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Blood eosinophils relate to atopy and not to tissue eosinophils in wheezing children

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To the editor

Asthma is a heterogeneous disease, with diverse underlying pathophysiology, usually characterized by an inflammatory response of the airways, often eosinophilic, which may differ among patients (1, 2). There is a current need for accessible indicators predicting eosinophilic airway inflammation for an accurate asthma management and monitoring response to biologic therapies. Several indicators of airway eosinophilia have been proposed including BAL, sputum and blood eosinophils, BAL ECP, serum IgE and FeNO (3-5). Blood eosinophils are currently used as a proxy for airway eosinophils to differentiate eosinophilic and non-eosinophilic phenotypes even if their accuracy is at best modest. Several studies, demonstrating discordance between airway and systemic eosinophilia, have questioned the clinical utility of these biomarkers in predicting asthma outcomes (3-7). ICS therapy and atopy could be further confounding factors. The relationship between blood and tissue eosinophils has been scarcely investigated in children (8), in particular with regard to its prognostic value.

In an attempt to clarify the accuracy of blood eosinophils and other T2 markers (BAL-eosinophils, BAL-ECP, serum IgE) as indicators of airway eosinophilia, we investigated a cohort of 103 children undergoing bronchoscopy with bronchial biopsies for appropriate clinical indications (58 wheezing/45 non-wheezing; details in Appendix Table S1).

Based on published data a threshold of 23 eosinophils/mm² was used to define airway eosinophilia in bronchial biopsies (9-11). Since there is no agreement on thresholds for blood eosinophilia in asthma, we considered a cut-off corresponding to the 75^o percentile of our cohort (367 eosinophils/μl). Tissue eosinophilia was present in 42 out of 58 (72%) wheezing children (W-TisEos⁺) and there were no differences in demographic and clinical characteristics between W-TisEos⁺ and those with low tissue eosinophils (W-TisEos⁻, n=16, Table1A). Importantly both W-TisEos⁺ and W-TisEos⁻ showed airway remodeling features of asthma (basement

membrane thickening and bronchial epithelial loss) when compared to children without wheezing (Figure 1A, B). Not surprisingly eosinophil degranulation in the airways was higher in W-TisEos⁺ than in W-TisEos⁻ ($p < 0.0009$, Table 1A)

The accuracy of blood eosinophils, serum IgE, BAL eosinophils and ECP in predicting tissue eosinophilia was examined by ROC analyses and Spearman rank's correlation coefficient. All four indicators had similar predictive accuracies, which were overall modest (AUC range 0.56-0.64, Figure 1C, Table S2, Table S3) and were weakly correlated to tissue eosinophils (r values ranging from 0.22 to 0.34, Figure S1). Furthermore, when we limited ROC analyses to wheezing children only none of the four potential indicators examined could predict tissue eosinophilia. As seen in Figure 1D only if blood eosinophils were above 367 cell/ μ l was it highly probable that tissue eosinophilia would also be present. Conversely, lower levels of blood eosinophils did not predict low tissue eosinophils: children with blood eosinophils below 100 cell/ μ l did have significant airway eosinophilic inflammation.

We then compared wheezing children with high blood eosinophils (W-BloodEos⁺, $n=17$; 29%) to those with low blood eosinophils (W-BloodEos⁻, $n=41$; 71%): no differences in demographic characteristics between the two groups were found. (Table 1B). A finding in our study that could explain in part the dissociation between blood and tissue eosinophils is that wheezing children with high blood eosinophils had higher prevalence of atopy ($p=0.03$), higher levels of total serum IgE ($p=0.008$), increased prevalence of rhinitis ($p=0.01$) and tended to have more sensitizations ($p=0.08$) in comparison to W-BloodEos⁻ (Table 1B). Moreover, blood eosinophils were significantly correlated to serum IgE in the whole cohort ($r=0.47$; $p < 0.0001$) independently to the presence of wheezing and tissue eosinophilia (Figure 1E). These findings suggest that blood eosinophils in children are largely a marker of

atopy and not of tissue eosinophilia. Along these lines, stratification by higher levels of blood eosinophils, independently from tissue eosinophils, results in the definition of an atopic phenotype with higher IgE, more rhinitis and more sensitizations (Table S6), which importantly was transversal to the whole cohort, being present both in wheezing and in non-wheezing children (TableS4, TableS5).

Finally, since wheezing is often transient in childhood, we looked at persistence of asthmatic symptoms after a five years follow-up. Among children who had wheezing at baseline, 59% still reported asthma symptoms at follow-up.

Emphasizing the probable discrepant role of tissue and blood eosinophils was the finding that tissue, but not blood eosinophils, were associated with persistence of symptoms over time in our population ($p=0.003$) (Figure S2). Furthermore, tissue, but not blood eosinophils, correlated with FeNO levels at follow-up (Figure S3).

Collectively, these results challenge the importance given to blood eosinophils as an indicator of tissue eosinophils in clinical practice.

To our knowledge this is the first time the relationship between blood and tissue eosinophils in children with wheezing has been reported. A previous study in children with severe therapy-resistant asthma treated with systemic steroids, also found that airway eosinophilia was poorly reflected by blood eosinophils (8), even though systemic steroids might have affected eosinophil counts. Our results in children with milder disease, mostly not treated with ICS, support the fact that blood eosinophils can't be taken as a marker of tissue eosinophilia.

The parallel analysis of tissue and blood eosinophils levels in our study suggests that blood eosinophils may have different pathogenic roles and clinical significance than tissue eosinophils in asthmatic children. In particular, airway eosinophils are a pathogenic trait of asthma -independent from atopy- which is important for the persistence of symptoms over time. Conversely, blood eosinophils are more closely

related to atopic status and IgE levels. Therefore phenotyping asthmatic patients based on blood eosinophils does not define the eosinophilic airway inflammation phenotype. Hence treatment decisions like tapering steroids or starting costly biological treatments should not be based solely on blood eosinophils.

