

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

Aline Stalder Siebeneichler ,¹ Desiree M Schumann,¹ Meropi Karakioulaki ,¹ Nora Brachsler,¹ Andrei M Darie,¹ Leticia Grize,¹ Thiago G Heck,² Michael Tamm,¹ Philipp Latzin,³ Daiana Stolz^{1,4,5}

To cite: Siebeneichler AS, Schumann DM, Karakioulaki M, et al. Single and multiple breath nitrogen washout compared with the methacholine test in patients with suspected asthma and normal spirometry. *BMJ Open Respir Res* 2024;**11**:e001919. doi:10.1136/bmjresp-2023-001919

► Additional supplemental material is published online only. To view, please visit the journal online (<https://doi.org/10.1136/bmjresp-2023-001919>).

Prior abstract presentation: 10o Health International Congress Unijui (April 2023, Ijuí, Brazil), accepted as a poster for the SSC/SSCS-SSP/SSTS Joint Annual Meeting 2023 (June 2023 in Basel, Switzerland) and accepted as a poster for the annual ERS congress (September 2023 in Milan, Italy).

Received 28 June 2023
Accepted 5 April 2024



© Author(s) (or their employer(s)) 2024. Re-use permitted under CC BY-NC. No commercial re-use. See rights and permissions. Published by BMJ.

For numbered affiliations see end of article.

Correspondence to
Dr Daiana Stolz;
daiana.stolz@uniklinik-freiburg.de

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 (N₂SBW) and nitrogen multiple breath washout (N₂MBW), may be pathological in patients with clinical suspicion of asthma but normal spirometry. Our aim was to assess whether N₂SBW and N₂MBW are associated with methacholine challenge test (MCT) results in this population. We also postulated that an alteration in S_{III} at N₂SBW could be detected before the 20% fall of forced expiratory volume in the first second (FEV₁) 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: N₂SBW (S_{III}), N₂MBW (Lung clearance index (LCI), S_{cond}, S_{acin}), MCT (FEV₁ and sGeff) as well as N₂SBW 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, N₂SBW was pathological in 10.6% at baseline and N₂MBW 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 N₂SBW measurements during the provocation phases (ρ 0.34–0.50) but no correlation with N₂MBW.

Conclusions Both MCT and N₂ 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.

INTRODUCTION

Asthma is a highly prevalent respiratory disease, estimated to affect 262 million people

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.

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_2 SBW) and nitrogen multiple breath washout (N_2 MBW).⁷ N_2 SBW is performed with a forced expiratory manoeuvre, which provides a nitrogen slope of phase III washout (S_{III}). N_2 MBW is performed at tidal breathing and provides the lung clearance index (LCI) as well as the evaluation of $S_{n_{III}}$ 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 patients¹⁵ 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_2 SBW and/or N_2 MBW 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_2 SBW 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) $\geq 70\%$. 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 practitioner (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 μ g) was performed and the test repeated after 15 min. Patients presenting FEV_1 /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_2 SBW test was performed in all patients followed by an N_2 MBW test and an MCT. Furthermore, one N_2 SBW 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 N₂SBW 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 N₂ washout calculated between 25%–75% of vital capacity).⁸ For the N₂MBW 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 (N₂) 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 N₂ 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 FEV₁ fell by ≥20%, named here as MCT₂₀. Alternatively, we also looked for a reduction of 40% in specific airway conductance (sGeff), named here as MCT₄₀.^{5, 19} Furthermore, we calculated the DRR for FEV₁ 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⁻¹¹; (b) the mean of Sacin in young asthmatics (mean 33 years old) is 0.080 L⁻¹²⁰; (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 N₂ 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 χ² 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

