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Association of $FEF_{25–75\%}$ Impairment with Bronchial Hyperresponsiveness and Airway Inflammation in Subjects with Asthma-Like Symptoms

Mario Malerba[a] Alessandro Radaeli[b] Alessia Olivini[a] Giovanni Damiani[a]
Beatrice Ragnoli[b] Valentina Sorbello[c] Fabio L.M. Ricciardolo[c]

[a]Department of Internal Medicine, University of Brescia, Spedali Civili di Brescia, and
[b]Emergency Department, Spedali Civili di Brescia, Brescia, and [c]Department of Clinical and Biological Sciences, University of Torino, San Luigi Hospital, Orbassano, Italy

Key Words
Forced expiratory flow at 25 and 75% of the pulmonary volume · Fractional exhaled nitric oxide · Bronchial asthma · Sputum eosinophilia · Bronchial hyperresponsiveness

Abstract
Background: Forced expiratory flow at 25 and 75% of the pulmonary volume ($FEF_{25–75\%}$) might be considered as a marker of early airway obstruction. $FEF_{25–75\%}$ impairment might suggest earlier asthma recognition in symptomatic subjects even in the absence of other abnormal spirometry values. ***Objectives:*** The study was designed in order to verify whether $FEF_{25–75\%}$ impairment in a cohort of subjects with asthma-like symptoms could be associated with the risk of bronchial hyperresponsiveness (BHR) and with airway inflammation expressed as fractional exhaled nitric oxide (FE_{NO}) and eosinophil counts in induced sputum. ***Methods:*** Four hundred adults with a history of asthma-like symptoms (10.5% allergic) underwent spirometry, determination of BHR to methacholine ($PD_{20}FEV_1$), FE_{NO} analysis and sputum induction. $FEF_{25–75\%}$ <65% of predicted or <–1.64 z-score was considered abnormal. ***Results:*** All subjects had normal FVC, FEV_1 and FEV_1/FVC, while $FEF_{25–75\%}$ was abnormal in 27.5% of them. $FEF_{25–75\%}$ (z-score) was associated with $PD_{20}FEV_1$ ($p < 0.001$), FE_{NO} ($p < 0.001$) and sputum eosinophils ($p < 0.001$). Patients with abnormal $FEF_{25–75\%}$ showed higher levels of FE_{NO} and eosinophils in induced sputum than did patients with normal $FEF_{25–75\%}$ ($p < 0.01$ and $p < 0.01$, respectively). Subjects with abnormal $FEF_{25–75\%}$ had an increased probability of being BHR positive (OR = 13.38; 95% CI: 6.7–26.7; $p < 0.001$). ***Conclusions:*** Our data show that abnormal $FEF_{25–75\%}$ might be considered an early marker of airflow limitation associated with eosinophilic inflammation and BHR in subjects with asthma-like symptoms, indicating a role for $FEF_{25–75\%}$ as a predictive marker of newly diagnosed asthma.

Introduction

Small airways seem to be involved in asthma pathogenesis [1, 2]. Recently, according to this idea, Perez et al. [3] demonstrated that, among moderate-to-severe asthmatics, patients with only small airway obstruction (compared to those with proximal airway obstruction) showed no correlations between small airway obstruction and

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Mario Malerba, MD
Department of Internal Medicine, University of Brescia
Spedali Civili di Brescia, Piazzale Spedali Civili 1
IT–25100 Brescia (Italy)
E-Mail malerba@med.unibs.it