A possible limitation of our study is that the cohort of children who underwent a clinically indicated bronchoscopy may not be representative of asthmatic children in general, and the concomitant diseases prompting the bronchoscopy could have influenced the results. However, these concomitant conditions were evenly present among the study groups and most likely did not affect the observed differences.

Despite these limitations, the results of our study bring up some challenging concepts that ought to be considered. First, there is no reliable indicator to assess tissue eosinophilia accurately. Second, we propose that blood eosinophils could be considered as a marker of atopic status rather than a characterizing feature of asthma. The third challenging finding is the identification of an important subset of children who do display eosinophilic inflammation in tissue, which is not associated to blood eosinophilia and atopy. This suggests that airway eosinophilic inflammation might be driven by different mechanisms in this subset of asthmatic children, who should be investigated further for the possible prognostic implications and response to treatment.

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FIGURE 1: **(A-B)** Boxplots reporting values of BM thickness and destroyed epithelium in W-TisEos⁺, W-TisEos⁻, and non-wheezing children (Nw). Bottom/top of box-plot: 25th/75th percentile; solid line: median; brackets: 10th/90th percentile. **(C)** Receiver Operating Characteristic (ROC) curve of peripheral blood eosinophils,

serum IgE , BAL eosinophils count and ECP levels in predicting bronchial biopsy eosinophilia (>23 cell/mm²). AUCs/best cut-offs in TableS2. **(D)** Scatterplots reporting values of blood eosinophils in wheezing children stratified according to presence of biopsy eosinophilia. Black dots represent W-TisEos⁺, white dots represent W-TisEos⁻. **(E)** Relationship between eosinophils in peripheral blood and serum IgE levels (expressed on logarithmic scale).

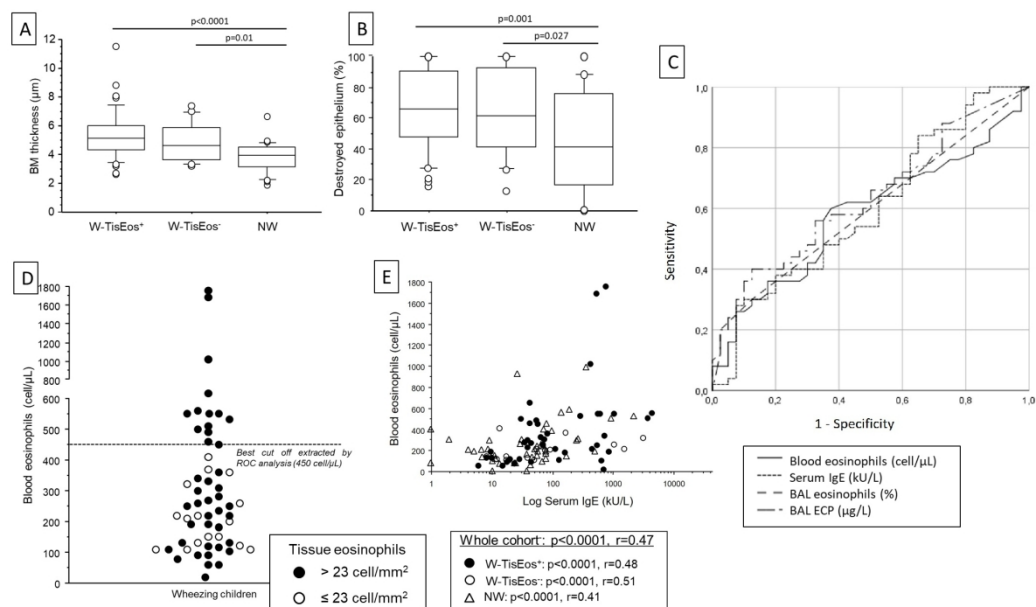
Table 1. Parallel comparison of clinical-demographical and pathological characteristics of wheezing children. Panel A shows the comparison of children with and without tissue eosinophilia (W-TisEos+ vs W-TisEos-). Panel B shows the comparison of children with and without blood eosinophilia (W-BloodEos+ vs W-BloodEos-). Panel C reports data of non-wheezing children (NW).

