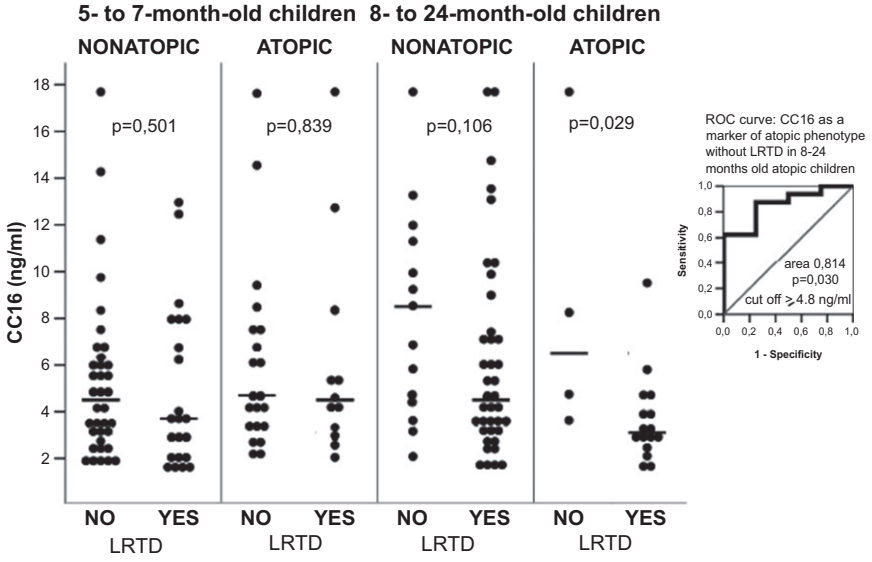
Atopic 8- to 24-month-old children with LRTD had lower serum CC16 values (Md=3.07 ng/mL, range 7.92, interquartile range 1.94, n=16) in relation to nonatopic children with LRTD at the same age (Md=4.53 ng/mL, range 16.1, interquartile range 4.03, n=39), Mann-Whitney *P*=.026. Atopic children with LRTD at 8-24 months of age also had lower serum CC16 values (3.07 ng/mL) in relation to atopic children without LRTD at the same age (6.51 ng/mL, 3.63-17.69; range 14.6, interquartile range 11.41, n=4), Mann-Whitney *P*=.029. In 8- to 24-month-old children with atopy, serum level CC16≥4.8 ng/mL indicates atopic phenotype without LRTD (75% specificity, 87.5% sensitivity, area 0.859). In atopic 5- to 7-month-old children, there was no difference in serum CC16 values related to LRTD (with LRTD CC16 Md=4.5 ng/mL, n=12 vs without LRTD CC16 Md=4.7 ng/mL, n=21, *P*=.839). Median values of serum CC16 related to atopy and LRTD in the age category (5-7 and 8-24 months) are shown in the table of the Figure 2.

3.2 | CC16 and type of LRTD

There was no correlation between serum CC16 and number of LRTD in atopic (Sp. ρ: −0.147, *P*=.455, n=28) or nonatopic group (Sp. ρ: 0.127, *P*=.330, n=61). In groups of children with different types of previously expressed LRTD (0=without LRTD, 1=wheezing, 2=pneumonia), in nonatopic children, there was a tendency of serum CC16 increase regardless of data on LRTD (statistically significant increase CC16 determined only in "without LRTD" group (Sp. ρ: 0.354, *P*=.013, n=49)), while in atopic children with previously expressed wheezing and pneumonia, there was a tendency of decreasing serum CC16 with age (Figure 3).

Kruskal-Wallis test showed that in nonatopic children, there was no significant difference in serum CC16 values between different types of previously expressed LRTD (0=without LRTD, 1=wheezing, 2=pneumonia), *P*=.755, while in atopic children, this difference was statistically significant, *P*=.040, but only in 8- to 24-month-old group (*P*=.023) (Figure 4), without any differences in 5- to 7-month-old atopic children (*P*=.975).

