Reference equations for pulmonary diffusing capacity of carbon monoxide and nitric oxide in adult Caucasians

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ABSTRACT The aim of this study was to determine reference equations for the combined measurement of diffusing capacity of the lung for carbon monoxide (CO) and nitric oxide (NO) (D_{LCONO}). In addition, we wanted to appeal for consensus regarding methodology of the measurement including calculation of diffusing capacity of the alveolo-capillary membrane (D_m) and pulmonary capillary volume (V_c).

D_{LCONO} was measured in 282 healthy individuals aged 18–97 years using the single-breath technique and a breath-hold time of 5 s (true apnoea period). The following values were used: 1) specific conductance of nitric oxide (θ_{NO})=4.5 $mL_{NO} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$; 2) ratio of diffusing capacity of the membrane for NO and CO (D_{mNO}/D_{mCO})=1.97; and 3) 1/red cell CO conductance ($1/\theta_{CO}$)=(1.30 +0.0041·mean capillary oxygen pressure)·(14.6/Hb concentration in $g \cdot dL^{-1}$).

Reference equations were established for the outcomes of D_{LCONO}, including D_{LCO} and D_{LNO} and the calculated values D_m and V_c. Independent variables were age, sex, height and age squared.

By providing new reference equations and by appealing for consensus regarding the methodology, we hope to provide a basis for future studies and clinical use of this novel and interesting method.

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Introduction

Marie KROGH [1] developed a method for measuring pulmonary gas exchange in Copenhagen a century ago. Since then, measurement of diffusing capacity of the lung for carbon monoxide (D_{LCO}) has been used in both work-up and monitoring of a wide variety of pulmonary disorders. Since the work of ROUGHTON and FORSTER [2] in 1957, the model for transfer of a gas from alveolus to blood has been described as consisting of two resistances in series:

$$1/D_L = 1/D_m + 1/(\theta_b \cdot V_c)$$

where $1/D_L$ is the total resistance for the specific gas in min·mmHg·mL$_{gas}^{-1}$, $1/D_m$ is the resistance to passive diffusion through the alveolocapillary membrane and $1/(-_b \cdot V_c)$ represents the resistance of gas uptake of the blood. D_m is the membrane conductance for a given gas (in mL$_{gas}$·min^{-1}·mmHg^{-1}), θ_b (blood conductance) is the amount of gas taken up by the blood per mmHg tension (in mL$_{gas}$·mL$_{blood}^{-1}$·min^{-1}·mmHg^{-1}) and V_c is the pulmonary capillary blood volume (in mL).

Using carbon monoxide (CO) as the inhaled gas, ROUGHTON and FORSTER showed how to determine values for D_m and V_c by solving the above equation with two unknown variables. The method required measurements of D_{LCO} at two or more different oxygen (O_2) tensions, since an increase in inhaled O_2 tension results in a decrease in θ_{CO} and thereby a decrease in the measured D_{LCO} [2]. In 1987, GUENARD *et al.* [3] proposed an alternative way of determining D_m and V_c, using CO and nitric oxide (NO) in one combined single-breath manoeuvre (D_{LCONO}), making measurements considerably more convenient and possibly also more precise and thereby more suitable for use in clinical work [4]. Using this test, as opposed to the standard D_{LCO} measurement, clinicians will obtain more detailed information about the pathoanatomy/pathophysiology underlying a low diffusion capacity, for example, if the defect is related primarily to D_m or V_c.

In the work of GUENARD *et al.* [3], θ_{NO} was assumed to be infinitely great, since it had been shown earlier that the reaction rate of NO with free haemoglobin (Hb) was 250–1400 times faster than for CO [2, 3, 5, 6]. However, in recent years the correctness of this assumption has been thoroughly debated and recent evidence points towards θ_{NO} being finite, with a value of 4.5 mL$_{NO}$·mL$_{blood}^{-1}$·min^{-1}·mmHg^{-1} [7–10]. This value is used in the present study.

Other disputed issues in this area of research are the true value of θ_{CO} and the value of $\alpha=D_{mNO}/D_{mCO}$. In the present study, FORSTER's [11] 1987 values for θ_{CO} measured at pH 7.4 are used together with α=1.97. The considerations underlying these choices are presented in the discussion.

To date, a small number of reference values for the D_{LCONO} method have been published, but most of these include a rather limited number of subjects (n=10–71) [6]. However, one study included 124 healthy adults with a mean±SD age of ~40±12 years) [12] and another study population comprised 130 subjects, of which only 17 were aged >60 years [13]. Furthermore, one larger study from 2008 includes 303 healthy adults aged 20–80 years and had a more uniform age distribution [14]. Recently, ZAVORSKY *et al.* [15] combined and reanalysed data from these three studies in order to achieve one combined set of reference equations. This procedure has the obvious benefit of reference values relying on a greater number of subjects, but considering a rather wide spectrum in the mean values between these studies, differences in methodology, *e.g.* breath-hold time, and the limited amount of data obtained from healthy people aged >60 years, there still is an obvious need for an additional larger study to reliably establish reference values for this new test before it can be used in the daily clinical work-up of patients.

Based on state-of-the-art methodology, the aim of this study is to establish new reference values for the D_{LCONO} measurement. In that respect, we also wish to achieve consensus regarding methodology of the measurement including calculation of D_m and V_c, so that these values can be of clinical use in the future.

Methods

Subjects

A sample of 282 healthy adults aged 18–97 years was recruited between September 11, 2013 and June 18, 2014. They were randomly chosen from the Copenhagen General Population Study, a large general population cohort study including >100 000 participants aged ⩾20 years who had been randomly selected from the Danish Civil Registration System. Details about this study have been published previously [16, 17]. In addition, participants aged 18–20 years were randomly selected from the Danish Civil Registration System. Subjects were selected in order to achieve a uniform age distribution. Inclusion criteria were age ⩾18 years, both parents of European origin, nonsmoker or former use of tobacco <1 pack-year, no known pulmonary or cardiovascular disease, no acute respiratory symptoms 4 weeks prior to investigation, no prior operation or radiation therapy of the chest, body mass index (BMI) <30 kg·m^{-2} and no pregnancy.

The subjects lived in Copenhagen or the surrounding area and comprised a socioeconomically heterogeneous group.

Ethics

All participants received written and verbal information about the study and gave their informed consent. The Danish Data Protection Agency and a Danish committee on health research ethics approved the study.