asthma history, scores of dyspnoea, asthma control or drug compliance. These conclusions would imply an underestimation, in treated asthmatic patients, of small airway dysfunction in routinely used lung function testing [3]. Forced expiratory flow at 25 and 75% of the pulmonary volume ($FEF_{25–75\%}$) is defined as the mean forced expiratory flow during the middle half of the FVC and measures average flow rates on an FVC segment that includes flow from medium-to-small airways [4]. $FEF_{25–75\%}$ indicates the status of the medium-sized and small airways particularly in subjects with normal FEV_1 and FEV_1/FVC [4, 5]. $FEF_{25–75\%}$ varies significantly in healthy subjects and is considered abnormal when it reaches values <65% of predicted [6–8]. Muñoz-López et al. [9] pointed out that $FEF_{25–75\%}$ is more appropriate than FEV_1 for evaluating bronchial hyperresponsiveness (BHR) to methacholine and for indicating small airway impairment earlier in subjects with asthma and/or allergic rhinitis. Compromised $FEF_{25–75\%}$ has been associated with BHR in patients with allergic rhinitis [10] and in subjects with respiratory symptoms suggestive of bronchial asthma [11]. BHR is the hallmark of bronchial asthma, and in particular BHR to indirect stimuli is related to the degree of airway inflammation (especially eosinophilic infiltration) [12]. Furthermore, in children with allergic rhinitis/asthma, abnormal $FEF_{25–75\%}$ has been associated with elevated levels of fractional exhaled nitric oxide ($FeNO$) [13], which is primarily derived from the respiratory epithelium and seems to reflect eosinophilic airway inflammation [14]. $FeNO$ measurement is a highly reproducible, safe, sensible, simple and rapid test [15–17]. Ciprandi et al. [18] demonstrated a strong and negative correlation between $FeNO$ and BHR in asthmatic children; these results confirmed the existence of a link between airway inflammation and BHR. Tossa et al. [19] as well proved that an increased $FeNO$ level was associated with BHR among apprentices.

The aim of our study was to verify whether abnormal $FEF_{25–75\%}$ in a cohort of subjects with asthma-like symptoms and normal FEV_1 and FEV_1/FVC could be associated with BHR and the degree of airway inflammation.

Subjects and Methods

Patients

From November 2011 to November 2013, 400 consecutive adult patients referred to the Respiratory Medicine Unit of the Department of Internal Medicine, University of Brescia, were studied in an outpatient setting. The patients complained of symptoms consistent with bronchial asthma: cough, chest tightness, dyspnoea or wheezing with nocturnal awakenings for >3 weeks with a normal chest X-ray and spirometry (FVC, FEV_1 and FEV_1/FVC) [20]. The exclusion criteria were use of any medication for cough, upper respiratory infection during the previous 6 weeks, use of corticosteroids during the previous 6 weeks, current smoking, any significant medical condition, a prior asthma diagnosis and the usual contraindications to methacholine challenge tests. The study was approved by the local ethics committee, and all participants provided written informed consent.

Recruited patients underwent the following procedures: clinical examination; symptom evaluation; skin prick testing; pulmonary function tests; determination of BHR to methacholine (methacholine challenge test); $FeNO$ analysis; sputum induction, and eosinophil count.

Skin Prick Test

Allergy was assessed by skin prick test positivity to the most common aeroallergens as stated by the European Academy of Allergy and Clinical Immunology [21].

Pulmonary Function Tests

Spirometry and maximal full flow-volume curves were obtained using a pneumotachograph with a volume integrator (CAD/Net system 1070; Medical Graphics Corporation, St. Paul, Minn., USA). The pulmonary function tests were performed following American Thoracic Society criteria [22]. All indices (FVC, FEV_1, FEV_1/FVC and $FEF_{25–75\%}$) were expressed as percent of predicted normal and z-scores. Predicted values and z-scores for the various indices were derived using prediction equations from the Global Lung Function Initiative (GLI-2012; http://www.lungfunction.org/) [23]. In particular, $FEF_{25–75\%}$ was categorized as (1) <65% of predicted or <–1.64 z-score (abnormal values) or (2) >65% of predicted or between –1.64 and +1.64 z-score (normal values) [23]. $FEF_{25–75\%}$ categorization by both criteria (% predicted and z-score) gave equal results. Only baseline or pre-bronchodilator data were analysed in the study.