In 8- to 24-month-old children with previously manifested pneumonia, there was no difference between atopic and nonatopic children in the age of first diagnosis of pneumonia (9.5 vs 11 months, *P*=.831) or in the time that has elapsed since pneumonia (4 vs 4.5 months, *P*=.442), but atopic children with pneumonia at this age had lower level of CC16 (2.9 ng/mL, n=6, range 1.81, interquartile range 1.13) in relation to nonatopic children with data on pneumonia at the same age (3.7 ng/mL, n=13, range 16.1, interquartile range 6.45), Mann-Whitney



Label	LRTD	ATOPIC,Md (n)	NONATOPIC,Md (n)	*Mann Whitney p*
LRTD	Total	4,09 (53)	4,61 (110)	*0,605*
	YES	3,65 (28)	4,09 (61)	*0,324*
	NO	4,68 (25)	5,00 (49)	*0,719*
	Mann-Whitney p	*0,051*	*p=0,465*	

Age	LRTD	ATOPIC	NONATOPIC	*Mann Whitney p*
5-7 months	Total	4,61 (33)	3,90 (58)	*0,223*
	YES	4,45 (12)	3,66 (22)	*0,292*
	NO	4,68 (21)	4,47 (36)	*0,418*
	Mann-Whitney p	*0,839*	*0,501*	
8-24 months	Total	3,51 (20)	5,33 (52)	*0,036*
	YES	3,07 (16)	4,53 (39)	*0,026*
	NO	6,51 (4)	8,53 (13)	*0,956*
	Mann-Whitney p	*0,029*	*0,106*	

FIGURE 2 Serum CC16 in atopic and nonatopic children in relation to LRTD in different age categories

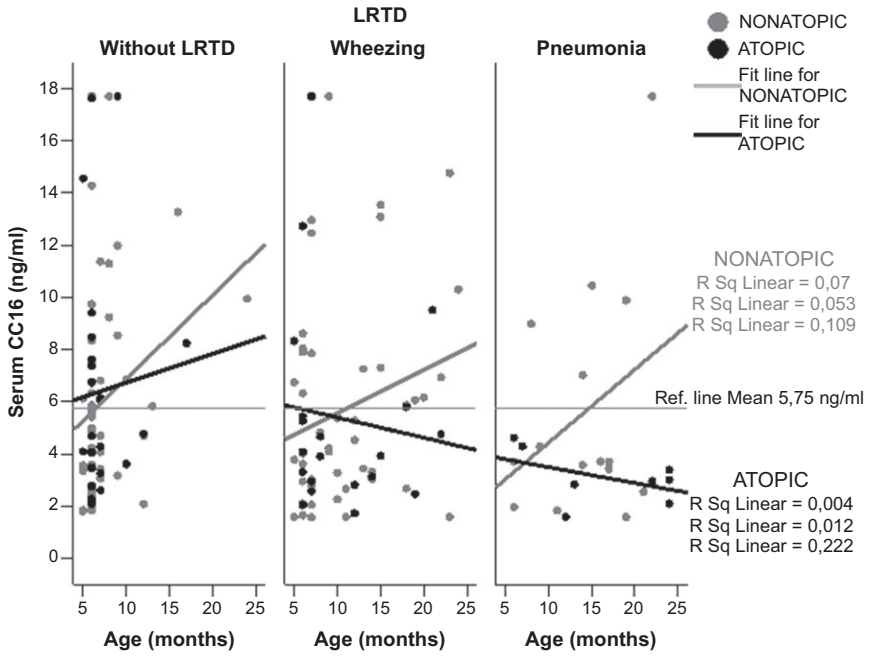


FIGURE 3 Serum CC16 in relation to age in atopic and nonatopic children without LRTD and with previously expressed wheezing or pneumonia

P=.029. Serum CC16≤3.4 ng/mL indicates atopy in the group of 8- to 24-month-old children with previously manifested pneumonia (sensitivity 100%, specificity 77%, and area 0.814).

