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Normative data for multiple breath washout outcomes in school-aged Caucasian children

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Take home message: This study provides reference values for nitrogen multiple breath washout outcomes in healthy Caucasian children from six to 18 years old, measured with a commercialy available device.

Keywords: reference values, children, multiple breath washout, lung clearance index, functional residual capacity, moment ratios

ABSTRACT

Background: The nitrogen multiple breath washout (N_2MBW) technique is increasingly used to assess the degree of ventilation inhomogeneity in school-aged children with lung disease. However, reference values for healthy children are currently not available. The aim of this study was to generate reference values for N_2MBW outcomes in a cohort of healthy Caucasian school-aged children.

Methods: N_2MBW data from healthy Caucasian school-age children between 6 and 18 years were collected from four experienced centers. Measurements were performed using an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and were analyzed with commercial software (Spiroware, 3.2.1, Eco Medics AG). Normative values and upper limits of normal were generated for lung clearance index at 2.5% ($LCI_{2.5\%}$) and at 5% ($LCI_{5\%}$), moment ratios (M_1/M_0 and M_2/M_0), and a prediction equation generated for functional residual capacity (FRC).

Results: Four hundred and eighty five trials from 180 healthy Caucasian children aged from 6 to 18 years were used for analysis. While LCI increased with age, this increase was negligible (0.04 units/year for $LCI_{2.5\%}$) and therefore fixed upper limits of normal were defined for this age group. These limits were 7.91 for $LCI_{2.5\%}$, 5.73 for $LCI_{5\%}$, 1.75 for M_1/M_0, and 6.15 for M_2/M_0 respectively. Height and weight were found to be independent predictors of FRC.

Conclusion: We report reference values for N_2MBW outcomes measured on a commercially available ultrasonic flowmeter device (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) in healthy school-aged children to allow accurate interpretation of ventilation distribution outcomes and FRC in children with lung disease.

INTRODUCTION

The multiple breath washout (MBW) test provides lung volume and ventilation distribution outcomes that are more sensitive than conventional lung function outcomes to detect lung disease in children with cystic fibrosis (CF) [1-3] and potentially other respiratory disorders [4, 5]. The lung clearance index (LCI) is a global marker of ventilation distribution derived from the MBW that is reproducible [6, 7], discriminates between health and disease [3], and correlates with the extent of structural lung disease [2, 8] in children with CF. These data have led to LCI being used as the primary endpoint in observational studies and interventional trials in patients with CF [9-11], and potentially as a future factor for the clinical surveillance of children with CF [12].

LCI is traditionally calculated as the number of lung turnovers required to washout a tracer gas to $1/40^{th}$ ($LCI_{2.5\%}$) of the starting tracer gas concentration [13]. The $LCI_{5\%}$ can also be calculated as the number of lung turnovers required to washout a tracer gas to $1/20^{th}$ of the initial tracer gas concentration [14, 15]. Moment ratios (M_1/M_0 and M_2/M_0) describe the degree of skewness of the washout curve and may be more sensitive to detect ventilation inhomogeneity in the periphery of the lung [2, 15]. In addition, the MBW test measures the functional residual capacity (FRC) of the lung which may indicate some degree of lung hyperinflation [1].

Despite the increasing use of MBW, reference values for MBW outcomes in children are scarce [16, 17]. Several commercially available and custom-made MBW devices are currently in use but outcomes are generally not interchangeable between them [18-20]. In addition, different software versions and system settings can influence the calculation of MBW outcomes [21, 22]. As a result, published normative values are only applicable for the specific device, software, and tracer gas used [17, 21, 23]. For this reason, studies evaluating MBW outcomes using commercially available equipment have required data collection in age-matched healthy controls [2, 12].

The aim of our study was to provide normative values for nitrogen MBW (N_2MBW) outcomes (LCI, moment ratios, FRC) measured with a commercially available device using the ultrasonic flowmeter

(Exhalyzer D, Eco Medics AG, Duernten, Switzerland) in school-aged children, and to investigate the association of MBW outcomes with anthropometric and physiological parameters, including the tidal volume and the equipment related dead space volume [24, 25]. To achieve this, we collected data from healthy children measured in four different centers with the same MBW device, protocol, and analysed using the same software and system settings.

METHODS

Study subjects

For this study we used MBW measurements from healthy school-aged Caucasian children collected between 2011 and 2016 from four pediatric centers specialized in N_2MBW, including Inselspital, Bern (Switzerland), SickKids, Toronto (Canada), University Children's Hospital, Heidelberg (Germany), and Telethon Kids Institute, Perth (Australia). We used the following exclusion criteria: non-Caucasians, age younger than 6 years or older than 18 years, chronic respiratory or cardiac disease, respiratory infection during the last four weeks prior to measurement, and other major systemic diseases with potential influence on lung function [26]. Healthy individuals from each center took part in prospective observational studies and therefore some data have been published previously [2, 8, 12, 18, 27, 28]. The study was approved from local ethic committees (Ethics Committee of the Canton of Bern, Switzerland, Research Ethics Board at SickKids Toronto, Canada, Ethics Committee of the University of Heidelberg, Germany, Princess Margaret Hospital Human Ethics Committee, Perth). Parents or caregivers provided informed written consent.

MBW measurements

We performed N_2MBW measurements using the ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and the software provided by the manufacturer (Spiroware 3.1.6) as previously described [12, 18]. All centers used the same equipment and performed the calibration and measurement using the same protocol (details provided in the online supplement).

MBW analysis

N_2MBW data originally recorded in Spiroware 3.1.6 were reloaded and analyzed using the updated version of the software provided by the manufacturer at the time the study was performed (Spiroware 3.2.1, Eco Medics AG). Further details are provided in the online supplement.

