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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 N2 on MBW_N2 outcomes in both healthy subjects and patients with CF and to assess whether it is possible to correct for tissue N2. The contribution of tissue N2 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 N2 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 N2-dependent and -independent techniques. Use of earlier washout cut-offs could reduce the influence of tissue N2. Applying tissue N2 corrections to LCI_N2 measurements did not significantly affect the interpretation of treatment effects reported in a previously published interventional trial. While tissue N2 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 (N2) 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 N2 in healthy adults from its accumulation during breathing of 100% oxygen for prolonged periods [5–11]. Based on these studies, the tissue N2 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
poorly-circulated and fat-containing tissues. Elimination rates were found to vary both within and between individuals.

Recently, Nielsen et al. applied a tissue N₂ 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 N₂ on the washout by subtracting 1% end-tidal concentration of N₂ 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 N₂ on MBW₅₂ outcomes in disease versus health, but they did not explore whether the contribution of tissue N₂ 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 N₂. Therefore, the impact of tissue N₂ 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 MBW₅₂ outcomes [2–4,12].

There are limited data to support correcting for the contribution of tissue N₂; thus it is not currently recommended as per American Thoracic Society/European Respiratory Society (ATS/ERS) consensus statement [12]. As MBW₅₂ 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 N₂ must be clarified in order to determine whether it is necessary to correct for its contribution to the MBW₅₂ test. The aim of this study was to estimate the magnitude of tissue N₂ 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 N₂ on the MBW₅₂ 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
MBW₅₂ measurements were performed using an open circuit, bias flow system (Exhalyzer D₂®, 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 (SF₆) as the tracer gas. MBW₅₂ 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\textsubscript{2} contribution

FRC measured by body plethysmograph (FRC\textsubscript{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\textsubscript{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\textsubscript{pleth} and FRC\textsubscript{N2} can be used to approximate the contribution of tissue N\textsubscript{2} to the MBW\textsubscript{N2}. Similarly, as SF\textsubscript{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\textsubscript{2} to FRC derived by gas dilution.

Tissue N\textsubscript{2} excretion equations

MBW\textsubscript{N2} assesses ventilation inhomogeneity by examining N\textsubscript{2} clearance over a series of breaths for the duration of the washout. To generate MBW\textsubscript{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\textsubscript{2}; CEV\textsubscript{N2}) must be measured. FRC and LCI are calculated when Cet\textsubscript{N2} falls below a predefined threshold (typically 2.5% of the initial CetN2).

\[
FRC = \frac{CEV\textsubscript{N2}}{(Cet\textsubscript{N2,initial} - Cet\textsubscript{N2,final}) - DS\textsubscript{pre}} \quad \text{Eq 1}
\]

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

where Cet\textsubscript{N2} is the end tidal concentration of nitrogen. Cet\textsubscript{N2, initial} is the end tidal concentration of N\textsubscript{2} in the first breath of the washout phase, and Cet\textsubscript{N2, final} is the end tidal concentration of N\textsubscript{2} in the first breath of the washout phase where Cet\textsubscript{N2} is less than the target threshold. DS\textsubscript{pre} is the equipment deadspace proximal to the sampling point of the apparatus.

In order to correct these values for tissue N\textsubscript{2} excretion, breath-by-breath end tidal body tissue N\textsubscript{2} concentration (Cet\textsubscript{N2,BT}) as well as the volume of body tissue nitrogen excreted over the washout (V\textsubscript{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).

\[
Cet\textsubscript{N2,BT} = \frac{V\textsubscript{N2,BT}}{V\text{Exp}} \quad \text{Eq 3}
\]

\[
FRC\textsubscript{corr} = \frac{(CEV\textsubscript{N2} - V\textsubscript{N2,BT})}{Cet\textsubscript{N2,initial} - (Cet\textsubscript{N2} - Cet\textsubscript{N2,BT})\textsubscript{final}} - DS\textsubscript{pre} \quad \text{Eq 4}
\]

\[
LCI\textsubscript{corr} = \frac{(CEV - V\textsubscript{N2,BT})}{FRC\textsubscript{corr}} \quad \text{Eq 5}
\]

where V\textsubscript{N2,BT} is the volume of body tissue nitrogen expired in breath i and V\text{Exp} is the net volume of expired gas in breath i. Cet\textsubscript{N2} is the end tidal concentration of nitrogen. Cet\textsubscript{N2,BT} is the end tidal concentration of nitrogen derived from the body tissues. Initial subscript
Table 1. Summary of tissue nitrogen correction equations.