In atopic children, univariate analysis of CC16≤3.4 ng/mL variance (dependent variable) ($F(3,49)=11.3$, $P<.001$) showed significance of age≥8 months ($P=.012$, η^2: 0.122), and mutual interaction

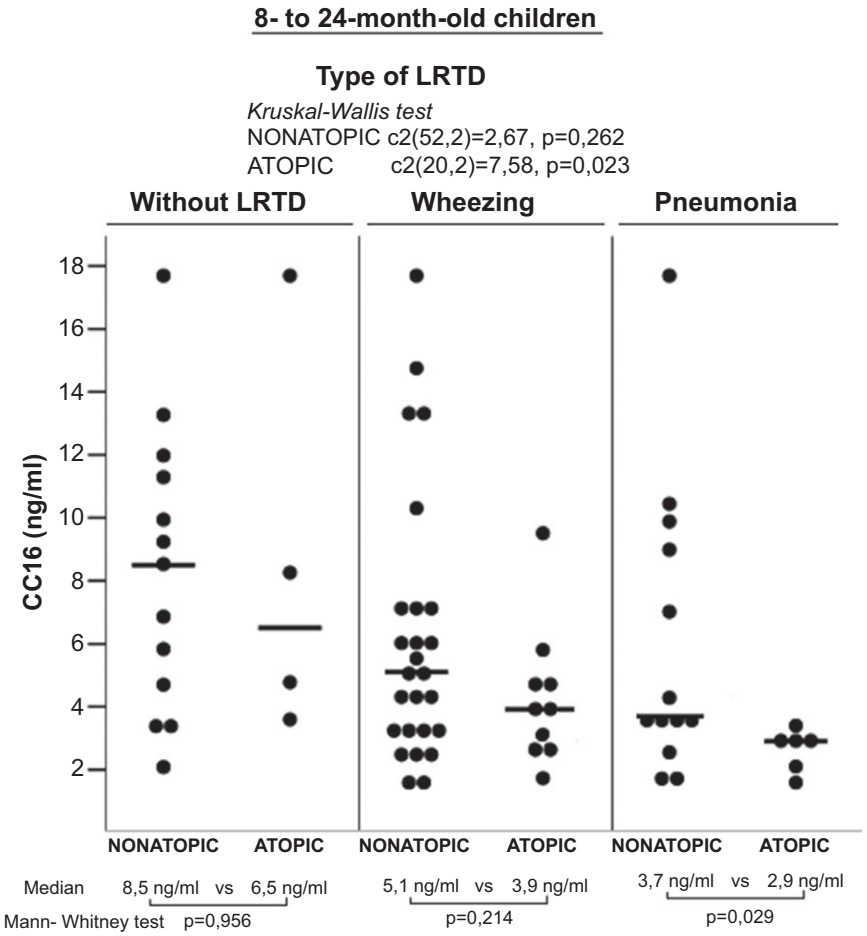


FIGURE 4 Serum CC16 in 8- to 24-month-old children with previously expressed different types of LRTD in relation to atopy

between age and pneumonia (P=.011, η^2: 0.124) without significance of only pneumonia (P=.272). Median age of children with CC16≤3.4 ng/mL was 12 months, while higher level of serum CC16 was found in atopic children at median age of 6 months (Mann-Whitney P=.013, area 0.700, for cutoff ≥11-month sensitivity 53%, specificity 82%).

3.3 | Low CC16 and atopic phenotype with chronic wheezing and pneumonia (early asthma-like phenotype)

