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DIAGNOSIS

Comparison of diagnostic validity of mannitol and methacholine challenges and relationship to clinical status and airway inflammation in steroid-naïve asthmatic patients

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ABSTRACT

Objectives: The purpose of this study was to demonstrate and compare the diagnostic validity of two bronchial challenges and to investigate their correlation with patient clinical status, atopy and inflammation markers. **Methods:** Eighty-eight patients, 47 women and 41 men, mean age 38.56 ± 16.73 years who presented with asthma related symptoms and were not on any anti-asthma medication, were challenged with mannitol and methacholine on separate days. Medical history regarding asthmatic symptoms, physical examination, skin prick tests and FeNO levels were also assessed. The clinical diagnosis of asthma was based on bronchodilator reversibility test. Results: Sixty-seven patients were diagnosed with asthma and 21 without asthma. Both methacholine ($P < 0.014$) and mannitol ($P < 0.000$) challenges were significant in diagnosing asthma. The positive/negative predictive value was 93.33%/41.86% for methacholine, 97.72%/45.45% for mannitol and 97.05%/45.45% for both methods assessed together. Worthy of note that 22% of asthmatics had both tests negative. There was a negative correlation between PC20 of methacholine and the FeNO level $P < 0.001$, and positive with the PD15 of mannitol $P < 0.001$ and the pre-test FEV₁ % pred $P < 0.005$, whereas PD15 of mannitol was negatively correlated with the FeNO level $P < 0.001$. Furthermore, dyspnea was the only asthmatic symptom associated with FeNO level $P < 0.035$ and the positivity of mannitol $P < 0.014$ and methacholine $P < 0.04$. **Conclusions:** Both challenge tests were equivalent in diagnosing asthma. Nevertheless, specificity appeared to be slightly higher in mannitol challenge.

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Introduction

Asthma is a chronic disease of the airways characterized by airway inflammation and bronchial hyperresponsiveness (BHR) to a variety of stimuli leading to frequently reversible airway obstruction. The diagnosis of asthma can be suspected by its clinical manifestations such as cough, wheezing, chest tightness and breathlessness in accordance with symptom precipitation by non-specific triggers (such as smoke, fumes, smells or exercise), deterioration overnight and also by the response to an appropriate asthma treatment [1]. Nevertheless, asthmatic symptoms are commonly accompanied by normal lung function tests and this results in under-diagnosis of the disease. Therefore, confirming or excluding asthma is of critical importance. In such patients, asthma diagnosis can be confirmed with the assessment of BHR.

Various bronchial challenge tests (BCTs) have been used so far for the assessment of BHR. They can be distinguished to direct BCTs (such as histamine or methacholine), where the agent used acts directly on the

airways smooth muscle cells causing bronchoconstriction, where BHR can be identified without the presence of airway inflammation [2,3]. Indirect BCTs such as hypertonic saline, exercise, adenosine monophosphate and mannitol, which act through release of bronchoconstricting mediators (histamine, prostaglandins, leukotrienes) from the airway inflammatory cells, generate a better and stronger relationship with airway inflammation [4,5].

The most common BCTs used so far in clinical practice are the methacholine challenge [6] and the subsequently introduced mannitol inhalation challenge test [7]. Methacholine is considered to be highly sensitive to identify BHR but still with grey areas. A negative test result of PC20 at 16mg/ml has been proposed adequate to rule out clinically current asthma [8,9]. A positive predictive value is 100% if PC20 is less than 1mg/ml. A PC20 between 1 and 16mg/ml is consistent with asthma but not always diagnostic. Cockcroft et al initially chose 8mg/ml, which identified all subjects with asthma. This cut-off point of 8mg/ml, has now been expanded to a borderline range of 4 to 16 mg/ml [10].

However, a negative methacholine test could frequently result in under-diagnosis of asthma. This test requires specific technical equipment (dosimeter, nebulizer) and it can be performed in certain pulmonary laboratories. Mannitol test is introduced in a standardized test kit that contains pre-filled mannitol capsules and a hand-held powder device, where the patient inhales escalating doses of powdered mannitol, according to a certain protocol, until either a drop of 15% of the FEV₁ baseline will be achieved (PD15), or a more than 10% drop of FEV₁ between two consecutive measurements. This test can be easily performed in any setting with safety and it takes no more than fifteen minutes to be completed with the use of a single spirometer [11]. BHR to mannitol is frequently but not always associated to high fraction of exhaled nitric oxide (FeNO) and high percentages of sputum eosinophils both used as markers of inflammation [12].