MBW outcomes and physiological indices

FRC, $LCI_{2.5\%}$, $LCI_{5\%,}$ and moment ratios (M_1/M_0 and M_2/M_0) were calculated according to current recommendations [13, 14]. The mean tidal volume (V_T) for each trial was provided by the software. In order to investigate the effect of breathing pattern [29] and equipment related dead space (V_d) [30] on MBW outcomes, the mean ratio of V_T to FRC (V_T/FRC, %), and V_d to V_T (V_d/V_T, %) were calculated per trial.

Quality control of MBW trials

Quality control of MBW trials was assessed by an experienced operator at the Inselspital, Bern (PA), according to the 2013 ATS/ERS MBW consensus guidelines and additional criteria proposed by Jensen and colleagues [13, 31]. Further details are provided in the online supplement. Tests with at least two technically acceptable trials with FRC values within 25% of the mean were included for analysis.

Statistics

Statistical analysis of the data was performed using R (version 3.4.3; R Foundation, Vienna, Austria) and Stata (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP). Graphs were generated using GraphPad Prism (version 5.0; GraphPad Software, San Diego California, USA). Normality of data distribution was assessed visually and using the Shapiro-Wilk test. The upper and lower limits of normal (ULN and LLN, respectively) correspond to the 97.5^{th} centile (mean ± 1.96SD), as previously described [17]. The intra-subject variability was defined as the mean relative difference for subjects with two trials [trial1-trial2/mean (%)] or the coefficient of variation [CV=SD/mean (%)] for those with at least three trials. We used one-way ANOVA tests with

Bonferroni post-hoc test for between-center comparisons. Multiple linear regression was performed to assess the associations of normally distributed primary outcomes ($LCI_{2.5\%}$, $LCI_{5\%,}$ $M_1/M_{0,}$ M_2/M_0 and FRC) with demographic (age, weight, height, sex) and physiological factors (V_T/FRC, Vd/V_T). All of the variables considered were selected for biological reasons. Multicollinearity between independent variables was assessed by checking the variance inflation factor and was not present in the final models. The homoscedasticity and normality of residuals for the models were assessed using White's test and Shapiro-Wilk test, respectively. In the final models, only significant factors were considered to facilitate easy application of the reference equations. The statistical significance level was set to a p-value < 0.05.

RESULTS

Study subjects

N_2MBW data from 285 healthy children were assessed for eligibility. Of them, 67 did not fulfil the inclusion criteria and 38 did not pass the quality control (success rate 82.6%) *(Figure 1)*. Thus, 485 MBW trials from 180 children were used for analysis: 82 children from the University Children's Hospital in Bern, Switzerland (mean age 10.8 years, range 6.0 -17.8), 33 children from the University Children's Hospital Heidelberg, Germany (mean age 12.0 years, range 7.1 -17.0), 28 children from the SickKids in Toronto, Canada (mean age 12.5 years, range 6.5 -17.2) and 37 children from the Telethon Kids Institute in Perth, Australia (mean age 9.5 years, range 6.0 -13.6). Of the 180 study participants, 38.9% had two acceptable MBW trials, 53.9% had three acceptable trials, and 7.2% had four or more acceptable trials.

Center differences

Study demographics and anthropometrics for each centre are reported in Table 1. Participants from Perth were significantly younger compared with those from Toronto and Heidelberg ($p < 0.001$) *(Supplemental Figure 1)*. Participants from Toronto had higher height z-scores compared with those from Heidelberg ($p = 0.02$) but there were no differences in weight z-score between centres. There were no differences in LCI or moment ratio outcomes between centers. There were significant

differences in FRC (L), whereby children from Toronto had higher FRC values compared to children in Perth (p = 0.02) *(Supplemental Figure 1)*.

Lung clearance index and moment ratios

In univariable regression analyses, $LCI_{2.5\%}$ was negatively associated with height (Coef. -0.005, 95% CI -0.0087; -0.0019, p=0.002), weight (Coef. -0.0049, 95% CI -0.0090; -0.0008, p=0.020), and age (Coef. -0.0225, 95% CI -0.0421; -0.0030, p=0.024), and positively associated with Vd/V_T (Coef. 0.0258, 95% CI 0.0093; 0.0422, p=0.002). No association was found between $LCI_{2.5\%}$ and sex or V_T/FRC (p > 0.05).

Similarly, $LCI_{5\%}$ and moment ratio outcomes were negatively associated with height, weight, and age *(Supplemental Table 1)*. In addition, Vd/V_T and V_T/FRC were both negatively associated with age *(Supplemental Figure 2)*, and $LCI_{5\%}$ and M_1/M_0 were positively associated with Vd/V_T and V_T/FRC *(Supplemental Table 1; Supplemental Figure 2)*. There was no evidence for associations of $LCI_{5\%}$ and moment ratios with sex (p > 0.05) *(Supplemental Table 1)*.

In a multivariable regression model age, Vd/V_T, and V_T/FRC were independently associated with LCI and moment ratio outcomes *(Supplemental Table 2)*. However, the regression coefficients for each of these independent predictors were small ($r \leq 0.10$). For $LCI_{2.5\%}$ the coefficient for age was 0.04, indicating that (after adjusting for V_d/V_T and V_T/FRC) $LCI_{2.5\%}$ would increase by 0.04 (95% CI: 0.01; 0.07) units per year. This equates to a maximum 0.5 unit change in $LCI_{2.5\%}$ over 12 years (from the age of 6 years to 18 years). Thus, in our judgement, the age related changes in LCI and moment ratios are negligible. Therefore, we report fixed upper limits of normal during this age interval *(Figure 2)*.

The mean lower and upper limits of normal for LCI and moment ratios are provided in Table 2. The upper limit of normal for $LCI_{2.5\%}$ was 7.91 and $LCI_{5\%}$ was 5.73. Upper limits of normal for M_1M_0 was 1.75 and M_2/M_0 was 6.15 *(Figure 2)*.

