



Double tracer gas single-breath washout: reproducibility in healthy subjects and COPD

Kim Husemann¹, Nina Berg¹, Jennifer Engel¹, Johannes Port²,
Christoph Joppek², Ziran Tao², Florian Singer³, Holger Schulz⁴ and
Martin Kohlhäuf¹

Affiliations: ¹Klinik Schillerhöhe, Center for Respiratory Medicine and Thoracic Surgery, Robert-Bosch-Krankenhaus-GmbH, Stuttgart, Germany. ²University of Stuttgart, Dept of Biomedical Engineering, Stuttgart, Germany. ³University Children's Hospital Zurich, Switzerland. ⁴Helmholtz Zentrum Munich, Institute of Epidemiology I, Neuherberg, Germany.

Correspondence: Kim Husemann, Klinik Schillerhöhe, Center for Respiratory Medicine and Thoracic Surgery, Robert-Bosch-Krankenhaus-GmbH, Solitudestr. 18, 70839 Gerlingen, Germany.
E-mail: kim.husemann@klinik-schillerhoehe.de

ABSTRACT The applicability and interpretation of inert tracer gas washout tests is hampered by the lack of feasible protocols and reproducibility data. We assessed feasibility, variability and reproducibility of a new easy to perform double tracer gas (DTG) single-breath washout (SBW) test and compared this with conventional nitrogen washouts.

In 40 healthy nonsmokers and 20 patients with stable chronic obstructive pulmonary disease (COPD), we performed three N₂ vital capacity SBWs, three N₂ multiple-breath washouts and three tidal DTG-SBW tests. Follow-up was after 1 week, 1 month and 6 months. Main outcomes were the lung clearance index (LCI) (N₂ multiple-breath washout), slope of phase III (dN₂) (N₂ vital capacity SBW) and slope of phase III (SIIIDTG) (DTG-SBW).

In healthy subjects, mean $\pm$ SD LCI at baseline was 6.94 ± 0.61 , dN₂ $0.99 \pm 0.42\%$ N₂ per litre and SIIIDTG $-0.206 \pm 0.108 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$. In COPD, LCI and dN₂ were significantly higher (LCI 12.23 ± 2.67 , dN₂ $7.43 \pm 5.38\%$ N₂ per litre; $p < 0.001$) and SIIIDTG significantly steeper ($-0.653 \pm 0.428 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$, $p < 0.001$). Reproducibility was high for main outcome parameters: the intraclass correlation coefficient over 6 months was 0.77 (0.86 in COPD) for LCI, 0.82 (0.89) for dN₂ and 0.83 (0.93) for SIIIDTG.

The tidal DTG-SBW is a reproducible test in healthy and COPD subjects that seems attractive for use in routine clinical settings.



@ERSpublications

DTG-SBW may play a role in detection and monitoring of small airway diseases or as an outcome in drug trials <http://ow.ly/x15bw>

This article has supplementary material available from www.erj.ersjournals.com

Received: May 19 2013 | Accepted after revision: May 14 2014

Clinical trial: This study is registered in the German Clinical Trials Register with identifier number DRKS00003541.

Support statement: This study is part of an investigator-initiated trial supported in equal parts by grants from Chiesi GmbH (Hamburg, Germany) and the Robert Bosch Foundation (grant number KKF-12-8; Robert-Bosch Stiftung GmbH, Stuttgart, Germany).

Conflict of interest: Disclosures can be found alongside the online version of this article at www.erj.ersjournals.com

Copyright ©ERS 2014

Introduction

Inert tracer gas washout (IGW) tests are simple and noninvasive tools to detect air trapping and ventilation inhomogeneity due to patchy small airway obstruction [1]. The multiple-breath washout (MBW) technique and the vital capacity single-breath washout (SBW) technique are the two principal tests established. Compared with MBW, the vital capacity SBW protocol is considerably shorter but requires good coordination and cooperation to perform the vital capacity manoeuvre at constant flow. Until recently, the lack of commercially available hard- and software hampered the use of gas washout tests in research and in the clinical setting. Thus, the recent American Thoracic Society/European Respiratory Society consensus statement on IGW testing stressed the need for feasibility and reproducibility studies [2].

The new double tracer gas (DTG)-SBW test may be more feasible than other techniques due to its easy and fast application requiring only a few tidal breaths [3]. The test is based on a tidal in- and exhalation of a dual tracer gas mixture containing helium and sulphur hexafluoride (He/SF₆ 5/1). An ultrasonic flow meter (USFM) quantifies molar mass during in- and expiration, and accurately reflects He and SF₆ washout patterns in the expired air [3].

The tracer gases SF₆ and He have frequently been used separately, while recent data for the simultaneous use of a SF₆-He dual tracer gas mixture in adults are rare [4–6]. The rationale behind using a dual tracer gas mixture is to assess specific ventilation inhomogeneity in distal airways. As the contribution of convection-dependent ventilation inhomogeneity for He and SF₆ is supposed to be equal, convection-diffusion-dependent ventilation inhomogeneity differs for He and SF₆, and is supposed to arise in different areas of the pre-acinar and acinar lung zones. While the simultaneous analysis of He and SF₆ washout (usually assessed as slope difference between SF₆ and He in a SBW) can be interpreted as a specific index for peripheral ventilation inhomogeneity, the comparable signal of a single tracer gas mainly provides information about overall ventilation inhomogeneity [7].

Parameters of the new tidal DTG-SBW were associated with global and acinar ventilation inhomogeneity indices in children with cystic fibrosis lung disease [8]. In this study, the tidal DTG-SBW was much faster on average (3 *versus* 30 min for three trials) and easier (success rate 92% *versus* 82%) [8]. Recent data suggest that the DTG-SBW may be a sensitive test to assess ventilation inhomogeneity in the lung periphery in different clinical conditions (*e.g.* detection of small airway dysfunction in children with mild asthma and normal spirometry [9] or improved gas mixing efficiency as an immediate treatment response to physiotherapy in children with mild cystic fibrosis lung disease [10]).

In the current study, we assessed the feasibility, variability and short/long-term reproducibility of the new tidal DTG-SBW-test in adult healthy nonsmokers and patients with stable chronic obstructive pulmonary disease (COPD). Classical and new outcome parameters of the test were determined, and their association with established N₂ washout indices and conventional lung function parameters was assessed. Variability and reproducibility estimates of all IGW outcomes were compared. Secondary end-points were differences in ventilation inhomogeneity between groups.

Materials and methods

Study design, study population and ethics

This study is part of a larger prospective cohort study (Evaluation of N₂ single- and multiple-breath washout *versus* a double-tracer single-breath technique in healthy adults and patients with small airway diseases; German Clinical Trials Register DRKS00003541). This trial was approved by the Ethics Committee of the Landesaerztekammer Baden-Wuerttemberg (AZ F-2011-073) and performed at the Klinik Schillerhoehe, Center for Respiratory Medicine and Thoracic Surgery, Robert-Bosch-Krankenhaus-GmbH, Stuttgart, Germany. 40 healthy nonsmokers and 20 patients with clinically stable COPD (spirometric grading Global Initiative for Chronic Obstructive Lung Disease (GOLD) 1–3) were recruited between April 2012 and August 2013 (table 1). COPD subjects had a clinical diagnosis of COPD according to GOLD with a post-bronchodilator forced expiratory volume in 1 s (FEV₁)/forced vital capacity ratio <0.7 and a smoking history ≥10 pack-years [11]. Patients with very severe airflow obstruction (FEV₁ <30% predicted), supplemental oxygen therapy or recent exacerbation were excluded. After written informed consent was obtained, all participants performed a routine lung function test (spirometry and body plethysmography); COPD subjects had an additional baseline measurement of single-breath diffusing capacity of the lung for carbon monoxide (DLCO). Subsequently, we performed three N₂ MBW, three N₂ vital capacity SBW and three DTG-SBW tests. Follow-up was after 1 week, 1 month and 6 months. Each visit included the same lung function tests. COPD patients continued their regular bronchodilator medication before each visit.

