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Original Article

Periostin and IFN-γ levels in serum and nasopharyngeal aspirate in infants with viral-induced wheezing – 2 year follow-up

Sirma Mileva[1], Ekaterina Ivanova-Todorova[2], Kalina Tumagelova-Yuzeir[2], Trifonova T. Ivelina[3], Neli S. Korsun[3], Venelin A. Alexiev[1]

[1]Pediatric Clinic, University Hospital Alexandrovska, Medical University, Sofia, Bulgaria; [2]Laboratory of Clinical Immunology, University Hospital "St. Ivan Rilski", Medical University, Sofia, Bulgaria; [3]Department of Virology, National Center of Infectious and Parasitic Disease, Sofia, Bulgaria.

ABSTRACT

Background. The present study assesses the immune response in children with viral-induced wheezing by examining the two factors-interferon-gamma (IFN-γ) and periostin in serum and nasopharyngeal aspirate (NPA). The aim was to find a pattern with the severity and frequency of wheezing episodes.

Methods. Sixty-nine infants (40 boys and 29 girls), with a mean age of 11.4±6 (2 - 23) months, hospitalized with a first or recurrent episode of bronchial obstruction were enrolled in this study. The serum and NPA concentrations of IFN-γ and periostin were assessed by ELISA methodology. Fifty of the children (72%) were followed for 2 years.

Results. We detected lower NPA IFN-γ production in boys, infants with atopic status, family history of asthma, and respiratory syncytial virus infection. Recurrent wheezing in children was associated with a twice lower concentration of IFN-γ in NPA compared to those with the first episode (7.1 vs. 14.8 pg/ml, p=0.05). Higher serum periostin level was established in children over 12 mo in the group of recurrent wheezers with persistent manifestations compared to those without symptoms during the follow-up (410.5 vs. 269.7 ng/ml, p = 0.03). Multivariate logistical regression model assessed high level of serum periostin, male gender, atopy, family history of asthma, and severity of the attack as significant risk factors for persistent compared to intermittent wheezing ($r^2 = 0.87$, p = 0.04).

Conclusions. Our results demonstrated that recurrent viral-induced wheezing is associated with decreased IFN-γ production and increased periostin response and their correlation with severity and persistence of symptoms were the main outcome measures.

Key words: viral-induced wheezing, periostin, interferon-gamma (IFN-γ).

It is a challenge to determine the likelihood of recurrent wheezing in infants. It remains unclear what differentiates children with the first episode of wheezing from those who will switch to asthma in the future. Among the most common reasons for viral-induced wheezing is respiratory syncytial virus (RSV). Although our understanding of its immunopathogenesis is increasing, there are still no objective criteria to assess the risk for asthma, and results are limited and controversial.

The theory of imbalance in the T-helper 1 / T-helper 2 (Th1/Th2) immune response is the most widely accepted. In primary RSV infection, a type I response develops in which natural killer (NK) cells and Th1 lymphocytes are sources of interferon-gamma (IFN-γ), a major cytokine of cell-mediated immunity. IFN-γ inhibits allergic reactions and is an important molecule in antiviral protection. The lack of sufficiently

✉ Sirma Mileva
sirmamileva@yahoo.com

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strong polarization of T cells in the Th1 direction may allow the development of an unregulated Th2 response in RSV infection. In recent years, periostin has been used as a systemic biomarker for the Th2 immune response and eosinophilic inflammation.[1] Periostin is an extracellular protein, first described in 1993, after extraction from the periosteum of mice.[2] The role of periostin in Th2 inflammation and asthma in many aspects, includes eosinophil accumulation, increased mediator expression, and airway remodeling.[3]

The study aimed to assess the IFN-γ and periostin levels in serum and nasopharyngeal aspirate (NPA) in wheezing infants and to find a pattern with the severity and frequency of wheezing episodes.