Up to date, numerous studies have demonstrated several epidemiological indices (such as sensitivity, specificity, negative and positive predictive values) comparing the two methods with BHR, using FeNO levels as inflammatory marker [13], as well as with a clinical diagnosis of asthma [14] or quality of life in asthmatics assessed by a validated questionnaire [15]. In most of these studies, sensitivity was more in favor of methacholine challenge compared to BHR, even for pediatric populations [16] or young athletes [17], while mannitol was shown more specific to BHR, reflecting a stronger association with airway inflammation.

The purpose of this study was to demonstrate the diagnostic validity of the two methods and their relationship with FeNO levels as an inflammatory marker and also with certain asthmatic symptoms, the atopic status and the presence of allergic rhinitis or any other relevant allergic disease.

Patients and methods

Patients

The study population consisted of 88 subjects, (47 women and 41 men) mean age 38.56 ± 16.73 with asthma related symptoms in the previous month but without previous diagnosis of asthma and without initiation of treatment. Subjects with any other known cardiopulmonary or systematic disease, which could influence airway limitation were excluded from participating in the study. One year was taken to recruit the 88 subjects.

Procedure

This cross-sectional study was conducted in the Outpatient Clinic for Asthma, Pulmonary Department, Aristotle University of Thessaloniki, at the "G. Papanikolaou"

General Hospital, Thessaloniki, Greece. The protocol was approved by the local Hospital Ethics Committee. All subjects were informed and gave written consent. Subjects recruited in the study when visited the Asthma Clinic either for a formal examination of asthma diagnosis or after the referral of another specialist for work-up of respiratory symptoms. Subjects were told not to use any long acting bronchodilators within 24 hours and short acting bronchodilators within 6 hours of any provocative testing, methacholine or mannitol challenges.

The study consisted of 3 visits to the clinic. On the first visit (V1) the following parameters were measured: demographic data, medical history, smoking habits, asthmatic symptoms. FeNO levels, spirometry, allergy skin test reactivity and physical examination were assessed by a pulmonary physician. According to GINA guidelines, the clinician diagnosis of asthma was established by the combination of at least a +12% (and at least 200 ml) increase in baseline FEV₁ after albuterol, along with new symptoms of coughing, wheezing, or shortness of breath over the past month, and no previous diagnosis of asthma [1]. Visit 2 and Visit 3 included methacholine and mannitol challenges, respectively. Visit 2 occurred 14–20 days after the first visit. To avoid any influence of the two provocation agents, there was an interval of at least 48 hours between the two challenges. All subjects were instructed to avoid drinking caffeine before admission in the morning of visits.

Spirometry

Spirometries were performed with the use of a portable spirometer (Spirobank®), standardized according to ATS/ERS recommendations [18], initially for the assessment of the FEV₁ baseline and FEV₁ measurements one minute after the inhalation of each incremental dose of mannitol or methacholine, until the test was terminated. Then a new spirometry was performed 15 minutes after administration of 400 mcg of inhaled salbutamol.

Bronchial challenge tests

The Methacholine challenge test was performed according to the method described by Cockcroft and co-workers [8,19,20]. The patient inhaled incremental doses of methacholine in wet aerosol form, until a drop of 20% of the FEV₁ baseline was obtained (PC₂₀), where the test was considered positive and the patient was assigned as having BHR.

Initially baseline FEV₁ was measured and the test was performed if FEV₁ was >60% of predicted. Then normal saline was administered and if a decrease of FEV₁ > 10% was not observed sequential inhalations of increasing

concentrations of methacholine (Sigma Chemicals, USA) ranging from 0.5 to 32 mg/ml were delivered. Aerosol was generated by a nebulizer (Sidestream Medic-aid Ltd., Sussex, UK, flow 9 lt/min). FEV₁ was measured after each inhalation step. The exact methacholine provocative concentration causing a 20% decrease in FEV₁ (PC₂₀) was estimated from a dose-response curve and was considered as a threshold value. The test was terminated when either the threshold value had been reached or the highest concentration of methacholine had been given. Patients were considered hyperresponsive if they reacted by bronchoconstriction to PC₂₀ ≤ 16 mg/ml. Positivity at 32 mg/ml was considered as negative for asthma [6,9,21,22].