Functional residual capacity

FRC values were right skewed and therefore natural log transformed FRC values and predictors were used in the models. Ln FRC was positively associated with ln height, weight, tidal volume, and age, and negatively associated with ln Vd/V_T in the univariate analysis *(Supplemental Table 1; Figure 3)*. There was no evidence of association with sex (p = 0.68) *(Supplemental Table 1)*. In a multivariable model, ln FRC was independently associated with ln height and ln weight. There were no differences in FRC z-scores between centres (p = 0.81). The full FRC prediction equation including the intercept for the model is provided in the online supplement.

$$\boldsymbol{Predicted\ FRC} = (\boldsymbol{e^{-18.18}}) \times (\boldsymbol{height^{3.98}}) \times (\boldsymbol{weight^{-0.32}})$$

$$\boldsymbol{z\text{-}score\ FRC} = \frac{\boldsymbol{ln}\ (\boldsymbol{Measured\ FRC - Predicted\ FRC})}{\boldsymbol{0.1632}}$$

Equation 1: FRC is expressed in L, height in cm, and weight in kg.

DISCUSSION

We report N_2MBW normative data for a pediatric Caucasian population between the age of six and 18 years using commercially available equipment and software. While a significant age dependency was observed for LCI and moment ratio outcomes, the magnitude of this effect was small during the age interval of 12 years. Therefore, fixed upper limits of normal for LCI and moment ratios can be used in this age group. FRC was independently predicted by both height and weight, and we provide FRC predicted values and z-score equation.

Comparison with the literature

The $LCI_{2.5\%}$ values in our cohort are slightly higher than previously reported for this age group [16, 17, 24]. However, it is difficult to perform a direct comparison with previous studies due to differences in equipment and software algorithms. Lum and colleagues reported an ULN for $LCI_{2.5\%}$ of 7.53 in healthy children who performed MBW measurements using a mass-spectrometer with sulfur hexafluoride (SF_6) as the tracer gas [17]. However, several studies have reported higher $LCI_{2.5\%}$ values

in N_2MBW compared with SF_6MBW [18, 32, 33]. These differences may be explained by different distribution of a resident gas compared with an exogenous gas in the lung tissue and/or by the contribution of N_2 diffusion from the lung tissue during the washout [18, 20]. Fuchs and colleagues reported an ULN for $LCI_{2.5\%}$ of 7.0 in healthy children who performed SF_6MBW using a device which utilizes a similar ultrasonic flowmeter measurement principle [16]. Recent data show that this device provides lower $LCI_{2.5\%}$ values compared to the device used in our study [19, 34]. In addition, Houltz and colleagues [24] reported an ULN for $LCI_{2.5\%}$ of 7.09 in healthy children who performed N_2MBW using the same equipment used in our study but they analysed their data using a custom-made software, so a direct comparison between the two datasets is not possible. These findings highlight the need for equipment and software specific normative data for MBW data.

Center differences

Slight differences in height and weight between centers were not surprising because the age distribution of the study participants differed between centers. Previous multi-center studies have reported differences in MBW indices using the same measuring equipment and protocol [17, 35]. In order to minimize the risk for between-center differences, the analysis was performed using the same software version, system settings, and equipment related dead space. We did not find any differences in LCI and moment ratio outcomes between centers. Small but statistically significant differences were found in FRC between centers. However, FRC z-scores were not different between centers. Therefore we hypothesize that any differences in FRC between centers were simply due to differences in demographic data.

Predictors of MBW outcomes

Previous studies have reported that $LCI_{2.5\%}$ is age-dependent from birth to adulthood [17, 25], however it is unclear whether this age-dependence continues during school-age and adolescence. Lum and colleagues reported that despite a small continuation of decline in $LCI_{2.5\%}$ throughout the entire pediatric range, changes were minimal once the child reached six years of age [17]. The authors reported that fixed upper limits of normal for $LCI_{2.5\%}$ could be used between the age of six and 19

years. We report small but statistically significant associations between age and LCI and moment ratios. However, the changes in LCI and moment ratios with age during the school-age period are minimal as shown in Figure 2, therefore we believe that upper limits of normal for LCI and moment ratios are appropriate throughout this age range.

We found that both height and weight independently predicted FRC in our cohort. This contrasts with data from Lum and colleagues who found that height, age, and sex were independent predictors of FRC in their cohort, however, the age range of their population extended from infancy to adolescence [17]. During the period from early childhood to adolescence, pubertal changes can result in higher variability in weight with increasing height. Therefore pubertal changes during this period likely influenced the association between FRC and weight, which appears to be independent of sex. Only seven out of 180 subjects in our study were classified as obese (≥ 2 body mass index z-scores) and therefore, it is possible that predicted FRC values could be underestimated in obese individuals.

Technical and physiological factors

In order to further understand the technical and physiological factors that may influence ventilation distribution outcome during childhood and adolescence, we investigated whether equipment related dead space and breathing pattern influenced results. It has been reported previously that Vd/V_T decreases with age during childhood [36] and Vd/V_T is positively associated with LCI outcomes [30]. We also found that Vd/V_T was positively associated with LCI and moment ratio outcomes. Users can ensure that the effect of Vd on MBW outcomes is minimized by using the appropriate dead space reducers recommended by the manufacturer. In addition, manufacturers of MBW devices should aim to further reduce equipment related dead space to avoid over-estimation of ventilation distribution outcomes in young children [13].

It has previously been shown that breathing pattern can influence MBW outcomes. Fixed 1L breathing protocols highly influenced LCI outcomes in children compared with relaxed breathing [29]. We found that the V_T/FRC ratio was negatively associated with age and positively associated with

ventilation distribution outcomes. These findings indicate that any small age-dependence of ventilation distribution outcomes in our study were likely to be influenced by age-dependent changes in Vd/V_T and V_T/FRC during the period from early childhood to adolescence.