Material and Methods

This prospective observational single-center study was carried out in the Pediatric Department of Alexandrovska University Hospital with the recruitment of patients for a one-year period from February 2018 to March 2019. Sixty-nine infants, aged 2 to 23 months, hospitalized with a first or recurrent episode (≥3) of bronchial obstruction were enrolled in this study. The inclusion criteria for the study were as follows: age 2–24 months, admission due to an acute bronchial obstruction diagnosed clinically based on tachypnea, expiratory dyspnea, prolonged expiration, and wheezing, born in gestational week 35 or later. Infants born less than 34 weeks gestation, those with bronchopulmonary dysplasia, congenital anomalies, cystic fibrosis, and other concomitant diseases (cardiac and neurological) were excluded.

We divided the patients into two groups, according to assessment:

- 30 children (44%) with one episode of bronchial obstruction up to 2 years of age, conditionally accepted as "internal control group"- first wheezing (FW) group.

- 39 children (56%) with three or more episodes of wheezing up to 2 years of age - "recurrent wheezing" (RW) group.

Medical history was collected at admission: family history of asthma (maternal, paternal, and first-line siblings); own atopic status - atopic dermatitis, allergy to cow's milk protein and other food allergens; duration, frequency, and severity of wheezing episodes. The disease severity was classified by Wainwright score[4] as mild in 5 (7%) cases, moderate in 53 (77%), and severe in 11 (16%) patients based on pulse oximetry, respiratory rate, and respiratory effort.

Paraclinical assessment at baseline included standard blood counts (white blood cells, differential count, C-reactive protein), nasopharyngeal aspirate (NPA), and blood sample collection done on the day of admission. Concentrations of periostin and IFN-γ in serum and NPA were determined by commercial enzyme-linked immunosorbent assay (ELISA, ABclonal, and R&D Systems) according to the manufacturer's directions. Detection limit for periostin was 0.004 ng/ml and 8.0 pg/ml for IFN-γ respectively. The viral etiology of the respiratory tract infections was determined using polymerase chain reaction (PCR) for a panel of 11 respiratory viruses (RSV A/B, human metapneumovirus (hMPV), influenza, and parainfluenza virus type (PIV) 1/2/3, rhinovirus (RV), adenovirus (AdV) and bocavirus (BoV)). Viral nucleic acids were extracted automatically from respiratory specimens using a commercial ExiPrep Dx Viral DNA/RNA kit (Bioneer, Korea). The detection and typing of influenza viruses were carried out by a real-time reverse transcriptase (RT) PCR method and the SuperScript III Platinum® One-Step qRT-PCR System (Invitrogen, Thermo Fisher Scientific, USA). The detection of RSV, hMPV, PIV 1/2/3, RV, AdV, and BoV was performed using singleplex real-time PCR assays and an AgPath-ID One-Step RT-PCR kit (Applied Biosystems, Thermo Fisher Scientific, USA).

The families were contacted by phone for information about new respiratory events and conducted treatment. Fifty of the parents (72%) responded to our phone survey and were prospectively followed-up during the next 2 years, the other 19 refused to be involved in the observation series.

The study protocol was approved by the Institutional Ethics Committee of the Sofia Medical University (No. 2781) and informed consent was obtained from the parents.

Statistical data processing was performed with the statistical package SPSS Version 23. The following were used: descriptive analysis, statistical dependence between qualitative variables (cross tabulation), parametric (t-test), and non-parametric methods (Mann-Whitney U-test, H-test of Kruskal-Wallis). The correlation of periostin and IFN–γ with the clinical variables was determined using Spearman's rank correlation coefficient. The odds ratio (OR) and 95% confidence interval (CI) were reported. Predictors of persistent wheezing were analyzed using a stepwise logistic regression model, with an inclusion $p < 0.05$ and an exclusion $p > 0.10$. The diagnostic values of IFN–γ and periostin levels to identify children with persistent wheezing were determined by receiver operating characteristic (ROC) curve analysis. Sensitivity and specificity were calculated for the selected cut-off point.

