Original Article

Bronchodilation Test with Inhaled Salbutamol Versus Bronchial Methacholine Challenge to Make an Asthma Diagnosis: Do They Provide the Same Information?

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What is already known about this topic? Surveys show that misdiagnosis of asthma is frequent in clinical practice. Bronchial methacholine challenge and reversibility to salbutamol are key tests in supporting an asthma diagnosis in patients with recurrent or chronic respiratory symptoms, even if positive response to these may also be present in chronic obstructive pulmonary disease.

What does this article add to our knowledge? It directly compares the 2 tests on the same population of patients. Being much less influenced by baseline airway caliber, positive methacholine challenge has a much greater occurrence than positive bronchodilation, though not selecting patients with different demographics or immuneinflammatory features.

How does this study impact current management guidelines? It reemphasizes that a lack of significant reversibility to salbutamol is common in patients with symptoms suspected to be due to asthma and should prompt the performance of a methacholine challenge to confirm the diagnosis. Our data also cast doubt on the utility of inflammatory biomarkers to make an asthma diagnosis.

BACKGROUND: Methacholine bronchial challenge and bronchodilation to salbutamol are key tests in clinical practice to make asthma diagnosis.
OBJECTIVE: To assess the concordance between the 2 tests and to see whether they actually identify the same population of asthmatics.
METHOD: We conducted a retrospective study using our asthma clinic database to see how methacholine bronchial challenge compared to bronchodilation to salbutamol in untreated patients with recurrent or chronic symptoms suspicious of asthma. We identified 194 untreated patients with baseline forced expiratory volume in 1 second (FEV_1) ≥70% predicted who had both a bronchodilation test with salbutamol and a methacholine bronchial challenge 7 to 14 days apart. A positive bronchial challenge was a provocative concentration of methacholine causing a 20% fall in FEV_1 ≤16 mg/mL, whereas a positive bronchodilation test was a reversibility to 400 μg inhaled salbutamol ≥12% from baseline and 200 mL.
RESULTS: Overall, asthma diagnosis was confirmed in 91% of cases leaving 9% of subjects with double negative tests. Isolated positive methacholine challenge was found in 71% of subjects, double positive tests in 17%, whereas isolated significant bronchodilation to salbutamol was rare (3%). There was no correlation between provocative concentration of methacholine causing a fall in FEV_1 of 20% (PC20M) and the magnitude of salbutamol reversibility ($P = .10$). Baseline FEV_1/forced vital capacity ratio inversely correlated with reversibility to salbutamol ($P < .001$) but not with PC20M ($P = .1$). No difference was found between the groups regarding demographic and immunoinflammatory features, including the proportion of eosinophilic asthma.
CONCLUSION: We conclude that methacholine challenge outperforms reversibility to salbutamol to diagnose asthma without selecting patients with distinct inflammatory profile. Baseline airway obstruction predicts magnitude of reversibility but not hyperresponsiveness. **(J Allergy Clin Immunol Pract 2020;8:618-25)**

Key words: *Asthma diagnosis; Methacholine hyperresponsiveness; Salbutamol reversibility*

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Abbreviations used
AUC- Area under the curve
FeNO- Fraction of exhaled nitric oxide
FEV_1- Forced expiratory volume in 1 second
FVC- Forced vital capacity
GINA- Global Initiative for Asthma
ICS- Inhaled corticoids
PC20M- Provocative concentration of methacholine causing a fall in FEV_1 of 20%

Asthma is a chronic airway disease characterized by excessive fluctuation of airway caliber over time. Asthma diagnosis in primary care remains a major issue with up to 30% of misdiagnosis.[1] In clinical practice, both the bronchodilating test and the bronchial provocation challenge have been proposed to ascertain the diagnosis. A threshold of 12% and 200 mL reversibility to salbutamol 400 μg has been advocated to claim significant forced expiratory volume in 1 second (FEV_1) reversibility and is currently endorsed by Global Initiative for Asthma (GINA) as a proof of asthma when combined with chronic respiratory symptoms.[2] Pivotal drug trials, which have established the value of maintenance treatment with inhaled corticoids (ICS) or leukotriene receptor antagonists, have often selected the asthmatic patients on the basis of reversibility to β_2 agonists. On the other hand, a provocative concentration of inhaled methacholine <16 mg/mL has also proved to be of great interest with diagnosis of mild-to-moderate asthma in routine practice[3] and in drug clinical trials.[4] Reversibility to salbutamol and constriction to methacholine may reflect different facets of airway lability. To the best of our knowledge, the relative performance of these tests to make an asthma diagnosis in untreated patients with recurrent or chronic respiratory symptoms has not been extensively studied on the same cohort of patients. The few studies dealing with that have shown that methacholine challenge had a better sensitivity than FEV_1 or peak exploratory flow rate reversibility to salbutamol in patients with baseline FEV_1 >65% to 80% predicted.[5,6] Based on sputum analysis of large asthma cohort, it is today accepted that asthma may show different airway inflammatory phenotypes.[7,8] Beyond the performance of the lung function tests to make asthma diagnosis, it also needs to be clarified whether, in case of discordance, the tests select patients with different demographic and inflammatory characteristics.

We have taken advantage of our large asthma clinic database to look at the relationship between the magnitude of the bronchodilation to salbutamol and the concentration of methacholine causing an FEV_1 fall of 20% in patients free of maintenance treatment and in whom asthma was suspected. Here we have compared the performance of bronchodilating test with salbutamol with that of bronchial methacholine challenge in 194 patients with baseline FEV_1 ≥70% predicted. Furthermore, we have compared the systemic and airway immunoinflammatory status of the patients diagnosed by the 2 tests.

METHODS

Study design

We conducted a retrospective cross-sectional study in our asthma clinic database at Liege CHU. Over a number of 1610 patients who were investigated from June 2006 until November 2018, we selected 194 untreated subjects with intermittent or chronic respiratory symptoms who were referred by 2 asthma-dedicated respiratory physicians to make an asthma diagnosis (Figure 1, Table I). All selected patients had baseline airway caliber ≥70% predicted and underwent both bronchodilation test with 400 μg inhaled salbutamol and a methacholine challenge performed 7 to 14 days apart. The bronchodilation test was performed by measuring FEV_1 reversibility on visit 1 after measuring fraction of exhaled nitric oxide (FeNO), followed by sputum induction and blood sampling, whereas the methacholine challenge was performed on visit 2. Asthma control and quality of life were assessed on visit 1 by self-administered questionnaires (Asthma Control Questionnaire[9] and Mini Asthma Quality of Life[10]) that the patient completed during the reversibility phase of the bronchodilation test. Asthma was diagnosed either by an FEV_1 reversibility of at least 12% and 200 mL from baseline or by a provocative concentration of methacholine ≤16 mg/mL. Additional analyses were performed using the bronchodilation threshold criterion of 9% FEV_1 predicted and the provocative concentration of methacholine causing a fall in FEV_1 of 20% (PC20M) threshold set at 8 mg/mL as recommended by some authors. The work was approved by the CHU Liege Ethics committee.

Bronchodilation test

At visit 1, each patient underwent the bronchodilation test irrespective of baseline FEV_1 and the FEV_1/forced vital capacity (FVC) ratio as a standard procedure. The best of 3 consecutive spirometry recordings was retained in accordance with the European Respiratory Society recommendation. Patients received 400 μg inhaled salbutamol administered by a metered-dose inhaler (Ventolin) + a spacer (Volumatic) one puff at a time into the spacer and spirometry was performed again 15 minutes later.

Methacholine challenge

At visit 2, each patient with baseline FEV_1 ≥70% predicted underwent a methacholine bronchial challenge. Methacholine chloride was purchased as powder (Provocholine 1280 mg; Metapharm, Brantford, Ontario, Canada) and dissolved in NaCl 0.9% by the hospital pharmacy to give appropriate concentrations. The methacholine challenge was performed by using a Hudson jet nebulizer (Hudson RCI; Micro Mist, Research Triangle Park, NC) activated by an airflow rate of 6 L/minute and delivering 0.3 mL/minute. Each patient successively inhaled for 1 minute quadrupling methacholine concentration starting from 0.06 mg/mL until a maximal concentration of 16 mg/mL. FEV_1 was measured 30 and 90 seconds after each inhaled concentration and the best value was retained. The test was stopped if FEV_1 had dropped by at least 20% from the baseline value. The PC20M was calculated by linear interpolation from the last 2 points of the curve. Those with a fall less than 20% at 16 mg/mL were arbitrarily given a value of 32 mg/mL to calculate correlations.