<table>
<thead>
<tr>
<th>Study (citation)</th>
<th>n</th>
<th>Age range</th>
<th>Equation used</th>
</tr>
</thead>
</table>
| Lundin, 1953 [9] | 7 | 16–42     | Rate of excretion (in mL/min) at time \( t \):
|                  |   |           | \( \delta N_2 = 37.3e^{-0.13t} + 13.9e^{-0.009t} + 4.82e^{-0.0024t} \) |
|                  |   |           | Integrating to derive \( V_{N_2} \) at time \( t \):
|                  |   |           | \( V_{N_2} = \int 37.3e^{-0.13t} + 13.9e^{-0.009t} + 4.82e^{-0.0024t} \) \( dt \) |
|                  |   |           | \( V_{N_2} = \frac{(37.3)}{(0.13)}(1 - e^{-0.13t}) + \frac{(13.9)}{(0.009)}(1 - e^{-0.009t}) + \frac{(4.82)}{(0.0024)}(1 - e^{-0.0024t}) \) |
|                  |   |           | Where \( t \) = time in minutes |
| Cournand, 1941 [6] | 30 | 9–44     | \( V_{N_2} = \frac{1}{\text{BSA}} \times ((96.5 \times \text{BSA}) + 35) \) |
|                  |   |           | Where \( t \) = time in seconds |
| ATS/ERS [16]     | NA | NA       | \( V_{N_2} = (96.5 \times \text{BSA}) + 35 \) |

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

\( V_{N_2} = \text{excreted volume of nitrogen (mL)} \)

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 \( (Cet_{N_2} - C_{N_2 BT}) \) is less than the target threshold.

Three different equations (Table 1, Fig 1) were used to derive \( V_{N_2 BT} \). 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 \( (C_{N_2 BT}) \). The ATS/ERS (22) equation is time-independent and is therefore only used to generate \( V_{N_2 BT} \) and not \( Cet_{N_2 BT} \). Therefore, corrected FRC values were generated from all three equations (with \( Cet_{N_2} \), 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\(_{N_2}\)/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\(_{p} \) and the difference in FRC and LCI measured by MBW\(_{N_2}\) and MBWSF\(_{0}\), when available. FRC and LCI values were also re-calcuated 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\(_{N_2}\) 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: \( F_1CO_2 \sim 0.0004, F_1O_2 = 0.2095, F_1N_2 = 0.7808, F_1Ar = 0.0093 \)) with a high precision gas mixture (\( F_2CO_2 = 0.0500, F_2O_2 = 0.9500; \) Praxair Canada, Mississauga ON). \( F_{N_2} \) of the mixed reference gas (\( F_{M,N_2} \)) was calculated using Dalton’s Law of partial pressures, the fractional concentrations of the reference gases and the measured \( F_1O_2 \) of the mixed gas (\( F_{M,O_2} \)).

\( F_{N_2}O_2 \) was measured using the Oxigraf laser oxygen analyzer (Oxigraf Inc, Sunnyvale CA, USA) within the Exhalyzer D\( ^R \). 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$^\text{®}$ was compared to F$_M$N$_2$ 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$_M$N$_2$) 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 Courmand and Lundin excretion equations improve the FRC$_{N2}$ agreement with plethysmography, the uncorrected FRC$_{N2}$ (FRC$_{uncorr}$) and the FRC$_{N2}$ corrected (FRC$_{Courmand}$ 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$_{Courmand}$ 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 Courmand and Lundin equations decreased the rate of rise of the FRC$_{N2}$ by 23–34%, but did
Fig 1. Tissue N₂ excretion equations used for correction of MBW₂ measurements. The three equations used to estimate the volume of N₂ excreted from the body tissues are plotted over a 7 minute time period. The Courmand 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 N₂ excreted using Courmand’s 1941 equation standardized to a 7 minute washout for all subjects.

