

RESEARCH LETTER

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Resolvin D1 and miR-146a are independent distinctive parameters in children with moderate and severe asthma

To the Editor,

To date, a large number of mediators and biomarkers have been detected in childhood asthma, with a potential role in monitoring the disease course. In particular, the increased expression of proinflammatory markers, such as exhaled nitric oxide (eNO), sputum eosinophils and serum interleukin-8 (IL-8), showed clinical relevance having been correlated with lack of symptom control, despite the regular controller treatment.¹ Lung function evaluation through spirometry, forced oscillation technique and multiple breath washout proved its role in asthma monitoring, especially for investigating small airway impairment in children with more severe symptoms.² Nonetheless, the relationships between airway inflammation, lung function and asthma severity in children need further investigation.

Recently, novel specialized pro-resolving mediators (SPMs), such as resolvins and lipoxins, have been recognized as crucial in promoting the resolution of lung inflammation.³⁻⁵ Within the family of resolvins, resolvin D1 transduces its biological action by interacting with two G protein-coupled receptors, G protein-coupled receptor 32 (GPR32) and lipoxin (ALX)/N-formyl peptide receptor (FPR)-2⁶; the latter receptor is shared with lipoxin A₄ (LXA₄).⁵ A decreased expression of ALX/FPR2 receptor, along with reduced levels of LXA₄, has been reported in the induced sputum of children with severe asthma (SA), indicating a dysregulation in pro-resolution pathway related to LXA₄-ALX/FPR2 axis.⁵ The involvement of resolvin D1 in asthma severity has not been investigated in children so far.

Increasing evidence suggests that miRNAs are crucially entailed in asthma. Indeed, lower miR-34a, miR-92b and miR-210 levels in nasal lavage have been associated with reduced FEV₁/FVC and FEF₂₅₋₇₅ in children with SA, suggesting their involvement in regulating airway inflammation.⁷ Moreover, miR-146a has been suggested as a potential molecular regulator for eosinophilic Th2 endotypes of asthma, given that a higher plasmatic level was found in mild-moderate asthmatic children than in healthy controls.⁸ It was reported that resolvin D1 regulates miR-146b, another member of the miR-146 family.⁹ To date, no clinical studies evaluated the potential implications of resolvin D1 and miR-146a for disease severity in asthmatic children.

Partial least squares discriminant analysis (plsDA) is a modern technique for multiparametric analysis that allows summarizing the multidimensional structure of many predictors, by identifying

uncorrelated "latent components" (LCs) of decreasing importance.¹⁰

This technique has been mainly used to define metabolomic profiles in children with asthma,¹¹ whereas no study applied plsDA to characterize children with moderate asthma (MA) and SA. Thus, the purpose of this study was to establish through plsDA the implications of resolvin D1 and miR-146a, along with proinflammatory and lung function parameters, for characterizing children with MA and with SA.

Herein, we show preliminary results obtained in a subgroup of children enrolled in an ongoing study (ClinicalTrials.gov ID: NCT02433275) approved by the local Ethics Committee (protocol no. 8-2014); informed consent was obtained from all parents before study entry. The inclusion criteria were as follows: age 6-11 years; controlled MA or SA according to GINA (www.ginasthma.org); regular controller therapy with inhaled corticosteroids (ICS) for at least 6 months; and no respiratory infections in the last month. The exclusion criteria were as follows: chronic comorbidities and active smoking. Sixteen MA children and sixteen SA children matched for age (9.7 ± 2.2 years old) and gender (10 males and six females) were included. Demographic characteristics were recorded. All children underwent clinical examination, atopy assessment and blood sample collection for measuring eosinophil and neutrophil counts, total IgE concentration, and serum pro-inflammatory (IL-8) and pro-resolving (resolvin D1 and miR-146a) parameters.

Determinations of serum resolvin D1 and IL-8 concentration were performed using commercial ELISA Kits. Total RNA was purified from serum using miRNeasy Serum/Plasma Kit; cel-miR-39-3p was used as spike-in. miR-146a-5p and cel-miR-39-3p were reversely transcribed using specific primers and the TaqMan miRNA RT Kit. qRT-PCR of miR-146a-5p transcript was carried out on StepOnePlus Real-Time PCR System using specific TaqMan microRNA assays. The results were expressed as $2^{-\Delta CT}$. ΔCT values were obtained subtracting the CT values of cel-miR-39-3p from the CT values of the miR-146a-5p in order to normalize the miR-146a-5p CT for each sample.

eNO was measured "offline" by an electrochemical sensor following international recommendations. Spirometry was performed according to ATS/ERS guidelines; z-scores were calculated. Twenty-three children (72% of the whole study sample, 11 with MA and 12 with SA) also underwent spirometry and filled the Childhood Asthma Control Test (C-ACT) at a three-month scheduled visit. Respiratory impedance was measured using the Quark i2m[®] Forced Oscillation Measurement system; the z-score for the area under the reactance curve (AUCX) was recorded. Multiple breath washout was performed according to ATS/ERS guidelines; z-scores for the Lung

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Clearance Index (LCI) and functional residual capacity (FRC) were recorded.

