Spirometric changes in bronchodilation tests as predictors of asthma diagnosis and treatment response in patients with $FEV_1 \geq 80\%$ predicted

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Key message

- $\Delta FEV_1\% > 3.5\%$ in bronchodilation tests(BDT) together with fractional exhaled nitric oxide (FENO) > 33 ppb predict a positive response to anti-asthma therapy (PRAT) and a diagnosis of asthma in patients with normal FEV_1.
- This predictive model of anti-asthma response can easily be applied in clinical practice, especially in primary care, and its credibility is bolstered by the finding of pathological changes in the lungs of patients with predicted PRAT.

Abbreviations

%: the improvement of spirometric indices as a percentage of baseline value;

Δ: increase of spirometric indices in BDT;

Δ%: the increase of spirometric indices as a percentage of baseline value in BDT;

ΔACT: ACT change from baseline to post-treatment ($\Delta ACT = ACT_2 - ACT_1$);

ACT_1: asthma control test at the first visit;

ACT_2: asthma control test at the second visit;

AUC: area under the curve;

BMI: body mass index;

ELISA: enzyme-linked immunosorbent assay;

EOS: eosinophils;

FEF25: forced expiratory flow at 25% of forced vital capacity;

FEF25-75: forced expiratory flow at 25% to 75% of forced vital capacity;

FEF50: forced expiratory flow at 50% of forced vital capacity;

FEF75: forced expiratory flow at 75% of forced vital capacity;

FENO: fractional exhaled nitric oxide;

FEV1: forced expiratory volume in one second;

FVC: forced vital capacity;

ICS: inhaled corticosteroid;

IL: interleukin.

LABA: long-acting β agonist;

ND: negative diagnosis asthma;

NFκB: Nuclear factor kappa-B;
NLR: negative likelihood ratios;
NPV: negative predictive values;
NRAT: negative response to anti-asthma treatment;
Odds Ratio: odds ratio of characteristic variables;
PCC: percentages correctly classified;
PD: positive diagnosis asthma;
PEF: peak expiratory flow;
PPV: positive predictive values;
PRAT: positive response to anti-asthma treatment;
TGF-β: transforming growth factor-β;
SD, suspect diagnosis asthma;
WBC: white blood cells.

ABSTRACT

Background: Many patients with mild asthma are undiagnosed and untreated for low diagnostic sensitivity of the bronchodilation test (BDT).

Objective: Investigating whether airway reversibility in BDT alone or together with fractional exhaled nitric oxide (FENO) can predict the response to anti-asthma therapy (RAT) in suspected asthma patients.

Methods: This study included patients with chronic recurrent asthma symptoms, normal forced expiratory volume in 1 second (FEV_1), and negative BDT. Inhaled corticosteroid (ICS) and long-acting β agonist (LABA) were given for 4 weeks. Positive RAT (PRAT) was defined as improved symptoms and increase of FEV_1 > 200 mL after ICS/LABA. Lung tissues from 19 patients with lung nodules, grouped by predicted RAT, were also analyzed.

Results: Of 102 patients, the PRAT group had higher FENO and greater absolute (Δ) and (Δ%) percent improvements of forced vital capacity, FEV_1, and forced expiratory flows (FEFs) in BDT than the negative RAT group. The AUCs of FENO, ΔFEV_1%, ΔFEF_{25-75}%, and ΔFEF_{75}% for PRAT were 0.703, 0.824, 0.736, and 0.710, with the

optimal cut-off values of 33 ppb, 3.50%, 15.26%, and 26.04%. A joint model of FENO and $\Delta FEV_1\%$ increased the AUC to 0.880. IL-4, IL-5, IL-13, and NFκB were higher in lung tissues of patients with predicted PRAT than with predicted NRAT.

Conclusion: $\Delta FEV_1\% > 3.50\%$ in BDT together with FENO > 33 ppb predicted PRAT and an asthma diagnosis in patients with normal FEV_1 and negative BDT. Evidence of pathological changes in the early stage of asthma increased the credibility of the predictive model.

Key words Bronchodilation test; Fractional exhale nitric oxide; Forced expiratory volume in 1 second; Forced expiratory flows; Pathology.

INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation and variable expiratory airflow limitation. The overall prevalence of asthma is 4.2%, with 45.7 million affected individuals in China.[1] In 50%–75% of cases, the asthma is mild and can be well controlled with low-intensity treatments (Steps 1 and 2) according to the Global Initiative for Asthma (GINA) classification.[2,3] Nonetheless, individuals with mild asthma have a 30%~40% risk of exacerbations that result in emergency care over a year.[2]

Early detection and treatment of inflammation effectively block the development of asthma and reduce the economic burden of the disease.[4] However, the mild symptoms and near-normal spirometry of mild asthma make it difficult to diagnose, and both patients and physicians tend to underestimate the severity of the condition.[5] A bronchodilation test (BDT) and bronchial provocation test (BPT) are usually recommended to detect the variability of airflow limitation, which has been the main objective hallmark of asthma diagnosis for decades.[6,7] Because the BPT is expensive, time-consuming, and entails a risk of severe bronchospasm, it is not available in all hospitals, especially primary hospitals.[8] BDT is more convenient, safe, and has higher specificity, but its sensitivity in mild asthma is low because a 12% improvement is difficult for patients with a normal baseline forced expiratory volume in 1 second (FEV_1) to achieve.[9] An increase in FEV_1 >200 mL and an improvement of BDT >12% after 1 to 3 months' anti-asthma treatments is also recommended as a diagnostic criteria of asthma in GINA 2019.[3] Unfortunately, there is a lack of evidence to define which patients might benefit from diagnostic treatment. For symptomatic improvement would be due to the placebo effect, ICS might be given inappropriately.[10] Therefore, it is essential to identify a convenient method with high sensitivity to predict which patients might benefit from diagnostic treatment.

