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Reference equations for pulmonary diffusing capacity of CO and NO in adult Caucasians

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Abstract:

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

Methods: $D_{L,CO,NO}$ was measured in 282 healthy individuals aged 18-97 years using the single breath technique and a breath-hold time of 5 seconds (true apnoea period). The following values were used: 1) specific conductance of NO (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 (Dm_{NO}/Dm_{CO}) = 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 $g\cdot dL^{-1}$).

Results: Reference equations were established for the outcomes of $D_{L,CO,NO}$ including diffusing capacity of CO ($D_{L,CO}$) and NO ($D_{L,NO}$) and the calculated values Dm and Vc. Independent variables were age, sex, height and age squared.

Conclusion: 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.

INTRODUCTION:

Approximately 100 years ago in Copenhagen, Marie Krogh developed a method for measuring pulmonary gas exchange [1]. Since then, measurement of pulmonary diffusing capacity for carbon monoxide ($D_{L,CO}$) has been used in both work up and monitoring of a wide variety of pulmonary disorders. Since the work of Roughton and Forster in 1957 [2] the model for transfer of a gas from alveolus to blood has been described as consisting of two resistances in series:

$$1/DL = 1/Dm + 1/(\theta_b \cdot Vc) \quad (1)$$

where 1/DL is the total resistance for the specific gas in $min \cdot mmHg \cdot mL_{gas}^{-1}$, 1/Dm is the resistance to passive diffusion through the alveolo-capillary membrane, and $1/(\theta_b \cdot Vc)$ represents the resistance of gas uptake of the blood. Dm is the membrane conductance for a given gas (in $mL_{gas} \cdot min^{-1} \cdot mmHg^{-1}$), θ_b (blood conductance) is the amount of gas taken up by the blood per mmHg tension (in $mL_{gas} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$), and Vc is the pulmonary capillary blood volume (in mL).

Using CO as the inhaled gas, Roughton and Forster showed how to determine values for Dm and Vc by solving the above equation with two unknown variables. The method required measurements of $D_{L,CO}$ at two or more different 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 $D_{L,CO}$ [2]. In 1987, Guenard et al [3] proposed an alternative way of determining Dm and Vc, using CO and NO in one combined single breath manoeuvre ($D_{L,CO,NO}$) 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 $D_{L,CO}$ measurement, the clinician will get more detailed information about the pathoanatomy/pathophysiology underlying e.g. a low diffusion capacity. That is if the defect is related primarily to Dm or Vc.

In the work of Guenard et al [3], θ_{NO} was assumed to be infinitely great since it had earlier been shown that the reaction rate of NO with free 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 $mL_{NO} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$ [7-10]. This value will be used in the present study.

Other disputed issues in this area of research are the true value of θ_{CO} and the value of $\alpha = Dm_{NO}/Dm_{CO}$. In the present study, Forster's [11] 1987 values for θ_{CO} measured at pH 7.4 are used together with an $\alpha = 1.97$. The considerations underlying these choices will be presented in the discussion.

To this date, a small number of reference values for the $D_{L,CO,NO}$ method have been published, but most of these include a rather limited number of subjects (10-71 subjects)[6]. However, one study included 124 healthy adults with a mean age of ~ 40 (standard deviation (SD) ~ 12 years) [12] and another study comprised 130 subjects of which only 17 were aged > 60 years [13]. Furthermore, one larger study from 2008 includes 303 healthy adults aged 20 to 80 years and has 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 amount 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 the state-of-the-art methodology, the aim of this study is to establish new reference values for the $D_{L,CO,NO}$ measurement. In that respect, we also wish to contribute to achieving consensus regarding methodology of the measurement including calculation of Dm and Vc, 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 from the 11th of September 2013 to the 18th of June 2014. They were randomly chosen from the Copenhagen General Population Study, a large general population cohort study including more than 100,000 participants aged ≥20 years who had been randomly selected from the Danish Civil Registration System. Details about this study have been previously published [16, 17]. In addition, participants 18 - 20 years of age 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, non-smoker or former use of tobacco below 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, BMI < 30, and no pregnancy. The subjects lived in Copenhagen or 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 have approved the study.

Measurement of diffusing capacities for CO and NO

Measurements of $D_{L,CO}$ and $D_{L,NO}$ 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 haemoglobin (Hb) (to nearest 0.1 mmol/L) of the participants were obtained. Hb was measured from capillary blood using HemoCue® Hb 201+; HemoCue Denmark. It has earlier been shown that Hb measured from a capillary blood sample closely resembles Hb measured from a venous blood sample from the vein of a forearm [18]. In addition, spirometry, bodyplethysmography and standard single breath $D_{L,CO}$ were performed in all subjects. Measurements were done at 20 meters above sea level. The $D_{L,CO,NO}$ test was performed as follows: After a minimum of 30 minutes 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: dead space = 56 ml, resistance to flow at 12L/sec = 0.9 cm H_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 time (BHT) of 5 seconds was performed (true apnoea period). The actual breath-hold time was calculated using the Jones and Mead method [19] and was found to be 6 seconds (SD = 0.44). The participants then did a fast expiration, and after a $V_{washout}$ = 0.6 litre, a V_{sample} = 0.6 litre was collected. The procedure was repeated after a 4 minutes wait. The measurements were considered as being acceptable if the difference between the two measures of $D_{L,CO}$ was < 10% or < 3 $mL \cdot min^{-1} \cdot mmHg^{-1}$ as recommended by ATS/ERS [20]. If this was not the case, additional measures (up to 5 in total) were performed, until the difference between the highest and second highest measure of $D_{L,CO}$ 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 $D_{L,CO}$, $D_{L,NO}$, K_{CO}, K_{NO} (diffusion capacity per unit alveolar volume for CO and NO respectively) and V_A (alveolar volume) were calculated. The gas used for the measurements consisted of 0.28% CO, 9.3% He, 20.9% O_2, and 69.52% N_2 (analysis uncertainty: ± 2.0% relative. Supplier: Linde Healthcare / AGA) which was mixed with 400 ppm NO/N_2 (analysis uncertainty: ± 5.0% relative. Supplier: 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% oxygen to empty any tubes that might have CO/He/NO gas in them, 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 repetitions of the $D_{L,CO,NO}$ measurement does not lead to a decrease in $D_{L,NO}$ values. Likewise he showed that up to 12 repetitions of the test could be performed without significantly lowering $D_{L,CO}$ values. Therefore potential accumulation of CO in the blood creating CO backpressure and thereby decreasing $D_{L,CO}$ measurements were not considered to be a problem in the present study.

