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Single and multiple breath nitrogen washout compared with the methacholine test in patients with suspected asthma and normal spirometry

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ABSTRACT

Background Methods used to assess ventilation heterogeneity through inert gas washout have been standardised and showed high sensitivity in diagnosing many respiratory diseases. We hypothesised that nitrogen single or multiple breath washout tests, respectively nitrogen single breath washout (N_2SBW) and nitrogen multiple breath washout (N_2MBW), may be pathological in patients with clinical suspicion of asthma but normal spirometry. Our aim was to assess whether N_2SBW and N_2MBW are associated with methacholine challenge test (MCT) results in this population. We also postulated that an alteration in S_{III} at N_2SBW could be detected before the 20% fall of forced expiratory volume in the first second (FEV_1) in MCT.

Study design and methods This prospective, observational, single-centre study included patients with suspicion of asthma with normal spirometry. Patients completed questionnaires on symptoms and health-related quality-of-life and underwent the following lung function tests: N_2SBW (S_{III}), N_2MBW (Lung clearance index (LCI), S_{cond}, S_{acin}), MCT (FEV_1 and sGeff) as well as N_2SBW between each methacholine dose.

Results 182 patients were screened and 106 were included in the study, with mean age of 41.8±14 years. The majority were never-smokers (58%) and women (61%). MCT was abnormal in 48% of participants, N_2SBW was pathological in 10.6% at baseline and N_2MBW abnormality ranged widely (LCI 81%, S_{cond} 18%, S_{acin} 43%). The dose response rate of the MCT showed weak to moderate correlation with the subsequent N_2SBW measurements during the provocation phases (ρ 0.34–0.50) but no correlation with N_2MBW.

Conclusions Both MCT and N_2 washout tests are frequently pathological in patients with suspicion of asthma with normal spirometry. The weak association and lack of concordance across the tests highlight that they reflect different but not interchangeable pathological pathways of the disease.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The role of the small airway in understanding respiratory diseases, including asthma is been established in the last years. Although technical improvements helped research to flourish in this field, there are still knowledge gaps to be fulfilled before we can implement this method into daily practice

WHAT THIS STUDY ADDS

⇒ To the best of our knowledge, this study is the first to assess nitrogen single breath washout and nitrogen multiple breath washout as well as MCT in patients with clinical suspicion of asthma and normal spirometry. This specific population is a reality in the pneumological practice and one of the most common steps in the investigation of asthma is to proceed to a bronchoprovocation test. There is, however, no gold standard test to diagnose or exclude asthma in this population. This study showed that a substantial part of these patients has a pathological N2washout and that this is not strongly associated to a positive MCT.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study reinforces the importance of investigating the small airway of patients with suspicion of asthma and normal spirometry. Further studies are necessary to understand the impact of this finding in the long term, but given the potential burden associated with untreated asthma N2 washout may become a complementary routine diagnostic tool in the future.

INTRODUCTION

Asthma is a highly prevalent respiratory disease, estimated to affect 262 million people worldwide and leads to 21.6 million disability adjusted life years.[1] The definition of the disease is based on a history of respiratory symptoms combined with a variable expiratory airflow limitation.[2] There is, however, no single test that is considered as the gold standard to confirm or exclude the diagnosis of asthma.[3 4] In clinical practice, the investigation of patients with clinical suspicion of

asthma with normal spirometry includes a methacholine challenge test (MCT). MCT is a safe test with a high negative predictive value for the diagnosis of asthma,[5] nevertheless, it is time consuming, demands trained personnel and the administration of a medication, resulting in an increased diagnostic cost.[3]

Small airways play an important role in the pathophysiology of asthma. Histological findings in severe asthmatic patients indicate that small airways present significantly more inflammation than larger airways.[6] Therefore, one could postulate that non-invasive methods assessing ventilation heterogeneity of the small airways would be a reasonable diagnostic tool. Several methods analysing different inert gases are available. If nitrogen is the inert gas analysed, there are two different methods available: nitrogen single breath washout (N_2SBW) and nitrogen multiple breath washout (N_2MBW).[7] N_2SBW is performed with a forced expiratory manoeuvre, which provides a nitrogen slope of phase III washout (S_{III}). N_2MBW is performed at tidal breathing and provides the lung clearance index (LCI) as well as the evaluation of Sn_{III} slopes from the first breath (S_{acin}) and from lung turnover 1.5 to 6 (S_{cond}).[7 8] Cosio *et al*, as early as 1978, demonstrated a clear association between histological small airway alterations and the S_{III} slope (S_{III}).[9] The assessment of small airway involvement by the analysis of inert gas washout in cystic fibrosis, bronchiolitis obliterans in graft versus host disease as well as COPD is gaining momentum,[10 11] as these methods become more standardised.[7 12]

In subjects with diagnosed asthma, Downie *et al* found a significant association between ventilation heterogeneity in the conducting airways (S_{cond}) and dose response rate (DRR) in the MCT.[13] In addition, Kjelberg *et al* reported a significant association between FEV_1, LCI, S_{acin} and S_{cond} in asthmatic patients.[14] S_{III} was higher in severe when compared with mild/moderate asthmatic patients[15] and, more recently, in the ATLANTIS study, S_{cond} showed a positive association with asthma severity.[16]

Inert gas washout lung function tests are still not incorporated in the daily clinical assessment of the asthmatic patient. It remains unclear whether they are more sensitive and could contribute to an earlier diagnosis of asthma than traditional lung function tests. We hypothesised that N_2SBW and/or N_2MBW are pathological in patients with suspicion of asthma but normal spirometry. Therefore, our main aim was to assess S_{III}, LCI, S_{cond} and S_{acin} in comparison to MCT in this population. In addition, we postulate that an alteration in S_{III} at N_2SBW could be detected before the 20% fall of FEV_1 in MCT.

MATERIALS AND METHODS

Study design, setting and participants

This is a single-centre, investigator-initiated, prospective, observational study performed at the University Hospital of Basel.