Measurement of diffusing capacities for CO and NO

Measurements of D_{LCO} and D_{LNO} were achieved simultaneously during a single-breath manoeuvre using Jaeger Masterscreen PFT pro (CareFusion, Hoechberg, Germany). Two identical sets of equipment were used. Before measurement of diffusing capacity, standing height (to nearest 1 mm), weight (to nearest 100 g) and Hb (to nearest 0.1 $mmol \cdot L^{-1}$) of the participants were obtained. Hb was measured from capillary blood using HemoCue® Hb 201+ (HemoCue, Brønshøj, Denmark). It has been shown that Hb measured from a capillary blood sample closely resembles Hb measured from a venous blood sample taken from the vein of a forearm [18]. In addition, spirometry, body plethysmography and standard single-breath D_{LCO} were performed in all subjects. Measurements were taken at 20 m above sea level. The D_{LCONO} test was performed as follows. After a minimum of 30 min without any form of straining physical activity participants sat down, were equipped with a nose clip, and, after automatic resetting of the device, started tidal breathing through a mouthpiece and filter (SpiroBac; Henrotech, Aartselaar, Belgium) (dead space 56 mL, resistance to flow at 12 $L \cdot s^{-1}$ 0.9 cmH_2O) connected to the pneumotach. After completing a few tidal breaths, subjects were requested to perform a full expiration followed by a rapid full inspiration during which a valve opened allowing them to inspire the test gases. Following that, a breath-hold of 5 s was performed (true apnoea period). The actual breath-hold time was calculated using the Jones and Meade [19] method and was found to be mean±SD 6±0.44 s. The participants then performed a fast expiration, and after a $V_{washout}$=0.6 L, a V_{sample}=0.6 L was collected. The procedure was repeated after a 4-min wait. The measurements were considered acceptable if the difference between the two measurements of D_{LCO} was <10% or <3 $mL \cdot min^{-1} \cdot mmHg^{-1}$, as recommended by American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines [20]. If this was not the case, additional measurements (up to five in total) were performed, until the difference between the highest and second highest measurement of D_{LCO} met the requirements. In the vast majority of tests, the repeatability criteria were obtained after only two measurements. From the two chosen measurements, mean values for D_{LCO}, D_{LNO}, diffusion capacity per unit alveolar volume for CO (K_{CO}) and for NO (K_{NO}) and V_A (alveolar volume) were calculated. The gas used for the measurements consisted of 0.28% CO, 9.3% helium (He), 20.9% O_2 and 69.52% nitrogen (N_2) (analysis uncertainty ±2.0% relative) (Linde Healthcare/AGA, Copenhagen, Denmark), which was mixed with 400 ppm NO/N_2 (analysis uncertainty ±5.0% relative) (Linde Healthcare/AGA) in an inspiratory bag just before inhalation. The resulting inspired concentrations are presented in table 1. Due to a procedure where the system was flushed with 100% O_2 to empty any tubes that might contain CO/He/NO gas, the O_2 concentration was higher in the inspiratory bag than in the initial gas tank. The inert gas, He, was used in the calculation of V_A by means of the He-dilution technique.

In our calculations we did not account for NO backpressure, since concentrations of endogenous exhaled NO at rest range between 11 and 66 ppb and therefore were considered negligible compared to our NO measurements, which were in the ppm range [21, 22]. In addition, Zavorsky [23] showed that up to 22

TABLE 1 Summary of methodology for the two diffusion capacity methods

	D_{LCONO} method	D_{LCO} method
Breath-hold time s[#]	5	10
Inhaled gas concentrations mean±SD	0.19±0.018% CO, 6.34±0.59% He, 22.36±0.71% O_2, 52±6 ppm NO, balance N_2	0.3% CO, 0.3% CH_4, 20.9% O_2, balance N_2
Inert gas	He	CH_4
Gas analyser	NO: CiTicel[¶] 7BNT electrochemical cell, CO: electrochemical cell, He: thermal conductivity, O_2: electrochemical cell	CO, CH_4: nondispersive infrared thermopile
Gas sampling method	Physical sample from collection bag	Virtual sample constructed from signals from flow and gas concentration

D_{LCONO}: combined diffusing capacity of the lung for carbon monoxide and nitric oxide; D_{LCO}: diffusing capacity of the lung for carbon monoxide; CO: carbon monoxide; He: helium; O_2: oxygen; NO: nitric oxide; N_2: nitrogen; CH_4: methane. [#]: true apnoea period; [¶]: CiTicel, City Technology, Nuremberg, Germany.

repetitions of the D_{LCONO} measurement does not lead to a decrease in D_{LNO} values. Likewise, he showed that up to 12 repetitions of the test could be performed without significantly lowering D_{LCO} values. Therefore, potential accumulation of CO in the blood creating CO backpressure and thereby decreasing D_{LCO} measurements were not considered to be a problem in the present study.

In addition, we performed the standard D_{LCO} measurement on all subjects. Apart from the methodological differences presented in table 1, the two procedures were performed in the same way.

In order to be able to differentiate between the two methods, outcomes from the D_{LCONO} measurement are denoted with "5s" and outcomes from the standard D_{LCO} measurement with "10s", *e.g.* V_{A10s} for V_A measured using the standard D_{LCO} method.

Quality control

The quality and reproducibility of the measurements were ensured by the following means. Each day the pneumotach was calibrated using the three-flow method with a calibrated 3-L syringe and the apparatus was calibrated for gas fractions using automated procedures for He, CO, O_2 and methane (CH_4). Furthermore, the linearity of the analysers was factory checked. In addition, by using three gases, with different concentrations of CO and NO, linearity was checked before start of the study, in the middle of the study and at its end. Moreover, biological control measurements, in which the same subject performed D_{LCONO} measurements on both pieces of equipment in order to detect fluctuations in values, were performed regularly and showed high levels of repeatability. Furthermore, accuracy of the V_A measurements was checked before start of the study, in the middle of the study and at its end. To our knowledge, no technique has been developed to check V_A obtained during the D_{LCONO} measurements. Therefore, the correctness of the V_A measurements pertaining to the standard D_{LCO} technique with CH_4 as the inert gas was checked both using the Hans Rudolph (Shawnee, KS, USA) D_{LCO} Simulator with EasyLab™ software [24] and using the JQM-syringe D_{LCO} test, in which a D_{LCO} test is performed using a 3-L calibration syringe. Important differences to a normal D_{LCO} test is the fact that the pneumotach is non-heated and that no corrections are made for carbon dioxide or ambient temperature and pressure, saturated with water vapour/body temperature, ambient pressure, saturated with water vapour. V_A measurements obtained using the D_{LCO} technique could later be compared with V_A measurements pertaining to the D_{LCONO} technique.

Both sets of equipment passed all the tests performed.