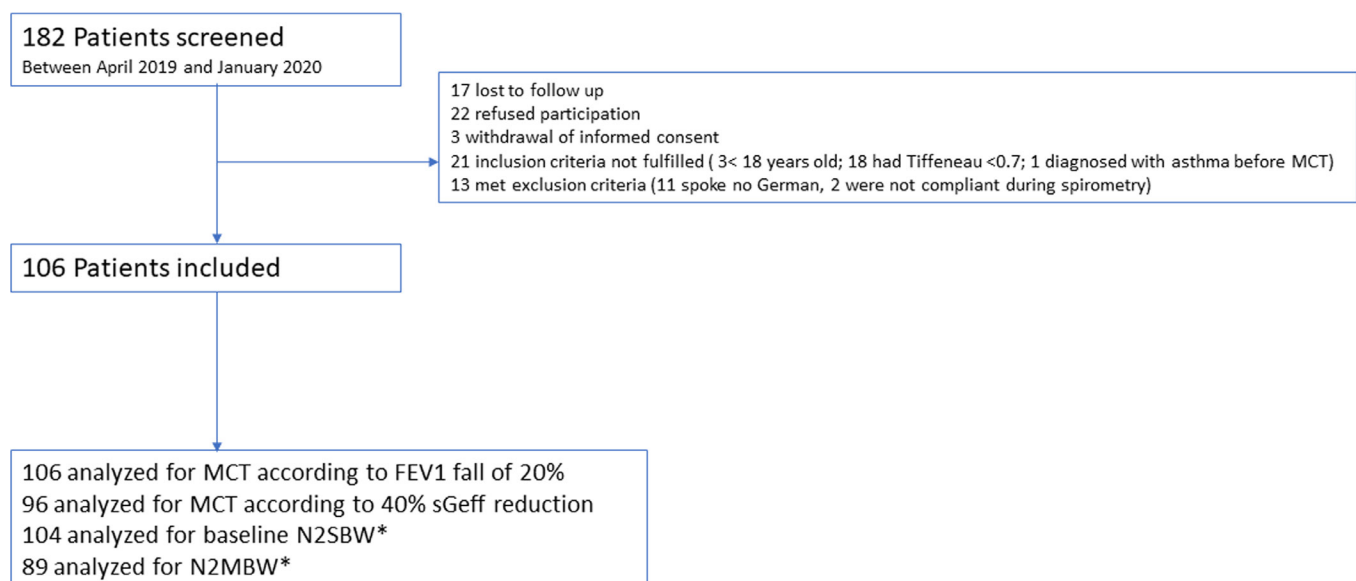


Figure 1 Study design. MCT, methacholine challenge test; N₂SBW, nitrogen single breath washout; N₂MBW, nitrogen multiple breath washout; Tiffeneau, forced expiratory volume in the first second (FEV₁)/forced vital capacity (FVC) ratio; sGeff, specific airway conductance. *Some curves needed to be excluded 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

Continued

Table 1 Continued

Characteristics	Average±SD
N=106	N (%)
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 ₂ MBW, nitrogen multiple breath washout; N ₂ SBW, nitrogen single breath washout; SABA, short-acting beta agonist; S _{acin} , S _{III} of the first breath; S _{cond} , S _{III} 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 FEV₁/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).

MCT₂₀ was positive in 48% (51) of the patients with an increase to 50% of subjects with MCT₄₀. N₂ 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 N₂ washout tests compared with positive versus negative MCT₂₀ 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 FEV₁ (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 groups²¹ 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 N₂ single breath washout (N₂SBW)

S_{III} showed a very low sensitivity (12%) and a high specificity (90.7%) for MCT according to 20% decrease in FEV₁, without improvement of these when MCT₄₀ 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 N₂ multiple breath washout (N₂MBW)

The sensitivity of N₂MBW 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).

N₂ single breath washout (N₂SBW) compared with N₂ multiple breath washout (N₂MBW)