https://doi.org/10.1371/journal.pone.0185553.g001

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

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

https://doi.org/10.1371/journal.pone.0185553.t002
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\textsubscript{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\textsubscript{N2} outcome. Outcomes were corrected by applying either the Cournand or Lundin tissue N\textsubscript{2} excretion equations.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Health</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (95% CI)</td>
<td>Mean difference (95% CI)</td>
</tr>
<tr>
<td>FRC\textsubscript{N2} (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cournand</td>
<td>0.11 (0.10; 0.13), p&lt;0.001</td>
<td>0.18 (0.15; 0.21), p&lt;0.001</td>
</tr>
<tr>
<td>Lundin</td>
<td>0.13 (0.12; 0.15), p&lt;0.001</td>
<td>0.19 (0.17; 0.20), p&lt;0.001</td>
</tr>
<tr>
<td>CEV\textsubscript{N2} (L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cournand</td>
<td>1.63 (1.36; 1.90), p&lt;0.001</td>
<td>4.41 (3.30; 5.52), p&lt;0.001</td>
</tr>
<tr>
<td>Lundin</td>
<td>1.57 (1.37; 1.76), p&lt;0.001</td>
<td>3.22 (2.40; 4.03), p&lt;0.001</td>
</tr>
<tr>
<td>LCI\textsubscript{N2}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cournand</td>
<td>0.35 (0.29; 0.42), p&lt;0.001</td>
<td>0.90 (0.63; 1.17), p&lt;0.001</td>
</tr>
<tr>
<td>Lundin</td>
<td>0.30 (0.23; 0.36), p&lt;0.001</td>
<td>0.41 (0.17; 0.65), p = 0.001</td>
</tr>
</tbody>
</table>

https://doi.org/10.1371/journal.pone.0185553.t003
Estimates of tissue $N_2$ contribution to CEV and LCI

Similar to $FRC_{N_2}$, application of tissue $N_2$ excretion equations to $MBW_{N_2}$ data resulted in lower CEV$_{N_2}$ and LCI$_{N_2}$ 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 $FRC_{N_2}$ for the contribution of tissue $N_2$, it was not used to correct LCI and CEV values.

When LCI as measured by $MBW_{N_2}$ and $MBW_{SF6}$ were compared within subjects ($LCI_{N_2} - LCI_{SF6}$), $LCI_{N_2}$ became disproportionately greater than $LCI_{SF6}$ as disease severity ($LCI_{N_2}$) increased ($r = 0.53$, $p<0.001$). Similar to $FRC_{N_2}$, there was a significant and positive
correlation observed between LCI\(N_2\) – LCI\(SF_6\) 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\(N_2\) 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\(N_2\) calculated at the 2.5% cut-off increased as disease severity \((LCI\(N_2\)) 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>
<thead>
<tr>
<th></th>
<th>Health</th>
<th>CF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncorrected slope (mL/min)</td>
<td>104.2 (97.9, 110.6)</td>
<td>125.3 (115.4, 135.2)</td>
</tr>
<tr>
<td>Corrected slope (Lundin)</td>
<td>77.4 (71.4, 83.5)</td>
<td>100.4 (90.1, 110.7)</td>
</tr>
<tr>
<td>Absolute diff (Lundin) (%)</td>
<td>26.8 (25.2, 28.4)</td>
<td>24.9 (23.4, 26.4)</td>
</tr>
<tr>
<td>Relative diff (Lundin) (%)</td>
<td>27.4 (25.0, 29.9)</td>
<td>22.5 (19.6, 25.3)</td>
</tr>
<tr>
<td>Corrected slope (Cournand)</td>
<td>70.5 (65.0, 76.0)</td>
<td>94.2 (84.9, 103.5)</td>
</tr>
<tr>
<td>Absolute diff (Cournand) (%)</td>
<td>33.7 (32.3, 35.1)</td>
<td>31.1 (29.6, 32.6)</td>
</tr>
<tr>
<td>Relative diff (Cournand) (%)</td>
<td>33.9 (31.9, 35.8)</td>
<td>26.7 (24.6, 28.8)</td>
</tr>
</tbody>
</table>

