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The Single-Breath Diffusing Capacity of CO and NO in Healthy Children of European Descent

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Abstract

Rationale: The diffusing capacity (D_L) of the lung can be divided into two components: the diffusing capacity of the alveolar membrane (Dm) and the pulmonary capillary volume (Vc). D_L is traditionally measured using a single-breath method, involving inhalation of carbon monoxide, and a breath hold of 8–10 seconds ($D_{L,CO}$). This method does not easily allow calculation of Dm and Vc. An alternative single-breath method ($D_{L,CO,NO}$), involving simultaneous inhalation of carbon monoxide and nitric oxide, and traditionally a shorter breath hold, allows calculation of Dm and Vc and the $D_{L,NO}/D_{L,CO}$ ratio in a single respiratory maneuver. The clinical utility of Dm, Vc, and $D_{L,NO}/D_{L,CO}$ in the pediatric age range is currently unknown but also restricted by lack of reference values.

Objectives: The aim of this study was to establish reference ranges for the outcomes of $D_{L,CO,NO}$ with a 5 second breath hold, including the calculated outcomes Dm, Vc, and the $D_{L,NO}/D_{L,CO}$ ratio, as well as to establish reference values for the outcomes of the traditional $D_{L,CO}$ method, with a 10 second breath hold in children.

Methods: $D_{L,CO,NO}$ and $D_{L,CO}$ were measured in healthy children, of European descent, aged 5–17 years using a Jaeger Masterscreen PFT. The data were analyzed using the Generalized Additive Models for Location Scale and Shape (GAMLSS) statistical method.

Measurements and Main Results: A total of 326 children were eligible for diffusing capacity measurements, resulting in 312 measurements of $D_{L,CO,NO}$ and 297 of $D_{L,CO}$, respectively. Reference equations were established for the outcomes of $D_{L,CO,NO}$ and $D_{L,CO}$, including the calculated values: Vc, Dm, and the $D_{L,NO}/D_{L,CO}$ ratio.

Conclusion: These reference values are based on the largest sample of children to date and may provide a basis for future studies of their clinical utility in differentiating between alterations in the pulmonary circulation and changes in the alveolar membrane in pediatric patients.

Introduction

The transfer factor of the lung for a gas, is often called the diffusing capacity of the lung (D_L). D_L for an inhaled gas reactive with hemoglobin is the flow of that gas from the alveoli to the blood for a unit difference in pressure. D_L can be divided into two components: the diffusing capacity of the pulmonary membrane (Dm) and the chemical reaction of the gas binding to the blood. The latter is determined by the specific conductance of blood for a given gas, Θ, and the capillary volume of the lung (Vc).

The single-breath method was first introduced in 1915 [1]. Today, the single-breath D_L of carbon monoxide (CO) using a breath-hold of 10 seconds ($D_{L,CO,10s}$) is the most frequently used method with the current ATS/ERS methodological guidelines [2].

In 1957, Roughton and Forster proposed a method of calculating Dm and Vc, using $D_{L,CO,10s}$, which required arterial samples and two respiratory maneuvers at two different oxygen tensions [3]. In 1987, Guénard, Varène and Vaida [4] proposed an alternative method ($D_{L,CO,NO}$) of determining Vc and Dm involving simultaneous inhalation of CO and nitric oxide (NO). Both CO and NO transfer are diffusion limited, but NO has approximately twice the physical diffusivity of CO, and the affinity to hemoglobin for NO (Θ_{NO}) is approximately 250 times greater [5]. The implications have been described in detail elsewhere, but in summary Θ_{NO} was previously assumed infinitely great [4]. However, recent studies have challenged this assumption, leading to proposal of a finite value of Θ_{NO}. The consequence of the use of a finite value for NO blood conductance is that $D_{L,NO}$ appears equally dependent on Dm and Vc as $D_{L,CO}$ is mainly dependent on Vc. [6], [7].

The calculation of Dm and Vc involves the resistance of the red blood cell to gas transfer (Θ_{gas}), but no consensus currently exists about the true value of Θ_{CO}.

With the previous assumption of an infinite value of Θ_{NO}, calculation of the $D_{L,NO}/D_{L,CO}$ ratio was thought to provide useful information about the differentiation between primary alveolar membrane impairment (low $D_{L,NO}/D_{L,CO}$ ratio) [8], [9], [10] or abnormalities of the pulmonary circulation (high $D_{L,NO}/D_{L,CO}$ ratio) [11], potentially providing additional insights into more specific factors affecting D_L [12]. Now that a finite value of Θ_{NO} has been determined, new interpretations of the ratio will be necessary.

Determination of Vc and Dm using $D_{L,CO,NO}$ requires a single respiratory maneuver and allows simultaneous determination of $D_{L,CO}$, $D_{L,NO}$, as well as

calculation of $D_{L,NO}/D_{L,CO}$, Dm, and Vc. In addition, $D_{L,CO,NO}$ generally involves a shorter breath-hold due to the fast disappearance of NO [4]. The present study used a breath-hold of 5 seconds ($D_{L,CO,NO,5s}$). Reference equations for these outcomes of $D_{L,CO,NO,5s}$ in children are scarce. A study involving 50 children over 8 years of age has been published [13], as well as a more recent study involving 85 healthy North African boys, aged 8–16 years [14] whereas two larger studies recently produced reference equations for the more frequently used outcomes of $D_{L,CO,10s}$ [15], [16].

Despite similarities in the performed respiratory maneuver $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ are two distinctly separate methods, with multiple methodological differences.

The primary goal of this study was to calculate reference equations for the outcomes of $D_{L,CO,NO,5s}$ including Dm, Vc, and the $D_{L,NO}/D_{L,CO}$ ratio, in healthy children. Since no consensus guidelines exist for $D_{L,CO,NO,5s}$ and previous data is limited, contemporary measurement of the frequently used $D_{L,CO,10s}$ was performed to allow assessment of correlation between these two substantially different techniques and to assess whether they could be used interchangeably, although, knowing for a fact, that significant methodological differences exist. The resulting measurements of $D_{L,CO,10s}$ allowed establishment of reference equations and comparison with existing published reference equations for $D_{L,CO,10s}$. Some of the results of this study have been previously reported in the form of an abstract [17].

Materials and Methods

The regional ethics committee of Copenhagen (''De Videnskabsetiske Komiteer i Region Hovedstaden'') approved the project, and all subjects and/or their parents provided written, informed consent (approval number: H-4-2011-111).

Design and Subjects

In this cross-sectional, single-center study, healthy children and adolescents aged between 5 and 17 years were recruited from December 2011 to August 2012 from a private combined elementary and high school in Copenhagen, a public elementary school in rural Denmark, and among the healthy siblings of patients, and the children of staff at the Danish Pediatric Pulmonary Service. Prior to participation, the children (>15 years) or their parents were asked to fill out a health questionnaire covering gestational age, previous or current pulmonary disease, atopic illness, allergies, and any additional diseases the child had had, as well as current and previous medications.

All participants were non-smokers, had two parents of European descent, and had no current pulmonary or cardiac disease, including any upper or lower respiratory infection 2 weeks prior to the measurements. Any use of bronchodilators, and in particular, use in the day previous to participation, was

considered an exclusion criterion. Furthermore, we excluded participants with FEV_1/FVC below the age- and weight-specific lower limit according to recent data [18] or who were unable to co-operate or perform adequate respiratory maneuvers.