TABLE 1 Clinical and demographical characteristics of healthy and chronic obstructive pulmonary disease (COPD) subjects

	Healthy	COPD	p-value
Subjects	40	20	
Age years	40.7 ± 10.3	66.2 ± 10.3	<0.001
Males/females	12:28	16:04	
Weight kg	69.0 ± 13.8	79.1 ± 16.7	<0.05
Height cm	169 ± 8	173 ± 9	0.068
BMI kg·m⁻²	24.1 ± 3.50	26.3 ± 4.1	<0.05
FVC % pred	102.2 ± 12.1	83.7 ± 13.9	<0.001
Z-score	0.18 ± 0.99	-0.93 ± 0.91	<0.001
FEV1 % pred	101.0 ± 11.5	55.2 ± 13.9	<0.001
Z-score	0.06 ± 0.92	-2.60 ± 0.80	<0.001
MMEF % pred	97.2 ± 18.2	26.6 ± 11.0	<0.001
Z-score	-0.14 ± 0.69	-2.46 ± 0.64	<0.001
DLCO % pred	ND	57.5 ± 19.4	
FRC_{MBW} L	3.21 ± 0.81	4.19 ± 1.27	<0.05
LCI	6.94 ± 0.61	12.23 ± 2.67	<0.001
S_{cond} L⁻¹	0.028 ± 0.026	0.045 ± 0.034	<0.05
S_{cond} × V_T	0.029 ± 0.029	0.046 ± 0.034	0.061
S_{acin} L⁻¹	0.058 ± 0.028	0.343 ± 0.139	<0.001
S_{acin} × V_T	0.060 ± 0.034	0.355 ± 0.150	<0.001
dN₂ % N₂ per L	0.99 ± 0.42	7.43 ± 5.38	<0.001
dN₂ × VC % N₂	3.82 ± 1.36	20.60 ± 6.84	<0.001
SIII_{DTG} g·mol⁻¹·L⁻¹	-0.206 ± 0.108	-0.653 ± 0.428	<0.001
SIII_{DTG} × V_T g·mol⁻¹	-0.204 ± 0.091	-0.635 ± 0.344	<0.001
AUC g·%·mol⁻¹	16.39 ± 5.37	33.04 ± 18.59	<0.001
AUC₆₀₋₉₀ g·%·mol⁻¹	2.69 ± 1.52	6.62 ± 4.92	<0.05
Peak g·mol⁻¹	0.59 ± 0.15	0.77 ± 0.30	<0.001
Peak volume %	19.00 ± 3.08	23.86 ± 5.09	<0.001
FWHM %	19.54 ± 7.82	36.71 ± 11.64	<0.001

Data are presented as n or mean ± SD. p-values derived from two-sample t-test. BMI: body mass index; FVC: forced vital capacity; FEV1: forced expiratory volume in 1 s; MMEF: maximal midexpiratory flow; DLCO: diffusing capacity of the lung for carbon monoxide; FRC_{MBW}: functional residual capacity measured by nitrogen multiple-breath washout; LCI: lung clearance index; S_{cond}: index of conductive ventilation inhomogeneity; V_T: tidal volume; S_{acin}: index of acinar ventilation inhomogeneity; dN₂: phase III slope of nitrogen single-breath washout; VC: vital capacity; SIII_{DTG}: phase III slope of double tracer gas single-breath washout; AUC: area under the curve for double tracer gas single-breath washout; AUC₆₀₋₉₀: area under the curve between 60% and 90% of expired volume for double tracer gas single-breath washout; peak: absolute peak concentration of the double tracer gas single-breath washout signal; peak volume: proportion of exhaled volume at peak; FWHM: full width at half maximum of double tracer gas single-breath washout; ND: not done.

Lung function procedures

Spirometry, body plethysmography and single-breath DLCO

We performed routine lung function testing with standard laboratory equipment (JAEGER MasterScreen; CareFusion, Hoechst, Germany) as recommended by current standards [12–14]. For all spirometric parameters, we used the recently published reference values of the Global Lungs Initiative [15]. Predicted values for DLCO were derived from those of COTES *et al.* [16].

N₂ vital capacity SBW and N₂ MBW

The classical N₂ vital capacity SBW and the N₂ MBW were performed using the commercially available device Exhalyzer D (Eco Medics AG, Duernten, Switzerland) according to recommendations [2]. Primary outcome parameters were the slope of phase III (dN₂) for N₂ vital capacity SBW, and functional residual capacity (FRC) and lung clearance index (LCI) for N₂ MBW. We additionally calculated MBW indices of conductive (S_{cond}) and acinar (S_{acin}) ventilation inhomogeneity originally described by VERBANCK *et al.* [17]. All SBW and MBW parameters were computed with the software provided with the Exhalyzer D (Spiroware version 3.1.6; Eco Medics AG). We applied volume correction to all slope III estimates to account for physiological variations in tidal volume (V_T): dN₂ × expiratory vital capacity, S_{cond} × V_T and S_{acin} × V_T.

Double tracer gas single-breath washout

The tidal DTG-SBW was performed with the Exhalyzer D and corresponding software for data recording. Setup, measurement and analysis were initially described by SINGER *et al.* [3]. The DTG mixture containing oxygen (21.0%), N₂ (47.7%), He (26.3%) and SF₆ (5.0%) had a similar molar mass to dry medical air (28.9 g·mol⁻¹), and was provided by Westfalen-AG (Muenster, Germany). During regular quiet breathing, the DTG was applied for a single tidal inhalation from FRC. In the pre-test phase, during the DTG in/expiration and the following inspiration, we recorded molar mass with a side-stream USFM, tidal flows with a mainstream USFM, and carbon dioxide and O₂ signals (fig. 1a).

The molar mass signal measured in side-stream mode (MM_{ss}) during ambient air breathing was used to calibrate the pre-test CO₂ signal, *i.e.* to transform the pre-test CO₂ signal by linear regression modelling into an additional molar mass signal (calculated molar mass signal (MM_{calc}); for details, see the online supplementary material). By “subtracting” MM_{calc} from the raw MM_{ss} during expiration of the DTG, the molar mass test signal reflecting the agglomerated SF₆ and He washout pattern was obtained (fig. 1a and b) [3]. The DTG expirogram (MM_{ss}-MM_{calc}) was plotted against expired volume (fig. 1c). The main outcome parameter was the phase III slope between 60% and 90% of the expired volume (SIIIDTG), which was determined by automated linear regression. SIIIDTG probably detects gas mixing efficiency near the acinar lung regions (diffusion-dependent ventilation inhomogeneity) [8, 9]. To account for variations in tidal volume (V_T), we also performed a volume correction for SIIIDTG (SIIIDTG × V_T) as proposed previously [8, 18].

We described visible dynamics of the simultaneous He/SF₆ washout signal by additional outcome parameters, tracking differences in the shape of the curve between healthy and COPD subjects. As an additional phase III parameter, we calculated the area under the curve between 60% and 90% of the expired volume (AUC₆₀₋₉₀). Parameters from tidal phase II were the absolute peak concentration of the signal (peak), the peak volume (percentage of exhaled volume at peak) and the area under the curve over the total expired volume (AUC). To describe the skewness of the DTG signal, we measured the full width at half maximum (FWHM). All DTG-SBW data analysis was performed with in-house customised software based on LabVIEW 2012 (National Instruments, Austin, TX, USA).

A detailed description of the equipment, procedures and analysis for all washout procedures, including an illustration of all outcome parameters for DTG-SBW, is provided in the online supplementary material.

Statistical analysis

Demographic data such as age, weight and height are reported as mean, standard deviation and range. Individual data for all IGW parameters were reported as mean and intra-individual standard deviation of three technically acceptable tests. Parameters between groups were compared using two-sample-t-tests.