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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\textsubscript{2} from body tissues affects MBW\textsubscript{N2} outcomes. The effects of tissue N\textsubscript{2} are greater in patients with longer washouts. This contribution of tissue N\textsubscript{2} to FRC\textsubscript{N2} and LCI\textsubscript{N2} is less pronounced at earlier cut-offs of the washout. Application of correction equations for tissue N\textsubscript{2} significantly reduced, but did not completely eliminate, the effect of tissue N\textsubscript{2} on MBW\textsubscript{N2} outcomes. Importantly, application of these tissue N\textsubscript{2} correction equations did not significantly alter treatment effects previously observed in interventional trials. Thus, while the excretion of tissue N\textsubscript{2} has a measurable effect on MBW\textsubscript{N2} outcomes, correction for tissue N\textsubscript{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...
of FRC, body plethysmography and inert gas washout are the most commonly used techniques [16,17]. In the current study, FRC$_{\text{N}_2}$ was compared to FRC$_{\text{pleth}}$ and FRC$_{\text{SF}_6}$ to estimate the contribution of tissue N$_2$ excretion. With FRC$_{\text{pleth}}$, the volume of all compressible intrathoracic gas is measured whereas only the volume of communicating lung units is measured with FRC$_{\text{N}_2}$. Therefore, FRC measured by gas-dilution technique (such as MBW$_{\text{N}_2}$) should be equal to or less than that measured by plethysmography in the absence of endogenous production of the tracer gas [17]. FRC$_{\text{SF}_6}$ 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$_{\text{N}_2}$.

We found that FRC$_{\text{N}_2}$ was systematically overestimated compared to FRC$_{\text{SF}_6}$ and, to a more variable extent, FRC$_{\text{pleth}}$ (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.

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

https://doi.org/10.1371/journal.pone.0185553.t005
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 O$_2$ 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 N$_2$ excretion was simultaneously performed on MBWN2 and MBWSF6 washouts and confirmed the time-dependent nature of tissue N$_2$ excretion and demonstrated higher rates of tissue N2 excretion during moderate exercise.”
Ideally, direct measurement of pulmonary $\text{N}_2$ excretion of tissue $\text{N}_2$ 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 $\text{N}_2$ excretions will be ideal for several reasons. First, even with modern technology, it is impossible to precisely isolate all of the $\text{N}_2$ 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 $\text{N}_2$ 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 $\text{N}_2$ excretion.

The extent to which the MBW$_{\text{N}_2}$ outcomes diverged from both MBW$_{\text{SF}_6}$ and MBW$_{\text{pleth}}$ 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 $\text{N}_2$ concentrations, thereby accentuating the relative contribution of excreted tissue nitrogen. Given this finding, we showed that the contribution of tissue $\text{N}_2$ can be minimized by calculating MBW$_{\text{N}_2}$ outcomes at earlier cut-offs of the washout, such as at the 5% normalized end-tidal concentration of $\text{N}_2$. 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$_{\text{N}_2}$ 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$_{\text{N}_2}$ 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 $\text{N}_2$ excretion could affect the results of previously reported interventional studies, we applied tissue $\text{N}_2$ correction equations to raw MBW$_{\text{N}_2}$ 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 $\text{N}_2$ 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 $\text{N}_2$ has a greater contribution in longer washouts. Taken together, these results suggest that non-correction for tissue $\text{N}_2$ 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 $\text{N}_2$.

In conclusion, MBW$_{\text{N}_2}$ outcomes are systematically different from MBW$_{\text{SF}_6}$ and plethysmography. We show that correction for tissue $\text{N}_2$ 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 $\text{N}_2$ 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$_{\text{N}_2}$ and MBW$_{\text{SF}_6}$ measurements using an appropriately tuned mass spectrometer) could elucidate the contribution of tissue $\text{N}_2$ to MBW$_{\text{N}_2}$ outcome measures. Until this is clarified, it should be recognized that the magnitude of treatment responses measured with MBW$_{\text{N}_2}$ may be over-estimated by tissue $\text{N}_2$. 

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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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References


