Standardisation and application of the single-breath determination of nitric oxide uptake in the lung

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ABSTRACT Diffusing capacity of the lung for nitric oxide (D_{LNO}), otherwise known as the transfer factor, was first measured in 1983. This document standardises the technique and application of single-breath D_{LNO}. This panel agrees that 1) pulmonary function systems should allow for mixing and measurement of both nitric oxide (NO) and carbon monoxide (CO) gases directly from an inspiratory reservoir just before use, with expired concentrations measured from an alveolar "collection" or continuously sampled *via* rapid gas analysers; 2) breath-hold time should be 10 s with chemiluminescence NO analysers, or 4–6 s to accommodate the smaller detection range of the NO electrochemical cell; 3) inspired NO and oxygen concentrations should be 40–60 ppm and close to 21%, respectively; 4) the alveolar oxygen tension (P_{AO_2}) should be measured by sampling the expired gas; 5) a finite specific conductance in the blood for NO (θNO) should be assumed as $4.5\ mL \cdot min^{-1} \cdot mmHg^{-1} \cdot mL^{-1}$ of blood; 6) the equation for 1/θCO should be $(0.0062 \cdot P_{AO_2}+1.16)$·(ideal haemoglobin/measured haemoglobin) based on breath-holding P_{AO_2} and adjusted to an average haemoglobin concentration (male $14.6\ g \cdot dL^{-1}$, female $13.4\ g \cdot dL^{-1}$); 7) a membrane diffusing capacity ratio (D_{MNO}/D_{MCO}) should be 1.97, based on tissue diffusivity.

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Development and selection of the task force panel

The initial application to create a standardisation document on diffusing capacity of the lung for nitric oxide (*D*LNO) began when a proposal was submitted to the European Respiratory Society (ERS) scientific committee in 2014. The proposal suggested that a task force be created to tackle important methodological considerations for the measurement of *D*LNO so that its measurement and interpretation of the results could be standardised. In October 2014, a revised application was submitted that included a panel of experienced physicians, physiologists, physician scientists and a technologist. In early 2015, the ERS science council and executive committee approved the expert panel and funded the task force. All conflicts of interest were declared and vetted.

The task force panel searched Medline (accessed *via* PubMed) in a literature search. We used the following main keywords in our search: "pulmonary diffusing capacity", "pulmonary diffusing capacity for nitric oxide", "*D*LNO" and "*T*LNO" (transfer factor of the lung for nitric oxide). We combined the results from each of the keywords and then filtered the search to list only human studies published in English between 1946 and 2016. The results yielded 4000 citations. The task force panel then reviewed the abstracts of these citations and identified 103 peer-reviewed articles as relevant to this document, and a further 47 as potentially relevant. Article relevance was determined through panel discussion and consensus. Abstracts from scientific conferences and articles that were not peer-reviewed were generally not included. However, two abstracts [1, 2], a dissertation [3] and a chapter from the *Handbook of Physiology* [4] were included due to their important historical and scientific significance with regard to *D*LNO. In all three face-to-face meetings, each panel member critiqued each section for content and appropriate references and debated several issues. This document is the culmination of compromise within the panel.

The history of single-breath *D*LNO or *T*LNO

Origins of D*LNO*

Initially, interest in nitric oxide (NO) uptake was toxicological. High concentrations of nitrogen dioxide (NO_2; 100 ppm) or NO (0.5–2%) when inhaled for 7–50 min caused death and lung damage in cases of accidental human exposure during anaesthetic procedures [5] or experimental animal exposure [6]. Interestingly an emphysema-like lesion had been described [6], fuelling speculation that "oxides of nitrogen" caused emphysema in smokers.

A group in Cambridge (UK), using an NO analyser based on the description of chemiluminescence [7] found that the half-life disappearance of 1000 ppm NO in whole smoke was 4.3 min (adjusting for the 14.4% oxygen concentration in smoke [8]) and when inhaled, almost all the NO completely diffused into the lungs [9]. This suggested that oxidation of inhaled NO was minimal and that emphysema was not caused by NO.

Next, they measured *D*LNO and the diffusing capacity of the lung for carbon monoxide (*D*LCO); these data were initially presented as abstracts in 1983–1984 [1, 2], and reported in BORLAND's doctoral project [3]. Subsequently, these *D*LCO and *D*LNO observations on varying breath-hold time and back tension were published in 1989 [10], in which the differences in *D*LCO and *D*LNO were undetectable within the sensitivity of the analyser (1 ppm). However, there was greater volume dependence of *D*LNO compared to *D*LCO, and independence of *D*LNO (but not *D*LCO) from hyperoxia [10].