Sputum induction, blood sampling, and FeNO measurement

Sputum was induced using an ultrasonic nebulizer (Devilbiss Ultraneb; Devilbiss Healthcare, Manheim, Germany)[11] and processed[12] as previously described. Briefly, sputum was induced by hypertonic saline (5%) combined with salbutamol in the nebulizer cup. Sputum was then processed using the whole expectorate technique. Dithiothreitol was used as the mucolytic agent. Sputum was considered as adequate for cell count when squamous cell count was <80%. Differential cell count was performed on cytospins after Diff-Quick staining by counting 400 cells. Blood was sampled by a venous puncture from the forearm in the morning between 9H00

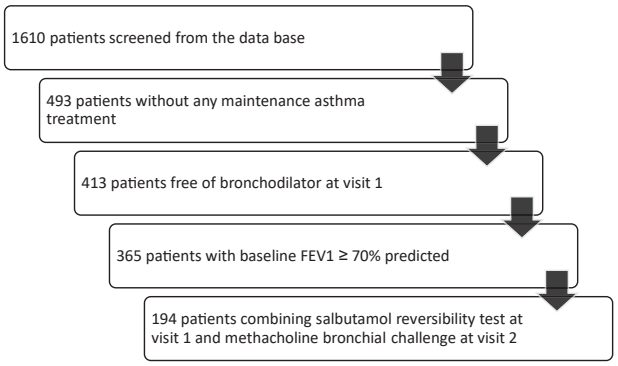

FIGURE 1. Flow chart of the patient selection process. *FEV_1*, Forced expiratory volume in 1 second.

and 12H00 at the end of visit 1 after the sputum induction. Hematological cell count, total and specific serum IgE, C reactive protein, and fibrinogen measurements were performed by the routine hospital laboratory. FeNO was measured using NiOX (Aerocrine, Solna, Sweden) at a flow rate of 50 mL/second.

Statistical analysis

Results are expressed as mean ± standard deviation or median (interquartile range) depending of the data distribution unless otherwise stated. Comparisons between multiple groups were performed by the Kruskal-Wallis test followed, when significant, by Dunn tests, for pairwise comparisons. Comparisons between proportions were performed by the χ^2 test. Correlations were assessed by calculating the Spearman coefficient of correlation. *P* values <5% were considered as statistically significant.

RESULTS

Proportion of patients diagnosed as being asthmatic according to the criterion chosen

Of the 194 patients, 176 (91%) had either positive bronchodilation to inhaled salbutamol or positive methacholine challenge leaving 18 patients being double negative (9%). Those with isolated positive methacholine challenge represented 71% of the subjects (137 of 194), whereas those combining both positive reversibility test and positive bronchial challenge represented 17% (33 of 194). The group diagnosed on the basis of an isolated positive bronchodilation was very limited, including only 6 patients that accounts for 3% of the cohort. Overall, the methacholine challenge identified 96% of asthmatics, whereas significant reversibility to inhaled salbutamol was observed in only 22% of patients receiving an asthma diagnosis. The concordance between the 2 tests was 19% in the patients receiving a diagnosis of asthma. For the whole cohort, there was no significant correlation between percentage of FEV_1 reversibility to salbutamol and PC20M ($r = -0.12$, $P > .05$) (Figure 2, *A*). Among the 41 patients who showed a reversibility from baseline ≥12%, only 2 had less than 200 mL. However, there were 84 patients who had a reversibility from baseline ≥200 mL, among whom 45 failed to reach the 12% reversibility. In these patients with exclusive 200 mL reversibility, FEV_1 averaged 93 ± 1.8% predicted and 3456 ± 127 mL (mean ± standard error of the mean), values which were significantly higher than those found in the 39 patients who had both 12% and 200 mL reversibility (87 ± 1.8%, $P < .05$ and 2527 ± 102 mL, $P < .0001$, respectively). If we choose the reversibility threshold of 9% predicted as the criterion of significant bronchodilation, we found 2 additional patients who qualified as being asthmatic, thereby leaving the group of double negative with only 16 patients. Overall, there were 61 patients who satisfied this bronchodilation criterion versus 39 patients for the criterion 12% from baseline and 200 mL. The group with 9% predicted included all the patients who had a 12% and 200 mL reversibility. When using the 9% predicted criterion, the concordance between methacholine challenge and bronchodilation to salbutamol reached 30% among those receiving an asthma diagnosis. As some guidelines recommend a PC20M threshold of 8 mg/mL in corticosteroid naïve patients to ascertain asthma (GINA), we also analyzed the results using that threshold. The number of patients in each group according to the different scenarios is given in Table II.

TABLE I. Demographic, functional, and inflammatory features of the whole asthma cohort

Age, y	49 ± 16	N = 194
Gender (F), n (%)	124 (64%)	N = 194
Tobacco NS/ES/CS, n (%)	109/41/44 (56%, 21%, 22%)	N = 194
BMI, kg/m^2	26 ± 4	N = 194
Atopy, Yes/No (%)	85/100 (44%, 56%)	N = 185
Prebronch FEV_1, % pred	94 ± 14	N = 194
Prebronch FVC, % pred	102 ± 14	N = 194
Prebronch FEV_1/FVC, %	77 ± 8	N = 194
Blood eosinophils, 1/μL	164 (92-256)	N = 190
Total IgE, kU/L	75 (25-220)	N = 188
FeNO, ppb	21 (12-36)	N = 183
Sputum eosinophils, %	1 (0-4.6)	N = 168

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV_1*, forced expiratory volume in 1 second; *FVC*, forced vital capacity; *IQR*, interquartile range; *NS*, non-smoker; *SD*, standard deviation.
Results are expressed as mean ± SD or median (IQR).

Relationship between baseline lung function and reversibility to salbutamol and hyperresponsiveness to methacholine

There was a significant inverse correlation between baseline airway obstruction assessed by the FEV_1/FVC ratio and the magnitude of reversibility to salbutamol ($r = -0.50$, $P < .0001$) (Figure 2, *B*). The strength of the relationship was much less between the FEV_1/FVC ratio and PC20M ($r = 0.15$, $P < .05$) (Figure 2, *C*). Likewise, % predicted FEV_1 correlated with reversibility to salbutamol ($r = -0.37$, $P < .0001$) and, to a lesser extent, to PC20M ($r = 0.25$, $P < .001$). However, % predicted FEV_1 failed to correlate with PC20M in the 33 patients who were both positive with salbutamol and methacholine challenge ($r = 0.05$, $P = .49$) (Figure E1, *A*, available in this article's Online Repository at www.jaci-inpractice.org), whereas % predicted FEV_1 was inversely correlated with the magnitude of reversibility in the same group ($r = -0.45$, $P < .01$) (Figure E1, *B*, available in this article's Online Repository at www.jaci-inpractice.org). Mean variation in baseline FEV_1 between the 2 visits was on average 5.5% ± 4.9% and did not correlate with PC20M ($r = -0.04$, $P > .05$) nor with the magnitude of bronchodilation to salbutamol ($r = 0.06$, $P > .05$). When drawing an receiver operating characteristic curve of % reversibility to predict positive methacholine challenge, we found a significant area under the curve (AUC) of 0.60

