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Correcting for tissue nitrogen excretion in multiple breath washout measurements

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Abstract

Nitrogen excreted from body tissues impacts the calculation of multiple breath nitrogen washout (MBW_{N2}) outcomes. The aim of this study was to determine the effect of tissue N_2 on MBW_{N2} outcomes in both healthy subjects and patients with CF and to assess whether it is possible to correct for tissue N_2. The contribution of tissue N_2 to MBW_{N2} outcomes was estimated by comparing MBW_{N2}-derived functional residual capacity (FRC_{N2}) to FRC measured by body plethysmography (FRC_{pleth}) and by comparing MBW outcome measures derived from MBW_{N2} and sulfur hexafluoride MBW (MBW_{SF6}). Compared to plethysmography and MBW_{SF6}, MBW_{N2} overestimated FRC and lung clearance index (LCI). Application of mathematical tissue N_2 corrections reduced FRC_{N2} values closer to FRC_{pleth} in health and reduced LCI_{N2} in both health and CF, but did not explain all of the differences observed between N_2-dependent and -independent techniques. Use of earlier washout cut-offs could reduce the influence of tissue N_2. Applying tissue N_2 corrections to LCI_{N2} measurements did not significantly affect the interpretation of treatment effects reported in a previously published interventional trial. While tissue N_2 excretion likely has an impact on MBW_{N2} outcomes, better understanding of the nature of this phenomenon is required before routine correction can be implemented into current MBW_{N2} protocols.

Introduction

Multiple breath nitrogen washout (MBW_{N2}) has been shown to be a feasible and sensitive test to measure ventilation inhomogeneity and detect early obstructive lung disease in children and adults [1,2]. Nitrogen (N_2) excreted from body tissues through the lungs can impact the calculation of MBW_{N2} outcomes, including the functional residual capacity (FRC) and lung clearance index (LCI) [3,4]. Several studies have measured the elimination of tissue N_2 in healthy adults from its accumulation during breathing of 100% oxygen for prolonged periods [5–11]. Based on these studies, the tissue N_2 excretion rate and accumulated volume over time was found to fit a multi-phase exponential curve with the early phases representing the desaturation of highly perfused tissues and the later phases representing the slower desaturation of

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poorly-circulated and fat-containing tissues. Elimination rates were found to vary both within and between individuals.

Recently, Nielsen *et al*. applied a tissue N_2 excretion equation to a simulated washout in a two compartment lung model with variable dead space and ventilation heterogeneity [3]. Yammine *et al*. used a different approach to illustrate the effect of tissue N_2 on the washout by subtracting 1% end-tidal concentration of N_2 evenly over the course of the washout for one healthy subject and one subject with cystic fibrosis (CF) [4]. These two studies confirmed that there is a greater effect of tissue N_2 on MBW_{N2} outcomes in disease versus health, but they did not explore whether the contribution of tissue N_2 can be adequately offset in measurements from subjects with a range of body size and lung disease severity. In patients with CF, increased ventilation inhomogeneity leads to greater washout duration, and in theory, longer washouts have a greater total contribution of tissue N_2. Therefore, the impact of tissue N_2 excretion likely introduces greater bias in a subject with significant lung disease compared to a healthy subject of similar size and leads to the overestimation of their FRC and other MBW_{N2} outcomes [2–4,12].

There are limited data to support correcting for the contribution of tissue N_2; thus it is not currently recommended as per American Thoracic Society/European Respiratory Society (ATS/ERS) consensus statement [12]. As MBW_{N2} develops into an increasingly important clinical research tool for the monitoring of CF lung disease and the assessment of treatment effects, the role of tissue N_2 must be clarified in order to determine whether it is necessary to correct for its contribution to the MBW_{N2} test. The aim of this study was to estimate the magnitude of tissue N_2 in both healthy pediatric and adult subjects and patients with CF across a range of disease severity and to assess the effect of applying correction factors for tissue N_2 on the MBW_{N2} test and on treatment effects in interventional trials.

Materials and methods

Study participants

Data were collected as part of four previously published studies [2,13–15]. Healthy participants without a history of respiratory disease or current acute respiratory tract symptoms were recruited from staff and families at the Hospital for Sick Children. Participants with a confirmed diagnosis of CF (defined by a positive newborn screening test or at least one clinical feature of CF in combination with either a documented sweat chloride >60 mEq/L by quantitative pilocarpine iontophoresis test or a genotype with two CF-causing mutations) were recruited from families attending a routine visit to the CF outpatient clinic at the Hospital for Sick Children or St. Michael's Hospital in Toronto, Canada. Informed written consent was obtained from the participant or parent/guardian for all subjects. The original studies were approved by the Research Ethics Board at the Hospital for Sick Children (REB #1000019945, #1000024909, and #1000023162) and St. Michael's Hospital (REB #12–139), Toronto, Canada.

Pulmonary function testing

MBW_{N2} measurements were performed using an open circuit, bias flow system (Exhalyzer D®, EcoMedics AG, Duernten, Switzerland) and associated software (Spiroware® 3.1 EcoMedics AG). A subgroup of subjects also performed MBW tests using a respiratory mass spectrometer system (AMIS 2000, Innovision A/S, Odense, Denmark), which used sulfur hexafluoride (SF_6) as the tracer gas. MBW_{SF6} traces were analyzed by a single trained observer using custom-written analysis software (TestPoint, Capital Equipment Corp., Billerica, MA, USA). All MBW trials were reviewed for quality control according to guidelines proposed in the ATS/ERS consensus statement [12]. In addition to MBW testing, subjects performed

plethysmographic lung volume measurements using the Vmax system (VIASYS CareFusion, San Diego, California, USA) according to ATS standards [16].

Estimates of tissue N_2 contribution

FRC measured by body plethysmograph (FRC_{pleth}) includes the volume of all compressible intrathoracic gas, whereas only the volume of communicating lung units is measured during MBW. Therefore, in healthy individuals, FRC measured by a gas-dilution technique (such as MBW_{N2}) should be equal to or less than that measured by plethysmography [17] in the absence of endogenous production of the tracer gas. Thus the differences between FRC_{pleth} and FRC_{N2} can be used to approximate the contribution of tissue N_2 to the MBW_{N2}. Similarly, as SF_6 is an exogenous, biologically inert gas that does not dissolve significantly in blood or other tissues, it was used as an indirect reference method to assess the magnitude of the contribution of tissue N_2 to FRC derived by gas dilution.

