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

ONLINE SUPPLEMENT

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1. List of acronyms

AUC	area under the curve over the total expired volume
AUC 60-90	area under the curve between 60% and 90% of the expired volume
BTPS	body temperature, ambient pressure, saturated with water vapor
CR	coefficient of repeatability
CV	coefficient of variation
dN_2	slope of phase III of single-breath nitrogen washout
DL_{CO}	transfer factor for carbon monoxide
DTG	double-tracer gas
DTG-SBW	double-tracer-gas single-breath washout
FEV_1	forced expiratory volume in 1 second
FRC	functional residual capacity
FRC_{MBW}	functional residual capacity measured by nitrogen multiple-breath washout
FVC	forced vital capacity
FWHM	full width at half maximum
He	helium
IGW	inert gas washout
LCI	lung clearance index
MBW	multiple-breath washout
MM	molar mass
MMEF	maximum midexpiratory flow
MMss	molar mass signal measured in sidestream
MMcalc	calculated molar mass signal
N_2	nitrogen
Peak	absolute peak concentration of the signal
Peak-volume	% of exhaled volume at peak
r^2	coefficient of determination
RV	residual volume
Sacin	index of acinar ventilation inhomogeneity
SBW	single-breath washout
Scond	index of conductive ventilation inhomogeneity
SD	standard deviation
SF_6	sulphur hexafluoride
SIII	slope of phase III
VC	vital capacity
TLC	total lung capacity
USFM	ultrasonic flowmeter
VI	ventilation inhomogeneity
Vt	tidal volume

2. Methods

2.1. Hardware for inert tracer gas washout

For all gas washout tests we used the commercially available and approved device Exhalyzer D® (Eco Medics AG, Duernten, Switzerland). The setup was recently described and validated against mass spectrometry and a new in vitro lung model [1, 2]. Tidal flows and derived volumes were measured in mainstream using an ultrasonic flow meter (USFM). A gas probe was conducted with a continuous flow of 200mL/min via sampling tube (Nafion®) to a second ultrasonic flowmeter measuring molar mass signal in the sidestream (MMss) with a frequency of 200 Hz [1, 3]. An infrared CO_2-sensor was inserted in the mainstream and an O_2 laser sensor in the sidestream. An additional dead space reducer in the flow head was used to minimize the post-capillary dead space to 26.9 mL. The total apparatus dead space was 50 mL. A continuous bypass-flow of 1 L/s (medical air or pure O_2 or double tracer gas) prevents re-inspiration of expired or ambient gas. Tidal flows and derived volumes were converted to body temperature, ambient pressure, and saturated with water vapor (BTPS) conditions. A bacterial filter was integrated in the mouthpiece and subjects used a soft nose clip during all tests.

2.2. Calibration and synchronization procedures

Prior to the measurements, a daily two-point calibration and verification of the flow and O_2 sensors and zero calibration of the CO_2 sensor were performed. The internal synchronization procedure of the Exhalyzer D® was described in detail by [2]. In brief, synchronization of the MM-signal was applied simultaneously with the synchronization of the O_2 and CO_2-signals during tidal breathing of 100% O_2. The O_2 sensor had a slower 10-90% response time (140 ms) than the CO_2 sensor (55 ms). To align their signals, a speeding algorithm was applied to the O_2 signal reducing its response to approximately 110 ms. Gas signals were synchronized to the flow signal using the re-inspired post-capillary dead space to produce a step response in CO_2 and O_2. The gas signal vectors were time shifted to the point in time when the post-capillary dead space had been inhaled such that a 50% change in gas signal deflection then occurred. This was repeated over a minimum of 5 washout breaths and median "delay times" for CO_2 (50 ms on average) and O_2 (565 ms on average) were applied automatically for signal alignment.

The above described recommended synchronization procedure of the Exhalyzer D® is based on signal matching during inspiration to ensure accurate calculation of re-inspired N_2 during N_2-MBW. For SBW tests focusing on expiratory signals alternative synchronization procedures during expiration might be more accurate. However, looking at the analysis of the DTG-SBW (see **OLS 2.5**, linear regression modelling using the pre-test CO_2-signal) we had an almost perfectly superimposed difference signal **(Figure 1)**. The improvement of synchronization procedures should be systematically assessed in further methodological studies.

2.3. Nitrogen vital-capacity single-breath washout

The classical SBW test was performed according to actual recommendations and involved a VC maneuver performed in three steps at low constant flows of approx. 0.5 L/s [4, 5]: 1. Exhalation to RV. 2. Inhalation of 100% oxygen to TLC. 3. Exhalation from TLC to RV. The test was accepted if there was no evidence of leaks and inspiratory and expiratory VC breaths did not vary more than 10%. The expiratory N_2-concentration was determined as function of expired volume. Main outcome parameter was the slope of phase III (dN_2) calculated between 25-75% of the expired volume (cut-offs manually adjustable, if required). As proposed by the current consensus on inert gas washout testing, we also reported dN_2 corrected for expiratory volume (= dN_2 x VC) [4]. The test was repeated 3 times with an interval of 3 minutes between each trial to reset alveolar N_2-concentrations. All analyses were done automatically with software provided by the manufacturer of the Exhalyzer D® (Spiroware® 3.1.6, Eco Medics AG; Duernten, Switzerland).