Mannitol test was introduced in a standardized test kit that contained pre-filled mannitol capsules and a hand-held powder device, where the patient inhaled escalating doses of powdered mannitol, according to a certain protocol, until either a drop of 15% of the FEV₁ baseline was achieved (PD15), or a more than 10% drop of FEV₁ between two consecutive measurements was observed. Mannitol challenges were performed, following a certain protocol and results were recorded either positive for BHR or negative. The provocative dose of mannitol that resulted to a positive test (15% decrease in PD15 - all negative tests recorded this variable with the maximum cumulative dose of 635 mg). The percentage of FEV₁ fall from the baseline value, when the mannitol test was terminated (FEV₁FALL) and the reduction dose rate of the FEV₁ (Response dose ratio-RDR, expressed in mg) were computed by the software accompanied by the Aridol test [8,9]. During methacholine and mannitol challenges, cough or any other adverse events were recorded.

Fractional exhaled nitric oxide

FeNO levels were measured with the Nitric Oxide Analyzer (DENOX CLD 88, ECO MEDICS) according to the American Thoracic Society Guidelines. The cut-off point of 20 ppb was defined as an increased level of FeNO at a flow rate of 0.05 L/s compliant to ATS Guidelines [23].

Skin prick tests

Modified skin prick tests (SPT) with allergen extracts including histamine as a positive control and diluent as a negative control were performed at V1 visit. SPTs were performed using allergens for house dust mites (*D. pteronyssinus*, *D. farinae*), animal dander, grass pollens, tree pollens and moulds (Allergopharma, Germany). The largest wheal diameter was measured 15 minutes after the test. The test was considered positive if a diameter of the wheal was at least 5 mm.

Statistical analysis

Values were expressed as mean ± sd. FEV₁ was expressed as % of predicted. Differences of parametric variables among patient groups were assessed by analysis of variance (one-way ANOVA, SPSS for Windows, release 10.0.1). Bonferoni adjustment was performed in order to correct the significant differences among multiple comparisons. Pearson's bivariate correlation coefficient was calculated and Bonferoni adjustment was performed in order to correct multiple correlation significance (SPSS for Windows, release 10.0.1). Bonferoni adjustment for an alpha level of 0.05 for 4 tests was lowered to 0.0125 and for 3 tests to 0.025. Non-parametric variables were compared using chi-square test (2001 version 5,0 McGraw-Hill) and Kruskal-Wallis test (SPSS for Windows, release 10.0.1).

Estimates of sensitivity, specificity, positive and negative predictive values were calculated for both methacholine and mannitol challenges, as well for the combination of both tests, for attaining diagnosis of asthma, by using the relevant equations:

$$\text{Sensitivity} = \frac{\text{true asthmatic cases with positive test}}{\text{all asthmatic cases}}$$

$$\text{Specificity} = \frac{\text{true non-asthmatic cases with negative test}}{\text{all non-asthmatic cases}}$$

$$\begin{aligned} \text{Positive Predictive Value (PPV)} \\ = \frac{\text{true asthmatic cases with positive test}}{\text{all cases with positive result}} \end{aligned}$$

$$\begin{aligned} \text{Negative Predictive Value (NPV)} \\ = \frac{\text{true non-asthmatic cases with negative test}}{\text{all cases with negative result}} \end{aligned}$$

These indices are expressed with their mean values ± SD, reflecting estimates of them deriving from the true population, by employing a Chi-square test with SPSS statistical software (version 17.0).

Correlations among age, gender, smoking status in pack years, symptoms of asthma, FeNO, atopy, the duration of symptoms and the BCT results have been calculated with the estimation of Spearman's correlation coefficient with SPSS and a multiple regression model was employed to identify any predictors that can be positively correlated with the presence of bronchial hyperresponsiveness, due to the methacholine or/and mannitol test result.

Results

Both methacholine and mannitol challenges were well tolerated by the patients. Of the 88 patients examined, 67 were diagnosed with asthma based on clinical symptoms

of asthma and reversible airflow obstruction. Then, 44 (50%) had a positive bronchial BCT and BHR to mannitol and 45 (51%) to methacholine. Of these 67 asthmatic patients, 43 (64%) had BHR to mannitol, 42 (62%) to methacholine and 34 (51%) to both tests. In total, 52 asthma patients (77%) had BHR to at least one of the two tests, whereas 15 (22%) did not have BHR to either mannitol or methacholine (Table 1). Seventy-four percent of the subjects had FEV₁ values within the normal range (FEV₁% pred $\geq$ 80%) and 66% reported only few asthmatic symptoms.