Calculation of D_m, $1/\theta_{CO}$ and V_c

As mentioned, we took as our starting point the formula proposed by ROUGHTON and FORSTER [2]:

$$1/D_L = 1/D_m + 1/(\theta_b \cdot V_c)$$

According to the most recent knowledge, θ_{NO} is considered to be finite with a value of 4.5 $mL_{NO} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$. Thereby the calculation of D_{mCO} is as follows:

$$D_{mCO} = (1/\alpha - 1/k)/(1/D_{LNO} - 1/(k \cdot D_{LCO}))$$

Where $\alpha = D_{mNO}/D_{mCO} = 1.97$ and $k = \theta_{NO}/\theta_{CO}$. It is important to realise that k is not a constant, since it changes with changes in Hb concentration and mean capillary oxygen pressure (P_{capO_2}) [3, 10, 25].

When calculating $1/\theta_{CO}$, FORSTER's [11] 1987 values for θ_{CO} measured at pH 7.4 were used:

$$1/\theta_{CO} = (1.30 + 0.0041 \cdot P_{capO_2}) \cdot (14.6/\text{Hb concentration in } g \cdot dL^{-1})$$

Most earlier publications in the field have used P_{capO_2}=100 mmHg. However, in the present study, the inspiratory fraction of O_2 was higher than in these studies due to the flushing procedure already described. In order to be able to compare our results with earlier results, we did a correction for O_2 as follows.

Presuming P_{capO_2}=100 mmHg, at standard Hb concentrations (males 14.6 $g \cdot dL^{-1}$, females 13.4 $g \cdot dL^{-1}$) [20], this provides the following values for $1/\theta_{CO}$: males 1.710 $mL_{blood} \cdot min \cdot mmHg \cdot mL_{CO}^{-1}$, females 1.863 $mL_{blood} \cdot min \cdot mmHg \cdot mL_{CO}^{-1}$

V_c was calculated using the following formula:

$$Vc = (1/\theta CO)(1 - \alpha/k)/(1/DLCO - \alpha/DLNO)$$

Again, $\alpha = DmNO/DmCO = 1.97$ and $k = \theta NO/\theta CO$.

As mentioned, the choices made in reference to these calculations are considered in more detail in the Discussion.

Correction for O_2

Largely, the O_2 correction was performed as described by MARTINOT *et al.* [10]. First, P_{capO_2} was calculated using the following equation:

$$PAO_2 - PcapO_2 = VO_2/DLO_2$$

Where P_{AO_2} was the alveolar oxygen tension measured in the expired sample and $V'O_2$ was the oxygen uptake calculated from the mass balance of oxygen between inspiration and expiration in the manoeuvre. The oxygen fraction measured in the sample volume (mid-expiratory) was assumed to be similar to the oxygen fraction in the residual volume at end-expiration. The diffusion capacity of the lung for oxygen (DLO_2) was assumed to be equal to $DLCO5s \times 1.23$.

For each subject we then calculated the $1/\theta CO$ value corresponding to their P_{capO_2} value and standard Hb. This $1/\theta CO$ value was used to calculate $DmCO$ and Vc in the high O_2 conditions described. Finally, $1/\theta CO$ corresponding to P_{capO_2}=100 mmHg and standard Hb was calculated, and by rearranging the ROUGHTON and FORSTER equation, this value and the calculated values of $DmCO5s$ and Vc were used to calculate $DLCO$ corresponding to P_{capO_2}=100 mmHg. Thereby, these $DLCO5s$ values were uncorrected for Hb.

Correction for Hb

Hb-corrected values for $DmCO$ and Vc (labelled "Hb-corr") were found by calculating the $1/\theta CO$ value corresponding to the P_{capO_2} value and measured Hb of each subject. This $1/\theta COHb\text{-}corr$ value was then used to calculate $DmCOHb\text{-}corr$ and $VcHb\text{-}corr$, as already described. Dm is regarded as being independent of Hb, but when estimating Dm from the $DLCONO$ measurement, Hb is to be taken into account since the calculation of Dm includes $DLCO$, which is dependent on Hb. In order to determine $DLCO5sHb\text{-}corr$, $1/\theta CO$ corresponding to P_{capO_2}=100 mmHg and standard Hb was calculated, and by rearranging the ROUGHTON and FORSTER equation, this value and the calculated values of $DmCOHb\text{-}corr$ and $VcHb\text{-}corr$ were used to calculate $DLCO5sHb\text{-}corr$ corresponding to P_{capO_2}=100 mmHg.

Statistical analyses

For demographics, ANOVA was applied to compare means of continuous variables.

Reference equations were established using stepwise model selection in multiple linear regression analysis according to the Akaike information criterion. Possible explanatory variables were age, age squared, sex and height. For equations in table 3, data were stratified by sex. The stepwise regression analysis was initially performed on the entire dataset. Second, data screening was conducted in two steps and based on the initial models. In step 1 of the data screening, cases with residuals $\geqslant$3.0 SD units above and below the predicted values (individual models for each outcome) were removed. In step 2, the same exclusion criterion was used in the regression analysis based on the reduced datasets. Note that an excluded case for one outcome can be included for the other outcomes. Finally, the stepwise regression analysis was performed on data without outliers. The model selection was unaffected by the data screening, since the initial model selection resulted in the exact same models as the model selection based on data without outliers.

To compare the outcomes according to different breath-hold times, Passing–Bablok regression analyses were performed. 95% confidence intervals were calculated using quantile nested bootstrap resampling.

The residual standard deviation (RSD) expresses the variation from the reference equation, and the predicted value $\pm 1.96 \times RSD$ approximates the 2.5th and 97.5th percentiles.

The plots of the reference equations were stratified by sex and present predicted values according to median height. The median height was based on quantile regression with age as explanatory variable.

All analyses were performed using the statistical software R (version 3.2.0; R Foundation, www.r-project.org).

Results

Baseline characteristics of the study population are presented in table 2. When expressed as % predicted values [26], we found no statistically significant difference in forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) or FEV_1/FVC ratio between females and males.

The age distribution of the study population is presented in figure 1. As seen, the age distribution was close to uniform and decreased only slightly for ages >85 years.

Reference equations for the D_{LCONO} measurement are presented in table 3. As seen, after stratification by sex, the independent variables were height, age and age squared, although not all independent variables were included in all equations. The introduction of age squared allows for an accelerated decrease in the dependent variable with increasing age (figure 2a and b).

The D_{LNO}/D_{LCO5s} ratio±SD was found to be 4.4±0.24 and was only marginally dependent on age and height (the latter relationship being nonsignificant). Linear regression analysis with age and height as the only variables showed p=0.00032 with a slope of −0.00251 for age and p=0.058 with a slope of −0.00284 for height (in cm). Adjusted r^2=0.0403 (after data screening).

The reference equations for D_{LCO5s} and D_{LNO} were compared to previously published reference equations for adults (figure 2a and b).