2.4. Nitrogen multiple-breath washout

The general procedure of the nitrogen multiple-breath washout was described in detail by Verbanck et al [6, 7] and Gustafsson [8]. Unlike the studies by Verbanck et al. subjects were not restricted to a tidal breathing volume of 1l. We performed all tests during relaxed tidal breathing. Once a regular breathing pattern was established (Vt varying less than 10%) and there was no evidence of leaks the subject was switched from room air to 100% oxygen during the washout. Flow/volume and N_2-signals were continuously recorded. The test was continued until the end-tidal N_2-concentration has dropped from 80% to just below 2% (1/40th of the initial N_2-starting concentration). After a time interval of 5 minutes for natural N_2-washin the test was repeated. We assured that all signals returned to baseline before starting the next measurement (e.g. COPD patients may need a longer N_2-washin interval). Quality criteria were as follows: continuous relaxed tidal breathing without evidence of leakage e.g. closed flow-volume-loops, no sudden N_2-rise during inspiration, no extreme flow drift after BTPS correction. We performed three technically acceptable trials. A trial was excluded and repeated, if FRC differs by > 25% from both of the other two washouts.

For data sampling and analysis (including SIII analysis) we used the software provided with the Exhalyzer D® (Spiroware® 3.1.6, Eco Medics AG; Duernten, Switzerland). Main outcome parameters were the functional residual capacity (FRC) and the LCI (ratio of cumulative expired volume divided by FRC). Additionally phase III slope analysis was performed for each expiration as described [6–8]: From the normalized phase III-slope (SnIII) curve throughout the washout procedure the two regional VI-indices Scond and Sacin were calculated: Scond was determined by linear regression of SnIII values between 1.5 and 6 lung turnovers (TO = cumulative expired volume divided by FRC). Sacin was calculated as SnIII of the first breath minus the contribution of Scond.* The post-test quality control included a visual inspection of all recorded breaths included in SIII analysis. The limits for SIII analysis automatically set to 50-95% of the expired volume could be adjusted manually to exclude phase II or phase IV contributions in individual breaths. Inadequate data points due to insufficient breath volume or artefacts (e.g. breath hold, cough, swallowing, cardiogenic oscillations) were excluded for SIII analysis. To account for variations in tidal volume due to our relaxed tidal breathing protocol we corrected slope-III-parameter for tidal volume (Vt) by multiplication with Vt as proposed previously [9, 10]. This correction is in accordance with the actual consensus document [4].

*** Algorithms of Scond and Sacin analysis (Spiroware version 3.1.6):**

Scond: slope of SnIII versus TO (between TO 1.5 und 6)

Scond Vt: slope of SnIII*Vt versus TO (between TO 1.5 und 6)

Sacin: firstBreathSnIII - (firstBreathTO * Scond)

Sacin Vt: (firstBreathSnIII*Vt) - (firstBreathTO * ScondVt)

Vt = (Vt insp.-Vt exp)/2

2.5. Double-tracer-gas single-breath washout

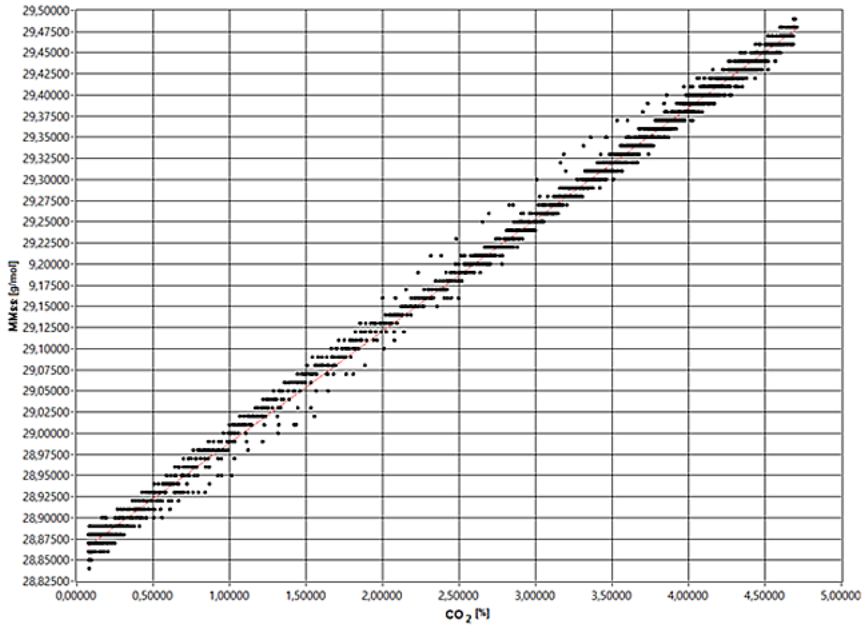
The tidal DTG-SBW measurement was described in detail by Singer [1, 11]. The double-tracer gas mixture (DTG) containing O_2 (21.0 %), N_2 (47.7%), He (26.3%), and SF_6 (5.0%) has similar MM compared to dry medical air (28.9 g/mol) and was provided by Westfalen-AG, Muenster, Germany.