Intra-visit reproducibility was assessed as distribution (mean ± SD) of the intraindividual coefficients of variation of three trials (intraindividual SD/mean). We calculated the intra-class correlation coefficient

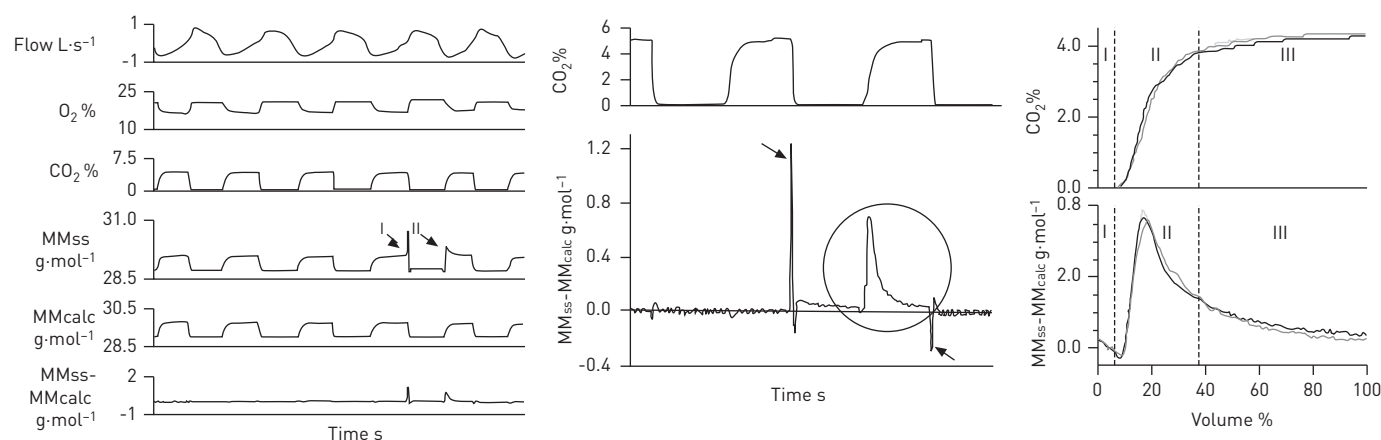


FIGURE 1 Synchronised raw data and dual tracer gas (DTG) test signal. a) Synchronised raw data of DTG single-breath washout. Flow (measured as body temperature, pressure and saturation), oxygen concentration, carbon dioxide concentration, molar mass signal measured in side-stream mode (MM_{ss}), calculated molar mass signal (MM_{calc}) and difference signal (MM_{ss}-MM_{calc}). I: start of inspiration of DTG; II: the following expiration shows a different MM_{ss} signal compared with the naturally exhaled MM_{ss} signal in the previous breaths. b) DTG test signal. CO₂ signal and MM_{ss}-MM_{calc} corresponding to the final DTG test signal (circled). Small artefacts in MM_{ss}-MM_{calc} between in- and expiration are due to gas and sensor dynamics during flow inversion. At the moment of inspiration of the DTG (upper arrow) and the subsequent inspiration (lower arrow), there are two spikes explained by gas de-mixing phenomena due to the technical set-up. c) Typical DTG signal of a healthy adult. Three expirograms of CO₂ and MM_{ss}-MM_{calc} on one test occasion. CO₂ and MM_{ss}-MM_{calc} were plotted against expired volume (% of total expired volume). Phase I: dead space; phase II: transitional phase; phase III: alveolar phase.

(ICC) in a two-way mixed model to assess intra-visit reproducibility (three individual trials at one test occasion) as well as inter-visit reproducibility (baseline, 1 week, 1 month and 6 months). The ICC compares within-subject variance to total variance across all measurements and subjects, and is appropriate to compare repeatability on untransformed data measured on different scales [19]. Differences between visits were assessed graphically by the method described by BLAND and ALTMAN [20], and quantified by the coefficient of repeatability representing a 95% range of differences between two measurements. The coefficient of repeatability was calculated as $1.96 \times \text{SD}$ of the differences between two measurements [20].

To describe the association between IGW indices and age and conventional lung function parameters, we performed simple linear regression.

To assess effects of breathing pattern and lung volume on DTG-SBW indices, we used a linear mixed-effects model with a random intercept to account for multiple observations. Variance components of random and fixed effects were assessed with the residual maximum likelihood method. All statistical analyses were performed using JMP (version 10.0; SAS Institute, Cary, NC, USA) for Windows and a p-value of 0.05 or less was regarded as significant.

Results

40 healthy nonsmokers (aged 20–64 years) and 20 patients with stable COPD (aged 53–83 years, FEV₁ 31–78% predicted) were recruited. The clinical and demographic baseline characteristics of both groups are outlined in table 1. 38 out of 40 healthy subjects (15 out of 20 COPD patients) completed the whole study. The test success rate (three technically acceptable tests at one occasion) was 88% for N₂ MBW (75% in COPD), 95% for N₂ vital capacity SBW (99% in COPD) and 97% for DTG-SBW (96% in COPD).

In the COPD group, indices of N₂ vital capacity SBW and N₂ MBW were significantly different from healthy adults while $S_{\text{cond}} \times V_T$ was of borderline significance (0.029 *versus* 0.046, $p=0.061$) (table 1). All outcome parameters describing the shape of the DTG-SBW curve (SIIIDTG ($\times V_T$), AUC, AUC_{60–90}, peak, peak volume and FWHM) were significantly different between both groups. The difference between SIIIDTG and SIIIDTG $\times V_T$ was small (mean $\pm$ SD difference $0.002 \pm 0.049 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$ (95% CI -0.018 – $0.014 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$) in healthy subjects; $0.018 \pm 0.146 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$ (95% CI -0.094 – $0.059 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$) in COPD).

In healthy subjects, we found an age-dependent increase in LCI ($r^2=0.34$, $p<0.001$; $\text{LCI}=5.529+0.0345 \times \text{age}$), and a marginal association between age and AUC ($r^2=0.11$, $p<0.05$; $\text{AUC}=9.39+0.172 \times \text{age}$). All other washout parameters did not correlate with age (online supplementary fig. 3).

The influence of tidal volume, flow and FRC on DTG-SBW main outcome parameters was assessed by multiple linear regression analysis in a mixed-effects model obtained across all subjects (with different breathing manoeuvres) (table 2 and online supplementary fig. 5). The breathing pattern (V_T and flow) was comparable in healthy and COPD subjects (mean $\pm$ SD V_T 1.05 ± 0.22 *versus* $1.05 \pm 0.21 \text{ L}$; expiratory flow -0.65 ± 0.19 *versus* $-0.69 \pm 0.17 \text{ L} \cdot \text{s}^{-1}$). According to our model, 69–81% of the total variance can be explained by clustering within healthy subjects (92–97% in COPD). Thus, only a minor proportion of the total variance can be attributed to the breathing manoeuvre itself. However, extreme values of V_T , flow or FRC may have considerable influence on DTG-SBW parameters (e.g. change of 1 L in V_T leads to change of $0.262 \text{ g} \cdot \text{mol}^{-1} \cdot \text{L}^{-1}$ in SIIIDTG in healthy subjects).

Typical DTG-SBW signals of COPD patients are shown in figure 2. SIIIDTG is steeper and AUC is higher compared with the signal in healthy nonsmokers (fig. 2 *versus* fig. 1c). Patient B in figure 2, with lower DLCO, presumably due to a higher extent of emphysema, shows a steeper slope (SIIIDTG) and a greater AUC than patient A despite their comparable airflow limitation (FEV₁ 40% pred). In the COPD group, DLCO as surrogate marker of emphysema extent correlates with $S_{\text{acin}} \times V_T$ ($r^2=0.41$, $p<0.01$), dN₂ ($r^2=0.21$, $p<0.05$) and main DTG-SBW indices (SIIIDTG ($\times V_T$): $r^2=0.33$ (0.31), $p<0.05$; AUC: $r^2=0.22$, $p<0.05$). FEV₁ as marker of airway obstruction shows no association to SIIIDTG ($\times V_T$) or other DTG-SBW indices (fig. 3; complete data including associations between DTG-SBW indices and N₂ washout indices in online supplementary tables 2 and 3).

Variability and reproducibility

The mean intra-visit coefficient of variation in healthy subjects was 4.4% for LCI (5.3% in COPD), 16.4% for dN₂ (7.8% in COPD) and 22.2% for SIIIDTG (10.1% in COPD) (table 3). $S_{\text{cond}} (\times V_T)$ and $S_{\text{acin}} (\times V_T)$ showed a clearly higher coefficient of variation (72.9% (73.1%) and 48.7% (44.4%), respectively). In COPD, with slightly higher absolute values for $S_{\text{cond}} (\times V_T)$ and considerably higher values for $S_{\text{acin}} (\times V_T)$, the mean coefficient of variation was 45.1% (29.8%) and 16.3% (8.8%), respectively. For additional DTG-SBW parameters, the mean intra-visit coefficient of variation was between 7% and 18% in both groups (data not shown).