Bronchial Hyperresponsiveness

The methacholine challenge test was performed according to international guidelines as a dose-response curve by increasing (doubling) doses of methacholine chlorohydrate every 3 min. Results were expressed as cumulative doses of methacholine provoking a 20% fall in FEV_1 (PD_{20}). A methacholine challenge test result was considered positive if the PD_{20} was <16.00 mg/ml [24].

Fractional Exhaled Nitric Oxide

$FeNO$ was determined with a high-resolution chemiluminescence NO analyser (Ecomedics AG Analyzer CLD88; Dürnten, Switzerland), whose limit of detection was 0.06 ppb, with a measurement range reaching 100 ppb. The measurements were performed in accordance with the ATS recommendations using a standardized procedure for on-line measurement of exhaled NO in adults [25]. $FeNO$ values between 4 and 20 ppb were considered within normal limits according to literature consensus, identifying 20 ppb as the cut-off point for a positive result [26].

Sputum Induction

After baseline FEV_1 and FVC measurements, the subjects were pre-treated with inhaled salbutamol (200 μg by metred-dose inhaler) and 10 min later inhaled a hypertonic (4.5%) nebulized ster-

ile saline solution for 3 periods of 5 min at most by means of an ultrasonic nebulizer (Ultraneb 2000; DeVilbiss, Somerset, Pa., USA). Nebulization was discontinued if one of the following symptoms occurred: wheezing, chest tightness or moderate-to-severe dyspnoea. Sputum was processed as previously reported [27]. The cut-off for an abnormal result was considered a sputum eosinophil count >3% (percentage of cells) [28].

Statistical Analysis

Values are expressed as medians (minimum–maximum) for continuous variables and as numbers and percentages for categorical variables. Spearman's rank method was applied if variables were not normally distributed. In order to obtain reliable assessments of the relationship between $FEF_{25-75\%}$ and the other variables, $FEF_{25-75\%}$ was considered both a continuous and a categorical variable.

BHR was categorized as 'negative' (threshold ≥16.0 mg/ml) or 'positive' (threshold <16.0 mg/ml) [24]. The χ^2 test was used to test the analysed categorical variables. The Mann-Whitney test was used to assess differences between categorical and continuous variables. OR for an effect of $FEF_{25-75\%}$ on BHR and relative 95% CI are shown. A p value ≤0.05 was considered statistically significant, and SPSS version 21 (IBM Corp., Armonk, N.Y., USA) was used for analysis.

The statistical power of the study was assessed with the PS Power and Sample Size program available for free on the Internet (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). Concerning BHR, the study had a power of 96% with an OR difference of 3.5 in discriminating between abnormal and normal $FEF_{25-75\%}$ groups.

Table 1. Patients' demographic, functional and biological characteristics

Age, years	29.6 (17.8–41.4)
Gender F/M	226/174 (56.5/43.5)
Smoking	
Non-smoker	323 (80.75)
Ex-smoker	77 (19.25)
Current smoker	0 (0)
Pack-years	2.7 (0–3.2)
Allergy	42 (10.5)
FVC, % predicted	106.99 (81.8–120.7)
FEV_1, % predicted	104.38 (82.6–116.5)
$FEF_{25-75\%}$, % predicted	93.55 (20.0–190.0)
$FEF_{25-75\%}$ (z-score) [range]	–0.398 (1.66) [–4.54–3.48]
$FEF_{25-75\%}$ category (by % predicted or z-score)	
Normal	290 (72.5)
Abnormal	110 (27.5)
BHR, mg/ml	14.10 (0.10–16.00)
BHR category	
Negative (>16 mg/ml)	176 (44.0)
Positive (<16 mg/ml)	224 (56.0)
FeNO, ppb	34.21 (12–80)
Sputum eosinophils, %	4.32 (0.00–21.01)

Data are expressed as medians (minimum–maximum) or n (%) unless indicated otherwise.