	Panel A: TISSUE ANALYSIS				Panel B: BLOOD ANALYSIS				Panel C
	W-TisEos+ (>23 cells/mm ²)	W-TisEos- (≤23 cells/mm ²)	<i>p</i>		W-BloodEos+ (≥ 367 cells/μL)	W-BloodEos- (< 367 cells/μL)	<i>p</i>		Non-Wheezing
Subjects, n (%)	42 (72%)	16 (28%)			17 (29%)	41 (71%)			45
Age (yrs)	5.9 ± 3	4.3 ± 2	n.s.		6.2 ± 3.5	5.1 ± 2.5	n.s.		4.9 ± 2.6
Gender male, n (%)	17 (40%)	7 (44%)	n.s.		9 (52%)	20 (49%)	n.s.		25 (55%)
Symptom pattern (multitrigger/episodic)	33/9	11/5	n.s.		13/4	31/10	n.s.		n.a.
Symptom onset (early/late)	29/13	13/3	n.s.		13/4	29/12	n.s.		n.a.
Subjects in ICS treatment, n (%)	14 (33%)	4 (25%)	n.s.		6 (35%)	12 (29%)	n.s.		7 (15%)
Daily fluticasone (mcg)	250 [200-400]	275 [100-1000]	n.s.		250 [200-600]	275 [100-1000]	n.s.		100 [50-1200]
Subjects in OCS treatment, n (%)	1 (2%)	0 (0%)	n.s.		0 (0%)	1 (2%)	n.s.		4 (9%)
Daily bethametasone (mg)	1.5	-			-	1.5			1 [0.5-1.5]
Atopy, n (%) **	13 (30%)	6 (37%)	n.s.		9 (52%)	10 (24%)	0.03		13 (28%)
Serum IgE (kU/L)	411 ± 862	393 ± 841	n.s.		741 ± 1264†	267 ± 565	0.05		141 ± 352
Rhinitis, Yes/No (%) [n=71/103]	7/22 (24%)	4/9 (30%)	n.s.		6/5 (54%) [‡]	5/26 (16%)	0.01		5/24 (17%)
Dermatitis, Yes/No (%) [n=96/103]	7/31 (18%)	2/14 (12%)	n.s.		4/10 (28%)	5/35 (12%)	n.s.		12/30 (28%)
Allergen sensitization, n	0 [0-3]	0 [0-3]	n.s.		1 [0-3]	0 [0-3]	0.08		0 [0-3]
Blood eosinophils (cell/μL)	371 ± 369	215 ± 101	n.s.		617 ± 422 [§]	185 ± 87	<.0001		258 ± 208
BAL									
Total cells (n.10 ³) [n=92/103]	162 [12-1800]	184 [37-970]	n.s.		243 [12-1470]	153 [37-1800]	n.s.		200 [1-1680]
Eosinophils (%) [n=95/103]	0 [0-10]	0 [0-4]	n.s.		0 [0-8]	0 [0-10]	n.s.		0 [0-9]