Results

Descriptive statistics

A total of 69 children with an average age of 11.4 months (range 2 to 23 months, SD ± 6 months) were enrolled. There was a slight predominance of boys in the gender distribution – 40 (58%) vs. 29 girls (42%). The full characteristics of the study subjects are outlined in Table I. We established a difference in the baseline demographic characteristics between the two groups – FW and RW, in terms of a higher age of enrolment, lower age of first wheezing, male gender prevalence, and older siblings in the RW group.

There were no differences between groups in terms of onset of symptoms, fever, indicators of inflammatory activity, and severity. Virological examination demonstrated RSV etiology of infection in 40 children (58%), RV in four, BoV virus in three, one child with hMPV, and 21 (30%) were negative.

Fifty of the children (72%) were followed for 2 years. Twenty-six (52%) of them were with persistent symptoms. The distribution by groups showed that in the RW group 23/33 (70%) were with persistent wheezing (RWP) compared to 3/17 (17%) in the FW group – OR 9.33 (7.3-39.4), p =0.001. As risk factors for persistence of manifestations were established: male gender – 20/26 (77%) vs. 12/24 (50%) OR=3.33 (0.99-11.21), p=0.04 and wheezing during the next 12 months, after the hospitalization – 69% (24/35)

Table I. Demographic characteristics.

	n (%)	Males	Average Age (months)	Family history of asthma	Atopy	Age of first wheezing (months)	Siblings
First wheezing (FW)	30 (49%)	12/30 (35%)	9 (2-23)	12/34 (35%)	5/34 (15%)	9.6 (3-23)	10/34 (29%)
Recurrent wheezing (RW)	39 (51%)	28/39 (80%)	11 (5-17)	13/35 (37%)	6/35 (17%)	6.7 (2-13)	23/35 (65%)
p =		0.001	0.001	0.5	0.5	0.003	0.003

of wheezers during the first year of follow-up were with persistent symptoms vs. 0/15 (0%) from patients without symptoms for that period, OR= 3.1 (1.95-5.19).

IFN-γ in serum and NPA

The mean IFN-γ level in serum was 14.28 (0-100.3) pg/ml and 10.93 (0-124.14) pg/ml in NPA. There was no relationship between the concentration of IFN-γ in serum and NPA. An increase in serum IFN-γ levels was observed with age – children over 18 months had 3 times higher IFN-γ 37,77 vs. 10.46 pg/ml, p=0.02. We detected lower IFN-γ in NPA in boys and infants with RSV infection. Recurrent wheezing in children was associated with a twice lower concentration of IFN-γ in NPA compared to those with the first episode (Table II). When distributed by gender, the male group is with lower IFN-γ NPA levels from the beginning (the first episode of wheezing), but not significant – boys 10.60 vs. girls 13.27 pg/ml, p=0.6. This discrepancy achieved statistical significance in the recurrent wheezing group – NPA IFN-γ levels: boys 5.48 vs. girls 15.11 pg/ml, p=0.008.

Periostin in serum and NPA

The mean serum periostin level was 418.73 (0-602.57) ng/ml. There was a significant inverse age correlation – periostin in serum decreased with age, Spearman r = - 0.51, p=0.005, Figure 1. The most significant decline was after 18 months, almost twice.

Table II. Comparison of IFN- γ and periostin levels in serum and NPA according to clinical variables in 69 participants.