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

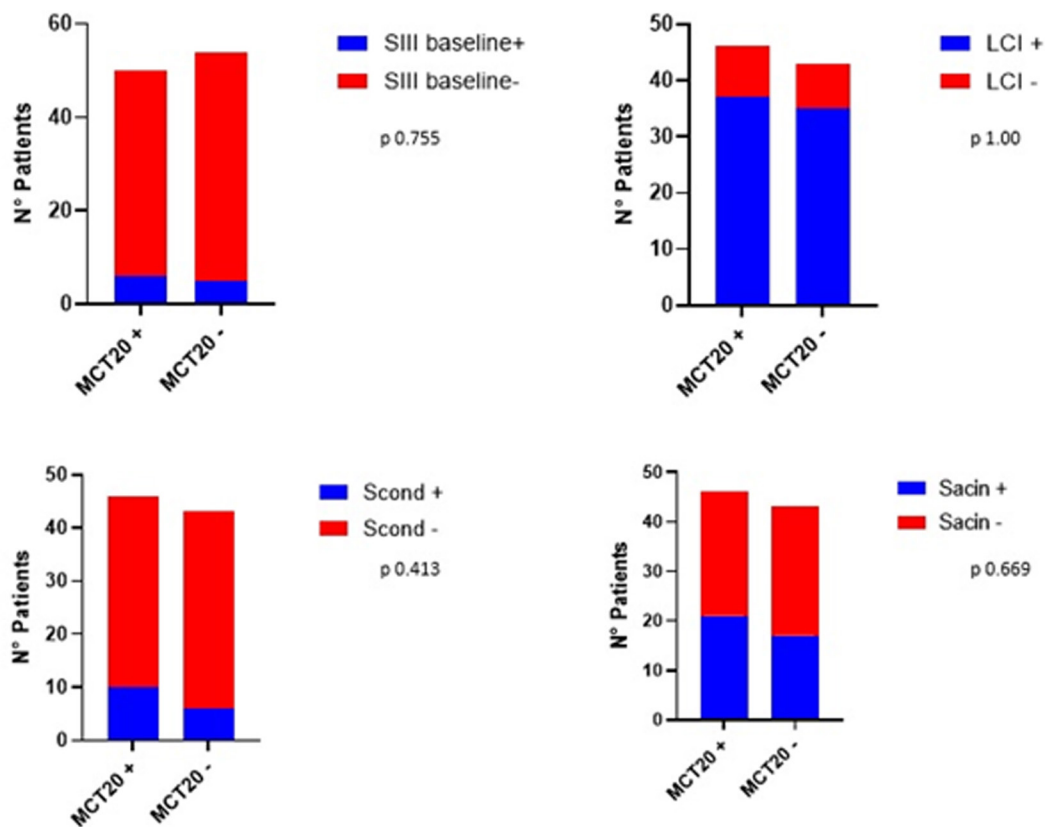


Figure 2 Comparison of N₂ washout test results in patients with positive versus negative MCT₂₀. LCI, lung clearance index from N₂MBW; MCT₂₀, methacholine challenge test according to ≥20% fall of FEV₁; N₂SBW, nitrogen single breath washout; N₂MBW, nitrogen multiple breath washout; S_{III}, slope III from N₂SBW at baseline; S_{cond}, S_{III} from lung turnover 1.5–6 in the N₂MBW; S_{acin}, S_{III} from first breath in N₂MBW. + 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 ₂ SBW – S _{III}			N ₂ MBW – LCI			N ₂ MBW – S _{cond}			N ₂ MBW – S _{min}		
	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 (99.8)	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 (99.6)	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)	
AQLO	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 FEV ₁ /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
SacinVT (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	0.11±0.019	0.103±0.007	0.761	0.104±0.007	0.062±0.003	<0.001
SecondVT (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. ACO, Asthma Control Questionnaire; AQLO, Asthma Quality of Life Questionnaire; BMI, body mass index; FEV₁, forced expiratory volume in the first second; FVC, forced vital capacity; LCI, lung clearance index; MCT, methacholine challenge test; N₂MBW, nitrogen multiple breath washout; N₂SBW, nitrogen single breath washout; S_{III}, S_{cond}, S_{min} of the first breath; S_{III}, S_{cond}, S_{min} between Lung turnover; SEM, SE of the mean; SIII, slope from Phase III N₂SBW; 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 ₂ SBW	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 ₂ MBW	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 ₂ MBW	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 ₂ MBW	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 MCT₂₀ (methacholine challenge test according to $\geq 20\%$ fall of FEV₁).

†Sensitivity, specificity, likelihood ratios and accuracy from washout methods calculated as compared with the reference standard MCT₄₀ (positive methacholine challenge test according to fall of $\geq 40\%$ of specific airway conductance).

‡Disease prevalence of 6%.²⁷

FEV₁, forced expiratory volume in the first second; MCT, methacholine challenge test; N₂MBW, nitrogen multiple breath washout; N₂SBW, nitrogen single breath washout; S_{Geff}, specific airway conductance; S_{III}, slope III from N₂SBW.

