



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

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This study provides reference values for nitrogen multiple breath washout outcomes in healthy Caucasian children from 6 to 18 years old, measured with a commercially available device <http://bit.ly/2rsab8v>

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## ABSTRACT

**Background:** The multiple breath nitrogen washout (N<sub>2</sub>MBW) 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<sub>2</sub>MBW outcomes in a cohort of healthy Caucasian school-aged children.

**Methods:** N<sub>2</sub>MBW data from healthy Caucasian school-age children between 6 and 18 years old were collected from four experienced centres. Measurements were performed using an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and were analysed with commercial software (Spiroware version 3.2.1, Eco Medics AG). Normative values and upper limits of normal (ULN) were generated for lung clearance index (LCI) at 2.5% (LCI<sub>2.5%</sub>) and at 5% (LCI<sub>5%</sub>) of the initial nitrogen concentration and for moment ratios (M<sub>1</sub>/M<sub>0</sub> and M<sub>2</sub>/M<sub>0</sub>). A prediction equation was generated for functional residual capacity (FRC).

**Results:** Analysis used 485 trials from 180 healthy Caucasian children aged from 6 to 18 years old. While LCI increased with age, this increase was negligible (0.04 units·year<sup>-1</sup> for LCI<sub>2.5%</sub>) and therefore fixed ULN were defined for this age group. These limits were 7.91 for LCI<sub>2.5%</sub>, 5.73 for LCI<sub>5%</sub>, 1.75 for M<sub>1</sub>/M<sub>0</sub> and 6.15 for M<sub>2</sub>/M<sub>0</sub>, respectively. Height and weight were found to be independent predictors of FRC.

**Conclusion:** We report reference values for N<sub>2</sub>MBW outcomes measured on a commercially available ultrasonic flowmeter device (Exhalyzer D, Eco Medics AG) in healthy school-aged children to allow accurate interpretation of ventilation distribution outcomes and FRC in children with lung disease.

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## Introduction

The multiple breath washout (MBW) test provides lung volume and ventilation distribution outcomes that are more sensitive than conventional lung function outcomes for detecting 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 test 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]. It may potentially also be a future factor for the clinical surveillance of children with CF [12].

LCI is traditionally calculated as the number of lung turnovers required to wash out a tracer gas to one fortieth (1/40th) of its starting concentration ( $LCI_{2.5\%}$ ) [13]. The number of lung turnovers required to wash out a tracer gas to one twentieth (1/20th) of its initial concentration ( $LCI_{5\%}$ ) has also been calculated [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 for detecting 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 multiple breath nitrogen washout ( $N_2$ MBW) outcomes (LCI, moment ratios and FRC) in school-aged children, as measured with a commercially available device (the Exhalyzer D ultrasonic flowmeter (Eco Medics AG, Duernten, Switzerland)) and to investigate the association of MBW outcomes with anthropometric and physiological parameters, including tidal volume ( $V_T$ ) and equipment-related dead space volume ( $V_D$ ) [24, 25]. To achieve this, we collected data from healthy children in four different centres using the same MBW device and protocol, and analysed these data 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 paediatric centres specialised in  $N_2$ MBW, 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 less than 6 years or greater than 18 years, chronic respiratory or cardiac disease, respiratory infection during the last 4 weeks prior to measurement and other major systemic diseases with potential influence on lung function [26]. Healthy individuals from each centre took part in prospective observational studies and therefore some data have been published previously [2, 8, 12, 18, 27, 28]. The study was approved by local ethics committees (Ethics Committee of the Canton of Bern, Bern, Switzerland; Research Ethics Board at SickKids, Toronto, Canada; Ethics Committee of the University of Heidelberg, Heidelberg, Germany; Princess Margaret Hospital Human Ethics Committee, Perth, Australia). Parents or caregivers provided informed written consent.

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### **MBW measurements**

We performed N<sub>2</sub>MBW measurements using an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and the software provided by the manufacturer (Spiroware version 3.1.6, Eco Medics AG) as previously described [12, 18]. All centres used the same equipment and performed the calibration and measurement using the same protocol (details are available in the supplementary material).

### **MBW analysis**

N<sub>2</sub>MBW data originally recorded in Spiroware version 3.1.6 were reloaded and analysed using the updated version of the software provided by the manufacturer at the time the study was performed (Spiroware version 3.2.1, Eco Medics AG). Further details are provided in the supplementary material.

### **MBW outcomes and physiological indices**

FRC, LCI<sub>2.5%</sub>, LCI<sub>5%</sub> and moment ratios ( $M_1/M_0$  and  $M_2/M_0$ ) were calculated according to current recommendations [13, 14]. The mean  $V_T$  for each trial was provided by the software. In order to investigate the effect of breathing pattern [29] and equipment-related  $V_D$  [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 (expressed as percentages).