TABLE 2 Effect of breathing pattern and lung volume on dual tracer gas single-breath washout parameters

	SIIDTG g·mol ⁻¹ ·L ⁻¹	AUC g·%·mol ⁻¹	Peak g·mol ⁻¹
Healthy adults			
Flow L·s ⁻¹	0.133 [0.056–0.210]***	10.43 [5.71–15.15]***	-0.49 [-0.65–0.33]***
V _T L	0.262 [0.181–0.345]***	-6.15 [-15.81– -8.26]***	-0.15 [-0.31–0.01]
FRC L	-0.028 [-0.049– -0.007]*	1.69 [0.63–2.74]*	0.06 [0.02–0.10]*
Constant	-0.307	10.43	0.25
Intraclass correlation	0.81	0.87	0.69
R ²	0.89	0.91	0.79
COPD patients			
Flow L·s ⁻¹	-0.228 [-0.543–0.088]	0.79 [-9.27–10.85]	0.03 [-0.20–0.26]
V _T L	0.494 [0.145–0.842]*	-2.84 [-14.16–8.47]	-0.04 [-0.30–0.22]
FRC L	-0.037 [-0.101–0.028]	1.65 [-0.44–3.74]	0.05 [0.00–0.09]
Constant	-1.203	29.90	0.66
Intraclass correlation	0.92	0.97	0.94
R ²	0.96	0.98	0.96

Data are presented as β (95% CI), unless otherwise stated. Effects of flow, tidal volume (V_T) and functional residual capacity (FRC) on main dual tracer gas single-breath washout parameters (phase III slope (SIIDTG), area under the curve (AUC) and absolute peak concentration (peak)) in 40 healthy adults (20 chronic obstructive pulmonary disease patients) calculated by fitting a linear mixed-effects model with random intercept. Calculations are based on averaged values of three individual measurements of all test occasions. Flow, V_T and FRC were considered as fixed effects, and individual subjects as random effects to account for repeated measurements. Estimates of random effects are not shown. The variance components of random and fixed effects were calculated with residual maximum likelihood. The intraclass correlation equals the proportion of total residual variance explained by random effects. For additional illustration of these effects, see online supplementary figure 5. R²: proportion of total quadratic variation that can be explained by fixed effects after correction for random effects. *: p<0.05; ***: p<0.001.

The intra-visit ICC was >0.7 for all gas washout parameters except S_{acin} (× V_T) in healthy adults (ICC 0.42 (0.52)) and S_{cond} (× V_T) in both groups (ICC 0.44 (0.62) in healthy subjects and 0.67 (0.66) in COPD). The intra-visit ICC was comparable for uncorrected and “volume”-corrected phase III slope indices in both groups (table 3).

The mean difference between tests was small for all three washouts, considering short-term as well as long-term reproducibility (table 4, fig. 4 and online supplementary fig. 6). The inter-visit ICC was >0.75 for all washout parameters except MBW slope III indices (S_{cond} (× V_T) and S_{acin} (× V_T)) in healthy subjects and S_{cond} (× V_T) in COPD. Inter-visit variability was comparable for uncorrected and volume-corrected phase III slope indices.

In healthy adults, the coefficient of repeatability ranged between 0.83 and 0.96 for LCI (corresponding to 11.9–13.8% of mean LCI). The coefficient of repeatability for dN₂ was between 0.39% and 0.89% N₂ per L (39.6–89.5% of mean dN₂), and for SIIDTG between 0.098 and 0.140 g·mol⁻¹·L⁻¹ (47.6–67.8% of mean SIIDTG). In COPD, the absolute coefficient of repeatability was higher for all washout parameters except for S_{cond} (× V_T) (table 3).

Discussion

This is the first study to analyse variability and long-term reproducibility of easily obtained measures of ventilation inhomogeneity based on commercially available hardware, the new tidal DTG-SBW, in adults. Our results show that the DTG-SBW is a feasible and reproducible test in subjects with and without COPD. It is easy to perform, with a success rate of ≥96%. However, analysis has to be performed offline using custom software.

Washout indices in healthy subjects

Parameters of conventional lung function and N₂ vital capacity SBW in healthy subjects are in accordance with previously published normative data [15, 21–23]. All N₂ MBW indices corresponded well to preliminary reference values obtained with the same equipment and protocol in adults [24]. A small, linear age-dependent increase in N₂ MBW parameters (LCI, S_{cond} and S_{acin}) was described before with the strongest association between age and LCI [25]. We confirmed a comparable rise in LCI (about 0.3 per 10 years). The possibly low sensitivity of our study to detect the age dependency of S_{cond} and S_{acin} may be attributed to the smaller sample size (n=40 versus n=120 [25]) with few individuals aged >55 years. Since

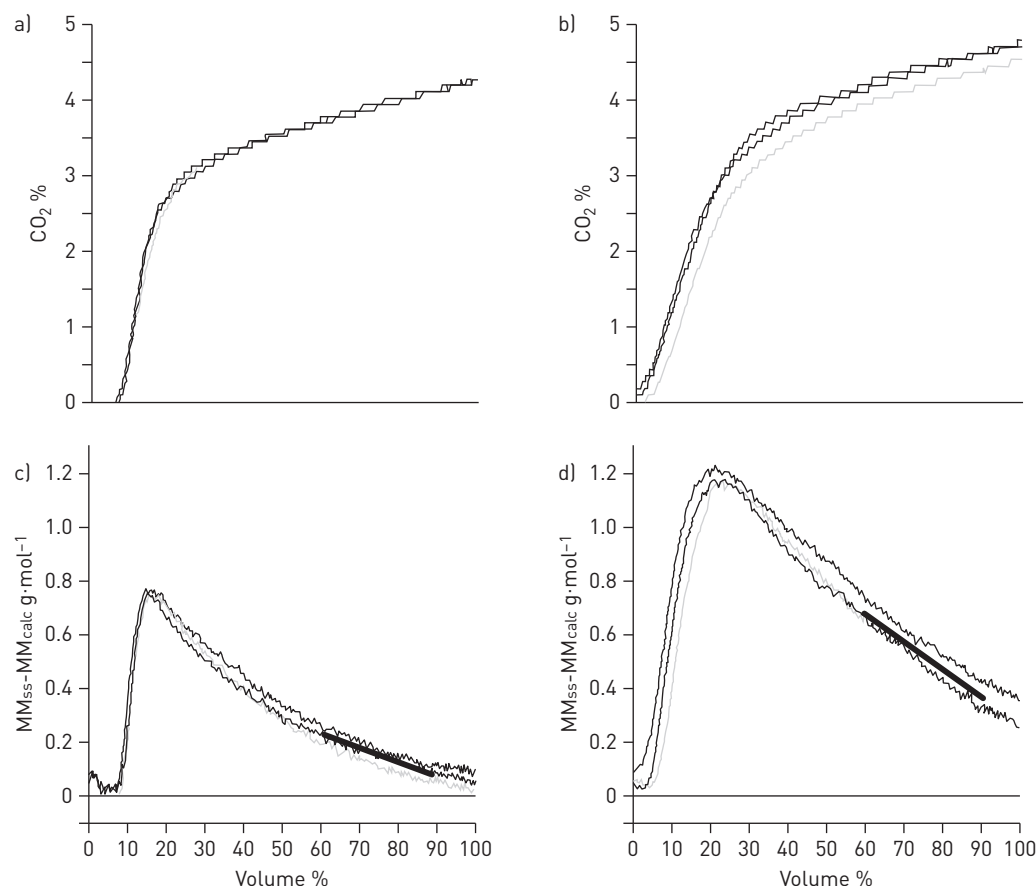


FIGURE 2 Typical dual tracer gas (DTG) single-breath washout (SBW) signals in chronic obstructive pulmonary disease (COPD). DTG-SBW signal in two COPD patients with comparable airflow limitation (postbronchodilator forced expiratory volume in 1 s 40% predicted) and different diffusing capacity of the lung for carbon monoxide (DLCO) as a noninvasive marker for emphysema extent. An expirogram of a, b) carbon dioxide and c, d) difference signal (MM_{ss}-MM_{calc}) at one test occasion (three trials) plotted against expired volume (% of total expired volume). a, c) Patient A: 73 years old, male, DLCO 63% predicted. b, d) Patient B: 65 years old, male, DLCO 42% predicted. Parameters of nitrogen vital capacity SBW and multiple-breath washout were comparable in both patients (phase III slope 5.0 versus 6.2%·L⁻¹; lung clearance index 13.7 versus 12.7; index of conductive ventilation inhomogeneity × tidal volume (V_T) 0.073 versus 0.068; index of acinar ventilation inhomogeneity × V_T 0.447 versus 0.443). However, the phase III slope of DTG-SBW signal between 60% and 90% of expired volume (SIIDTG; black solid line) was markedly steeper (-0.404 versus -1.352 g·mol⁻¹·L⁻¹), and absolute peak concentration of the DTG-SBW signal and area under the curve of the DTG-SBW signal clearly greater (0.75 versus 1.17 g·mol⁻¹ and 29 versus 63 g·%·mol⁻¹, respectively) in patient B.

only a marginal association between AUC (DTG-SBW) and age was detectable, we suggest the association between age and DTG-SBW indices be verified in a larger series.