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 N₂MBW 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, N₂MBW and N₂SBW (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 N₂ 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 N₂SBW and N₂MBW, ranging from 10.6% for S_{III} from N₂SBW up to 81% for LCI from N₂MBW. There are few data in the literature comparing N₂SBW and N₂MBW. Our findings reinforce the conclusion from Kjelberg *et al*²² suggesting N₂MBW to be more sensitive than N₂SBW 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 than in N₂MBW 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 N₂ 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 ₂ MBW Sacin	N ₂ MBW Scand	N ₂ MBW LCI 2.5	N ₂ SBW S _{III} baseline	N ₂ SBW S _{III} Prov1	N ₂ SBW S _{III} Prov2	N ₂ SBW S _{III} Prov3	N ₂ SBW S _{III} Prov4	N ₂ SBW S _{III} Prov5
N ₂ MBW Sacin	ρ=-0.005 p=0.963 N=89									
N ₂ MBW Scand	ρ=-0.009 p=0.935 N=89	ρ=-0.032 p=0.768 N=89								
N ₂ MBW 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 ₂ SBW 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 ₂ SBW 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 ₂ SBW 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 ₂ SBW 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 ₂ SBW 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 ₂ SBW 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 ₂ SBW 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; □, 0.10–0.39 weak correlation; ■, 0.40–0.69 moderate correlation; ■, 0.70–0.89 strong correlation; ■, 0.9–1.0 very strong correlation.

DRR, methacholine dose response rate; N₂<MBW, nitrogen multiple breath washout; N₂SBW, nitrogen single breath washout; Prov 1 to 6, subsequent methacholine dose