Subjects were breathing normal ambient air provided by an ambient air bypass (1L/s) in an open ventilation circuit. After at least five regular tidal breaths (flow < 1L/s, volumes within 10%) monitored by online flow-volume loops, the DTG was switched to the ventilation circuit. During the test phase probands inhaled a tidal volume of the DTG from functional residual capacity (FRC) and exhaled back to FRC. The molar mass signals (MMss), flow, CO_2 and O_2-signals were recorded in the pretest- phase, during the DTG in-/expiration and the following inspiration. The test was repeated 3 times with an interval of at least 10 breaths of room-air between each trial.

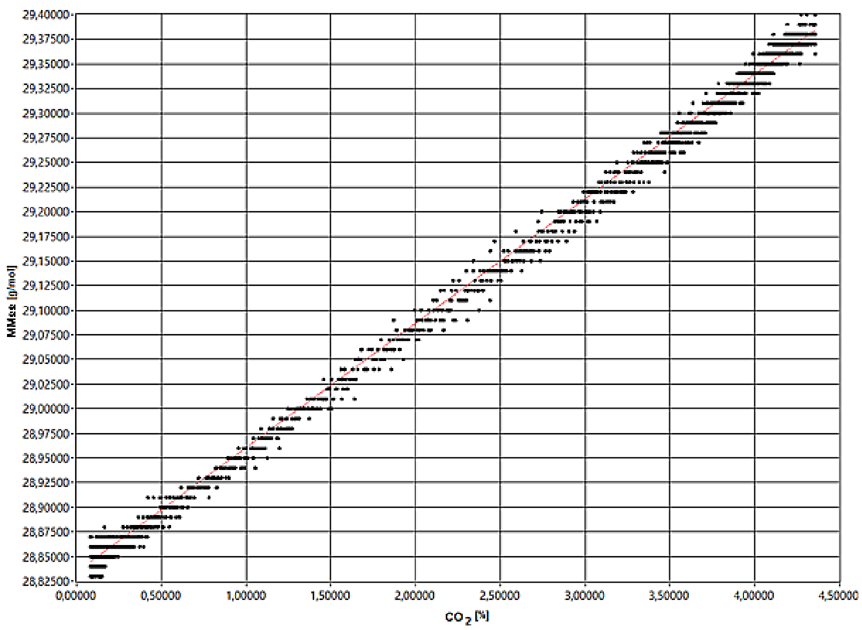
We analyzed the synchronized raw data (flow, O_2, CO_2, MMss) of the pretest phase (at least 5 breaths), the DTG in/expiration and the following inspiration with software customized in-house based on LabVIEW™ 2012, National instruments. The MMss during in- and expiration of ambient air is nearly exactly proportional to the actual CO_2-signal (R2 > 0.99). This was shown previously by Singer for healthy adults [1] and by own analysis in adult nonsmokers and COPD-patients **(Figure 1a/b).** As consequence, the MM expected during ambient air breathing could be calculated by „transformation“, i.e. linear regression modeling, using the pre-test CO_2-signal (= MMcalc).

The MMss during expiration of the DTG differs from the MMss during expiration of ambient air and depends on the cumulative O_2-, N_2-, CO_2-, SF_6-, and He - fraction. To „extract“ the DTG-test-signal we „subtracted“ the MMcalc during expiration of the DTG from the raw MMss. This difference signal (MMss-MMcalc) was plotted against expired volume and reflects SF_6- and He-washout as measured by mass spectrometry [1]. However, the test signal is a composite signal and does not allow to differentiate between the individual fractions of exhaled gases.

Figure 1 a/b. Association of MMss (g/mol) and CO_2 (%) during tidal breathing of ambient air
1a) Healthy nonsmoker. $R^2 > 0.99$