ECP (μg/L) [n=96/103]	57 ± 72	15 ± 14	n.s.		69 ± 77	36 ± 57	n.s.		26 ± 44
Neutrophils (%) [n=95/103]	16 [0-88]	4 [1-44]	n.s.		16 [1-24]	11 [0-88]	n.s.		14 [0-91]
Lymphocytes (%) [n=94/103]	6 [1-34]	5 [1-26]	n.s.		5 [1-24]	6 [1-34]	n.s.		4 [0-16]
Macrophages (%) [n=94/103]	74 [6-95]	78 [49-95]	n.s.		74 [25-95]	76 [6-95]	n.s.		79 [4-100]
BIOPSY									
BM thickness (μm) [n=103/103]	5.3 ± 1.7*	4.8 ± 1.3*	n.s.		5.2 ± 0.9 ^ψ	5.1 ± 1.8 ^ψ	n.s.		3.7 ± 0.97
Destroyed epith. (%) [n=97/103]	66 ± 26 [#]	64 ± 29 [#]	n.s.		69 ± 26 [§]	64 ± 27 [§]	n.s.		44 ± 32
Eosinophils (cell/mm ²)	109 [25-620] [§]	7 [0-23]	<.0001		134 [0-448] [¶]	38 [0-620] [¶]	n.s.		12 [0-304]
Free Eosinophil Granules score (%) [n=103/103]	50 [0-100] [‡]	0 [0-25]	.0009		50 [0-100] [∇]	25 [0-100] [∇]	n.s.		0 [0-75]

Mast cells (cell/mm ²) [n=96/103]	239 [0-800]	139 [12-650]	n.s.		279 [0-755]	177 [0-800]	n.s.		100 [0-650]
CD4 ⁺ lymph. (cell/mm ²) [n=80/103]	321 [0-1014]	124 [0-739]	n.s.		115 [0-1014]	216 [0-981]	n.s.		245 [0-1108]
Neutrophils (cell/mm ²) [n=99/103]	209 [0-1023]	141 [0-611]	n.s.		201 [0-1023]	141 [0-611]	n.s.		134 [0-925]
Macrophages (cell/mm ²) [n=96/103]	117 [0-597]	84 [0-253]	n.s.		126 [0-597]	100 [0-430]	n.s.		88 [0-467]

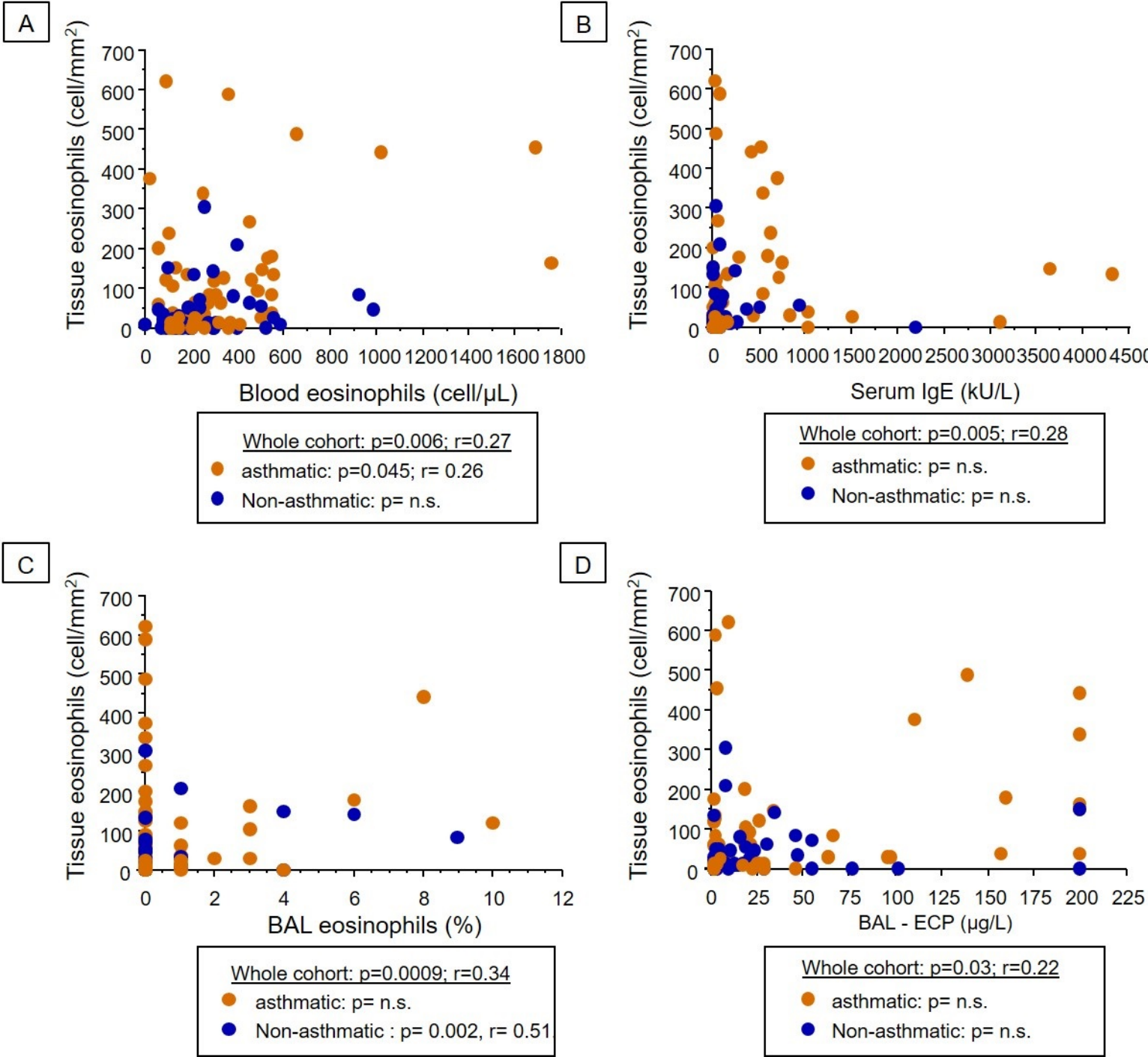
Data are expressed as mean $\pm$ standard deviation, mean [range] or count (percentage). The p-values in table are referred to the Mann-Whitney-U or Chi-square/Fisher exact test parallel comparison between W-TisEos+ vs W-TisEos- and W-BloodEos+ and W-BloodEos-. ** The presence of atopy was defined by an increase of total (higher than the age-related normal levels) and specific IgE.