Strengths and limitations

This study comprises the largest sample of healthy Caucasian children collected using a commercially available MBW device. All four centers have extensive experience in MBW testing and collected MBW data using the same equipment and measurement protocol. In addition, all the measurements were quality controlled and analysed using the same software version, equipment dead space volume, and system settings. However, the sample size is still relatively small for the generation of reference values. The quality control has been performed by a single operator, which could have produced a bias in the study. We did not report phase III slope indices as these estimates require additional breath-by-breath quality control, which is not yet standardised. We did not include preschool children in this cohort for several reasons, including the limited number of preschool visits available in our data set, lack of preschool MBW standardization at the time of measurement, and differences in interfaces used for testing at this age (facemask vs mouthpiece).

Recommendations for the use of reference values

MBW outcomes are considered to be device and tracer gas specific [18-20, 37], thus these reference values have been generated for N_2MBW data collected on the Eco Medics Exhalyzer D device. It is possible that the upper limits of normal we report for LCI and moment ratios are appropriate for other MBW systems but further work is needed to address this. Our data were analysed using the Spiroware software version 3.2.1 which uses different signal processing algorithms compared with previous versions (described in the online supplement) which have known influences on outcomes [21, 37]. Ideally, the most robust approach for Spiroware users would be to reload their raw data (A-files) collected in previous software versions into the new software version in order to reanalyze them. If this is not feasible, the users should recruit age-matched healthy controls at their own centre. In addition, as we only included subjects of Caucasian origin and it is unclear whether MBW indices

differ with ethnicity at this age range [17, 38], we cannot generalize these data to other ethnic groups. Future studies that include children of other ethnicities are needed.

Conclusion

This study provides reference values for N_2MBW outcomes in Caucasian school-aged children measured on the commercially available Eco Medics AG ultrasonic flowmeter device. Definition of the upper limits of normal over a wide age range will allow appropriate interpretation of MBW outcomes in the pediatric clinical setting.

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TABLES

	Bern	Toronto	Heidelberg	Perth	Total
Subjects (males)	82 (38)	28 (19)	33 (20)	37 (15)	180 (92)
No of trials	224	98	86	84	492
Age, years	10.8 (3.8)	12.5 (3.1)	12.0 (2.6)	9.5 (2.0)	11.0 (3.3)
Weight, kg	39.8 (17.0)	47.5 (15.5)	43.1 (16.0)	34.2 (10.4)	40.5 (15.9)
Weight, z-score	0.21 (0.83)	0.34 (0.93)	0.01 (1.02)	0.27 (0.77)	0.21 (0.87)
Height, cm	144.8 (21.9)	155.9 (17.8)	148.5 (14.6)	139.1 (12.8)	146.0 (19.1)
Height, z-score	0.40 (1.00)	0.69 (1.40)	-0.10 (1.06)	0.51 (0.69)	0.38 (1.05)
BMI	18.0 (3.2)	19.0 (3.6)	19.0 (2.8)	17.3 (2.7)	18.2 (3.1)
BMI, z-score	0.09 (0.90)	0.19 (1.03)	0.25 (1.20)	0.04 (0.83)	0.12 (0.97)
FRC, L	1.77 (0.89)	2.19 (0.87)	1.78 (0.62)	1.50 (0.38)	1.78 (0.78)

Table 1: Demographic and anthropometric characteristics of the study population per center. Data are presented as mean (SD). BMI: Body Mass Index; FRC: functional residual capacity. Weight, height and BMI z-scores were calculated according to CDC growth charts [39].

	Mean (SD)	LLN	ULN	Within-test intra-subject variability, % (SD)
$LCI_{2.5\%}$	7.04 (0.45)	6.16	7.91	4.77 (3.16)
$LCI_{5\%}$	5.10 (0.32)	4.47	5.73	4.47 (2.89)
M_1/M_0	1.58 (0.09)	1.40	1.75	4.10 (2.67)
M_2/M_0	4.97 (0.60)	3.79	6.15	8.07 (5.22)