Independently, Daniel Bargeton and Hervé Guénard in Paris had speculated that the ROUGHTON and FORSTER equation ($1/D_{LCO}=1/D_M+1/\theta CO\cdot V_C$) [11] could be solved using a single manoeuvre with simultaneous measurement of carbon monoxide (CO) and NO uptake. *D*M is the diffusing capacity, dependent on molecular diffusion only, of the membranes separating the alveolar epithelial surface from the red cell (also called the alveolar–capillary membrane conductance), *V*C is the total volume of blood in the lung capillaries exposed to alveolar air in millilitres and θCO is the number of millilitres of gas taken up by the red cells in 1 mL of blood per minute per 1 mmHg of partial pressure of dissolved gas between the plasma and interior of the red cell (also called the specific conductance in the blood for CO) [11]. The reciprocals ($1/D_{LNO}$ or $1/D_{LCO}$, $1/D_M$ and $1/\theta\cdot V_C$) are the total diffusion (or transfer) resistance and the membrane and red cell or blood resistance, respectively. GUÉNARD *et al.* [12] published their formula for *D*M and *V*C from simultaneous single-breath *D*LNO and *D*LCO in 1987.

Evolution of D*LNO (1989–2016)*

Early work had shown that mean *D*LNO exceeded mean *D*LCO by 4.3–5.3-fold [10, 12]. In other words, the transfer resistance for NO ($1/D_{LNO}$) from alveolar gas to capillary blood was about one-fifth of that for CO; this difference could not be wholly explained by the two-fold greater tissue diffusivity of NO *versus* CO. The physiological challenge was to find the reasons for this difference, and to test the notion, originally held, that the specific conductance in the blood for NO (θNO) was quasi-infinite and that the transfer resistance from plasma to haemoglobin (Hb) capture was close to zero.

Over the subsequent two decades, it was demonstrated that there was "significant blood resistance to nitric oxide transfer in the lung" [13, 14], and that θNO was finite. In clinical studies, the fact that *D*LNO, unlike *D*LCO, was relatively independent of changes in the inspired oxygen concentration, and thus alveolar oxygen pressure [15, 16] and haematocrit [17], which operate through variations in the θ value for blood, seemed to support the original notion that θNO was "effectively" infinite, and that *D*LNO is a surrogate for the alveolar membrane diffusing capacity, *i.e.* *D*LNO=*D*MNO=1.97·*D*LCO; this view is still held by some [18, 19]. But the current consensus is that *D*LNO is weighted, but not dominated, by the membrane gas conductance, while the *D*LCO is dominated by θCO [20]. The *D*LNO/*D*LCO ratio has been studied in several clinical situations [21]. The uptake pathways for inhaled NO and CO from the alveolus to the red cell in the pulmonary capillary are presented in figure 1.

Determinants of NO uptake

Reaction of NO and CO with capillary blood

The reaction of Hb in solution with NO is extremely rapid (nearly 1500 times faster than CO) [22]. More importantly, the reaction of NO with Hb solutions is 500–1000 times faster than its reaction with blood from animal [23] or human [24] sources. Therefore, θNO cannot be "infinite", as originally thought [10, 12] or more recently claimed [18, 19]. Further support for a "finite" θNO value comes from physiological experiments where the red cell was "by-passed", either by adding free Hb (by haemolysis) or a haem-based blood substitute to the membrane oxygenator perfusate, or by exchange transfusion of dogs with chemically stabilised bovine haemoglobin (Oxyglobin™). In every case, *D*LNO increased as Hb or its haem substitute became more accessible to inhaled NO [13]. The site of red cell resistance could lie in plasma, the red cell membrane or the interior of the cell. Borland *et al.* [14] altered each barrier in turn. Only changing the red cell interior appeared to alter *D*LNO [14].