Most patients (55) were non-smokers, 17 patients were smokers and 16 were ex-smokers. Thirty-eight patients suffered from atopy, 48 from rhinitis and 17 from gastroesophageal reflux disease (GERD). Regarding the asthmatic symptoms, the most frequent was cough apparent in 70 patients, followed by dyspnea in 48, wheezing in 21 and chest tightness in 7. Most of the patients had more than one symptom, whereas 22 had night symptoms (Table 2).

The mean decrease of FEV₁ during mannitol and methacholine challenge was $11.4 \pm 7.4\%$ and $18 \pm 7.3\%$, respectively, and the mean improvement of FEV₁, 15 minutes after inhalation of salbutamol, was $11.35 \pm 7.4\%$ for mannitol and $14.1 \pm 8\%$ for methacholine. Fifteen minutes following bronchodilation, 40 (90.1%) patients recovered from mannitol and 39 (88.6%) from methacholine challenge whereas 30 minutes after bronchodilation all patients except for one responded. In addition, cough was manifested in 55 patients during mannitol and in 15 during the methacholine test, but none of them interrupted the procedure (Table 3).

FeNO > 20 ppb was measured in 30 patients while 26 of them had asthma. Of these 30 patients, 27 had positive methacholine and 26 had positive mannitol (Table 4). In Table 5, there are the classifications of asthmatic and non-asthmatic cases with positive or negative test result to methacholine, mannitol or the combination of the two challenges. According to the equations of sensitivity, specificity, PPV and NPV, their estimates to the population are seen in Table 6.

Concerning correlations, both methacholine and mannitol challenges were significant in diagnosing asthma $p < 0.014$ and $p < 0.001$, respectively. There was a negative correlation between PC20 of methacholine and the FeNO level $p < 0.001$ and positive with the PD15 of mannitol $p < 0.001$ and the pre-test FEV₁% pred $p < 0.005$, whereas PD15 of mannitol was negatively correlated with the FeNO level $p < 0.001$. Furthermore, dyspnea was the only asthmatic symptom associated with FeNO level $p < 0.035$ and the positivity of mannitol $p < 0.014$ and methacholine $p < 0.04$. According to the correlation coefficients analyzed, there were no significant correlations among the

variables age, gender, smoking status, pack year history, atopy, symptoms of asthma and their duration, and the bronchial provocation test results (Table 7).

A multiple regression model was also applied to investigate whether any of the symptoms could predict the presence of BHR according to bronchial challenge tests. The analysis showed that none of them was statistically significant to enter the regression model by the stepwise method.

Discussion

An accurate diagnosis of asthma is essential for receiving an appropriate treatment. Although the use of bronchial challenge tests aids in diagnosing asthma and understanding its pathophysiology, however, the absence of a "gold standard" (objective evidence), preserves the major difficulty in assessing methacholine and mannitol validity.

In the present study we investigated the diagnostic properties of mannitol and methacholine challenge test and showed that both methods were significant in diagnosing asthma. These findings support the conclusions of a recent review confirming that the responses to both direct and indirect measures of BHR are correlated with clinically defined asthma, although direct methods have usually a weak relationship with airway inflammation [24]. Moreover, it was not surprising that the responsiveness to both challenge tests showed a significant correlation. This may occur as both tests are considered to cover parts of the underlying hyperresponsiveness and thus, when a patient is positive in mannitol challenge, is highly likely to be also positive in methacholine test. This is usually the case when the studies consist of more selected samples of asthmatic patients [25].

Most of comparative studies regarding the diagnostic sensitivity have demonstrated that the challenge tests of indirect stimuli are less sensitive compared to the tests with direct stimuli [13,26,27]. However, our results indicated that mannitol and methacholine tests were equally sensitive as 48.8% of the subjects with asthma had a positive mannitol test response compared to 47.7% of the methacholine test. This finding is in accordance with other authors, which demonstrated that the sensitivity of direct and indirect bronchial challenge tests for a clinical diagnosis of asthma are equivalent [28,29]. Moreover, Sverrild et al. proposed that methacholine may not be as highly sensitive in population samples [13]. These findings may be the trigger of reevaluating the hypothesis that direct challenges are more sensitive for clinical asthma.