Results

Demographic, functional and biological patient characteristics are shown in table 1; the numbers of males and females were equally balanced, and patients were ex-smokers or non-smokers, with respiratory symptoms during the previous 6 months (symptoms might have been absent at the time of enrolment). Forty-two patients (10.5%) were sensitized to perennial and/or pollen allergens. Concerning spirometric parameters, in all subjects FVC, FEV_1 and FEV_1/FVC were within normal reference ranges, while $FEF_{25-75\%}$ was abnormal in 27.5% of the subjects, as extrapolated by percent of predicted or z-score data (a comparison of the data between subjects with normal and those with abnormal $FEF_{25-75\%}$ is shown in table 2). The median value of FeNO was 34.2 ppb (12–80), and the median sputum eosinophil count was 4.32 cells (0.0–21.0); 176 patients (42.0%) were negative to BHR (table 1). No significant differences in demographic or functional and biological data were observed when grouping patients according to gender, age, atopy or smoking habit.

A significant moderate positive correlation between BHR (expressed as $PD_{20}FEV_1$ to methacholine) and $FEF_{25-75\%}$ z-score (Spearman's coefficient = 0.339, $p < 0.001$; fig. 1) and percent of predicted (table 3) was noted, despite the wide scattering of data due to the large sample size. Significant strong negative correlations were observed between $FEF_{25-75\%}$ (z-score) and both FeNO (fig. 2; table 3) and sputum eosinophil count (fig. 3; table 3) (Spearman's coefficient = –0.729, $p < 0.001$, and Spearman's coefficient = –0.813, $p < 0.001$, respectively) as well as between BHR and both FeNO (Spearman's coefficient = –0.851, $p < 0.001$; fig. 4; table 3) and sputum eosinophil count (Spearman's coefficient = –0.760, $p < 0.001$; table 2). Finally, a strong positive correlation was found between FeNO and sputum eosinophil count (Spearman's coefficient = –0.944, $p < 0.001$; table 3).

Patients with positive BHR had a median FeNO value of 46.3 ppb (12–80); this result was significantly different ($p < 0.001$) from that of BHR-negative patients, who showed a lower median value of 19.4 ppb (14–32). Similarly, patients with abnormal $FEF_{25-75\%}$ had significantly ($p < 0.001$) higher values of FeNO [53.55 ppb (25–80)] than patients with normal $FEF_{25-75\%}$ [21.60 ppb (12–61)], as shown in table 2. Moreover, the sputum eosinophil

Table 2. Demographic, functional and biological characteristics of the patients according to normal or abnormal $FEF_{25-75\%}$

	Abnormal $FEF_{25-75\%}$ (n = 110)	Normal $FEF_{25-75\%}$ (n = 290)	p
Age, years	27.9 (18.4–41.4)	31.15 (17.8–41.4)	n.s.
Gender F/M	66/44 (58/42)	160/130 (55/45)	n.s.
Allergy	14 (12.7)	28 (9.6)	n.s.
FVC, % predicted	98.87 (97.1–120.7)	100.37 (81.8–107.8)	n.s.
FEV_1, % predicted	98.51 (96.4–116.5)	99.44 (82.6–106.1)	n.s.
BHR, mg/ml	7.80 (0.10–16.00)	16.00 (0.10–16.00)	<0.001
FeNO, ppb	53.55 (25–80)	21.60 (12–61)	<0.001
Sputum eosinophils, %	9.86 (2.16–21.01)	1.21 (0.00–7.83)	<0.001

Data are expressed as medians (minimum–maximum) or n (%). n.s. = Not significant.

Table 3. Correlation coefficients between variables

	BHR		FeNO		Eosinophils	
	S	p	S	p	S	p
$FEF_{25-75\%}$ (% pred.)	0.270	<0.001	–0.660	<0.001	–0.752	<0.001
$FEF_{25-75\%}$ (z-score)	0.339	<0.001	–0.729	<0.001	–0.813	<0.001
BHR			–0.851	<0.001	–0.760	<0.001
FeNO					0.944	<0.001

S = Spearman's correlation coefficient; % pred. = % predicted.