Methods

Height and weight were measured without shoes to the nearest 0.1 cm and 100 grams, respectively, using standard stadiometers (Seca, Hamburg, Germany) and scales. Age was calculated by difference between date of birth and participation date, and was recorded to decimal accuracy.

Hemoglobin concentration was measured by a finger stick blood sample test (The HemoCue Hb 201+; HemoCue, Denmark) in all participants unless the child refused. Correction for hemoglobin concentration is not imperative in healthy children, as variations within the normal range do not significantly affect $D_{L,CO}$ [19]. In children who refused hemoglobin measurement, we assumed normal values of 13.4 g/dL (8.3 mmol/L) for females, as well as males up to 15 years of age, and 14.6 g/dL (9.0 mmol/L) for males >15 years of age according to ATS/ERS guidelines [2].

Measurements of lung function

Spirometry, $D_{L,CO,NO,5s}$, and $D_{L,CO,10s}$ were performed using the Jaeger Masterscreen PFT pro (CareFusion, Hoechberg, Germany). Two identical sets of equipment were used at the three locations: one was used at the two participating schools and the other at the Danish Pediatric Pulmonary Service. Two experienced technicians performed all of the measurements. For most participants, spirometry and measurements of diffusing capacity were performed in a single sitting, but occasionally it required two sittings due to weariness with decreasing ability to perform technically acceptable measurements, especially with the younger children. If a participant was not able to make technically acceptable measurements in all three pulmonary function tests during the first sitting, they were invited back a second time. Spirometry always preceded the diffusing capacity measurements; $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ were performed in a random order except in the youngest children, in whom $D_{L,CO,NO,5s}$ was measured first because it was the primary goal of this study.

Participants breathed through a single-use mouthpiece with a built-in bacterial/viral filter (Spirobach, Tyco, Healthcare, Italy) connected to the pneumotachograph.

Diffusing capacity measurements

Participants were instructed to breathe normally. Following two to three normal breaths, participants performed a deep expiration and then a complete and fast inspiration. Following a breath-hold, a complete and smooth expiration was performed. As stated in the introduction $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ are performed

with a identical respiratory maneuver, with the exception of breath-hold time, but it is important to clarify that they are two distinctly separate methods, contained within one equipment setup, with differences in test gasses, gas analyzers and sampling techniques.

See Table 1 for specific methodological differences between $D_{L,CO,NO,5s}$, and $D_{L,CO,10s}$.

Quality control was performed separately for the two methods. Having unacceptable measurements for one method did not exclude the participant from attempting to perform the other method. The average of two acceptable tests for each method was reported and included in data analysis.

We required at least 4 minutes between each measurement, to allow adequate elimination of the test gases. Discard and sample volume were each 600 ml in both $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$. For children with a VC <1.5 L we reduced the discard volume to 500 ml [2]. The gas concentration curves were viewed prior to sample collection to confirm that dead space washout was complete.

Breath-holding time was calculated using the Jones and Mead method for both $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ [20].

The instrument dead space for both $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ ($V_{D,\ ins}$) was 130 ml, and the anatomical dead space ($V_{D,an}$) was calculated according to Cotes formula from 1993 as $V_{D,\ an}$=2.2 ml/kg $*$ weight in kg [21].

Alveolar volume (V_A) was calculated using the following formula:

$$V_A = (FI_{gas})/(FA_{gas}) * (V_{IN}\text{-}V_{D,\ an}\text{-}V_{D,\ ins})$$

where FI_{gas} is the inspiratory fraction of inert gas (Methane or Helium for $D_{L,CO,10s}$ and $D_{L,CO,NO,5s}$ respectively) and FA_{gas} is the alveolar fraction of inert gas. V_{IN} is the inspiratory volume.

All measurements were performed at sea level. $D_{L,CO}$ and the diffusing capacity for CO per unit of alveolar volume ($D_{L,CO}/V_A$=K_{CO}) were corrected for

Table 1. Summary of methodology.

	$D_{L,CO,NO,5s}$	$D_{L,CO,10s}$
Breath-hold	5 seconds	10 seconds
Gas mixture	0.3% CO, 9% He, 20.9% O_2, 69.8% N_2 mixed with 400 ppm NO/O_2*	0.3% CO, 0.3% CH_4, 20.9% O_2, and balanced N_2
Inert gas	Helium	Methane
Gas analyzer†	NO: CiTicel 7BNT electrochemical cell, CO: Electrochemical Cell, He: Thermal Conductivity, O_2: Electrochemical Cell	CO, CH_4: Non-dispersive infrared thermopile
Gas sampling method	Physical sample from collection bag	Virtual sample constructed from flow and gas concentration signals.
CO_2 -correction‡	4,5%	-

$D_{L,CO,NO,5s}$ represents the single-breath diffusing capacity for NO and CO with a 5-second breath-hold. **$D_{L,CO,10s}$** represents the single-breath diffusing capacity for CO with a 10-second breath-hold. Inert gas was used to measure the alveolar volume (V_A).
*The concentration of NO in inspired gas was approximately 50 PPM according to the standard settings of the equipment.
†City Tech. Ltd produced all gas analyzers.
‡CO_2 correction is applied due to cross-sensitivity of the Helium Analyser with CO_2.

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hemoglobin concentration when available. $D_{L,NO}$ and the diffusing capacity for NO per unit of V_A ($D_{L,NO}/V_A=K_{NO}$) were not corrected for hemoglobin concentration [6].

$D_{L,CO,NO5s}$ and $D_{L,CO,10s}$ were performed according to current ATS/ERS guidelines [2], though we considered a ratio between inspiratory volume and FVC (V_{IN}/FVC) >80% as sufficient, in contrast to a ratio >85%. The vital capacity (VC) was not measured in our subjects, but FVC acquired during spirometry was assumed to be equivalent to the VC, as FVC has been shown to not differ significantly from VC in healthy subjects [22], [23].

Both $D_{L,CO,10s}$ and $D_{L,CO,NO,5s}$ result in the measurement of $D_{L,CO}$, V_A, and K_{CO}. In addition, $D_{L,CO,NO,5s}$ produces measurements of $D_{L,NO}$, K_{NO}, and allows calculation of Dm, Vc, and $D_{L,NO}/D_{L,CO(5s)}$. To differentiate between the two methods, $D_{L,CO,10s}$ outcomes are denoted with "10s" and $D_{L,CO,NO,5s}$ outcomes with "5s" in this paper, e.g., $V_{A,10s}$ for V_A measured using $D_{L,CO,10s}$.

Quality control of equipment

Volume and gas calibration and biological quality control was performed daily prior to the measurements. Calibration syringes were tested for volume accuracy and were in accordance with ATS/ERS standards [2]. Gas-analyzers were factory checked and quality controlled for linearity as required for the $D_{L,CO,10s}$ method before start of the study and after completion of the study in both sets of equipment, and were found in accordance with ATS/ERS standards. A quality control report on both sets of equipment is provided in Supporting Information. **Appendix S1.** Biological quality control of measurements using both $D_{L,CO,10s}$ and $D_{L,CO,NO,5s}$ in addition to assessments of volumes demonstrated high levels of repeatability within subjects, between session and between equipment setups during the entire study period.