### **Quality control of MBW trials**

Quality control of MBW trials was assessed by an experienced operator at the Inselspital, Bern (P. Anagnostopoulou) according to the 2013 American Thoracic Society (ATS)/European Respiratory Society (ERS) MBW consensus guidelines [13] and additional criteria proposed by JENSEN *et al.* [31]. Further details are provided in the supplementary material. 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 (The R Project for Statistical Computing, [www.r-project.org](http://www.r-project.org)) and Stata statistical software, release 14 (StataCorp LP, College Station, TX, USA). Graphs were generated using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, 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 97.5th percentile ( $\text{mean} \pm 1.96\text{SD}$ ), as previously described [17]. The intra-subject variability was defined as the mean relative difference for subjects with two trials ( $(\text{trial}_1 - \text{trial}_2) / \text{mean}$  (%)) or the coefficient of variation (CV;  $\text{SD} / \text{mean}$  (%)) for those with at least three trials. We used one-way ANOVA with a Bonferroni *post-hoc* test for between-centre comparisons. Multiple linear regression was performed to assess the associations of normally distributed primary outcomes (LCI<sub>2.5%</sub>, LCI<sub>5%</sub>,  $M_1/M_0$ ,  $M_2/M_0$  and FRC) with demographic factors (age, weight, height and sex) and physiological factors ( $V_T/FRC$  and  $V_D/V_T$ ). 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 the 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  $p < 0.05$ .

## **Results**

### **Study subjects**

N<sub>2</sub>MBW data from 285 healthy children were assessed for eligibility. Of these, 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 years), 33 children from the University Children’s Hospital Heidelberg, Germany (mean age: 12.0 years; range: 7.1–17.0 years), 28 children from the SickKids in Toronto, Canada (mean age: 12.5 years; range: 6.5–17.2 years) and 37 children from the Telethon Kids Institute, Perth, Australia (mean age: 9.5 years; range: 6.0–13.6 years). 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.

### **Centre 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 ( $p < 0.001$ ) (supplementary figure S1). 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 centres; however, there were significant differences in FRC, whereby

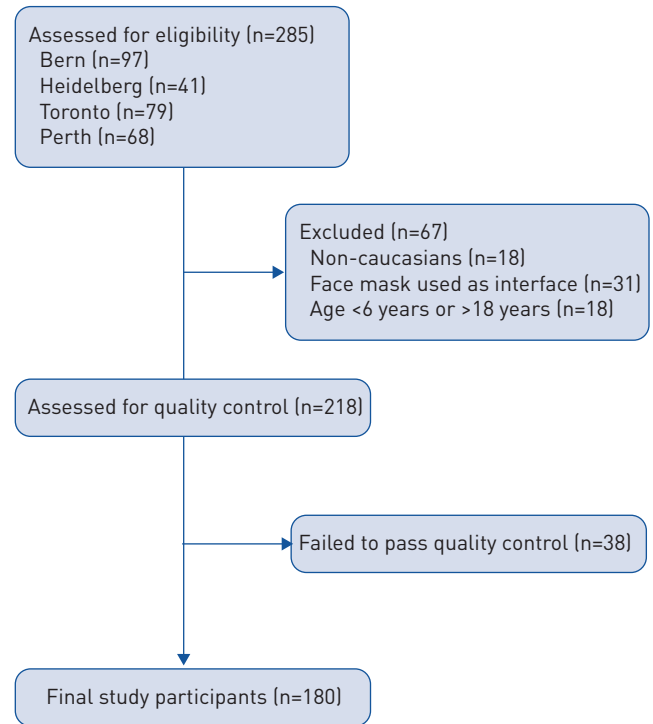


FIGURE 1 Flow chart of healthy subjects who participated in the study

children from Toronto had higher FRC values compared to children from Perth ( $p=0.02$ ) (supplementary figure S1).