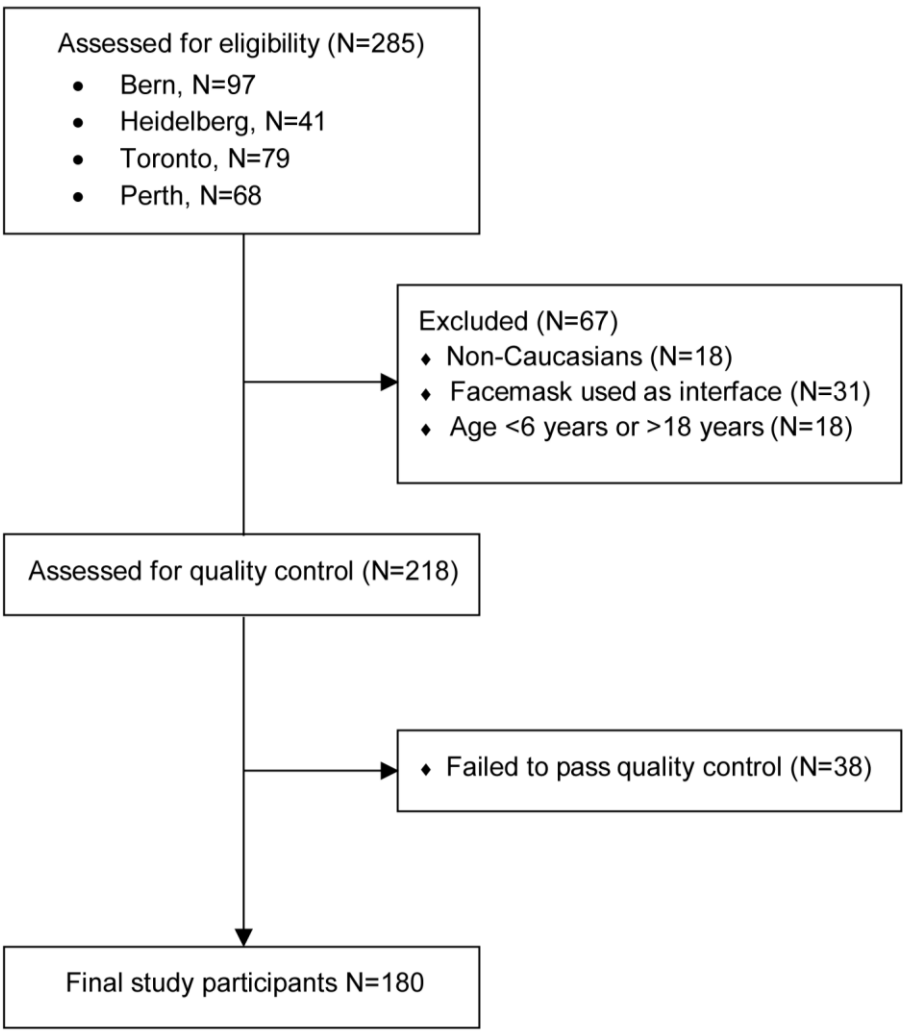
Table 2: Mean (SD) values, upper limit of normal (ULN), lower limit of normal (LLN), and intra-subject variability (SD) in multiple breath nitrogen washout outcomes from 180 healthy children. $LCI_{2.5\%}$ and $LCI_{5\%}$ = Lung clearance index at 2.5% and 5% of the initial nitrogen concentration, respectively. M_1/M_0 = moment ratio 1 and M_2/M_0 = moment ratio 2. ULN and LLN were calculated as mean ± 1.96SD. The intra-subject variability was calculated as % difference [(trial1-trial2)/mean%] for subjects with two trials, and coefficient of variation (CV=SD/mean%) for subjects with ≥ three trials.

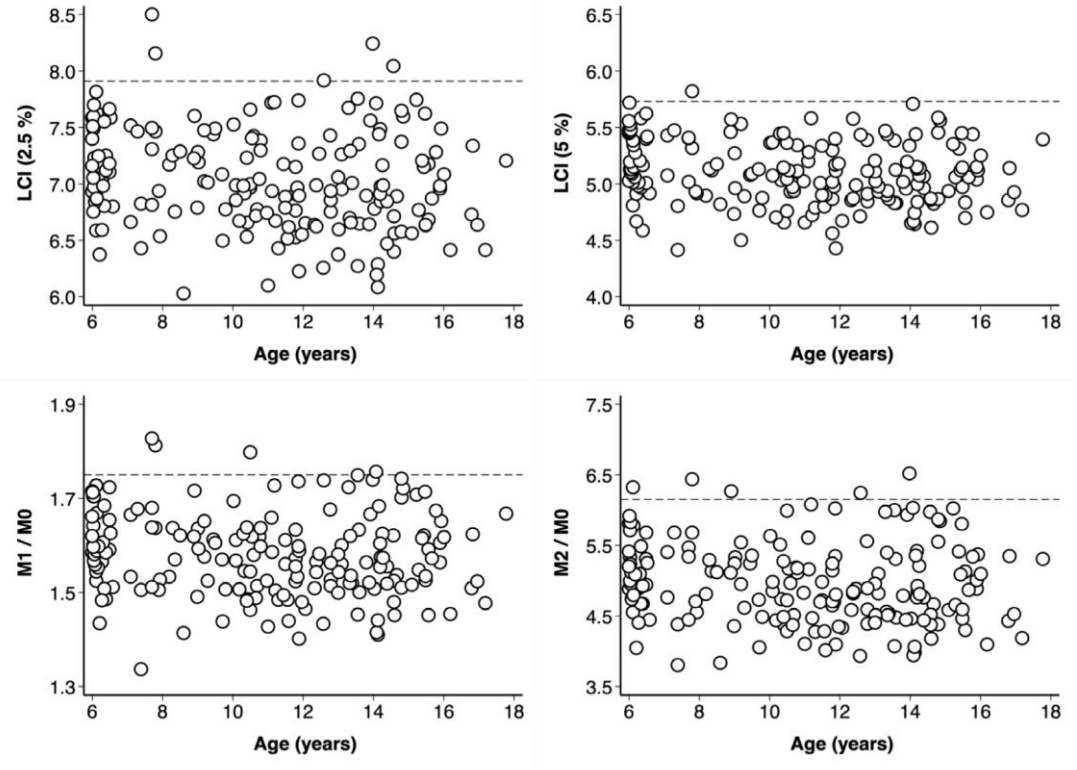
FIGURE TITLES

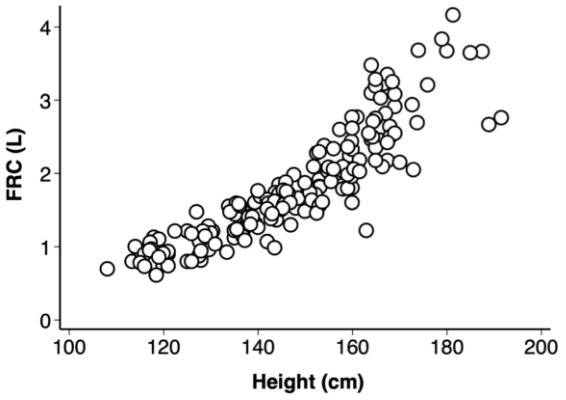
Figure 1: Flow chart of healthy subjects who participated in the study.