	IFN-γ, serum (pg/mL)	P-value	Periostin, serum (ng/mL)	P-value	IFN-γ NPA (pg/mL)	P-value	Periostin NPA (ng/mL)	P-value
Age		**0.02**		0.005		0.2		0.1
2-12 mo	10.46		445.74		9.65		2.15	
12-18 mo	11.43		410.93		6.91		1.22	
>18 mo	37.77		239.44		7.07		0.01	
Gender		0.7		0.2		**0.007**		0.7
Male	10.00		419.85		7.03		1.81	
Female	17.34		404.40		16.07		1.74	
Atopy		0.8		0.1		0.2		0.9
Yes	10.94		440.81		7.01		1.79	
No	24.32		404.98		11.50		1.73	
Family asthma		0.4		0.1		0.1		0.3
Yes	20.29		443.05		8.77		1.62	
No	9.99		390.75		12.01		2.09	
Disease severity		0.6		**0.02**		0.4		0.4
Mild	17.20		401.80		1.60		1.60	
Moderate	15.15		422.41		1.12		1.12	
Severe	14.00		448.78		1.48		1.48	
Respiratory viruses		0.7		0.7		**0.01**		0,15
RSV	9.61		477.34		4.99		1.74	
Other than RSV	12.81		356.21		14.4		3.36	
Wheezing groups		0.5		0.3		**0.05**		**0.01**
FW	16.50		440.65		14.8		0.65	
RW	11.66		382.12		7.1		2.84	

FW: first wheezing, IFN-γ: interferon-gamma, NPA: nasopharyngeal aspirate, RW: recurrent wheezing

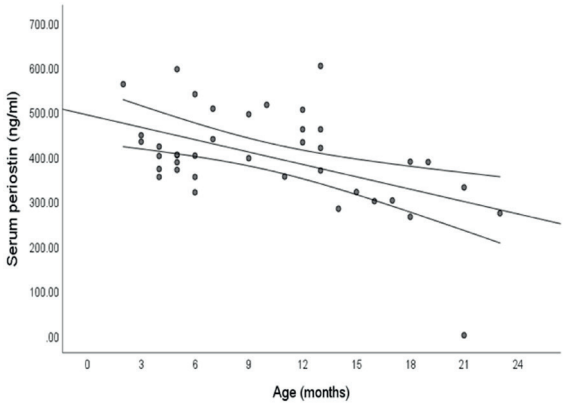

Fig. 1. Inverse correlation of the serum periostin by age.

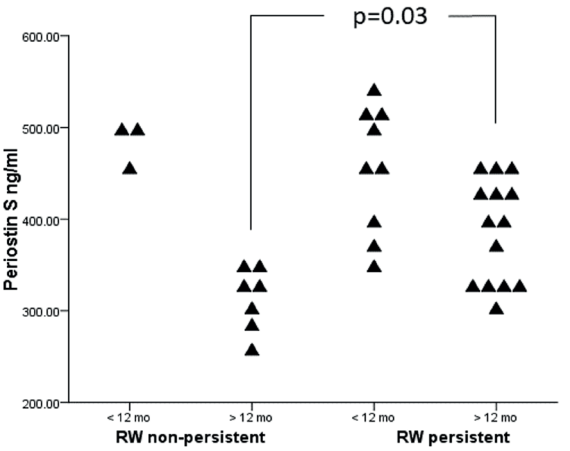

Fig. 2. Serum periostin level in recurrent persistent wheezers and non-persistent groups according to age distribution.

No differences in periostin levels were found according to gender. Slightly higher periostin concentration was reported in children with a family history of asthma, and atopy, but without statistical significance. A correlation between the higher serum periostin and severity of disease was observed, see Table II.

The mean periostin level in NPA was lower compared to serum - 1.75 (0-32.9) ng/ml. There was no correlation between periostin levels in serum and NPA. No significant differences in periostin NPA concentration were found according to age, gender, family history of asthma, atopic status, and severity of clinical manifestation. Children with RW were with a higher level of periostin in NPA- 2.84 vs. 0.65 ng/ml in the FW group, p = 0.01, see Table II.

We established higher serum periostin levels in children over 12 mo in the group of recurrent wheezers with persistent manifestations RWP (+) compared to those without symptoms during the follow-up RWP (-) - 410.5 vs. 269.7 ng/ml, r = 0.50, p = 0.03, as can be seen in Figure 2.