**Lung clearance index and moment ratios**

In univariable regression analyses,  $LCI_{2.5\%}$  was negatively associated with height (coefficient  $-0.005$ , 95% CI  $-0.0087$  to  $-0.0019$ ;  $p=0.002$ ), weight (coefficient  $-0.0049$ , 95% CI  $-0.0090$  to  $-0.0008$ ;  $p=0.020$ ) and age (coefficient  $-0.0225$ , 95% CI  $-0.0421$  to  $-0.0030$ ;  $p=0.024$ ), and positively associated with  $V_D/V_T$  (coefficient  $0.0258$ , 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 (supplementary table S1). In addition,  $V_D/V_T$  and  $V_T/FRC$  were both negatively associated with age

TABLE 1 Demographic and anthropometric characteristics of the study population per centre

	Bern	Toronto	Heidelberg	Perth	Total
<b>Subjects n</b>	82	28	33	37	180
Male sex n	38	19	20	15	92
<b>Trials n</b>	224	98	86	84	492
<b>Age years</b>	10.8±3.8	12.5±3.1	12.0±2.6	9.5±2.0	11.0±3.3
<b>Weight<sup>#</sup></b>					
In kg	39.8±17.0	47.5±15.5	43.1±16.0	34.2±10.4	40.5±15.9
By z-score	0.21±0.83	0.34±0.93	0.01±1.02	0.27±0.77	0.21±0.87
<b>Height<sup>#</sup></b>					
In cm	144.8±21.9	155.9±17.8	148.5±14.6	139.1±12.8	146.0±19.1
By z-score	0.40±1.00	0.69±1.40	-0.10±1.06	0.51±0.69	0.38±1.05
<b>BMI<sup>#</sup></b>					
In kg·m <sup>-2</sup>	18.0±3.2	19.0±3.6	19.0±2.8	17.3±2.7	18.2±3.1
By z-score	0.09±0.90	0.19±1.03	0.25±1.20	0.04±0.83	0.12±0.97
<b>FRC L</b>	1.77±0.89	2.19±0.87	1.78±0.62	1.50±0.38	1.78±0.78

Data are presented as mean±SD unless otherwise stated. BMI: body mass index; FRC: functional residual capacity; CDC: Centers for Disease Control and Prevention. #: weight, height and BMI z-scores were calculated according to CDC growth charts [32].

(supplementary figure S2), while  $LCI_{5\%}$  and  $M_1/M_0$  were positively associated with  $V_D/V_T$  and  $V_T/FRC$  (supplementary table S1 and supplementary figure S2). There was no evidence for association of  $LCI_{5\%}$  and moment ratios with sex ( $p>0.05$ ) (supplementary table S1).

In a multivariable regression model age,  $V_D/V_T$  and  $V_T/FRC$  were independently associated with LCI and moment ratio outcomes (supplementary table S2). 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 units·year<sup>-1</sup> (95% CI 0.01–0.07). This equates to a maximum 0.5 unit change in  $LCI_{2.5\%}$  over 12 years (from 6 years to 18 years of age). Thus, in our judgement, the age related changes in LCI and moment ratios are negligible. Therefore, we report fixed ULN during this age interval (figure 2).

The mean LLN and ULN for LCI and moment ratios are provided in table 2. The ULN for  $LCI_{2.5\%}$  was 7.91 and for  $LCI_{5\%}$  was 5.73. For  $M_1/M_0$  the ULN was 1.75 and for  $M_2/M_0$  was 6.15 (figure 2).

**Functional residual capacity**

FRC values were right skewed and therefore natural log (ln) transformed FRC values and predictors were used in the models.  $\ln(FRC)$  was positively associated with  $\ln(\text{height})$ ,  $\ln(\text{weight})$ ,  $\ln(V_T)$  and  $\ln(\text{age})$ , and negatively associated with  $\ln(V_D/V_T)$  in the univariate analysis (figure 3 and supplementary table 1). There was no evidence of association with sex ( $p=0.68$ ) (supplementary table 1). In a multivariable model,  $\ln(FRC)$  was independently associated with  $\ln(\text{height})$  and  $\ln(\text{weight})$ . 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 supplementary material. See also equations 1 and 2 (where FRC is expressed in L, height in cm and weight in kg).

$$\text{Predicted FRC} = e^{-18.18} \cdot \text{height}^{3.98} \cdot \text{weight}^{-0.32} \tag{1}$$

$$\text{z-score FRC} = \frac{\ln(\text{measured FRC} - \text{predicted FRC})}{0.1632} \tag{2}$$