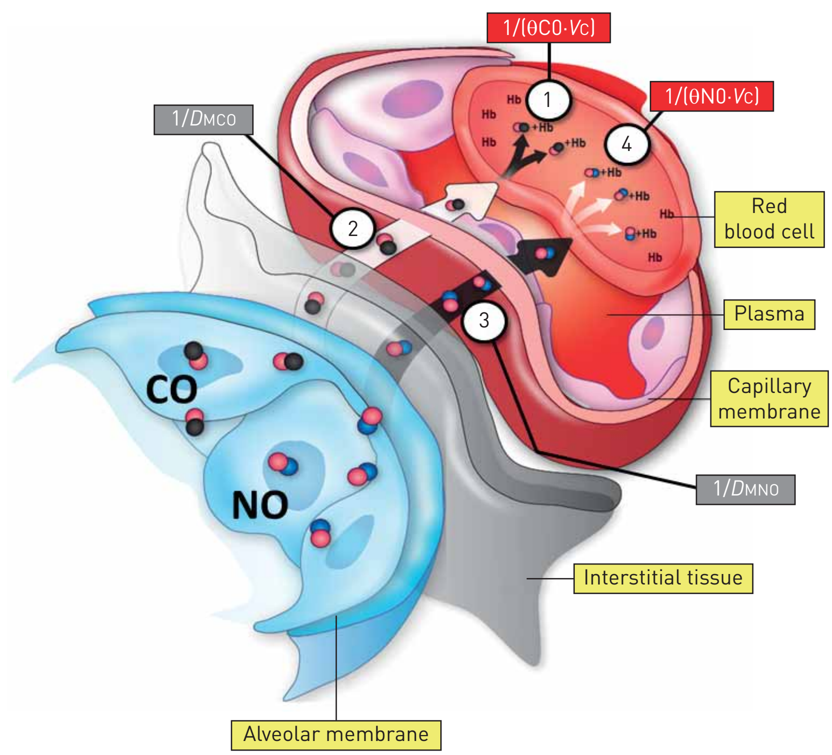

FIGURE 1 Diagram of the uptake pathways for inhaled nitric oxide (NO) and carbon monoxide (CO) from the alveolar membrane to their combination with haemoglobin (Hb) within the red blood cell, in terms of the Roughton–Forster equation, $1/D_L=1/D_M+1/(\theta \cdot V_c)$, where $1/D_L$ is the total resistance to NO or CO uptake, $1/D_M$ is the resistance from the alveolar membrane to the red cell membrane (membrane resistance) and $1/(\theta \cdot V_c)$ is the diffusion and chemical combination resistance (red cell resistance) within the erythrocyte (1). The chief barrier to CO uptake is within the red cell (~70–80%); the ~25% remaining resistance to CO diffusion is located in the alveolar membrane (2). The main resistance barrier for NO lies between the alveolar and red blood cell membranes (~60%; 3), with the red cell resistance (4) comprising ~40% of the resistance to NO diffusion, as observed by Borland *et al.* [13]. Specifically, the red cell interior is the determinant part of the membrane resistance to NO [14]. Reproduced and adapted from [20] with permission from the publisher.

An optimal value for θCO

Due to competitive binding between CO and oxygen for Hb-accessible sites, there is a strong association between θCO and mean pulmonary capillary oxygen tension (P_{O_2}) (1/θCO, the resistance to CO uptake by blood, increases as P_{O_2} increases). The ideal alveolar P_{O_2} has been taken as a surrogate for mean lung capillary P_{O_2} [16]; the difference is small in normoxia in healthy lungs, but increases in disease due to ventilation–perfusion and/or diffusion–perfusion heterogeneity. The relationship between 1/θCO and alveolar (capillary) P_{O_2} is usually expressed as:

$$1/\theta \mathrm{CO} = (\mathrm{a} \cdot P_{\mathrm{O}_2} + \mathrm{b}) \cdot (\text{ideal Hb} \div \text{measured Hb}) \quad (1)$$

where the units for 1/θCO are mL of CO·(mL blood·min·mmHg)$^{-1}$; “a” is the slope, a temperature- and pH-dependent coefficient linked to the kinetics of CO combining with Hb (the “reactive” coefficient); “b” is the y-intercept, or “diffusion” coefficient (now thought to be mostly within the red cell [14]); and (ideal Hb ÷ measured Hb) is the standardised normal Hb concentration as a proportion of the subject’s actual Hb value. Eight published equations (for human blood) have been reviewed in recent publications, but they differ in terms of pH and rapid-reaction methodology [16, 19, 25]. There is considerable interstudy variation in both “a” and “b” coefficients (equation 1), but methodological differences probably explain most of the variability. For example, REEVES and PARK [26] exposed static, non-flowing blood to step changes of P_{O_2} and carbon monoxide tension; their “reactive” coefficient was 50–90-fold less than found by other methodologies, and their findings have not been replicated. Clearly, differences in the coefficients in equation 1 will influence the calculation of D_{MCO} using the classical Roughton–Forster multistep alveolar P_{O_2} method. For example, upon exercise, and depending on the 1/θCO *versus* P_{O_2} equation used, D_{MCO} may vary from 48 to 128 mL·min^{-1}·mmHg^{-1} and for V_C from 104 to 212 mL [27]. In the literature, several versions of equation 1 are used or recommended, notably ROUGHTON and FORSTER [11], FORSTER [4] and REEVES and PARK [26]. Thus, reported values of D_{MCO} and pulmonary capillary blood volume are inconsistent, although the directly measured D_{LNO} and D_{LCO} should be available for others to calculate D_{MCO} and V_C using their favoured different equations.