Tissue N_2 excretion equations

MBW_{N2} assesses ventilation inhomogeneity by examining N_2 clearance over a series of breaths for the duration of the washout. To generate MBW_{N2} outcomes, the total volume of exhaled gas (net cumulative expired volume; CEV) and the total volume of inert gas expired per breath (cumulative expired volume of N_2; CEV_{N2}) must be measured. FRC and LCI are calculated when Cet_{N2} falls below a predefined threshold (typically 2.5% of the initial CetN2).

$$FRC = \frac{CEV_{N2}}{(Cet_{N2,initial} - Cet_{N2,final})} - DS_{pre} \quad \text{Eq 1}$$

$$LCI = \frac{CEV}{FRC} \quad \text{Eq 2}$$

where Cet_{N2} is the end tidal concentration of nitrogen. $Cet_{N2,\ initial}$ is the end tidal concentration of N_2 in the first breath of the washout phase, and $Cet_{N2,\ final}$ is the end tidal concentration of N_2 in the first breath of the washout phase where Cet_{N2} is less than the target threshold. DS_{pre} is the equipment deadspace proximal to the sampling point of the apparatus.

In order to correct these values for tissue N_2 excretion, breath-by-breath end tidal body tissue N_2 concentration ($Cet_{N2\ BT}$) as well as the volume of body tissue nitrogen excreted over the washout ($V_{N2\ BT}$) are subtracted from Eqs 1 and 2 (Eqs 3–5). The volume of tissue nitrogen was generated for the entire breath (from the start of inhalation to the end of exhalation).

$$\mathrm{Cet}_{\mathrm{N2,BT}} = \frac{V_{N2,BTi}}{VExp_i} \quad \text{Eq 3}$$

$$FRC_{corr} = \frac{(CEV_{N2} - V_{N2,BT})}{Cet_{N2,initial} - (Cet_{N2} - Cet_{N2,BT})_{final}} - DS_{pre} \quad \text{Eq 4}$$

$$LCI_{corr} = \frac{(\mathrm{CEV} - V_{N2,BT})}{FRC_{corr}} \quad \text{Eq 5}$$

where $V_{N2,BTi}$ is the volume of body tissue nitrogen expired in breath i and $VExp_i$ is the net volume of expired gas in breath i. Cet_{N2} is the end tidal concentration of nitrogen. $Cet_{N2\ BT}$ is the end tidal concentration of nitrogen derived from the body tissues. Initial subscript

Table 1. Summary of tissue nitrogen correction equations.

Study (citation)	n	Age range	Equation used
Lundin, 1953 [9]	7	16–42	Rate of excretion (in mL/min) at time *t*: $\frac{dV_{N2}}{dt} = 37.3e^{-0.45t} + 13.9e^{-0.056t} + 4.82e^{=0.0054t}$ Integrating to derive V_{N2} at time *t*: $V_{N2} = \int 37.3e^{-0.45t} + 13.9e^{-0.056t} + 4.82e^{-0.0054t}dt$ $V_{N2} = \left(\frac{37.3}{0.45}\right)(1 - e^{-0.45t}) + \left(\frac{13.9}{0.056}\right)(1 - e^{-0.056t}) + \left(\frac{4.82}{0.0054}\right)(1 - e^{-0.0054t})$ Where t = time in minutes
Cournand, 1941 [6]	30	9–44	$V_{N2} = \frac{t}{420} \times [(96.5 \times BSA) + 35]$ Where t = time in seconds
ATS/ERS [16]	NA	NA	V_{N2} = (96.5 × *BSA*) + 35

$BSA = Body\ Surface\ [m^2] = \frac{Weight[kg]^{0.425} \times Height[cm]^{0.725} \times 71.84}{10000}$

VN_2 = excreted volume of nitrogen (mL)

NA = Not applicable

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indicates the first breath of the washout phase, and final subscript indicates the first breath of the washout phase where (Cet_{N2}- $Cet_{N2\ BT}$) is less than the target threshold.

Three different equations (Table 1, Fig 1) were used to derive ($V_{N2\ BT}$). Cournand's body size-dependent Eq (6) and Lundin's three-phase exponential excretion rate Eq (14) are time-dependent and calculate the end tidal tissue N_2 concentration ($Cet_{N2\ BT}$). The ATS/ERS (22) equation is time-independent and is therefore only used to generate $V_{N2\ BT}$ and not $Cet_{N2\ BT}$. Therefore, corrected FRC values were generated from all three equations (with $Cet_{N2,\ final}$ being uncorrected in the ATS/ERS equation), but corrected LCI values were only generated from the time-dependent equations.

To assess whether the breath-by-breath calculated FRC achieves a plateau, linear regression slopes of the FRC_{N2}/time curves were calculated for the second half all uncorrected and corrected washouts.

Comparisons of the corrected and uncorrected FRC and LCI results were made with FRC_{pleth} and the difference in FRC and LCI measured by MBW_{N2} and $MBWSF_6$, when available. FRC and LCI values were also re-calculated from the Cournand and Lundin-corrected measurements at the standard MBW end-point of 2.5% normalized end-tidal N_2 concentration, as well as for earlier end-points of 5%, 9%, 12%, and 18% normalized end-tidal N_2 concentration. These end-points were chosen to reflect previous studies that evaluated earlier cut-offs and existing software algorithms [18].

Accuracy of derived nitrogen concentration

Since N_2 concentration values generated by the Exhalyzer D are derived from O_2 and CO_2 concentrations and not directly measured, our results may be biased if these derived values are inaccurate. To ensure the accuracy of the derived N_2 values over the range observed during a MBW_{N2} test, we compared the C_{ET} N_2 calculated by the Spiroware software to a set of reference gases generated by blending medical air (compressed on site with presumed gas concentrations: F_1CO_2 = 0.0004, F_1O_2 = 0.2095, F_1N_2 = 0.7808, F_{1Ar} = 0.0093) with a high precision gas mixture (F_2CO_2 = 0.0500, F_2O_2 = 0.9500; Praxair Canada, Mississauga ON). FN_2 of the mixed reference gas (F_MN_2) was calculated using Dalton's Law of partial pressures, the fractional concentrations of the reference gases and the measured FO_2 of the mixed gas (F_MO_2).

F_MO_2 was measured using the Oxigraf laser oxygen analyzer (Oxigraf Inc, Sunnyvale CA, USA) within the Exhalyzer D®. The accuracy of the Oxigraf analyzer was confirmed against a

paramagnetic oxygen analyzer (Servomex 570A, Servomex, Sugar Land TX, USA). The reported FN_2 from the Exhalyzer D® was compared to F_MN_2 over the range of FN_2 observed in a washout (0.01–0.8).

Statistical analysis

Study population characteristics and lung function measurements were summarized as mean and standard deviation (SD). Group differences were calculated using two-sample t-tests, whereas differences in outcomes within the same subject were compared using paired t-tests. The agreement between outcomes within the same subject was assessed using Bland-Altman plots. Pearson correlations were used to determine the correlation between two outcomes. All statistical analysis was conducted using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Accuracy of derived nitrogen concentration

The absolute difference between FN_2 reported by the Exhalyzer D and the reference concentrations (F_MN_2) was measured over the full range of washout nitrogen concentrations. The mean absolute difference was 0.064% (95% CI -0.032 to 0.16). All measured differences (n = 14) were less than 0.12%. Therefore, we considered the $CetN_2$ derived by the Exhalyzer D to accurately reflect the true $CetN_2$.