A plsDA model was run with two LCs, for which the percentages of variance explained for the predictors (χ^2R^2 , where X refers to the panel of potentially distinctive features) and the outcome (χ^2R^2 , where Y refers to asthma severity) were calculated. Spearman's ρ was calculated for each parameter to allow interpretation of the first two LCs. Feature contribution was considered good if the variable importance in the projection (VIP) was larger than one. Statistical analyses were performed with R 3.6.1.

Resolvin D1 and miR-146a levels, along with eosinophil and neutrophil counts and FEV₁/FVC, were significantly different in MA and SA (Table S1). The first two LCs (cumulative $\chi^2R^2 = 28\%$) explained a highly significant percentage of asthma severity ($\chi^2R^2 = 76\%$, permutation test P -value $< .001$). The first LC (LC1, $\chi^2R^2 = 14\%$) was positively correlated with resolvin D1 and FEV₁/FVC, while it was negatively correlated with eosinophil count and FVC. The second LC (LC2, $\chi^2R^2 = 14\%$) was positively correlated with neutrophil count, FEV₁, FVC and FRC, while it was negatively correlated with miR-146a, FEV₁/FVC and LCI (Figure 1). Resolvin D1 had the highest ability to characterize MA and SA; miR-146a, eosinophil and neutrophil counts and FEV₁/FVC had a fair ability too (VIP ≥ 1 , Figure 1). Figure 2A highlights a marked group separation.

Concerning children who underwent the three-month scheduled visit, mean ΔC -ACT was 0.65 (data not shown). ΔFEV_1 z-scores were calculated and compared (t test) between children with low and high resolvin D1 values (below and above the median of 80 pg/mL) and between children with low and high miR-146a (below and above the median of 0.0124 2^{- ΔCT}). Mean ΔFEV_1 z-scores were significantly different between children with low and high resolvin D1 values (low = -0.44 vs high = 0.48, $P = .028$, Figure 2B), while mean ΔFEV_1 z-scores did not change significantly between children with low and high miR-146a (low = 0.22 vs high = -0.24, $P = .289$, Figure 2C).

Our study showed that resolvin D1, associated with LC1, exhibited the highest discriminant ability in distinguishing MA and SA. The latter, although clinically controlled, displayed an unresolved airway inflammation as a possible result of a defective generation of resolvin D1. This finding confirms previous evidence indicating a reduction in SPMs in uncontrolled airway inflammation in children with SA.⁵

miR-146a was associated with LC2, suggesting that it is an independent distinctive parameter in MA and SA children. Previous studies reported that plasma miR-146a concentrations correlated with blood eosinophil levels and higher doses of ICS, suggesting an up-regulation of miR-146a related to asthma severity.⁸ In our study, FRC, LCI and neutrophil count correlated with miR-146a (LC2) but not with resolvin D1 (LC1). Indeed, FRC and LCI are functional parameters related to small airway disease and to structural lung abnormalities. Thus, the relationship between these two parameters and miR-146a might be useful to early track children with progressive airway remodelling. Concerning the correlation of miR-146a with neutrophils, previous data demonstrated a negative association between miR-146a expression and the number of neutrophils in the airways¹² and our data confirmed that the reduced expression of miR-146a may contribute to the development of neutrophilic phenotype of asthma.