In addition, using Pearson's r and Passing–Bablok regression we compared D_{LCO}, K_{CO} and V_A from the D_{LCONO} and standard D_{LCO} methods, respectively (figure 3a–c). As expected, in all three cases 10-s and 5-s values were strongly correlated with Pearson's r>0.9. However, when using Passing–Bablok regression the 10-s and 5-s methods were shown to be slightly different from each other, since 1 was not included in the 95% confidence interval for slope in any of the three cases. For V_A, Passing–Bablok regression showed that V_{A10s} was systematically higher than V_{A5s} by a constant of 0.01, and proportionally higher by a factor of 1.04. In addition, we found V_{A10s} to be significantly higher with the mean±SD of the difference being 0.28±0.25 L (p<0.01).

The mean red cell fraction of the total resistance for CO uptake, that is the fraction that $1/(\theta_{CO} \cdot V_c)$ constitutes of the total resistance $1/D_{LCO}$, was found to be 72.3%. For NO, the corresponding value $((1/(\theta_{NO} \cdot V_c))/(1/D_{LNO}))$ was 39.3%.

TABLE 2 Characteristics of the study population

	Females	Males
Subjects n	142	140
Age years	53.4±22.6 (18–97)¶	54.1±22.3 (18–97)
Height cm	165.4±7.2 (148.8–183.8)**	179.4±8.1 (155.6–197.5)
Weight kg	64.6±9.0 (45.1–97.0)**	78.5±11.0 (52.0–108.8)
BMI kg·m^2	23.6±2.7 (18.1–29.8)¶	24.4±2.6 (18.0–30.0)
Hb g·dL^{-1}	13.26±1.13 (10.47–15.95)**	14.62±1.36 (11.28–19.01)
FEV_1 L	3.00±0.79 (1.22–4.66)**	4.08±1.02 (1.43–6.31)
FEV_1 % pred [26]	114.6±21.7 (80.9–235.2)¶	109.6±16.7 (68.8–166.9)
FEV_1 Z-score	0.9±1.0 (−1.6–3.7)¶	0.6±1.1 (−2.4–3.7)
FVC L	3.76±0.86 (1.91–5.83)**	5.24±1.22 (1.96–8.50)
FVC % pred [26]	113.1±17.3 (84.5–197.4)¶	111.0±14.9 (78.8–171.5)
FVC Z-score	0.84±0.99 (−1.32–3.30)¶	0.79±1.05 (−1.67–3.77)
FEV_1/FVC % [26]	79.2±7.1 (60.7–95.7)¶	77.8±7.3 (55.4–98.8)
FEV_1/FVC Z-score	0.0±1.0 (−2.7–2.3)¶	−0.3±1.2 (−3.8–3.9)
V_{A5s} L	4.8±0.7 (3.1–7.5)**	6.4±1.1 (3.4–8.6)
V_{A5s} % pred [27]	102.7±12.5 (74.7–135.0)**	96.2±11.9 (65.3–121.8)
D_{LCO10s} mL·min^{-1}·mmHg^{-1}	22.1±5.1 (10.1–34.6)**	30.5±7.8 (11.4–46.1)
D_{LCO10s} % pred [28]	90.1±12.0 (59.7–120.1)**	98.2±14.3 (60.3–150.6)
TLC$^{\#}$L	5.5±0.8 (3.8–8.0)**	7.5±1.1 (4.2–10.1)
TLC$^{\#}$ % pred [29]	106.5±11.7 (77.4–136.2)**	102.9±10.8 (78.8–129.4)

Data are presented as mean±SD (range), unless otherwise stated. Range: lowest to highest value; BMI: body mass index; Hb: haemoglobin; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; V_{A5s}: alveolar volume (from the D_{LCONO} (combined diffusing capacity of the lung for carbon monoxide and nitric oxide) method); D_{LCO10s}: diffusing capacity of the lung for carbon monoxide (from the standard 10-s method); TLC: total lung capacity (from body plethysmography). #: 1 female and 1 male were excluded from the TLC calculations because they did not undergo the body plethysmography measurement; ¶: nonsignificant; **: p<0.01.

In order to examine sex differences in lung structure, regressions were performed for V_c/V_{A5s} and D_{mCO}/V_{A5s} (table 4). Both of these ratios were affected by sex, but in opposite directions. That is, V_c/V_{A5s} was generally lower in males than in females while D_{mCO}/V_{A5s} was higher in males. This suggests that there is a sex difference both in the structure of the alveolocapillary membrane and in the capillary blood volume when normalised to V_A.

Agreement between our two sets of equipment was evaluated for D_{LCO5s} and D_{LNO} measurements after adjustment for the known independent variables. No significant difference was found.

Discussion

The present study is one of the largest of its kind to present reference equations for the combined D_{LCONO} measurement. In particular, the group of subjects aged >70 years is unparalleled in earlier studies. In addition, it is first large-scale standalone study performed on a single uniform population to present

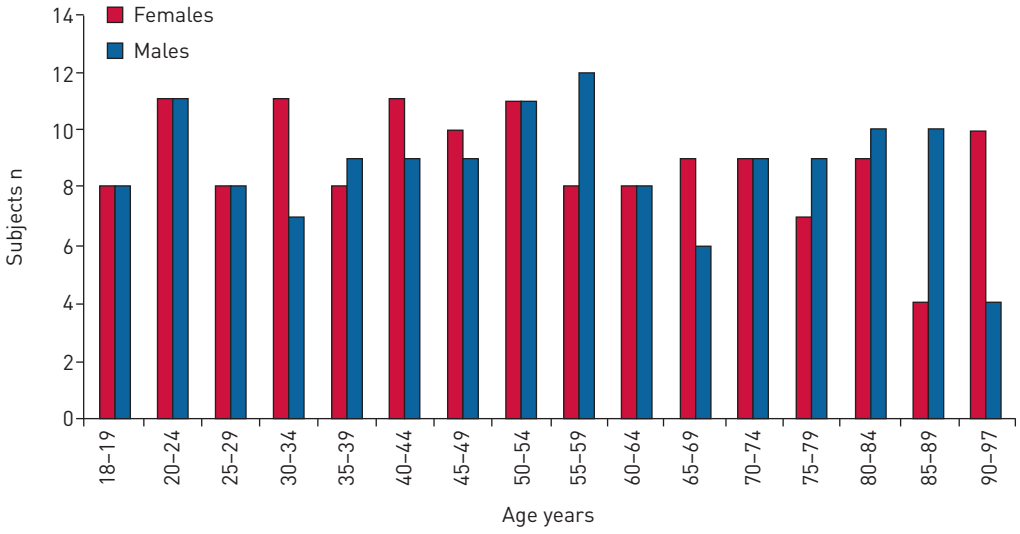

FIGURE 1 Age distribution of the study population.