Calculation of Θ and Vc

Roughton and Forsters' 1/ΘCO value at pH 7.4 was used [24]:

$$1/\Theta CO = (1.30 + 0.0041 * PaO_2) * (14.6/\text{Hb concentration in g/dL})$$

PaO_2 was set at 100 mmHg. Using the standard hemoglobin concentrations, 1/ΘCO was found to be 1.71 for females and for males <15 years of age, and 1.86 for males >15 years of age.

A Dm_{NO}/Dm_{CO} ratio (α) of 1.97 [4] was used, and Vc was determined by isolating 1/Vc in the following equation:

$$1/D_{L,CO} = 1/Dm_{CO} + 1/\Theta CO * Vc$$

The calculations above assume the previously acknowledged infinite value of Θ_{NO}. Recently a finite value of Θ_{NO} has been accepted as more accurate, and was therefore used in this study. The finite value of Θ_{NO} leads to the following equations:

$$Vc_{finite} = 0.744 * Vc_{infinite}$$

and

$$Dm_{finite} = 0.377/(1/D_{L,NO} - 0.13/D_{L,CO})$$

[25]

Statistical analysis

The primary outcomes for $D_{L,CO,NO,5s}$ were considered to be $D_{L,CO,5s}$, $K_{CO,5s}$, $V_{A,5s}$, $D_{L,NO}$, K_{NO}, and the calculated outcomes $D_{L,NO}/D_{L,CO,5s}$, and Vc, Dm for the finite value of Θ_{NO}. Primary outcomes for $D_{L,CO,10s}$ were $D_{L,CO,10s}$, $K_{CO,10s}$, and $V_{A,10s}$. Reference equations were established using Generalized Additive Models for Location Scale and Shape (GAMLSS) with extended capabilities compared to the simpler, generalized linear models. The GAMLSS regression analysis allows the median or mean value (*mu*), the variability (*sigma*), and the skewness (*nu*) of the outcome variable to change with the explanatory variables. Possible distributions for the GAMLSS models were normal distribution (linear regression with *mu* and *sigma*), gamma distribution (*mu* and *sigma*), or the Box-Cox Cole and Green (BCCG) distribution (*mu*, *sigma*, and *nu*). The latter is suitable for skewed data.

Stepwise model selection was carried out using the Generalized Akaike Information Criterion (GAIC). Possible explanatory variables in the selection of *mu*, *sigma*, and *nu* were age, sex, height, and cube of height, as well as any two-way interaction between these variables for mu. Goodness of fit was assessed by 'worm plots' and Q statistics [26], [27]. For all three distributions we investigated models with log mu links, log sigma links and for the Box-Cox Cole Green distribution identity nu links. Measurements not meeting ATS quality criteria (>10% difference between to measurements, and V_{IN}/FVC between 80% and 85%) were included after evaluating the influence and leverage of the resulting data points in ordinary linear regression analysis [28], [29], [30], [31]. All analyses were performed using the statistical software R (version 3.0.2; R Foundation, http://www.r-project.org) including the GAMLSS package.

Results

See figure 1 for the inclusion flow chart. Baseline characteristics are provided in table 2. The populations in our three locations were similar in all regards. See **Figure S1** for the age distribution.

Conformity between the two sets of equipment for $D_{L,CO,10s}$ was evaluated using a paired t-test (p=0.62) and a Bland-Altman plot (mean difference=0.06). See figure 2.

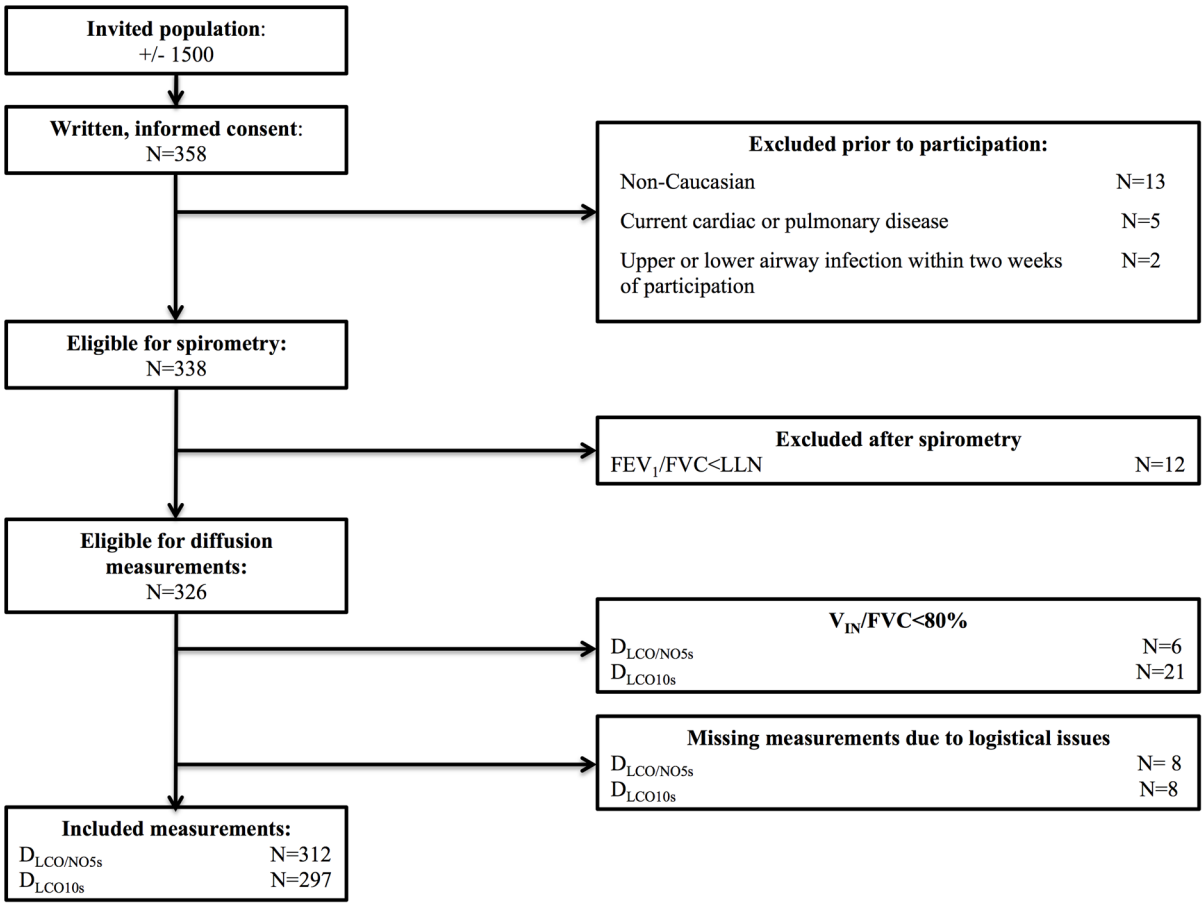

Figure 1. Inclusion flowchart. Invitations to participate were sent to approximately 1500 children, of which 358 participants and/or their parents provided informed consent.

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Table 2. Baseline characteristics at the three locations.