The dilemma, in terms of which equation should be recommended, has been addressed, in part, by a recent publication from GUÉNARD *et al.* [16], who tested the published 1/θCO *versus* P_{O_2} equations for a constant D_{MCO}/V_C ratio when normal subjects, at rest, were exposed to inspired oxygen concentrations of 13.3% and 18.9%. The equations that best predicted an unchanging D_{MCO} and V_C, using the one-step NO–CO technique with a finite θNO, were HOLLAND [28], ROUGHTON and FORSTER [11] and FORSTER [4], but not REEVES and PARK [26]. A “best-fit” optimal solution was given by the equation provided by GUÉNARD *et al.* [16]:

$$1/\theta \mathrm{CO} = (0.0062 \cdot \mathrm{P_A O_2} + 1.16) \cdot (\text{ideal Hb} \div \text{measured Hb}) \quad (2)$$

The “a” and “b” coefficients are not dissimilar from existing published values, with the exclusion of REEVES and PARK [26]. Accordingly, we agree with using equation 2 in this document, since there is insufficient information, at the present time, to choose between the existing published 1/θCO *versus* P_{O_2} equations derived *in vitro*.

An optimal value for θNO

Using the same continuous flow rapid mixing apparatus as the 1957 θCO measurements [11], θNO can be calculated as 4.5 mL·min^{-1}·mmHg^{-1}·mL^{-1} of blood [29]. Less direct estimates have ranged from 3.0 mL·min^{-1}·mmHg^{-1}·mL^{-1} (humans, *in vivo*; GUÉNARD *et al.* [16]) to 4.0 mL·min^{-1}·mmHg^{-1}·mL^{-1} (membrane oxygenator, *in vitro*; BORLAND *et al.* [30]) to <4.5 min^{-1}·mmHg^{-1} (dog, *in vivo*, exchange transfusion; BORLAND *et al.* [13]). The consensus is that θNO should be taken as 4.5 mL·min^{-1}·mmHg^{-1}·mL^{-1} of blood. The influence of inspired oxygen concentration (and thus alveolar P_{O_2}) on D_{LNO} (and therefore θNO) is small [15, 16], and for clinical purposes, can be ignored. Similarly, the influence of Hb concentration >5–7 g·dL^{-1} on D_{LNO} is too small to matter [14, 17, 31].

Alveolar–capillary membrane diffusing capacity for NO and the α-ratio

The alveolar–capillary membrane diffusing capacity (D_M) is that part of the NO (or CO) uptake pathway where molecular diffusion, driven by the diffusion pressure gradient between the alveolus and the plasma, is the dominant mode of transport. Anatomically, this pathway encompasses the surfactant lining layer, alveolar epithelium, interstitium, capillary endothelium, plasma and the Hb molecule within erythrocytes under the term blood–gas barrier (figure 1). Physiologically, in terms of the ROUGHTON–FORSTER equation [11], D_M is the y-axis intercept, at zero P_{O_2}, on a plot of 1/D_{LCO} *versus* 1/θCO; this definition does not extend to NO, which is effectively P_{O_2}-independent [15]. An important determinant of D_{MNO}

and D_{MCO} is the matching of alveolar NO and CO concentrations to the distribution of pulmonary capillary red cells. Uptake of either CO or NO will be compromised if the alveolar capillaries contain few or no erythrocytes. Two major reasons for the increase in D_{MNO} and D_{MCO} upon exercise are 1) capillary recruitment due to increased blood flow or pressure and 2) more homogeneous erythrocyte distribution, which improves the physical matching between tissue and erythrocyte membrane surfaces [32, 33].

The determinants of D_M are tissue diffusivity (a "lumped" parameter for the entire blood–gas barrier) and the pressure gradient between the alveolus and plasma for both NO and CO. Diffusivity for a gas in tissue is the ratio of its solubility in tissue divided by the square root of its molecular weight. NO and CO have similar molecular weights (30 and 28 $g \cdot mol^{-1}$), but NO has about twice the solubility of CO [34]. The diffusivity ratio (NO/CO) is generally taken as 1.97 [34] and is termed α. Thus, $D_{MNO}=\alpha \cdot D_{MCO}$. Until more data become available on NO and CO tissue diffusivities in the lung tissue itself, this ERS task force agrees to retain 1.97 as the D_{MNO}/D_{MCO} ratio.