TABLE 3 Reference equations for the diffusing capacity of the lung for carbon monoxide and nitric oxide

	Subjects after data screening n	Multiple linear regression equation[#]	Adjusted r^2	Residual standard error
Females				
D_{LCO5s} mL·min^{-1}·mmHg^{-1}	141	−3.58+0.192·height−0.00166·age^2	0.766	2.8
K_{CO5s}[¶] mL·min^{-1}·mmHg^{-1}·L^{-1}	140	6.35−0.0316·age	0.649	0.524
D_{LNO} mL·min^{-1}·mmHg^{-1}	142	−2.36+0.766·height−0.00753·age^2	0.796	11.4
K_{NO}[+] mL·min^{-1}·mmHg^{-1}·L^{-1}	141	36.5−0.153·age−0.0476·height	0.718	2.07
V_{A5s} L	141	−3.55+0.0466·height+0.0391·age−0.000426·age^2	0.534	0.488
V_c mL	141	−13.8+0.527·height−0.00421·age^2	0.693	8.70
D_{mCO} mL·min^{-1}·mmHg^{-1}	142	3.76+0.591·height−0.00620·age^2	0.744	10.8
Males				
D_{LCO5s} mL·min^{-1}·mmHg^{-1}	139	−5.01+0.252·height−0.00258·age^2	0.812	3.69
K_{CO5s}[¶] mL·min^{-1}·mmHg^{-1}·L^{-1}	139	7.88−0.0107·height−0.000345·age^2	0.733	0.487
D_{LNO} mL·min^{-1}·mmHg^{-1}	138	5.72+0.970·height−0.0125·age^2	0.824	16.6
K_{NO}[+] mL·min^{-1}·mmHg^{-1}·L^{-1}	139	38.8−0.0689·height−0.00168·age^2	0.777	2.07
V_{A5s} L	138	−7.90+0.0387·age+0.0774·height−0.000442·age^2	0.588	0.687
V_c mL	138	−23.8+0.645·height−0.00547·age^2	0.767	9.31
D_{mCO} mL·min^{-1}·mmHg^{-1}	138	50.4+0.623·height−0.0123·age^2	0.743	19.6

To obtain lower and upper limits of normal (corresponding to the 2.5th and the 97.5th percentile, respectively) subtract or add 1.96·residual standard error to the equation. D_{LCO5s}: diffusing capacity of the lung for carbon monoxide; K_{CO5s}: diffusion capacity per unit alveolar volume for carbon monoxide; D_{LNO}: diffusing capacity of the lung for nitric oxide; V_{A5s}: alveolar volume; V_c: capillary volume; D_{mCO} =diffusing capacity of the alveolar membrane for carbon monoxide. [#]: age in years, height in cm; [¶]: $K_{CO5s}=D_{LCO5s}/V_{A5s}$; [+]: $K_{NO}=D_{LNO}/V_{A5s}$.

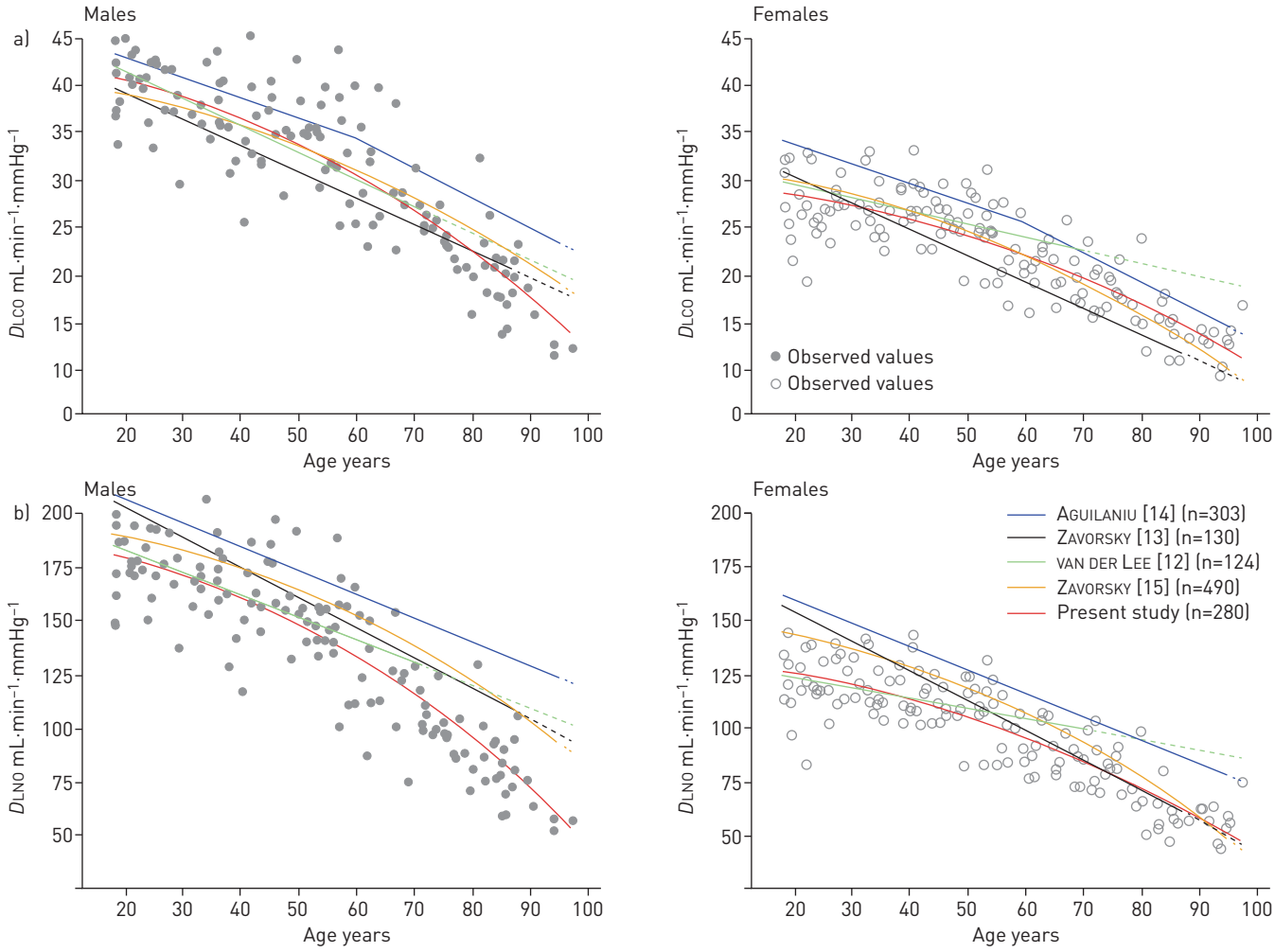

FIGURE 2 a) Diffusing capacity of the lung for carbon monoxide (D_{LCO}) and b) nitric oxide (D_{LNO}) compared to previously published reference equations [12–14]. For each age group, median anthropometric values from our subjects were inserted into the reference equations and the predicted values were depicted as a function of age. Dots represent values measured on each of the subjects. Breath-hold time (true apnoea period) was 5 s in the present study and 5 s in the study by Zavorsky *et al.* [13], 4 s in the study by Aguilaniu *et al.* [14] and 10 s in the study by van der Lee *et al.* [12].