Estimates of tissue N_2 contribution to FRC

Characteristics of study participants included are shown in Table 2. Healthy subjects and individuals with CF did not differ in age or lung volumes measured by either MBW_{N2} or body plethysmography. As expected, LCI measured by MBW_{N2} was significantly higher in patients with CF.

FRC measured by the MBW_{N2} gas dilution technique (FRC_{N2}) should be smaller than or equal to, but not exceed, FRC measured by body plethysmography (FRC_{pleth}). However, healthy subjects who performed both techniques had FRC_{N2} values that were on average greater than FRC_{pleth} (mean difference 0.21L; 95% CI 0.12 to 0.29, $p<0.001$). In contrast, the relationship between FRC_{N2} and FRC_{pleth} was inconsistent in subjects with CF (mean difference 0.06; 95% CI -0.10 to 0.21, $p = 0.44$). FRC_{N2} values were recalculated by applying the three tissue N_2 excretion equations. Application of all three tissue N_2 excretion equations decreased FRC_{N2} values compared to FRC_{pleth} in health and CF (Fig 2).

Given that the Cournand and Lundin excretion equations improve the FRC_{N2} agreement with plethysmography, the uncorrected FRC_{N2} (FRC_{uncorr}) and the FRC_{N2} corrected ($FRC_{Cournand}$ and FRC_{Lundin}) were then compared within subjects (Table 3).

The within-subject difference in FRC as measured by MBW_{N2} and MBW_{SF6} (FRC_{N2} – FRC_{SF6}) were also compared with the estimated contribution of tissue N_2 to FRC_{N2}. The difference between FRC_{N2} and FRC_{SF6} was positively correlated with increased washout time ($r = 0.69$, $p<0.001$). FRC_{N2} became disproportionately larger than FRC_{SF6} as the contribution of tissue N_2 as estimated by FRC_{uncorr}–$FRC_{Cournand}$ increased ($r = 0.68$, $p<0.001$) (Fig 3).

When plotted against washout time, the breath-by-breath calculation of FRC_{N2} did not plateau as would be expected in a closed system, but rather continued to increase throughout the washout (representative examples from health and CF shown in Fig 4). This is consistent with continuous tissue N_2 excretion. Breath-by-breath correction of the FRC_{N2} values using the Cournand and Lundin equations decreased the rate of rise of the FRC_{N2} by 23–34%, but did

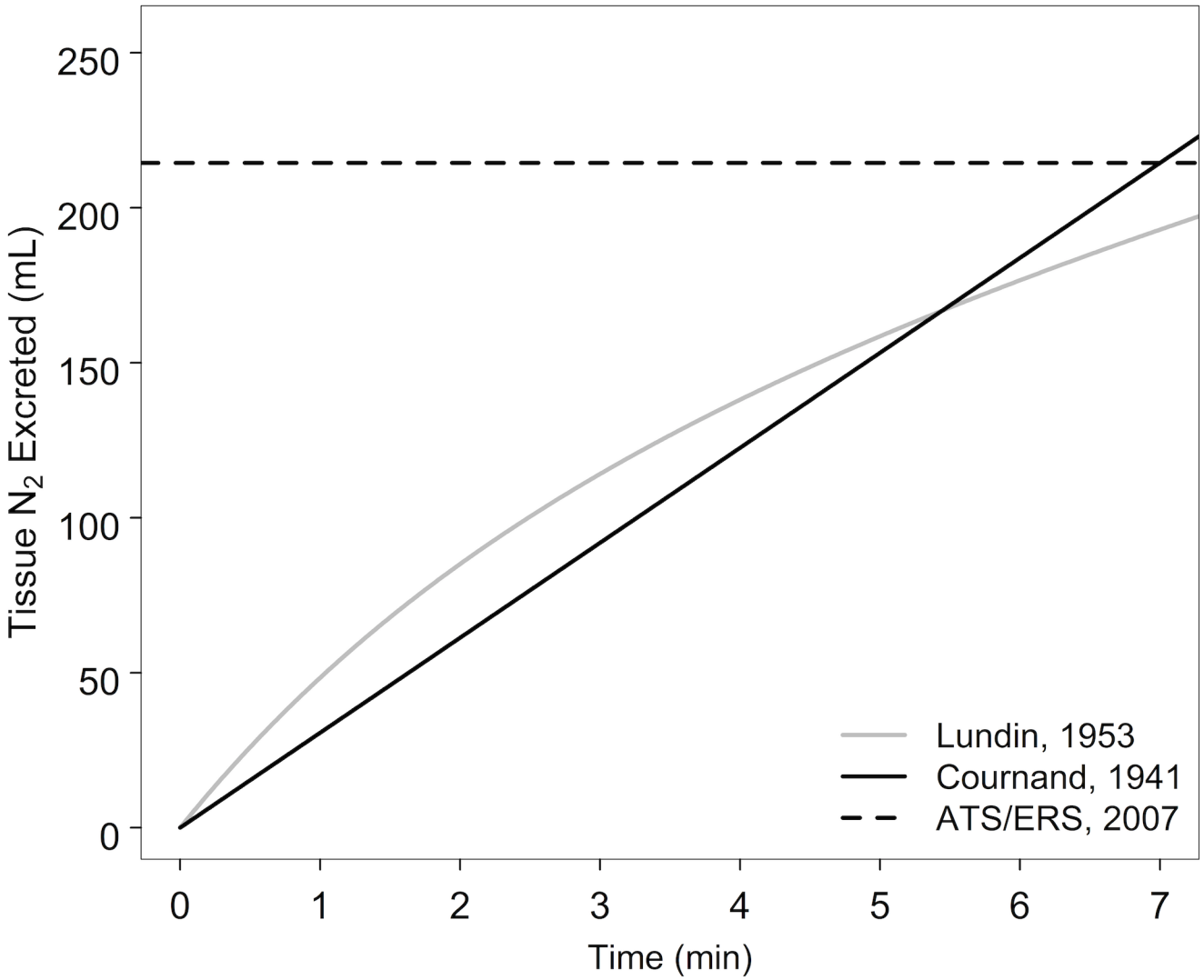

Fig 1. Tissue N_2 excretion equations used for correction of MBW_{N2} measurements. The three equations used to estimate the volume of N_2 excreted from the body tissues are plotted over a 7 minute time period. The Cournand 1941 equation was adjusted for a constant excretion rate and plotted for a subject with the average body size of the subjects measured in the Lundin 1953 study. The ATS/ERS equation calculates the volume of tissue N_2 excreted using Cournand's 1941 equation standardized to a 7 minute washout for all subjects.