An "empirical" value (α) for D_{MNO}/D_{MCO}

Several groups have measured D_{MCO} using the Roughton–Forster multistep alveolar P_{O_2} method and related it to D_{MNO} (assuming an "infinite" θNO, so that $D_{LNO}=D_{MNO}$). This "D_{MNO}/D_{MCO}" ratio was significantly greater than the 1.97 predicted from the tissue diffusivity ratio (α), and varied from 2.06 to 4.4, depending on the equation used [19, 25, 35, 36]. Even higher values of α would have been obtained if a finite value for θNO had been used. Since θNO has a finite value (and the evidence is overwhelming) this empirical D_{MNO}/D_{MCO} ratio (α) merely states the fact that D_{MCO} calculated from the simultaneous one-step NO–CO method (with or without a finite θNO value) is significantly greater than D_{MCO} calculated by the classical Roughton–Forster multistep alveolar P_{O_2} method. When recalculating data from a study that used a rebreathing technique [36], with a finite θNO and GUÉNARD's 1/θCO equation (equation 2) [16], the results show that the D_{MCO} from the simultaneous one-step NO–CO method was 1.25 times greater than the D_{MCO} calculated by the classical Roughton–Forster multistep alveolar P_{O_2} method, at rest and upon exercise. With other equations, with or without an infinite θNO, the discrepancy was even greater.

With the simultaneous one-step NO–CO method, D_{MNO} could be overestimated if there was significant bronchial uptake of NO, due to its greater solubility, in relation to CO. But the bronchial diffusing capacity for NO is a trivial fraction of the alveolar NO diffusing capacity in normal subjects. Again, θNO would have to double (to 9.0 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot mL^{-1}$) to reduce D_{MNO} sufficiently in the simultaneous one-step NO–CO method. One probable reason for the D_{MCO} discrepancy lies in the methods of calculation. Many of the measurements are common to both methods (D_{LNO}, D_{LCO}, θNO and θCO at a nominal P_{O_2} (100 mmHg)), but the simultaneous one-step NO–CO method uses the diffusivity ratio constant, α (1.97) whereas the Roughton–Forster multistep alveolar P_{O_2} method extrapolates the 1/θCO–P_{O_2} equation to zero P_{O_2} to obtain the intercept (1/D_{MCO}). Experimentally, the 1/θCO–O_2 relationship appears to be linear (see figure 3 of FORSTER's article [4]), but REEVES and PARK [26] found that θCO doubled at P_{O_2} <40 mmHg, possibly due to CO binding of unliganded Hb sites *versus* the HbO_2 replacement reaction at higher P_{O_2}. Nonlinearity of the 1/θCO–P_{O_2} relationship could lead to overestimation of the zero P_{O_2} intercept and underestimation of D_{MCO} with the Roughton–Forster multistep alveolar P_{O_2} method. It is an area clearly in need of further research.

NO in the gas phase

Airway uptake of inhaled NO in the single breath hold is negligible (∼0.02%) (supplementary appendix A). Within the acinus, the dominant mode of gas transport is molecular diffusion. Gas phase diffusion coefficients are inversely proportional to the square root of the molecular weight of the gas, so there is no significant difference between NO and CO. This means that gas phase resistance as a proportion of total transfer resistance (from respiratory bronchiole or alveolar duct to capillary blood) will be greater for NO than for CO, but the effects in normal lungs will be negligible. When gas phase diffusion resistance was experimentally increased using pneumonectomy, a density-dependent reduction of D_{LNO} was observed [37]. There was no consistent effect with D_{LCO} because of its slower alveolar uptake. Gas phase diffusion resistance diminishes as the convection–diffusion "quasi-stationary" front moves peripherally towards the alveoli [38]; a rapid inspiration from residual volume to total lung capacity (TLC) promotes such a peripheral location. Thus, in the single-breath technique, gas phase diffusion limitation of D_{LNO} will be small (∼5% of total 1/D_{LNO}) [39].

NO blood uptake is diffusion dependent

Like CO, the uptake of NO is diffusion limited on the basis of a low, dimensionless $DL/\beta\dot{Q}$ value (the Bohr integral or diffusion/perfusion conductance ratio) where D_L is the diffusing capacity, β is the capacitance coefficient (either the water or plasma solubility or the instantaneous slope of the dissociation curve of gases reacting with Hb) and $\dot{Q}$ is pulmonary blood flow (*i.e.* cardiac output). GIBSON and ROUGHTON [40] have published the only known NO/NOHb dissociation curve showing near linearity, with a half saturation at 0.2 mmHg, therefore β=2.5 $mmHg^{-1}$ and hence DL/β, or rather *D*LNO/B$\dot{Q}$ at rest = 150/(2.5·5000) = 0.012. With exercise, the ratio is even lower since the increase in $\dot{Q}$ is much greater than the increase in *D*LNO. This low value of ~0.012 (at rest) indicates that the diffusive rather than the perfusive conductance is the rate-limiting step in alveolar NO uptake. The demonstration of a constant *D*LNO with a 25-fold variation in blood flow in an oxygenator model with a constant membrane surface area favours diffusion rather than perfusion limitation [30].