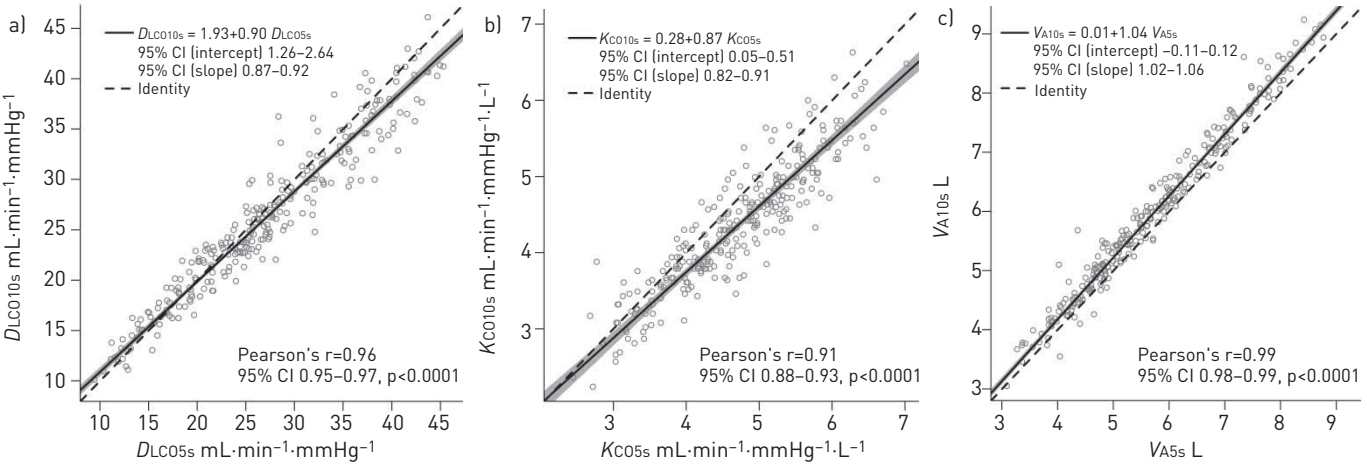

FIGURE 3 Comparison of a) diffusing capacity of the lung for carbon monoxide from the standard 10-s method (D_{LCO10s}) and from the combined method (D_{LCO5s}); b) carbon monoxide transfer coefficient from the standard 10-s method (K_{CO10s}) and from the combined method (K_{CO5s}); and c) alveolar volume from the standard 10-s method (V_{A10s}) and from the combined method (V_{A5s}). Passing–Bablok regressions are shown in the upper left corner of each figure.

TABLE 4 Reference equations for capillary volume (V_c)/alveolar volume (V_{A5s}) and diffusing capacity of the alveolar membrane for carbon monoxide (D_{mCO})/V_{A5s}

	Subjects after data screening n	Multiple linear regression equation[#]	Adjusted r^2	Residual standard error
V_c/V_{A5s} mL·L^{-1}	279	15.4–0.0391·age–0.972·sex–0.000352·age^2	0.577	1.56
D_{mCO}/V_{A5s} mL·min^{-1}·mmHg^{-1}·L^{-1}	278	31.8–0.151·age+2.21·sex–0.0432·height	0.678	2.28

[#]: age in years, sex: male=1, female=0, height in cm. To obtain lower limit of normal and upper limit of normal (corresponding to the 2.5th and the 97.5th percentile, respectively) subtract or add 1.96·residual standard error to the equation.

reference equations for D_{mCO} and V_c based on a finite value of 4.5 mL$_{NO}$·mL$_{blood}^{-1}$·min^{-1}·mmHg^{-1} for the conductance of NO (θ_{NO}) [7].

Furthermore, apparently we are the first to find a small but statistically significant relationship between the D_{LNO}/D_{LCO} ratio and age. This might be a consequence of the relatively large number of older people included in the present study. However, although it is statistically significant, it is worth noting that the change with age is minor, especially compared to the standard deviation of the values. So for clinical purposes D_{LNO}/D_{LCO} can be regarded as an age-independent variable with a mean value of 4.4.

As mentioned in the Methods section, Hb measurements were performed in all participants, but correction for Hb proved to have no or only minor effect on the overall results. It did not significantly change the mean of any of the main outcomes, apart from D_{mCO} which increased slightly from 100.4 to 101.7 mL$_{CO}$·min^{-1}·mmHg^{-1}. Nor did the Hb correction improve the adjusted r^2 for any of the reference equations, and consequently only reference equations for non-Hb corrected values are presented. Our observations on this point are in concordance with those of STAM *et al.* [30] and ZAVORSKY [23], the latter finding changes in D_{LCO} of only ~3%.

Comparison to other reference equations

As seen in figure 2, the reference equations of the present study estimate comparable, but for D_{LNO} somewhat lower values, to those obtained if using one of the other reference equations for adults [12–15, 31]. As described in the Methods section, quality control of measurements was an integrated part of this study and both sets of equipment passed all tests performed. In addition, the fact that no statistically significant difference was found when comparing measurements from the two sets of equipment strengthens our belief that both measured correctly throughout the study.

Of the five reference equations compared in figure 2, AGUILANIU *et al.* [14] produced the highest predicted values. Several circumstances might work together to explain the difference between their results and the results from the present study. Firstly, AGUILANIU *et al.* [14] performed their measurements at two different sites (the cities of Grenoble and Bordeaux) and reported significant effects on both D_{LCO} and D_{LNO}, which were lower in Grenoble than in Bordeaux (mean differences 8.5% and 13.2%, respectively). In figure 2 we have used their equations based on the entire population, which therefore results in higher values than if the Grenoble equations had been used. In addition, although they performed at least two acceptable tests for each subject, apparently AGUILANIU *et al.* [14] used only values from the test with the greater D_{LCO}, which differs from the ATS/ERS recommendation, according to which the mean of two acceptable measurements should be reported [20]. In the present study we used the mean of two acceptable measurements, and this difference in procedures contribute to the observed discrepancy between the studies. Other possible explanations of the observed differences might be that different equipment was used, as well as differences in the populations studied. Finally, deviations in the simultaneously measured V_A might have a rather large impact on D_{LCO} and D_{LNO}. But as AGUILANIU *et al.* [14] did not present information about V_A, comparison with V_A from the present study and its potential influence on the other presented values cannot be made.