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Table 2. Characteristics of study participants. Values are presented as mean (SD) unless otherwise indicated. P value indicates group difference between health and CF.

	Health (n = 43)	CF (n = 35)	Mean difference (95% CI)	P-value
Age (years)	16.5 (5.5)	16.2 (8.3)	0.3 (-2.9 to 3.6)	0.83
Females (%)	60.5	60.0	0.5 (-21 to 22)	0.96
Height (cm)	163.0 (15.8)	157.5 (16.8)	5.5 (-1.9 to 12.9)	0.14
Weight (kg)	57.8 (20.6)	51.7 (17.0)	6.1 (-2.4 to 14.6)	0.16
FRC_{pleth} (L)	2.29 (0.88)	2.49 (1.06)	-0.2 (-0.72 to 0.33)	0.47
FRC_{N2} (L)	2.59 (0.92)	2.46 (0.96)	0.13 (-0.31 to 0.55)	0.58
LCI	6.88 (0.49)	12.04 (3.60)	-5.16 (-6.40 to -3.91)	<0.001

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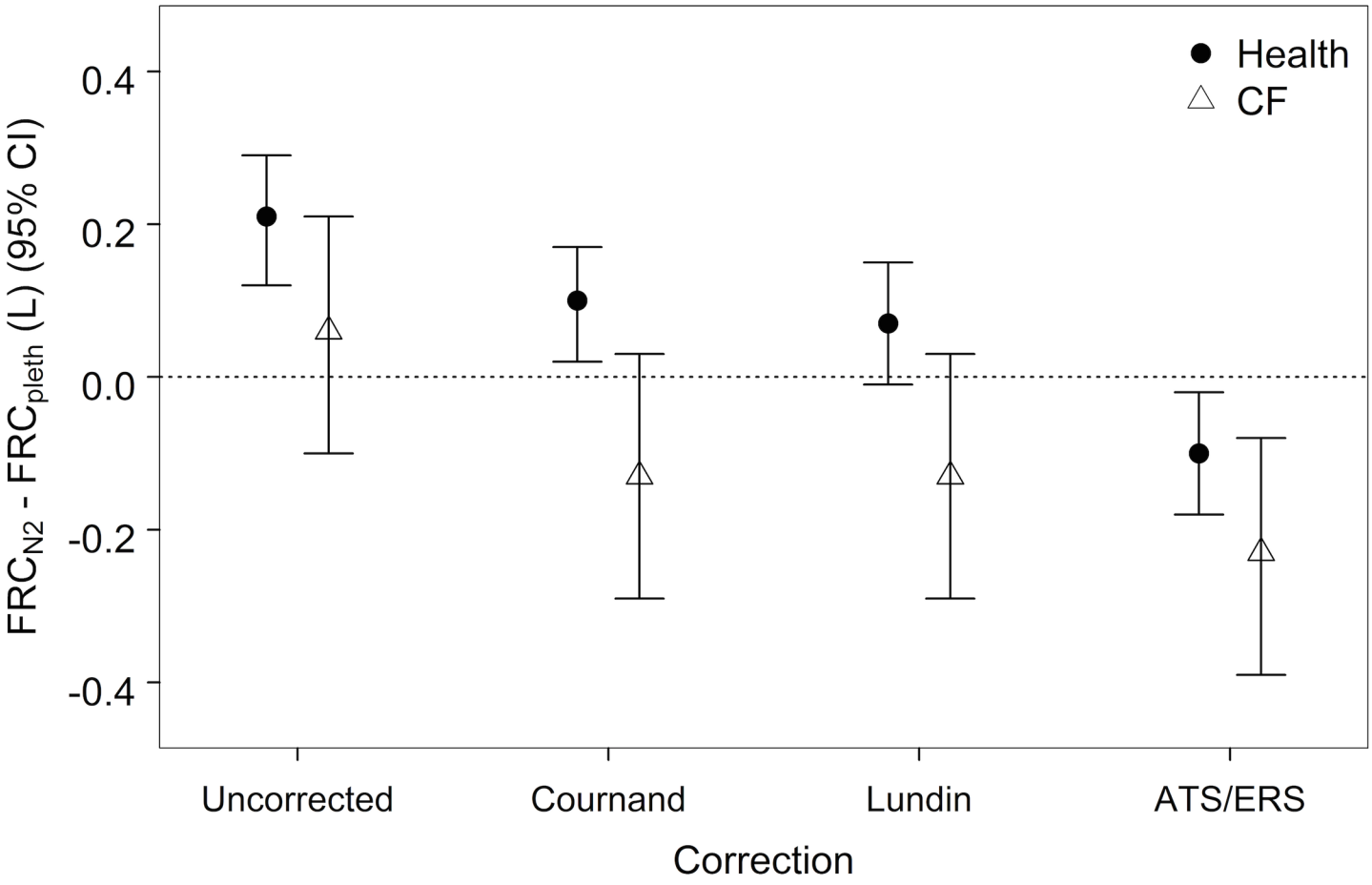

Fig 2. Within-subject difference in FRC measured by multiple breath nitrogen washout (FRC_{N2}) and body plethysmography (FRC_{pleth}). The mean within-subject difference FRC_{N2}—FRC_{pleth} decreased after the three tissue N_2 excretion equations were applied to washout data from healthy and CF populations.

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not reduce it to zero (Fig 4, Table 4). The absolute and relative magnitudes of the decrease in the FRC/time slope were greater in healthy subjects than in those with CF for both the Lundin and Cournand equations (Table 4).

Table 3. Estimates of tissue N_2 contribution to MBW outcomes at the 2.5% washout cut-off. Values are presented as the mean within-subject difference (95% CI) of the uncorrected–corrected MBW_{N2} outcome. Outcomes were corrected by applying either the Cournand or Lundin tissue N_2 excretion equations.

Outcome	Health *Mean difference (95% CI)*	CF *Mean difference (95% CI)*
FRC_{N2} (L)		
Cournand	0.11 (0.10; 0.13), p<0.001	0.18 (0.15; 0.21), p<0.001
Lundin	0.13 (0.12; 0.15), p<0.001	0.19 (0.17; 0.20), p<0.001
CEV_{N2} (L)		
Cournand	1.63 (1.36; 1.90), p<0.001	4.41 (3.30; 5.52), p<0.001
Lundin	1.57 (1.37; 1.76), p<0.001	3.22 (2.40; 4.03), p<0.001
LCI_{N2}		
Cournand	0.35 (0.29; 0.42), p<0.001	0.90 (0.63; 1.17), p<0.001
Lundin	0.30 (0.23; 0.36), p<0.001	0.41 (0.17; 0.65), p = 0.001