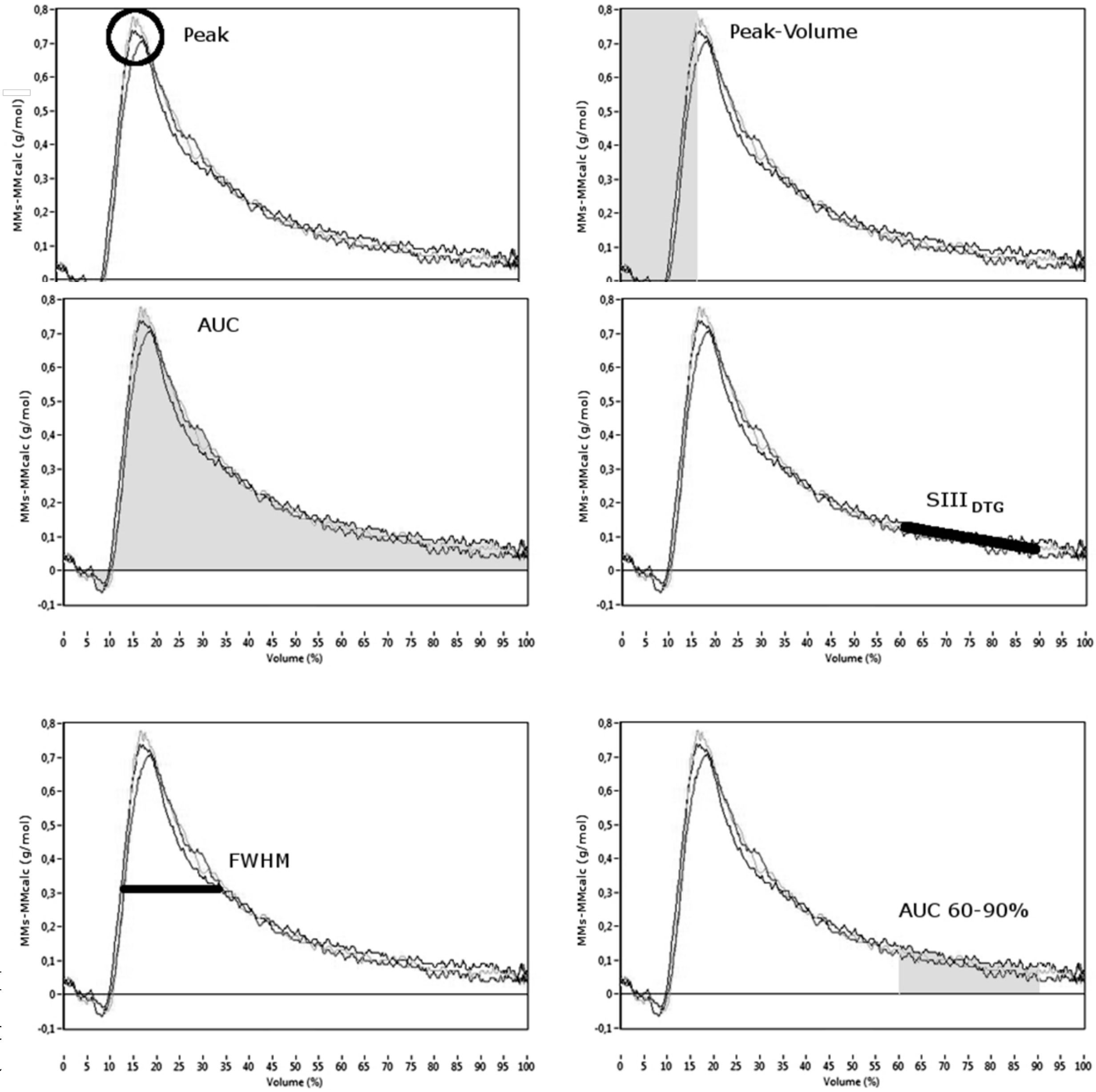
1b) Patient with COPD. $R^2 > 0.99$



MMss (g/mol) plotted against CO_2 (%) in a healthy nonsmoker (1a) and a COPD patient (1b). Linear regression of all data points during pretest breaths of 12 tidal SBW tests.

As main outcome parameter of the MMss-MMcalc expirogram the phase III slope between 60% and 90% of the expired volume ($SIII_{DTG}$) was determined by linear regression. As recommended by the current consensus document on inert gas washout testing, we also reported $SIII_{DTG}$ corrected for tidal volume ($SIII_{DTG}$ x Vt) [4, 11]. As additional phase III parameter we calculated the area under the curve between 60% and 90% of the expired volume (AUC 60-90). Parameters from tidal phase II were the absolute peak concentration of the signal (Peak), the peak-volume (% of exhaled volume at peak) and the area under the curve (AUC). To describe the skewness of the DTG-signal we measured the full width at half maximum (FWHM). **Figure 2** illustrates all proposed DTG outcome parameters.