As seen in figure 2, only the predicted values from the present study, the study by AGUILANIU *et al.* [14] and the combined dataset by ZAVORSKY *et al.* [15] take into account the observed accelerated loss of diffusing capacity with age. The reason why ZAVORSKY *et al.* [13] and VAN DER LEE *et al.* [12] have only one slope, when values are fitted for age, might be that the number of old people in their studies have been too low to reliably detect this accelerated change with age. In the case of VAN DER LEE *et al.* [12], the slopes depicted in figure 2 are markedly less steep than those from the other studies, which is probably also an effect of the relatively young population in that study. Also notable is the rather high value of 5.1 for the D_{LNO}/D_{LCO} ratio found by ZAVORSKY *et al.* [13] (current consensus is that the ratio is in the range of 4.3–4.9 [6]; values from present study = 4.4, VAN DER LEE *et al.* [12]= 4.5 and AGUILANIU *et al.* [14]= 4.75).

If this is not a consequence of actual differences between different study populations, it can result from measures of D_{LNO} being too high or measures of D_{LCO} being too low or a mixture of the two. We like to think that the first possibility has had the greatest significance, since ZAVORSKY *et al.*'s [15] combined values for D_{LCO} fit almost perfectly with the values from the present study. It should be mentioned that methods, equipment and of course study populations were different in all four studies discussed. In relation to differences in study population, it has been shown that differences in physical activity status have an impact on diffusion parameters [31–34]. Likewise, differences in exposure to air pollution might affect lung function. These aspects have not been analysed in the present study, but they might explain some of the observed variation between studies.

D_{LCONO} *method* versus *standard* D_{LCO} *method*

As seen in figure 3, some differences can be observed between values obtained from the D_{LCONO} method and the standard D_{LCO} method.

The largest difference is in V_A, with V_{A5s} being generally lower than V_{A10s}. In part, this difference might be a result of inadequate mixing of the inert gas with the alveolar gas since short breath-hold times have been shown to lower the measured V_A in some patient groups and in healthy subjects [35–37]. Other important possible reasons for the observed difference are the differences in methodology between the two methods (see table 1). For example, the inert gas used in the calculation of V_A is not the same (He *versus* CH_4). The two gases might have different distributions in the lung and different solubility in tissue owing to their physical properties, and this might lead to differences in the measured V_A.

K_{CO} has been shown to increase with decreasing breath-hold time [37]. When looking at D_{LCO}, this increase in K_{CO} will tend to counteract the effect of a decreasing V_A on D_{LCO}. Indeed, classically D_{LCO} is thought to increase with decreasing breath-hold time, which is shown in studies where breath-hold time is the only factor being changed (that is, same methodology in all other aspects) [37, 38]. In the present study, K_{CO5s} is generally larger than K_{CO10s}, as seen in figure 3b. And, as described above, this increase is seen to "compensate" for the decrease in V_A, thereby resulting in D_{LCO5s} being slightly but significantly larger than D_{LCO10s} (mean±SD difference=0.85±2.3 $mL \cdot min^{-1} \cdot mmHg^{-1}$). In summary, as seen in table 1 the two methods differ in a number of ways, and more research is needed in order to determine how these differences in methodology influence V_A, K_{CO} and D_{LCO}. What is certain is that D_{LCO} measured using the two different methods cannot be used interchangeably, that is, specific reference material has to be used for each of the two methodologies.

Sex difference in V_c/V_A, D_{mCO}/V_A *and* D_{LNO}/D_{LCO5s}

Both V_c/V_A and D_{mCO}/V_A were to some extend affected by sex, although in opposite directions: V_c/V_A was generally slightly higher in females, while D_{mCO}/V_A was lower. This suggests that there is a sex difference both in the alveolocapillary membrane and in the pulmonary capillary volume when normalised to V_A. However, this observed sex difference is affected by the method used for correcting for Hb. This should be kept in mind if subsequent studies are to compare similar results with the results presented here.

In contrast, D_{LNO}/D_{LCO5s} showed no sex difference. However, it is important to note that the D_{LCO5s} values used in this calculation were not corrected for Hb. Since Hb is generally lower in females, the resultant values for D_{LCO5s} should be lower for this reason alone. Therefore, if no sex difference existed between the alveolocapillary membrane and the pulmonary capillary volume when normalised to V_A, then we would expect D_{LNO}/D_{LCO5s} to be higher in females than in males, which was not the case in the present study.

D_{LNO}/D_{LCO}

It has been pointed out that the D_{LNO}/D_{LCO} ratio might be the best way to assess the relationship between D_{mCO} and V_c. The main argument has been the former lack of consensus regarding the true values of θ_{CO}, θ_{NO} and α used in the calculation of D_{mCO} and V_c, since the D_{LNO}/D_{LCO} ratio has the advantage of being independent of these values [6]. Certainly, the ratio can tell us something about the relationship between D_{mCO} and V_c, and in the case of a low measured D_{LCO} value it could point in the direction of the parameter (D_{mCO} or V_c) predominantly accountable for the decrease. However, caution should be exercised when looking at the ratio alone, since an apparently normal value could result from both D_{LNO} and D_{LCO} being low, and in addition a low ratio could of course either result from a low value of D_{LNO} or a high value of D_{LCO}, while the opposite could apply to a high ratio. Furthermore, the scatter of the normal values for the ratio is rather large (mean±SD 4.4±0.24), and for patients the scatter of values is also found to be large [39, 40]. Obviously, this might result in difficulties differentiating between normal and pathological values. The usage of specific values for D_{mCO} and V_c could overcome some of these challenges and in addition it could provide a more detailed view of the resistances associated with lung

diffusion. But as mentioned, if this is to become reality, consensus has to be made regarding the calculation of D_{mCO} and V_c. As discussed later, this might be achievable today.

θ_{CO}, θ_{NO} and α

In recent years, most scientists have agreed that the most correct values for θ_{CO} are those presented by FORSTER [11] in 1987, and thereby not the 1957 values presented by ROUGHTON and FORSTER [2]. Forster himself argued that these new values were more correct, particularly since they were measured at a physiological pH of 7.4 and not pH 8.0 like the 1957 values [9, 12, 14]. In 2016, GUÉNARD *et al.* [41] tested several of the available $1/\theta_{CO}$ *versus* P_{capO_2} equations by exposing 10 normal subjects to two different inspiratory oxygen concentrations while measuring D_{LNO} and D_{LCO}. Several of the equations managed to keep changes in the D_m/V_c ratio at a minimum during changes in P_{capO_2}, among these the equation proposed by FORSTER [11]. On the basis of these results, GUÉNARD *et al.* also proposed a new "best-fit" equation. This equation is used in the work by ZAVORSKY *et al.* [15], since they found that there is still insufficient information to decisively choose between the existing published $1/\theta_{CO}$ *versus* P_{capO_2} equations derived *in vitro*. However, it is important to note that very little difference is seen in values for D_m and V_c when comparing this new equation to the equation by FORSTER. Therefore, in the present study we have decided to continue with the *in vitro* FORSTER equation.