Patients referred to the Clinic of Respiratory Medicine and Pulmonary Cell Research between April 2019 and January 2020 were included in the study if they fulfilled the following inclusion criteria: aged 18 years or more, clinical suspicion of asthma and with normal spirometry defined as FEV_1/forced vital capacity (FVC) ≥70%. The exclusion criteria: exacerbation in the previous 2 weeks, patient unable to perform spirometry, current pregnancy, known aortic aneurysm, heart attack or stroke in the last 3 months, eye surgery in the last month and inability to participate due to language barrier or dementia. Patients included in the study were referred to the respiratory medicine department (in most cases by the general practician (GP)) for further evaluation due to respiratory symptoms. The suspicion of asthma was determined by the GP or by the attending physician at the respiratory department. None of these patients had a previous asthmatic diagnosis. All patients underwent a full lung function testing including spirometry. In case of obstruction, bronchial dilatation with short-acting B2-agonist (Salbutamol 4 puffs 100 µg) was performed and the test repeated after 15 min. Patients presenting FEV1/FVC ratio <0.7 before OR after bronchodilatation were excluded from the study. Patients or the public were not involved in the design, conduct, reporting or dissemination plans of the study.

Study assessments

Patients self-completed the following six questionnaires to ascertain symptoms: Asthma Control Questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ), Work Productivity and Activity Impairment Questionnaire-General Health (WPAI-GH), Reflux Severity Index (RSI), Gastroesophageal Reflux Disease questionnaire (GERDq) and Leicester cough questionnaire. Physical examination assessing vital signs, oedema and lung auscultation was performed and documented by a respiratory physician. A medical history pertaining to family history of asthma, smoking status, comorbidities, concomitant medication, vaccinations and symptoms was also taken. A skin prick test evaluating the response to 16 main regional inhalant allergens was performed (see online supplemental appendix 2).

Patients under treatment with long-acting beta-agonist (LABA)/LAMA and/or inhaled corticosteroids (ICS) were instructed to discontinue this at least 48 hours prior to the study assessments, antihistamines at least 24 hours before assessments and SABA/SAMA at least 12 hours before lung function testing.

The N_2SBW test was performed in all patients followed by an N_2MBW test and an MCT. Furthermore, one N_2SBW test was performed between each methacholine dose increment. All tests were performed according to the guidelines for inert gas washout measurement of the European Respiratory Society/American Thoracic Society,[7] except for the number of repetitions of each test modality, due to a time limitation (see online supplemental appendix 1). In brief, all patients were instructed to sit in an upright position and wear the nose clip. For

the N_2SBW test, subjects were instructed to place their mouth around the mouthpiece of the device and to make a maximal inhalation to total lung capacity from a source of 100% oxygen concentration and exhaled fully in a constant flow. The ventilation inhomogeneity was assessed by S_{III} (slope phase of phase III N_2 washout calculated between 25%–75% of vital capacity).[8] For the N_2MBW test, patients were instructed to inhale through the mouth from a source of 100% oxygen from functional residual capacity and perform a series of multiple breaths at tidal volume, until nitrogen (N_2) concentration decreased to 1/40th (2.5%) of its initial value. The estimation of the ventilation inhomogeneity was obtained from the LCI (number of lung turnovers needed to achieve the N_2 washout) as well as S_{cond} (Sn_{III} between lung turnover 1.5 and 6, due to convection-dependent inhomogeneity (ICD)) and S_{acin} (Sn_{III} for diffusion-convection-dependent variable from first breath).[7] The measurements were performed with the Exhalyzer D (Eco Medics AG, Durenten, Switzerland) using Spiroware V.3.1 software. For analysis, the updated Spiroware V.3.3 was used.[17]

MCT was performed according to the recommendations of the European Respiratory Society, including the instruction of individuals to discontinue the maintenance inhaler in order to decrease the chance of a false-negative methacholine provocation test.[5 18] In brief, a baseline spirometry was performed with the patient instructed to sit in an upright position, with a nose clip closing the nose and the mouth sealed around the mouthpiece. The patient breathed normally, took a fast, full inhalation and exhaled forcefully. The procedure was repeated after inhaling NaCl 0.9%, and then methacholine at a cumulative dose of 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, utilising the Carefusion Spirometry PC Software. A positive methacholine test was reached when FEV_1 fell by ≥20%, named here as MCT_{20}. Alternatively, we also looked for a reduction of 40% in specific airway conductance (sGeff), named here as MCT_{40}.[5 19] Furthermore, we calculated the DRR for FEV_1 fall of 20% predicted.[13]

Statistical analysis

The sample size was estimated according to the following assumptions: (a) the mean of Sacin in normal population is 0.072±0.025 L^{-1}[11]; (b) the mean of Sacin in young asthmatics (mean 33 years old) is 0.080 L^{-1}[20]; (c) two-tailed alpha value of 5%; (c) statistical power of 80%. Using these assumptions, it is estimated that 77 cases would be necessary to compare Sacin in the N_2 washout test in non-asthmatics to asthmatics. Adjusting for non-compliance and loss to follow-up of 20%, the final sample size required was 97 cases.

The statistical analysis was performed using IBM-SPSS-Statistics V.25 and SAS V.9.4 (SAS Institute, Cary, North Carolina) and the graphics presented in the study were obtained using GraphPad Prism V.9.5.1. A p value <0.05 was considered significant. All tests were two tailed. The χ^2 test was used to calculate differences in dichotomous variables and the Mann-Whitney U test to calculate differences in continuous variables. All associations were conducted using Spearman's correlation test. Furthermore, we performed a McNemar's test to look for agreement between S_{III} and MCT during the provocation phases. Results are presented as mean±SD or SEM (respectively, SD and SE of the mean).

An abnormal reading for LCI, S_{cond}, S_{acin} and S_{III} was defined as a value >1.96 z-score at baseline. DRR was