Figure 2. Illustration of DTG outcome parameters



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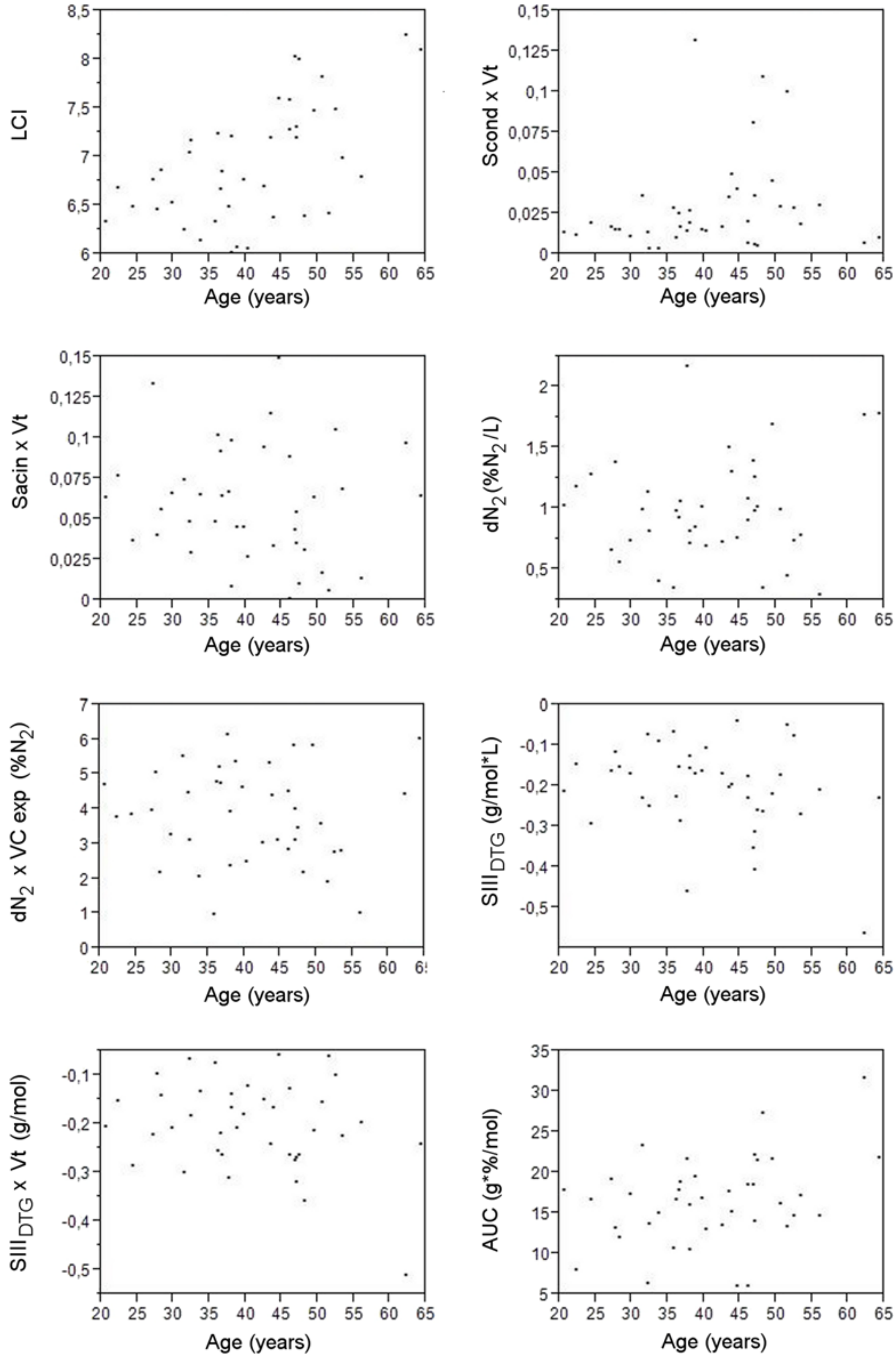
MMss-MMcalc (g/mol) plotted against expired volume (% of total expired volume). Peak: absolute peak concentration of the DTG-SBW signal; Peak-Volume: % of exhaled volume at peak concentration of DTG-SBW signal; AUC: area under the curve (DTG-SBW) over the total expired volume; $SIII_{DTG}$: Phase III slope of double-tracer single-breath washout; FWHM: full width at half maximum of DTG-SBW signal; AUC 60-90: area under the curve between 60% und 90% of expired volume.

3. Additional results

3.1. Age-dependency of gas washout parameters in healthy subjects

Figure 3.



Washout parameters of N_2-MBW, N_2-VC-SBW and DTG-SBW plotted against age in healthy subjects. A significant association was found for LCI (r^2= 0.34, p< 0.0001) and AUC (r^2 = 0.11, p=0.037). Corresponding regression equations: LCI: 5.529 + 0.0345*Age (years); AUC: AUC (g*%/mol) = 9.390 + 0.172*Age (years). All other parameters showed no significant association with age in our group of 40 healthy subjects. LCI: lung clearance index; Scond: index of conductive ventilation inhomogeneity; Sacin: index of acinar ventilation inhomogeneity; dN_2: phase III slope of nitrogen single-breath washout; dN_2 x VC: dN_2 x expiratory vital capacity; $SIII_{DTG}$: Phase III slope of double-tracer single-breath washout; Vt: tidal volume; AUC: area under the curve (DTG-SBW).