		Private school, Copenhagen	Pediatric Pulmonary Service, Copenhagen	Public school, rural Denmark
N		159	55	112
Sex (male)	N (%)	82 (51.6)	24 (43.6)	55 (49.1)
Age (y)	Mean (SD) [range]	11.4 (3.3) [5–17]	11.5 (4.4) [5–17]	10.3 (2.9) [5–16]
Height (cm)	Mean (SD) [range]	152.7 (19.3) [104.9–187.6]	150.5 (24.2) [107.4–186.8]	145.5 (16.8) [108.0–182.0]
Weight (kg)	Mean (SD) [range]	45.2 (16.3) [18.8–93.6]	45.9 (19.2) [14.8–81.5]	40.1 (14.8) [18.1–101.2]
FEV_1 (Z-score)	Mean (SD) [range]	1.17 (0.93) [−1.05–3.62]	0.73 (0.83) [−1.29–2.82]	1.14 (0.93) [−1.20–3.38]
FVC (Z-score)	Mean (SD) [range]	1.16 (0.95) [−0.89–3.97]	0.62 (0.81) [−1.01–2.50]	1.11 (1.04) [−1.31–4.10]

FEV_1=forced expiratory volume in one second, FVC=forced vital capacity, SD=standard deviation.

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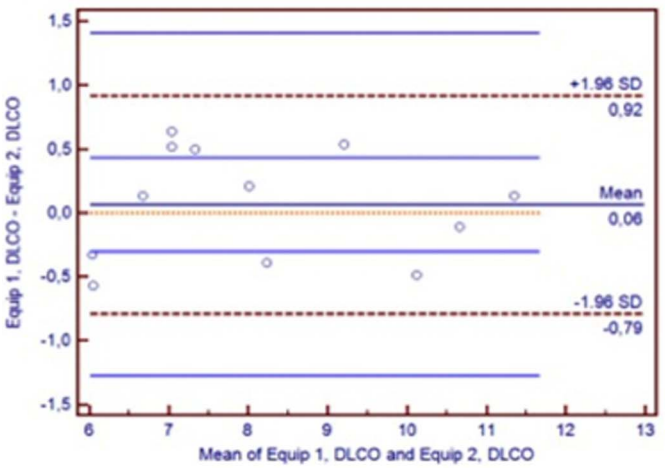
	Sub 1	Sub 2	Sub 3	Sub 4	Sub 5	Sub 6	Sub 7	Sub 8	Sub 9	Sub 10	Sub11	Sub 12
Equip1. D_{LCO}	9.47	5.87	10.6	8.11	9.87	5.76	6.73	11.41	7.58	7.3	8.04	7.36
Equip 2. D_{LCO}	8.93	6.2	10.71	7.9	10.36	6.33	6.6	11.28	7.08	6.78	8.43	6.72

Result paired t-test: 0.62



Bland-Altman plot:
Mean difference 0.06

Figure 2. Comparison using Bland and Altman plots of results in 12 subjects assessed by the two sets of equipment used. One at the Pediatric Pulmonary Service, Copenhagen and the other at the two schools involved.

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Reference equations

Reference equations, as well as the sigma for all outcomes, are presented in table 3. In addition please see the provided excel spreadsheet, that allows calculation of predicted reference values.

For $D_{L,CO,5s}$,, $K_{CO,5s}$, $D_{LCO,10s}$, $K_{CO,10s,}$ and $D_{L,NO}/D_{L,CO,5s}$, reference equations were produced for both hemoglobin-corrected and non-corrected values. Vc and Dm were calculated based on the finite value of Θ_{NO}.

Example of calculation:

The reference equation for $D_{L,CO,5s}$ is:

$$D_{L,CO,5s} = \exp(0.9440 + 0.0205^{*}A + 0.0908^{*}S + 1.6233^{*}10\hat{}-7^{*}H\hat{}3)$$

A 10-year-old boy, 140 cm tall has a predicted $D_{L,CO,5s}$ of:

$$D_{L,CO,5s} = \exp(0.9440 + 0.0205^{*}10 + 0.0908^{*}1 + 1.6233^{*}10\hat{}-7^{*}140\hat{}3)$$

$$= 5.4(\mathrm{mmol/min})/\mathrm{kPa}.$$

Table 3. Reference equations for $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$.

$D_{L,CO,NO,5s}$	Model	Equation (=mu)	Coefficient of variation (=Sigma)
$D_{L,NO}$, (mmol/min)/kPa*	Gamma	exp(1.3145+0.0214*A−0.0058*S+0.0119*H−1.2893*10^ −8 *H^3+2.7070*10^−8*S*H^3)	exp(−2.2490)
K_{NO}, ((mmol/min)/kPa)/L‡	Normal	exp(1.2672+1.1168*S+0.0098*H−1.8280*10^−7*H^ 3−0.0117*S*H+1.9769*10^−7 *S*H^3)	exp(−0.3370)
$D_{L,CO,5s}$, (mmol/min)/kPa§	Gamma	exp(0.9440+0.0205*A+0.0908*S+1.6233*10^−7*H^3)	exp(−2.2521)
$D_{L,CO,5s,hb\text{-}corr}$, (mmol/min)/kPa	BCCG	exp(0.6392−0.0570*A+0.0922*S+0.0062*H+0.0005*A*H)	exp(−2.2678)
$K_{CO,5s}$, ((mmol/min)/kPa)/L ll	Gamma	exp(0.9567+0.0576*S−0.0028*H)	exp(−2.3644)
$K_{CO,5s,hb\text{-}corr}$, ((mmol/min)/kPa)/L	Gamma	exp(1.6187+0.0526*S−0.0092*H+8.8280*10^−8*H^3)	exp(−2.3626)
$V_{A,5s}$, L†	Gamma	exp(−0.6939−0.0181*A+0.0409*S+0.0111*H+0.0003*A*H)	exp(−2.5047)
Vc, ml**	Gamma	exp(2.7298−0.0729*A−0.0268*S+0.0066*H+0.0126*A*S+0.0005*A*H)	exp(−2.1027)
Dm, (ml/min)/mmHg††	Gamma	exp(2.0825+0.0329*A+0.0573*S+0.0123*H)	exp(−1.9359)
$D_{L,NO}/D_{L,CO,5s}$	Normal	exp(0.9407+0.0458*A+0.0039*H−0.0003*A*H)	exp(−1.3398)
$D_{L,CO,10s}$	Model	Equation(=mu)	Coefficient of variation (=Sigma)
$D_{L,CO,10s}$, (mmol/min)/kPa	Gamma	exp(1.0826+0.0178*A+0.0948*S+1.5419*10^−7*H^3)	exp(−2.1883)
$D_{L,CO,10s,hb\text{-}corr}$, (mmol/min)/kPa	Gamma	exp(0.6371−0.0435*A+0.0939*S+0.0070*H+0.0004*A*H)	exp(−2.2356)
$V_{A,10s}$, L	Gamma	exp(−1.2915−0.1265*A+0.0509*S+0.0213*H−2.4645*10^−7 *H^3+0.0009*A*H)	exp(−2.4837)
$K_{CO,10s}$, ((mmol/min)/kPa)/L	Gamma	exp(1.2115+0.0576*S−0.0043*H)	exp(−2.2442)
$K_{CO,10s,hb\text{-}corr}$, ((mmol/min)/kPa)/L	Gamma	exp(1.2825+0.0566*S−0.0047*H)	exp(−2.2597)

The GAMLSS model was used with a gamma distribution for all outcomes except $D_{L,NO}/D_{L,CO,5s}$ which had a normal distribution, and $D_{L,CO,5s,hb\text{-}corr}$ that had a Box-Cox-Cole-Green distribution (BCCG). H=height in cm, A=age in years, S=sex (male=1, female=0),
*D_{LNO}=diffusing capacity for NO,
†V_A=alveolar volume,
‡$K_{NO}=D_{LNO}/V_A$,
§D_{LCO}=diffusing capacity for CO, ll $K_{CO}=D_{L,CO}/V_A$,
**Vc=capillary volume,
††Dm=diffusing capacity of the alveolar membrane.
The notation $_{(10s)}$ and $_{(5s)}$ indicates if the outcomes were found using the $D_{L,CO,10s}$ method or the $D_{L,CO,NO,5s}$ method. ''hb-corr''=values corrected for hemoglobin concentration.