Comparison with the control group (NW): † p=0.005; £ p=0.02; ¥ p<0.0001; * p<0.0001 for W-TisEos+ and p=0.01 for W-TisEos-; ψ p<0.0001 for both W-BloodEos+ and W-BloodEos-; # p=0.001 for W-TisEos+ and p=0.027 for W-TisEos-; ; \$ p=0.008 for W-BloodEos+ and p=0.005 for W-BloodEos-; § p=0.0004; ¶ p<0.0001 for W-BloodEos+ and p=0.01 for W-BloodEos-; ‡ p=0.0003; ∇ p=0.03 for W-BloodEos+ and p=0.05 for W-BloodEos;



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Figure S1



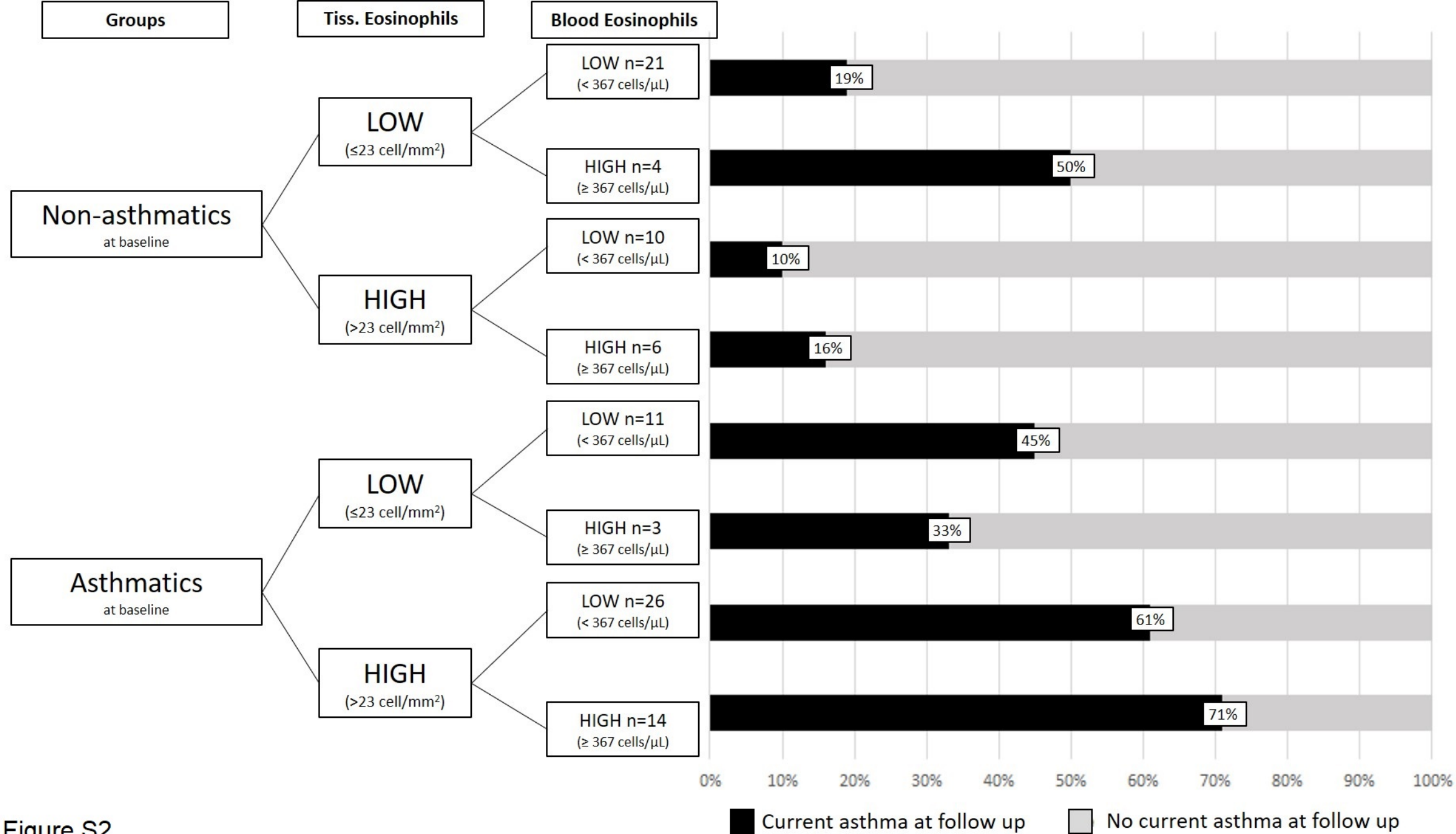
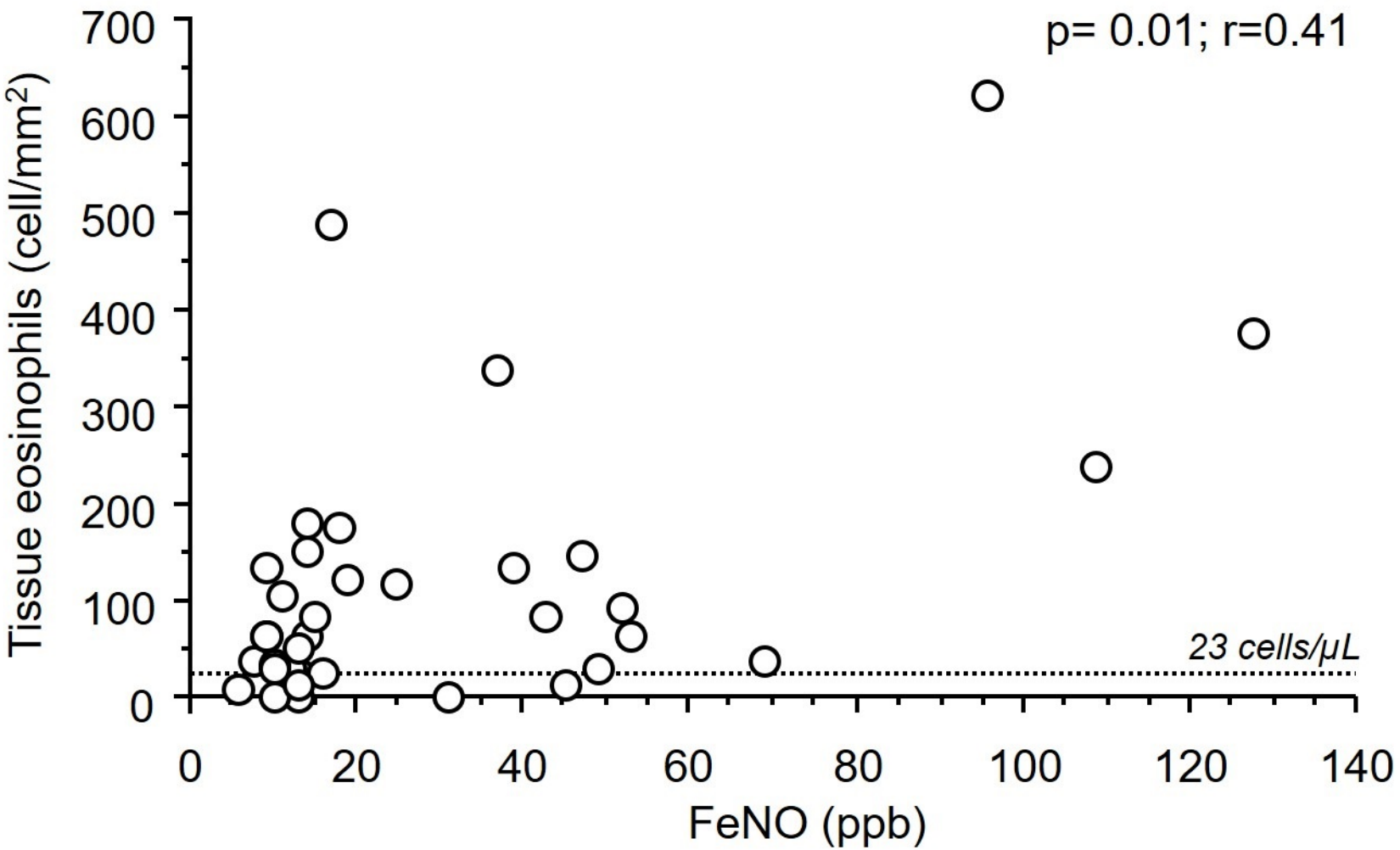


Figure S2

Figure S3



APPENDIX S1

Blood eosinophils relate to atopy and not to tissue eosinophils in wheezing children

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METHODS

Subjects

Children were recruited at Woman's and Child Health Department, University of Padova, Italy, from 2002 to 2014. All children underwent bronchoscopy for appropriate clinical indications according to guidelines (1, 2), as summarized in TableS1. Written consent was obtained from children's parents. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital. At baseline, all children underwent routine blood tests, including complete blood cell count and total and specific IgE. The presence of atopy was defined by an increase of total (higher than the age-related normal levels) and specific IgE ($>0.35\text{KU/L}$; IMMunoCAp, Phadia, Sweden). In particular, specific IgE for the following aeroallergens were investigated in all children: house dust mite (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*), moulds (*Alternaria alternata*), cat dander and grass pollens (*Lolium perenne*, *Poa pratensis*, *Phleum pratense*, *Dactylis glomerata* and *Cynodon dactylon*).