To our knowledge, the majority of research literature concerning the specificity of bronchial provocation for the clinical diagnosis of asthma has established that the inhaled dry-powder mannitol has significantly higher

Table 1. Patients' clinical characteristics and spirometry.

	SEX	AGE	SMOK	P/Y	COUGH	DYS	WHE	CH TIGH	NIGHT	RHI	ATOP	FEV ₁ (ml)	FEV ₁ %	DFEV1 %	DFEV1 (ml)	FeNO	METH	PC20	MAN	PD15	ASTHMA
1	M	34	Y	3	Y	Y	N	N	N	Y	Y	4600	120	2.0	90	58.3	—	32.0	—	635	N
2	M	58	N	0	Y	N	N	N	N	Y	N	3060	89	4.0	120	21.2	+	5.8	—	635	N
3	M	32	EX	5	Y	N	N	N	N	Y	N	4430	95	3.5	150	16.5	—	32.0	—	635	N
4	F	21	N	0	Y	N	N	N	N	Y	Y	3120	85	2.5	80	4.4	—	32.0	—	635	N
5	M	43	N	0	Y	N	N	N	N	N	N	3960	99	3.5	140	10.5	—	32.0	—	635	N
6	M	34	EX	3	Y	Y	N	N	N	Y	Y	4280	89	4.2	180	26.2	+	16.0	—	635	N
7	M	12	N	0	Y	N	N	N	N	N	Y	4720	99	1.5	100	5.1	—	32.0	—	635	N
8	M	22	Y	11	Y	N	Y	N	N	Y	N	4780	95	2.0	100	23.6	—	32.0	—	635	N
9	F	39	N	0	Y	N	Y	N	Y	N	N	3070	95	1.5	50	5.3	—	32.0	—	635	N
10	M	45	EX	5	N	Y	N	N	N	N	N	3480	92	1.2	45	6.7	—	32.0	—	635	N
11	M	44	N	0	Y	Y	N	N	N	Y	Y	3080	69	6.0	25	31.3	+	3.9	—	635	N
12	F	32	Y	12	Y	Y	N	N	N	Y	Y	2870	96	1.0	80	11.1	—	32.0	—	635	N
13	F	49	EX	17	Y	Y	N	Y	N	N	N	2130	81	1.5	30	5.6	—	32.0	—	635	N
14	F	26	N	0	Y	Y	N	N	N	N	Y	3930	104	2.0	65	4.1	—	32.0	—	635	N
15	F	32	N	0	Y	N	N	N	N	Y	Y	3040	101	4.0	110	3.9	—	32.0	—	635	N
16	F	54	EX	7	Y	N	Y	N	N	N	Y	2790	98	1.5	40	5.2	—	32.0	—	635	N
17	M	56	Y	23	N	N	N	N	N	Y	Y	3550	96	2.0	70	6.4	—	32.0	—	635	N
18	M	43	EX	5	Y	Y	N	Y	N	Y	N	3210	91	2.6	85	9.6	—	32.0	—	635	N
19	M	12	N	0	Y	N	N	N	N	N	Y	4720	99	2.5	120	5.1	—	32.0	—	635	N
20	F	39	N	0	Y	N	N	N	Y	N	N	3070	95	1.0	30	5.3	—	32.0	—	635	N
21	M	12	N	0	Y	N	N	N	N	N	Y	4720	99	0.5	10	5.1	—	32.0	—	635	N
22	F	29	N	0	N	Y	Y	N	N	N	Y	3770	95	12.7	480	50.5	—	32.0	—	344	Y
23	M	31	N	0	Y	Y	N	N	N	Y	Y	4240	89	12.5	530	24.5	+	3.8	+	265	Y
24	M	14	N	0	N	Y	N	N	N	N	Y	2950	87	13.8	410	6.8	—	32.0	+	507	Y
25	M	63	N	0	N	N	N	N	Y	Y	Y	3680	100	15.2	560	32.8	+	8.5	+	66	Y
26	M	16	N	0	Y	Y	N	N	N	Y	Y	3110	80	14.7	460	16.4	+	6.0	+	619	Y
27	F	44	Y	10	Y	Y	N	N	N	Y	Y	2180	80	13.7	300	23.4	+	5.9	—	635	Y
28	F	23	N	0	Y	Y	N	N	Y	Y	Y	3020	86	12.5	380	35.2	+	3.8	+	99	Y
29	M	18	EX	8	N	Y	N	N	N	N	N	3590	78	13.6	490	4.2	+	4.8	—	635	Y
30	F	66	N	0	Y	Y	N	N	N	N	N	1910	76	16.6	310	31.2	+	5.1	+	293	Y
31	F	47	EX	4	Y	Y	Y	N	N	Y	Y	3230	96	13.0	420	18.1	—	32.0	—	635	Y
32	M	20	Y	3	N	Y	N	N	N	Y	Y	4470	97	13.6	610	7.5	+	8.5	—	614	Y
33	F	32	N	0	Y	N	N	N	Y	N	N	3080	87	14.2	440	12.7	+	8.5	—	635	Y
34	F	17	N	0	Y	Y	N	N	N	Y	Y	3040	90	12.6	380	9.41	—	32.0	+	348	Y
35	F	52	Y	25	Y	Y	N	N	N	N	Y	1940	69	12.5	245	15.1	—	32.0	+	544	Y
36	M	15	N	0	Y	Y	N	N	Y	Y	N	3060	74	14.5	445	4.7	—	32.0	+	230	Y
37	F	25	N	0	Y	Y	N	N	N	Y	Y	2900	82	15.8	360	17.9	+	7.9	+	475	Y
38	M	60	EX	17	Y	Y	N	N	N	N	N	2470	69	16.5	410	27.8	+	5.8	+	475	Y
39	F	17	N	0	Y	N	N	N	N	N	N	2630	78	14.6	385	21.7	+	16.0	+	249	Y
40	M	29	EX	12	Y	N	N	N	N	N	N	4420	105	13.9	615	4.95	—	32.0	—	635	Y
41	M	16	N	0	Y	Y	Y	N	Y	Y	Y	3530	92	12.6	445	5.3	—	32.0	—	635	Y
42	M	13	N	0	Y	Y	N	N	Y	N	N	4350	94	15.2	665	6.3	—	32.0	—	635	Y
43	F	75	N	0	Y	Y	N	N	Y	Y	N	2570	93	14.7	380	4.7	—	32.0	—	635	Y
44	F	52	N	0	Y	Y	N	N	Y	N	N	2060	79	16.0	330	9.1	—	32.0	—	635	Y
45	F	34	N	0	Y	N	N	N	Y	N	N	2160	97	13.6	285	4.9	—	32.0	—	635	Y
46	F	33	N	0	Y	Y	N	N	N	Y	Y	2500	80	20.8	520	18.9	+	7.6	+	61	Y
47	F	31	N	0	Y	Y	N	N	Y	Y	Y	2880	88	12.8	370	4.8	—	32.0	—	635	Y
48	F	39	N	0	Y	N	N	Y	N	Y	N	4110	124	14.0	575	16.4	—	32.0	+	99	Y