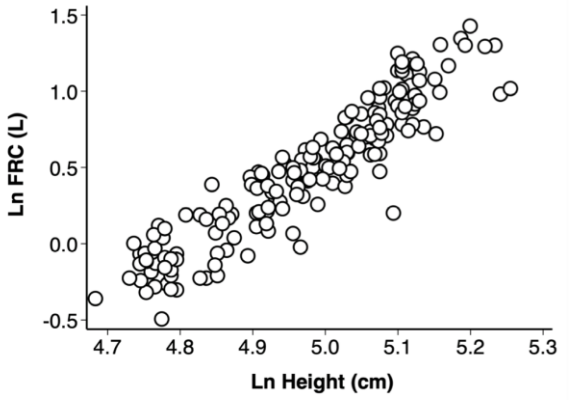
Figure 2: Relationship between age and lung clearance index at 2.5% ($LCI_{2.5\%}$), lung clearance index at 5% ($LCI_{5\%}$), moment ratio 1 (M_1/M_0), and moment ratio 2 (M_2/M_0). Dashed lines indicate upper limit of normal.

Figure 3: Relationship between functional residual capacity (FRC) and height.


Assessed for eligibility (N=285)
Bern, N=97
Heidelberg, N=41
Toronto, N=79
Perth, N=68
Excluded (N=67)
Non-Caucasians (N=18)
Facemask used as interface (N=31)
Age <6 years or >18 years (N=18)
Assessed for quality control (N=218)
Failed to pass quality control (N=38)
Final study participants N=180


LCI (2.5 %)
8.5
8.0
7.5
7.0
6.5
6.0
6
8
10
12
14
16
18
Age (years)
LCI (5 %)
6.5
6.0
5.5
5.0
4.5
4.0
6
8
10
12
14
16
18
Age (years)
M1 / M0
1.9
1.7
1.5
1.3
6
8
10
12
14
16
18
Age (years)
M2 / M0
7.5
6.5
5.5
4.5
3.5
6
8
10
12
14
16
18
Age (years)


FRC (L)
4
3
2
1
0
100
120
140
160
180
200
Height (cm)


Ln FRC (L)
1.5
1.0
0.5
0.0
-0.5
4.7
4.8
4.9
5.0
5.1
5.2
5.3
Ln Height (cm)

Normative data for multiple breath washout outcomes in school-aged Caucasian children

Pinelopi Anagnostopoulou, Philipp Latzin, Renee Jensen, Mirjam Stahl, Alana Harper, Sophie Yammine, Jakob Usemann, Rachel E. Foong, Ben Spycher, Graham L. Hall, Florian Singer, Sanja Stanojevic, Marcus Mall, Felix Ratjen, Kathryn A. Ramsey

ONLINE SUPPLEMENT

METHODS

MBW measurements

Each subject was tested in a single visit and performed at least two trials. During the test the child sat in an upright position wearing a nose-clip and was asked to breath regularly through a snorkel-like mouthpiece connected to a bacterial filter (Air Safety Eco Slimline, No. 4222/01), spirette, and dead space reducer (set 2 for subjects ≤35 kg, set 3 for subjects >35 kg). The washout was stopped following at least three tidal breaths below 1/40th of the pre-phase end-tidal N_2 concentration [13].

MBW analysis

The original temperature and pressure conditions were applied to the data and the software corrected appropriately for equipment-related dead space. The pre-capillary dead space was 33.3 ml for all measurements collected with both set 2 and set 3 [29]. The post-capillary dead space was either 9.5 ml for set 2 (children up to 35kg) or 22 ml for set 3 (children greater than 35kg).

Differences in the analysis between Spiroware 3.1.6 and Spiroware 3.2.1

The open-circuit hardware we used for N_2MBW (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) measures F_{N2} indirectly based on Dalton's law [E1]. A side-stream oxygen (O_2) sensor and a mainstream carbon dioxide (CO_2) sensor measure gas concentrations. These two signals must be aligned in order to allow the calculation of the N_2 signal. A static synchronization method has been used in previous software versions including Spiroware version 3.1.6 [E1]. During a measurement, flow, O_2, and CO_2 signals are not stable throughout the measurement and depend on the breathing pattern of the subject. Spiroware version 3.2.1 constantly calculates new delay times based on the breathing pattern (dynamic delay correction, DDC) [E2].

Apart from the different synchronization method, the calculation method of the re-inspired N_2 volume differs also between the two software versions. In Spiroware 3.1.6 this is calculated from the post-capillary dead space (number of breaths x post-capillary dead space) [E3], while Spiroware 3.2.1 uses the integral between inspiratory flow and N_2 concentration to calculate it [E4].

Reloading measurements in Spiroware 3.2.1

To ensure that our data were analyzed using the new DDC synchronization method we reloaded the raw data (recorded in Spiroware 3.1.6) into Spiroware 3.2.1. First, we performed a new synchronization of the signals by reloading the raw data (A-files) from one measurement per subject into the channel synchronization tool in Spiroware. Five consecutive breaths of good quality from the pre-washout phase were used to generate new static delay times. Each trial was then reloaded manually into Spiroware 3.2.1 to ensure calculation of the DDC was applied. The quality of the signal alignment was checked by visual control. The original temperature and pressure conditions were applied to the data and the software corrected appropriately for equipment-related dead space.

Quality control of MBW trials

The following criteria were used to assess quality of the entire dataset of MBW measurements after reloading in SPW 3.2.1: no evidence of a leak, stable pre-washout phase, regular breathing pattern during the washout, and at least three consecutive breaths with the end tidal concentration of N_2 below 2.5% of the pre-washout phase N_2 concentration[E3]. In addition, trials with a drift in end tidal CO_2 concentration out of the range of 4-6% across the washout were excluded[E5]. Tests with at least two technically acceptable trials with FRC values within 25% of the mean were included for analysis.