Risk factors for persistent wheezing

The analysis of risk factors for persistent wheezing during the 2 years follow-up by univariate logistic regression did not find a statistically significant difference in relation to the age, virus etiology, and NPA IFN-γ levels, Table III. The following factors were identified as potential risk factors: males with OR (95% CI) of 3.33 (0.99-11.21), p = 0.04, atopic status with OR (95% CI) of 3.11 (0.71-13.51), p=0.1, family history of asthma with OR (95% CI) of 2.33 (0.74-7.34), p=0.1, disease severity with OR (95% CI) of 4.05 (0.75-21.9), p=0.1, and serum periostin with OR (95% CI) of 5.62 (1.72-12.5), p=0.001, see Table III.

Multivariable regression and ROC analyses for wheezing persistence

The risk factors to predict persistent wheezing, with a p-value of 0.1 for inclusion, were further analyzed by logistic multivariable regression and by receiver operating characteristic (ROC) curve. High level of serum periostin, male gender, atopy, family history of asthma, and severity of the attack was significantly associated with persistent compared to intermittent wheezing, as can be seen in Table IV. Analysis, using ROC curves, identified the decreased NPA IFN-γ levels (AUC: 0.65, 95% CI: 0.51-0.78, p=0.03) with sensitivity 77% and specificity 67% respectively, and serum periostin with the best cut-off value of 310.92 ng/mL (AUC: 0.80, 95% CI: 0.45-1.00, P < .001) obtained with 81% sensitivity and 67% specificity respectively, see Figure 3.

Table III. Comparison of data between patients with and without persistent wheezing during the follow-up.

	PW (-) n=24 (%)	PW (+) n=26 (%)	OR (95% CI)	p
Age (mo)	10.42 (±6.35)	13 (±5.79)	1.07 (0.97-1.29)	0.2
Sex (males)	12 (50%)	20 (59%)	3.33 (0.99-11.21)	0.04
Atopy	3 (12%)	8 (31%)	3.11 (0.71-13.51)	0.1
Family asthma	8 (33%)	14 (54%)	2.33 (0.74-7.34)	0.1
Severe disease	2 (8%)	7 (27%)	4.05 (0.75-21.9)	0.1
RSV (+)	16 (67%)	14 (54%)	0.58 (0.17-1.83)	0.3
RW	10 (42%)	23 (88%)	0.73 (2.51-45.81)	0.001
IFN-γ serum (pg/mL)	14.26 (0-100.3)	11.38 (0-32.8)	1.11 (0.92-1.34)	0.3
IFN-γ NPA (pg/mL)	10.12 (0-61.2)	7.34 (0-49)	1.59 (0.95-3.02)	0.2
Periostin Serum (ng/mL)	292.5 (256.0-602.5)	411.3 (273-488)	5.62 (1.72-12.5)	0.001
Periostin NPA (ng/mL)	1.63 (0-11.44)	3.03 (0-32.9)	1.04 (0.93-1.38)	0.4

PW(+), patients with persisten wheezing episodes; PW(−), patients without persistent wheezing episodes, data are expressed as number and % of positive cases, mean ± SD or median (minimum-maximum).

Table IV. Multivariate logistical regression model on the involved in the follow-up patients for risk of persistence in children with recurrent wheezing.

	Univariate		Multivariate	
	OR (95% CI)	p	OR (95% CI)	p
Age (mo)	1.07 (0.97-1.29)	0.2		
Sex (males)	3.33 (0.99-11.21)	0.04*	2.98 (0.84-10.55)	0.009
Atopy	3.11 (0.71-13.51)	0.1*	2.44 (0.53-11.17)	0.2
Family asthma	2.33 (0.74-7.34)	0.1*	1.5 (0.48-4.65)	0.3
Severe disease	4.05 (0.75-21.9)	0.1*	0.46 (0.093-2.35)	0.35
RSV (+)	0.58 (0.17-1.83)	0.3		
RW	0.73 (2.51-45.81)	0.001**		
IFN-γ serum (pg/mL)	1.11 (0.92-1.34)	0.3		
IFN-γ NPA (pg/mL)	1.59 (0.95-3.02)	0.2		
Periostin Serum (ng/mL)	5.62 (1.72-12.5)	0.001*	4. 22 (1.55-11.82)	0.004
Periostin NPA (ng/mL)	1.04 (0.93-1.38)	0.4		