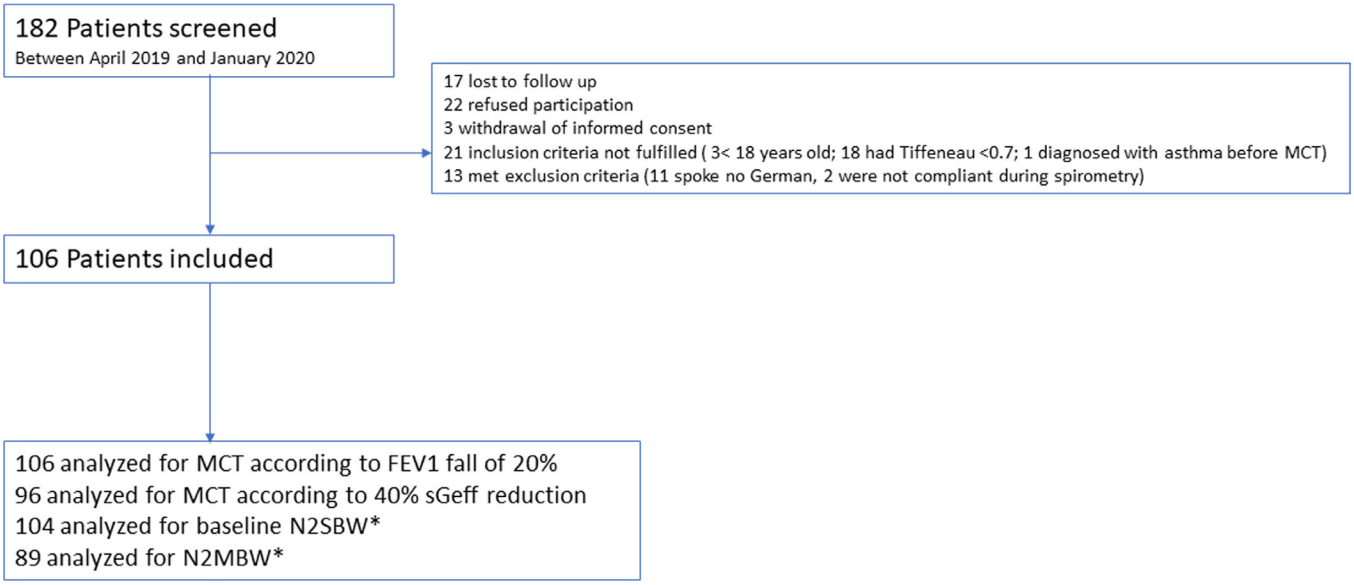

Figure 1 Study design. MCT, methacholine challenge test; N_2SBW, nitrogen single breath washout; N_2MBW, nitrogen multiple breath washout; Tiffeneau, forced expiratory volume in the first second(FEV_1)/forced vital capacity (FVC) ratio; sGeff, specific airway conductance. *Some curves needed to be excluded after the quality control of washout tests, see online supplemental appendix 1.

Table 1 Study subjects demographics

Characteristics	Average±SD
N=106	N (%)
Age (years)	41.8±14.0
Sex : male	41 (39)
Body mass index (kg/m^2)	26±6.0
Smoking status (N=102)	
Current smoker	21 (20.6)
Ex-smoker	22 (21.6)
Never-smoker	59 (57.8)
Positive family history of asthma (n=97)	40 (41)
Comorbidities	
Arterial hypertension	16 (15.1)
Depression	9 (8.5)
Diabetes mellitus	5 (4.7)
Connective tissue disease	4 (3.8)
AIDS	3 (2.8)
Chronic kidney disease	2 (1.9)
Malignant solid tumour	2 (1.9)
Peripheral vascular disease	1 (0.9)
Symptoms	
Cough	64 (60.4)
Sputum	20 (18.9)
Sneeze	16 (15.2)
Wheeze	15 (14.3)
Referred symptom trigger	
Sport	36 (35.0)
Emotional stress	16 (15.7)
Current respiratory medication	
ICS	21 (19.8)
LABA	17 (16)
LAMA	2 (1.9)
SABA	9 (8.5)
Nasal steroid	9 (8.5)
Antihistamine	6 (5.7)
Antitussive	1 (0.9)
Oral steroid	1 (0.9)
Non-respiratory medication	47 (44)
Lung function tests (pre-BD)	
FEV_1 (% pred)	97.9±10.2
FVC (% pred)	102.4±11.8
TLC (% pred)	103.0±11.2
DLCO (% pred)	91.7±13.5
FeNO (ppb)	21.0±1.7
S_{III} (%N/L)	1.59±0.98
LCI (CEV/FRC)	7.81±1.41
S_{cond} (/L)	0.02±0.02
S_{acin} (/L)	0.10±0.06
Lung function tests (pos BD)	
FEV_1 (% pred)	97.8±10.4
FVC (% pred)	99.2±19.6
Prick test performed (N=102)	83 (81.4)

BD, bronchodilator; CEV, cumulative expired volume; DLCO, diffusing capacity of carbon monoxide; FEV_1, forced expiratory volume in the first second; FRC, forced vital capacity; FVC, forced vital capacity; ICS, inhaled corticosteroids; LABA, long-acting beta agonist; LAMA, long-acting muscarinic; LCI, lung clearance index; N_2MBW, nitrogen multiple breath washout; N_2SBW, nitrogen single breath washout; SABA, short-acting beta agonist; S_{acin}, S_{nIII} of the first breath; S_{cond}, S_{nIII} between Lung turnover 1.5 and 6; TLC, total lung capacity.

calculated from the final step in the test as a percentage of decrease in FEV_1/methacholine dose (µmol). A constant of 3 was added to allow log transformation of zero and negative values. Higher DRR values indicate more severe airway hyperresponsiveness.[13]

RESULTS

In total, 182 patients with suspicion of asthma referred to the University Hospital of Basel were screened and 106 were included in the study (figure 1). The average age was 41.8±14 years and there was a majority of women (61%), and never-smokers (57.8%; table 1). Family history of asthma was reported by 41% of patients. The most common reported symptoms were cough (60%) and sputum (19%), physical activity was reported as a trigger for symptoms in 35% of patients, followed by emotional stress in 15%. ICS were already prescribed as treatment in 20% of patients, LABA in 16%, and 81% of the patients were allergic to at least one of the tested inhalant allergens in the skin prick test (table 1).

MCT_{20} was positive in 48% (51) of the patients with an increase to 50% of subjects with MCT_{40}. N_2 washout tests, on the other hand, showed very heterogeneous outcomes: S_{III} was pathological in only 10.6% of the patients at baseline, S_{cond} in 18%, S_{acin} in 43% and LCI in 81% of the study population. The distribution of abnormal versus normal N_2 washout tests compared with positive versus negative MCT_{20} is graphically demonstrated in figure 2.

Patients with positive MCT were more commonly women (74.5% vs 49.1%, p=0.009) with a significantly higher DRR (89.5 vs 5.7% fall FEV1/mmol methacholine+3, p<0.001; table 2). Patients with pathological S_{III} at baseline presented a lower predicted FEV_1 (91% vs 98.5%, p=0.018), and patients with pathological S_{III} as well as pathological S_{cond} and S_{acin} were significantly older than their non-pathological counterparts (table 2). Other lung volumes in the spirometry did not differ

significantly between the groups. Respiratory symptoms, assessed through ACQ, were mild in all groups[21] and the difference in quality of life due to asthma symptoms, chronic cough, reflux symptoms and work impairment (respectively assessed through ACQLQ, Leicester Questionnaire, GERD/RSI and WPAI-GH) between groups was not statistically significant.