In addition, we also performed the standard $D_{L,CO}$ 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 $D_{L,CO,NO}$ measurement are denoted with ''5s'' and outcomes from the standard $D_{L,CO}$ measurement with ''10s'' – e.g. $V_{A,10s}$ for V_A measured using the standard $D_{L,CO}$ method.

Table 1. Summary of methodology for the two diffusion capacity methods:

	$D_{L,CO,NO}$ method	$D_{L,CO}$ method
Breath-hold time*	5 seconds	10 seconds
Inhaled gas concentrations (SD)	0.19 % CO (0.018), 6.34 % He (0.59), 22.36 % O_2 (0.71), 52 ppm NO (6), and balance N_2	0.3% CO, 0.3% CH_4, 20.9% O_2, and balance N_2
Inert gas	Helium	Methane
Gas analyser	NO: CiTicel 7BNT electrochemical cell, CO: electrochemical cell, He: thermal conductivity, and O_2: electrochemical cell	CO, CH_4: Non-dispersive infrared thermopile
Gas sampling method	Physical sample from collection bag	Virtual sample constructed from signals from flow and gas concentration

* True apnoea period.

Quality control

The quality and reproducibility of the measurements were ensured by the following means: 1) Each day the pneumotach was calibrated using the three-flow method with a calibrated 3 litre syringe and the apparatus was calibrated for gas fractions using automated procedures for He, CO, O_2, and CH_4. 2) Linearity of the analysers was factory checked. In addition, by using 3 gases with different concentrations of CO and NO respectively, 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 $D_{L,CO,NO}$ measurements on both equipments 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 currently been developed to check V_A obtained during the $D_{L,CO,NO}$-measurements. Therefore the correctness of the V_A-measurements pertaining to the standard $D_{L,CO}$-technique with methane as the inert gas was checked both using the Hans Rudolf DLco Simulator with EasyLab™ Software [24] and using the JQM-syringe $D_{L,CO}$ test in which a $D_{L,CO}$ test is basically performed by the use of a 3 L calibration syringe. Important differences to a

normal $D_{L,CO}$ test is the fact that the pneumotach is non-heated and that no corrections for CO_2 or ATPS-BTPS are made. V_A-measurements obtained by the $D_{L,CO}$-technique could later be compared with V_A-measurements pertaining to the $D_{L,CO,NO}$-technique.
Both sets of equipment passed all the tests performed.

Calculation of Dm, 1/θ$_{CO}$ and Vc
As mentioned, we took as our starting point the formula proposed by Roughton and Forster [2]:

$1/D_L = 1/Dm + 1/(\theta_b \cdot Vc)$ (1)

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 Dm_{CO} is as follows:

$Dm_{CO} = (1/\alpha - 1/k)/(1/ D_{L,NO} - 1/(k \cdot D_{L,CO}))$ (2)

Where $\alpha = Dm_{NO}/Dm_{CO} = 1.97$, and $k = \theta_{NO}/\theta_{CO}$. It is important to realize that k is not a constant, since it changes with changes in Hb concentration and mean capillary oxygen pressure ($PcapO_2$) [10, 3, 25].

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

$1/ \Theta_{CO} = (1.30+0.0041 \cdot PcapO_2) \cdot (14.6/\text{Hb concentration in } g \cdot dL^{-1})$ (3)

A $PcapO_2$ = 100 mmHg has been used in most of earlier publications in the field. 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 described in the following paragraph.
Presuming that $PcapO_2$ were = 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}·min·mmHg·mL_{CO}^{-1}, Females: 1.863 mL_{blood}·min·mmHg·mL_{CO}^{-1}

Vc was calculated by use of the following formula:

$$Vc = (1/\theta_{CO})(1-\alpha/k)/(1/D_{L,CO} - \alpha/D_{L,NO}) \quad (4)$$

Again, $\alpha = Dm_{NO}/Dm_{CO} = 1.97$, 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 done as recently described by Martinot JB et al [10]. First, $PcapO_2$ was calculated using the following equation:

$$PAO_2 – PcapO_2 = VO_2/D_{,LO2}$$

Where PAO_2 was the alveolar oxygen partial pressure measured in the expired sample. VO_2 was the oxygen uptake and was 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 oxygen ($D_{L,O2}$) was assumed to be equal to $D_{L,CO,5s}$ x 1.23.

For each subject we then calculated the $1/\theta_{CO}$ value corresponding to their $PcapO_2$ value and standard Hb. This $1/\theta_{CO}$ value was used to calculate Dm_{co} and Vc in the conditions of high O2 as described in the paragraph above. Finally, $1/\theta_{CO}$ corresponding to $PcapO_2$ = 100mmHg and standard Hb was calculated, and by rearranging the Roughton and Forster equation, this value and the calculated values of $Dm_{CO,5s}$ and Vc were used to calculate $D_{L,CO}$ corresponding to $PcapO_2$ = 100mmHg. Thereby, these $D_{L,CO,5s}$-values were uncorrected for Hb.