Respiratory symptoms were recorded at baseline by respiratory paediatricians who collected a detailed clinical history, visited the child and administered parental interviews focused on the presence of respiratory symptoms, the treatment during the previous 12 months and the presence of allergic manifestations.

Wheezing was defined as repeated episodes of wheezing, particularly at night and early morning, that were present even apart from colds and responsive to prescribed bronchodilators, as previously reported (3, 4). Following previous publications, late and early onset wheezing were defined according to the onset of symptoms after or before age of 3 (5). Episodic and multitrigger wheeze were defined according to the ERS 2008 Task Force: episodic wheeze is wheezing during discrete time periods, often in association with clinical evidence of a viral cold, with absence of wheeze between episodes, while multiple-trigger wheeze is wheezing that shows discrete exacerbations, but also symptoms between episodes (6).

Considering that wheezing in children can be transient, we analysed our cohort looking at the persistence of asthma symptoms after a five years follow-up. At follow-up, a respiratory paediatrician interviewed the children's parents or the study subjects and conducted a detailed clinical investigation to diagnose or exclude the presence of current asthma. Pulmonary function tests (MICRO MEDICAL®, Superspiro, UK) and FeNO measurements (NIOX VERO®, Aerocrine, Solna Sweden) were performed at follow-up. Asthma persistence at follow-up was based on a history of repeated episodes of wheezing, breathlessness or cough, particularly at night or in the early morning (that were present even apart from colds and were responsive to prescribed bronchodilators) in association with at least one of the following: a) treatment with regular or as-needed asthma medications; b) a doctor diagnosis of asthma ever; c) presence of airflow obstruction reversible to bronchodilators.

Bronchial biopsies/BAL Full details of bronchoscopy, bronchial biopsies sampling, processing and analysis have been previously described (4,7,8). Eosinophils, neutrophils, mast cells, macrophages, and CD4+ T lymphocytes were assessed in the subepithelium by immunohistochemistry, as previously described (4,7), and expressed as number of positive cells/mm² of examined subepithelium. The presence of free eosinophil granules was assessed using a semiquantitative score [0: no granules/scarce– 1 low/moderate density – 2 high density] and expressed as percentage of the highest score. BAL was carried out as previously described (9).

Statistical analysis

Results were expressed using mean±SD or median [range] for continuous variables, and counts and percentages for categorical variables. For continuous variables, normal distributions were tested using the Shapiro-Wilk test. Comparisons among groups were evaluated with either Kruskal-Wallis or ANOVA tests followed by Student's t-tests or Mann-Whitney-U test, as appropriate. Distributions of categorical variables were compared with the χ^2 -test or with Fisher's exact test, when sample size was small ($n < 5$). Correlation coefficients were calculated using the non-parametric Spearman's rank method. A multivariate logistic regression following the univariate logistic analyses, was performed to detect the strongest predictors of tissue eosinophilia. The covariates included in the final models were those that were significantly different in the univariate analyses. ROC curve analyses were performed to determine diagnostic accuracy in predicting tissue eosinophilia, with the Youden method to detect the optimal cut off. All analyses were performed using SPSS (version x64 3.3.3 for Windows). All statistical tests were two-tailed and statistical significance was assumed for P value <0.05.

Table S1. Clinical indications to bronchoscopy (according to the ERS Guidelines, ref. 1,2)

	Whole Cohort n=103
Recurrent/persistent pneumonia	49 (48%)
Chronic cough	26 (25%)
Severe therapy-resistant wheezing	13 (13%)
Stridor	8 (8%)
Suspected tracheomalacia	2 (2%)
Bronchiectasis	3 (3%)
Asphyxia episodes	1 (1%)
Interstitial pneumonia	1 (1%)

Table S2. ROC Analysis

Indicator	Best cut-off	NPV	PPV	AUC	p
WHOLE COHORT					
Blood eosinophils (cell/ μ L)	220	0.45 - 0.6	0.56 - 0.75	0.64	0.009
Serum IgE (kU/L)	19.4	0.36 - 0.51	0.62 - 0.81	0.63	0.02
BAL eosinophils (%)	2	0.3 - 0.5	0.56 - 0.8	0.56	n.s.
BAL ECP (μ g/L)	30.9	0.37 - 0.5	0.6 - 0.8	0.62	0.03
WHEEZERS					
Blood eosinophils (cell/ μ L)	450	0.21 - 0.34	0.77 - 1	0.63	0.07
Serum IgE (kU/L)	19.4	0.29 - 0.5	0.5 - 0.7	0.57	n.s.
BAL eosinophils (%)	2	0.14 - 0.31	0.51-0.87	0.46	n.s.
BAL ECP (μ g/L)	63.5	0.21 - 0.36	0.75 - 1	0.62	n.s.

Table S3. Univariate and multivariate logistic regression analysis of the four indicators in predicting the tissue eosinophilia.

UNIVARIATE			
Indicators	Rho		p
Blood eosinophils (cell/ μ L)	0.27		0.006
Serum IgE (kU/L)	0.28		0.005
BAL eosinophils (%)	0.34		0.0009
BAL ECP (μ g/L)	0.22		0.03
MULTIVARIATE			
Indicators	OR	95% CI	p
Blood eosinophils (cell/ μ L)	1.6	-	n.s.
Serum IgE (kU/L)	0.06	-	n.s.
BAL eosinophils (%)	1.0	-	n.s.
BAL ECP (μ g/L)	2.6	0.001 – 0.004	0.009

Table S4. Demographic, clinical and pathological characteristics of non-wheezing children (NW) stratified according to tissue eosinophilia.