Methacholine challenge test (MCT) compared with N_2 single breath washout (N_2SBW)

S_{III} showed a very low sensitivity (12%) and a high specificity (90.7%) for MCT according to 20% decrease in FEV_1, without improvement of these when MCT_{40} was used as reference (table 3). The DRR from MCT depicted a weak correlation with S_{III} at baseline and a weak to moderate correlation with the subsequent S_{III} during provocation phases 1 to 6 (table 4). Patients with positive MCT also showed significantly higher S_{III} at baseline (1.79 /L vs 1.41 /L, p 0.002) (table 2).

Analysing S_{III} over the bronchoprovocation phases, there was an increase in the proportion of pathological tests, especially around provocation phases 3–4 with a significant association between the tests in provocation phases 1, 3, 4 and 5 (figure 3). Further analysis for agreement between the tests showed a slight agreement in most provocation phases (1,2 and 6), with phase 3, 4 and 5 reaching a fair to moderate agreement (kappa coefficient 0.238–0.503) (table 5).

Methacholine challenge test (MCT) compared with N_2 multiple breath washout (N_2MBW)

The sensitivity of N_2MBW outcomes for a pathologic test ranged from 21.7% with S_{cond} to 80.4% with LCI (table 3). The highest specificity (86.1%) was observed with S_{cond} (table 3). There was no association between DRR and LCI, S_{cond} or S_{acin} (table 4).

N_2 single breath washout (N_2SBW) compared with N_2 multiple breath washout (N_2MBW)

S_{III} showed a moderate association with LCI at baseline (ρ 0.528) as well as after each methacholine provocation phase (table 4). S_{acin} showed a moderate correlation with S_{III} at baseline (ρ 0.548), that persisted except in provocation phase 1, where this was weak (ρ 0.394). S_{cond} showed only a weak correlation to S_{III}, except at provocation phase 6, where it increased to moderate (table 4).

Patients with pathological S_{III} at baseline showed a markedly elevated LCI and S_{acin} than the patients with

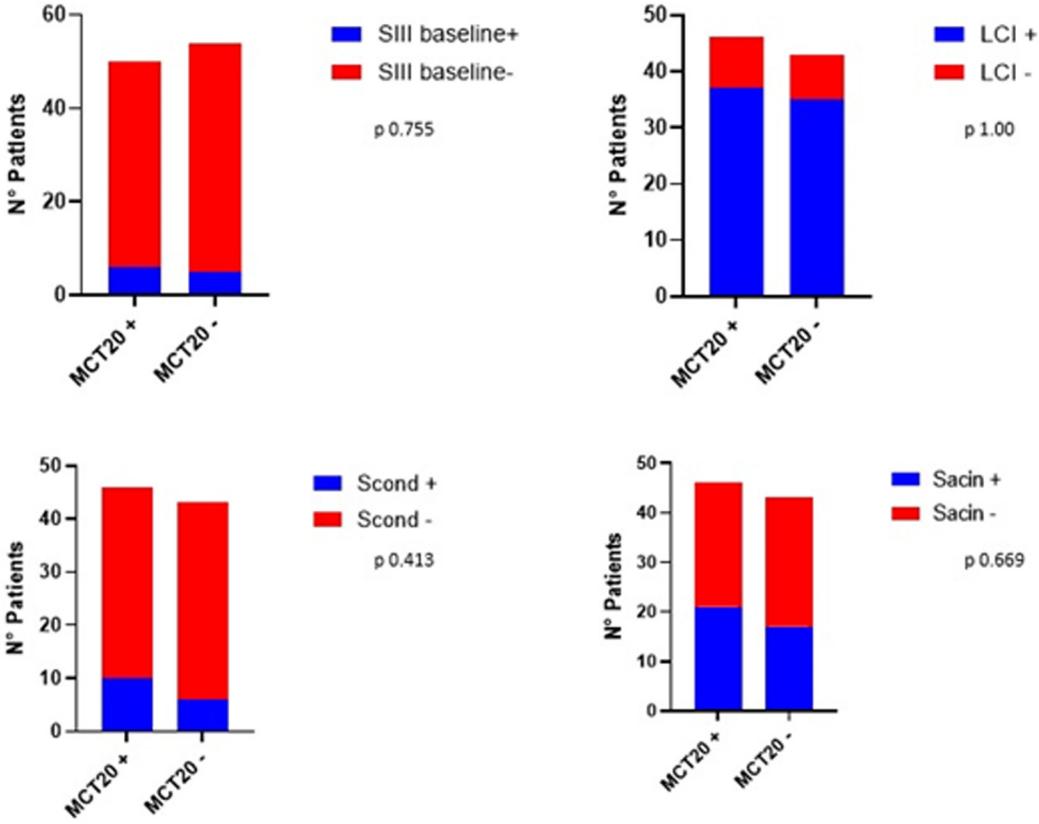

Figure 2 Comparison of N_2 washout test results in patients with positive versus negative MCT_{20}. LCI, lung clearance index from N_2MBW; MCT_{20}, methacholine challenge test according to≥20% fall of FEV_1; N_2SBW, nitrogen single breath washout; N_2MBW, nitrogen multiple breath washout; S_{III}, slope III from N_2SBW at baseline; S_{cond}, Sn_{III} from lung turnover 1.5–6 in the N_2MBW; S_{acin}, Sn_{III} from first breath in N_2MBW. + stands for patients with a positive/pathological test (>1.96 z-score) and – stands for the patients with a negative/normal test.