Correction for Hb

Hb corrected values for Dm_{CO} and Vc (in the following labelled with “hb-corr”) were found by calculating the $1/\theta_{CO}$ value corresponding to the $PcapO_2$ value and measured Hb of each subject. This $1/\theta_{CO,hb\text{-}corr}$ value was then used to calculate $Dm_{co,hb\text{-}corr}$ and $Vc_{,hb\text{-}corr}$ as already described. The actual Dm of a person is regarded as being independent of Hb – but when estimating Dm from the $D_{L,CO,NO}$-measurement, Hb is to be taken into account since the calculation of Dm includes $D_{L,CO}$, which is dependent on Hb. In order to determine $D_{L,CO,5s,hb\text{-}corr}$, $1/\theta_{CO}$ corresponding to $PcapO_2$ = 100mmHg and standard Hb was calculated, and by rearranging the Roughton and Forster equation, this value and the calculated values of $Dm_{CO,hb\text{-}corr}$ and $Vc_{,hb\text{-}corr}$ were used to calculate $D_{L,CO,5s,hb\text{-}corr}$ corresponding to $PcapO_2$ = 100mmHg.

Statistical analyses

For demographics, analysis of variance 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 (AIC). Possible explanatory variables were age, age squared, sex, and height. For equations in table 3, data was stratified by sex. The stepwise regression analysis was initially performed on the entire data set. Secondly, data screening was conducted in 2 steps and based on the initial models. In step 1 of the data screening, cases with residuals ≥ 3.0 standard deviation 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 data sets. 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% CIs were calculated using quantile nested bootstrap resampling.

The residual standard deviation (RSD) expresses the variation from the reference equation, and the predicted value ±1.96×RSD approximates the 2.5th and 97,5th percentile.

The plots of the reference equations were stratified by sex and presents 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, http://www.r-project.org).

RESULTS:

Baseline characteristics of the study population can be seen in table 2. When expressed as % predicted values [26], we found no statistically significant difference in FEV_1, FVC or FEV_1/FVC between females and males.

Table 2. Characteristics of the study population

	Females (n=142) Mean (SD) [range]	Males (n=140) Mean (SD) [range]
Age (years)	53.4 (22.6) [18-97]^{ns}	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/m2)	23.6 (2.7) [18.1-29.8]^{ns}	24.4 (2.6) [18.0-30.0]
Hb ($g \cdot dL^{-1}$)	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]^{ns}	109.6 (16.7) [68.8-166.9]
FEV_1 (Z-score)	0.9 (1.0) [-1.6-3.7]^{ns}	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]^{ns}	111.0 (14.9) [78.8-171.5]
FVC (Z-score)	0.84 (0.99) [-1.32-3.30]^{ns}	0.79 (1.05) [-1.67-3.77]

FEV_1/FVC (%) [26]	79.2 (7.1) [60.7-95.7] ns	77.8 (7.3) [55.4-98.8]
FEV_1/FVC (Z-score)	0.0 (1.0) [-2.7-2.3] ns	-0.3 (1.2) [-3.8-3.9]
$V_{A,5s}$ (L)	4.8 (0.7) [3.1-7.5]*	6.4 (1.1) [3.4-8.6]
$V_{A,5s}$ (%pred) [27]	102.7 (12.5) [74.7-135.0]*	96.2 (11.9) [65.3-121.8]
$D_{L,CO,10s}$ (ml·min^{-1}·mmHg^{-1})	22.1 (5.1) [10.1-34.6]*	30.5 (7.8) [11.4-46.1]
$D_{L,CO,10s}$ (%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]

*$p<0.01$; nsnot significant

Range = lowest to highest value.

BMI = body mass index, FEV_1 = forced expiratory volume in one second, FVC = forced vital capacity, $V_{A,5s}$ = alveolar volume (from the $D_{L,CO,NO}$ method), $D_{L,CO,10s}$ = diffusing capacity for CO (from the standard 10s-method), TLC = total lung capacity (from bodyplethysmography).

§1 female and 1 male have been excluded from the TLC- calculations because they did not perform the bodyplethysmography measurement.

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 above 85.

Reference equations for the $D_{L,CO,NO}$ measurement are presented in table 3. As seen, after having stratified 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 dependant variable with increasing age (figure 2a-b).

Table 3. Reference equations for $D_{L,CO,NO}$:

a) Women:

	n after data screening	Multiple linear regression equation	Adjusted r^2	Residual standard error
$D_{L,CO,5s}$, ml·min^{-1}·mmHg^{-1} *	141	-3.58 + 0.192*height – 0.00166*age^2	0.766	2.8
$K_{CO,5s}$, ml·min^{-1}·mmHg^{-1}·L^{-1} **	140	6.35 – 0.0316*age	0.649	0.524
$D_{L,NO}$, ml·min^{-1}·mmHg^{-1} #	142	-2.36 + 0.766*height – 0.00753*age^2	0.796	11.4
K_{NO}, ml·min-	141	36.5 – 0.153*age – 0.0476*height	0.718	2.07

^{1}·mmHg^{-1}·L^{-1} ##				
$V_{A,5s}$, L §	141	-3.55 + 0.0466*height + 0.0391*age – 0.000426*age^2	0.534	0.488
Vc, ml §§	141	-13.8 + 0.527*height – 0.00421*age^2	0.693	8.70
Dm_{CO}, ml·min^{-1}·mmHg^{-1} &	142	3.76 + 0.591*height – 0.00620*age^2	0.744	10.8

b) Men:

	n after data screening	Multiple linear regression equation	Adjusted r^2	Residual standard error
$D_{L,CO,5s}$, ml·min^{-1}·mmHg^{-1} *	139	-5.01 + 0.252*height – 0.00258*age^2	0.812	3.69
$K_{CO,5s}$, ml·min^{-1}·mmHg^{-1}·L^{-1} **	139	7.88 – 0.0107*height – 0.000345*age^2	0.733	0.487
$D_{L,NO}$, 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
$V_{A,5s}$, L §	138	-7.90 + 0.0387*age + 0.0774*height – 0.000442*age^2	0.588	0.687
Vc, ml §§	138	-23.8 + 0.645*height – 0.00547*age^2	0.767	9.31
Dm_{CO}, ml·min^{-1}·mmHg^{-1} &	138	50.4 + 0.623*height – 0.0123*age^2	0.743	19.6

Age in years. Height in cm.
To obtain Lower Limit of Normal (LLN) and Upper Limit of Normal (ULN) (corresponding to the 2,5th and the 97,5th percentile respectively) subtract or add 1.96*residual standard error to the equation.
*$D_{L,CO,5s}$=diffusing capacity for CO,
**$K_{CO,5s}$=$D_{L,CO,5s}/V_{A,5s}$,
#$D_{L,NO}$=diffusing capacity for NO,
##K_{NO}=$D_{LNO}/V_{A,5s}$,
§$V_{A,5s}$=alveolar volume,
§§Vc=capillary volume,
&Dm_{CO}=diffusing capacity for CO of the alveolar membrane.