NPA: nasopharyngeal aspirate, RW: recurrent wheezing

Discussion

Pediatricians need biomarkers that allow early recognition of infants at risk of persistent wheezing. We examined two factors, IFN-γ-associated with viral immune response, and periostin- responsible for the Th2 immune reaction.

The age-dependent reduction in serum IFN-γ level that we observed - three times lower in the age group below 18 mo, suggests more frequent and severe respiratory infections in small infants. Reduced local interferon concentrations would be expected to permit increased viral replication, greater cytopathic effects, and increased shedding into the respiratory tract.[5,6]

Early studies have demonstrated a lower production of interferon in nasal washes during RSV infections when compared to other viral infections.[7] Our results confirm that RSV infection is associated with a significant decrease in IFN-γ responses in the NPA. This is in agreement with other authors and their suggestions for the suppression of Th1 cytokine response in the respiratory tract during RSV infections.[8] The protective role of IFN-γ in RSV

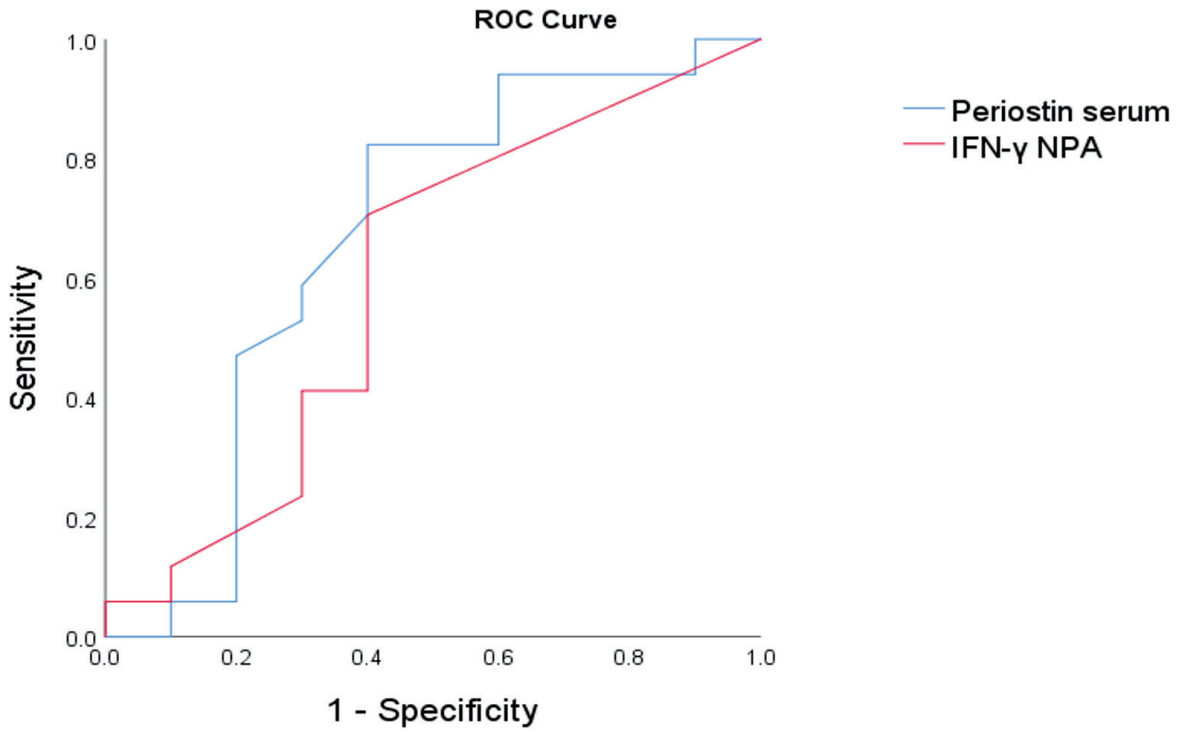

Fig. 3. ROC curve analyses of the sensitivity and specificity for the prediction of persistent wheezing by IFN-γ NPA (AUC: 0.65, 95% CI: 0.51-0.78, p=0.03) and serum periostin (AUC: 0.80, 95% CI: 0.45-1.00, P < .001).

infection can explain the association between reduced IFN-γ production and lower systemic proliferative responses to the severity of the disease and the need for mechanical ventilation.[9]

There is a lot of data that lower IFN-γ production is not a temporary condition, it was either present prior to bronchiolitis or induced by bronchiolitis and persists.[5,7,8] Renzi et al.[5] have shown that infants with the lowest IFN-γ production at the time of bronchiolitis are the most likely to develop asthma afterward and have abnormal pulmonary function later.

Twice the lower levels of IFN-γ in nasopharyngeal aspirate reported in our male group could be speculatively accepted as one of the reasons why males belong to the risk group for recurrent wheezing. In support of the proven risk factors for asthma, we and other authors have demonstrated reduced IFN-γ in NPA to viral infection in children with atopy and a family history of asthma, regardless of the viral agent.[10]