Table 4. Cross tabulation between BHR and $FEF_{25-75\%}$ (categorized)

	$FEF_{25-75\%}$	
	normal	abnormal
BHR		
Normal	166	10
Abnormal	124	100

With abnormal $FEF_{25-75\%}$, the OR for BHR positivity was 13.38 (95% CI: 6.7–26.7, p < 0.001).

count in BHR-negative and normal-$FEF_{25-75\%}$ patients was significantly lower than that in BHR-positive and abnormal-$FEF_{25-75\%}$ patients [0.6 cells (0–3.1) in BHR-negative vs. 6.5 cells (0.0–21.0) in BHR-positive patients, p < 0.001; 1.2 cells (0.0–7.8) in normal-$FEF_{25-75\%}$ vs. 9.89 cells (2.1–21.0) in abnormal-$FEF_{25-75\%}$ patients, p < 0.001]. Of the BHR-positive and BHR-negative patients, 13.4 and 6.8%, respectively, were allergen sensitized, and this difference was statistically significant (χ^2 test, p = 0.03). Subjects with abnormal $FEF_{25-75\%}$ had an increased probability of being BHR positive (OR = 13.38; 95% CI: 6.7–26.7; p < 0.001) (table 4).

Discussion

In the present study, a cohort of subjects with asthma-like symptoms and normal FVC, FEV_1 and FEV_1/FVC showed a relationship between $FEF_{25-75\%}$ values and BHR to methacholine – the hallmark of bronchial asthma – and between $FEF_{25-75\%}$ values and markers of eosinophilic airway inflammation such as FeNO and sputum eosinophils. Moreover, patients with abnormal $FEF_{25-75\%}$, which is considered a reliable marker of early airflow limitation [4–8], had higher FeNO and sputum eosinophil levels than patients with normal $FEF_{25-75\%}$. We also pointed out that subjects with asthma-like symptoms and abnormal

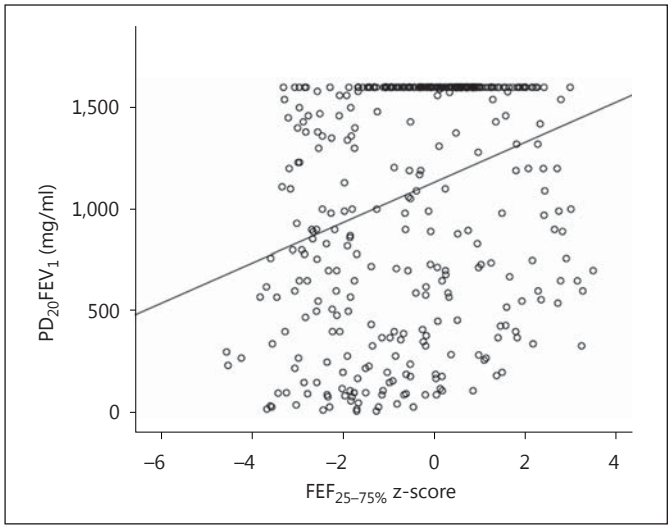

Fig. 1. Overall patients' relationship between BHR and $FEF_{25–75\%}$ z-score. S = 0.339, p < 0.001.