Table 2. Patients' symptoms, respiratory function and airway inflammation.

	FEV ₁ %pred < 80%	FeNO > 20 ppb
Dyspnea	17 (35.4%)	21 (43.7%)
Cough	15 (21.4%)	23 (32.8%)
Wheezing	7 (33.3%)	8 (38%)
Chest tightness	0	2 (28.5%)
Night symptoms	4 (18%)	5 (22.7%)

Table 3. Mean scores (mean) and standard deviations (SD) of the included variables.

	Mean	SD	CI
Age	38.56	16.73	
P/Y	3.96	6	
FeNO (ppb)	18.5	± 14.6	14.8–22.6
FEV ₁ (ml)	3209	± 846	2983–3435
FEV ₁ pred%	88.5	± 12.6	85.2–91.9
PC20 methacholine	12.1	± 9.5	9.6–14.3
Decrease of FEV ₁ (%)	18	± 7.3	16–19.9
B/D methacholine of FEV ₁ (%)	14.1	± 8	12–16.1
PD15 mannitol	454.2	± 228.2	385.7–510.2
Decrease of FEV ₁ (%)	11.45	± 7.4	9.5–13.4
B/D mannitol of FEV ₁ (%)	11.35	± 7.2	9.4–13.1

Note. (B/D = bronchodilation after methacholine or mannitol test).