The ratio $D_{L,NO}/D_{L,CO,5s}$ was found to be 4.4 (SD = 0.24) and was only marginally dependent on age and height (the latter relation being insignificant). Linear regression analysis having 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-squared = 0.0403 (after data screening).

The reference equations for $D_{L,CO,5s}$ and $D_{L,NO}$ were compared to previously published reference equations for adults (figure 2a-b).

In addition, using Pearson's r and Passing Bablok regression we compared $D_{L,CO}$, K_{CO} and V_A from the $D_{L,CO,NO}$ and standard $D_{L,CO}$ method, respectively (figure 3a-c). As expected, in all three cases 10s and 5s-values were strongly correlated with Pearson's $r > 0.9$. However, when using Passing Bablok regression the 10s and 5s-methods were shown to be slightly different from each other since 1 was not included in the 95% CI for slope in any of the three cases. For V_A Passing Bablok regression showed that $V_{A,10s}$ was systematically higher than $V_{A,5s}$ by a constant of 0.01, and proportionally higher by a factor of 1.04. In addition, we found $V_{A,10s}$ to be significantly higher with the mean of the difference being 0.28 L (SD = 0.25, $p<0.01$).

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

In order to examine sex differences in lung structure, regressions were performed for $Vc/V_{A,5s}$ and $Dm_{CO}/V_{A,5s}$ (table 4). As seen, both of these ratios were affected by sex – but in opposite directions. That is, $Vc/V_{A,5s}$ was generally lower in males than in females while $Dm_{CO}/V_{A,5s}$ was higher in males. This suggests, that there is a sex difference both in the structure of the alveolo capillary membrane and in the capillary blood volume when normalised to V_A.

Table 4. Reference equations for $Vc/V_{A,5s}$ and $Dm_{CO}/V_{A,5s}$:

	n after data screening	Multiple linear regression equation	Adjusted r^2	Residual standard error
$Vc^{*}/V_{A,5s}^{**}$, $ml\cdot L^{-1}$	279	15.4 – 0.0391*age – 0.972*sex – 0.000352*age^2	0.577	1.56
$Dm_{CO}^{\&}/V_{A,5s}$, $ml\cdot min^{-1}\cdot mmHg^{-1}\cdot L^{-1}$	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 (LLN) and Upper Limit of Normal (ULN) (corresponding to the $2{,}5^{th}$ and the $97{,}5^{th}$ percentile respectively) subtract or add 1.96*residual standard error to the equation.
*Vc=capillary volume,
**$V_{A,5s}$=alveolar volume,
&Dm_{CO}=diffusing capacity for CO of the alveolar membrane.

Agreement between our two sets of equipment was evaluated for $D_{L,CO,5s}$ and $D_{L,NO}$ 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 $D_{L,CO,NO}$ measurement. In particular, the group of subjects aged >70 years is unparalleled in earlier studies. It is also the first large scale standalone study performed on a single uniform population to present reference equations for Dm_{CO} and Vc based on a finite value of 4.5 $mL_{NO} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$ for the conductance of NO (θ_{NO}) [7].
Furthermore, apparently we are the first to find a small but statistically significant relation between the ratio $D_{L,NO}/D_{L,CO}$ and age. This might be a consequence of the relatively large amount of old people included in the present study. However, though 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 $D_{L,NO}/D_{L,CO}$ 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 none or only minor effect on the overall results. It did not significantly change the mean of any of the main outcomes apart from Dm_{CO} that increased slightly from 100.4 to 101.7 $mL_{CO} \cdot min^{-1} \cdot mmHg^{-1}$. 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. and Zavorsky, the latter finding changes in $D_{L,CO}$ of only about 3% [30, 23].

Comparison to other reference equations

As seen in figure 2a-b, the reference equations of the present study estimate comparable but for $D_{L,NO}$ somewhat lower values than those obtained if using one of the other reference equations for adults [14, 13, 12, 31, 15]. As described in the Methods section, quality control of measurements was an integrated part of this study from its beginning to its end 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 they both measured correctly throughout the study.
Of the five reference equations compared in figure 2a-b, 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. First of all, Aguilaniu et al. performed their measurements at two different sites (the cities of Grenoble and Bordeaux) and reported significant effects on both $D_{L,CO}$ and $D_{L,NO}$, which were lower in Grenoble than in Bordeaux (mean differences 8.5% and 13.2%, respectively). In figure 2a-b 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, though they performed at least two acceptable tests for each subject, apparently Aguilaniu et al. used only values from the test with the greater $D_{L,CO}$, which is different 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 rather large impact on $D_{L,CO}$ and $D_{L,NO}$. But as Aguilaniu et al. 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 from figure 2a-b, only the predicted values from the present study, the study by Aguilaniu et al. and the combined dataset by Zavorsky et al (2017) take into account the observed accelerated loss of diffusing capacity with age. The reason why Zavorsky et al. (2008) and Van der Lee et al. 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 2a-b are markedly less steep than those from the other studies, which is probably also an effect of the relatively young population in that study. Notable is also the rather high value of 5.1 for the $D_{L,NO}/D_{L,CO}$ ratio found by Zavorsky et al. (2008) [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, obviously it can either result from measures of $D_{L,NO}$ being too high or measures of $D_{L,CO}$ 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 combined values from 2017 for $D_{L,CO}$ 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 earlier been shown that differences in physical activity status have an impact on diffusion parameters [32-34, 31]. 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.

$D_{L,CO,NO}$ method vs standard $D_{L,CO}$ method

As seen from figure 3a-c, some differences can be observed between values obtained from the $D_{L,CO,NO}$ method and the standard $D_{L,CO}$ method respectively.