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''A'' is the age in years, ''S'' is the sex (1 for males and 0 for females), and ''H'' is the height in cm.

When creating a ''best-fit'' model for the $D_{L,NO}/D_{L,CO,5s}$ ratio as a function of height, we saw that the ratio increased with height for the youngest participants and reached a plateau around age 14 (figure 3).

Our reference values for $D_{L,CO,10s}$ and $K_{CO,10s}$ were comparable to published reference values (figure 4a–b) [16], [19].

We have provided an Excel calculation sheet based on both GAMLSS regression and linear regression, and an example of calculation. The excel sheet is provided as **Appendix S2**.

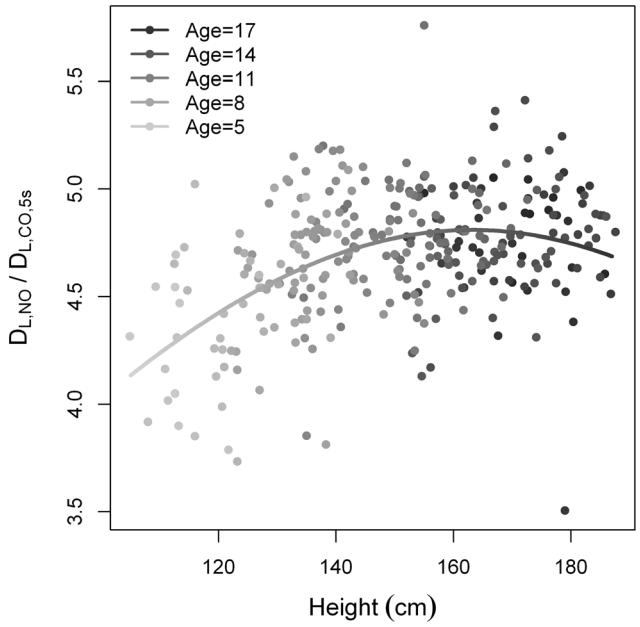

Figure 3. Scatter plot and curve of $D_{L,NO}/D_{L,CO,5s}$ versus height. Dot colors indicate participant age (light gray indicates the youngest and black dots the oldest).

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Quality Control

Repeatability of measurements in 5 to 8-year-olds and the V_{IN}/FVC ratio

Young children were less likely to meet the guideline requiring less than 10% variation between two measurements of $D_{LCO,5s}$, inspiratory volume ($V_{IN,5s}$), $D_{L,CO,10s}$, and $V_{IN,10s}$. Including the mean of two measurements, not complying with ATS/ERS guidelines did not alter the reference equations (**Figure S2, Figure S3, Figure S4 and Figure S5.**).

Using the same procedure as described for the repeatability of measurements, we found little evidence that observations of V_{IN}/FVC between 80% and 85% should be excluded (**Figure S6 and Figure S7**).

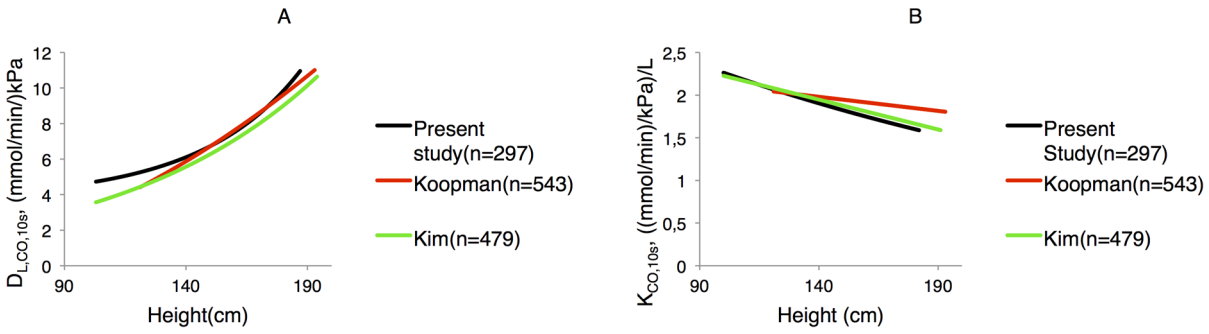

Figure 4. (a) $D_{L,CO,10s}$ and (b) $K_{CO(10s)}$ compared to recent reference equations [16], [15]. The reference equations are plotted as a function of height. All other variables were kept constant.

doi:10.1371/journal.pone.0113177.g004

The influence of a given data point, such as an outlier, cannot be evaluated using residuals or Z-scores, as highly influential points will force the regression line close to it, resulting in a small residual and Z-score. We found little evidence that participants who deviated from ATS/ERS guidelines should be excluded from the estimation of reference equations for $D_{L,CO,5s}$ and $D_{L,CO,10s}$, as the resulting data points were not highly influential, and excluding them did not alter the Z-scores. Therefore, including them in the data analysis was acceptable.

$D_{L,CO,5s}$ vs. $D_{L,CO,10s}$

$D_{L,CO,10s}$ was significantly higher than $D_{L,CO,5s}$ (paired t-test $p<0.0001$) but as expected, $D_{L,CO,10s}$ and $D_{L,CO,5s}$ were strongly correlated ($r=0.98$, $p<0.0001$). Similarly, using the Passing Bablok regression, we found a systematic difference, as well as a proportional difference (figure 5).

When plotting $D_{L,CO,10s}$ and $D_{L,CO,5s}$ as a function of height, we found $D_{L,CO,10s}>D_{L,CO,5s}$. (Figure 6) as well as $V_{A,10s}>V_{A,5s}$ (Figure 7).