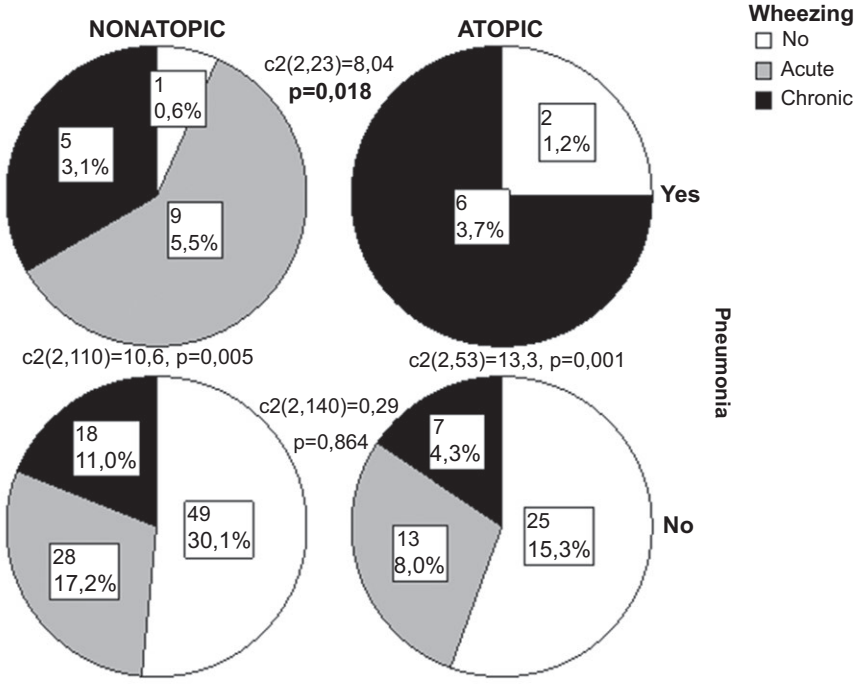
In the group of atopic children with pneumonia (n=8), chronic wheezing was manifested in 75% (n=6/8) and pneumonia without wheezing in 25% (n=2/8), while in the group of nonatopic children with pneumonia (n=15), there were data about acute wheezing in 60% (n=9/15), chronic wheezing in 33.3% (n=5/15), and pneumonia without wheezing in 6.7% (n=1/15) cases; Contingency table 2×3, P=.018, Cramers V 0.591 (Figure 5).

Atopic children with previously manifested pneumonia and chronic wheezing had lower level of serum CC16 in regard to nonatopic children with the same phenotype (P=.068), that was statistically significant in 8- to 24-month-old group (atopic Md=2.8 ng/mL, range 1.81, interquartile range 1.34, n=5 vs nonatopic 9.9 ng/mL, range 15.1, interquartile range 10.9, n=5, P=.047). In the group of 8- to 24-month-old children with previously manifested pneumonia and chronic wheezing, serum CC16≤3.4 ng/mL indicates atopy (sensitivity 100%, specificity 80%, area 0.880) (Figure 6).

Atopic 8- to 24-month-old children with data on pneumonia, chronic wheezing, and CC16≤3.4 ng/mL (considered as "low serum CC16 early asthma-like atopic endotype" at 8- to 24-month-old atopic children) (n=5) in relation to other atopic children with previously expressed other LRTD phenotypes (n=23) were the following:

- older (22 vs 7 months, Mann-Whitney P=.013, area 0.848, for cutoff ≥12.5-month sensitivity 80%, specificity 70%, for cutoff ≥21.5-month sensitivity 60%, specificity 91%),
- with older age of the first LRTD (9 months vs 5 months, P=.039, area 0.800, for cutoff ≥7.5-month sensitivity 80%, specificity 83%),
- with a larger number of LRTD (6× vs 2×, P=.004, area 0.891, for cutoff ≥3.5× sensitivity 80%, specificity 83%).

In the group of atopic 8- to 24-month-old children with the "low serum CC16 early asthma-like atopic endotype with pneumonia and chronic wheezing," temporal sequences of expression of LRTD were the following: first pediatric examination due to acute wheezing at 9 months of age, while chronic wheezing and pneumonia were diagnosed concurrently (less than 1 month interval), and median 4 months after the first wheezing.



Label	Categories	ATOPIC, n=53 Pneumonia YES n=8	ATOPIC, n=53 Pneumonia NO n=45	p	NONATOPIC, n=110 Pneumonia YES n=15	NONATOPIC, n=110 Pneumonia NO n=95	p
Type of wheezing	Chronic	**75% (6)**	15,6% (7)		33,3% (5)	18,9% (18)	
	Acute	0 (0)	28,9% (13)	**0,001**	**60% (9)**	29,5% (28)	**0,005**
	Non	25% (2)	55,6% (25)		6,7% (1)	51,6% (49)	

FIGURE 5 Distribution of different types of wheezing in atopic and nonatopic children in relation to pneumonia