The largest difference is in V_A with $V_{A,5s}$ being generally lower than $V_{A,10s.}$ 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 earlier 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, which is used in the calculation of V_A, is not the same (helium vs. methane). The two gases might have different distributions in the lung and different solubility in tissue owing to their respective physical properties, and this might lead to differences in the measured V_A.

K_{CO} has earlier been shown to increase with decreasing breath-hold time [37]. When looking at $D_{L,CO}$, this increase in K_{CO} will tend to counteract the effect of a decreasing V_A on $D_{L,CO}$. Indeed, classically $D_{L,CO}$ 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, $K_{CO,5s}$ is generally larger than $K_{CO,10s}$ as seen from figure 3b. And as described above, this increase is seen to "compensate" for the decrease in Va, thereby resulting in $D_{L,CO,5s}$ being slightly but significantly larger than $D_{L,CO,10s}$ (mean difference = 0.85 ml/min/mmHg, SD = 2.3). In summary, as seen from 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 $D_{L,CO}$. What is certain is that DL,CO measured with 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 Vc/V_A, Dm_{CO}/VA and $D_{L,NO}/D_{L,CO,5s}$

Both Vc/V_A and Dm_{CO}/V_A were to some extend affected by sex, though in opposite directions seeing that Vc/V_A was generally slightly higher in females while Dm_{CO}/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, $D_{L,NO}/D_{L,CO,5s}$ showed no sex difference. However, it's important to note that the $D_{L,CO,5s}$ values used in this calculation were not corrected for Hb. Since Hb is generally lower in females, the resultant values for $D_{L,CO,5s}$ 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 one would expect $D_{L,NO}/D_{L,CO,5s}$ to be higher in females than in males, which was not the case in the present study.

$D_{L,NO}/D_{L,CO}$

Earlier it has been pointed out that the $D_{L,NO}/D_{L,CO}$ ratio might be the best way to assess the relationship between Dm_{CO} and Vc. 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 Dm_{CO} and Vc, since the $D_{L,NO}/D_{L,CO}$ ratio has the advantage of being independent of these values [6]. Most certainly, the ratio can tell us something about the relationship between Dm_{CO} and Vc, and in the case of a low measured $D_{L,CO}$ value it could point in the direction of the parameter (Dm_{CO} or Vc) predominantly being accountable for the decrease. However, caution should be exercised when looking at the ratio alone, since an apparently normal value could result from both $D_{L,NO}$ and $D_{L,CO}$ being low, and in addition a low ratio could of course either result from a low value of $D_{L,NO}$ or a high value of $D_{L,CO}$, while the opposite could apply to a high ratio. Furthermore, as mentioned the scatter of the normal values for the ratio is rather large (mean = 4.4, SD = 0.24), and for patients the scatter of values is found to be large as well [39, 40]. Obviously, this might result in difficulties differentiating between normal and pathological values. The usage of specific values for Dm_{CO} and Vc 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 Dm_{CO} and Vc. As discussed in the paragraph below this might be achievable today.

θ_{CO}, θ_{NO}, and α

In the 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 by Roughton and Forster [2]. Forster himself argued that these new values were more correct in particular 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. tested several of the available $1/\theta_{CO}$ versus $PcapO_2$ equations by exposing 10 normal subjects to two different inspiratory oxygen concentrations while measuring $D_{L,NO}$ and $D_{L,CO}$. Several of the equations managed to keep changes in the Dm/Vc ratio at a minimum during changes in $PcapO_2$ – 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. 2017, since they find that there is still insufficient information to decisively choose between the existing published $1/\theta_{CO}$ versus $PcapO_2$ equations derived in vitro. However, it is important to note that very little difference is seen in values for Dm and Vc when comparing this new equation to the equation by Forster. Therefore, in the present study we have decided to stick 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]. Earlier, some researchers have forced α to higher values in order to achieve a better fit of Dm_{CO} and Vc values obtained from the $D_{L,CO,NO}$ method with values obtained from the oxygen two-step Roughton-Forster $D_{L,CO}$ method. For example in this way Tamhane et al. [41] found $D_{L,NO}/Dm_{CO}$(two-step) = 2.42. An explanation for this might be that in their calculations they used θ_{NO} = infinite (thereby assuming $D_{L,NO} = Dm_{NO}$) together with the 1957-values for θ_{CO}. If instead they had used θ_{NO} = finite, Dm_{NO} would not be equal to $D_{L,NO}$ but would exceed this value with around 70-80% (according to values from the present study and Hughes and Bates [42]). This would lead to an apparent $Dm_{NO}/Dm_{CO} \sim 4.1$ to 4.3. However, using 1987-values for θ_{CO} increases Dm_{CO} ~ twofold compared to the 1957 values thereby leading to α values that might be better in concordance with the theoretical value of 1.97 [42]. In any case, since α is defined as the physical diffusivity ratio between NO and CO, the approach by Tamhane et al. cannot be correct [6, 41].

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 $D_{L,CO,NO}$ measurement as a possible means of determining Dm_{CO} and Vc. 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 et al. [7, 43] 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 [44] in 1958. Borland et al. 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 heme based oxyglobin in anaesthetised dogs increased $D_{L,NO}$ considerably while $D_{L,CO}$ hardly changed [43, 7]. Today, this has led researchers previously regarding θ_{NO} as being infinite to consider it as being finite with the value of 4.5 $mL_{NO} \cdot mL_{blood}^{-1} \cdot min^{-1} \cdot mmHg^{-1}$ [4, 39, 6].