Blood flow

Pulmonary blood flow (*i.e.* cardiac output) increases with exercise intensity. In healthy subjects, there is a linear increase in *D*LNO of ~16–22 $mL \cdot min^{-1} \cdot mmHg^{-1}$ for every 1.0 $L \cdot min^{-1}$ increase in oxygen uptake [40–42], or ~5–7 $mL \cdot min^{-1} \cdot mmHg^{-1}$ for every 1.0 $L \cdot min^{-1}$ increase in cardiac output [35, 36] (figure 2c). Pulmonary sarcoidosis reduces the slope to ~2.2 $mL \cdot min^{-1} \cdot mmHg^{-1}$ per 1.0 $L \cdot min^{-1}$ increase in cardiac output [35]. The increase in *D*LNO (and *D*LCO) with exercise is not due to increased blood flow as such, but rather to recruitment of *V*C and better matching between tissue and erythrocyte surfaces, and to a lesser extent the recruitment of alveolar–capillary membrane surface area. The correlation of *D*LCO with pulmonary blood flow is tighter than that of *D*LNO with blood flow (figure 2c), suggesting that *D*LCO is more sensitive than *D*LNO to alveolar microvascular recruitment.

In healthy subjects, inhalation of 40 ppm NO for 5 min changed the distribution of blood flow [43], with the redistributed flow favouring the dependent regions. Nevertheless, in terms of whole-body pulmonary gas exchange responses, a 10-min inhalation of 20 ppm NO does not alter oxygen uptake, arterial oxygen pressure, arterial oxyhaemoglobin saturation or the alveolar-to-arterial oxygen pressure difference at rest or during exercise, in either normoxic or hypoxic conditions [44]. In addition, rebreathing NO for 16 s does not change the measured *D*LCO or pulmonary blood flow [36].

Back tension

The endogenous alveolar NO concentration is ~8–20 ppb during tidal breathing [45] and ~100–140 ppb in the nose [46]. The mean±SD fraction of NO from a single-breath exhalation at 50 $mL \cdot s^{-1}$ is significantly higher in asthmatics (73±11 ppb) compared with healthy subjects (35±4 ppb) [47]. Using inhaled NO concentrations of 40–60 ppm and a nose-clip should avoid back tension interference. The presence of NO does not affect the measured *D*LNO [10, 48, 49] or *D*LCO [10, 50].

Heterogeneity

A drawback of the single-breath *D*LNO (and *D*LCO) measurement is that the exhaled sample (500–1000 mL) is not truly representative of the actual dispersion of function within even normal lungs. For example, with rapid gas analysers, uneven concentrations of NO, CO and inert gases (helium (He), methane (CH_4), *etc.*) exist within the alveolar sample, as shown by sloping alveolar plateaus of concentrations *versus* time or