Although disease severity has been recognized to have a great influence on serum IL-8 levels, in the current study IL-8 concentrations were found to have a poor discriminant ability, likely due to the clinically well-controlled status of the enrolled patients.¹³

Among lung function parameters, only FEV₁/FVC showed a fair discriminant ability. Indeed, a relatively larger decrease in FEV₁ compared with FVC is recognized to be more indicative of an obstructive pattern than decreased FEV₁ alone. Accordingly, FEV₁/FVC has been recognized as one of the most meaningful functional parameters in children with persistent asthma.¹⁴ Notably, resolvin D1 exhibited a

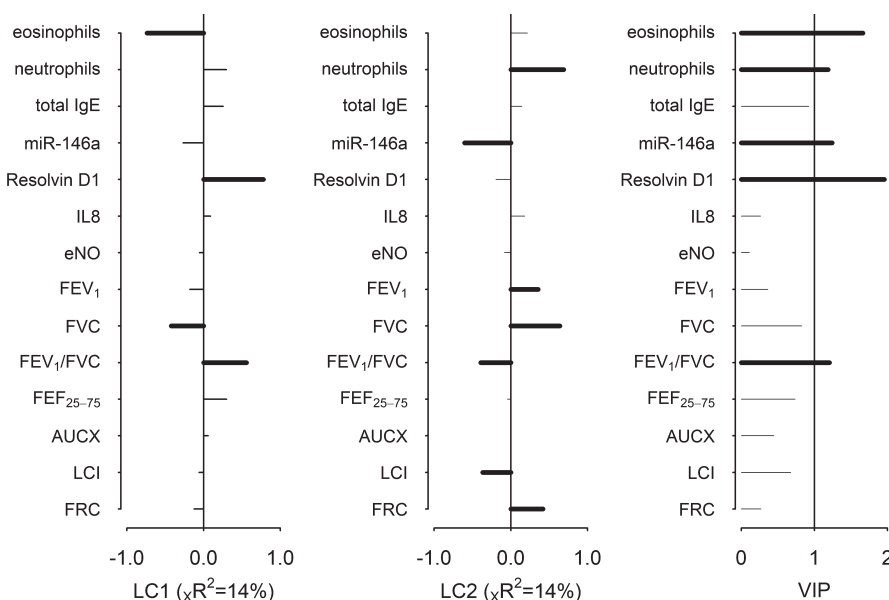


FIGURE 1 Correlations between the two latent components (LCs) from the plsDA model and the panel of parameters, and scores of variable importance in the projection (VIP). Significant correlations ($P \leq .05$) and good variable contributions ($VIP \geq 1$) are in bold

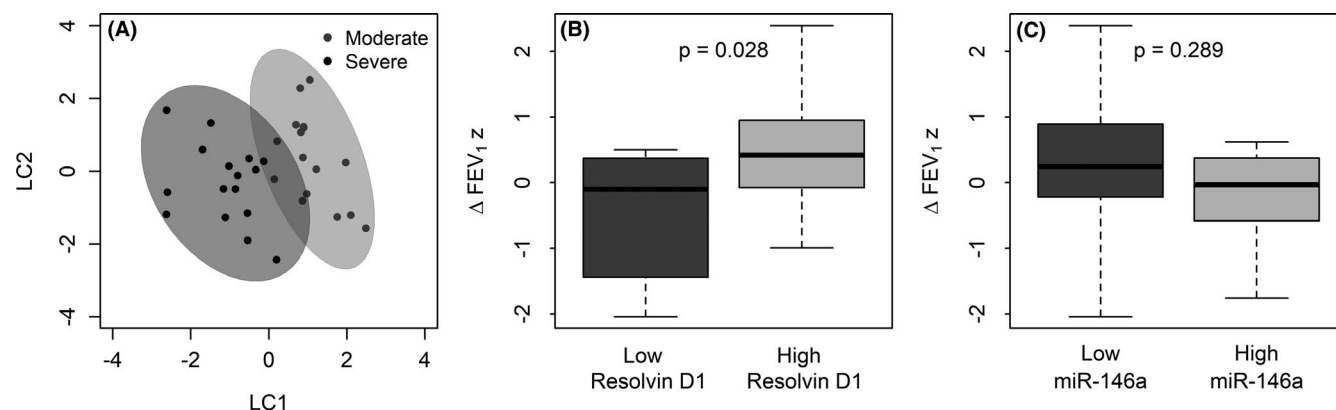


FIGURE 2 A, Scatter plot of the two latent components (LCs) from the plsDA model and 95% confidence ellipses for moderate (light grey points) and severe (dark grey points) asthmatic children. B, Box plot of ΔFEV_1 z-score in children with low and high resolvin D1 values (below and above the median of 80 pg/mL). C, Box plot of ΔFEV_1 z-score in children with low and high miR-146a (below and above the median of $0.0124 \cdot 2^{-\Delta\text{CT}}$)

potential for predicting short-term lung function modification, as suggested by its relationship with ΔFEV_1 z-scores.