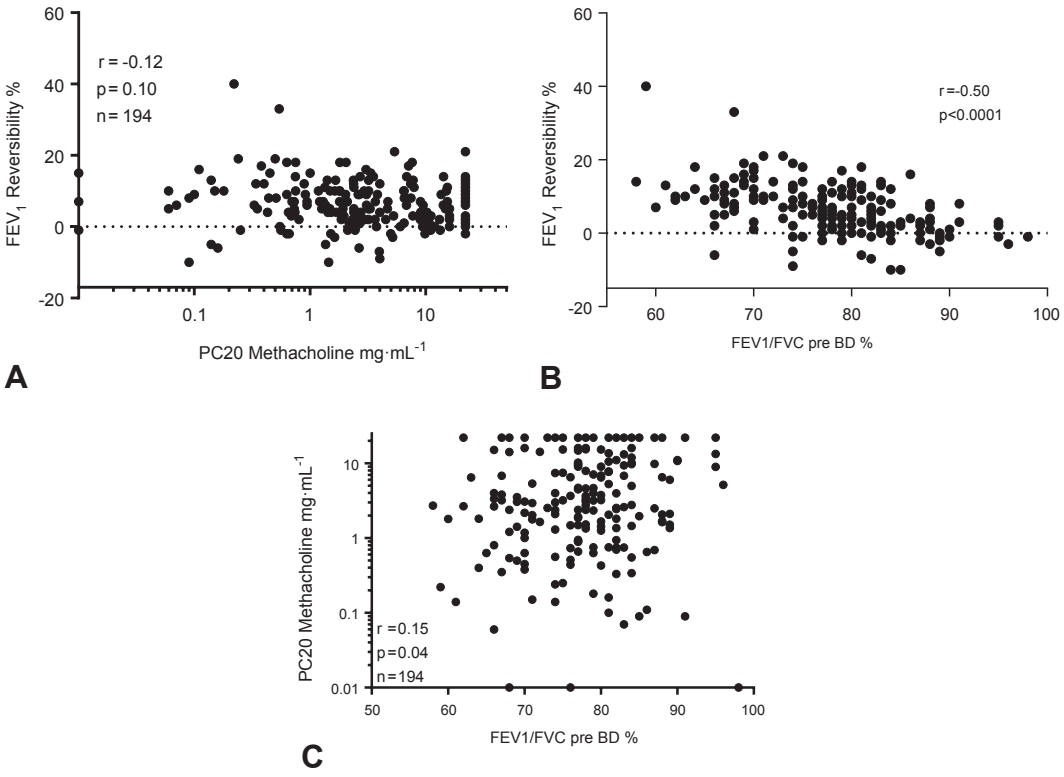

FIGURE 2. **A**, Relationship between PC20M and salbutamol reversibility. **B**, Relationship between the prebronchodilation ratio FEV_1/FVC and salbutamol reversibility. **C**, Relationship between the prebronchodilation ratio FEV_1/FVC and PC20M. The *r* value is the Spearman rank coefficient of correlation (n = 194). *PC20M*, Provocative concentration of methacholine causing a fall in FEV_1 of 20%; *FEV_1*, forced expiratory volume in 1 second; *FVC*, forced vital capacity.

TABLE II. Number of patients in each group according to the criteria used to define significant salbutamol reversibility test and methacholine challenge

	Salbutamol − Methacholine −	Salbutamol + Methacholine −	Salbutamol − Methacholine +	Salbutamol + Methacholine +	% Concordance* among asthmatics
Reversibility ≥12% and 200 mL PC20M ≤16 mg/mL	18	6	137	33	19
Reversibility ≥9% predicted PC20M ≤16 mg/mL	15	9	117	53	30
Reversibility ≥12% and 200 mL PC20M ≤8 mg/mL	46	7	109	32	21
Reversibility ≥9% predicted PC20M ≤8 mg/mL	40	13	92	49	32

PC20M, Provocative concentration of methacholine causing a fall in FEV_1 of 20%; *FEV_1*, forced expiratory volume in 1 second.
*Concordance is the double positive/all asthmatics ratio.

($P = .04$) when hyperresponsiveness was defined as PC20M <8 mg/mL but not as PC20M ≤16 mg/mL (AUC 0.53, $P > .05$). The cutoff of reversibility to predict PC20M ≤8 mg/mL was 3.5% with a sensitivity of 72% and a specificity of 47% (Table E1, available in this article's Online Repository at www.jaci-inpractice.org).

Relationship between inflammatory features and reversibility to salbutamol and hyperresponsiveness to methacholine

There was a significant inverse relationship between sputum eosinophils % and PC20M ($r = -0.28$, $P < .001$) (Figure 3, *A*) and also between FeNO and PC20M ($r = -0.24$, $P < .01$) (Figure 3, *B*). By contrast, no significant association was found between sputum eosinophils % or FeNO and reversibility to salbutamol ($r = 0.11$, $P > .05$ and $r = 0.14$, $P > .05$, respectively). Total serum IgE was correlated with reversibility to salbutamol ($r = 0.17$, $P < .05$) (Figure 3, *C*) but not with PC20M ($r = 0.05$, $P > .05$).

Comparison between the groups of patients according to the diagnostic test

The comparison between the 3 groups with a confirmed asthma diagnosis showed a significant difference for baseline

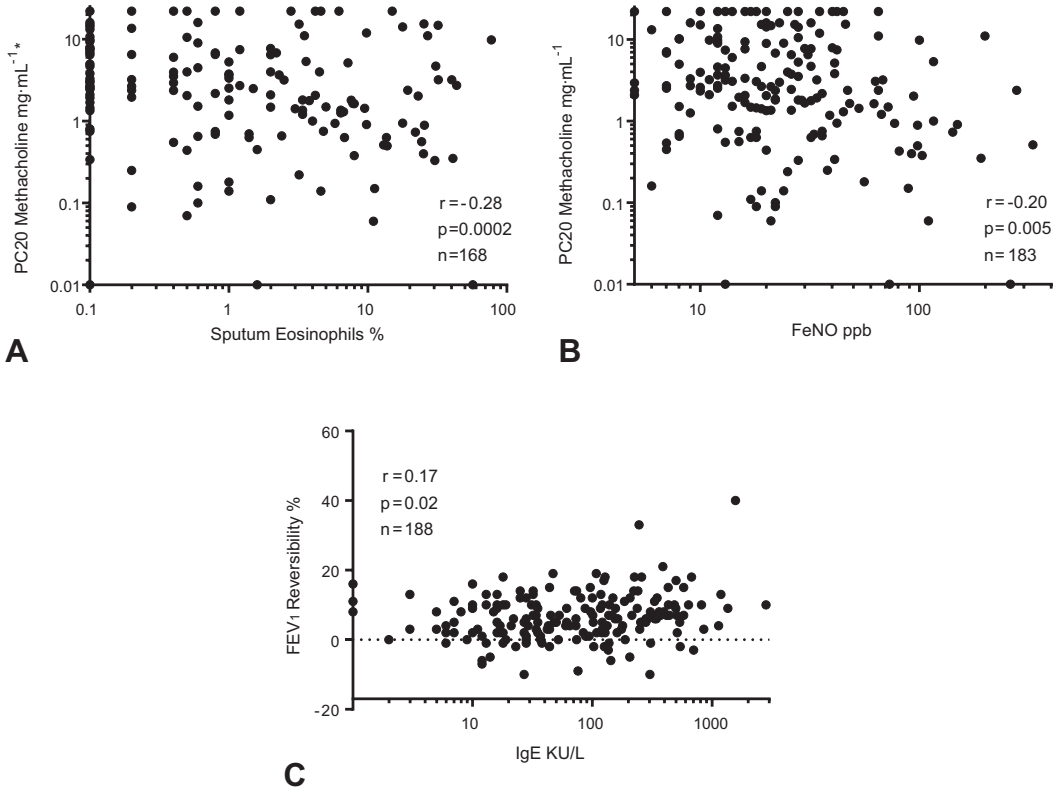

FIGURE 3. **A**, Relationship between sputum eosinophils and PC20M (n = 168). **B**, Relationship between FeNO and PC20M (n = 183). **C**, Relationship between serum total IgE and salbutamol reversibility (n = 188). The *r* value is the Spearman rank coefficient of correlation. *PC20M*, Provocative concentration of methacholine causing a fall in FEV_1 of 20%; *FeNO*, fraction of exhaled nitric oxide; *FEV_1*, forced expiratory volume in 1 second.

airway caliber with the lowest FEV_1 ($P < .001$) and FEV_1/FVC ratio ($P < .001$) found in the group combining both a positive reversibility test and a positive methacholine challenge (Table III, and Figure E2, available in this article's Online Repository at www.jaci-inpractice.org). There was no difference between the groups regarding the FeNO values or the proportion of patients with FeNO above 25 ppb. No difference between the groups was found regarding asthma control or quality of life (data not shown). As for the immunoinflammatory parameters no significant difference was noted between the groups either for the blood (Table IV) or the sputum parameters (Table V). The proportion of eosinophilic asthma defined as a sputum eosinophil count $\geq$3% was comparable in patients identified by positive PC20M (35%, 52 of 148) and those identified by reversibility to salbutamol (37%, 12 of 39). Using the criteria of 9% predicted to define significant bronchodilation and 8 mg/mL to define significant airway hyperresponsiveness does not alter the finding (36%, 19 of 53 and 34%, 42 of 122, respectively). Adopting a definition of reversibility based on an exclusive 200 mL improvement did not change the overall results except that it selected slightly younger patients in the reversible group. Detailed analyses of the demographic, lung function, and inflammatory parameters in each subgroup according to the different scenarios are given in Tables E2 to E13 (available in this article's Online Repository at www.jaci-inpractice.org).