Recurrent wheezing according to our results was associated with a twice lower concentration of IFN-γ in NPA compared to those with the first episode and the lowest values were found in the male group. This confirms the concept that decreased levels of IFN-γ at early age determine susceptibility to common viral diseases and an altered viral immune response which is a risk for atopic asthma.[11] The imbalance between Th1 and Th2 responses in early life may create opportunities for the development of a Th2 response and the absence of a normal IFN-γ producing CD8+ T-cell response may skew towards a Th2 phenotype.[12]

During the last years, periostin was imposed as a systemic biomarker for the Th2 immune response and eosinophilic inflammation.[13] High osteoblast activity during linear growth in childhood causes extremely high levels of periostin- approximately 2- to 3-fold higher in children than in adult levels, with the highest values up to two years of age.[13] We observed

an inverse correlation between serum periostin and age with a double-declining after 18 mo of age.

García-García et al.[14] showed increased concentrations of periostin in NPA during viral bronchiolitis in RSV, HRV, HBoV, and hMPV-infected infants compared to healthy controls.

High levels of nasal periostin in infants with bronchiolitis suggest that respiratory viruses alter the immune response to Th2 and prove the presence of eosinophilic inflammation even at an early age.[15] These results indicate that periostin could be a biomarker for the prognosis of bronchiolitis. This was confirmed by data for high levels of periostin in children with severe pulmonary hypertension compared to those with mild form during RSV bronchiolitis.[16]

The observed positive relationship between severity of bronchoobstructive events and serum periostin levels in our study indicates that severe infection correlates with an altered profile of mediators to the Th2 immune response. Periostin plays a role in neonatal lung remodeling as well, because prolonged hyperoxic lung injury upregulates the expression of periostin, which in turn stimulates ectopic accumulation of myofibroblasts, followed by alveolar simplification.[17]

The role of periostin in asthma and type 2 inflammatory responses is an area of active research. Anderson and colleagues[13] demonstrated that high serum periostin level at the age of 2 years is a risk factor for asthma by school age. Results of several previous childhood studies have demonstrated an association between serum periostin levels and asthma. Multivariable logistic regression analysis demonstrated an association between serum periostin levels and asthma severity in children a value of 52 ng/mL emerged as the best cut-off level to differentiate children with severe asthma with high sensitivity and negative predictive values.[18]