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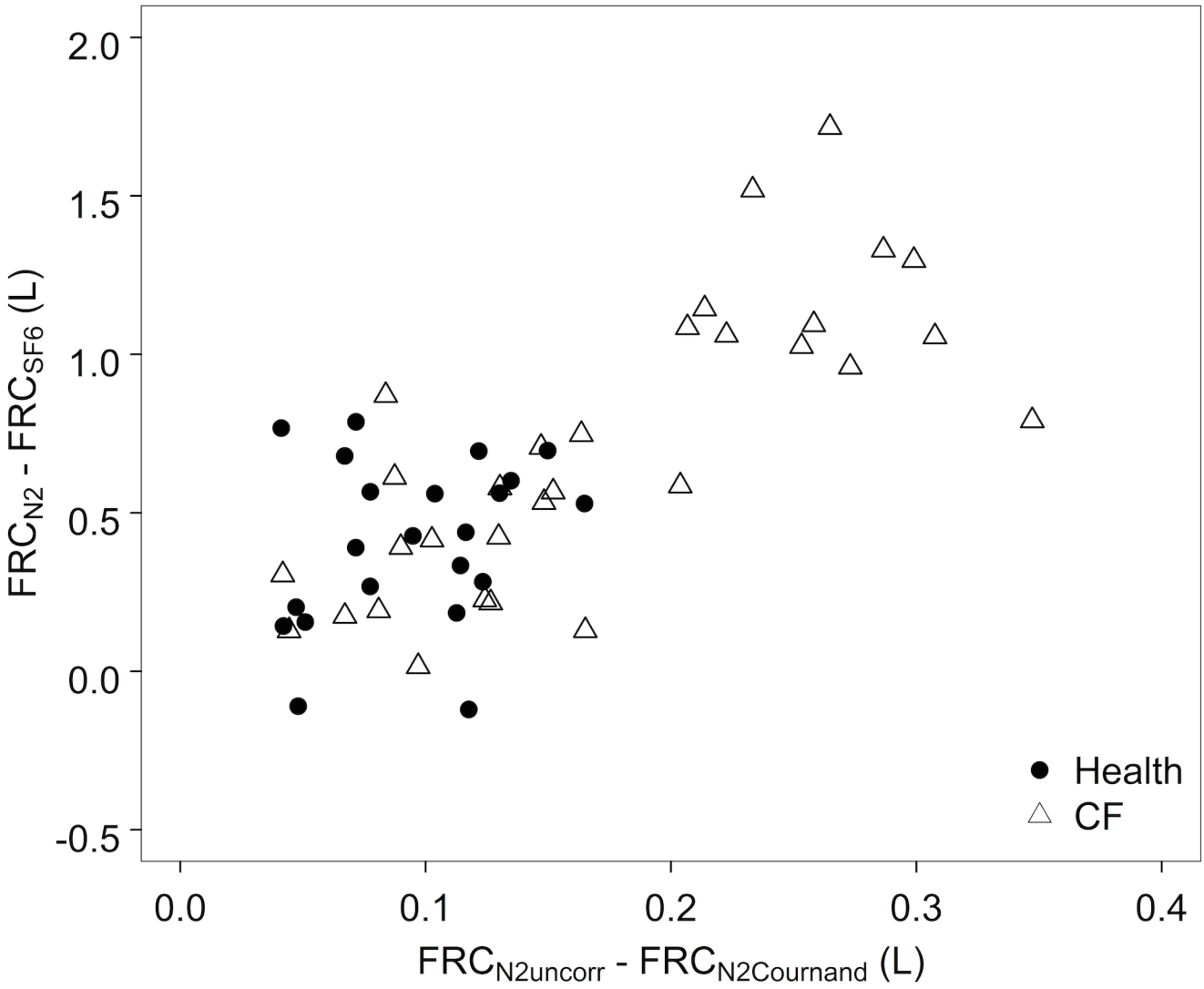

Fig 3. Relationship between the contribution of tissue N_2 to FRC_{N2} and the difference between FRC as measured by MBW_{N2} and MBW_{SF6}. FRC_{N2} became disproportionately greater than FRC_{SF6} as the contribution of tissue N_2 estimated by the within-subject difference FRC_{N2}—$FRC_{Cournand}$ increased.

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Estimates of tissue N_2 contribution to CEV and LCI

Similar to FRC_{N2}, application of tissue N_2 excretion equations to MBW_{N2} data resulted in lower CEV_{N2} and LCI_{N2} values (Table 2). Application of the Cournand excretion equation shortened the washout by an average of 2.9 breaths in health (95% CI 2.5 to 3.3, $p<0.001$) and 7.6 breaths in CF (95% CI 6.3 to 8.8, $p<0.001$). Similar results were observed for the Lundin equation (2.9 and 5.9 breaths in health and CF, respectively). Since the ATS/ERS correction is not time-dependent and only corrects FRC_{N2} for the contribution of tissue N_2, it was not used to correct LCI and CEV values.

When LCI as measured by MBW_{N2} and MBW_{SF6} were compared within subjects (LCI_{N2} – LCI_{SF6}), LCI_{N2} became disproportionately greater than LCI_{SF6} as disease severity (LCI_{N2}) increased ($r = 0.53$, $p<0.001$). Similar to FRC_{N2}, there was a significant and positive

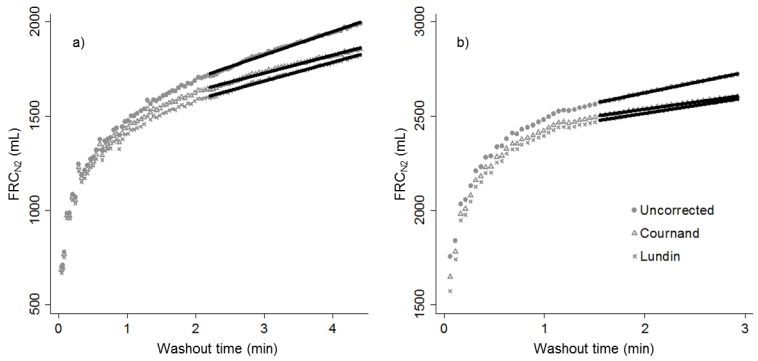

Fig 4. Representative examples of FRC_{N2} plotted against washout time in a) a healthy subject and b) a patient with CF. Application of the Cournand and Lundin tissue N_2 excretion equations resulted in a less pronounced increase in the calculation of FRC_{N2} over the course of the washout, but a plateau was never achieved. Linear regressions of these curves over the second half of the washout are shown, demonstrating the slopes that are reported in Table 4.

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correlation observed between LCI_{N2} –LCI_{SF6} and the effect of tissue N_2 as estimated by LCI_{uncorr}–$LCI_{Cournand}$ (r = 0.55, p<0.001).