The purpose of our research is to investigate whether changes in spirometric indices alone or combined with fractional exhaled nitric oxide (FENO) can help with diagnosing mild asthma and predicting response to anti-asthma treatment (RAT) in

patients with variable asthmatic respiratory symptoms, FEV_1 ≥80% predicted, and negative BDT.

MATERIALS AND METHODS

The study protocol was approved by the Institutional Review Board at Shanghai General Hospital (NO.2018KY186), and registered on chictr.org.cn (NO. ChiCTR2000029065). Written informed consent was obtained from each subject.

PART I

Study design

An open-label, single-center, prospective study was performed at the Pulmonary Outpatient Clinic of Shanghai General Hospital (Shanghai, China). Consecutive patients with chronic recurrent wheeze, shortness of breath, chest tightness, and/or cough (asthma symptoms), FEV_1 ≥80% predicted, and negative BDT were enrolled. All underwent an asthma control test (ACT), FENO, echocardiography, and high-resolution computed tomography (HRCT) scan and were given 4 weeks of ICS and a long-acting β-agonist (LABA). Follow-up spirometry and ACT were performed after the ICS/LABA treatment. Improvement of symptoms was reported by telephone or WeChat weekly (Figure 1).

Participants

Inclusion criteria were as follows: 16-75 years old, with recurrent asthma symptoms lasting more than 8 weeks, normal cardiac structure and function, normal chest HRCT confirmed by two specialists, normal complete blood count results except for eosinophils, FEV_1/forced vital capacity (FVC) >0.7, FEV_1% ≥80% predicted after administration of salbutamol, and negative BDT according to GINA standards (increase in FEV_1 ≤12% and/or ≤200 mL from baseline).

Exclusion criteria included a respiratory infection in the 8 weeks before screening; cigarette smoking (including current smoking, cessation within 2 months, or smoking history over 10 pack-years); pregnancy; concomitant systemic respiratory disease, including chronic obstructive pulmonary disease; or other significant medical

problems as determined by the principal investigator. Potential participants were also excluded if they had used short-acting drugs (e.g., the β-agonist salbutamol or the anticholinergic agent ipratropium bromide) within 1 day, long-acting drugs (e.g., salmeterol or formoterol, aminophylline, or slow-release β-agonists) within 2 weeks, oral or inhaled steroids within 4 weeks, or oral β-blockers or angiotensin-converting enzyme inhibitors within 4 weeks prior to screening.

Spirometry, BDT, and FENO measurements

Spirometry, including bronchodilation tests, was performed by the same technologist with the same spirometer (Jaeger Co, Hoechberg, Germany) in accordance with the specifications and performance criteria recommended in the American Thoracic Society (ATS)/European Respiratory Society (ERS) Standardization of Spirometry.[11] Participants underwent spirometry before and 15 minutes after inhaling salbutamol. The response to the bronchodilator was expressed as the percentage change relative to the pre-bronchodilator value of FEV_1 ($\Delta FEV_1\%$), FVC (ΔFVC%), and forced expiratory flows (FEFs; ΔFEFs%) and as the absolute change of FEV_1 (ΔFEV_1), ΔFVC, and ΔFEFs).

FENO (NIOX MINO, Aerocrine AB, Solna, Sweden) was calculated with a mathematical model by measuring exhaled NO at a standard flow rate of 50 mL/s at a flow rate of 50 mL/s.

Assessment of asthma control and symptom improvement

Asthma control was assessed in all patients with the ACT. This questionnaire consists of five questions, each scored on a 5-point scale from 1 to 5 (from reporting the symptom all the time or very frequently to never reporting it).[12] Therefore, the total ACT score is between 5 and 25, with lower scores representing poorer asthma control. ACT score were categorized as follows: ACT < 16 (uncontrolled), 16 ≤ ACT ≤ 19 (poorly controlled), 20 ≤ ACT ≤24 (well-controlled), and ACT = 25 (complete asthma control).[13]

Drugs and group definition

Inhaled salbutamol (Vetolin, salbutamol sulphate inhaled aerosol, Registration

ID: JX20080307, 400 μg, GlaxoSmithKline) was used for the BDT. Budesonide and formoterol fumarate powder (160 μg budesonide and 4.5 μg formoterol per puff, one puff twice per day, AstraZeneca) was used as the anti-asthma treatment. All of the participants were educated with the "teach-back" method[14] to ensure that they used the inhaler correctly.

After 4 weeks of therapy, patients were divided into three groups according to their RAT: positive diagnosis of asthma (PD) (FEV_1 improved >200 mL and >12%), suspected diagnosis of asthma (SD) (FEV_1 improved >200 mL and ≤12%), and negative diagnosis of asthma (ND) (FEV_1 improved ≤200 mL and <12%). Improved symptoms were defined as improvement of ACT by one or more categories from baseline or post-treatment ACT (ACT_2) >19.

Statistical analysis

Data analysis was performed with SPSS software version 23.0 (SPSS Inc., Chicago, Illinois, USA) except for the receiver operating characteristic (ROC) contrast estimation and ROC contrast test, which were performed with SAS Proc LOGISTIC version 9.4 (SAS Institute Inc., Cary, NC, USA). Baseline data are presented descriptively. Normally distributed data are presented as mean ± standard deviation. Normality of distribution was checked with the Kolmogorov-Smirnov test.