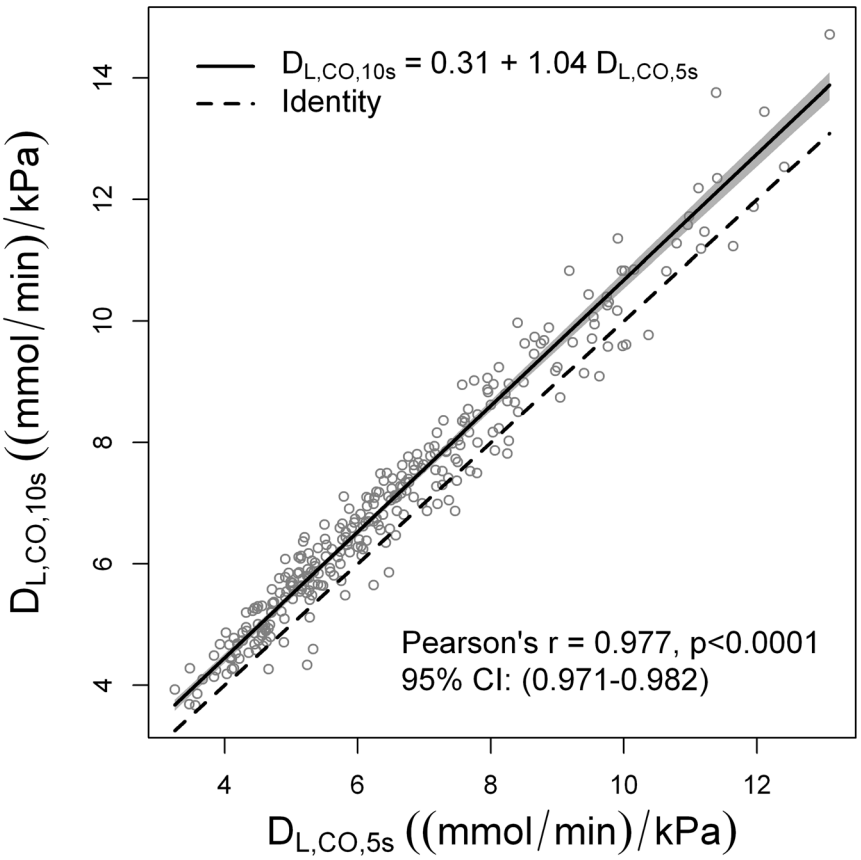

Figure 5. Comparison of $D_{L,CO,5s}$ and $D_{L,CO,10s}$. $D_{L,CO,5s}$ and $D_{L,CO,10s}$ were strongly correlated, with a Pearson's r=0.977. Passing Bablok regression showed that $D_{L,CO,10s}$ was systematically higher by a constant of 0.31, and proportionally higher by a factor of 1.04.

doi:10.1371/journal.pone.0113177.g005

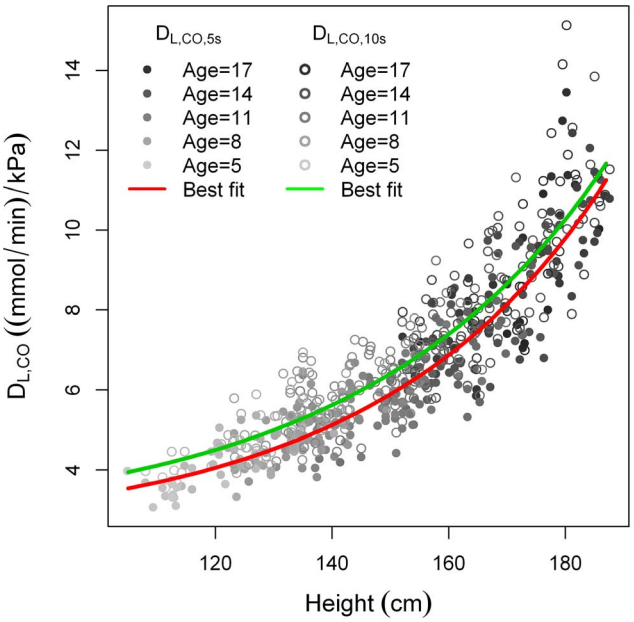

Figure 6. $D_{L,CO,5s}$ and $D_{L,CO,10s}$ plotted as a function of height.

doi:10.1371/journal.pone.0113177.g006

Vc and Dm

Vc and Dm both increase with height. (Figure 8 and 9).

Discussion

This is the first study to establish reference equations for the outcomes of $D_{L,CO,NO,5s}$, including the calculated outcomes: Vc, Dm, and the $D_{L,NO}/D_{L,CO,5s}$ ratio, in a large group of healthy children of European descent. The measurement and evaluation of Vc and Dm can potentially provide valuable information about the causes of decreased diffusing capacity and the development and progression of lung disease or vascular disorders from the age of 5 years.

Vc and Dm are not entirely accepted as robust parameters, partially due to the lack of reference equations, which limits their clinical and scientific use. A more problematic issue is the current lack of agreement regarding the true value of ΘCO and the relationship with arterial oxygen pressure. The calculated Vc is dependent on this value and will vary depending on which equation is used. The equation utilized in this paper was based on measurements performed at pH 7.4 [24], for conventional reasons, and because it is closer to a physiological value. Finally, another topic of debate is α, the ratio of NO to CO diffusivity. In the present study, a physical α value of 1.97 was used [4], but an alternative empiric value of 2.42 has been proposed [4], [32].

The $D_{L,NO}/D_{L,CO,5s}$ ratio has been proposed as a measure of the relative properties of Dm and Vc [33]. Previous studies have concluded that the $D_{L,NO}/D_{L,CO,5s}$ ratio

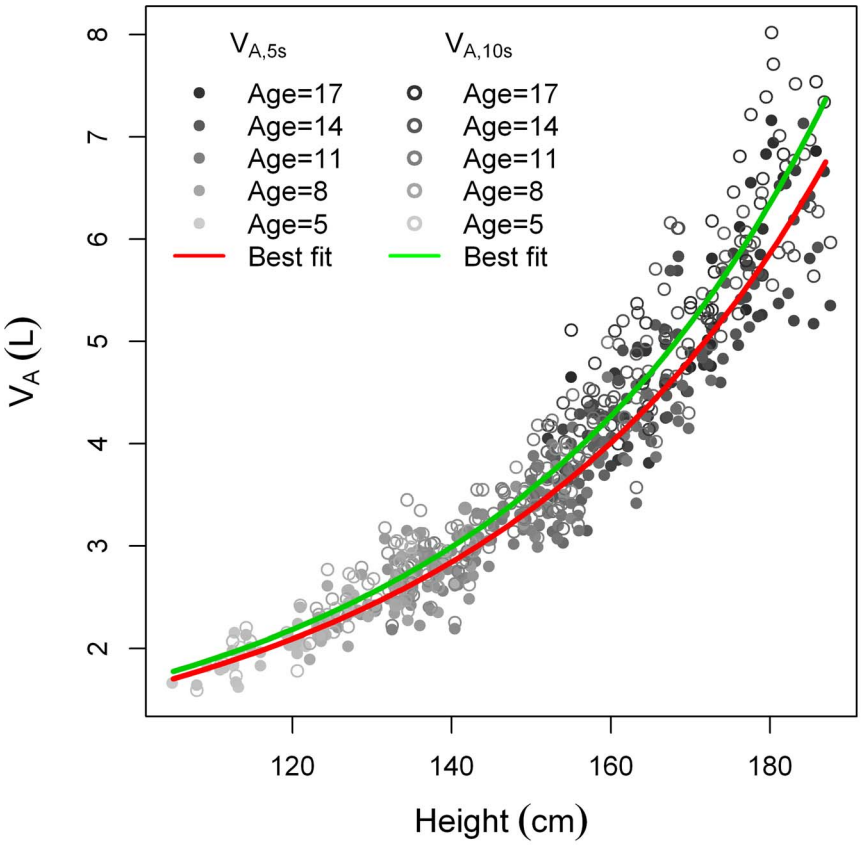

Figure 7. Va_{5s} and Va_{10s} plotted as a function of height.

doi:10.1371/journal.pone.0113177.g007

in adults is independent of age [12], [34]. Figure 3 is produced via a "best fit"-model for the available data, and may not reflect the true bio-physical relationship between height and this ratio. That being said, we found that the ratio increased with height until mid pubertal age at approximately 14 years and then reached a plateau.

We have shown that both Vc and Dm increase with height(Figure 8 and 9). As stated in the introduction, according to current opinion the diffusing capacity of NO ($D_{L,NO}$) reflects both Dm and Θ_{CO}*Vc, whereas $D_{L,CO}$ primarily reflects Vc. With increasing height $D_{L,NO}$ will increase relatively more than $D_{L,CO}$ leading to the $D_{L,NO}/D_{L,CO,5s}$ reaching a plateau around 140 cm.