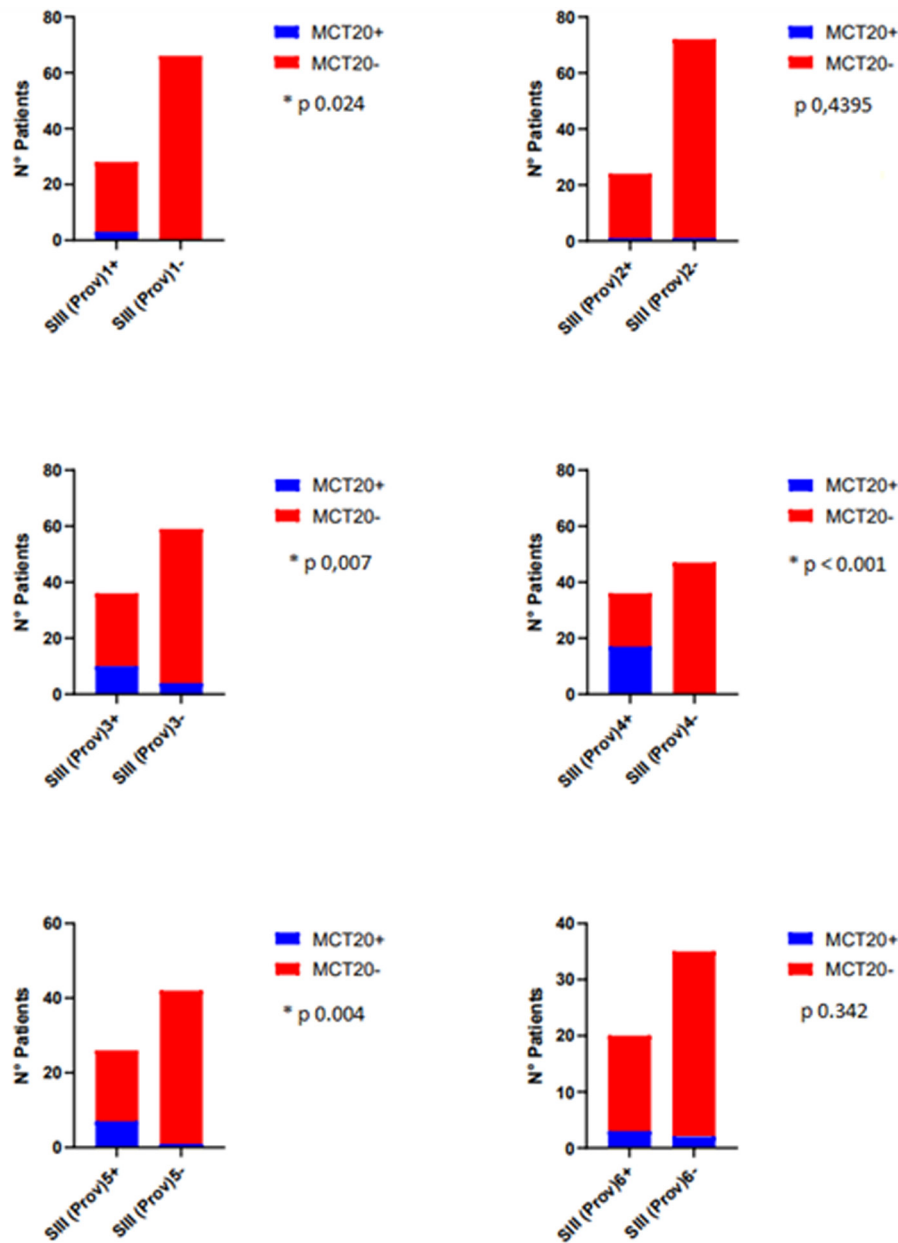


Figure 3 Number of patients with MCT₂₀₊ and MCT₂₀₋ in various bronchoprovocation phases. MCT₂₀₊, methacholine challenge test according to $\geq 20\%$ fall of FEV₁; Prov1, bronchoprovocation with methacholine dose 0.1 mg/mL; Prov2, bronchoprovocation with methacholine dose 0.2 mg/mL; Prov3, bronchoprovocation 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} N₂SBW and FEV₁ 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% (MCT₂₀) to 50% of participants (MCT₄₀). The highest sensitivity for a positive was observed with LCI N₂MBW (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 N₂SBW and no association with N₂MBW outcomes. While repeated

S_{III} N₂SBW determinations along the provocation phases depicted a moderate association to DRR of MCT, and the prevalence of pathological S_{III} N₂SBW 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 MCT₂₀ and S_{III} z-score (all available pairs for each of the steps)

Step	MCT ₂₀		S _{III} z-score \geq 1.96		Agreement			
	Result	n (%)	Negative n (%)	Positive n (%)	P value*	Kappa†	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 \leq 0.20 slight agreement; 0.20<Kappa \leq 0.40 fair agreement; 0.40<Kappa \leq 0.60 moderate agreement; 0.60<Kappa \leq 0.80 substantial agreement; 0.80<Kappa \leq 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 N₂ 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. It might be possible that repeated measures of N₂ washout 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 N₂SBW.

In conclusion, the findings of this study highlight that MCT as well as S_{acin} and LCI from N₂MBW 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.

Author affiliations

¹Clinic of Pneumology, University Hospital Basel, Basel, Switzerland

²UNIJUI, Ijuí, RS, Brazil

³Inselspital, Kinderklinik, University of Bern, Bern, Switzerland

⁴Clinic of Pneumology, University of Freiburg, Freiburg, Germany

⁵Faculty of Medicine, University of Freiburg, Freiburg, Germany

Acknowledgements The authors are very thankful to the patients for their willingness to participate in the study. They also appreciate the competence and diligence of the health care professionals of University Hospital of Basel, which made this study possible. ASS is the guarantor of this manuscript's content.

Contributors All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. DS is the guarantor who accepts full responsibility for the work and/or the conduct of the study, had access to the data, and controlled the decision to publish. All authors listed here contributed substantially to the conception and study design, data analysis and interpretation, and the writing of the manuscript.

Funding This study was fully supported with the research budget from the Clinic of Respiratory Medicine and Pulmonary Cell Research, University Hospital Basel, Switzerland.

Competing interests DS (second author) is currently employed at Tillotts Pharma AG, however, during the period of the study was an employee of the University Hospital of Basel. AMD has received a grant from University Hospital Basel, PL has a grant/contract to Vertex and OM Pharma. DS (last author) reports grants from Astra-Zeneca AG, Curetis AG, BostonScientific, Novartis AG, GSK AG, Roche AG, Zambon, Pfizer, Schwabe Pharma AG, Vifor AG. Other authors have no conflict of interest to declare.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

Ethics approval The study was approved by the Ethics Committee of Northwest/Central Switzerland (EKNZ 2018-02086). Participants gave informed consent to participate in the study before taking part.