Washout indices in COPD

This is the first study, using commercially available equipment, to show that in COPD with moderate-to-severe airflow limitation, classical measures of global and acinar ventilation inhomogeneity are significantly elevated. This is in accordance with previously described washout data in COPD [26–29]. The DTG-SBW signal markedly differs from the signal in healthy nonsmokers but seems comparable to the pattern observed in children with cystic fibrosis lung disease [8]. In these patients, SIIDTG was significantly steeper than in healthy controls (-0.457 versus -0.088 g·mol⁻¹·L⁻¹), and correlated with LCI and S_{acin} as marker of global and acinar ventilation inhomogeneity [8]. In COPD subjects, we observed a comparably steep slope III (SIIDTG -0.653 versus -0.206 g·mol⁻¹·L⁻¹ in healthy) and a greater AUC (reflecting greater SF₆ contribution to the signal). Both parameters were associated with DLCO as marker of peripheral airway disease and emphysema extent. SIIDTG × V_T and AUC were correlated with S_{acin} as index of acinar ventilation inhomogeneity, but not with global markers of ventilation inhomogeneity (dN₂ and LCI; online supplementary material). In a former study using mass spectrometry for simultaneous He/SF₆ washout in 27 adult smokers, the SF₆-He slope difference could differentiate between subjects with/without histologically confirmed emphysema during one of several tidal breathing manoeuvres (FRC+V_T=1.5 L)

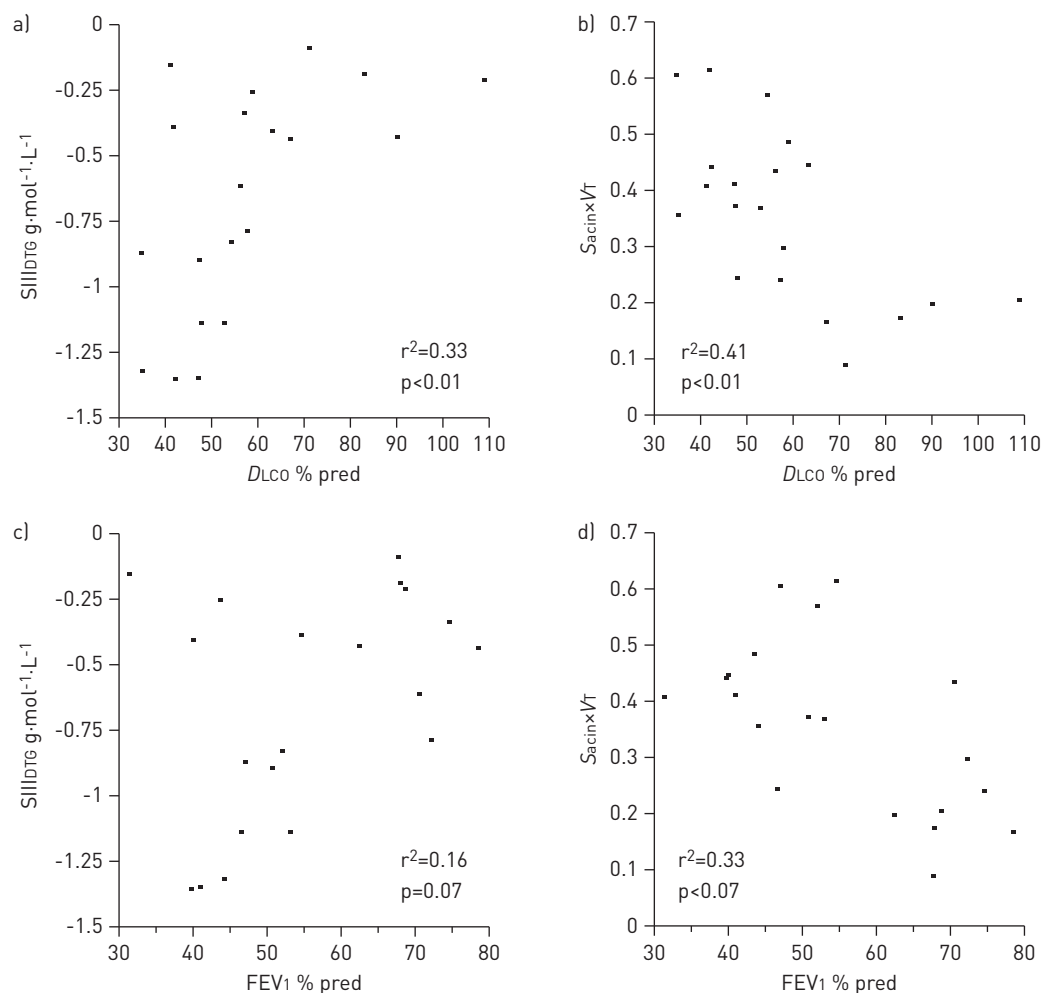


FIGURE 3 Association between phase III slope of double-tracer gas single-breath washout signal (SIIIDTG) and index of acinar ventilation inhomogeneity (Sacin) $\times$ tidal volume (VT) and conventional lung function in chronic obstructive pulmonary disease. Baseline a, c) SIIIDTG and b, d) Sacin $\times$ VT are plotted against a, b) diffusing capacity of the lung for carbon monoxide (DLCO) and c, d) forced expiratory volume in 1 s (FEV1). r^2 : coefficient of determination.

[5]. In contrast, the global ventilation inhomogeneity index N₂ slope (classical N₂ vital capacity SBW) was not significantly different in subjects with/without emphysema [5].

In the context of previous clinical DTG-SBW data and former studies on simultaneous He/SF₆ washout [3, 5, 8, 30], we assume that the main outcome parameters of the DTG-SBW (SIIIDTG and AUC) probably reflect structural acinar changes in our COPD patients.

Reproducibility of washout indices

LCI showed a low intra-visit variability assessed by the coefficient of variation (mean <5% in healthy adults) in accordance with other published data [24, 31]. The intra-visit coefficient of variation of DTG-SBW indices ranged between 7% and 23% (22.2% for SIIIDTG). In healthy children and children with cystic fibrosis lung disease, the intra-test variability of SIIIDTG was 18.5% and 23.9%, respectively, consistent with our data [8]. In contrast, phase III parameters of N₂ MBW (Scond and Sacin) showed a clearly higher intra-visit coefficient of variation. Particularly in healthy adults with values near zero, small variations in phase III slope lead to high coefficients of variation. In COPD, the coefficient of variation of phase III indices is considerably lower due to higher absolute values. This phenomenon was also observed by HORSLEY *et al.* [32]: the coefficient of variation of intra-visit repeats of Scond $\times$ VT was lower in cystic fibrosis than in healthy volunteers (32.7% versus 59.5%) and overall variability of phase III slope indices was consistent with our data. To better compare variability of our untransformed data on different scales, we also assessed the ICC. Intra-visit reproducibility measured by ICC was good (>0.7) for the majority of washout parameters in both groups, except for phase III slope indices of MBW in healthy adults and Scond ($\times$ VT) in COPD