3.2. Association between gas washout indices and conventional lung function in COPD patients

Table 2. Association between gas washout parameters and DL_{CO} (FEV_1)

Parameter	DL_{CO} (% pred.)	FEV_1 (% pred.)
LCI	n.s.	n.s.
Scond x Vt	n.s.	n.s.
Sacin x Vt	**0.41 (0.003)**	**0.33 (0.007)**
dN_2 (%N_2/L)	**0.21 (0.04)**	**0.26 (0.02)**
$SIII_{DTG}$ (g/mol*L)	**0.33 (0.008)**	n.s.
$SIII_{DTG}$ x Vt (g/mol)	**0.31 (0.01)**	n.s.
AUC (g*%/mol)	**0.22 (0.037)**	n.s.
AUC 60-90 (g*%/mol)	**0.24 (0.03)**	n.s.
FWHM (%)	**0.41 (0.002)**	n.s.
Peak –Volume (%)	**0.33 (0.008)**	n.s.
Peak (g/mol)	n.s.	n.s.

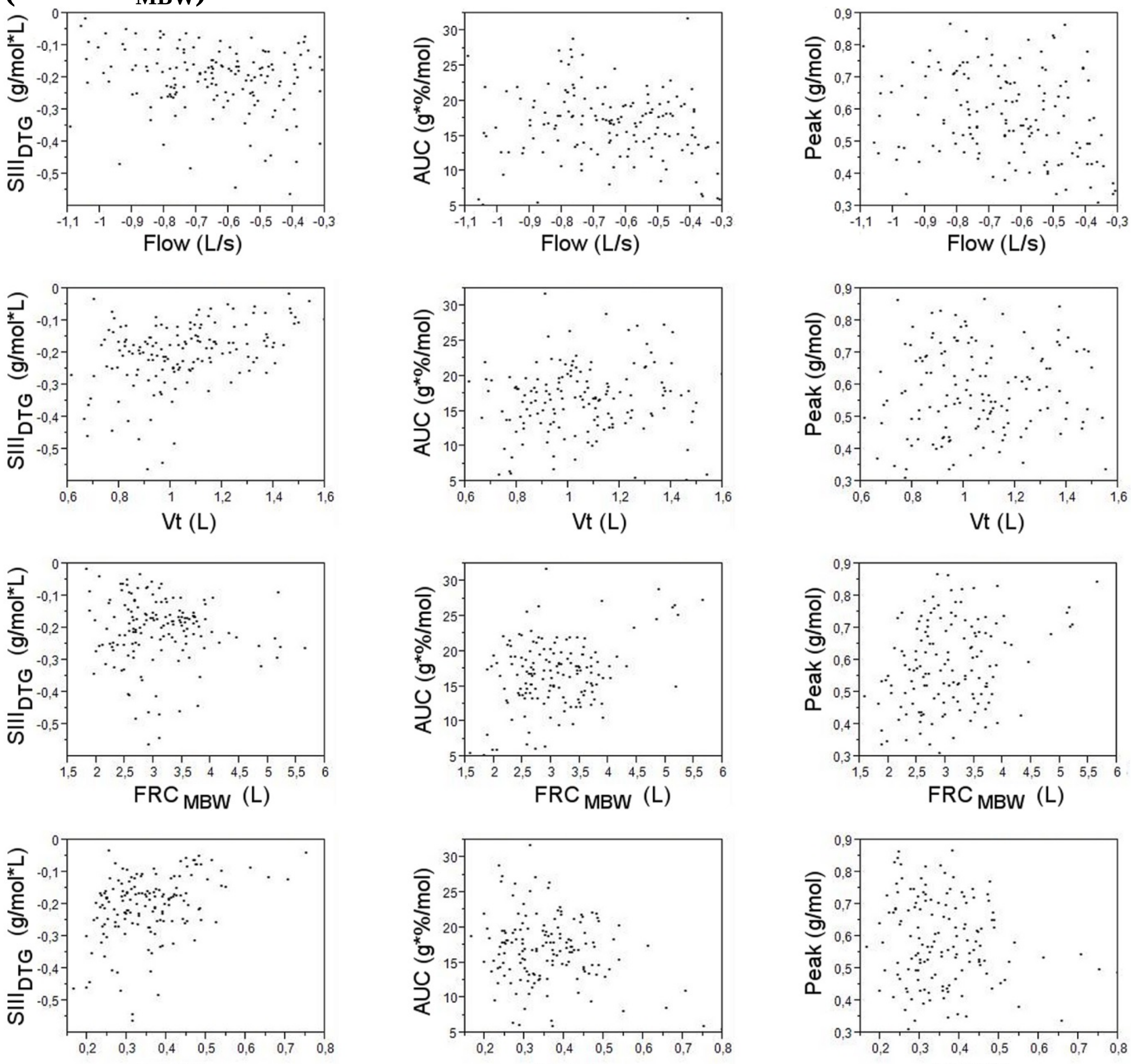
Table 3. Association between DTG_{SBW}-indices and N_2-washout-indices

Parameter	LCI	Scond x Vt	Sacin x Vt	dN_2
SIII $_{DTG}$ (g/mol*L)	n.s.	n.s.	n.s.	n.s.
$SIII_{DTG}$ x Vt (g/mol)	n.s.	n.s.	**0.24 (0.03)**	n.s.
AUC (g*%/mol)	n.s.	n.s.	**0.25 (0.024)**	n.s.
AUC60-90 (g*%/mol)	n.s.	n.s.	**0.21 (0.04)**	n.s.
FWHM (%)	n.s.	n.s.	**0.25 (0.03**	n.s.
Peak-Volume (%)	n.s.	n.s.	n.s.	**0.29 (0.01)**
Peak (g/mol)	n.s.	n.s.	**0.21 (0.04)**	n.s.