Impact of tissue N_2 at earlier washout cut-offs

With application of the Cournand tissue N_2 excretion equation, the effect of tissue N_2 (LCI_{uncorr}–$LCI_{Cournand}$) decreased when LCI_{N2} was calculated at earlier cut-offs of the washout (Fig 5). Compared to the traditional cut-off of 2.5% normalized end-tidal concentration of N_2, the difference between corrected and uncorrected LCI (LCI_{uncorr}–$LCI_{Cournand}$) was less pronounced at the 5% cut-off and was no longer significant by the 9% cut-off. While the effect of tissue N_2 (LCI_{uncorr}–$LCI_{Cournand}$) on LCI_{N2} calculated at the 2.5% cut-off increased as disease severity (LCI_{N2}) increased (r = 0.61, p<0.001) (Fig 6A), this relationship was not observed at the 5% cut-off (r = 0.17, p = 0.13) (Fig 6B).

Impact of tissue N_2 correction on interventional trial outcomes

Both the Cournand and Lundin equations were applied to MBW data of an observational study investigating the effect of ivacaftor on LCI in children with class 3 mutations in CF [14] (Table 5). The Lundin-corrected treatment effect was significantly smaller than the uncorrected value (p = 0.01) and the Cournand-corrected difference showed a similar trend

Table 4. Average slopes of the second half of all uncorrected, Lundin-corrected and Cournand-corrected FRC_{N2}/breath number curves (depicted graphically in Fig 4) for healthy subjects and those with CF. Average paired difference (uncorrected-corrected) in absolute and relative (percent of uncorrected slope) terms are shown. Data are expressed as mean 95% confidence interval) unless otherwise stated.

	Health	CF
Uncorrected slope (mL/min)	104.2 (97.9, 110.6)	125.3 (115.4, 135.2)
Corrected slope (Lundin)	77.4 (71.4, 83.5)	100.4 (90.1, 110.7)
Absolute diff (Lundin)	26.8 (25.2, 28.4)	24.9 (23.4, 26.4)
Relative diff (Lundin) (%)	27.4 (25.0, 29.9)	22.5 (19.6, 25.3)
Corrected slope (Cournand)	70.5 (65.0, 76.0)	94.2 (84.9, 103.5)
Absolute diff (Cournand)	33.7 (32.3, 35.1)	31.1 (29.6, 32.6)
Relative diff (Cournand) (%)	33.9 (31.9, 35.8)	26.7 (24.6, 28.8)

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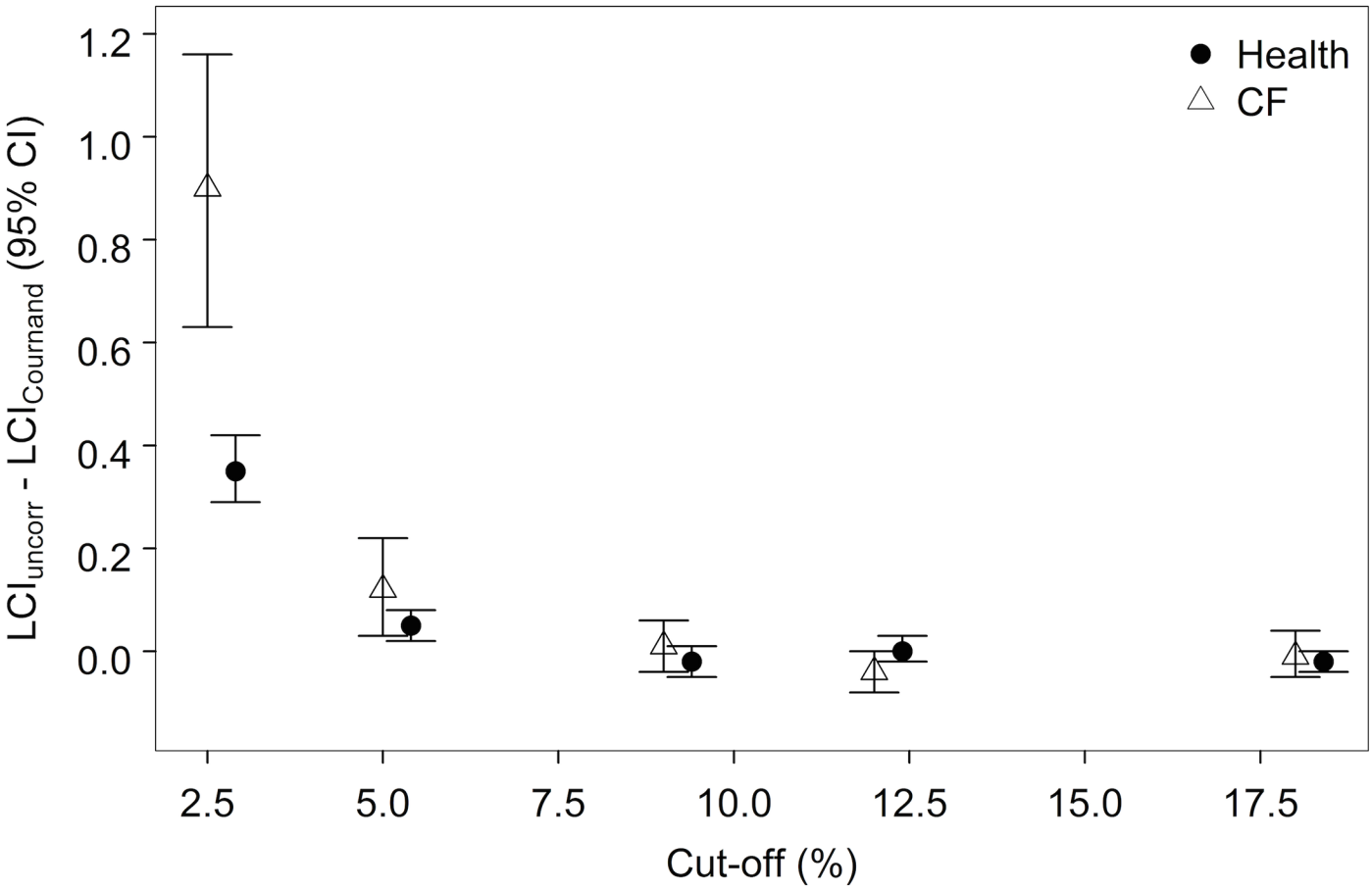

Fig 5. Within-subject difference between uncorrected LCI_{N2} and LCI_{N2} corrected for tissue N_2 excretion ($LCI_{Cournand}$) at different washout cut-offs. The difference was progressively smaller when calculated at earlier cut-offs of the washout in both health and CF.

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(p = 0.11). This change in treatment effect was driven by a greater negative correction in pre-treatment LCI than post-treatment LCI by both Lundin (pre-treatment correction -0.9 [-1.3, -0.5] units; post-treatment correction -0.6 [-0.8, -0.3] units) and Cournand (pre-treatment correction -1.3 [-1.8, -0.8] units; post-treatment correction -0.9 [-1.3, -0.4] units) equations. Neither correction equations changed the direction or significance of the treatment effect.