TABLE 3 Main gas washout parameters and intra-visit reproducibility

	Baseline	1 week	1 month	6 months	CV %	ICC [#]
Healthy adults n	40	40	40	38		
LCI	6.94 ± 0.61	7.09 ± 0.64	7.12 ± 0.74	7.05 ± 0.66	4.4 ± 3.0	0.71
FRCMBW L	3.21 ± 0.81	3.03 ± 0.76	2.97 ± 0.73	2.92 ± 0.71	7.7 ± 6.6	0.82
Scond L ⁻¹	0.028 ± 0.026	0.026 ± 0.020	0.021 ± 0.017	0.028 ± 0.029	72.9 ± 41.8	0.44
Scond × Vt	0.029 ± 0.029	0.027 ± 0.024	0.023 ± 0.019	0.028 ± 0.030	73.1 ± 41.9	0.62
Sacin L ⁻¹	0.058 ± 0.028	0.058 ± 0.029	0.057 ± 0.039	0.055 ± 0.041	48.7 ± 78.2	0.42
Sacin × Vt	0.060 ± 0.034	0.065 ± 0.036	0.058 ± 0.037	0.058 ± 0.041	44.4 ± 34.4	0.52
dN ₂ % N ₂ per L	0.99 ± 0.42	1.03 ± 0.43	1.23 ± 0.68	1.10 ± 0.46	16.4 ± 16.5	0.80
dN ₂ × VC % N ₂	3.82 ± 1.36	3.81 ± 1.27	4.26 ± 1.73	4.04 ± 1.31	14.1 ± 11.9	0.84
SIIDTG g·mol ⁻¹ ·L ⁻¹	-0.206 ± 0.108	-0.195 ± 0.089	-0.205 ± 0.109	-0.216 ± 0.121	22.2 ± 31.4	0.80
SIIDTG × Vt g·mol ⁻¹	-0.204 ± 0.091	-0.199 ± 0.089	-0.201 ± 0.096	-0.212 ± 0.110	20.7 ± 30.5	0.82
AUC g·%·mol ⁻¹	16.40 ± 5.38	16.87 ± 5.37	17.18 ± 5.88	17.17 ± 6.27	17.5 ± 52.5	0.93
COPD patients n	20	19	17	15		
LCI	12.23 ± 2.67	12.40 ± 3.48	12.07 ± 2.88	12.42 ± 2.62	5.3 ± 5.4	0.89
FRCMBW L	4.19 ± 1.27	4.30 ± 1.00	4.13 ± 1.23	4.20 ± 1.66	5.9 ± 5.8	0.87
Scond L ⁻¹	0.045 ± 0.034	0.042 ± 0.031	0.050 ± 0.035	0.047 ± 0.032	45.1 ± 32.0	0.67
Scond × Vt	0.046 ± 0.034	0.045 ± 0.031	0.048 ± 0.033	0.043 ± 0.029	29.8 ± 22.4	0.66
Sacin L ⁻¹	0.343 ± 0.139	0.330 ± 0.143	0.348 ± 0.153	0.384 ± 0.156	16.3 ± 15.8	0.86
Sacin × Vt	0.355 ± 0.150	0.354 ± 0.160	0.367 ± 0.167	0.354 ± 0.162	8.8 ± 8.5	0.87
dN ₂ % N ₂ per L	7.43 ± 5.38	7.40 ± 4.01	7.44 ± 4.00	7.17 ± 3.41	7.8 ± 5.7	0.81
dN ₂ × VC % N ₂	20.60 ± 6.84	22.01 ± 7.92	22.18 ± 7.49	21.46 ± 6.80	7.1 ± 5.1	0.83
SIIDTG g·mol ⁻¹ ·L ⁻¹	-0.653 ± 0.428	-0.635 ± 0.419	-0.721 ± 0.447	-0.820 ± 0.459	10.1 ± 9.0	0.95
SIIDTG × Vt g·mol ⁻¹	-0.630 ± 0.344	-0.601 ± 0.325	-0.664 ± 0.305	-0.726 ± 0.353	9.6 ± 9.8	0.96
AUC g·%·mol ⁻¹	33.07 ± 18.59	32.32 ± 18.36	24.50 ± 18.46	25.43 ± 20.18	7.9 ± 14.1	0.97

Data are presented as mean ± SD unless otherwise stated. Intra-visit reproducibility was assessed as distribution (mean ± SD) of the intraindividual coefficient of variation (CV) of three single measurements of all subjects and all test occasions. ICC: intraclass correlation coefficient; LCI: lung clearance index; FRCMBW: functional residual capacity measured by nitrogen multiple-breath washout; Scond: index of conductive ventilation inhomogeneity; Vt: tidal volume; Sacin: index of acinar ventilation inhomogeneity; dN₂: phase III slope of nitrogen single-breath washout; VC: vital capacity; SIIDTG: phase III slope of double tracer gas single-breath washout; AUC: area under the curve (double tracer gas single-breath washout). #: calculated from three individual trials on the same day.

patients (ICC 0.42–0.66). A recent study similarly found a poor intra-visit reproducibility of Scond (ICC 0.23) in 18 patients with moderate-to-severe asthma [33].

Over a period of 6 months, the long-term reproducibility of most washout indices was good (ICC >0.75), except for Scond (× Vt) in both groups and for Sacin (× Vt) in healthy adults. Others reported comparable reproducibility of LCI (ICC 0.70–0.91), but better reproducibility of Sacin and Scond (ICC 0.78–0.95) over a period of 8 days in 21 subjects [34]. In contrast to our study, the MBW was performed with a fixed 1-L breathing protocol, which may partly explain their lesser variability in phase III slope indices.

Intra- and inter-visit reproducibility of all DTG-SBW indices was high in both groups. The better reproducibility of SIIDTG compared with Scond and Sacin in our study is somewhat counterintuitive, as the latter estimates are derived from slope values throughout a MBW procedure, whereas DTG-SBW is based on averaged slope values from three single breaths. We frankly acknowledge that from the current dataset, we cannot definitely explain this phenomenon. In the COPD group, the differences in variability between phase III slope indices of MBW and SIIDTG are definitely smaller, suggesting that absolute values of phase III slopes have an impact on our calculated variability indices. Technical differences between both tests may contribute as well as differences in phase III slope analysis (automated phase III slope analysis using Spiroware 3.1.6 (pre-set limits 50–95%) with the option for manual correction in MBW *versus* automated analysis of SIIDTG (pre-set limits 60–90%) with custom software).

The observed variability of the DTG-SBW parameters appeared to be to some extent explained by the variability of the uncontrolled breathing manoeuvre, *i.e.* flow rate, Vt or individual lung volume. Therefore, we also considered a volume correction for SIIDTG (SIIDTG × Vt) as proposed by SINGER *et al.* [8]. Due to the fact that healthy and COPD subjects had comparable Vt around 1 L (mean Vt 1.05 L in both groups), the correction factor (Vt) was close to 1 and had only minor impact on phase III slope estimates. The variability of SIIDTG could not be reduced by this volume correction. This is in accordance with the finding that, for all phase III slope indices in our study, the proposed volume correction (multiplication by Vt) did