Table 2 Population characteristics with normal versus abnormal methacholine challenge tests and normal versus abnormal nitrogen washout tests

	Methacholine – MCT_{20}			$N_2SBW – S_{III}$			$N_2MBW – LCI$			$N_2MBW – S_{cond}$			$N_2MBW – S_{acin}$		
	Abnormal n (%); mean±SEM	Normal n (%); mean±SEM	P value	Abnormal n (%); mean±SEM	Normal n (%); mean±SEM	P value	Abnormal n (%); mean±SEM	Normal n (%); mean±SEM	P value	Abnormal n (%); mean±SEM	Normal n (%); mean±SEM	P value	Abnormal n (%); mean±SEM	Normal n (%); mean±SEM	P value
Age (years)	39.76±1.99	43.69±1.86	0.120	55.36±2.83	39.99±1.42	0.001	41.61±1.62	36.12±2.71	0.133	48.8±2.46	38.8±1.58	0.004	41.80±1.36	35.75±1.64	<0.001
BMI ($kg.m^2$)	25.42±0.73	26.53±0.90	0.807	27.44±2.5	25.80±0.59	0.677	26.05±0.76	24.95±0.92	0.970	28.4±1.92	25.3±0.64	0.083	26.00±0.59	25.48±0.76	0.649
Gender: Male	13 (25.5)	28 (50.9)	0.009	4 (36.4)	37 (39.8)	1.000	26 (36.1)	6 (35.3)	1.000	7 (43.8)	25 (34.2)	0.568	14 (36.8)	18 (35.3)	0.880
Smoking status															
Never-smoker	24 (50.0)	35 (64.8)	0.235	4 (36.4)	53 (59.6)	0.314	38 (55.1)	13 (76.5)	0.199	7 (43.7)	44 (62.9)	0.322	19(50)	32 (66.7)	0.257
Ex-smoker	11 (22.9)	11 (20.4)		4 (36.4)	18 (20.2)		16 (23.2)	1 (5.9)		5 (31.3))	12 (17.1)		10 (26.3)	7 (14.6)	
Current smoker	13 (27.1)	8 (14.8)		3 (27.3)	18 (20.2)		15 (21.7)	3 (17.6)		4 (25)	14 (20)		9 (23.7))	9 (18.8)	
AQLQ	5.49±0.16	5.75±0.17	0.155	5.56±0.35	5.64±0.125	0.605	5.53±0.14	5.70±0.31	0.572	5.27±0.33	5.63±0.14	0.290	5.63±0.12	5.66±1.17	0.439
ACQ	1.08±0.15	0.85±0.15	0.134	1.09±0.27	0.93±0.11	0.507	1.06±0.12	1.00±0.30	0.618	1.28±0.25	0.99±0.13	0.269	0.96±0.10	1.04±0.16	0.860
Leicester Questionnaire	16.31±0.68	16.8±0.65	0.524	16.93±1.63	16.60±0.49	0.863	16.53±0.59	15.37±1.14	0.295	14.73±1.45	16.66±0.55	0.187	16.58±0.47	16.14±0.70	0.729
GERD	2.51±0.45	2.96±0.53	0.684	1.64±0.65	2.96±0.39	0.237	2.70±0.40	3.35±1.12	0.908	3.00±0.91	2.79±0.43	0.475	2.75±0.35	3.00±0.54	0.859
WPAI-GH															
% work time missed due to health	0.013±0.01	0.06±0.03	0.462	0.00±0.00	0.04±0.02	0.263	0.05±0.00	0.03±0.02	0.559	0.04±0.04	0.04±0.03	0.556	0.037±0.02	0.026±0.01	0.241
% impairment while working due to health	21.7±3.8	23.7±3.8	0.800	12.9±2.8	24.2±3.0	0.155	24.5±3.7	21.5±4.5	0.877	25.8±6.57	23.26±3.37	0.592	22.81±2.65	22.67±3.49	0.734
% overall work impairment due to health	21.1±4.1	21.1±3.3	0.702	13.3±3.3	22.1±2.9	0.391	21.9±3.4	21.8±4.7	0.980	28.84±5.75	21.1±3.26	0.351	21.09±2.58	22.20±3.39	0.343
% activity impairment due to health	28.6±4.1	28.8±3.3	0.885	20.9±4.8	29.4±2.8	0.399	29.5±3.2	30.0±7.1	0.727	23.75±4.37	31.09±3.45	0.505	28.72±2.59	28.37±3.76	0.554
RSI	11.50±1.47	11.88±1.50	0.941	8.73±2.51	12.06±1.17	0.308	11.42±1.23	15.81±2.30	0.174	16.07±3.33	11.43±1.19	0.221	11.71±1.05	12.84±1.57	0.455
FEV_1 (%predicted±SEM)	96.1±1.5	99.5±1.3	0.130	91.00±2.94	98.48±1.04	0.018	97.57±1.15	95.59±3.10	0.715	94.6±1.93	97.75±1.26	0.181	97.89±0.99	98.69±1.36	0.139
FVC (%predicted±SEM)	101.9±1.8	102.8±1.5	0.795	98.36±3.48	102.56±1.22	0.166	101.47±1.38	101.18±3.42	0.863	100.4±2.70	101.6±1.45	0.712	102.4±1.15	102.9±1.65	0.215
TLC (%predicted±SEM)	103.5±12.1	102.5±1.4	0.740	99.55±4.33	103.24±1.13	0.256	102.33±1.31	102.24±3.08	0.938	99.2±2.24	103.0±1.38	0.248	103.0±1.09	104.0±1.43	0.096
FeNO (ppb±SEM)	22.3±2.6	19.9±2.4	0.254	28.91±10.30	20.27±1.57	0.859	22.94±2.40	17.88±2.39	0.421	20.1±2.87	22.4±2.36	0.940	21.0±1.75	20.4±2.24	0.709
DRR (% fall FEV1/mmol methacholine+3)	89.48±11.88	5.72±0.23	<0.001	48.06±19.74	46.54±7.65	0.895	58.21±9.82	24.55±0.23	0.230	55.25±22.48	51.02±8.73	0.906	61.41±13.63	44.60±9.99	0.648
LCI (2.5%norm)	7.84±0.20	7.78±0.23	0.774	10.59±0.59	7.46±0.09	<0.001	8.14±0.16	6.43±0.10	<0.001	8.28±0.38	7.71±0.16	0.129	7.81±0.15	7.15±0.11	<0.001
Sacin*VT (L^{-1})	0.104±0.01	0.105±0.01	0.908	0.20±0.02	0.09±0.006	<0.001	0.12±0.007	0.05±0.005	<0.001	0.111±0.019	0.103±0.007	0.761	0.104±0.007	0.062±0.003	<0.001
Scond*VT (L^{-1})	0.024±0.003	0.024±0.002	0.967	0.03±0.006	0.02±0.002	0.107	0.02±0.002	0.03±0.007	0.242	0.051±0.005	0.018±0.001	<0.001	0.024±0.002	0.026±0.003	0.528
SIII ($\%N_2.L^{-1}$)	1.79±0.13	1.41±0.14	0.002	3.76±0.28	1.33±0.06	<0.001	1.67±0.12	1.16±0.11	0.064	2.16±0.29	1.46±0.10	0.012	1.59±0.10	1.15±0.08	<0.001

The χ^2 test was used to calculate differences in dichotomous variables and the Mann-Whitney U test to calculate differences in continuous variables.
ACQ, Asthma Control Questionnaire; AQLQ, Asthma Quality of Life Questionnaire; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in the first second; FVC, forced vital capacity; LCI, lung clearance index; MCT, methacholine challenge test; N_2MBW, nitrogen multiple breath washout; N_2SBW, nitrogen single breath washout; S_{acin}, S_{cond}, S_{III} of the first breath; S_{cond}, S_{III} between Lung turnover 1; SEM, SE of the mean; SIII, slope from Phase III N_2SBW; TLC, total lung capacity; VT, tidal volume.