	NW-TisEos ⁺	NW-TisEos ⁻	p-value
Subjects, n (%)	16	29	
Age (yrs)	4.4 ± 2.3	5.1 ± 2.8	n.s.
Gender male, n (%)	9 (56%)	16 (55%)	n.s.
Subjects in ICS treatment, n (%)	4 (25%)	3 (10%)	n.s.
Daily fluticasone (mcg)	100 [50-300]	450 [50-1200]	n.s.
Subjects in OCS treatment, n (%)	2 (12%)	2 (6%)	n.s.
Daily bethametasone (mg)	0.75 [0.5-1]	1.25 [1-1.5]	n.s.
Atopy, n (%)	6 (37%)	7 (24%)	n.s.
Serum IgE (kU/L)	162 ± 252	129 ± 401	n.s.
Blood eosinophils (cell/μL)	341 ± 272	212 ± 149	n.s.
BAL			
Total cells (n x 10 ³) [n=42/45]	240 [0.2-1680]	200 [20-935]	n.s.
Eosinophils (%) [n=42/45]	0 [0-9]	0 [0-4]	n.s.
ECP(μg/L) [n=42/45]	31 ± 48	22 ± 42	n.s.
Neutrophils (%) [n=42/45]	25 [1-91]	12.5 [0-90]	n.s.
Lymphocytes (%) [n=42/45]	5 [0-15]	4 [0-16]	n.s.
Macrophages (%) [n=42/45]	68 [5-96]	80 [4-100]	n.s.
BRONCHIAL BIOPSIES			
BM thickness (μm) [n=45/45]	4 ± 1	3.6 ± 2.8	n.s.
Destroyed epith. (%) [n=45/45]	50 ± 34	40 ± 32	n.s.
Mast cells (cell/mm ²) [n=42/45]	143 [32-650]	79 [0-620]	n.s.
CD4 ⁺ lymphocytes (cell/mm ²) [n=36/45]	236 [0-813]	253[11-1108]	n.s.
Neutrophils (cell/mm ²) [n=42/45]	183 [15-925]	121 [0-742]	n.s.
Macrophages (cell/mm ²) [n=42/45]	134 [0-467]	76 [0-366]	n.s.

Data are expressed as mean ± standard deviation, mean [range] or count (percentage). The p-value is referred to the Mann-Whitney-U test or Chi-square/Fisher exact test comparison between NW-TisEos⁻ and NW-TisEos⁺.

Table S5. Demographic, clinical and pathological characteristics of non-wheezing children (NW) stratified according to blood eosinophilia

	NW-BloodEos ⁺	NW-BloodEos ⁻	p-value
Subjects, n (%)	10	35	
Age (yrs)	4.2 ± 1.7	5.0 ± 2.8	n.s.
Gender male, n (%)	6 (60%)	19 (54%)	n.s.
Subjects in ICS treatment, n (%)	2 (20%)	5 (14%)	n.s.
Daily fluticasone (mcg)	650 [100-1200]	100 [0-450]	
Subjects in OCS treatment, n (%)	1 (10%)	3 (8%)	n.s.
Daily bethametasone (mg)	0.5	1 [1-1.5]	
Atopy, n (%)	6 (60%)	7 (20%)	0.01

Serum IgE (kU/L)	411 ± 683	168 ± 84	0.008
BAL			
Total cells (n x 10 ³) [n=42/45]	127 [50-935]	220 [0.2-1680]	n.s.
Eosinophils (%) [n=42/45]	0 [0-9]	0 [0-6]	n.s.
ECP (µg/L) [n=42/45]	37 ± 58	22 ± 39	n.s.
Neutrophils (%) [n=42/45]	11 [0-68]	15 [0-91]	n.s.
Lymphocytes (%) [n=42/45]	6.5 [1-15]	4 [0-16]	n.s.
Macrophages (%) [n=42/45]	77 [4-97]	79 [5-100]	n.s.
BRONCHIAL BIOPSIES			
BM thickness (µm) [n=45/45]	3.8 ± 0.7	3.7 ± 1.0	n.s.
Destroyed epith. (%) [n=45/45]	50 ± 29	42 ± 34	n.s.
Eosinophils (cell/mm ²) [n=45/45]	50 [0-207]	9 [0-304]	n.s.
Mast cells (cell/mm ²) [n=42/45]	197 [56-650]	84 [0-620]	n.s.
CD4 ⁺ lymphocytes (cell/mm ²) [n=36/45]	319 [36-524]	200 [0-1108]	n.s.
Neutrophils (cell/mm ²) [n=42/45]	110 [0-507]	137 [0-925]	n.s.
Macrophages (cell/mm ²) [n=42/45]	144 [0-354]	82 [0-476]	n.s.

Data are expressed as mean ± standard deviation, mean [range] or count (percentage).
The p-value is referred to the Mann-Whitney-U test or Chi-square/Fisher exact test comparison between NW-BloodEos- and NW-BloodEos+.

Table S6 Demographic, clinical and pathological characteristics of children stratified according to the combined values of tissue and blood eosinophils

	GROUP A TisEos ⁺ /BloodEos ⁺	GROUP B TisEos ⁺ /BloodEos ⁻	GROUP C TisEos ⁻ /BloodEos ⁺	GROUP D TisEos ⁻ /BloodEos ⁻	p
Subjects, n (%)	20 (19%)	38 (37%)	7 (7%)	38 (37%)	
Age (yrs)	6.1 ± 3.4	5.2 ± 2.6	3.7 ± 0.7	5 ± 2.7	n.s.
Gender male, n (%)	12 (60%)	14 (36%)	3 (42%)	25 (65%)	n.s.
Indication to FBS					
Chronic cough	6 (30%)	6 (15%)	2 (28%)	12 (31%)	n.s.
Severe wheezing	1 (5%)	8 (21%)	0 (0%)	4 (10%)	
Recurrent Pneumonia	10 (50%)	19 (50%)	4 (55%)	16 (42%)	
Other	3 (15%)	5 (14%)	1 (17%)	6 (17%)	
Wheezing subjects, n (%)	14 (70%)*	28 (73%)*	3 (43%)	13 (34%)	0.02
Symptom pattern (multitrigger/episodic)	11/3	22/6	22/6	9/4	n.s.
Symptom onset (early/late)	10/4	19/9	19/9	10/3	n.s.
Subjects in ICS treatment, n (%)	6 (30%)	12 (31%)	2 (28%)	5 (13%)	n.s.
Daily fluticasone (mcg)	225 [100-600]	300 [50-1000]	800 [400-1200]	250 [50-450]	n.s.
Subjects in OCS treatment, n (%)	1 (5%)	2 (5%)	0 (0%)	2 (5%)	n.s.
Daily bethametasone (mg)	0.5	1.25 [1-1.5]	-	1.25 [1-1.5]	n.s.
Atopy, n (%)	11 (55%) [‡]	8 (21%)	4 (57%) [¶]	9 (23%)	0.01