RESULTS

FRC equation

The full regression equation for FRC is given below. The standard deviation of the residuals for the model was 0.1632.

Ln FRC = -18.18016 + 3.98197*ln(height) – 0.31707*ln(weight)

Univariate Analysis	Coefficient	95% Confidence interval	p-value
$LCI_{2.5\%}$			
Height (cm)	-0.0053	-0.0087; -0.0019	**0.002**
Weight (kg)	-0.0049	-0.0090; -0.0008	**0.020**
Age (y)	-0.0225	-0.0421; -0.0030	**0.024**
Vd/V_T (%)	0.0258	0.0093; 0.0422	**0.002**
V_T/FRC (%)	0.0040	-0.0013; 0.0093	0.135
Sex	0.1413	-0.0113; 0.2712	0.133
$LCI_{5\%}$			
Height (cm)	-0.0046	-0.0070; -0.0023	**<0.001**
Weight (kg)	-0.0040	-0.0069; -0.0011	**0.007**
Age (y)	-0.0178	-0.0316; -0.0039	**0.012**
Vd/V_T (%)	0.0160	0.0042; 0.0278	**0.008**
V_T/FRC (%)	0.0073	0.0037; 0.0109	**<0.001**
Sex	0.0867	-0.0058; 0.1796	0.066
M_1/M_0			
Height (cm)	-0.0009	-0.0016; -0.0002	**0.009**
Weight (kg)	-0.0008	-0.0016; 0.0002	0.060
Age (y)	-0.0030	-0.0069; 0.0009	0.133
Vd/V_T (%)	0.0050	0.0017; 0.0083	**0.003**
V_T/FRC (%)	0.0010	-0.0001; 0.0020	0.069
Sex	0.0274	-0.0015; 0.0532	0.382
M_2/M_0			
Height (cm)	-0.0068	-0.0114; -0.0023	**0.003**
Weight (kg)	-0.0058	-0.0114; -0.0003	**0.040**
Age (y)	-0.0249	-0.0514; 0.0016	0.065
Vd/V_T (%)	0.0161	-0.0066; 0.03871	0.164
V_T/FRC (%)	0.0147	0.0079; 0.0215	**<0.001**
Sex	0.1475	-0.02858; 0.3236	0.100
Ln FRC (L)			
Ln Height (cm)	3.0997	2.9136; 3.286	**<0.001**
Ln Weight (kg)	0.9668	0.8778; 1.0558	**<0.001**
Ln Age (y)	1.1836	1.0857; 1.2814	**<0.001**
Ln Vd/V_T (%)	-0.6429	-0.8042; -0.4815	**<0.001**
Ln V_T (L)	0.6918	0.5694; 0.8142	**<0.001**
Sex	-0.0270	-0.1580; 0.1039	0.684

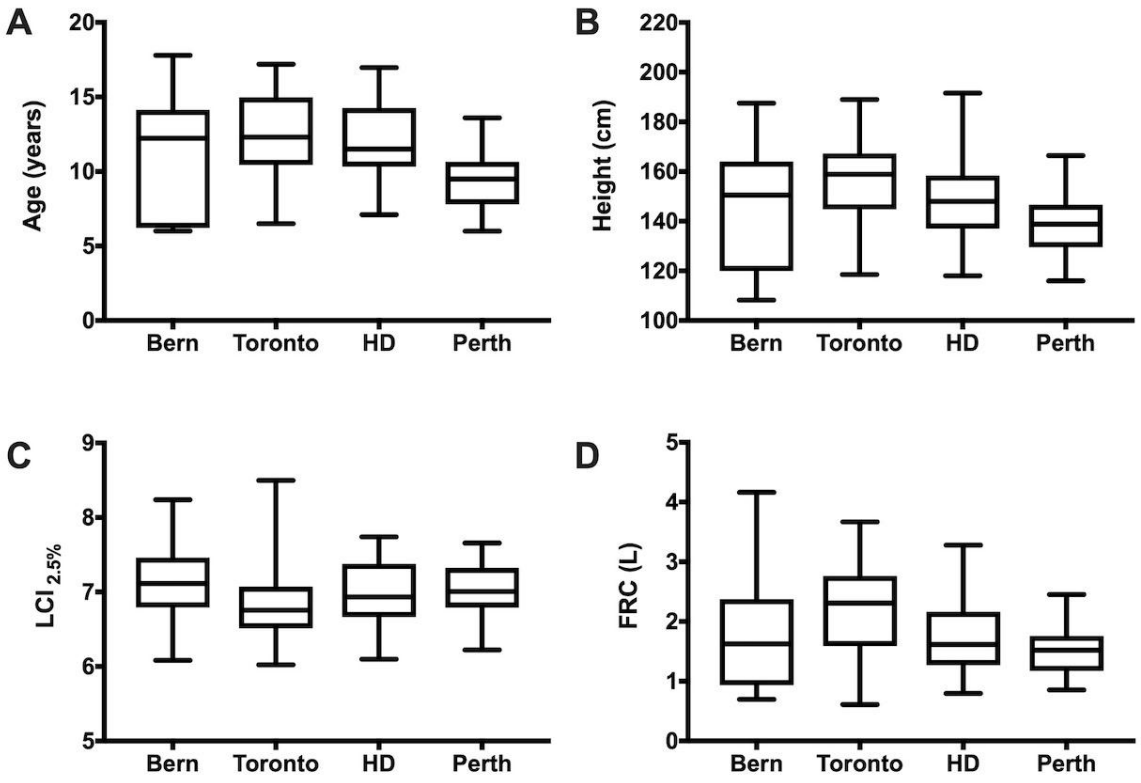
Supplemental Table 1: Univariate linear regression model describing the association between lung clearance index at 2.5% ($LCI_{2.5\%}$) and 5% ($LCI_{5\%}$), moment ratios 1 (M_1/M_0) and 2 (M_2/M_0), and functional residual capacity (FRC) with demographic and physiological parameters. V_T/FRC (%): tidal volume/functional residual capacity, Vd/V_T (%): dead space volume / tidal volume.