Demographic data were analyzed with one-way analysis of variance (ANOVA) if normally distributed, or by Kruskal-Wallis if not, and the difference between two groups were analyzed with Student-Newman-Keuls. The spirometric indices in the three groups were analyzed with multivariate ANOVA.

Increase in FEV_1 by >200 mL and improvement of symptoms from baseline after 4 weeks of anti-inflammatory was defined as positive RAT (PRAT). The prediction performance of each variable was measured as the AUC of the ROC derived from the logistic regression models.[15] The optimal value giving the highest sum of sensitivity and specificity was used as a cut-off value to judge the PRAT. Positive predictive values (PPV), negative predictive values (NPV), and percentages correctly classified were calculated for each cut-off value.[16-18] The corresponding contrast values,

confidence intervals, and p values were also calculated. Furthermore, a multiple logistic model of the 2 variables was fitted, and the resultant AUC of this multiple logistic model was used as a measure of the joint prediction performance. We use the chi-square test proposed by DeLong et al to determine whether the multiple logistic model would significantly improve the prediction performance, defined as the AUC, relative to the marginal models.[19]

The threshold for statistical significance for all analyses was set at $p < 0.05$.

PART II

Study design

To investigate the possible pathological characteristics of patients with RAT, patients with lung nodules who accepted pneumectomy were also included in this study. The patients were grouped by predicted RAT according to the predictive model verified in Part I, and their lung biopsy tissues were analyzed.

Participants

Inclusion criteria were as follows: 16-75 years old; had lung nodules; accepted pneumectomy; completed the spirometry, BDT, FENO, and complete blood cell count before surgery; and had $FEV_1\% \geq 80\%$, $FEV_1/FVC > 0.7$, and a maximum diameter of the pulmonary nodule <3 mm. Exclusion criteria were the same as those in Part I.

Subjects with $\Delta FEV_1\% > 3.50\%$ in BDT and FENO >33 parts per billion (ppb) and who matched the criteria of the predictive model established in our study were classified into the predicted PRAT group, and patients with lower $\Delta FEV_1\%$ or FENO than the criteria of the predictive model were classified into the predicted negative RAT (NRAT) group.

Pathological section preparation, cells count, and cytokine analysis

During the lobectomy, lung tissue at least 5 cm away from the pulmonary nodules was resected, and micrographs of hematoxylin-eosin (HE)-stained slides were collected. Stained slices were analyzed by two pathologists who were blinded to group assignment. Eosinophils, macrophages, lymphocytes, and neutrophils within the epithelial and submucosal areas were counted.

About 200 mg of lung tissue was washed, homogenized with an electric homogenizer for about 5 minutes, and centrifuged at 3000 × *g* at 4°C for 15 min. The supernatants were subjected to Enzyme-linked immunosorbent assays (ELISAs) for the following cytokines: nuclear factor kappa-B (NF-κB)(40096, Active Motif, California, USA), interleukin (IL) 4 (EL10026, Anogen, Ontario, Canada), IL-5 (EL10035, Anogen, Ontario, Canada), IL-6 (EL10023, Anogen, Ontario, Canada), IL-8 (EL10008, Anogen, Ontario, Canada), IL-13 (EL10054, Anogen, Ontario, Canada), and transforming growth factor-β_1 (TGF-β_1)(EL10029, Anogen, Ontario, Canada).

Statistical analysis

Fisher's exact test was performed for the analysis of inter-group differences for categorical variables. The independent t-test or Mann-Whitney test was performed for inter-group comparisons for continuous variables.

The threshold for statistical significance for all analyses was set at $p<0.05$.

RESULTS

PART I

Demographic and clinical characteristics data

Between April 1, 2020, and June 30, 2020, 110 patients with recurrent asthma symptoms were enrolled, of whom 102 patients completed the 4-week treatment and scheduled spirometry at the second visit (within 7 days before or after the end of the 4-week treatment); 8 patients were excluded because they did not attend the second visit on time (Figure 1). Data from the 102 patients who completed the study were analyzed.

There were 46 patients in the ND group (45.1%), 27 patients in the SD group (26.5%), and 29 patients in PD group (28.4%). All spirometric indices increased after bronchodilation in all 3 groups; they increased further after the 4-week treatment period in the PD and SD groups but not in the ND group (Figure 2).

Most demographic data and clinical features did not differ between the three groups at baseline (Table 1). However, FEV_1%pred and FEFs%pred (except

$FEF_{75\%}$pred) were lower in the PD group ($p<0.05$). The PD and SD groups had higher baseline FENO than the ND group ($p<0.05$ for both), and baseline ACT score was significantly higher in the ND group than in the PD group ($p<0.05$).

The baseline spirometric indices did not differ significantly between the three groups at baseline (p=0.065, multivariate ANOVA in Table S1 in Supplementary File). Post-treatment spirometric indices in SD group were higher than ND group (p=0.017, multivariate ANOVA in part 1 in Supplementary File). Absolute and percentage increases from baseline to the post-treatment measurement were significantly different among the three groups (p<0.05 for all; part 2 in Supplementary File). Improvements of each spirometric index from baseline to after bronchodilation and treatment are shown in part 2 of Supplementary File.

After the 4-week treatment, the ACT score was significantly higher in the PD group and SD group than the ND group (p<0.05 for both). We therefore classified the SD and PD groups as the PRAT group and the ND group as NRAT. ACT categories in the NRAT and PRAT groups before and after treatment are shown in Figure 3. The symptom recovery time was longer in the ND group compared with the PD group (p<0.05, Table 1).