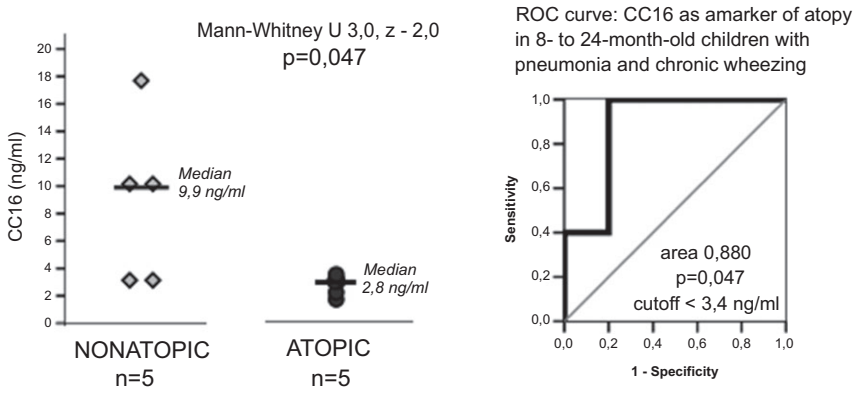


FIGURE 6 Serum CC16 in 8- to 24-month-old children with previously expressed pneumonia and chronic wheezing in relation to atopy

4 | DISCUSSION

This study has confirmed the hypothesis that serum CC16 decreases with age in atopic children, but also found age-dependent increases in nonatopic children. These results indicate that serum CC16 may also be an indicator of postnatal lung growth, as Clara cells appear to play an indispensable role in the lung development as critical airway progenitor cells.[16,17] During lung development, branching of distal bronchioles and alveolarization (present during first several years of life) are regulated by the expression of transcriptional factors, which also regulate the differentiation of Clara cells and production of surfactant in terminal bronchioles.[16] After an acute injury, the respiratory epithelium is capable of regeneration without remodeling or fibrosis,[18] and in this process, Clara cells proliferate and differentiate into ciliary epithelial cells in bronchioles, as well as in type 2 pneumocyte in alveoli, with maintenance of airway epithelium after injury.[17,18] It is known that air pollution exposures occurring during the critical periods of lung growth are especially damaging,[19] as well as that the airway epithelium in asthmatics functions abnormally and it is sensitive to oxidative damage.[20,21] Repeated damage of the airway barrier with agents associated with lowering of serum CC16 during childhood (air pollution, ozone, chlorine metabolites) may increase TH2 response and the

penetration of allergens in the lung, which leads to the development of atopic inhalant sensitization.[6,22-25]

Second hypothesis indicates that children with data on previously expressed LRTD have lower serum CC16 in relation to children without LRTD, confirmed in 8- to 24-month-old group of atopic children. The majority of asthmatics may be atopic, but only a minority of those with atopy or atopic disease (including those reactive to inhaled allergen) will ever develop asthma.[4] High anti-inflammatory airway capacity, with high level of CC16 serum, in older subgroup of atopic children may be the reason for absence of clinical manifestation of LRTD. Our results indicate that serum CC16≥4.8 ng/mL at ≥8 months of age may be the marker of atopic phenotype without LRTD, but these "atopic phenotypes without LRTD" are determined only in one-fifth of atopic group at 8-24 months of age. However, there was no difference in serum CC16 in relation to manifestation of LRTD in younger atopic children (probably due a still large lung developmental potential in ≤7 months of age), as well as in all nonatopic children.