DISCUSSION

Reversibility test and bronchial challenge are pivotal lung function tests to ascertain excessive airway caliber fluctuation to confirm asthma diagnosis in patients with recurrent/chronic respiratory symptoms. Our study shows that positive challenge to methacholine was more often observed than positive reversibility test to salbutamol in patients with suggestive symptoms and baseline FEV_1 greater than 70% predicted. An isolated positive methacholine challenge was found in 71% of cases, whereas the isolated significant reversibility has a rare occurrence of 3%. The group that combined both a positive methacholine challenge and a significant reversibility to salbutamol represented 19% of the cohort and had clearly more obstructed airways as indicated by lower FEV_1 together with a lower FEV_1/FVC ratio, but, interestingly, displayed similar systemic and airway inflammatory features.

Although it is generally accepted that either a reversibility test or a bronchial challenge is sufficient to ascertain asthma diagnosis in patients with suggestive symptoms, the concordance between the 2 tests using standard criteria was rather weak in our study, not overpassing one fifth of the cohort. It slightly rises when adopting the more inclusive 9% predicted reversibility criterion for bronchodilation but does not exceed one third. The poor sensitivity of bronchodilating test to make an asthma diagnosis has already been previously highlighted both at the population level[13] and in secondary care center studies.[5,6] Besides, the present study did not find significant correlation between the

TABLE III. Demographic and functional features according to the reversibility test to salbutamol and the methacholine bronchial challenge

	Salbutamol− Methacholine− N = 18	Salbutamol+ Methacholine− N = 6	Salbutamol− Methacholine+ N = 137	Salbutamol+ Methacholine+ N = 33	*P* value
Age, y	46 (34-62)	56 (51-61)	49 (35-63)	55 (40-63)	.69
Gender (F), n (%)	9 (50%)	4 (33%)	89 (64%)	22 (66%)	.64
Tobacco NS/ES/CS, n (%)	11/5/2 (61%, 28%, 11%)	2/2/2 (33%, 33%, 33%)	76/29/32 (55%, 21%, 23%)	19/5/8 (58%, 15%, 24%)	.75
Atopy, Yes/No (%)	6/8 (43%)	2/4 (33%)	65/70 (49%)	13/19 (41%)	.78
BMI, kg/m^2	26 (23-29)	24 (23-28)	25 (22-29)	24 (22-30)	.93
PreFEV$_1$, % pred	100 ± 15	92 ± 8	96 ± 14	86 ± 11*†	.001
PreFVC, % pred	104 ± 14	105 ± 7	99 ± 14	100 ± 12	.95
PreFEV$_1$/FVC, %	80 ± 9	77 ± 5	79 ± 7	72 ± 7*†	<.0001
PostFEV$_1$, % pred	104 ± 14	105 ± 9	99 ± 14	100 ± 12	.38
Reversibility, % from baseline	5 ± 4	14 ± 3	4 ± 5	16 ± 6	ND
PC20M, mg/mL	>16	>16	2.5 (0.94-6.5)	1.66 (0.45-3.52)	ND
FeNO, ppb	19 (12-31)	17 (10-32)	21 (12-38)	23 (16-61)	.69
FeNO >25 ppb, %	6/18 (33)	2/5 (40)	54/128 (42)	13/32 (41)	.91

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV$_1$*, forced expiratory volume in 1 second; *FVC*, forced vital capacity; *IQR*, interquartile range; *ND*, not determined; *NS*, non-smoker; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
Results are expressed as mean ± SD or median (IQR).
*$P < .05$ vs group salbutamol−, methacholine+.
†$P < .05$ vs group salbutamol−, methacholine−.

TABLE IV. Blood immuneinflammatory features according to reversibility test to salbutamol and methacholine bronchial challenge

	Salbutamol− Methacholine− N = 18	Salbutamol+ Methacholine− N = 5	Salbutamol− Methacholine+ N = 134	Salbutamol+ Methacholine+ N = 33	*P* value
Total leukocytes, 1/μL	6790 (5300-8850)	7860 (5930-8330)	6840 (5810-8020)	7200 (5910-8280)	.88
Neutrophils, 1/μL	3618 (2336-5130)	3169 (2888-5173)	3603 (2838-4584)	4031 (3046-4971)	.61
Lymphocytes, 1/μL	2486 (2061-2967)	2502 (2307-2672)	2437 (1882-2981)	2341 (1849-2732)	.73
Monocytes, 1/μL	643 (468-723)	433 (379-683)	533 (400-669)	473 (362-530)	.06
Eosinophils, 1/μL	176 (111-239)	90 (57-183)	173 (99-288)	139 (82-209)	.19
Basophils, 1/μL	27 (20-39)	33 (22-34)	31 (20-50)	33 (28-52)	.36
IgE, kU/L	99 (28-128)	240 (222-348)	61 (21-188)	100 (32-246)	.22
Fibrinogen, g/L	3.3 (2.8-3.9)	3 (2.8-3.4)	3.2 (2.6-3.6)	3.3 (2.8-3.5)	.81
CRP, mg/L	2.1 (0.2-3)	2.9 (1.8-3.7)	1.4 (0.4-3.9)	1 (0.2-2.9)	.58

CRP, C reactive protein; *IQR*, interquartile range.
Results are expressed as median (IQR).
P value calculated by the Kruskal-Wallis test (the group salbutamol+ methacholine− is too small to undertake statistical analysis, and data are just given for information).

TABLE V. Sputum cell counts according to reversibility test to salbutamol and methacholine bronchial challenge

	Salbutamol− Methacholine− N = 16	Salbutamol+ Methacholine− N = 4	Salbutamol− Methacholine+ N = 120	Salbutamol+ Methacholine+ N = 28	*P* value
Total nonsquamous cell, 10^6/g	1.78 (0.85-3.68)	1.62 (0.73-11.13)	1.09 (0.48-2.32)	0.92 (0.59-1.78)	.28
Squamous cell, %	10 (2-20)	15 (4-37)	16 (6-33)	19 (6-29)	.38
Viability, %	79 (68-83)	83 (73-87)	72 (59-82)	63 (55-82)	.13
Macrophages, %	29 (10-59)	19 (13-27)	24 (11-40)	30 (14-47)	.36
Neutrophils, %	62 (34-81)	71 (49-83)	59 (39-83)	52 (26-77)	.44
Lymphocytes, %	1.6 (0.5-2.2)	1.6 (0.5-2.2)	1.3 (0.6-3)	2.3 (0.8-4.1)	.18
Eosinophils, %	0.3 (0-2)	1.8 (0.4-3.7)	0.9 (0.2-6.4)	1.6 (0.2-3.8)	.52
Epithelial cells, %	1.5 (1-3)	6 (1-20)	2.4 (1.2-5.9)	4.3 (1.5-8.1)	.11

IQR, Interquartile range.
Results are expressed as median (IQR).
P value calculated by the Kruskal-Wallis test (the group salbutamol+ methacholine− is too small to undertake statistical analysis and data are just given for information).

severity of responsiveness to methacholine assessed by the PC20M and the magnitude of reversibility to 400 μg inhaled salbutamol. This may appear to be surprising given the mechanisms through which both salbutamol and methacholine are influencing airway caliber. Both agents bind to receptors at the smooth muscle cell surface to induce either a relaxation for salbutamol or a contraction for methacholine. There are, however, several possible explanations for the discrepancy seen in our study. First, the *in vivo* pharmacologic design was totally different. Although reversibility was assessed after a single administration of one optimal dosage of salbutamol, the PC20M was determined after multiple inhalations of quadrupling concentrations of methacholine. Therefore, although the bronchodilation may be considered as the maximal response to salbutamol, the PC20M reflects the sensitivity of the airways to methacholine rather than the maximal pharmacologically induced airway obstruction. Second, the pharmacologic pathway leading to airway smooth muscle contraction or relaxation is likely depending on receptor density on the cell surface and on the intracellular signal transduction, 2 steps that may show intrinsic variability according to the mediator.