Predictive values of single measurements

There were 56 patients in the PRAT group (55% of the total; 27 SD and 29 PD) and 46 in the NRAT group (45%). Patients with PRAT had significantly higher ΔFVC, ΔFEV_1, ΔFEF_{50}, ΔFEF_{75}, ΔFEF_{25-75}, ΔFVC%, ΔFEV_1%, ΔFEF_{25}%, ΔFEF_{50}%, ΔFEF_{75}%, and ΔFEF_{25-75}%, than those with NRAT (p<0.05 for all). The baseline FENO was also higher in the PRAT group than in the NRAT group (p<0.001; part 3 in Supplementary File).

The prognostic value of these variables for RAT prediction was calculated by AUC (Table 2). The two largest AUCs were the ΔFEV_1 (0.833, 95% CI 0.753 to 0.913) and ΔFEV_1% (0.824, 95% CI 0.741 to 0.906), taking the optimal cut-off values of 100 mL and 3.50%, respectively. The AUCs of FENO, ΔFEF_{25-75}%, and ΔFEF_{75}% for PRAT were 0.703, 0.736, and 0.710 with cut-off values of 33 ppb, 15.26%, and 26.04%, respectively.

Predictive value of joint models: FENO & $\Delta FEV_1\%$ or $\Delta FEFs\%$

In evaluated joint models, FENO and $\Delta FEV_1\%$ had the highest AUC (0.880; Table 3), taking the cut-off values of 33 ppb for FENO and 3.50% for $\Delta FEV_1\%$, significantly higher than the AUC of $\Delta FEV_1\%$ alone (p=0.034). The AUC for FENO combined with $\Delta FEF_{25-75}\%$ was 0.803. The AUC of FENO combined with $\Delta FEF_{75}\%$ was 0.793 (Figure 4).

PART II

Clinical characteristics of subjects enrolled

Nineteen subjects who met the inclusion criteria were enrolled, 11 in the PRAT group and 8 in the NRAT group. In the PRAT group, 4 subjects had a positive BDT. There was no significant inter-group difference for age, sex, BMI, smoking history, eosinophils in blood, or baseline spirometric indices, except for $FEF_{25}\%$, which was significantly lower in the predicted PRAT group (p=0.033), and FENO, which was higher in the predicted PRAT group than in the NRAT group (p=0.006; Table 4).

Pathology and ELISA analysis of cytokines

The concentrations of IL-4, IL-5, IL-13, and NFκB in the predicted PRAT group were significantly higher than in the predicted NRAT group (p=0.001, 0.017, 0.032, and 0.026, respectively; Figure 5). There were no inter-group differences for IL-6 and IL-8 (p>0.05; Figure 5).

Photomicrographs of lung tissue in predicted PRAT and predicted NRAT group are shown in Figure 6. The groups did not differ significantly in the number of inflammatory cells, including macrophages, neutrophils, lymphocytes, and eosinophils (Table 4).

DISCUSSION

We investigated whether the airway reversibility detected by BDT combined with FENO in patients with $FEV_1 \geq 80\%$ predicted and negative BDT. The joint model of $\Delta FEV_1\% > 3.50\%$ and FENO >33 ppb predicted PRAT, and pathological evidence

of an imbalance in inflammatory cytokines supported the presence of asthma in these patients. $\Delta FEF_{25-75}\%$ or $\Delta FEF_{75}\%$ combined with FENO also had predictive value for PRAT. Most spirometric indices in the PD group were lower than those in the ND and SD groups, and small-airway dysfunction was more severe, which was consistent with previous study that small-airway dysfunction is a reminder of bronchial hyperresponsiveness.[20] Addressing increases of spirometric indices in BDT and small-airway dysfunction may mitigate the decrease in general health, vitality, and mental health problems caused by undiagnosed asthma.[21]

FEV_1 is an essential spirometric marker indicating airway obstruction in the central airway, with a small degree of variation in the healthy population. It is used to evaluate the variability of airflow limitation in BDT and BPT. However, the sensitivity of the diagnostic criteria is low in mild asthma; because the percentage change of FEV_1 in BDT is negatively correlated with baseline FEV_1,[22] patients with mild asthma and $FEV_1 \geq 80\%$ predicted rarely meet the current standard for diagnosis of asthma: FEV_1 improved >12% in BDT, even if the absolute change is much more than 200 mL. BPT is a good alternative choice for those patients. Unfortunately, few hospitals in China, especially primary hospitals, are currently able to conduct BPT.

GINA recommended a combination of variable respiratory symptoms and expiratory airflow limitation to confirm an asthma diagnosis. Any increase in spirometry after initiating treatment for asthma control can help to confirm the diagnosis of asthma.[3] Airway reversibility may be demonstrated only after over 2-8 weeks of anti-inflammatory therapy,[7] which provides time for reducing airway wall edema and glandular hyperplasia or remodeling the airway. In our study, 28.43% of patients with negative BDT could be definitively diagnosed with asthma after treatment. In addition, the ACT score improved in 26.49% patients with negative BDT and FEV_1 that improved ≤12% but >200 mL after ICS/LABA treatment. We defined those patients as showing PRAT, together with patients who showed improvement of FEV_1 >200 mL and >12% after treatment. Our results indicate that negative BDT is not a sufficient reason to avoid anti-asthma medication, especially for patients with

normal central airway function.