Table 3 Sensitivity and specificity of nitrogen washout tests as compared with methacholine challenge tests

		MCT_{20}*	MCT_{40}†
S_{III} baseline N_2SBW	Sensitivity	12%	10.4%
	Specificity	90.7%	89.1%
	Positive likelihood ratio	1.30	0.96
	Negative likelihood ratio	0.97	1.01
	Accuracy‡	86.02%	84.4%
LCI N_2MBW	Sensitivity	80.4%	79.1%
	Specificity	18.6%	18.9%
	Positive likelihood ratio	0.99	0.98
	Negative likelihood ratio	1.05	1.11
	Accuracy‡	22.3%	22.5%
S_{cond} N_2MBW	Sensitivity	21.7%	20.9%
	Specificity	86.1%	83.8%
	Positive likelihood ratio	1.56	1.29
	Negative likelihood ratio	0.91	0.94
	Accuracy‡	82.2%	80%
S_{acin} N_2MBW	Sensitivity	45.7%	41.9%
	Specificity	60.5%	62.2%
	Positive likelihood ratio	1.15	1.11
	Negative likelihood ratio	0.90	0.94
	Accuracy‡	59.6%	60.9%

*Sensitivity, specificity, likelihood ratios and accuracy from washout methods calculated as compared with the reference standard MCT_{20} (methacholine challenge test according to ≥20% fall of FEV1).
†Sensitivity, specificity, likelihood ratios and accuracy from washout methods calculated as compared with the reference standard MCT_{40}(positive methacholine challenge test according to fall of ≥40% of specific airway conductance).
‡Disease prevalence of 6%.[27]
FEV1, forced expiratory volume in the first second; MCT, methacholine challenge test; N_2MBW, nitrogen multiple breath washout; N_2SBW, nitrogen single breath washout; SGeff, specific airway conductance; SIII, slope III from N_2SBW.

S_{III} within normal range, respectively, LCI 10.6 versus 7.5, p<0.001 and S_{acin} 0.20 versus 0.09/L, p<0.001. Moreover, both pathological S_{cond} and S_{acin} groups showed significantly higher S_{III} at baseline (respectively, 2.16 vs 1.46, p=0.012 and 1.59 vs 1.15, p<0.001; table 2).

Nitrogen multiple breath washout test

Within the N_2MBW test, S_{acin} was strongly correlated to LCI (ρ 0.759; table 4). Participants with pathological LCI showed a significantly higher S_{acin} (0.12 vs 0.05/L, p<0.001; table 2). S_{cond} showed no significant association with LCI, while S_{acin} and S_{cond} depicted only a weak association (table 4).

DISCUSSION

To the best of our knowledge, this is the first study that compares MCT, N_2MBW and N_2SBW (including measurements across methacholine doses) in a large population of patients with suspicion of asthma and with normal spirometry. This study also provided a thorough assessment of symptoms and quality-of-life using various questionnaires (ACQ, AQLQ, WPAI-GH, Leicester cough questionnaire, RSI and GERDq). Our results indicate that both ventilation inhomogeneity, specially LCI and S_{acin}, assessed by N_2 washout, as well as airway hyperresponsiveness, assessed by MCT were present in a significant proportion of the participants. We did not find a significant association of symptoms and spirometric values to pathological nitrogen washout outcomes.

We observed a diverse prevalence of pathological tests across N_2SBW and N_2MBW, ranging from 10.6% for S_{III} from N_2SBW up to 81% for LCI from N_2MBW. There are few data in the literature comparing N_2SBW and N_2MBW. Our findings reinforce the conclusion from Kjelberg *et al*[22] suggesting N_2MBW to be more sensitive than N_2SBW to diagnose small airway disease and, therefore, S_{III} seems less promising for clinical indications. One could postulate that this difference is due to the fact that CDI and diffusion-convection interaction dependent inhomogeneity, particularly its non-gravitational component, contributes to S_{III} in a lesser degree then in N_2MBW assessments.[7 22] We similarly observed a stronger correlation between S_{III} with S_{acin} as well as LCI then with S_{cond}.[22] What exactly is the contribution of the CDI component in asthma remains a valid but unanswered question. When dividing our study patients in pathological versus non pathological N_2 washout groups, patients within pathological LCI and S_{acin} groups showed increased mean S_{III}, S_{acin} and LCI but no increased mean S_{cond}. Zell-Baran *et al*[23] found that patients with small airway involvement due to different environmental exposures and pulmonary diseases in military deployers also had higher LCI and S_{acin} but the same was not seen with S_{cond}.

Nevertheless S_{cond} was pathological in 18% of our participants, a similar prevalence then reported in patients with Asthma Global Initiative for Asthma (GINA) class 1 in the ATLANTIS cohort,[16] where the authors found that the involvement of small airways, including S_{cond}, increased according to higher GINA stratification groups. Our proportion of pathological S_{acin} of 43% was, on the other hand, similar to that found in patients with GINA 5 in ATLANTIS (40.9%). We did have a significantly older population in both groups, pathological S_{acin} and S_{cond}, and this may be a confounding factor, once increased age was previously associated with ventilation inhomogeneity.[22] Furthermore, our study included 20.6% of current smokers, while in the ATLANTIS cohort, this proportion was of only 3% and S_{cond} and S_{acin} are known to be altered in smokers as well, even when spirometry values are normal.[24 25]

Inert gas washout methods are sensitive tests that do not require the administration of a provocative agent.[7]