The significantly higher values of periostin in the nasopharyngeal aspirate that we obtained in children with recurrent wheezing compared to those with the first attack, support the possible role of this protein in asthma development (2.84 vs. 0.65 ng/ml $p = 0.01$). We established higher serum periostin levels in children over 12 mo in the group of recurrent wheezers with persistent manifestations RWP (+) compared to those without symptoms during the follow-up RWP (-) (410.5 vs. 269.7 ng/ml, $r = 0.50$, $p = 0.03$). Our ROC curves analysis determining persistent wheezing children identified decreased IFN-γ NPA levels (AUC: 0.65, 95% CI: 0.51-0.78, p=0.03) with 77% sensitivity and 67% specificity, and serum periostin (AUC: 0.80, 95% CI: 0.45-1.00, $P < .001$) with the best cut-off value of 310.92 ng/mL obtained with 81% sensitivity and 67% specificity. Results of multivariable logistic regression analysis demonstrated an association between high serum periostin level, male gender, atopy, family history of asthma, and severity of the attack with a significant risk of persistent compared to intermittent wheezing, $r^2 = 0.87$, $p = 0.04$.

The mechanism of periostin's action is associated with its pleiotropic effects on airway epithelial cell function and on the development of airway fibroblasts, which promote airway remodeling in children with asthma.[19] Periostin appears to contribute to several pathogenic processes in asthma, including subepithelial fibrosis, eosinophil recruitment, and mucus production from goblet cells.[20] This is the reason why the gene coding for periostin is among one of the most highly up-regulated genes in asthma.[21] The increased secretion of periostin in the respiratory epithelium in children with asthma, compared to atopic and healthy controls, supports the hypothesis that periostin gene expression leads to subepithelial remodeling even in the growing pediatric lung.[19]

According to a lot of authors, periostin may be a useful biomarker for asthma diagnosis in children.[22,23] First, it allows patients with asthma to be distinguished from controls and is especially useful for a target small age group, with recurrent wheezing, who are unsuitable for lung function testing or FeNO

measurement.[22] On one hand, serum periostin level is accepted as a predictor of impaired forced expiratory volume 1 (FEV1) in asthmatic children and is comparable with FeNO in its usefulness as a biomarker for the diagnosis of pediatric asthma.[22,23] On the other hand, due to the insufficient data for the periostin levels in healthy children, without an accepted normal range, the study's interpretations are limited and contradictory, especially for preschool age.[3,24,25]

Contrary to the majority of reports in the study conducted by Castro-Rodriguez et al.[24], there wasn't a significant difference in periostin levels between preschool children with positive and negative asthma predictive index. Similar are the results in a more recent study that explores the role of periostin in young children with wheezing episodes for the prediction of asthma development. They did not confirm periostin as a predictive factor for future asthma in young children.[25]

There are several limitations of the current study that should be acknowledged. Selection of the examined children, with the severity of symptoms requiring treatment at a hospital, does not allow to draw generalized conclusions. As a limiting factor, we also consider the lack of a control group, which hinders a broader analysis of the established correlations. The short follow-up period of 2 years does not allow us to verify the observed correlations in children with a confirmed diagnosis of asthma. Long-term follow-up with repeated measurements over time is necessary in order to verify the diagnostic and prognostic value of these parameters and to confirm that the changes are not temporary events and could persist.

Our results demonstrated that recurrent viral-induced wheezing is associated with decreased IFN-γ production and increased periostin response and their correlation with severity and persistence of symptoms were the main outcome measures. Assessing the antiviral immune response would allow the identification of risk groups in children and the creation of new, more effective preventive strategies and therapeutic behaviors. The practical significance of the problem, as well as the limited number of studies in childhood, are a prerequisite for active scientific interest.

Ethical approval

The study was approved by the Ethics Committee of the Sofia Medical University (No.2781).

Author contribution

The authors confirm their contribution to the paper as follows: study conception and design: SM, EIT; data collection: KTY, SM, IT; analysis and interpretation of results: SM, NK; draft manuscript preparation: VA, SM. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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