Table 4. FeNO levels with BHR to mannitol and methacholine.

	Mannitol (+)	Methacholine (+)	Non-responders
FeNO > 20 ppb	26	27	3
Medium FeNO in pt. with > 20 ppb	31.1	29.8	23.6
Medium FeNO in all pt.	19.5	22.6	10.9

Table 5. Asthmatic and non-asthmatic patients with positive or negative test to Bronchial Challenge Tests.

		Asthma diagnosis		
		Positive	Negative	Total
Methacholine test*	Positive	42	3	45
	Negative	25	18	43
	Total	67	21	88
Mannitol test**	Positive	43	1	44
	Negative	24	20	44
	Total	67	21	88
Methacholine and Mannitol test*	Positive	52	3	55
	Negative	15	18	33
	Total	67	21	88

Note. * $p < 0.05$. ** $p < 0.001$.

Table 6. Results of specificity, sensitivity, PPV and NPV (mean ± SD) of the BCTs.

	Methacholine test	Mannitol test	Combination of the two tests
Sensitivity	62.68 ± 50.32 %	64.17 ± 47.34 %	64.7 ± 45.1 %
Specificity	85.71 ± 41.88 %	95.23 ± 22.94 %	93.75 ± 41.88 %
PPV	93.3 ± 32.28 %	97.72 ± 15.8 %	97.05 ± 28.49 %
NPV	35.71 ± 48.5 %	45.45 ± 50.67 %	45.45 ± 50.8 %

Table 7. Statistical analysis.

		FEV ₁ %pred	FeNO (ppb)	PC20 Methacholine	PD15 Mannitol
Gender	F	88.8	14.9	14.7	445
	M	90.5	17.1	14.5	460
Rhinitis	—	88	17.5	13.3	468
	+	90.5	15.7	15.5	439
GERD	—	90.3	14.4	15.5	454
	+	84.6	13.5	12.9	436
Cough	—	86.1	19.3	12.4	418
	+	90.2	15.1	15.3	458
Dyspnea	—	91.2	13.7*	16.1*	422*
	+	88	17.6	13.4	475
Wheezing	—	90.4	14.6	15.5	462
	+	86.3	20	13.3	415
Chest tightness	—	89.1	15.9	13.9	457
	+	93.4	15.2	15.4	394
Night symptoms	—	89.7	16.1	14.2	461
	+	88.8	13.3	15.9	423
Atopy	—	88.1	15.5	13.9	442
	+	90.4	16.3	15.1	481
Cough during mannitol	—	91.2	15.3	452	14
	+	88.6	16.1	450	14.7
Methacholine	—	92.9*	10.2**	32	553**
	+	85.4	22.6	5.2	328
Mannitol	—	90.9	11.9**	19.6**	635
	+	88.1	19.5	9.9	2.96

Note. * $p < 0.05$. ** $p < 0.001$.

diagnostic specificity than a direct test such as methacholine [30]. The most rational interpretation for this was presented by the mechanism by which mannitol acts on the airway smooth muscle [31]. The inhalation of mannitol stimulates indirectly the release of inflammatory mediators from airway cells; therefore, for many years it was considered that mannitol was more appropriate for neural and cellular contribution to establish the diagnosis of asthma in epidemiological studies [27]. These findings were in accordance with our results as the specificity of mannitol was more than 10% higher compared to methacholine. Similarly, a recent study by Sverrild et al. found that mannitol was significantly more specific in identifying clinical asthma compared to methacholine (98% vs 80%) [13]. In both studies, specificity values were shown higher than previously reported. This may be attributed to the fact that our subjects at the entry of the study were untreated with ICS and might have had higher probability of asthma.

The combined assessment of diagnostic properties of methacholine and mannitol tests was not previously investigated. The results of the present study revealed that the sensitivity/specificity of the two combined challenge tests were equal to those values of mannitol alone. These findings could possibly suggest that it is not necessary to perform both provocation tests as usually occurs in clinical practice because mannitol alone is sufficient to

confirm the diagnosis of active asthma. However, it was previously supported that the two tests are complementary and not interchangeable in practice [32].