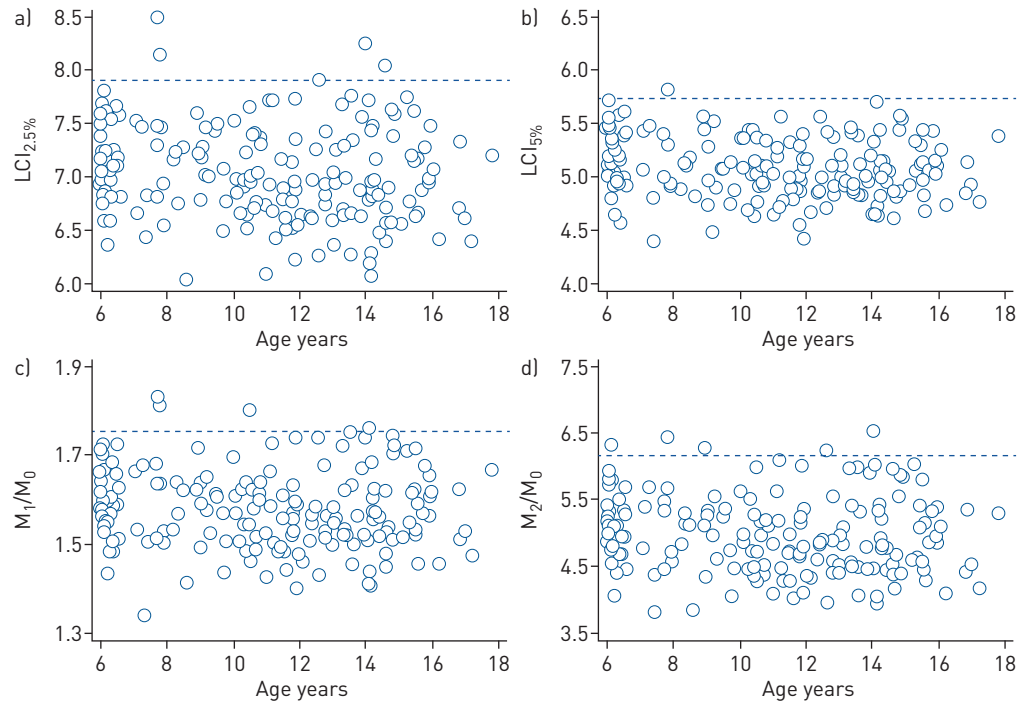


FIGURE 2 Relationship between age and lung clearance index (LCI) at (a) 2.5% ( $LCI_{2.5\%}$ ), (b) 5% ( $LCI_{5\%}$ ), (c) moment ratio one ( $M_1/M_0$ ) and (d) moment ratio two ( $M_2/M_0$ ). Dashed lines indicate the upper limit of normal (ULN)

TABLE 2 Variability in multiple breath nitrogen washout (N<sub>2</sub>MBW) outcomes from 180 healthy children

Outcome	Mean±sd	LLN <sup>#</sup>	ULN <sup>#</sup>	Within-test intra-subject variability %±sd
LCI <sub>2.5%</sub>	7.04±0.45	6.16	7.91	4.77±3.16
LCI <sub>5%</sub>	5.10±0.32	4.47	5.73	4.47±2.89
M <sub>1</sub> /M <sub>0</sub>	1.58±0.09	1.40	1.75	4.10±2.67
M <sub>2</sub> /M <sub>0</sub>	4.97±0.60	3.79	6.15	8.07±5.22

Data are presented as mean±sd unless otherwise stated. LLN: lower limit of normal; ULN: upper limit of normal; LCI<sub>x</sub>: lung clearance index at x% of the initial nitrogen concentration; M<sub>1</sub>/M<sub>0</sub>: moment ratio one; M<sub>2</sub>/M<sub>0</sub>: moment ratio two. <sup>#</sup>: LLN and ULN were calculated as mean±1.96sd. The intra-subject variability was calculated as the % difference  $[(\text{trial}_1 - \text{trial}_2) / \text{mean} (\%)]$  for subjects with two trials and as the coefficient of variation (CV;  $\text{sd} / \text{mean} (\%)$ ) for subjects with three or more trials.

## Discussion

Normative N<sub>2</sub>MBW data for a paediatric Caucasian population between the ages of 6 and 18 years is reported using commercially available equipment and software. While a significant age dependency is observed for LCI and moment ratio outcomes, the magnitude of this effect is small during the age interval of 12 years. Therefore, fixed ULN for LCI and moment ratios can be used in this age group. FRC was independently predicted by both height and weight, and FRC predicted values and a z-score equation are provided.

### Comparison with the literature

The LCI<sub>2.5%</sub> 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 *et al.* [17] reported an ULN for LCI<sub>2.5%</sub> of 7.53 in healthy children who performed MBW measurements using a mass spectrometer with sulfur hexafluoride (SF<sub>6</sub>) as the tracer gas. However, several studies have reported higher LCI<sub>2.5%</sub> values in N<sub>2</sub>MBW compared with SF<sub>6</sub> MBW [18, 33, 34]. 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 nitrogen diffusion from the lung tissue during the washout [18, 20]. FUCHS *et al.* [16] reported an ULN for LCI<sub>2.5%</sub> of 7.0 in healthy children who performed SF<sub>6</sub> MBW using a device which utilises a similar ultrasonic flowmeter measurement principle. Recent data show that this device provides lower LCI<sub>2.5%</sub> values compared to the device used in our study [19, 35]. In addition, HOULTZ *et al.* [24] reported an ULN for LCI<sub>2.5%</sub> of 7.09 in healthy children who performed N<sub>2</sub>MBW using the same equipment used in our study; however, they analysed their data using custom-made software and as such 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.