Meta-analysis that included 10 relevant studies on the impact of polymorphism of CC16 on asthma risk showed an atopy-independent increased risk for developing asthma in CC16 genotype associated with lower level of serum CC16,[10,13,25], but significance of CC16 as a biomarker of acute or chronic pulmonary diseases is still a matter of research and dispute.[26] In this study, there was no difference in serum CC16 between atopic and nonatopic children with only wheezing phenotype, although there is a tendency of age-dependent decrease in serum CC16 in atopic and increase in nonatopic children. These results indicate that serum CC16 must be evaluated according to age, type of LRTD, and atopy, due to different causes of low serum CC16 in younger nonatopic children with wheezing (for example smaller caliber of the airways in transient wheezing[2]) that increases with age, and on the other site, low serum CC16 in older atopic children (may be due to lung development alterations with weaker anti-inflammatory response), which continues to decrease with age. This study showed that in ≥8-month-old children with previously manifested pneumonia, atopic children had lower serum CC16 in relation to nonatopic children (decision marker CC16≤3.4 ng/mL). Predictors of CC16≤3.4 ng/mL in atopic group of children were age, and interaction of age with pneumonia. In the subgroup of atopic children with low serum CC16, weaker anti-inflammatory epithelial potential may be a primary state (atopic children with low CC16 were 12 months old), and as a result, later in life, they may have manifestation of severe LRTD such as pneumonia with chronic course of wheezing (in children at the median age of 22 months). Weaker anti-inflammatory response of the respiratory bronchial epithelium to common environment agents in subgroup of atopic children with low serum CC16, as well as the lack of Clara cell-dependent regeneration after infection or injury may be the basis of circulus vitiosus that leads to early genesis of asthma.

Pediatric asthma has been of great interest of researching efforts to find a valuable time to interrupt remodeling, but there is also the heterogeneity of pathogenesis leading to remodeling in asthma.[27,28] Disturbance of airways reparation is considered as one of the possible mechanisms for development of airway remodeling in childhood [29] that might be present early in life regardless of long-standing inflammation.[5] Thinning of basal membrane of the airway epithelium (initial macroscopic sign of remodeling) was not observed in children with wheezing under the age of 1 year,[30] and the risk period for the formation of airway's remodeling could be the second year of life.[31] Clara cells at the bronchiolar alveolar duct junction (BADJ) have been proposed to serve as bronchiolar alveolar stem cells.[32,33] Chronic respiratory damage reduces the proliferative potential of Clara cells and leads to their transdifferentiation into mucus-producing cells,[7,16,17] as well as to peribronchiolar fibrosis independent of myofibroblast proliferation or differentiation.[33] Development of subepithelial fibrosis in primary and secondary alveolar septum during early childhood may also be associated with impairment of permeability of alveo-capillary membrane in large areas of the lungs with reduced leakage of CC16 in circulation.[34] However, further studies are needed to define the serum CC16 as a marker of early airway remodeling.

Allergic sensitization early in life is an important risk factor for persistent wheezing and asthma development, but not all children who wheeze early in life will have asthma later in life.[2,5] Early segmentation of children with asthma and other wheezy disorders remains the main research challenge today [35] and serum CC16 may help in differentiation of infants with transient wheezing from children who will develop asthma.[10,13] Significant increase in serum CC16 during lung development in nonatopic children with appropriate bronchial anti-inflammatory response due to greater recovery potential of Clara cells progenitor pool after injury and adequate development of imunoregulatory mechanisms in the lung [36,37] may be associated with overgrowth of wheezing later in life. Atopy-associated alterations in lung development (end-organ alterations) are considered as one of the critical steps in the pathogenesis of asthma.[3,4] Our results showed that in atopic children, there is an association of pneumonia, chronic bronchial diseases, and low serum CC16 (≤3.4 ng/mL) and all that together may indicate early atopic asthma-risk endotype that is determined in 3.1% of children up to 2 years of age, that is, 9.4% atopic children, that is, in 17.9% of atopic children with LRTD. Low CC16 asthma-risk atopic endotype in ≥12.5-month-old atopic children is characterized with first LRTD at ≥7.5 months of age and higher number of LRTD episodes (≥4×), wherein pneumonia and diagnosis of chronic bronchial diseases were manifested concurrently, in the interval less than 1 month.

Our results showed that for early determining the "low CC16 asthma-risk atopic endotype," it is necessary to design a study including children in the second year of age, along with taking samples from symptom-free children before vaccination/revaccination, measuring serum-specific IgE and CC16 as well as the categorization of LRTD by type (acute, chronic wheezing and pneumonia), and not only by the number of LRTD. Correlation of serum CC16 with available and accessible biomarkers of inflammation and immunoregulatory response in children, as well as humoral response to infection in the critical period of lung development during early childhood, remain to be determined.

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