Multivariate Analysis	Coefficient	95% Confidence interval	p-value
$LCI_{2.5\%}$			
Age (y)	0.04	0.01; 0.07	0.02
Vd/V_T (%)	0.07	0.04; 0.10	<0.001
V_T/FRC (%)	0.02	0.01; 0.02	<0.001
$LCI_{5\%}$			
Age (y)	0.04	0.02; 0.06	<0.001
Vd/V_T (%)	0.07	0.05; 0.08	<0.001
V_T/FRC (%)	0.02	0.01; 0.02	<0.001
M_1/M_0			
Age (y)	0.01	0.01; 0.02	<0.001
Vd/V_T (%)	0.02	0.01; 0.02	<0.001
V_T/FRC (%)	0.004	0.00; 0.005	<0.001
M_2/M_0			
Age (y)	0.07	0.03; 0.10	<0.01
Vd/V_T (%)	0.10	0.06; 0.14	<0.001
V_T/FRC (%)	0.03	0.02; 0.04	<0.001
Ln FRC (L)			
ln Height (cm)	3.98	3.43; 4.54	<0.001
ln Weight (kg)	-0.32	-0.51; -0.13	<0.01

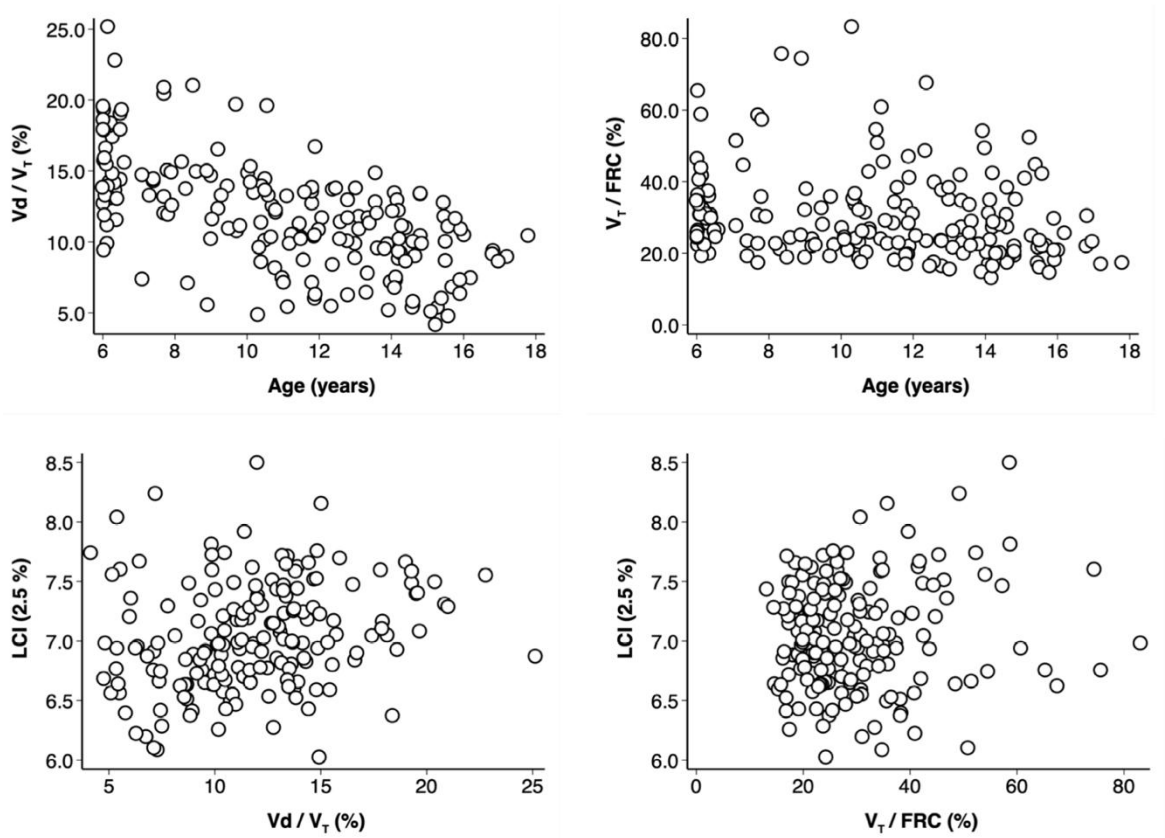
Supplemental Table 2: Final multiple linear regression model describing the association between lung clearance index at 2.5% ($LCI_{2.5\%}$) and 5% ($LCI_{5\%}$), moment ratios 1 (M_1/M_0) and 2 (M_2/M_0), and functional residual capacity (FRC) with demographic and physiological parameters. V_T/FRC (%): tidal volume/functional residual capacity, Vd/V_T (%): dead space volume / tidal volume.

Figures

Supplemental Figure 1: Distribution of age, height, lung clearance index at 2.5% ($LCI_{2.5\%}$) and functional residual capacity (FRC) per study center. HD indicates Heidelberg.



Supplemental Figure 2: Association of Vd/V_T (%) and V_T/FRC (%) with age, and association of LCI 2.5% with Vd/V_T (%) and V_T/FRC (%) in 180 healthy children.



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E5. Jensen R, Stanojevic S, Klingel M, Pizarro ME, Hall GL, Ramsey K, Foong R, Saunders C, Robinson PD, Webster H, Hardaker K, Kane M, Ratjen F. A Systematic Approach to Multiple Breath Nitrogen Washout Test Quality. PLoS One 2016: 11: e0157523.