It should be also mentioned that in our study, methacholine and mannitol failed to identify BHR in 22% of the subjects. However, a high frequency of negative methacholine and mannitol tests has also been previously reported in patients with a given diagnosis of asthma [33]. Although a negative bronchial challenge test may prompt the need for investigation of other possible causes, a negative test should not be definitely relied upon to rule out asthma in a person with typical asthma symptoms, as it may also simply suggest that the person has mild asthma with no need for ICS or does not have BHR to the above mediators [34]. In such a case, an alternative test may be needed to confirm the diagnosis [35]. In our study, the diagnosis of asthma was documented in all patients by a positive bronchodilation test.

Concerning the relationship of the two BCTs, in the present study, a negative correlation between PC20 of methacholine and PD15 of mannitol with FeNO levels was found. These associations were similar for both mannitol and methacholine challenge as a FeNO level greater than 20 ppb was seen in 90% of the asthmatic patients with BHR to mannitol and 86.6% of the subjects with BHR to methacholine. These findings were also observed in previous research suggesting that the absence of inhaled steroids might be the reason for this positive association [15]. In a group of asthma patients treated with inhaled steroids, the level of FeNO was not associated with positive results to any of the two methods [16,36]. Contrary, other relevant studies have shown that the relationship between the degree of airway inflammation and BHR to mannitol is stronger compared to that of methacholine [4,13]. Recently, Anderson et al. reported that mannitol challenge reflects underlying inflammation using FeNO and direct BHR using methacholine in persistent asthmatics receiving inhaled corticosteroids [37].

Despite previous investigations demonstrating that neither symptoms nor physician estimates could predict BHR [26], our findings revealed that dyspnea was the only symptom associated with the positivity of mannitol, methacholine and FeNO levels. This may be explained by the fact that the patients who had symptoms of dyspnea appeared to have worse respiratory function (35.4% of them had $FEV_1 < 80\%$ of predicted) and increased airway inflammation (43.7% had $FeNO > 20$ ppb).

In everyday practice, the majority of patients undergoing challenge tests present with cough as predominant symptom and although a correlation with challenge tests was expected, it did not emerge in our results. Concerning findings of other studies, challenge tests may not be able to differentiate cough-variant asthma from eosinophilic

bronchitis concluding that negative results may not be useful to the underlying diagnosis of cough [2]. Regarding cough occurring during mannitol challenge, it has been reported to be associated to the positivity of the process [38]. This fact was not observed in our study, as the number of patients positive and negative to the test was equal and none of them interrupted the procedure.

Concerning the relationship of BCT to the $FEV_1\%$ of predicted in the present study, it appeared to be correlated only with the positivity of methacholine but not with mannitol. The lower the values in $FEV_1\%$ of predicted were, the more likely a positive methacholine might be anticipated. In another previous work of our team, $FEV_1\%$ of predicted was found to be correlated with both challenge tests; however, recent studies comparing the two methods failed to demonstrate this relationship [39].

The present study has evaluated a group of selected patients admitted to a specialized clinic for asthma and allergies, with respiratory symptoms suspicious for bronchial asthma and good lung function. We consider that the investigation included a representative sample of patients with different characteristics i.e., smokers, atopic, with rhinitis etc. Our findings suggest that both mannitol and methacholine are safe challenge tests indicated for the diagnosis of asthma. Additionally, an advantage of mannitol includes the safety profile due to its progressive dose-response challenge providing the option to interrupt the procedure before any severe decrease in FEV_1 . However, mannitol has many advantages over other challenges in clinical practice. It is a practical and simple well-tolerated method to apply and does not involve specialized equipment. It is also less time-consuming compared to other methods.

There are possible limitations to our study. Although we had statistically significant results with this sufficient number of patients, our results on validity of the two challenges could have been stronger, if we had included a higher number of patients. Another possible limitation is the long period of patient recruitment aiming to include patients with relevant reversibility to bronchodilation. Furthermore, as we did not focus on atopy, skin prick tests to specific allergens were not included in the analysis. However, in patients with monosensitivity to pollen, BCTs have been performed in the respective pollen season.

Conclusions

Both bronchial challenge tests were safe and equivalent in diagnosing asthma in steroid-naïve patients, although specificity values appeared to be higher in mannitol challenge. A test as highly specific as inhaled dry-powder mannitol is adequate to be applied alone in order to

confirm BHR in specialized outpatient clinics and can be widely used as a main diagnostic tool for asthma.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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