TABLE 4 Inter-visit reproducibility of main washout parameters

	Healthy subjects			COPD patients		
	Difference from baseline		ICC overall [#]	Difference from baseline		ICC overall [#]
	Absolute	% mean		Absolute	% mean	
LCI						
1 week	-0.15 ± 0.45	12.8		0.38 ± 1.14	18.3	
1 month	-0.18 ± 0.49	13.8		0.42 ± 1.75	28.1	
6 months	-0.10 ± 0.42	11.9	0.77	0.16 ± 1.17	18.7	0.86
Scond L⁻¹						
1 week	0.002 ± 0.021	144.9		-0.003 ± 0.025	106.7	
1 month	0.007 ± 0.024	166.7		0.001 ± 0.029	124.4	
6 months	-0.003 ± 0.024	168.0	0.46	-0.004 ± 0.020	82.2	0.71
Scond × Vt						
1 week	0.002 ± 0.019	128.4		-0.004 ± 0.028	117.2	
1 month	0.006 ± 0.027	179.3		-0.007 ± 0.029	121.8	
6 months	-0.002 ± 0.023	158.6	0.53	0.001 ± 0.019	79.8	0.67
Sacin L⁻¹						
1 week	-0.001 ± 0.024	81.4		0.010 ± 0.053	30.2	
1 month	0.001 ± 0.035	118.6		-0.003 ± 0.081	46.6	
6 months	0.003 ± 0.030	102.2	0.59	-0.045 ± 0.107	61.1	0.80
Sacin × Vt						
1 week	-0.005 ± 0.031	102.7		-0.001 ± 0.055	30.3	
1 month	0.002 ± 0.030	99.7		-0.007 ± 0.075	41.6	
6 months	0.003 ± 0.033	107.8	0.61	-0.005 ± 0.101	56.0	0.86
dN₂ % N₂ per L						
1 week	-0.04 ± 0.20	39.6		0.11 ± 2.08	54.8	
1 month	-0.24 ± 0.45	89.5		0.05 ± 2.19	57.7	
6 months	-0.12 ± 0.33	65.3	0.82	0.22 ± 3.32	87.6	0.89
SIIIDTg g·mol⁻¹·L⁻¹						
1 week	-0.011 ± 0.050	47.6		0.007 ± 0.122	36.8	
1 month	<0.001 ± 0.059	56.0		0.040 ± 0.136	40.7	
6 months	0.009 ± 0.071	67.8	0.83	0.125 ± 0.149	44.9	0.93
SIIIDTg × Vt g·mol⁻¹						
1 week	-0.005 ± 0.041	39.2		0.013 ± 0.092	28.5	
1 month	-0.002 ± 0.050	48.4		0.039 ± 0.082	25.5	
6 months	0.007 ± 0.002	60.0	0.82	0.094 ± 0.116	36.2	0.95
AUC g·%·mol⁻¹						
1 week	-0.47 ± 3.38	40.3		-0.01 ± 4.09	24.2	
1 month	-0.78 ± 3.02	36.1		0.10 ± 3.59	21.3	
6 months	-0.73 ± 3.21	38.3	0.84	-1.32 ± 4.76	28.3	0.97

Data are presented as mean ± sd unless otherwise stated. COPD: chronic obstructive pulmonary disease; CR: coefficient of repeatability; ICC: intraclass correlation coefficient; LCI: lung clearance index; Scond: index of conductive ventilation inhomogeneity; Vt: tidal volume; Sacin: index of acinar ventilation inhomogeneity; dN₂: phase III slope of nitrogen single-breath washout; SIIIDTg: phase III slope of double tracer gas single-breath washout signal; AUC: area under the curve (double tracer gas single-breath washout). [#]: calculated over 6 months (four test occasions).

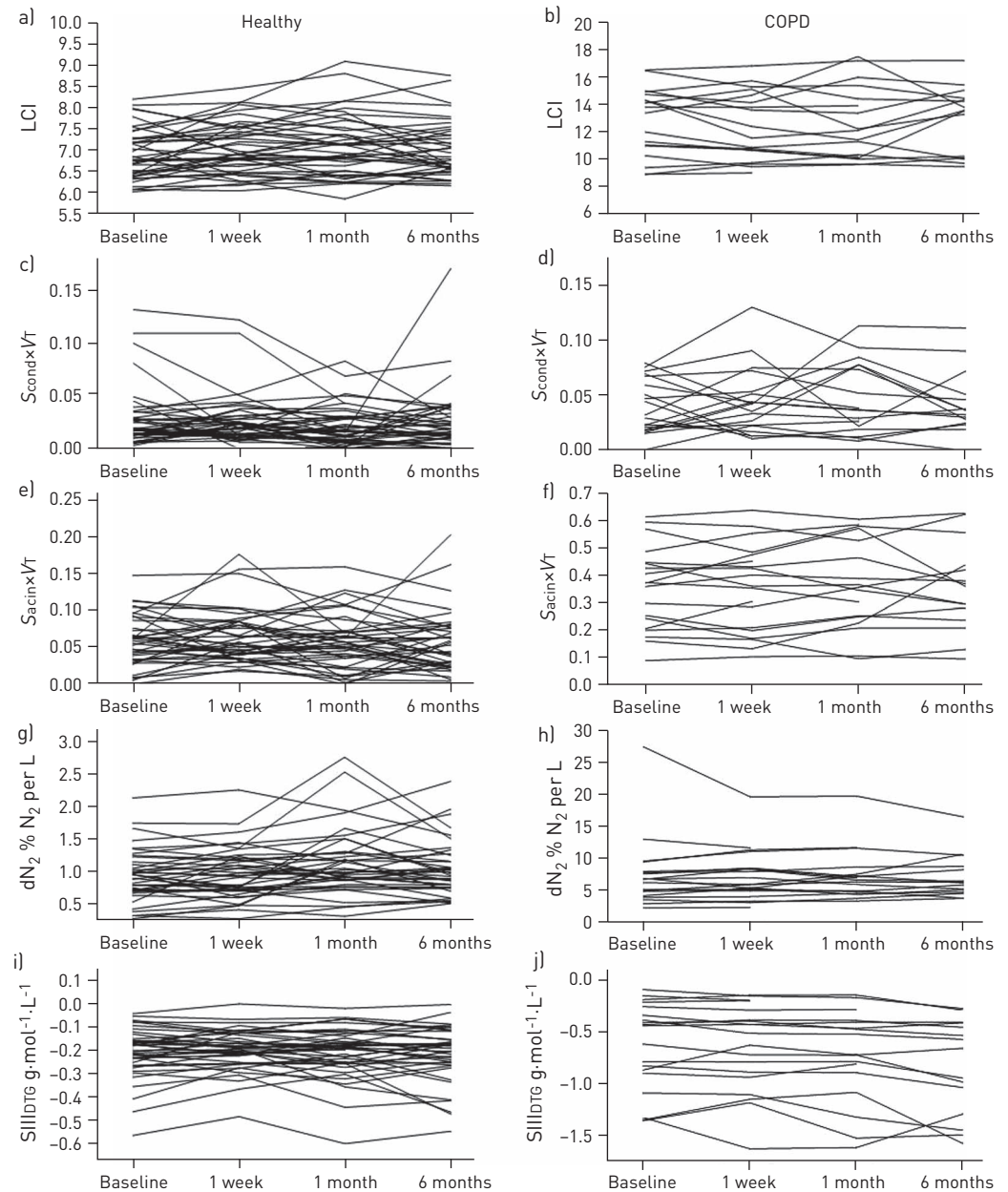


FIGURE 4 Reproducibility of main washout parameters. Means of a, b) lung clearance index (LCI), c, d) conductive ventilation inhomogeneity ($S_{\text{cond}} \times V_T$), e, f) acinar ventilation inhomogeneity ($S_{\text{acin}} \times V_T$), and phase III slope of g, h) nitrogen single-breath washout (dN_2) and i, j) dual tracer gas single-breath washout (SIIIDTG) plotted per test occasion and subject in a, c, e, g, i) healthy adults and b, d, f, h, j) chronic obstructive pulmonary disease patients. Intraindividual changes between visits (baseline, 1 week, 1 month and 6 months) are tracked *via* connecting lines.

not improve reproducibility. Thus, variability of V_T alone seems not to explain variability of phase III slope indices.

According to our calculated model, the greatest part of the variability in DTG indices is explained by clustering within subjects, not by the breathing pattern itself. Breathing pattern and lung volumes seem to influence DTG-SBW outcomes to some extent in healthy subjects and almost not at all in COPD patients. This reassures us that the different DTG-SBW values observed in COPD are indeed due to the underlying pathology and not due to a difference in breathing pattern. The minor effects of V_T , flow or FRC in healthy adults (or of V_T in COPD) do not seem clinically relevant in the physiological range of tidal flow and V_T (online supplementary fig. 5). It may be of interest to study this aspect in a more systematic approach performing different breathing manoeuvres in the same subjects.

The calculation of the coefficient of repeatability provides clinically important data helping to interpret changes in measures of ventilation inhomogeneity over time: in healthy subjects, the coefficient of repeatability for LCI was between 0.83–0.96. Translated into clinical conditions, this means that the limit of agreement was within 1 unit and a difference >1 between two LCI measurements (corresponding to a change over 12–14% of mean LCI) suggests a significant change of this parameter. Similar data on LCI reproducibility have been reported using the same device in children with cystic fibrosis and healthy controls [35]. In COPD patients, the coefficient of repeatability of ventilation inhomogeneity indices was generally higher due to presumably higher variation of lung function in obstructive lung disease. In clinically stable COPD patients LCI may vary by 2–3 units (18–28%) across 6 months.

Limitations

We acknowledge that we have measured only a small group of COPD patients not matched for age and sex with our healthy subjects. The latter factors could have influenced the comparison of ventilation inhomogeneity indices between groups. Further longitudinal data in larger cohorts are required to adjust for possible age confounding and to better characterise the variability in chronic lung disease.