Baseline airway obstruction was a good predictor of reversibility to salbutamol as demonstrated by the convincing inverse relationship between the FEV_1/FVC ratio, and to a lesser extent, between FEV_1% predicted and the magnitude of FEV_1 reversibility to salbutamol. Though being expected, our finding has not been reported previously in a large cohort of patients with mild disease and preserved baseline airway caliber. Our data are in keeping with a previous study conducted on a limited number of patients using peak expiratory flow as in an index to assess bronchodilation to salbutamol.[14] The obvious inverse correlation between reversibility to salbutamol and the baseline FEV_1/FVC ratio somewhat contrasts with what is observed for methacholine challenge, in which case the strength of the relationship between baseline FEV_1 or FEV_1/FVC and PC20M appears to be rather weak. The relationship between baseline airway caliber and direct bronchial hyperresponsiveness has been extensively studied both in asthmatics and in normal subjects, and it was shown that the lower the baseline airway caliber the higher the level of bronchial hyperresponsiveness to methacholine or histamine.[15] However, as shown in our study, some asthmatics with normal baseline % predicted FEV_1 may sometimes exhibit severe bronchial hyperresponsiveness, the determinants of which may partly involve airway inflammation and, more probably, abnormal smooth muscle contraction velocity.[15,16] Although PC20M was found to inversely correlate with sputum eosinophils and FeNO in our cohort, which is in keeping with previous studies[17,18] and supports a partial role of airway inflammation on bronchial hyperresponsiveness,[19] it is striking that the same relationship was not verified for the reversibility to salbutamol. However, the latter correlated with total serum IgE, which, in his turn, failed to correlate with methacholine responsiveness. Why serum IgE would influence airway reversibility remains uncertain, but we have previously shown that serum IgE strongly correlated with sputum IgE.[20] We could speculate that a high amount of IgE in airway mucosa may prime mast cells to release larger amounts of newly formed constricting mediators such as cysteinyl-leukotrienes, thereby leading to a heightened airway smooth muscle tone.

According to our study, it is evident that relying exclusively on significant reversibility to salbutamol as a sign of airway lability may result in missing a significant proportion of mild-to-moderate asthmatics encountered in daily practice. Therefore, one key question is to know whether patients diagnosed by different criteria of excessive airflow fluctuation may actually show different demographic or immuneinflammatory features. Asthma is now recognized as a heterogeneous disease with patients featuring several inflammatory phenotypes making them respond differently to maintenance asthma treatment, and in particular, to inhaled corticoids.[21] Our study shows, for the first time, that there is no striking difference between the subjects selected on the basis of reversibility to inhaled salbutamol and those based on airway constriction to inhaled methacholine in terms of systemic and airway inflammation. In particular, it is reassuring that the proportion of eosinophilic asthma phenotype (featuring a sputum eosinophil $\geq$3%), which generally predicts good response to ICS in routine practice,[22] is not different between the patients selected on the basis of a PC20M $<$16 mg/mL and those selected on the basis of an FEV_1 reversibility $\geq$12% and 200 mL after salbutamol inhalation. On the other hand, it is worth noting that the proportion of eosinophilic asthma found in this population of mild-to-moderate untreated asthmatics, which is approaching 35% whichever the criterion used, is close to that reported by McGrath in a similar asthma population[22,23] and much lower than that reported in a population of severe asthma[24] where eosinophilic phenotype may reach 60% despite heavy treatment with inhaled and sometimes oral corticoids. The combination of all these studies, which investigated a large number of patients, further supports the role of airway eosinophilic inflammation in grading asthma clinical severity, as already shown 20 years ago on a smaller cohort.[25] Whether mild-to-moderate noneosinophilic asthmatics, which represents the majority of patients in the present study, and also presumably in primary care setting,[26] all need to receive regular treatment with ICS, is a key question that needs to be resolved in prospective large-scale clinical trials. The characteristics of our patients, including FEV_1 and sputum eosinophils, are very close to those described by Boushey et al[4] in a clinical trial showing that intermittent, instead of continuous, use of ICS may be appropriate to control for exacerbation in mild persistent asthma.

Finally, the fact that inflammatory profile from blood and sputum was not significantly different between those with asthma and those in whom diagnosis could not be confirmed suggests that it is highly hazardous to rely on inflammatory markers to make asthma diagnosis in patients with preserved baseline lung function. This is in keeping with the similar FeNO values found between the groups. Not denying the importance of qualifying the inflammatory pattern to phenotype asthma, our data argue against its utility in ruling in or ruling out the disease.

One limitation of the present study is the narrow spectrum of disease severity assessed in our cohort. No doubt that bronchodilating test would have shown increased sensitivity if we had accepted symptomatic patients with baseline FEV_1 less than 70% predicted. Although focused on mild-to-moderate patients, we, however, believe that our study has clinical relevance as this group of patients is the most frequently encountered in daily practice, and particularly in primary care where asthma diagnosis proved to be wrong in at least 30% of cases.[1] Another limitation

is the retrospective design of the study, which precludes assessment of the reproducibility of bronchodilating test to salbutamol and PC20M. The latter has been well studied and was shown to be generally within one doubling dilution.[3] By contrast, there are not many data on the repeatability of bronchodilating test in mild asthma, but the reversibility to bronchodilating agents was found to be highly variable over time in chronic obstructive pulmonary disease.[27] It would have been of interest to compare the reversibility after inhaled salbutamol found at visit 1 with the time to recovery after inhalation of salbutamol at the end of the methacholine challenge, but prolonged accurate monitoring of FEV_1 recovery is not performed in our routine practice.

We conclude that methacholine challenge greatly outperforms reversibility with salbutamol to make asthma diagnosis in patients with baseline FEV_1 greater than 70% predicted. The asthmatics diagnosed by positive methacholine challenge combined with positive reversibility to salbutamol exhibit a higher degree of airway obstruction but no different inflammatory status as compared with those diagnosed by methacholine challenge alone.

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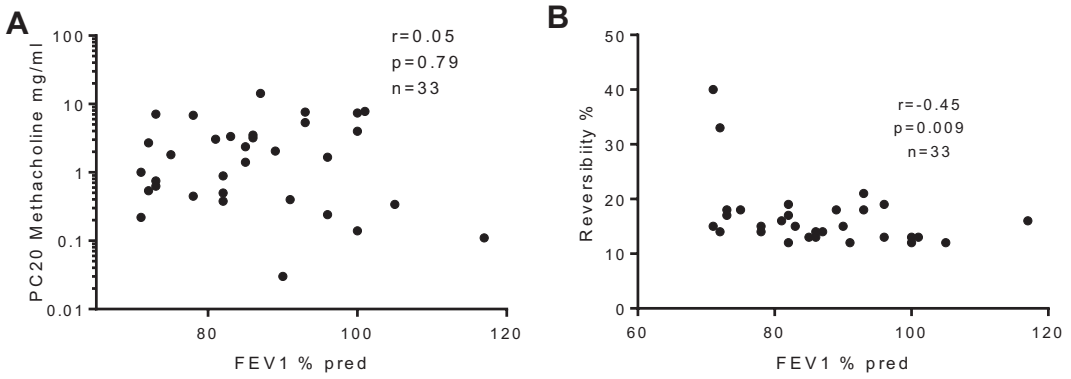

FIGURE E1. Relationship between % predicted FEV_1 and PC20M (**A**) and FEV_1 reversibility (**B**) in the 33 subjects positive to both salbutamol and methacholine challenge. *FEV_1*, Forced expiratory volume in 1 second; *PC20M*, provocative concentration of methacholine causing a fall in FEV_1 of 20%.