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 Dm and re-calculation of values obtained from the oxygen two-step Roughton-Forster $D_{L,CO}$ method suggests that this fraction is more likely to be around 75-80% [45, 42]. Some well-known features about $D_{L,CO}$ argue in favour of the view that $1/(\theta_{CO} \cdot Vc)$ should be the most important rate limiting factor for CO transfer. These are 1) anaemia, increase of HbCO, and/or raising of $PcapO_2$ lower $D_{L,CO}$, and 2) $D_{L,CO}$ is low in some pulmonary vascular conditions with normal vital capacity [42, 46, 47]. 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 $D_{L,CO,NO}$ measurement is the breath-hold time. Standard breath-hold time for the $D_{L,CO}$ measurement is 10 sec, but this is not suitable for the combined $D_{L,CO,NO}$ measurement since NO transfer is about 4.5 times faster than for CO. This leads to very low concentrations of NO after 10 sec, 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 sec (true apnoea period), which is in concordance with earlier studies [48, 13, 14].

Caution with automated procedures

The presented data has been achieved by the use of equipment using 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 this date, several studies have pointed at the added value of $D_{L,CO,NO}$ compared to measurement of $D_{L,CO}$ 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 [49, 50, 40]. Unfortunately, a lack of concordance concerning the $D_{L,CO,NO}$ method and computation of Dm_{CO} and Vc complicates the interpretation and especially 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 $D_{L,NO}/D_{L,CO}$, which according to the studies mentioned shows alterations specific to different pulmonary disorders. However, being able to reliably measure Dm_{CO} and Vc 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. This way, it seems achievable to use information obtained from the $D_{L,CO,NO}$ 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 $D_{L,CO,NO}$ 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 Dm_{CO} and Vc derived from the $D_{L,CO,NO}$ measurement and using todays 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 $D_{L,CO,NO}$ measurement and its ability to determine Dm_{CO} and Vc 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 has to be made. 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.

Conflict of interest

None declared

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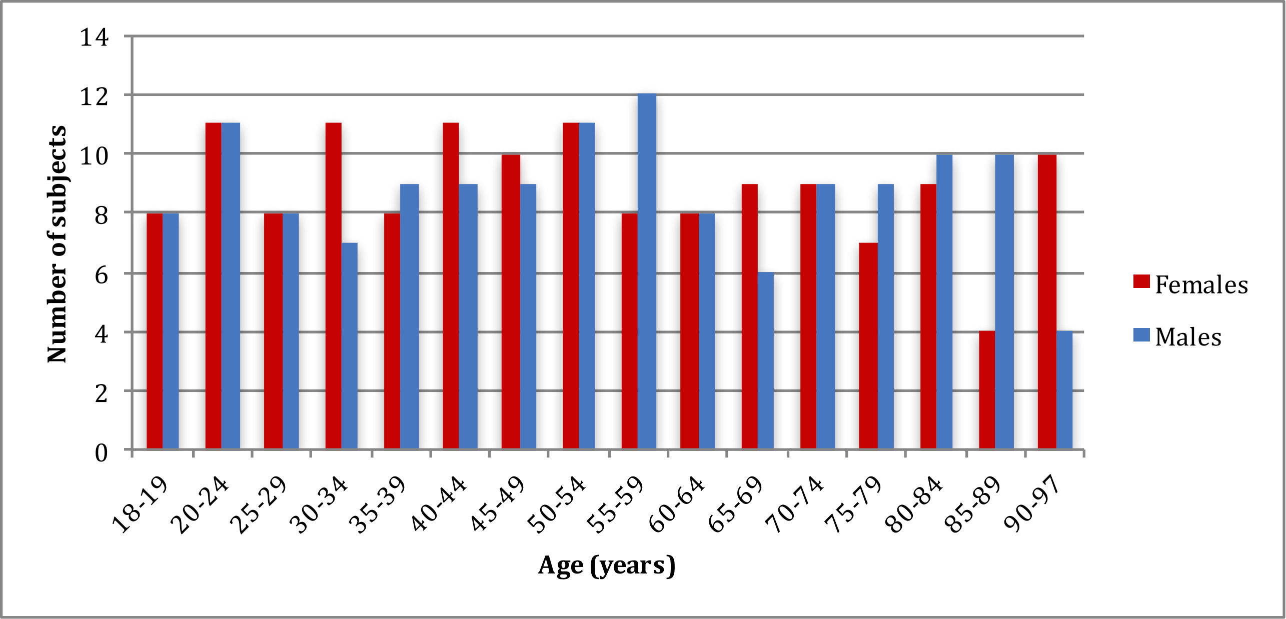


Number of subjects
14
12
10
8
6
4
2
0
18-19
20-24
25-29
30-34
35-39
40-44
45-49
50-54
55-59
60-64
65-69
70-74
75-79
80-84
85-89
90-97
Age (years)
Females
Males