Due to the clinical and empirical approach of our study, it was not possible to determine the exact location of regional ventilation inhomogeneity leading to altered DTG-SBW parameters in COPD or to provide a definite physiological interpretation of the DTG-SBW signal. Thus the predictive value of DTG indices for structural airway changes needs to be determined.

Another limiting factor of our study is the use of the potent greenhouse gas SF₆. Due to shorter test duration and the absence of a vital capacity manoeuvre in the tidal DTG-SBW, the overall consumption of SF₆ is considerably lower compared with conventional SBW and MBW tests.

Conclusion

SIIDTG as a potential marker of acinar ventilation heterogeneity seems attractive as its natural variability is considerably lower than that of, for example, S_{cond} and S_{acin} in adults with and without COPD. Change in SIIDTG >68% across 6 months exceeds natural variability (coefficient of repeatability) and thus is clinically relevant, with 95% probability. The DTG-SBW is feasible and reproducible in healthy subjects as well as in patients with COPD, and can be performed with commercially available hardware. The short test time and the absence of controlled breathing manoeuvres are further advantages. However, custom software is required for analysis. The DTG-SBW may play a future role in early and specific disease detection (e.g. bronchiolitis obliterans and phenotyping of early COPD), monitoring of diseases with small airway dysfunction (e.g. asthma) or as outcome parameters in drug trials to improve small airway function.

Acknowledgements

The authors would like to thank Mr Rudolf Isler (Eco Medics AG, Duernten, Switzerland) for his valuable technical assistance and Prof. Philipp Latzin (University Children's Hospital, Basel, Switzerland) and Dr Per Gustafsson (Dept of Paediatrics, Central Hospital, Skövde, Sweden) for their scientific support.

References

- 1 Robinson PD, Goldman MD, Gustafsson PM. Inert gas washout: theoretical background and clinical utility in respiratory disease. *Respiration* 2009; 78: 339–355.
- 2 Robinson P, Latzin P, Verbanck S, *et al.* Consensus statement for inert gas washout measurement using multiple and single breath tests. *Eur Respir J* 2013; 41: 507–522.
- 3 Singer F, Stern G, Thamrin C, *et al.* Tidal volume single breath washout of two tracer gases—a practical and promising lung function test. *PLoS ONE* 2011; 6: e17588.
- 4 Estenne M, Van Muylem A, Knoop C, *et al.* Detection of obliterative bronchiolitis after lung transplantation by indexes of ventilation distribution. *Am J Respir Crit Care Med* 2000; 162: 1047–1051.
- 5 Van Muylem A, De Vuyst P, Yernault JC, *et al.* Inert gas single-breath washout and structural alteration of respiratory bronchioles. *Am Rev Respir Dis* 1992; 146: 1167–1172.
- 6 Van Muylem A, Paiva M, Estenne M. Involvement of peripheral airways during methacholine-induced bronchoconstriction after lung transplantation. *Am J Respir Crit Care Med* 2001; 164: 1200–1203.
- 7 Van Muylem A, Baran D. Overall and peripheral inhomogeneity of ventilation in patients with stable cystic fibrosis. *Pediatr Pulmonol* 2000; 30: 3–9.
- 8 Singer F, Stern G, Thamrin C, *et al.* A new double-tracer gas single-breath washout to assess early cystic fibrosis lung disease. *Eur Respir J* 2013; 41: 339–345.
- 9 Singer F, Abbas C, Yammine S, *et al.* Abnormal small airways function in children with mild asthma. *Chest* 2014; 145: 492–499.
- 10 Abbas C, Singer F, Yammine S, *et al.* Treatment response of airway clearance assessed by single-breath washout in children with cystic fibrosis. *J Cyst Fibros* 2013; 12: 567–574.
- 11 Global Initiative for Chronic Obstructive Lung Disease. Global Strategy for the Diagnosis, Management and Prevention of COPD. www.goldcopd.org/uploads/users/files/GOLD_Report2014_Feb07.pdf Date last updated: February 7, 2014.
- 12 Miller MR, Hankinson J, Brusasco V, *et al.* Standardisation of spirometry. *Eur Respir J* 2005; 26: 319–338.

- 13 Wanger J, Clausen JL, Coates A, *et al.* Standardisation of the measurement of lung volumes. *Eur Respir J* 2005; 26: 511–522.
- 14 Macintyre N, Crapo RO, Viegi G, *et al.* Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J* 2005; 26: 720–735.
- 15 Quanjer PH, Stanojevic S, Cole TJ, *et al.* Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. *Eur Respir J* 2012; 40: 1324–1343.
- 16 Cotes JE, Chinn DJ, Quanjer PH, *et al.* Standardization of the measurement of transfer factor (diffusing capacity). *Eur Respir J* 1993; 6: Suppl. 16, 41–52.
- 17 Verbanck S, Schuermans D, Van Muylem A, *et al.* Ventilation distribution during histamine provocation. *J Appl Physiol* 1997; 83: 1907–1916.
- 18 Aurora P, Kozłowska W, Stocks J. Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. *Respir Physiol Neurobiol* 2005; 148: 125–139.
- 19 Chinn S. Statistics in respiratory medicine. 2. Repeatability and method comparison. *Thorax* 1991; 46: 454–456.
- 20 Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986; 1: 307–310.
- 21 Sixt R, Bake B, Oxhøj H. The single-breath N₂-test and spirometry in healthy non-smoking males. *Eur J Respir Dis* 1984; 65: 296–304.
- 22 Hedenström H, Malmberg P, Agarwal K. Reference values for lung function tests in females. Regression equations with smoking variables. *Bull Eur Physiopathol Respir* 1985; 21: 551–557.
- 23 Hedenström H, Malmberg P, Fridriksson HV. Reference values for lung function tests in men: regression equations with smoking variables. *Ups J Med Sci* 1986; 91: 299–310.
- 24 Houlitz B, Green K, Lindblad A, *et al.* Tidal N₂ washout ventilation inhomogeneity indices in a reference population aged 7–70 years. *Eur Respir J* 2012; 40: Suppl. 56, 694s.
- 25 Verbanck S, Thompson BR, Schuermans D, *et al.* Ventilation heterogeneity in the acinar and conductive zones of the normal ageing lung. *Thorax* 2012; 67: 789–795.
- 26 Verbanck S, Schuermans D, Van Muylem A, *et al.* Conductive and acinar lung-zone contributions to ventilation inhomogeneity in COPD. *Am J Respir Crit Care Med* 1998; 157: 1573–1577.
- 27 Verbanck S, Schuermans D, Vincken W. Small airways ventilation heterogeneity and hyperinflation in COPD: response to tiotropium bromide. *Int J Chron Obstruct Pulmon Dis* 2007; 2: 625–634.
- 28 Cuttillo A, Bigler AH, Perondi R, *et al.* Exercise and distribution of inspired gas in patients with obstructive pulmonary disease. *Bull Eur Physiopathol Respir* 1981; 17: 891–901.
- 29 Stuart-Andrews CR, Kelly VJ, Sands SA, *et al.* Automated detection of the phase III slope during inert gas washout testing. *J Appl Physiol* 2012; 112: 1073–1081.
- 30 Van Muylem A, Paiva M, Baran D, *et al.* Structural change of the acinus during growth assessed by single-breath tracer gas washouts. *Pediatr Pulmonol* 1996; 22: 230–235.
- 31 Fuchs SI, Eder J, Ellemunter H, *et al.* Lung clearance index: normal values, repeatability, and reproducibility in healthy children and adolescents. *Pediatr Pulmonol* 2009; 44: 1180–1185.
- 32 Horsley AR, Macleod KA, Robson AG, *et al.* Effects of cystic fibrosis lung disease on gas mixing indices derived from alveolar slope analysis. *Respir Physiol Neurobiol* 2008; 162: 197–203.
- 33 Gonem S, Corkill S, Singapuris A, *et al.* Between-visit variability of small airway obstruction markers in patients with asthma. *Eur Respir J* 2014 [In press DOI: 10.1183/09031936.00001814].
- 34 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: 684–689.
- 35 Singer F, Kieninger E, Abbas C, *et al.* Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. *Pediatr Pulmonol* 2013; 48: 739–746.