Discussion

In agreement with previous studies, these data suggest that excretion of N_2 from body tissues affects MBW_{N2} outcomes. The effects of tissue N_2 are greater in patients with longer washouts. This contribution of tissue N_2 to FRC_{N2} and LCI_{N2} is less pronounced at earlier cut-offs of the washout. Application of correction equations for tissue N_2 significantly reduced, but did not completely eliminate, the effect of tissue N_2 on MBW_{N2} outcomes. Importantly, application of these tissue N_2 correction equations did not significantly alter treatment effects previously observed in interventional trials. Thus, while the excretion of tissue N_2 has a measurable effect on MBW_{N2} outcomes, correction for tissue N_2 using currently available approaches cannot be recommended at the present time.

FRC is an integral component of the calculation of LCI by MBW and therefore a reliable FRC is required to derive a reliable LCI. While there is no gold standard for the determination

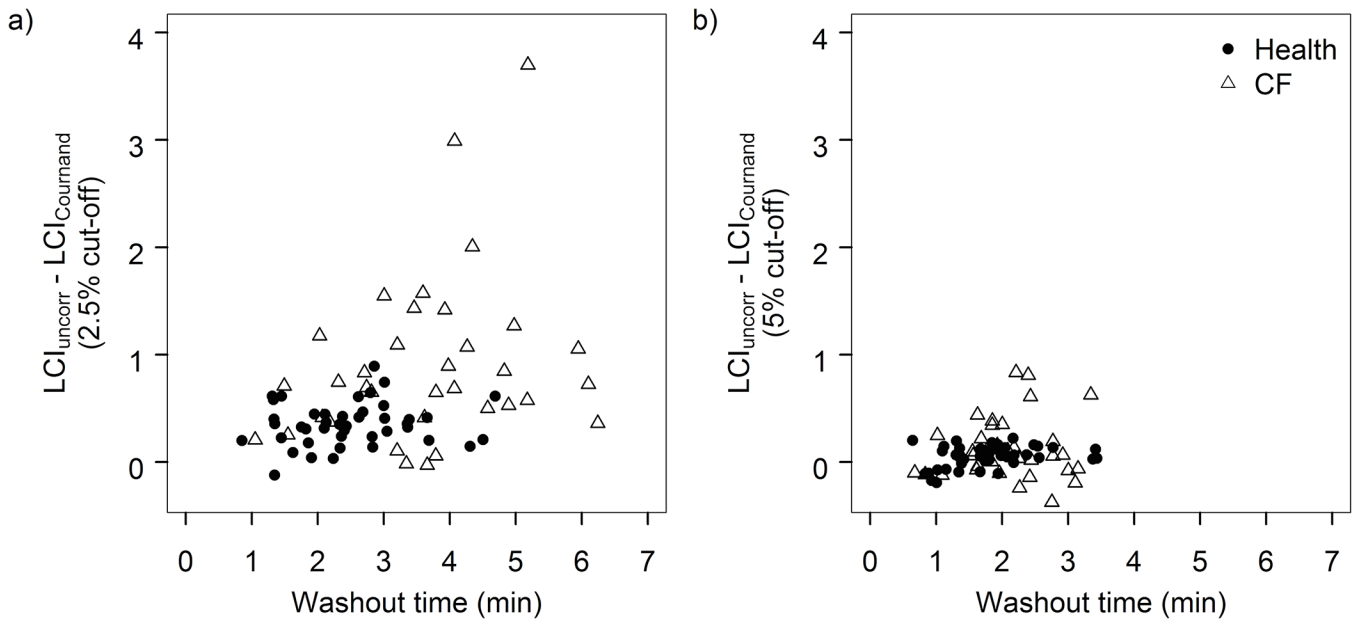

Fig 6. Relationship between the contribution of tissue N_2 to LCI_{N2} and length of washout calculated at a) the traditional 2.5% washout cut-off and b) the 5% washout cut-off. The contribution of tissue N_2 to LCI_{N2} calculated at the 2.5% cut-off (LCI_{uncorr}–$LCI_{Cournand}$) increased as washout time increased. However, this relationship was no longer observed at the earlier 5% cut-off.

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of FRC, body plethysmography and inert gas washout are the most commonly used techniques [16,17]. In the current study, FRC_{N2} was compared to FRC_{pleth} and FRC_{SF6} to estimate the contribution of tissue N_2 excretion. With $FRC_{pleth,}$ the volume of all compressible intrathoracic gas is measured whereas only the volume of communicating lung units is measured with FRC_{N2}. Therefore, FRC measured by gas-dilution technique (such as MBW_{N2}) should be equal to or less than that measured by plethysmography in the absence of endogenous production of the tracer gas [17]. FRC_{SF6} is also calculated using a gas-dilution technique, and because it is an exogenous, biologically inert gas that does not dissolve significantly in blood or other tissues, it was used as comparator to assess for the contribution of tissue N_2 excretion to FRC_{N2}.

We found that FRC_{N2} was systematically overestimated compared to FRC_{SF6} and, to a more variable extent, FRC_{pleth} (Figs 2 and 5). This suggests that there is a systematic difference between these tests and that the observed differences were not entirely due to intrinsic differences between the MBW and plethysmographic techniques. While our analyses focused on the potential effect of tissue nitrogen excretion on this overestimation, there are other explanations for this disparity that could contribute to the observed differences that were not assessed in the current study, such as testing order, technical inconsistencies in the MBW equipment, and physical differences between SF_6 and N_2 tracer gases.

Table 5. Effect of applying Lundin and Cournand correction equations to previously published observational MBW data. Data are shown as pre-treatment and post-treatment LCI with paired treatment effect. Values are presented as mean (SD) unless otherwise indicated.

	Pre-treatment LCI	Post-treatment LCI	Treatment effect mean difference (95%CI)
Ivacaftor [14]			
Uncorrected	13.7 (3.7)	11.6 (4.1)	-2.2 (-3.0, -1.3)
Lundin	12.8 (3.8)	11.0 (3.9)	-1.8 (-2.6, -0.9)
Cournand	12.4 (3.6)	10.7 (3.6)	-1.8 (-2.8, -0.7)

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The order of tests could have inadvertently biased the results through effects of tissue hysteresis or other unknown mechanisms. In the original study, all plethysmographic testing was performed after the MBW testing and the order of MBW_{SF6} and MBW_{N2} was randomized [2]. All MBW-based outcomes can be affected by errors in gas concentration measurement, flow-gas signal alignment, dead-space correction and other device-specific settings [19–21]. In this study, we used working-group recommended equipment and software settings on both the Exhalyzer D and AMIS 2000 devices and applied standardized quality control criteria to each MBW trial. We also confirmed the accuracy of the N_2 concentration calculation (as FN_2 is derived from measured O2 and CO_2 concentrations using the Exhalyzer device) across a range of gas standards. Despite our attempts to minimize technical software or device-specific inconsistencies, these cannot be completely ruled out as sources of systematic error that could contribute to the discrepancies observed.