The lower $D_{L,NO}/D_{L,CO,5s}$ in younger and smaller children may be due to a greater rate of capillary growth compared to lung surface growth or to a relatively thicker membrane in the young. As height increases with age, a compensatory relatively larger increase in Dm would result in an increasing ratio. Alveolarization has been shown to continue through out childhood and adolescence [35] and could help explain the increase in Dm. The literature on this topic is scarce, and future studies are needed to understand and interpret the effect of age and height on the $D_{L,NO}/D_{L,CO,5s}$ ratio.

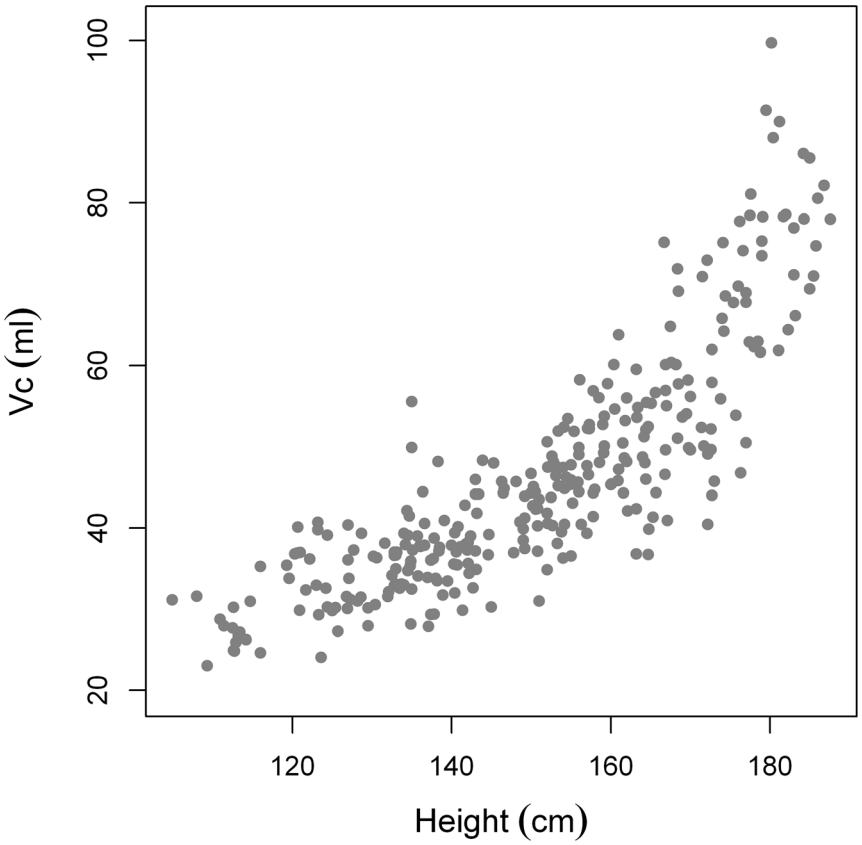

Figure 8. Vc plotted as a function of height.

doi:10.1371/journal.pone.0113177.g008

$D_{L,CO,10s}$

The reference values calculated in the present study for $D_{L,CO,10s}$ were slightly higher than existing, published reference values. One possible reason for this difference is that the present study population included children with both parents of European descent, whereas Koopman et al. included children with only one parent of European descent [16]. Ethnic differences in D_L in adults are small, but well established [36], [37]. Another reason for the difference is the pulmonary function equipment; the equipment used in the present study and by Koopman et al. were very similar, whereas the apparatus' used by Kim et al. [19] at their two locations were from two different manufacturers. Furthermore, even with the same apparatus, differences in software including various corrections, may lead to the observed differences.

Our results stress the importance of creating reference equations specific for a single population, or at least validating existing reference equations prior to implementing them in a laboratory setting.

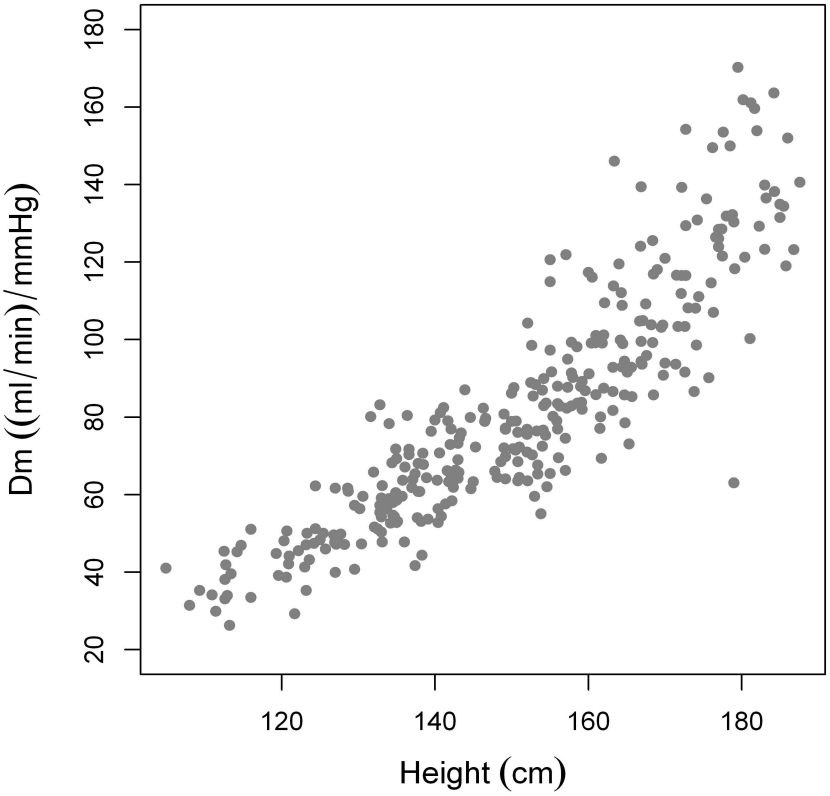

Figure 9. Dm plotted as a function of height.

doi:10.1371/journal.pone.0113177.g009

$D_{L,CO,5s}$ *vs.* $D_{L,CO,10s}$

Although the primary purpose of measuring $D_{L,CO,10s}$ was to secure a meaningful correlation to the much more scarcely described $D_{L,CO,NO,5s}$ technique, we secondarily wished to compare $D_{L,CO}$ measured by the two techniques. As expected we found a significant, systematic difference between $D_{L,CO,5s}$ and $D_{L,CO,10s}$. The difference in $D_{L,CO}$ can be caused by a number of factors, as the two methods vary in a number of ways. See table 1. First, methane and helium may have different distributions in the lung owing to their respective physical properties; they have also different solubility in tissue. This may lead to a difference in V_A and a resulting difference in $D_{L,CO}$ as $D_{LCO}=K_{CO}*V_A$. Second, the sample method varies, with a physical gas sample being collected in the case of $D_{L,CO,NO,5s}$, whereas a virtual sample was constructed from flow and gas concentration signals in the case of $D_{L,CO,10s}$. Finally, we speculate if the difference in the kinetics of NO and CO in binding with hemoglobin may play a roll.