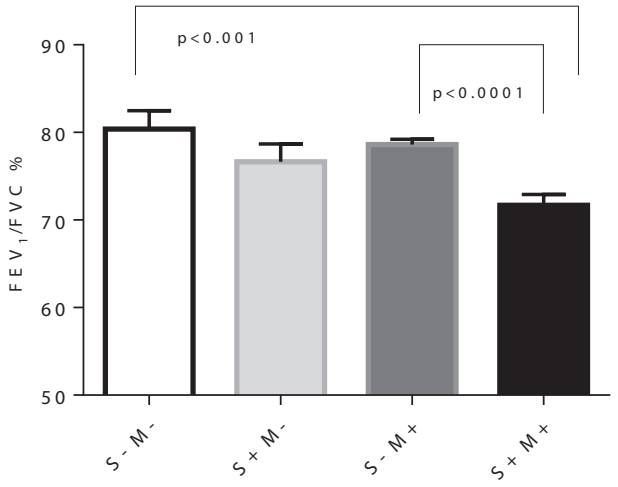

FIGURE E2. Comparison of prebronchodilation FEV_1/FVC % in the 4 groups according to reversibility to salbutamol and methacholine bronchial challenge. S−M− are patients with reversibility <12% or 200 mL and PC20M >16 mg/mL (n = 18), S+M− are patients with reversibility to salbutamol ≥12% and 200 mL but PC20 >16 mg/mL (n = 6), S−M+ are patients with reversibility to salbutamol <12% or <200 mL but PC20M ≤16 mg/mL (n = 137), S+M+ are patients with reversibility to salbutamol ≥12% and 200 mL and PC20 ≤16 mg/mL (n = 33). The group S+M− is too small to allow for comparison. *FEV_1*, Forced expiratory volume in 1 second; *FVC*, forced vital capacity; *PC20M*, provocative concentration of methacholine causing a fall in FEV_1 of 20%.

TABLE E1. ROC curves of reversibility with inhaled salbutamol 400 μg to predict positive PC20M

Reversibility	AUC	*P* value	Cutoff	Sensitivity, %	Specificity, %
PC20M ≤16 mg/mL					
mL from baseline	0.53	.69	ND	ND	ND
% from baseline	0.53	.66	ND	ND	ND
% predicted	0.53	.68	ND	ND	ND
PC20M ≤8 mg/mL					
mL from baseline	0.58	.08	115 mL	67	54
% from baseline	0.60	.04	3.5%	72	47
% predicted	0.58	.07	7.5%	43	73

AUC, Area under the curve; *ND*, not determined; *PC20M*, provocative concentration of methacholine causing a fall in FEV_1 of 20%; *ROC*, receiver operating characteristic.

TABLE E2. Demographics and lung function features when asthma was diagnosed by the criteria: Reversibility ≥ 12% and 200 mL and/or PC20M ≤ 8 mg/mL

Reversibility ≥12% and 200 mL PC20M ≤8 mg/mL	Salbutamol− Methacholine− N = 46	Salbutamol+ Methacholine− N = 7	Salbutamol− Methacholine+ N = 109	Salbutamol+ Methacholine+ N = 32	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Age, y	48 ± 15	58 ± 9	48 ± 17	49 ± 16	–	–
Gender (F), n (%)	28 (61%)	5 (71%)	70 (64%)	21 (66%)	–	–
Tobacco NS/ES/CS, n (%)	29/9/8 (63%/20%/17%)	3/2/2 (43%/23%/23%)	58/26/25 (54%/24%/23%)	19/8/5 (59%/25%/16%)	–	–
Atopy, Yes/No (%)	28/17 (62%)	3/3 (50%)	51/51 (50%)	13/19 (41%)	–	–
BMI	24 (23-29)	23 (23-27)	26 (22-29)	24 (22-30)	–	–
PreFEV$_1$, % pred	100 ± 13†	91 ± 8	94 ± 14*	86 ± 12	<.0001	.0221
PreFVC, % pred	104 ± 12	100 ± 12	101 ± 15	101 ± 14	–	–
PreFEV$_1$/FVC, %	80 ± 8†	76 ± 5	78 ± 7†	72 ± 7	<.0001	.0006
PostFEV$_1$, % pred	103 ± 12	104 ± 7	98 ± 14	100 ± 12	–	–
Reversibility % from baseline	3.9 ± 3.6	14 ± 3	4.3 ± 5	17 ± 6	–	–
PC20M, mg/mL	15 (11-22)	>16	1.9 (0.7−3.2)	1.5 (0.4−3.4)	–	–
FeNO, ppb	20 (12-28)	18 (16-28)	22 (13-40)	24 (16-68)	–	–

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV$_1$*, Forced expiratory volume in 1 second; *FVC*, forced vital capacity; *NS*, non-smoker; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
"–": nonsignificant Kruskal-Wallis test.
*<.01 vs S+M+.
†<.0001 vs S+M+.

TABLE E3. Blood immuneinflammatory features when asthma was diagnosed by the criteria: Reversibility ≥ 12% and 200 mL and/or PC20M ≤ 8 mg/mL

Reversibility ≥12% and 200 mL PC20M ≤8 mg/mL	Salbutamol− Methacholine− N = 45	Salbutamol+ Methacholine− N = 6	Salbutamol− Methacholine+ N = 107	Salbutamol+ Methacholine+ N = 32	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total leukocytes, 1/μL	6.7 (5.8-8.6)	8.1 (5.9-8.7)	6.9 (5.8-8)	7.2 (5.7-8.2)	–	–
Neutrophils, 1/μL	3523 (2461-5027)	4179 (2950-5173)	3657 (2841-4593)	4025 (3029-4957)	–	–
Lymphocytes, 1/μL	2550 (2102-2995)	2587 (2307-3102)	2429 (1860-2962)	2306 (1836-2720)	–	–
Monocytes, 1/μL	529 (399-655)	490 (379-683)	538 (411-675)	472 (361-526)	–	–
Eosinophils, 1/μL	175 (113-239)	90 (57-183)	171 (97-292)	141 (82-216)	–	–
Basophils, 1/μL	31 (20-42)	34 (22-52)	31 (20-49)	33 (27-50)	–	–
IgE, kU/L	73 (27-126)	231 (32-348)	61 (18-214)	104 (32-246)	–	–
Fibrinogen, g/L	3.3 (2.7-3.6)	3 (2.9-3.4)	3.1 (2.6-3.7)	3.3 (2.9-3.6)	–	–
CRP, mg/L	1.2 (0.2-3.2)	2.8 (0.9-3.1)	1.5 (0.6-3.9)	1 (0.2-3.2)	–	–

CRP, C reactive protein; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E4. Sputum cells counts when asthma was diagnosed by the criteria: Reversibility ≥ 12% and 200 mL and/or PC20M ≤ 8 mg/mL

Reversibility ≥12% and 200 mL PC20M ≤8 mg/mL	Salbutamol− Methacholine− N = 38	Salbutamol+ Methacholine− N = 5	Salbutamol− Methacholine+ N = 99	Salbutamol+ Methacholine+ N = 27	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S−M− vs S+M−
Total nonsquamous cells, 10^6/g	1.2 (0.6-2.7)	1.2 (0.9-2.1)	1.1 (0.5-2.7)	1 (0.6-1.8)	–	–	–
Squamous cells, %	15 (4-27)	4 (4-25.2)	16 (6-35)	20 (6-30)	–	–	–
Viability, %	69 (60-82)	83 (83-87)	73 (60-83)	59 (54-80)	–	–	–
Macrophages, %	30 (13-56)	17 (11-20)	21 (10-39)	31 (15-47)	–	–	–
Lymphocytes, %	1.4 (0.5-3.5)	1.4 (0.4-1.8)	1.3 (0.6-3)	2.4 (1-4.3)	–	–	–
Neutrophils, %	56 (33-77)	79 (63-86)	63 (42-83)	50 (24-76)	–	–	–
Eosinophils, %	0.5 (0-3.5)	0.8 (0-2.8)	1 (0.2-6.4)	1.6 (0.4-4)	–	–	–
Epithelial cells, %	2 (1-5.5)	2 (1.4-10)	2.4 (1.2-5)	4.6 (1.4-8.8)	–	–	–

PC20M, Provocative concentration of methacholine causing a fall in FEV_1 of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E5. Demographics and lung function features when asthma was diagnosed by the criteria: Reversibility ≥ 9% predicted and/or PC20M ≤ 16 mg/mL