### Centre differences

Slight differences in height and weight between centres were not surprising because the age distribution of the study participants differed between these centres. Previous multi-centre studies have reported differences in MBW indices using the same measuring equipment and protocol [17, 36]. In order to

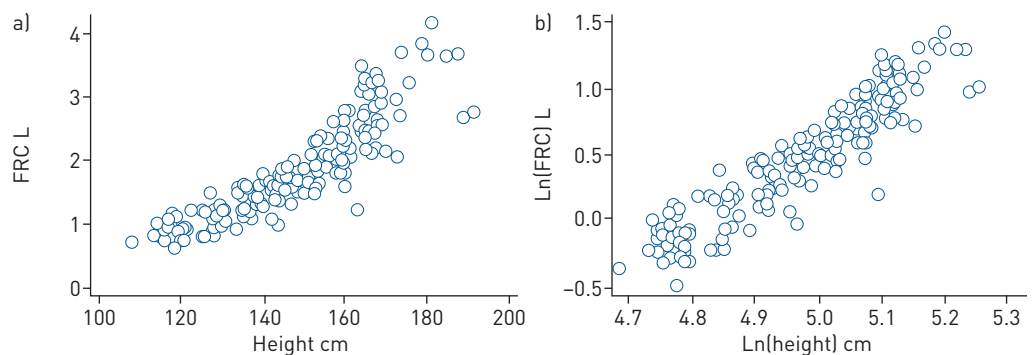


FIGURE 3 Relationship between functional residual capacity (FRC) and height expressed (a) linearly or (b) as natural logarithms

minimise the risk for between-centre differences, the analysis was performed using the same software version, system settings and equipment-related  $V_D$ . We did not find any differences in LCI and moment ratio outcomes between centres. Small but statistically significant differences were found in FRC between centres; however, FRC z-scores were not different between centres. Therefore we hypothesise that any differences in FRC between centres 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 *et al.* [17] reported that despite a small continuation of decline in  $LCI_{2.5\%}$  throughout the entire paediatric range, changes were minimal once the child reached 6 years of age. The authors reported that fixed ULN could be used for  $LCI_{2.5\%}$  between the ages of 6 and 19 years. We report small but statistically significant associations between age and LCI as well as between age 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 ULN 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 *et al.* [17], who found that height, age and sex were independent predictors of FRC in their cohort. It should be noted, however, that the age range of their population extended from infancy to adolescence. 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 (body mass index (BMI) z-scores  $\geq 2$ ) 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  $V_D$  and breathing pattern influenced results. It has been reported previously that  $V_D/V_T$  decreases with age during childhood [37] and that  $V_D/V_T$  is positively associated with LCI outcomes [30]. We also found that  $V_D/V_T$  was positively associated with LCI and moment ratio outcomes. Users can ensure that the effect of  $V_D$  on MBW outcomes is minimised by using the appropriate  $V_D$  reducers recommended by the manufacturer. In addition, manufacturers of MBW devices should aim to further reduce equipment-related  $V_D$  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 1 L 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  $V_D/V_T$  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 centres 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  $V_D$  and system settings. However, the sample size is still relatively small for the generation of reference values. In addition, 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. Furthermore, 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 standardisation at the time of measurement and differences in interfaces used for testing at this age (*i.e.* facemask *versus* mouthpiece).

#### **Recommendations for the use of reference values**

MBW outcomes are considered to be device and tracer gas specific [18–20, 38] and these reference values have been generated from  $N_2$ MBW data collected on the Eco Medics Exhalyzer D device. It is possible that the ULN 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 Spiroware version 3.2.1, which uses different signal processing algorithms compared with previous versions (as described in the supplementary material) and which has a known influence on outcomes [21, 38]. 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 re-analyse 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, 39], we cannot generalise these data to other ethnic groups. Future studies that include children of other ethnicities are needed.

### Conclusion

This study provides reference values for N<sub>2</sub>MBW outcomes in Caucasian school aged children measured on the commercially available Eco Medics AG ultrasonic flowmeter device. Definition of the ULN over a wide age range will allow appropriate interpretation of MBW outcomes in the paediatric clinical setting.

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