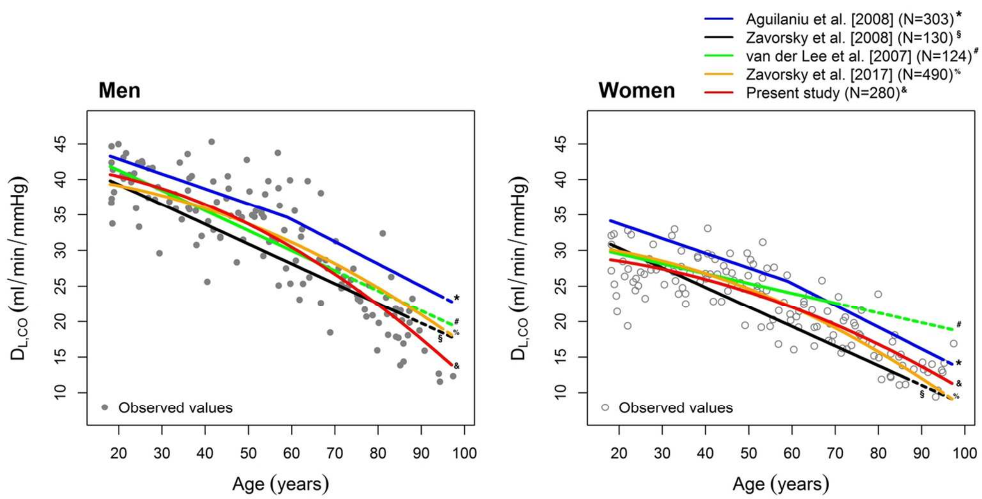
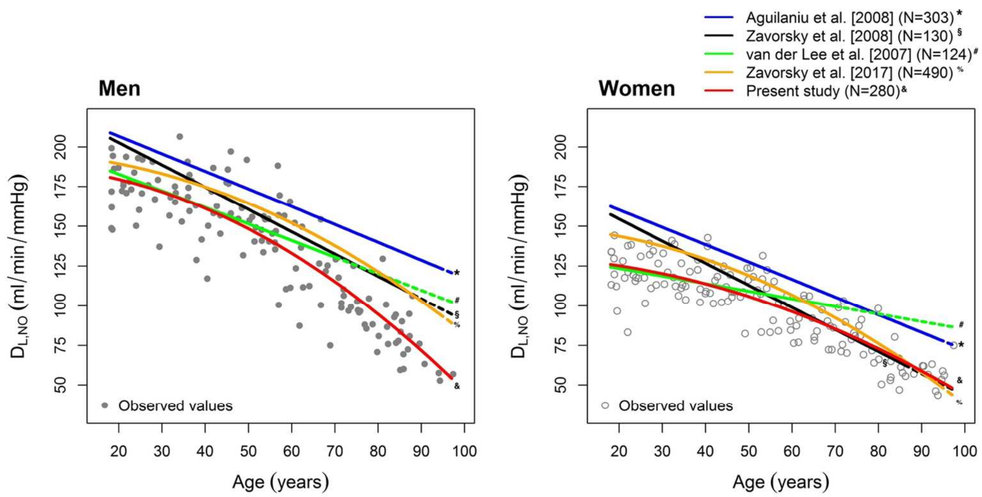
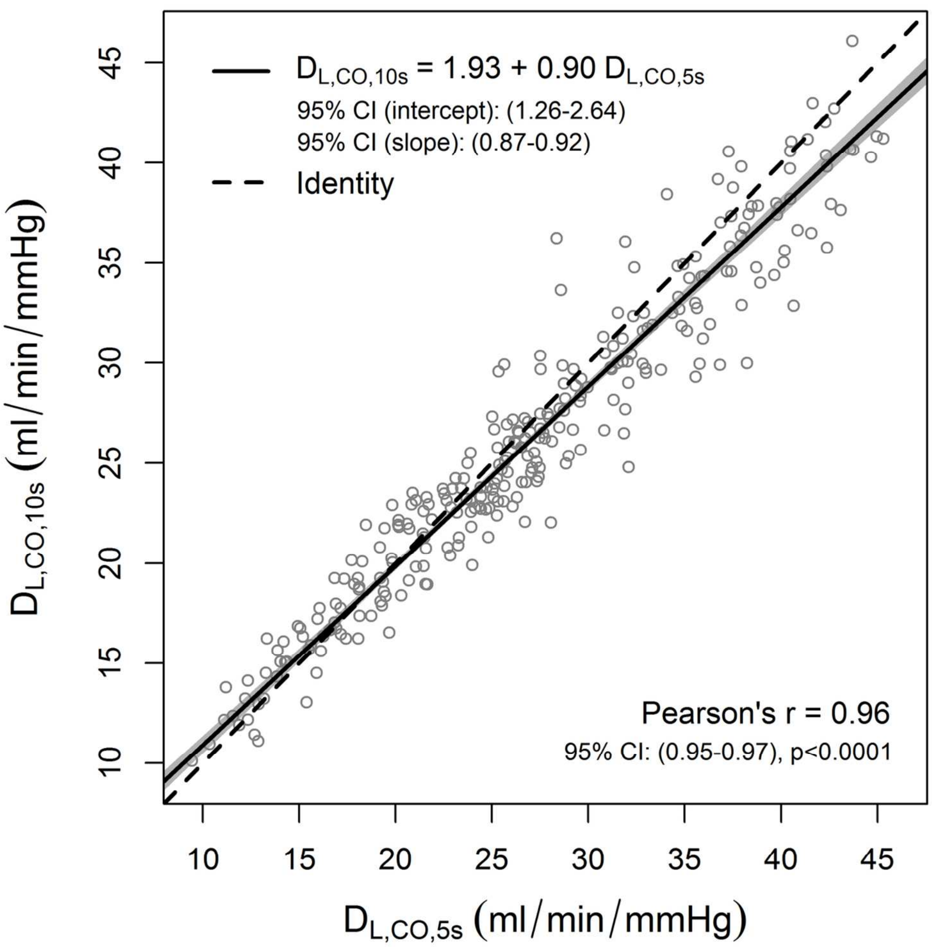
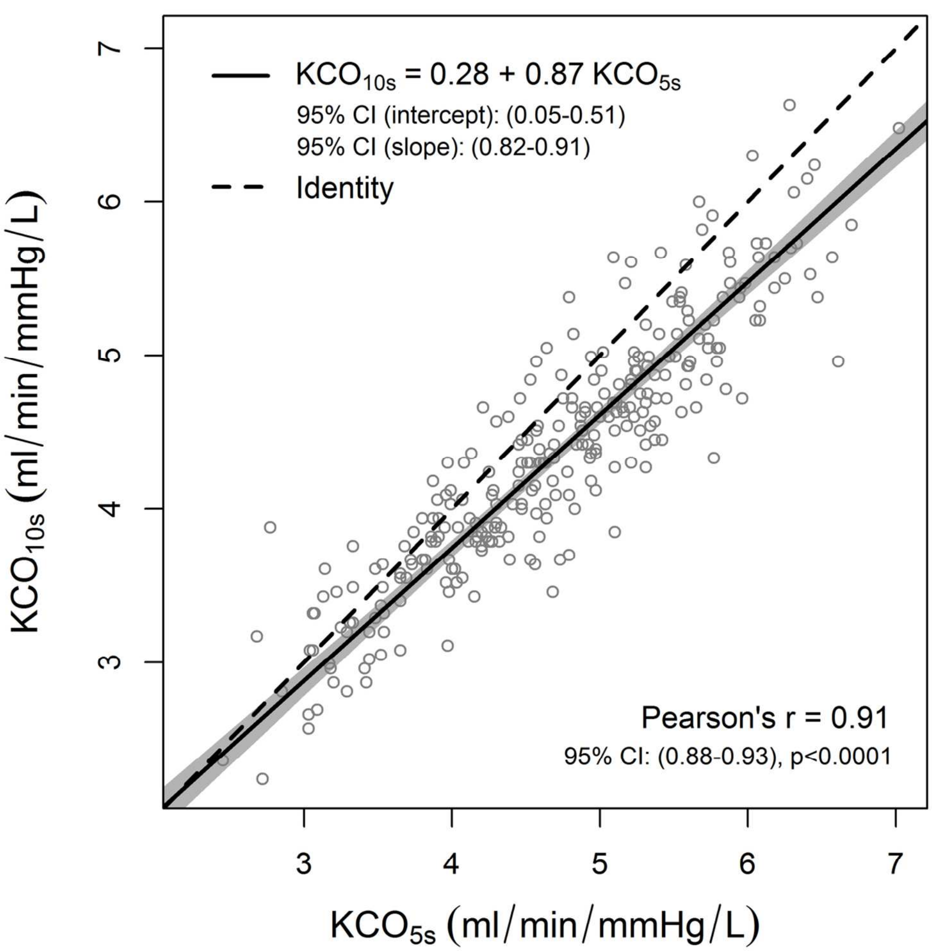