Reversibility ≥9% predicted PC20M ≤16 mg/mL	Salbutamol− Methacholine− N = 15	Salbutamol+ Methacholine− N = 9	Salbutamol− Methacholine+ N = 108	Salbutamol+ Methacholine+ N = 62	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S−M− vs S+M−
Age, y	48 ± 14	52 ± 16	48 ± 17	49 ± 16	–	–	–
Gender (F), n (%)	9 (60%)	4 (44%)	74 (68%)	37 (60%)	–	–	–
Tobacco NS/ES/CS, n (%)	10/3/2 (67%/20%/13%)	3/4/2 (33%/44%/22%)	62/23/23 (57%/21%/21%)	33/11/17 (53%/18%/27%)	–	–	–
Atopy, Yes/No (%)	11/4 (73%)	3/5 (33%)	54/50 (50%)	27/31 (44%)	–	–	–
BMI	27 (23-30)	23 (23-27)	26 (22-29)	25 (22-29)	–	–	–
$PreFEV_1$, % pred	102 ± 14*	91 ± 10	97 ± 14*	88 ± 11	.0059	.0012	–
PreFVC, % pred	103 ± 13	102 ± 13	102 ± 15	102 ± 13	–	–	–
$PreFEV_1$/FVC, %	83 ± 7†	74 ± 7‡	80 ± 7†	73 ± 7	.0003	<.0001	<.0002
$PostFEV_1$, % pred	105 ± 13	103 ± 11	99 ± 15	100 ± 11	–	–	–
Reversibility % from baseline	3.6 ± 3.1	13 ± 3	2.6 ± 4.1	13 ± 5	–	–	–
PC20M, mg/mL	>16	>16	3 (1-7)	1.8 (0.6-3.5)	–	–	–
FeNO, ppb	20 (12-40)	16 (14-28)	20 (12-34)	23 (17-48)	–	–	–

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV_1*, forced expiratory volume in 1 second; *FVC*, forced vital capacity; *NS*, non-smoker; *PC20M*, provocative concentration of methacholine causing a fall in FEV_1 of 20%.
"–": nonsignificant Kruskal-Wallis test.
*<.01 vs S+M+.
†<.001 vs S+M+.
‡<.0001 vs S−M+.

TABLE E6. Blood immuneinflammatory features when asthma was diagnosed by the criteria: Reversibility ≥ 9% predicted and/or PC20M ≤ 16 mg/mL

Reversibility ≥9% predicted PC20M ≤16 mg/mL	Salbutamol− Methacholine− N = 15	Salbutamol+ Methacholine− N = 8	Salbutamol− Methacholine+ N = 105	Salbutamol+ Methacholine+ N = 62	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total leukocytes, 1/μL	6.9 (5.3-8.9)	7.3 (5.8-9.1)	6.8 (6.1-8)	7.2 (5.5-8.2)	–	–	–
Neutrophils, 1/μL	3523 (2336-5130)	3728 (2919-5280)	3677 (2841-4538)	3797 (2837-4820)	–	–	–
Lymphocytes, 1/μL	2422 (2061-2967)	2526 (2282-2994)	2431 (1989-2976)	2345 (1849-2981)	–	–	–
Monocytes, 1/μL	638 (468-723)	541 (376-716)	520 (391-670)	492 (378-620)	–	–	–
Eosinophils, 1/μL	172 (111-239)	136 (88-190)	167 (101-264)	162 (84-253)	–	–	–
Basophils, 1/μL	27 (17-39)	34 (21-40)	32 (21-50)	32 (21-44)	–	–	–
IgE, kU/L	97 (28-126)	231 (107-493)	61 (23-179)	87 (19-246)	–	–	–
Fibrinogen, g/L	3.3 (2.7-3.9)	3 (2.8-3.9)	3.2 (2.7-3.7)	3.2 (2.6-3.6)	–	–	–
CRP, mg/L	2.3 (0.7-2.8)	2.8 (0.2-4.4)	1.5 (0.6-3.9)	1.1 (0.2-2.6)	–	–	–
Epithelial cells, %	1.6 (1-3.3)	1.5 (1.2-10)	2.3 (1-5.9)	3.2 (1.4-7)	–	–	–

CRP, C reactive protein; *PC20M*, provocative concentration of methacholine causing a fall in FEV_1 of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E7. Sputum cells counts when asthma was diagnosed by the criteria: Reversibility ≥ 9% predicted and/or PC20M ≤ 16 mg/mL

Reversibility ≥9% predicted PC20M ≤16 mg/mL	Salbutamol− Methacholine− N = 13	Salbutamol+ Methacholine− N = 7	Salbutamol− Methacholine+ N = 93	Salbutamol+ Methacholine+ N = 56	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total nonsquamous cells, 10^6/g	2.3 (1.4-4.5)	1.2 (0.8-2.1)	1.1 (0.5-2.4)	1 (0.6-1.8)	–	–	–
Squamous cells, %	18 (3-20)	4 (3-25.2)	16 (6-31)	18 (6-34)	–	–	–
Viability, %	78 (66-83)	83 (75-89)	72 (57-82)	70 (57-83)	–	–	–
Macrophages, %	33 (14-58)	17 (10-34)	26 (11-40)	20 (11-43)	–	–	–
Lymphocytes, %	1.7 (0.6-2.2)	1.4 (0-2.4)	1.6 (0.6-3)	1.6 (0.6-3.8)	–	–	–
Neutrophils, %	62 (38-77)	76 (34-87)	58 (37-82)	60 (41-81)	–	–	–
Eosinophils, %	0.2 (0-1.2)	0.8 (0-2.8)	0.9 (0.2-6.4)	1 (0-4.4)	–	–	–
Epithelial cells, %	1.6 (1-3.3)	1.5 (1.2-10)	2.3 (1-5.9)	3.2 (1.4-7)	–	–	–

PC20M, Provocative concentration of methacholine causing a fall in FEV_1 of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E8. Demographics and lung function features when asthma was diagnosed by the criteria: Reversibility ≥ 9% predicted and/or PC20M ≤ 8 mg/mL

Reversibility ≥9% predicted PC20M ≤8 mg/mL	Salbutamol− Methacholine− N = 40	Salbutamol+ Methacholine− N = 13	Salbutamol− Methacholine+ N = 83	Salbutamol+ Methacholine+ N = 58	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Age, y	49 ± 15	52 ± 15	48 ± 18	49 ± 16	–	–
Gender (F), n (%)	27 (67%)	6 (46%)	56 (67%)	35 (60%)	–	–
Tobacco NS/ES/CS, n (%)	27/6/7 (67%/15%/17%)	5/5/3 (38%/38%/23%)	45/20/18 (54%/24%/22%)	31/10/16 (53%/17%/28%)	–	–
Atopy, Yes/No (%)	26/14 (65%)	5/6 (38%)	39/40 (47%)	25/30 (43%)	–	–
BMI	24 (23-29)	25 (23-27)	26 (23-29)	25 (22-29)	–	–
PreFEV$_1$, % pred	101 ± 13†	91 ± 9	96 ± 15*	88 ± 11	.0002	.0145
PreFVC, % pred	103 ± 12	102 ± 11	101 ± 16*	102 ± 14	–	.0036
PreFEV$_1$/FVC, %	82 ± 7‡	74 ± 7	79 ± 7‡	72 ± 7	<.0001	<.0001
PostFEV$_1$, % pred	104 ± 12	103 ± 10	98 ± 15	100 ± 12	–	–
Reversibility % from baseline	3 ± 2.8	12 ± 3	2.6 ± 4.4	14 ± 5	–	–
PC20M, mg/mL	15 (10-16)	>16	1.9 (0.7-3.3)	1.7 (0.5-3.2)	–	–
FeNO, ppb	21 (12-32)	16 (13-24)	20 (12-34)	25 (17-52)	–	–

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV$_1$*, forced expiratory volume in 1 second; *FVC*, forced vital capacity; *NS*, non-smoker; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
"–": nonsignificant Kruskal-Wallis test.
*<.01 vs S+M+.
†<.001 vs S+M+.
‡<.0001 vs S+M+.