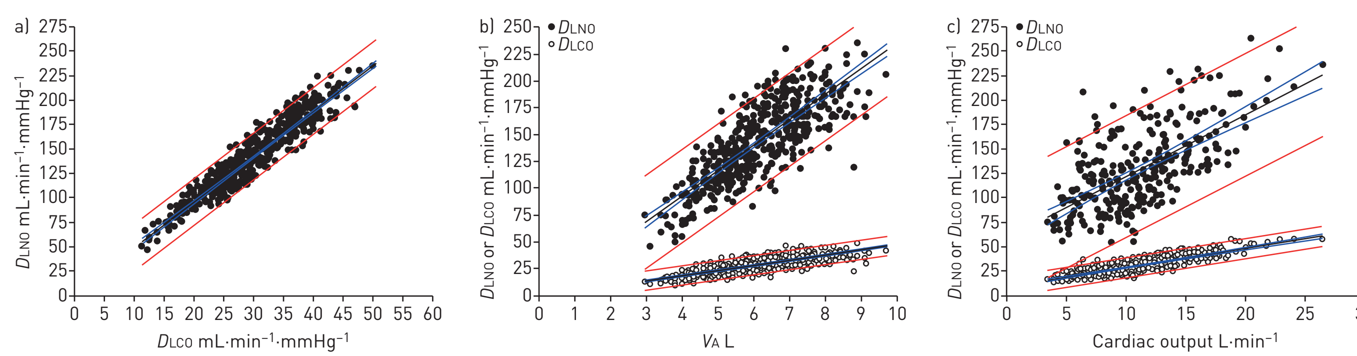

FIGURE 2 a) The association between diffusing capacities of the lung for nitric oxide (*D*LNO) and carbon monoxide (*D*LCO) measured at rest (single-breath; average breath-hold time was ~6 s). Several published studies were used [57, 106, 107]. *D*LNO=4.65·(*D*LCO)+3.8, R^2 0.90, standard error of the estimate (SEE) 11.8, p<0.001, 95% CI of the slope 4.51–4.79; n=493 healthy subjects. b) The association between pulmonary diffusing capacity and alveolar volume (*V*A) measured at rest (single-breath; average breath-hold time was ~6 s). Several published studies were used [57, 106, 107]. *D*LNO=23.0·(*V*A)+2.4, R^2 0.64, SEE 21.9, p<0.001, 95% CI of the slope 21.4–24.5; n=493. *D*LCO=4.63·(*V*A)+1.55, R^2 0.62, SEE 4.5, p<0.001, 95% CI of the slope 4.31–4.94; n=493. All healthy subjects. c) The association between pulmonary diffusing capacity and cardiac output (Q) measured at rest and during exercise by rebreathing. Data from two published studies [35, 109], including ~45% of previously unpublished data. *D*LNO=6.3·(Q)+58.2, R^2 0.42, SEE 31.3, p<0.001, 95% CI of the slope 5.5–7.2; n=76, four data points per subject. *D*LCO=2.0·(Q)+9.0, R^2 0.71, SEE 5.3, p<0.001, 95% CI of the slope 1.8–2.1; n=76, four data points per subject. All healthy subjects. When using rebreathing manouvres, *D*LCO is more tightly associated with cardiac output than *D*LNO (comparison of correlation coefficients z-statistic 5.52, p<0.01); however, *D*LNO is more tightly related to alveolar volume compared to *D*LCO (comparison of correlation coefficients z-statistic 2.27, p=0.023). The association between *D*LNO and *D*LCO in relation to *V*A during rebreathing manouvres (r=0.73 between *D*LNO *versus* *V*A, and r=0.63 between *D*LCO *versus* *V*A) is not shown here.

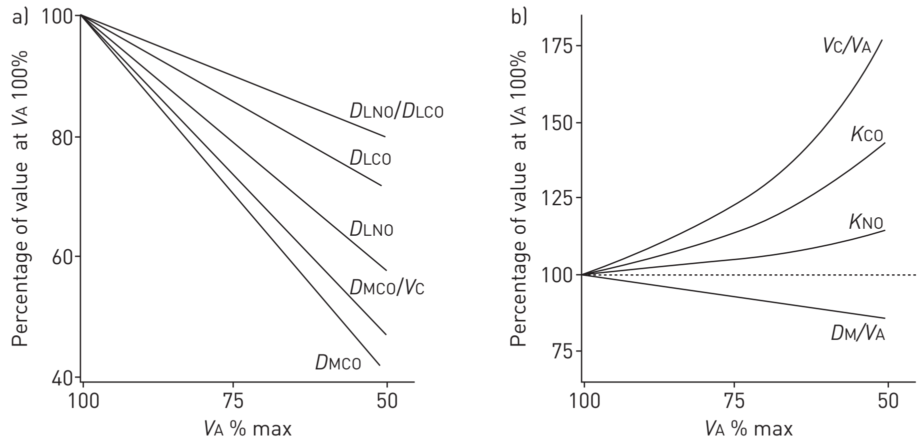

FIGURE 3 Plots of a) pulmonary diffusing capacity for nitric oxide (D_{LNO}) and pulmonary diffusing capacity for carbon monoxide (D_{LCO}), their ratio (D_{LNO}/D_{LCO}), alveolar–capillary membrane diffusing capacity for carbon monoxide (D_{MCO}) and the D_{MCO} to pulmonary capillary blood volume (V_C) ratio (D_{MCO}/V_C), as they relate to the percentage of maximal alveolar volume (V_A) (x-axis) compared to their percentage value at maximal V_A (y-axis); and b) rates of alveolar uptake for NO and CO per unit time and pressure, K_{NO} and K_{CO} (mathematically equivalent to D_{LNO}/V_A and D_{LCO}/V_A, respectively), and the membrane diffusing capacity (D_M) and pulmonary capillary volume (V_C), both per unit alveolar volume (V_A) (D_M/V_A and V_C/V_A), as the expansion of the lung is changed voluntarily in normal subjects (100% of maximal V_A, which is approximately total lung capacity, and 50% of maximal V_A, which is approximately functional residual capacity). Note in a) that with diminishing lung expansion (ΔV_A), ΔD_{LNO} is better related to membrane diffusing capacity (ΔD_{MCO}) and $\Delta D_{MCO}/V_C$ change than the D_{LCO} change. In b), ΔK_{CO} is a better reflection of changes in the pulmonary microcirculation (capillary volume per unit alveolar volume, V_C/V_A) than the K_{NO}; decrease of D_M/V_A with V_A change suggests isotropic change as alveolar dimensions reduce with concomitant thickening of the alveolar–capillary membranes. Interrupted line (in b) signifies no change with change of V_A. Data from [21, 57].