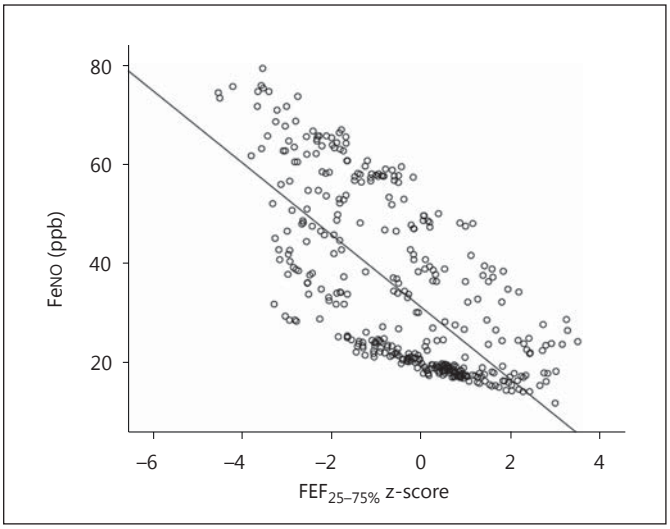

Fig. 2. Overall patients' relationship between $FEF_{25–75\%}$ z-score and FeNO. S = –0.729, p < 0.001.

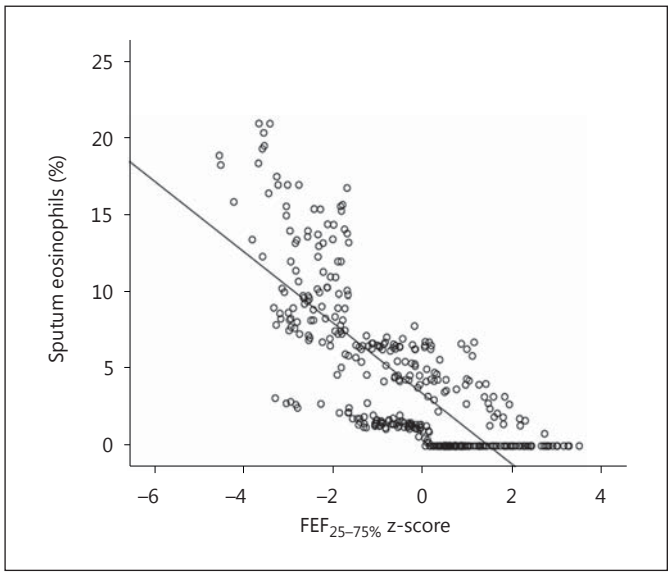

Fig. 3. Overall patients' relationship between $FEF_{25–75\%}$ z-score and sputum eosinophils. S = –0.813, p < 0.001.

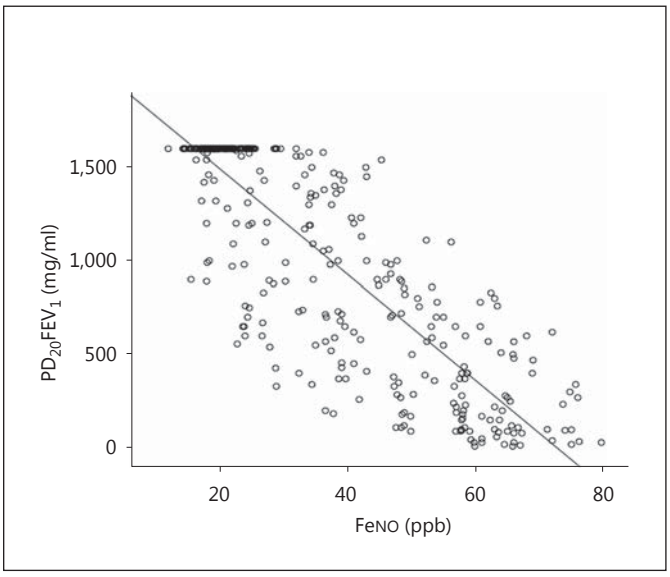

Fig. 4. Overall patients' relationship between BHR and FeNO. S = –0.851, p < 0.001.