Older studies on $D_{L,CO,10s}$ focusing on varying breath-hold times, keeping all other factors constant, have shown that breath-hold time alone, influences K_{CO}, leading to a decreased $D_{L,CO}$ with an increased breath-hold time [38]. This is in

contrast to our findings, but apparently the mentioned differences in methodology other than breath-hold, have a greater impact on $D_{L,CO}$.

In summary, the two methods vary in a number of ways and $D_{L,CO}$ measured using $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ cannot be used interchangeably for monitoring pulmonary disease. More research is required to determine how the mentioned factors combine to influence $D_{L,CO}$. A given value of $D_{L,CO}$ can only be evaluated using reference equations produced with the same methodology and breath-hold time as recently confirmed [39].

CO and NO backpressure

The participants performed two or three tests, and rarely up to six repetitions of both $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$, resulting in a maximum of 12 tests in a single sitting. Repeating measurements of $D_{L,CO,10s}$ leads to an accumulation of CO in the blood, creating CO backpressure and decreasing $D_{L,CO}$. However, recent work by Zavorsky showed that up to 12 tests can be performed in adults without significantly lowering the $D_{L,CO}$. Furthermore, in regards to $D_{L,CO,NO,5s}$, up to 22 repetitions does not lead to a decrease in $D_{L,NO}$ [40]. Taking this into account, we have no reason to suspect CO or NO backpressure to be of influence in the present study.

Quality control

Measuring lung function in this age group requires extra time and effort, but it is feasible. Most of the young children were able to perform the measurements according to ATS/ERS guidelines, but some had greater variability between measurements than normally accepted. This difference was partially due to the limited attention span of the children, who were not always able to perform repeated tests if the first two measurements did not comply with the ATS/ERS standard of a maximum 10% difference between measurements. We included measurements with greater variability, as they did not affect the estimated reference equations. Accepting greater variation in children makes sense if the alternative is to discard measurements completely.

The ATS/ERS guidelines recommend an acceptance criterion of $V_{IN}/VC \geq 85\%$ for adults. The recommendation is based on D_{LCO10s} measured in a large group of adults, where 72%, 86%, and 92% of the participants were able to achieve a V_{IN}/VC ratio of 90%, 85%, and 80%, respectively. Therefore, the recommended ratio, i.e., 85%, is a relatively arbitrary value and the guidelines state that $V_{IN}/VC < 85\%$ may still have clinical utility [2].

Although most of our participants were able to inhale to more than 85% of FVC, some were not, despite multiple attempts and prompting and otherwise performing an adequate maneuver.

We found no differences between reference equations including measurements with $V_{IN}/FVC > 80\%$ and reference equations only including $V_{IN}/FVC > 85\%$.

In summary, we accepted measurements that did not meet ATS/ERS quality criteria because these measurements had no effect on the resulting equations. In the future, specific pediatric guidelines for both $D_{L,CO,NO,5s}$ and $D_{L,CO,10s}$ would be relevant.

Strengths and limitations

The primary strength of this study is the large and acceptable age distribution of healthy children and adolescents from varying demographic backgrounds. Furthermore, this study was completed in two laboratory setups with identical equipment, as described in the online supplement. The same two technicians performed all measurements, resulting in a high level of repeatability and a systematic approach. In addition, we included children as young as 5 years of age, expanding our ability to adequately evaluate advanced pulmonary function in this age group. Finally, our calculated reference equations for $D_{L,CO,10s}$ corresponded well to recently published equations, in particular those of Koopmans et al. [16]

In hindsight, it would have been beneficial to include a ''young adult'' group, 18–22 years old, in this study, as it would open up the possibility of bridging reference equations to include children, adolescents, young adults, and adults.

For the youngest children with a VC$<$1.5 liters, we reduced the discard volume to 500 ml. If the VC is even lower, as in the case of disease, this method may not be suitable. Multiple other techniques exist for $D_{L,CO}$. These include the steady state method, particularly suitable for infants or anaesthetized patients, or the rebreathing and intrabreath method, that both require cooperation, but can be performed in patients with lower lung volumes [41]. So far these modifications have not been applied to $D_{L,CO,NO}$.

Conclusion

This study is the first to create pediatric reference equations for the outcomes $D_{L,CO,5s}$, $D_{L,NO}$, and the calculated outcomes $D_{L,NO}/D_{L,CO,5s}$, Vc, and Dm measured by $D_{L,CO,NO,5s}$ in healthy children and adults, of European descent. These equations are based on a large population with a broad age range, including children as young as 5 years of age. We expect that the present reference equations can be applied to similar populations throughout Europe, Australia and North America.

We hope that having reliable reference equations for Dm, Vc, and $D_{L,NO}/D_{L,CO,5s}$ will lead to improved diagnostic evaluation and provide a monitoring tool for the treatment of children presenting with diffuse interstitial lung disease, whether it is a pure alveolocapillary membrane disturbance or pulmonary micro vascular disease. In particular, we believe that the $D_{L,NO}/D_{L,CO,5s}$ ratio has great potential, as it is independent of the assumptions and models used to calculate Vc and Dm, that may be easily questionable. However, the clinical utility of Vc, Dm, and $D_{L,NO}/D_{L,CO,5s}$ still needs to be evaluated in future studies. We acknowledge that multicenter studies are required for external validation of these results. We

invite researchers to compare their results, in children with well known pathological features of the lung, with the results of this study. This will achieve increased understanding of the physiological meaning of the described measurements and their application in the early detection and monitoring of diseases.

Supporting Information

Figure S1. Age and gender distribution of participants.
doi:10.1371/journal.pone.0113177.s001 (TIFF)

Figure S2. Quality control. Participants with more than 10% difference between two independent measurements of $D_{L,CO,5s}$ were evaluated, as this is in contrast to ATS/ERS guidelines.
doi:10.1371/journal.pone.0113177.s002 (TIFF)

Figure S3. Quality control. Participants with more than 10% difference between two independent measurements of inspiratory volume ($V_{IN,5s}$) were evaluated, as this is in contrast to ATS/ERS guidelines.
doi:10.1371/journal.pone.0113177.s003 (TIFF)

Figure S4. Quality control. Participants with more than 10% difference between two independent measurements of $D_{L,CO,10s}$ were evaluated, as this is in contrast to ATS/ERS guidelines.
doi:10.1371/journal.pone.0113177.s004 (TIFF)

Figure S5. Quality control. Participants with more than 10% difference between two independent measurements of inspiratory volume $V_{IN,10s}$ were evaluated, as this is in contrast to ATS/ERS guidelines.
doi:10.1371/journal.pone.0113177.s005 (TIFF)

Figure S6. Quality control. Measurements of $V_{IN,5s}$/FVC between 80% and 85% were evaluated as ATS/ERS requires values $>$85%.
doi:10.1371/journal.pone.0113177.s006 (TIFF)

Figure S7. Quality control. Measurements of $V_{IN,10s}$/FVC between 80% and 85% were evaluated as ATS/ERS requires values $>$85%.
doi:10.1371/journal.pone.0113177.s007 (TIFF)

Appendix S1. Age distribution.
doi:10.1371/journal.pone.0113177.s008 (DOCX)

Appendix S2. Excel worksheet.
doi:10.1371/journal.pone.0113177.s009 (XLSX)

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Author Contributions

Conceived and designed the experiments: AT BH KN JM. Performed the experiments: BH AT. Analyzed the data: AT BH KN JLM JM FB. Wrote the paper: AT BH KN FB JLM JM.

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