All data (Table 2, 3) were calculated from mean baseline values of 20 COPD patients by simple linear regression. Values represent the coefficient of determination r^2 of statistically significant relations ($p < 0.05$) (n.s.: no statistically significant relationship was identified).

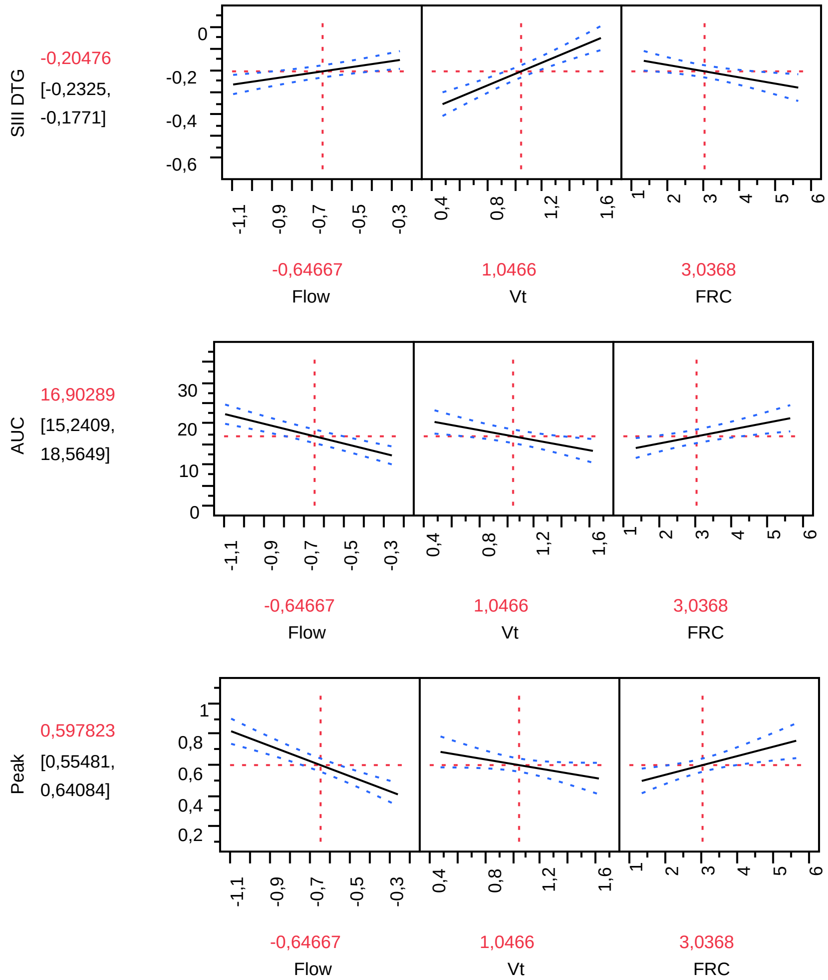
3.3. Association between DTG indices and breathing pattern in healthy subjects

Figure 4.
Association between $SIII_{DTG}$, AUC, Peak and Flow, Vt, FRC_{MBW} (Vt/FRC_{MBW})



$SIII_{DTG}$ (g/mol*L), AUC (g*%/mol) and Peak (g/mol) plotted vs. peak expiratory tidal flow (L/s) and tidal volume Vt (L) of all individual tests (averaged values of three individual trials) in 40 healthy subjects. $SIII_{DTG}$: Phase III slope of double-tracer single-breath washout signal; AUC: area under the curve (DTG-SBW). Peak: absolute peak concentration of the DTG-SBW signal.