To our knowledge, this is the first report addressing the possible implications of serum resolvin D1 and miR-146a in discriminating asthma severity in children. However, the main limitation of this study is the small sample size. Indeed, models trained using many features and few samples can be prone to overfitting, that is they may have very good performances (goodness of fit) on the training set but poor out-of-sample performances. For this reason, in this study, the significance of $\sqrt{R^2}$ was demonstrated through a permutation test.¹⁰

In conclusion, plsDA identified distinctive features in children with controlled MA and SA. In particular, resolvin D1 had the highest ability in distinguishing these two conventional phenotypic groups of asthmatics and exhibited a potential for predicting short-term lung function variation in children who report a well-controlled status.

Although we acknowledge that the role of other SPMs in the regulation of asthma inflammation and control should be further investigated, our findings suggest that an insufficient generation of resolvin D1 may contribute to an unbalanced inflammation in asthmatic children whose spirometry parameters reveal short-term variation. miR-146a could identify further endotypes in both MA and SA, who may be more prone to long-term remodelling processes leading to lung function impairment. Further investigations are warranted to confirm these preliminary results.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

RG and GF contributed to the acquisition and interpretation of data, drafting of the manuscript and final approval. SF contributed to the analysis and interpretation of data, drafting of the manuscript and final approval. SDV contributed to the acquisition and interpretation of data, drafting of the manuscript and final approval. EP and

SLG contributed to conception and design, interpretation of data, drafting of the manuscript and final approval. All the authors are accountable for all aspects of the work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Table S1. Children characteristics by asthma severity.

	Moderate n=16 (50%)	Severe n=16 (50%)	p-value [§]
Age, years, mean (SD)	9.6 (2.4)	9.8 (2.2)	0.804
Male gender, no. (%)	10 (62.5)	10 (62.5)	1.000
Age at diagnosis, years, mean (SD)	7.7 (2.4)	7.1 (2.3)	0.448
Atopy [†] , no. (%)	12 (75.0)	15 (93.8)	0.333
Current passive smoke, no. (%)	3 (18.8)	6 (37.5)	0.433
Treatment, no. (%)			0.484
ICS alone	2 (12.5)	0 (0.0)	
ICS-LABA	14 (87.5)	16 (100.0)	
ICS (FP) dose, $\mu\text{g}\cdot\text{day}^{-1}$, range	250-400	500-1000	-
≥ 2 attacks in the last year, no. (%)	3 (18.8)	6 (37.5)	0.433
>0 hospitalizations ever, no. (%)	3 (18.8)	5 (31.2)	0.685
C-ACT score, mean (SD)	24.0 (2.3)	23.8 (2.5)	0.766
Eosinophil count,	367.21 (118.95)	519.45 (139.25)	0.002
Neutrophil count,	3311.97 (808.60)	2782.16 (613.71)	0.045
Total Immunoglobulin E (IgE), IU/ml	392.89 (295.55)	249.93 (196.75)	0.118
miR-146a, $2^{-\Delta\text{CT}}$	0.013 (0.004)	0.018 (0.008)	0.042
Resolvin D1, pg/ml	160.56 (74.16)	60.78 (53.66)	<0.001
Interleukine-8 (IL-8), pg/ml	5.97 (7.91)	5.98 (12.26)	0.999
Exhaled nitric oxide (eNO), ppb	18.06 (13.82)	18.57 (11.89)	0.912
FEV ₁ , z-score	-0.76 (0.97)	-0.62 (1.07)	0.709
FVC, z-score	-0.66 (1.19)	-0.22 (1.15)	0.295
FEV ₁ /FVC, z-score	0.05 (0.84)	-0.65 (0.97)	0.038
FEV ₂₅₋₇₅ , z-score	-0.53 (0.75)	-0.95 (1.02)	0.197
AUCX, z-score	0.33 (0.88)	0.08 (1.27)	0.523
LCI, z-score	1.04 (1.72)	1.56 (1.49)	0.360
FRC, z-score	0.91 (1.22)	1.15 (1.78)	0.662

ICS: Inhaled Corticosteroids; LABA: Long-Acting Bronchodilators; FP: Fluticasone Propionate; C-ACT: Childhood Asthma Control Test. [†]At least one positive skin prick test among: *Dermatophagoides* mix, grass mix, *Parietaria judaica*, olive, dog and cat dander, alternaria, and *Blattella germanica*, plus a positive (histamine 1%) and a negative (saline) control. [§]t-test for quantitative variables, exact Fisher's test for categorical variables; significant p-values are in bold.