$FEF_{25–75\%}$ had a high probability of being BHR positive (OR = 13.38). These findings strengthen the hypothesis that early airflow limitation, expressed as abnormal $FEF_{25–75\%}$, is probably related to eosinophilic airway inflammation. In the literature, $FEF_{25–75\%}$ was validated as a marker able to predict high FeNO levels in asthmatic children [13] and correlated with FeNO in terms of percent change in improvement after 6 weeks of inhaled corticosteroid treatment in controlled asthmatic children [29]. In addition, Rao et al. [30] observed that in children, a low $FEF_{25–75\%}$ associated with a normal FEV_1 is linked to increased asthma severity, systemic steroid use and asthma exacerbations. Using the percent change in $FEF_{25–75\%}$ from baseline might be helpful in identifying

bronchodilator responsiveness in asthmatic children with normal FEV_1 [30]. In our study, $FEF_{25–75\%}$, expressed both as z-score and percent of predicted, is strongly related to sputum eosinophil and FeNO values in subjects with asthma-like symptoms. In addition, subjects with abnormal $FEF_{25–75\%}$ showed a higher number of sputum eosinophils and higher FeNO values than subjects with normal $FEF_{25–75\%}$, suggesting that $FEF_{25–75\%}$ could be considered a sensitive spirometric marker associated with eosinophilic airway inflammation in newly diagnosed adult asthma cases with normal FEV_1 and FVC.

We found a marked correlation between FeNO and sputum eosinophils confirming previous reports on the capability of FeNO to reflect eosinophilic airway inflammation at mucosal and luminal levels [14, 31]. In particular, concerning this aspect, we point out that blockade of interleukin-5, a key cytokine in eosinophil differentiation/maturation in the bone marrow as well as in recruitment/activation at sites of allergic inflammation, was expected to deplete eosinophils and improve symptoms in subjects with asthma. Surprisingly, a humanized monoclonal antibody against interleukin-5 (mepolizumab) substantially reduced eosinophil levels in peripheral blood and sputum in relation to a reduction of asthma exacerbations but did not appear to have pharmacodynamic effects on FeNO levels [32, 33]. Taken together, these data suggest (1) that although targeting eosinophils can significantly reduce the rate of asthma exacerbations, the precise roles of eosinophils in the biology of asthma are still unclear, and (2) that FeNO is mainly dependent on epithelium-derived inducible NO synthase production, which could be influenced by different stimuli/triggers such as viruses and eosinophil-derived mediators in the airways [34]. Thus, FeNO might be considered a marker of asthmatic airway dysfunction not only related to airway eosinophilia.

$FEF_{25–75\%}$ has been found to correlate with functional imaging assessment of small airway function [35] as well as indices of ventilation heterogeneity, which are markers of peripheral airway function, obtained with multiple nitrogen washout in normal subjects [36] and with double-tracer gas single-breath washout in mild asthmatics [37]. $FEF_{25–75\%}$ percent of predicted has been demonstrated to correlate better with air trapping in asthmatic subjects than do FEV_1 percent of predicted and FEV_1/FVC percent of predicted [6]. Furthermore, in the archetypal small airways disease – that is, obliterative bronchiolitis – $FEF_{25–75\%}$ is considered the most sensitive functional marker of early diagnosis [38, 39]. Finally, a paper by Bergeron et al. [40] concerning airway remodelling showed a significant reduction in the expression of smooth muscle α-actin in the small airways by analysing transbronchial biopsy specimens before and after 6 weeks of treatment with extra-fine inhaled corticosteroid. In fact, α-actin expression was correlated with improved $FEF_{25–75\%}$, suggesting an association between $FEF_{25–75\%}$ values and histology. Thus, on the basis of all this evidence, we may speculate that in the present study, abnormal $FEF_{25–75\%}$ in symptomatic subjects with normal FEV_1 can be considered a marker of early airway obstruction without involvement of the proximal/central airways. Pisi et al. [41] suggested investigating small airway dysfunction in asthmatic patients with normal FEV_1 values by using an impulse oscillometry system as an alternative to spirometry.