Concerning the true value of α, in line with most other researchers we consider the true value to be 1.97, since this is the theoretical value representing the relationship between the physical solubilities of NO and CO in plasma taking into account their molecular weight [3, 25]. Some researchers have forced α to higher values in order to achieve a better fit of D_{mCO} and V_c values obtained from the D_{LCONO} method with values obtained from the oxygen two-step Roughton–Forster D_{LCO} method. For example, in this way TAMHANE *et al.* [42] found D_{LNO}/D_{mCO} (two-step)=2.42. An explanation for this might be that in their calculations they used θ_{NO}=infinite (thereby assuming $D_{LNO}=D_{mNO}$) together with the 1957 values for θ_{CO}. If instead they had used θ_{NO}=finite, D_{mNO} would not be equal to D_{LNO}, but would exceed this value by ~70–80% (according to values from the present study and HUGHES and BATES [43]). This would lead to an apparent D_{mNO}/D_{mCO} ~4.1–4.3. However, using 1987 values for θ_{CO} increases D_{mCO} approximately two-fold compared to the 1957 values, thereby leading to α values that might be better in concordance with the theoretical value of 1.97 [43]. In any case, since α is defined as the physical diffusivity ratio between NO and CO, the approach by TAMHANE *et al.* [42] cannot be correct.

Much debate has been focused on the correct value of θ_{NO}. In 1987, GUENARD *et al.* [3] assumed $1/\theta_{NO}$ to be negligible when they first introduced the single-breath D_{LCONO} measurement as a possible means of determining D_{mCO} and V_c. Since then, many researchers have regarded θ_{NO} as being infinitely great with reference to the very fast reaction rate of NO with free Hb. However, in recent years experiments *in vitro* as well as *in vivo* conducted by BORLAND and colleagues [7, 44] have consolidated the *in vitro* value of θ_{NO}=4.5 $mL_{NO}\cdot mL_{blood}^{-1}\cdot min^{-1}\cdot mmHg^{-1}$ first presented by CARLSEN and COMROE JR [45] in 1958. In addition, BORLAND and colleagues [7, 44] showed that red blood cell lysis in a membrane oxygenator model of CO and NO transfer or substitution of red blood cells with cell-free haem-based oxyglobin in anaesthetised dogs increased D_{LNO} considerably while D_{LCO} hardly changed. This has led researchers who previously regarded θ_{NO} to be infinite to consider it finite with a value of 4.5 $mL_{NO}\cdot mL_{blood}^{-1}\cdot min^{-1}\cdot mmHg^{-1}$ [4, 6, 39].

According to the work of ROUGHTON and FORSTER from 1957 [2], the red blood cell fraction of the total resistance to CO uptake was estimated to be ~50%. However, newer evidence including morphometric measurements of D_m and re-calculation of values obtained from the oxygen two-step Roughton–Forster D_{LCO} method suggests that this fraction is more likely to be ~75–80% [43, 46]. Some well-known features of D_{LCO} argue in favour of the view that $1/(\theta_{CO}\cdot V_c)$ should be the most important rate-limiting factor for CO transfer: 1) anaemia, increase of carboxyhaemoglobin and/or raising of P_{capO_2} all lower D_{LCO}; and 2) D_{LCO} is low in some pulmonary vascular conditions with normal vital capacity [43, 47, 48]. Values from the present study support this more current view of the distribution of resistances for CO uptake, since the average red blood cell fraction of the total resistance in our study was 72.3%. In addition, the same fraction for NO uptake was 39.3%, which parallels the value of 37% presented by BORLAND *et al.* [7].

Breath-hold time

Another area that needs to be consistent between studies using the D_{LCONO} measurement is the breath-hold time. Standard breath-hold time for the D_{LCO} measurement is 10 s, but this is not suitable for the combined D_{LCONO} measurement, since NO transfer is ~4.5 times faster than for CO. This leads to very low concentrations of NO after 10 s, which is therefore undetectable by electrochemical cells. Use of a more sensitive chemiluminescent analyser circumvents this problem, but adds considerably to the expense. In the present study we chose a breath-hold time of 5 s (true apnoea period), which is in concordance with earlier studies [13, 14, 49].

Caution with automated procedures

The data presented have been obtained by using of equipment and largely automated procedures. This has some obvious advantages regarding effectiveness and ease of use. However, since we experienced more than one incident where these automated procedures did not comply with our needs and where manual correction of data therefore was needed, we would like to call attention to the fact that caution has to be taken when using such automated procedures.

Clinical implications

To date, several studies have pointed at the added value of D_{LCNO} compared to measurement of D_{LCO} alone when examining patients with different pulmonary disorders. This ranges from pulmonary vascular diseases such as chronic thromboembolic pulmonary hypertension to sarcoidosis and cystic fibrosis [40, 50, 51]. Unfortunately, a lack of concordance concerning the D_{LCNO} method and computation of D_{mCO} and V_c complicates the interpretation and in particular, comparison of results. As proposed by HUGHES and VAN DER LEE [6], a way to circumvent some of these discrepancies is to look mainly at the ratio of D_{LNO}/D_{LCO}, which according to the studies mentioned shows alterations specific to different pulmonary disorders. However, being able to reliably measure D_{mCO} and V_c and by comparing the results between studies and with the updated reference material presented in this study, we hope that future studies will be able to provide more information on the pathoanatomy and pathophysiology of pulmonary disorders. It seems achievable to use information obtained from the D_{LCNO} measurement in the everyday clinical work-up of patients.

Conclusion

The present study is one of the largest to date to present reference equations for the D_{LCNO} measurement. In particular, subjects >70 years of age are very well represented, which is exceedingly important as an increasing number of patients are in this age group. In addition, it is the first large-scale standalone study performed on a single uniform population to present reference equations for D_{mCO} and V_c derived from the D_{LCNO} measurement and using current state-of-the-art methodology in the computation of these two measures.

We found age, sex, height and age squared to be independent explanatory variables of the main outcomes. However, the four explanatory variables were not independent predictors of all outcomes. For all outcomes, we found an accelerated loss of capacity with age, which is represented by a negative value of the parameter for the independent variable age squared present in all the reference equations.

We believe that the D_{LCNO} measurement and its ability to determine D_{mCO} and V_c has great potential in future research and diagnostics of pulmonary disorders. Yet, in order to reap the full benefits of this technique, in addition to reliable reference equations, consensus concerning methods and computations must be reached. In recent years, much has changed in this field, but finally agreement seems to be within arm's reach. Therefore, we urge future studies to use this newest methodology as it is presented in this article.

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