The most important result from our study is that certain degrees of improvement of FEV_1 combined with FENO can predict RAT, even if the criteria for asthma diagnosis are not met. This finding can support physicians in accurately identifying patients with asthma, confidently administering experimental anti-asthma treatments, and reducing the abuse of glucocorticoids. $\Delta FEV_1\%$ >3.50% and FENO >33 ppb both predicted PRAT and were associated with pathological evidence of Th1/Th2 imbalance. Several studies[23,24] used BDT to predict ICS therapy responsiveness in children with mild-to-moderate asthma. Predicted values of ΔFEV_1 >7.5% or >10% were used to predict response to ICS. This difference in cut-off value may be that the patients included in our study were adults and not all had a clear diagnosis of asthma.

FENO is widely accepted as a biomarker of eosinophilic airway inflammation,[25] and increased FENO has been demonstrated in mild asthma.[26] In a placebocontrolled trial, FENO>47 ppb predicted good responses to ICS in patients with non-specific respiratory symptoms and insignificant bronchodilator reversibility.[27] In our study, the optimal value of FENO for clinical prediction was >33 ppb with a sensitivity and specificity of 55.36% and 47.65%, respectively. The difference in cut-off values was likely due to the different characteristics of the patients and treatment drugs. The AUC of FENO alone was relatively low in the PRAT group, but when combined with BDT, its predictive accuracy improved significantly. Assessment of airway inflammation and reversibility simultaneously may therefore contribute to the diagnosis and treatment of asthma.

Small-airway reversibility is another predictor of RAT that we addressed. After salbutamol inhalation, the small-airway resistance decreased in concert with improvement of central airway obstruction.[28] Thus, the small airways contribute to resistance in the entire airway of patients with obstructive airways disease[29] and are deeply involved in the pathogenesis of asthma. Small-airway dysfunction appears at an early stage and is involved in the mildest forms of asthma.[30] Recent studies have demonstrated that FEF_{25-75} is more sensitive as an indicator of symptomatic asthma

than FEV_1[31-33] and is useful in predicting bronchial hyperresponsiveness.[20] We also found that $\Delta FEF_{75}\%$ and $\Delta FEF_{25-75}\%$ were associated with an increased likelihood of PRAT, although their predictive values were lower than that of ΔFEV_1. Despite this, we believe that further exploration of the correlation between small-airway indices and the improvement of clinical symptoms after application of ultrafine-particle drugs may provide more effective clinical evidence for the diagnosis of asthma and individualized treatment.

ACT is a brief, easy-to-administer, validated, patient-based index of asthma control.[12] We used the improvement of ACT by one or more stages after treatment or ACT_2 >19 as the criteria for PRAT. In our study, patients in the PD group had a shorter symptom recovery time than those in the ND group, and short symptom recovery time was related to anti-asthma therapy, other than self-cure.

Analysis of lung biopsy tissues from patients with lung nodules who accepted pneumectomy were also included in our study. IL-4, IL-5, IL-13, and NFκB were higher in lung tissue of the predicted PRAT group than of the predicted NRAT group ($p<0.05$). IL-4, IL-5, and IL-13 are typical T_2 type cytokines,[34] and activation of the NFκB pathway is always involved in the pathogenesis of asthma. The ELISA analysis in our study provided evidence that Th1/Th2 imbalance occurs in patients with baseline normal FEV_1, FENO >33 ppb, and improvement of $FEV_1\%$ >3.50%, despite a negative BDT. This evidence that pathological changes occur in the early stage of asthma increases the credibility of the predictive model.

There are a number of limitations regarding our study. First, the sample size was relatively small, and a large multicenter clinical study will be necessary to confirm the results. Second, patients in the SD group will need further observations to make a definitive diagnosis. The correlation between small-airway parameters and the improvement of clinical symptoms needs further exploration, and the use of ultrafine-particle drugs may provide more effective clinical evidence for diagnosing asthma and individualizing treatment, because ultrafine inhaled drugs may be deposited more effectively in the small airways than drugs with larger particles.[35]

In conclusion, our research found that the airway responsiveness after administration of salbutamol (improvement of FEV_1 >3.50%) combined with FENO measurements >33 ppb can predict PRAT in patients with mild asthma and negative BDT. Attention to small-airway improvement can also improve the diagnosis and control of asthma. In primary hospitals or institutions without access to BPTs, conducting BDT and FENO can guide decisions on which patient should receive anti-asthma therapy.

Contributors

HH and MZ conceived of and designed entire study. XZ, DY, YX, and LH contributed to data collection. CL performed spirometry, BDT, and FENO. YZ, and PZ were involved in interpreting clinical data. WB and YZ performed the ELISAs, cell counts, and pathological analysis. HH, JY, and ZH performed statistical analyses. HH and WB wrote the manuscript, supervised by MZ. All authors critically reviewed and approved the final version.

All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Table 1 Demographic data, clinical features in ND, SD, and PD group