TABLE E9. Blood immuneinflammatory features when asthma was diagnosed by the criteria: Reversibility ≥ 9% predicted and/or PC20M ≤ 8 mg/mL

Reversibility ≥9% predicted PC20M ≤8 mg/mL	Salbutamol− Methacholine− N = 39	Salbutamol+ Methacholine− N = 12	Salbutamol− Methacholine+ N = 81	Salbutamol+ Methacholine+ N = 58	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total leukocytes, 1/μL	6.7 (5.8-8.8)	7.5 (5.8-8.5)	6.9 (6.1-8)	7.2 (5.5-8.2)	–	–
Neutrophils, 1/μL	3711 (2461-5075)	3330 (2919-5072)	3657 (2878-4538)	3818 (2837-4820)	–	–
Lymphocytes, 1/μL	2506 (2102-2976)	2611 (2282-3210)	2429 (1910-2962)	2337 (1832-2732)	–	–
Monocytes, 1/μL	509 (381-655)	590 (406-716)	551 (417-681)	488 (373-620)	–	–
Eosinophils, 1/μL	175 (116-239)	102 (79-190)	161 (97-278)	169 (88-256)	–	–
Basophils, 1/μL	31 (20-52)	33 (21-40)	31 (21-49)	32 (22-44)	–	–
IgE, kU/L	55 (27-124)	222 (32-638)	73 (21-206)	84 (19-242)	–	–
Fibrinogen, g/L	3.3 (2.6-3.7)	3.2 (2.9-3.4)	3.2 (2.7-3.7)	3.1 (2.6-3.6)	–	–
CRP, mg/L	1.2 (0.2-3.2)	1.4 (0.6-3.1)	2.3 (0.7-4)	1.1 (0.2-2.9)	–	–

CRP, C reactive protein; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E10. Sputum cells counts when asthma was diagnosed by the criteria: Reversibility $\geq$ 9% predicted and/or PC20M $\leq$ 8 mg/mL

Reversibility ≥9% predicted Methacholine ≤8 mg/mL	Salbutamol− Methacholine− N = 32	Salbutamol+ Methacholine− N = 11	Salbutamol− Methacholine+ N = 74	Salbutamol+ Methacholine+ N = 52	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total nonsquamous cells, 10^6/g	1.4 (0.4-3)	0.9 (0.8-1.7)	1.2 (0.5-2.7)	1.1 (0.6-1.9)	–	–
Squamous cells, %	17 (6-30)	4 (4-25.2)	16 (6-29)	18 (6-37)	–	–
Viability, %	67 (60-82)	83 (63-89)	73 (60-83)	70 (57-82)	–	–
Macrophages, %	30 (13-58)	17 (10-34)	25 (10-39)	20 (12-44)	–	–
Lymphocytes, %	1.4 (0.6-2.8)	1.4 (0-4.4)	1.6 (0.6-3)	1.6 (0.6-3.7)	–	–
Neutrophils, %	56 (30-74)	76 (46-87)	59 (39-82)	60 (39-81)	–	–
Eosinophils, %	0.5 (0-5.2)	0.5 (0-2.8)	1 (0.2-5.8)	1.2 (0.1-4.9)	–	–
Epithelial cells, %	2 (1-5.8)	2 (1.4-10)	2.2 (1-4.8)	3.2 (1.4-7)	–	–

"–": nonsignificant Kruskal-Wallis test.

TABLE E11. Demographics and lung function features when asthma was diagnosed by the criteria: Reversibility $\geq$ 200 mL and/or PC20M $\leq$ 16 mg/mL

Reversibility ≥200 mL Methacholine ≤16 mg/mL	Salbutamol− Methacholine− N = 12	Salbutamol+ Methacholine− N = 12	Salbutamol− Methacholine+ N = 98	Salbutamol+ Methacholine+ N = 72	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S+M− vs S−M−	*P* values Dunn test S+M− vs S−M+
Age, y	48 ± 15	51 ± 15	52 ± 17	44 ± 15	–	.0155	–	–
Gender (F), %	9 (75%)	4 (33%)	75 (76%)	36 (50%)	–	–	–	–
Tobacco NS/ES/CS, n (%)	9/2/1 (75%/17%/8%)	4/5/3 (33%/42%/25%)	55/24/19 (56%/24%/19%)	40/10/21 (56%/14%/29%)	–	–	–	–
Atopy, Yes/No (%)	2/10 (16%)	4/8 (33%)	27/71 (29%)	34/38 (47%)	–	–	–	–
BMI	27 ± 5	25 ± 3.8	26 ± 5	25.6 ± 4.6	–	–	–	–
PreFEV$_1$, % pred	105 ± 13	90 ± 9.8	96 ± 14	91 ± 13	.0091	–	–	–
PreFVC, % pred	105 ± 12	100 ± 14	100 ± 15	104 ± 13	–	–	–	–
PreFEV$_1$/FVC, %	85 ± 5	73.9 ± 6.7	80 ± 7	73.2 ± 6.5	<.0001	<.0001	.0024	.0440
PostFEV$_1$, % pred	108 ± 13	101 ± 11	98 ± 14	102 ± 13	–	–	–	–
Reversibility % from baseline	2.6 ± 2.6	11.6 ± 3.8	2.4 ± 4.4	12.1 ± 5.7	–	–	–	–
PC20M, mg/mL	>16	>16	2.1 (0.9-6)	1.6 (0.7-6.5)	–	–	–	–
FeNO, ppb	19 (12-27)	17 (13-35)	18 (12-32)	26 (18-56)	–	.0330	–	–

BMI, Body mass index; *CS*, current smoker; *ES*, ex-smoker; *FeNO*, fraction of exhaled nitric oxide; *FEV$_1$*, forced expiratory volume in 1 second; *FVC*, forced vital capacity; *NS*, non-smoker; *PC20M*, provocative concentration of methacholine causing a fall in FEV$_1$ of 20%.
"–": nonsignificant Kruskal-Wallis test.

TABLE E12. Blood immuneinflammatory features when asthma was diagnosed by the criteria: Reversibility ≥ 200 mL and/or PC20M ≤ 16 mg/mL

Reversibility ≥200 mL Methacholine ≤16 mg/mL	Salbutamol− Methacholine− N = 12	Salbutamol+ Methacholine− N = 11	Salbutamol− Methacholine+ N = 96	Salbutamol+ Methacholine+ N = 71	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total leukocytes, 1/μL	7.5 ± 3	7.8 ± 2.2	7.2 ± 2	7.1 ± 2.5	–	–	–
Neutrophils, 1/μL	4156 ± 2518	4326 ± 1885	3904 ± 1521	3889 ± 1679	–	–	–
Lymphocytes, 1/μL	2504 ± 873	2570 ± 495	2500 ± 759	2499 ± 1045	–	–	–
Monocytes, 1/μL	599 ± 255	634 ± 227	551 ± 193	511 ± 169	–	–	–
Eosinophils, 1/μL	157 ± 76	197 ± 117	212 ± 168	191 ± 136	–	–	–
Basophils, 1/μL	25 ± 15	39 ± 24	34 ± 19	36 ± 21	–	–	–
IgE, kU/L	79 (22-126)	222 (97-362)	43 (22-143)	113 (32-332)	–	.0823	.0702
Fibrinogen, g/L	3.3 (2.7-4.2)	3 (2.8-3.3)	3.3 (2.9-3.8)	3 (2.6-3.4)	–	–	–
CRP, mg/L	1.9 (0.2-2.8)	2.8 (0.9-4.4)	2.4 (0.8-4.3)	1 (0.2-2.4)	–	.0206	–

CRP, C reactive protein.
"–": nonsignificant Kruskal-Wallis test.
IgE: Kruskal-Wallis = 0.012.

TABLE E13. Sputum cells counts when asthma was diagnosed by the criteria: Reversibility ≥ 200 mL and/or PC20M ≤ 16 mg/mL

Reversibility ≥200 mL Methacholine ≤16 mg/mL	Salbutamol− Methacholine− N = 9	Salbutamol+ Methacholine− N = 9	Salbutamol− Methacholine+ N = 83	Salbutamol+ Methacholine+ N = 66	*P* values Dunn test S−M− vs S+M+	*P* values Dunn test S−M+ vs S+M+
Total nonsquamous cells, 10^6/g	2.1 (0.9-4.5)	1.6 (0.8-2.7)	1.2 (0.5-2.8)	0.9 (0.5-1.5)	–	–
Squamous cells, %	18 (8-20)	4 (1-25)	15 (5-26)	21 (8-38)	–	–
Viability, %	79 (66-83)	82 (70-83)	73 (61-84)	66 (56-80)	–	–
Macrophages, %	29 (19-39)	19 (10-58)	21 (10-36)	30 (13-46)	–	–
Lymphocytes, %	1.7 (0.5-2.2)	1.1 (0.2-2)	1.5 (0.8-2.8)	1.7 (0.5-4)	–	–
Neutrophils, %	62 (55-77)	70 (30-87)	65 (39-83)	52 (39-78)	–	–
Eosinophils, %	0.2 (0-1.2)	1 (0-2.8)	0.8 (0.2-4.6)	1.3 (0-5.8)	–	–
Epithelial cells, %	1.4 (1-1.6)	2.3 (1.4-10)	2.1 (1-6)	3.4 (1.4-7)	–	–

"–": nonsignificant Kruskal-Wallis test.