In our population, abnormal $FEF_{25–75\%}$ was associated with an increased BHR as measured by OR, indicating that $FEF_{25–75\%}$ could be a simple and non-invasive marker of suspected BHR in patients with asthma-like symptoms and normal spirometric values. Small airways disease, assessed by peripheral airway resistance, has recently been associated with excessive bronchoconstriction in asthmatic patients [42]. Our data support the role of $FEF_{25–75\%}$ as a marker of early airway obstruction and as a risk factor for BHR positivity and severity in agreement with previous reports on allergic [43–45] and rhinitic subjects [10]; therefore, in clinical practice a more rational approach should also include measurements of $FEF_{25–75\%}$ [46]. In agreement with other authors, we suggest a combined approach towards asthma follow-up, involving clinical aspects, functional parameters ($FEF_{25–75\%}$) and inflammatory biomarkers (FeNO) [46–48].

In the current study, a negative correlation between BHR and FeNO/sputum eosinophils has been shown; in addition, BHR-positive symptomatic subjects showed higher FeNO than BHR-negative subjects. BHR to nonspecific bronchoconstrictor stimuli is a key feature of asthma and could be associated with the inflammatory process of the disease. Several studies have shown that BHR to indirect stimuli such as bradykinin, mannitol and adenosine is related to FeNO or eosinophilic inflammation [12, 49–51], whilst a relationship between BHR and direct stimuli such as methacholine and FeNO or eosinophilic inflammation exists [19, 52] but is expressed to a lesser extent [51, 53] or remains controversial [49, 50, 54, 55]. Finally, on the basis of the present data on FeNO in BHR-positive compared to BHR-negative patients, it can be postulated that FeNO might also be a marker potentially able to identify BHR-positive patients with new-onset asthma, and that the link between BHR and FeNO/

eosinophilic inflammation is strictly involved in the pathogenesis of early airflow limitation as detected in newly diagnosed adult asthma cases.

In this study, we also found a small number of subjects with asthma-like symptoms to have allergen sensitization (10.5%); in particular, the number of allergic subjects with positive BHR was significantly higher than that of allergic subjects with negative BHR. These results are in agreement with those of a previous study which demonstrated that the attributable fraction of new-onset asthma (at an age of 20–44 years) to atopy varied from 12 to 21%, indicating that only a relatively small proportion of new-onset asthma in adults is linked to atopy [56]. In addition, a study based on the Italian population (aged 20–44 years) showed that the prevalence of asthma and asthma-like symptoms has increased in the past 20 years, and that this increase has mainly been due to non-atopic subjects, suggesting that this trend might reflect changes in population exposures [57].

The clinical relevance of the present study is based on an adequate number of enrolled patients. Unfortunately, in this study, data concerning the clinical diagnosis of persistent/seasonal rhinitis were not collected in the medical records, and thus the authors were not able to correlate this major risk factor for asthma [58] with the analysed variables. Another limitation of this study is intrinsically connected with the study type (cross-sectional), which does not estimate patients' progression; however, this limitation is well balanced by the large size of the chosen sample. An additional limitation might be the absence of post-bronchodilator data, even if reversibility testing is not mandatory for subjects with an FEV_1 >80% of predicted. Despite the fact that the weak reproducibility of $FEF_{25–75\%}$ measurements represents one of the most important limitations for clinical use, we want to emphasize that the measure of $FEF_{25–75\%}$ is the index of early airflow limitation most widely available in routine practice for identifying new-onset asthmatics [59]. Finally, it might have been useful to have other measures of small airway function, such as an impulse oscillometry system, nitrogen washout and plethysmography; however, these tools have been unavailable in our own routine clinical practice.

In conclusion, the present study shows that abnormal $FEF_{25–75\%}$ might be considered an early functional marker of airflow limitation associated with eosinophilic inflammation and BHR in symptomatic subjects, suggesting a predictive role as a marker of newly diagnosed asthma.

Financial Disclosure and Conflicts of Interest

The authors declare no conflict of interest.

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