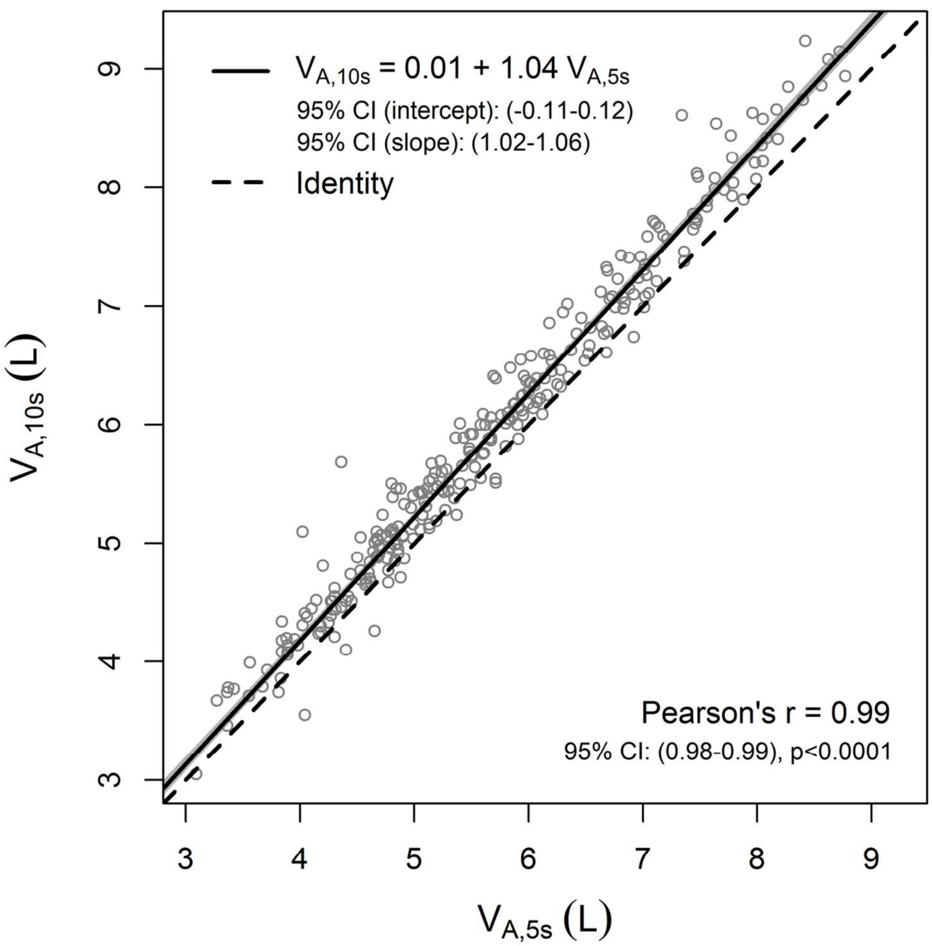
Figure 2. (a) DL,CO and (b) DL,NO compared to previously published reference equations [14, 13, 12]. For each age group, median anthropometric values from our subjects were inserted into the reference equations and the predicted values were depicted as a function of age. Dots represent values measured on each of the subjects. BHT (true apnoea period) was 5 sec in the present study and in the study by Zavorsky et al., 4 sec in the study by Aguilaniu et al., and 10 sec in the study by Van der Lee et al.


Aguilaniu et al. [2008] (N=303) *
Zavorsky et al. [2008] (N=130) §
van der Lee et al. [2007] (N=124) #
Zavorsky et al. [2017] (N=490) %
Present study (N=280) &
Men
Women
D_L,NO (ml/min/mmHg)
Age (years)
Observed values
200
175
150
125
100
75
50
20
30
40
50
60
70
80
90
100



Caption : Figure 3. Comparison of (a) DL,CO,10s and DL,CO,5s, (b) KCO,10s and KCO,5s, and (c) VA,10s and VA,5s. Passing Bablok regressions are shown in the upper left corner of each figure


KCO10s = 0.28 + 0.87 KCO5s
95% CI (intercept): (0.05-0.51)
95% CI (slope): (0.82-0.91)
Identity
Pearson's r = 0.91
95% CI: (0.88-0.93), p<0.0001
KCO10s (ml/min/mmHg/L)
KCO5s (ml/min/mmHg/L)
3
4
5
6
7
3
4
5
6
7



$V_{A,10s}$ = 0.01 + 1.04 $V_{A,5s}$
95% CI (intercept): (-0.11-0.12)
95% CI (slope): (1.02-1.06)
Identity
Pearson's r = 0.99
95% CI: (0.98-0.99), p<0.0001
$V_{A,10s}$ (L)
$V_{A,5s}$ (L)
3
4
5
6
7
8
9