Table 4 Correlation between lung function tests

	DRR	N_2MBW Sacin	N_2MBW Scond	N_2MBW LCI 2.5	N_2SBW S_{III} baseline	N_2SBW S_{III} Prov1	N_2SBW S_{III} Prov2	N_2SBW S_{III} Prov3	N_2SBW S_{III} Prov4	N_2SBW S_{III} Prov5
N_2MBW Sacin	ρ=−0.005 p=0.963 N=89									
N_2MBW Scond	ρ=−0.009 p=0.935 N=89	ρ=−0.032 p=0.768 N=89								
N_2MBW LCI 2.5	ρ=0.076 p=0.481 N=89	ρ=0.759 p<0.001 N=89	ρ=0.081 p=0.449 N=89							
N_2SBW S_{III} baseline	ρ=0.297 p=0.002 N=104	ρ=0.548 p<0.001 N=87	ρ=0.248 p=0.021 N=87	ρ=0.528 p<0.001 N=87						
N_2SBW S_{III} Prov1	ρ=0.418 p<0.001 N=94	ρ=0.394 p<0.001 N=79	ρ=0.272 p=0.015 N=79	ρ=0.427 p<0.001 N=79	ρ=0.788 p<0.001 N=93					
N_2SBW S_{III} Prov2	ρ=0.396 p<0.001 N=97	ρ=0.505 p<0.001 N=80	ρ=0.250 p=0.025 N=80	ρ=0.554 p<0.001 N=80	ρ=0.794 p<0.001 N=96	ρ=0.885 p<0.001 N=89				
N_2SBW S_{III} Prov3	ρ=0.436 p<0.001 N=95	ρ=0.469 p<0.001 N=78	ρ=0.212 p=0.062 N=78	ρ=0.577 p<0.001 N=78	ρ=0.751 p<0.001 N=95	ρ=0.817 p<0.001 N=86	ρ=0.908 p<0.001 N=91			
N_2SBW S_{III} Prov4	ρ=0.504 p<0.001 N=83	ρ=0.436 p<0.001 N=67	ρ=0.218 p=0.076 N=67	ρ=0.518 p<0.001 N=67	ρ=0.802 p<0.001 N=83	ρ=0.855 p<0.001 N=78	ρ=0.896 p<0.001 N=80	ρ=0.891 p<0.001 N=82		
N_2SBW S_{III} Prov5	ρ=0.354 p=0.003 N=68	ρ=0.438 p<0.001 N=55	ρ=0.323 p=0.016 N=55	ρ=0.439 p<0.001 N=55	ρ=0.762 p<0.001 N=67	ρ=0.879 p<0.001 N=62	ρ=0.866 p<0.001 N=66	ρ=0.898 p<0.001 N=67	ρ=0.909 p<0.001 N=66	
N_2SBW S_{III} Prov6	ρ=0.341 p=0.011 N=55	ρ=0.552 p<0.001 N=42	ρ=0.436 p=0.004 N=42	ρ=0.607 p<0.001 N=42	ρ=0.797 p<0.001 N=54	ρ=0.923 p<0.001 N=50	ρ=0.888 p<0.001 N=54	ρ=0.908 p<0.001 N=54	ρ=0.908 p<0.001 N=53	ρ=0.931 p<0.001 N=55

Spearman rho correlation coefficient: ▭, 0.00–0.10 negligible correlation; ▭, 0.40–0.69 moderate correlation; ▭, 0.10–0.39 weak correlation; ▭, 0.70–0.89 strong correlation; ▭, 0.9–1.0 very strong correlation.
DRR, methacholine dose response rate; N_2<MBW, nitrogen multiple breath washout; N_2SBW, nitrogen single breath washout; Prov 1 to 6, subsequent methacholine dose
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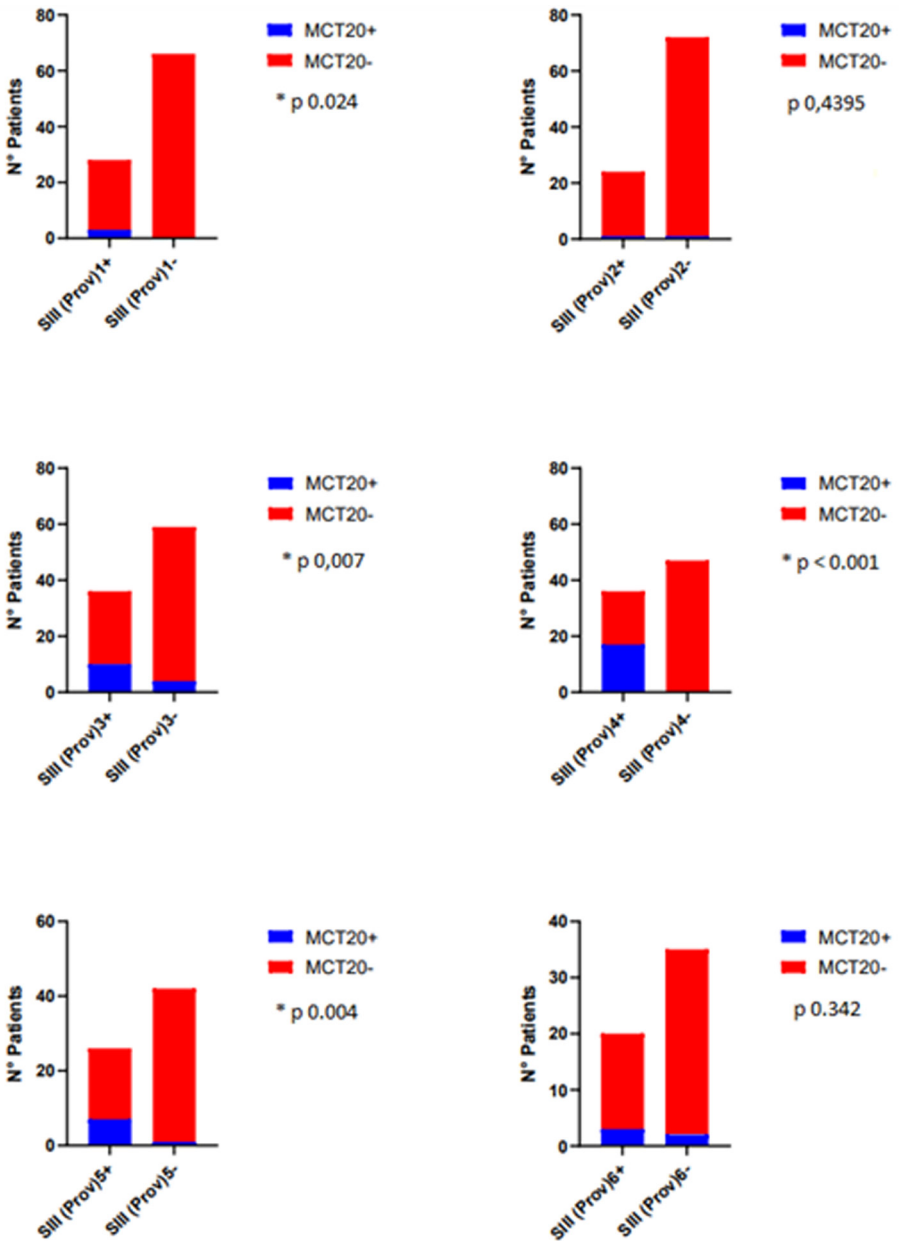