Serum IgE (kU/L)	696 ± 1175 [§]	156 ± 235	397 ± 792	190 ± 565	.0015
Rhinitis, Yes/No (%) [n=71/103]	8/5 (61%)	2/24 (7%)	2/2 (50%) [‡]	4/24 (14%)	.0006
Dermatitis, Yes/No (%) [n=96/103]	5/12 (29%)	5/31 (13%)	1/5 (16%)	10/27 (27%)	n.s.
Allergen sensitization, n	1 [0-3] [©]	0 [0-3]	1 [0-3]	0 [0-3]	0.05
Blood eosinophils (cell/μL)	695 ± 396 [≈]	187 ± 91	457 ± 93 [†]	168 ± 69	<.0001
BAL					
Total cells (n x 10 ³) [n=92/103]	134 [12-1470]	180 [200-1800]	200 [50-935]	200 [20-970]	n.s.
Eosinophils (%) [n=95/103]	0 [0-9]	67 [0-10]	0 [0-4]	0 [0-4]	n.s.
ECP (μg/L) [n=96/103]	60 ± 73	44 ± 63	46 ± 68	20 ± 35 [§]	0.02
Neutrophils (%) [n=95/103]	12 [0-63]	19 [0-91]	21 [0-68]	11 [0-90]	n.s.
Lymphocytes (%) [n=94/103]	5 [1-24]	5 [0-34]	5 [1-12]	5 [0-26]	n.s.
Macrophages (%) [n=94/103]	77 [25-95]	74 [5-96]	74 [4-97]	80 [4-100]	n.s.
BIOPSY					
BM thickness (μm) [n=103/103]	4.9 ± 1.7	4.9 ± 1.8	4.1 ± 0.7	4.1 ± 1.3	n.s.
Destroyed epith. (%) [n=97/103]	62 ± 30	62 ± 28	63 ± 27	47 ± 33	n.s.
Eosinophils (cell/mm ²) [n=103/103]	126 [25-488] [¥]	67 [25-620] [£]	8 [0-23]	8 [0-23]	<0.0001
Free Eosinophil Granules score (%)	62 [0-100] [€]	50 [0-100] [¥]	0 [0-0]	0 [0-75]	0.005
Mast cells (cell/mm ²) [n=96/103]	313 [0-755]	198 [0-800]	192 [28-650]	58 [0-620]	n.s.
CD4 ⁺ lymph. (cell/mm ²) [n=80/103]	236 [0-1014]	285 [0-981]	208 [0-524]	110 [0-1108]	n.s.
Neutrophils (cell/mm ²) [n=99/103]	127 [0-1023]	219 [15-925]	21 [0-68]	56 [0-742]	n.s.
Macrophages (cell/mm ²) [n=96/103]	313 [0-597]	115 [0-467]	145 [0-354]	56 [0-366]	n.s.

Data are expressed as mean ± standard deviation, mean [range] or count (percentage).

The p-value is referred to the Kruskal-Wallis or Chi-square/Fisher exact test parallel comparison between TisEos+/ BloodEos+ (GROUP A) vs TisEos+/BloodEos- (GROUP B) vs TisEos-/BloodEos+ (GROUP C) vs TisEos-/BloodEos- (GROUP D).

*Higher than group D p=0.01; ‡ higher than group B (p=0.01) and group D (p=0.02); ¶ higher than group B p=0.06; § higher than group B (p=0.007) and D (p=0.004); ¶ higher than group B (p=0.0001) and group D (p=0.003); ± higher than group B (p=0.06); © higher than group B (p=0.01); ≈ higher than group B and group D (p<0.0001 for both); † higher than group B and group D (p<0.0001 for both); § lower than group A (p=0.005) and group B (p=0.02); ¥ higher than group C and D (p<0.0001 for both); £ higher than group C (p=0.007) and D (p<0.0001); € higher than group C (p=0.02) and D (p=0.008); x higher than group C (p=0.07) and D (p=0.006)

Figure S1 Relationship between eosinophils in airway submucosa and peripheral blood eosinophils (1a), serum IgE levels (1b), BAL eosinophils count (1c) and ECP levels (1d) in wheezing (orange dots) and non-wheezing children (blue dots). A significant but modest positive correlation emerged between tissue eosinophils and the four indicators considered.

Figure S2 Outcome after a 5-yrs follow-up in wheezing and non-wheezing children, stratified according to tissue (>23 cell/mm²) and blood (>367 cell/ μ L) eosinophilia measured at baseline. The flow chart on the left shows the stratification of wheezing and non-wheezing children according to levels of tissue and blood eosinophils at baseline. The bar graph on the right represents the prevalence of “current asthma” (black) after a five-years follow-up in each subpopulation.

Figure S3 Relationship between eosinophils in airway submucosa at baseline and FeNO levels at follow-up in wheezing children. A significant, but modest, positive correlation emerged between baseline tissue eosinophils and FeNO levels at follow-up.

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