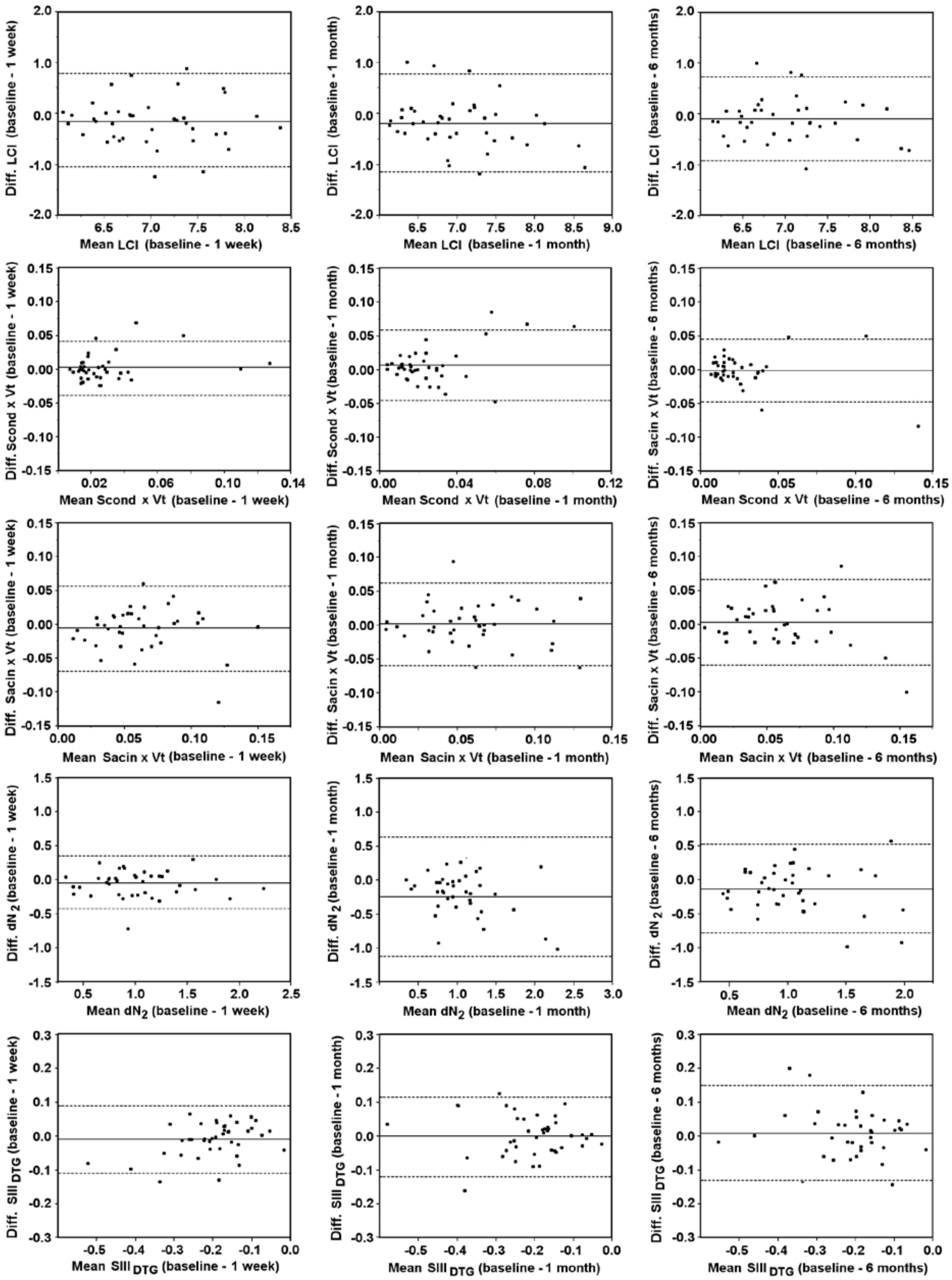
Figure 5. Effects of breathing pattern on DTG-SBW-indices in healthy subjects



Effects of changes in flow, tidal volume or FRC (measured by MBW) on DTG-SBW parameters ($SIII_{DTG}$, AUC, Peak) calculated by fitting a linear mixed effects model with random intercept (detailed results see **table 4** in the manuscript). Red dotted lines show prediction of $SIII_{DTG}$ (AUC, Peak) for a flow of 0.65L/s, tidal volume about 1L and FRC about 3l (mean flow, Vt, FRC of all measurements). In this case, the model predicts a $SIII_{DTG}$ about – 0.205 g/mol*L (95% CI: -0.177 - -0.233 g/mol*L in square brackets). A change of 1L in Vt without changes in Flow or FRC will rise $SIII_{DTG}$ about 0.262 g/mol*L (see Table 4 in the manuscript). In 90% of all measurements Vt was between 700 and 1500ml. Extreme Vt values may have considerable effect on $SIII_{DTG}$ and should be avoided. In the same way, extreme values of flow seem to have considerable effects on AUC and Peak and should be avoided. Blue dotted lines show 95% confidence interval of prediction line. $SIII_{DTG}$: Phase III slope of double-tracer single-breath washout signal; AUC: area under the curve. Peak: absolute peak concentration of the DTG-SBW signal.

3.4. Inter-visit reproducibility illustrated by Bland-Altman plots in healthy subjects.

Figure 6. Bland Altman plots for main washout indices



Difference of gas washout parameters (LCI, Scond x Vt, Sacin X Vt, dN2 (%/L) and $SIII_{DTG}$ (g*mol/L)) between two test occasions plotted versus mean value of two test occasions. Solid line: mean difference between two test occasions. Dotted lines: upper and lower limits of agreement (+/-1.96 x SD of the differences). LCI: lung clearance index; Scond: index of conductive ventilation inhomogeneity; Sacin: index of acinar ventilation inhomogeneity; Vt: tidal volume; dN_2: phase III slope of nitrogen single-breath washout; $SIII_{DTG}$: phase III slope of double-tracer gas single-breath washout signal.

4. References OLS

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