Figure 3 Number of patients with MCT_{20+} and MCT_{20-} in various bronchoprovocation phases. MCT_{20}, methacholine challenge test according to≥20% fall of FEV_1; Prov1, bronchoprovocation with methacholine dose 0.1 mg/mL; Prov2, bronchoprovocation with methacholine dose 0.2 mg/mL; Prov3, bronchoprovocation with methacholine dose 0.4 mg/mL; Prov4, bronchoprovocation with methacholine dose 0.8 mg/mL; Prov 5, bronchoprovocation with methacholine dose 1.6 mg/mL; Prov 6, bronchoprovocation with methacholine dose 3.2 mg/mL. + stands for patients with a positive/pathological test (>1.96 z-score) and – stands for the patients with a negative/normal test. *p<0.05.

Previous studies have found a correlation between S_{III} N_2SBW and FEV_1 in COPD patients,[26] thus, they potentially represent an appealing alternative to volume change measurement in MCT. Airway hyperresponsiveness, assessed by MCT, was present in 48% (MCT_{20}) to 50% of participants (MCT_{40}). The highest sensitivity for a positive was observed with LCI N_2MBW (80.4%) and the highest specificity was reached by S_{III} N2MBW (90.7%), but looking to the tests correlations, DRR from MCT showed a weak correlation only to S_{III} from N_2SBW and no association with N_2MBW outcomes. While repeated S_{III} N_2SBW determinations along the provocation phases depicted a moderate association to DDR of MCT, and the prevalence of pathological $S_{III}N_2$SBW increased during the process, the agreement between tests, however, was low in most provocation phases, so it did not add to a simplification or shortening of the MCT test. Methacholine provocation test is a direct method to trigger airway hyper-responsiveness and is considered a characteristic but not a specific feature of asthma, that is less specific than indirect provocation tests, for example.[8] Pathophysiological pathways involved in asthma are complex,

Table 5 Measuring agreement between MCT_{20} and S_{III} z-score (all available pairs for each of the steps)

	MCT_{20}		SIII z-score≥1.96		Agreement			
Step	Result	n (%)	Negative n (%)	Positive n (%)	P value* Exact McNemar's test	Kappa† coefficient	95% CI for kappa	P value Exact test
NaCl	Negative	106 (100)	85 (88.54)	11 (11.45)				
	Positive	0 (0.00)	–	–				
Prov 1	Negative	101 (95.28)	66 (72.52)	25 (27.47)	<0.0001	0.1442	−0.0045 to 0.2929	0.02444
	Positive	5 (4.716)	0 (0)	3 (100)				
Prov 2	Negative	99 (98.01)	71 (75.53)	23 (24.46)	<0.0001	0.04	−0.0806 to 0.1606	0.9999
	Positive	2 (1.980)	1 (50)	1 (50)				
Prov 3	Negative	84 (84.84)	55 (67.90)	26 (32.09)	<0.0001	0.2384	0.0607 to 0.4160	0.0074
	Positive	15 (15.15)	4 (28.57)	10 (71.42)				
Prov 4	Negative	69 (80.23)	47 (71.21)	19 (28.78)	<0.0001	0.5033	0.3331 to 0.6735	<0.0001
	Positive	17 (19.76)	0 (0)	17 (100)				
Prov 5	Negative	61 (88.40)	41 (68.33)	19 (31.66)	<0.0001	0.2827	0.0853 to 0.4801	0.0039
	Positive	8 (11.59)	1 (12.5)	7 (87.5)				
Prov 6	Negative	55 (90.16)	33 (66)	17 (34)	0.0007	0.1106	−0.0954 to 0.31661	0.34150
	Positive	6 (9.836)	2 (40)	3 (60)				

*If p<0.05 then 'no agreement'.
†Kappa<0 then 'no-agreement'; 0<Kappa≤0.20 slight agreement; 0.20<Kappa≤0.40 fair agreement; 0.40<Kappa≤0.60 moderate agreement; 0.60<Kappa≤0.80 substantial agreement; 0.80<Kappa≤1.00 almost perfect agreement.

multifactorial and not yet fully understood.[8 12] So far, we know that both ventilation heterogeneity and airway hyperresponsiveness represent important features of the disease, but this study highlights that the patients not necessarily present both of them simultaneously and there might not be used interchangeably in the disease. Further studies focusing in this methods and patient phenotyping could hopefully improve our understanding of the involved mechanisms.

Important limitations of our study include the fact that performance of N_2 washout tests could only be compared with methacholine test and not to a definite gold standard to diagnose asthma, for instance, typical remodelling in endobronchial tissue. Therefore, the sensitivity and specificity of these tests are relative. In addition, our study population may be quite heterogeneous as it is expected to include asthmatic patients and healthy subjects. Furthermore, our tests were performed at a single timepoint, and patients with asthma display variable pathology over time. If might be possible that repeated measures of N_2washout might add more information than a single test. Nevertheless, one could expect that by causing bronchoconstriction generally one could potentiate pathological S_{acin} and S_{cond} values during MCT. Finally, patients reaching the threshold for MCT were not further provoked, preventing the additional evaluation of the S_{III} outcome in N_2SBW.

In conclusion, the findings of this study highlight that MCT as well as S_{acin} and LCI from N_2MBW are frequently pathological in patients with suspicion of asthma and a normal spirometry. However, nitrogen washout test cannot yet replace MCT for asthma diagnosis. It is tempting to hypothesise that the weak correlation and lack of concordance between the tests might imply that these tests reflect different but not interchangeable pathological pathways of the disease.

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