Characteristics and variables	ND (n = 46)	SD (n = 27)	PD (n = 29)	P value
Age (years)§	44.7±14.9	39.6±13.9	39.8±15.2	0.240
Gender, male (n, %)§	17 (37.0%)	16 (59.3%)	11 (37.9%)	0.163
Height (cm)§	164.7±7.2	167.7±8.6	166.1±8.4	0.283
Weight (kg)§	61.2±11.9	63.3±11.3	65.0±12.7	0.389
BMI (kg/m^2)§	22.41±2.92	22.45±3.17	23.48±3.75	0.332
Former smoker (n, %)	5 (10.9%)	1 (3.7%)	2 (6.9%)	0.650
Cough (n, %)	32 (69.6%)	20 (74.1%)	22 (75.9%)	0.847
Nocturnal symptoms (n, %)	4 (8.7%)	2 (7.4%)	6 (20.7%)	0.242
Shortness of breath (n, %)	3(6.5%)	5(18.5%)	1(3.5%)	0.168
Chest tightness (n, %)	18 (39.1%)	8 (29.6%)	8 (27.6%)	0.531
Wheeze (n, %)	15 (32.6%)	4 (14.8%)	8 (27.6%)	0.266
Dyspnea (n, %)	9 (19.6%)	3 (11.1%)	5 (17.2%)	0.743
Allergic rhinitis (n, %)	14 (30.4%)	14 (51.9%)	12 (41.4%)	0.191
Former skin allergy (n, %)	8(17.4%)	11 (40.7%)	9 (31.0%)	0.093
FENO (ppb)¶	**19 [17]**	**40 [27] †**	**30 [30] ※**	**0.001**
ACT_1¶	**18 [3]**	**17 [3]**	**16 [3] ※**	**0.009**
ACT_2¶	**21 [3]**	**23 [2] †**	**23 [2] ※**	**<0.001**
ΔACT ¶	**3 [3]**	**5 [3]**	**7 [3] ※**	**<0.001**
WBC ($*10^9/L$)§	6.36±1.50	6.31±1.38	6.58±1.52	0.756
EOS%¶	2.1 [2.4]	1.4 [1.7]	1.9 [2.4]	0.257
EOS ($*10^9/L$)¶	0.12 [0.14]	0.11 [0.29]	0.13 [0.20]	0.454
Symptom recovery time (days)¶	**19 [34]**	**7 [16]**	**7 [6.5] ※**	**0.002**
FVC%pred§	99.4±12.7	100.6±13.1	96.9±11.9	0.520
FEV_1%pred§	**96.3±10.0**	**98.2±11.6**	**90.2±12.5 ※♯**	**0.020**
PEF%pred§	**94.0±14.9**	**93.7±12.0**	**85.0±17.7 ※♯**	**0.030**
FEF_{25}%pred§	**94.5±16.1**	**94.2±15.6**	**81.4±17.7 ※♯**	**0.002**
FEF_{50}%pred§	**80.3±20.2**	**83.9±18.4**	**64.6±17.2 ※♯**	**<0.001**
FEF_{75}%pred§	68.8±26.7	71.6±25.9	58.6±25.9	0.141
FEF_{25-75}%pred§	**74.9±19.4**	**79.7±18.7**	**62.0±20.4 ※♯**	**0.002**

ND, negative diagnosis of asthma; SD, suspected diagnosis of asthma; PD, positive diagnosis of asthma; FENO, fractional exhaled nitric oxide; ACT_1, asthma control test at the first visit; ACT_2, asthma control test at the second visit; ΔACT, ACT change from baseline ($\Delta ACT = ACT_2 - ACT_1$). WBC, white blood cells; EOS, eosinophils; BMI, body mass index; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; PEF, peak expiratory flow; FEF_{25}, forced expiratory flow at 25% of forced vital capacity; FEF_{50}, forced expiratory flow at 50% of forced vital capacity; FEF_{75}, forced expiratory flow at 75% of forced vital capacity; FEF_{25-75}, forced expiratory flow at 25% to 75% of forced vital capacity; %pred, spirometric indices as a percentage of predicted value. § Mean ± standard deviation values; ¶ Median [IQR] values; †, the difference between ND and SD was statistically significant; ※, the difference between ND and PD was

statistically significant; ♯ the different between SD and PD was statistically significant; Statistical significance is shown by bold font.

Table 2. Optimal cut-off values for the prediction of positive response to anti-asthma treatment (PRAT)

Characteristics and variables	AUC	Cut-off values*	Sensitivity %	Specificity %	PPV %	NPV %	PCC %	Odds ratio	95% CI	P value
FENO	**0.703**	**33**	**47.65**	**55.36%**	**82.61%**	**79.49%**	**60.32%**	**1.039**	**(1.013,1.066)**	**0.003**
ΔFVC	**0.682**	**50**	**66.47**	**66.07%**	**69.57%**	**72.55%**	**62.75%**	**1.004**	**(1.001,1.007)**	**0.008**
ΔFEV_1	**0.833**	**100**	**67.39**	**82.14%**	**73.91%**	**79.31%**	**77.27%**	**1.013**	**(1.007,1.019)**	**< 0.001**
ΔFEF_{50}	**0.700**	**460**	**64.13**	**66.07%**	**71.74%**	**74.00%**	**63.46%**	**1.001**	**(1.000,1.002)**	**0.004**
ΔFEF_{75}	**0.727**	**260**	**84.12**	**62.50%**	**76.09%**	**76.09%**	**62.50%**	**1.002**	**(1.001,1.004)**	**0.002**
ΔFEF_{25-75}	**0.747**	**430**	**58.82**	**64.29%**	**80.43%**	**80.00%**	**64.91%**	**1.002**	**(1.001,1.003)**	**0.001**
ΔFVC%	**0.665**	**2.5237**	**66.47**	**46.43%**	**82.61%**	**76.47%**	**55.88%**	**1.144**	**(1.034,1.264)**	**0.009**
ΔFEV_1%	**0.824**	**3.4965**	**70.00**	**83.93%**	**78.26%**	**82.46%**	**80.00%**	**1.455**	**(1.247,1.697)**	**< 0.001**
ΔFEF_{25}%	**0.620**	**5.5066**	**0.65**	**60.71%**	**63.04%**	**66.67%**	**56.86%**	**1.037**	**(1.002,1.073)**	**0.039**
ΔFEF_{50}%	**0.710**	**17.6471**	**43.79**	**62.50%**	**78.26%**	**77.78%**	**63.16%**	**1.049**	**(1.018,1.081)**	**0.002**
ΔFEF_{75}%	**0.710**	**26.036**	**56.95**	**62.50%**	**73.91%**	**74.47%**	**61.82%**	**1.030**	**(1.012,1.049)**	**0.001**
ΔFEF_{25-75}%	**0.736**	**15.2610**	**65.29**	**73.21%**	**69.57%**	**74.55%**	**68.09%**	**1.058**	**(1.026,1.091)**	**< 0.001**

AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value; PCC, percentage correctly classified; odds ratio, odds ratio of characteristic variables; 95% CI, 95% confidence interval of odds ratio; P Value, the p value of logistic regression test.* The cut-off points were selected by maximizing the sum of sensitivity and specificity. Δ, increase of spirometric parameters in BDT; Δ, spirometric indices%, increase in spirometric indices as a percentage of baseline value. The other abbreviations are as defined for Table 1.

Table 3. Predictive values of different joint models in predicting PRAT

Characteristics and variables	AUC	95% CI (AUC)	Sensitivity %	Specificity %	PPV %	NPV %	PCC %	Contrast	95% CI (Contrast)	*P* Value
ΔFEV_1 + ΔFEV_1%	0.832	(0.752,0.913)	83.93%	78.26%	82.46%	80.00%	81.37%	-0.001	(–0.016 to 0.014)	0.877
ΔFEV_1 + ΔFEF_{25-75}	0.838	(0.759,0.917)	80.36%	78.26%	81.82%	76.60%	79.41%	0.005	(–0.008 to 0.018)	0.468
ΔFEV_1% + ΔFEF_{25-75}%	0.825	(0.743,0.907)	80.36%	78.26%	81.82%	76.60%	79.41%	0.001	(–0.010 to 0.013)	0.812
ΔFEV_1 + ΔFEF_{50}	0.830	(0.749,0.911)	77.59%	80.43%	83.33%	74.00%	78.85%	-0.003	(–0.008 to 0.002)	0.181
ΔFEV_1% + ΔFEF_{50}%	0.823	(0.741,0.906)	83.93%	78.26%	82.46%	80.00%	81.37%	-0.000	(–0.001 to 0.000)	0.480
ΔFEV_1 + ΔFEF_{75}	0.841	(0.764,0.918)	67.86%	89.13%	88.37%	69.49%	77.45%	0.008	(–0.020 to 0.036)	0.579
ΔFEV_1% + ΔFEF_{75}%	0.830	(0.749,0.910)	78.57%	78.26%	81.48%	75.00%	78.43%	0.006	(–0.014 to 0.026)	0.551
FENO + ΔFEV_1	0.870	(0.799,0.941)	78.57%	82.61%	84.62%	76.00%	80.39%	0.037	(–0.012 to 0.086)	0.136
FENO + ΔFEV_1%	**0.880**	**(0.812,0.949)**	**89.29%**	**73.91%**	**80.65%**	**85.00%**	**82.35 %**	**0.057**	**(0.004** to **0.109)**	**0.034**
FENO + ΔFEF_{25-75}	0.779	(0.687,0.872)	75.00%	78.26%	80.77%	72.00%	76.47%	0.033	(–0.029 to 0.095)	0.300
FENO + ΔFEF_{25-75}%	0.803	(0.716,0.891)	73.21%	80.43%	82.00%	71.15%	76.47%	0.067	(–0.002 to 0.136)	0.055
FENO + ΔFEF_{75}	0.766	(0.673,0.859)	78.57%	65.22%	73.33%	71.43%	72.55%	0.039	(–0.037 to 0.115)	0.312
FENO + ΔFEF_{75}%	**0.793**	**(0.704,0.882)**	**80.36%**	**71.74%**	**77.59%**	**75.00%**	**76.47 %**	**0.084**	**(0.012** to **0.155)**	**0.022**
FENO + ΔFEF_{50}	0.759	(0.664,0.855)	67.86%	80.43%	80.85%	67.27%	73.53%	0.057	(–0.018 to 0.131)	0.136

The abbreviations are as defined for Tables 1 and 2.

Bold font indicates statistical significanc

Table 4 Demographic data in the predicted NRAT group and predicted PRAT group in Part II.

Characteristics and variables	predicted NRAT (n = 8)	predicted PRAT (n = 11)	P value
Age (years)§	56.13±6.47	59.55±6.62	0.277
Gender, male (n,%)	5 (62.5%)	5 (55.5%)	0.650
Height (cm)§	164.25±7.25	162.09±6.64	0.509
Weight (kg)§	69.00±12.09	60.64±7.76	0.083
BMI (kg/m^2)§	25.42±3.03	23.13±2.94	0.116
Fomer smoker (n,%)	4 (50.0%)	2 (18.2%)	0.319
FVC%pred§	106.31±13.06	93.50±14.83	0.068
FEV_1%pred§	101.20±13.07	89.98±17.53	0.146
FEF_{25}%pred§	**100.40±18.30**	**78.72±21.33**	**0.033**
FEF_{50}%pred§	86.83±20.10	76.27±31.80	0.422
FEF_{75}%pred§	83.40±33.81	82.58±42.10	0.964
FEF_{25-75}%pred§	88.05±26.46	73.65±28.78	0.281
FENO (ppb)§	**18.50±6.97**	**45.36±23.27**	**0.006**
NFκB(ng/mg protein)§	**1.21±0.64**	**1.97±0.69**	**0.026**
p38 MAPK (OD/mg protein)§	0.23±0.12	0.22±0.15	0.827
IL-4 (pg/mg protein)§	**1.03±0.41**	**3.99±2.28**	**0.001**
IL-5 (pg/mg protein)¶	**1.70 [1.05]**	**3.38 [3.29]**	**0.017**
IL-6 (pg/mg protein)¶	3.47 [9.38]	10.56 [16.10]	0.137
IL-8 (pg/mg protein)¶	29.89 [33.54]	37.81 [65.06]	0.322
IL-13 (pg/mg protein)¶	**2.53 [1.69]**	**3.86 [7.54]**	**0.032**
TGF-β_1 (pg/mg protein)	135.34±31.43	108.82±55.76	0.244
Macrophages in lung tissue (/10 HPF)¶	7.17 [13.00]	4.00 [14.67]	0.535
Lymphocytes in lung tissue (/10 HPF)§	1.75±1.65	1.36±1.67	0.624
Neutrophils in lung tissue (/10 HPF)§	2.08±1.59	3.55±2.31	0.141
EOS in lung tissue (/10 HPF)¶	0.00 [0.00]	0.00 [0.00]	0.816
EOS in the blood ($*10^9$/L)¶	0.17 [0.13]	0.09 [0.09]	0.083
EOS% in the blood (%)¶	3.45 [3.18]	2.40 [2.7]	0.385