Provenance and peer review Not commissioned; externally peer-reviewed.

Data availability statement Data are available upon reasonable request.

Supplemental material This content has been supplied by the author(s). It has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>.

ORCID iDs

Aline Stalder Siebeneichler <http://orcid.org/0000-0003-3726-7481>

Meropi Karakioulaki <http://orcid.org/0000-0002-1067-8582>

REFERENCES

- GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990-2019: a systematic analysis for the global burden of disease study 2019. *Lancet* 2020;396:1204–22.
- Reddel HK, Bacharier LB, Bateman ED, et al. Global initiative for asthma strategy 2021: executive summary and rationale for key changes. *Eur Respir J* 2022;59:2102730.
- Louis R, Satia I, Ojanguren I, et al. European respiratory society guidelines for the diagnosis of asthma in adults. *Eur Respir J* 2022;53:2101585.
- MacNeil J, Loves RH, Aaron SD. Addressing the misdiagnosis of asthma in adults: where does it go wrong? *Expert Rev Respir Med* 2016;10:1187–98.
- Crapo RO, Casaburi R, Coates AL, et al. Guidelines for methacholine and exercise challenge testing-1999. This official statement of the American Thoracic society was adopted by the ATS board of directors, July 1999. *Am J Respir Crit Care Med* 2000;161:309–29.
- Balzar S, Wenzel SE, Chu HW. Transbronchial biopsy as a tool to evaluate small airways in asthma. *Eur Respir J* 2002;20:254–9.
- Robinson PD, Latzin P, Verbanck S, et al. Consensus statement for inert gas washout measurement using multiple- and single- breath tests. *Eur Respir J* 2013;41:507–22.
- Kaminsky DA, Chapman DG. Asthma and lung mechanics. *Compr Physiol* 2020;10:975–1007.
- Cosio M, Ghezzi H, Hogg JC, et al. The relations between structural changes in small Airways and pulmonary-function tests. *N Engl J Med* 1978;298:1277–81.
- Nyilas S, Baumeler L, Tamm M, et al. Inert gas washout in bronchiolitis obliterans following hematopoietic cell transplantation. *Chest* 2018;154:157–68.
- Stolz D, Mkorombindo T, Schumann DM, et al. Towards the elimination of chronic obstructive pulmonary disease: a lancet commission. *Lancet* 2022;400:921–72.
- Usemann J, Yammine S, Singer F, et al. Inert gas Washout: background and application in various lung diseases. *Swiss Med Wkly* 2017;147:w14483.
- Downie SR, Salome CM, Verbanck S, et al. Ventilation heterogeneity is a major determinant of airway hyperresponsiveness in asthma, independent of airway inflammation. *Thorax* 2007;62:684–9.
- Kjellberg S, Houlitz BK, Zetterström O, et al. Clinical characteristics of adult asthma associated with small airway dysfunction. *Respir Med* 2016;117:92–102.
- van Veen IH, Sterk PJ, Schot R, et al. Alveolar nitric oxide versus measures of peripheral airway dysfunction in severe asthma. *Eur Respir J* 2006;27:951–6.
- Postma DS, Brightling C, Baldi S, et al. Exploring the relevance and extent of small Airways dysfunction in asthma (ATLANTIS): baseline data from a prospective cohort study. *Lancet Respir Med* 2019;7:402–16.
- Wylter F, Oestreich M-A, Frauchiger BS, et al. Correction of sensor crosstalk error in Exhalyzer D multiple-breath washout device significantly impacts outcomes in children with cystic fibrosis. *J Appl Physiol (1985)* 2021;131:1148–56.
- Coates AL, Wanger J, Cockcroft DW, et al. ERS technical standard on bronchial challenge testing: general considerations and performance of methacholine challenge tests. *Eur Respir J* 2017;49:1601526.
- Kraemer R, Smith H-J, Sigrist T, et al. Diagnostic accuracy of methacholine challenge tests assessing airway hyperreactivity in asthmatic patients-A multifunctional approach. *Respir Res* 2016;17:154.
- Verbanck S, Schuermans D, Paiva M, et al. The functional benefit of anti-inflammatory aerosols in the lung periphery. *J Allergy Clin Immunol* 2006;118:340–6.
- Validity of Outcome Measures. Canadian agency for drugs and technologies in health. 2017. Available: <https://www.ncbi.nlm.nih.gov/books/NBK476090/> [Accessed 20 Mar 2023].
- Kjellberg S, Viklund E, Robinson PD, et al. Utility of single versus multiple breath washout in adult asthma. *Clin Physiol Funct Imaging* 2018;38:936–43.
- Zell-Baran LM, Krefft SD, Moore CM, et al. Multiple breath washout: a noninvasive tool for identifying lung disease in symptomatic military deployers. *Respir Med* 2021;176:106281.
- Kurz JM, Frey J, Auer R, et al. Influence of ventilation inhomogeneity on diffusing capacity of carbon monoxide in smokers without COPD. *ERJ Open Res* 2021;7.
- Verbanck S, Schuermans D, Meysman M, et al. Noninvasive assessment of airway alterations in smokers: the small Airways revisited. *Am J Respir Crit Care Med* 2004;170:414–9.
- Boeck L, Gensmer A, Nyilas S, et al. Single-breath washout tests to assess small airway disease in COPD. *Chest* 2016;150:1091–100.
- Jaun F, Capponi J, Jochmann A, et al. Swiss severe asthma registry (SSAR)- good asthma control and high number of exacerbations. How does this fit? *European Respiratory Journal* 2021;58.