The intrinsic properties of MBW_{SF6} and MBW_{N2} tests could also have contributed to these differences. The molecular properties of SF_6 and N_2 likely result in differences in their diffusion-convection fronts, which could potentially impact MBW outcomes [22]. MBW_{SF6} requires a wash-in equilibration phase as SF_6 is an exogenous tracer gas, and while standardized quality control techniques were implemented to attempt to ensure complete SF_6 washing, it is possible that incomplete wash-in of the SF_6 could result in altered excretion kinetics. Finally, the 100% oxygen washout phase in MBW_{N2} could also theoretically have pro-atelectatic effects, thus altering pulmonary gas flow dynamics. While simultaneous direct measurements of N_2 and SF_6 on the same device would permit an ideal comparison of these two MBW systems, unfortunately, high O_2 concentration impairs the ability of the AMIS 2000 respiratory mass spectrometer to measure N_2 concentrations and can therefore not be used to measure the two gases in the context of a 100% oxygen washout. Overall, our results need to be interpreted in the context of these potential limitations; nevertheless, the consistent overestimation of FRC_{N2} when compared to FRC_{pleth} and FRC_{SF6} suggests that tissue N_2 likely contributes to this phenomenon.

Both FRC_{N2} and LCI_{N2} decreased significantly upon application of the tissue N_2 excretion equations in both healthy subjects and subjects with CF, with greater differences observed in CF. The estimates of the contribution of tissue N_2 to FRC_{N2} and LCI_{N2} are similar to those previously predicted by a two-compartment lung model including variable ventilation heterogeneity and dead space effects [3]. However, the difference between FRC_{SF6} and FRC_{N2} was significantly greater than the degree of correction applied by either Lundin or Cournand equations (Fig 3). Also, application of the correction equations only decreased the time-dependent-rise in FRC_{N2} by ~30% (Fig 4; Table 4). These findings suggest either that the equations used in this study underestimate the amount of tissue N_2 excretion, or that there are other factors in addition to tissue N_2 secretion that are driving this difference.

The Lundin tissue N_2 excretion equation is based on the average of measurements derived from healthy adults, therefore its application to MBW_{N2} data derived from subjects of varying size is limited. Compared to the Cournand equation, which was derived from subjects ranging from 9 to 44 years old and adjusts for a subject's body size, the Lundin equation may overestimate the effect of tissue N_2 excretion in smaller pediatric subjects. Although the Cournand equation may introduce less error overall in MBW_{N2} measurements from subjects with a range of body size, it assumes a constant rate of N_2 excretion from the body tissue which is unlikely to be the case in subjects of varying body composition and between health and disease. In a recently published study [23], the rate of tissue N2 excretion was simultaneously performed on MBWN2 and MBWSF6 washouts and confirmed the time-dependent nature of tissue N2 excretion and demonstrated higher rates of tissue N2 excretion during moderate exercise."

Ideally, direct measurement of pulmonary N_2 excretion of tissue N_2 with modern equipment across a range of ages, body compositions and disease states would allow us to generate an optimal correction equation. However, due to the long duration of the testing and uncomfortable testing setup, replications of these early studies would be extremely challenging to conduct today, especially in children [6,9]. Furthermore, no mathematical correction for tissue N_2 excretions will be ideal for several reasons. First, even with modern technology, it is impossible to precisely isolate all of the N_2 in the lungs that was excreted from the body tissue, especially during the beginning of the washout when the relative proportion is very small; the derived equations are reflections of this imprecision. Second, the contribution of N_2 from the body tissue is likely dependent not only on time and body size, but also on factors such as cardiac output, tissue perfusion, body fat content, ventilation homogeneity, and dead space [3,6,8,23–26]. Any number of these physiological factors could be altered in a disease like CF and could confound the estimation of tissue N2 excretion.

The extent to which the MBW_{N2} outcomes diverged from both MBW_{SF6} and MBW_{pleth} was related to the length of the washout. This correlation makes intuitive sense, since individuals with longer washouts (greater ventilation inhomogeneity) spend a longer time at lower end-tidal N_2 concentrations, thereby accentuating the relative contribution of excreted tissue nitrogen. Given this finding, we showed that the contribution of tissue N_2 can be minimized by calculating MBW_{N2} outcomes at earlier cut-offs of the washout, such as at the 5% normalized end tidal concentration of N_2. Using an earlier cut-off of the washout has the additional benefit of shortening the total time it takes to perform an MBW test; however, there is some evidence that there may be a trade-off with decreased sensitivity to treatment efficacy [18]. Nevertheless, the use earlier cut-off for MBW_{N2} did not affect the significance of treatment effects observed in a study of Ivacaftor treatment [14], suggesting that the sensitivity of an MBW cut-off may depend upon the effect size of the intervention. The optimal MBW_{N2} cutoff for interventional studies may depend on study design and treatment.

Finally, to address the practical question of whether or not the correction for tissue N_2 excretion could affect the results of previously reported interventional studies, we applied tissue N_2 correction equations to raw MBW_{N2} data from a study that assessed the effect of ivacaftor on LCI [14]. Overall, this study had a large treatment effect (-2.2 LCI units) and we found that applying tissue N_2 correction equations attenuated the treatment response, but did not change the significance or direction of the treatment effect. This attenuation of the treatment response occurred primarily by reducing the post-treatment LCI by a greater amount than the pre-treatment LCI and is likely a reflection of the observation that tissue N_2 has a greater contribution in longer washouts. Taken together, these results suggest that non-correction for tissue N_2 release may result in marginally overestimated treatment effects. While this does not significantly affect the results of the studied trial, it is conceivable that smaller treatment effects could be amplified by non-correction for tissue N_2.

In conclusion, MBW_{N2} outcomes are systematically different from MBW_{SF6} and plethysmography. We show that correction for tissue N_2 excretion using previously derived equations can reduce, but not eliminate, these differences. This suggests that either there are other physiologic/experimental factors contributing to this difference, or that the correction equations that were used underestimate the quantity of tissue N_2 excretion. Given our data, we suggest that there is currently inadequate knowledge of the true rate of pulmonary tissue nitrogen excretion to suggest a standard correction equation for this phenomenon in the calculation of MBW outcomes. Further study (ideally simultaneous MBW_{N2} and MBW_{SF6} measurements using an appropriately tuned mass spectrometer) could elucidate the contribution of tissue N_2 to MBW_{N2} outcome measures. Until this is clarified, it should be recognized that the magnitude of treatment responses measured with MBW_{N2} may be over-estimated by tissue N_2

excretion, however, application of correction equations in this study did not change the direction or significance of the treatment effects of a previously studied intervention.

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