NRAT, negative response to anti-asthma treatment; HPF, high-power field. The other abbreviations are as defined for Tables 1 and 2. § Mean±SD values; ¶ Median [IQR] values.

Figure 1 Flow chart showing the course of study.

ND, negative diagnosis of asthma; SD, suspected diagnosis of asthma; PD, positive diagnosis of asthma; BDT, bronchodilator test; HRCT, high-resolution computed tomography scan; FEV_1, forced expiratory volume in 1 second; FENO, fractional exhaled nitric oxide; ACT, asthma control test; ICS/LABA, inhaled corticosteroid and long-acting β agonist.

Figure 2 Dynamic changes of spirometric variables in the three groups at baseline, after bronchodilation, and after 4 weeks of treatment.

ND, negative diagnosis of asthma; SD, suspected diagnosis of asthma; PD, positive diagnosis of asthma; After BD, after bronchodilation; After 4 weeks, after 4 weeks of treatment; FEV_1, forced expiratory volume in one second; FEF_{25}, forced expiratory flow at 25% of forced vital capacity; FEF_{50}, forced expiratory flow at 50% of forced vital capacity; FEF_{75}, forced expiratory flow at 75% of forced vital capacity; FEF_{25-75}, forced expiratory flow at 25% to 75% of forced vital capacity; %pred, spirometric actual measured value as a percentage of predicted value.

The mean values of spirometric variables in the ND, SD, and PD groups are shown at three times. The spirometric indices in the three groups were analyzed with one way ANOVA, and the difference between two groups were analyzed with Student-Newman-Keuls. *, the difference between ND and SD was statistically significant; #, the difference between ND and PD was statistically significant; @, the difference between SD and PD was statistically significant.

Figure 3. ACT categories in the NRAT and PRAT groups.

PRAT, positive response to anti-asthma treatment; NRAT, negative response to anti-asthma treatment; ACT_1, asthma control test at the first visit; ACT_2, asthma control test at the second visit.

Figure 4 ROC curves of the joint models predicting PRAT.

ROC curves for the joint models of (A) FENO and ΔFEV1%, (B) FENO and ΔFEF_{25-75}%, and (C) FENO and ΔFEF_{75}% in predicting PRAT.

(A) n=102; $AUC_{\Delta FEV1\% + FENO}$ = 0.880 (95% CI, 0.812 to 0.949); AUC_{FENO} = 0.703 (95% CI, 0.601 to 0.805); $AUC_{\Delta FEV1\%}$= 0.833 (95% CI, 0.741 to 0.906).

(B), n=102; $AUC_{FENO+\Delta FEF25-75\%}$ = 0.803 (95% CI, 0.716 to 0.891); AUC_{FENO} = 0.703 (95% CI, 0.601 to 0.805); $AUC_{\Delta FEF25-75\%}$ = 0.736 (95% CI, 0.638 to 0.834).

(C), n=102; $AUC_{FENO+\Delta FEF75\%}$ = 0.793 (95% CI, 0.704 to 0.882); AUC_{FENO} = 0.703 (95% CI, 0.601 to 0.805); $AUC_{\Delta FEF75\%}$ = 0.710 (95% CI, 0.607 to 0.812)

PRAT, positive response to anti-asthma treatment; ΔFEV_1%, the increase of forced expiratory in 1 second as a percentage of baseline value in a bronchodilation test (BDT). ΔFEF_{25-75}%, the increase of forced expiratory flow at 25% to 75% of forced vital capacity as a percentage of baseline value in BDT; FENO, fractional exhaled nitric oxide; ROC, receiver operating characteristic; AUC, area under the curve.

Figure 5 Inflammatory cytokines and cells in lung tissues from patients in the predicted PRAT and predicted NRAT groups in Part II.

PRAT, positive response to anti-asthma treatment; NRAT, negative response to anti-asthma treatment; positive, predicted PRAT group; negative, predicted NRAT group. IL, interleukin. NFκB, Nuclear factor kappa-B

p values obtained with the Mann-Whitney test.

Figure 6 Photomicrographs of lung tissue in the predicted PRAT and predicted NRAT groups in Part II.

Panel A-B, 100×; Panel C-D, 400×; A, C, predictive NRAT group; B, D, predictive PRAT group; Scale bar: 200 μm. PRAT, positive response to anti-asthma treatment; NRAT, negative response to anti-asthma treatment; Yellow arrows mark the eosinophils in the lung tissue.