Annex 1:**Quality criteria for N₂ washout lung function tests.**1) N₂ Single Breath Washout (N₂SBW) – general principles for quality control during the test performance:

- Patients were instructed to sit in an upright position and to wear the nose use a nose-clip for the examination
- The position of the mouthpiece and noseclip was controlled through the lung function technician to avoid leakage.
- Patients inhaled 100% O₂ in a closed system and were advised to breath in complete and on constant flow and after to completely exhale to a constant flow to their residual volume, receiving guidance from the lung functional technician during the process.
- At least 3 maneuvers were performed with at least 2 minutes pause between
- Software gives active feedback during the maneuver to check for its quality and guide the lung function technician's orientation to the patient.
- The examination was performed using a flow reducer coupled to the system, as demonstrated in the picture (red piece).

*Quality control after test performed*

- Curves were proofed individually for relevant leak through graphical visualization.
- Inspiratory and expiratory vital capacity (VC) should express less than 20% difference within one trial. Inspiratory VC as well as expiratory VC were compared between different trials and should not differ more then 10% (reproducibility).

2) N₂ Multiple Breath Washout (N₂MBW) – general principles for quality control during the test performance:

- Patient were instructed to sit in an upright position and to wear the nose use a nose-clip for the examination.
- The position of the mouthpiece and nose clip was controlled through the lung function technician to avoid leakage.
- Patients inhaled 100% O₂ in a closed system, were advised to exhale completely (until reach of functional residual capacity) and then instructed to breathe in and out constantly at tidal volume. Patients received guidance from the lung function technician during the examination, who would be checking a “real time” feedback from the software during the process.
- At least 2 maneuvers evaluated as acceptable by the software were performed.
- The examination was performed without flow reducer

Quality control after test performed

- Curves for N₂ concentration over time as well as volume and flow over time were checked individually for leakage and/or discrepancies.

3) N₂ Single Breath Washout (N₂SBW) between MCT Dose increment:

Same procedure described in number 1, except for the number of maneuvers. Here just one maneuver was performed in order to avoid trespassing the ideal time between the MCT doses.

Appendix 2

Performed skin prick test



Stallergenes Alyostal Prick

Universitätsspital Basel Pneumologie	Patient Data
---	--------------

Positive control (histamin)
Negative control (saline solution)
Grass mixture
Rye
Birch
Hazel
Alder
Ash
Ribwort
Mugwort
Ambrosia
Derm Pteronyssinus
Derm. Farinae
Dog hair
Cat hair
Aspergillus fumigatus
Cladosporium
Penicillum mix

Criteria for a positive test: welt of ≥ 3 mm circumference of diameter (Heinzerling et al., 2013).