volume. There are two ways in which the effect of heterogeneity on D_{LNO} (and D_{LCO}) has been assessed: first by modelling the distribution and uptake in a theoretical lung [51, 52]; and second by observing the effect of different breath-hold times on D_{LNO} and D_{LCO} in normal subjects and patients [53, 54].

Following the work of Cotton *et al.* [55], Tsoukias *et al.* [52] demonstrated that the lungs fill sequentially, the first gas to be inspired being the last gas to be expired (first in, last out), and that the longer residence times for the first inspired gas would increase its alveolar NO uptake (this effect would be greater for NO because of its more rapid uptake than CO). Thus, the later the expired gas portion was sampled, the higher the calculated D_{LNO}. Note that the more familiar parallel model with slow and fast ventilated compartments, where the "slow" is "last in, last out" has identical functional implications. Piiper and Sikand [51] used the classical two-compartment parallel model in which D_{LCO} and alveolar volume (the compartment or total lung volume (V_A)) during breath holding could be varied independently, and breath-hold time could also be altered. Note that D_{LNO} is the product of V_A during breath holding and K_{NO} (rate of change of NO from alveolar gas, per unit pressure of NO, and equivalent to D_{LNO}/V_A). If K_{NO} was uniform but alveolar volume was uneven between the two compartments, D_{LNO} was less than if alveolar volume had been evenly distributed, but this underestimation was independent of breath-hold time [51]. If both K_{NO} and alveolar volume were unevenly distributed, D_{LNO} would be underestimated and this deficit would increase as breath-hold time was prolonged [51].

In the second approach, breath-hold time was varied for simultaneous D_{LNO} and D_{LCO} measurements in normal subjects and patients with airflow obstruction. D_{LNO} and D_{LCO} decrease as breath-hold time is prolonged [54] because the decrease in K_{NO} (and K_{CO}) at a longer breath-hold time (more weight being given to the low K_{NO} and K_{CO} compartments) outweighs the increase in alveolar volume (more time for inert gas equilibration at 10 s breath-hold). D_{LNO} and D_{LCO} are affected similarly, so the effect of heterogeneity on the D_{LNO}/D_{LCO} ratio is small in normal subjects.

There is no recognised method that "corrects" the D_{LNO} and D_{LCO} for the effects of heterogeneity. Rather than analysing a "mixed", and possibly unrepresentative, "alveolar" sample, modern rapid gas analysers can measure concentrations in real time throughout expiration for NO, CO and inert gases, so that the effects of dispersion (a sloping "alveolar plateau") can be recognised. Whether rapid gas analysers will permit a heterogeneity "correction" remains a subject for further research. What is already known is that heterogeneity of compartmental alveolar volume leads to underestimation of the overall V_A measured at full inflation, in relation to a separately measured estimate of TLC [56]. In normal subjects who use the single-breath method and a 10 s breath-hold time, the mean±SD V_A to TLC ratio is 0.94±0.07 [56, 57], which is slightly less than 1.0, mostly due to sequential heterogeneity. Alveolar volumes from a single-breath test <85% of the TLC (measured using a body plethysmograph) is associated with airflow obstruction [56]. One way of correcting for this mixing defect would be to calculate D_{LNO} as $K_{NO}\times$TLC, where TLC is measured separately. Nonetheless, this has not found favour as it presumes that the K_{NO} in the "inaccessible" units is the same as in the well-ventilated parts of the lung; which is unlikely to be the case for conditions such as emphysema.

In summary, heterogeneity becomes an issue when D_{LNO} and D_{LCO} at 10 s breath hold is compared to 5 s breath hold. There is a tendency for a trade-off between an increase in V_A and a decrease in K_{NO} and K_{CO} (or *vice versa*) at 10 s *versus* 5 s, so that dispersion of V_A and D_L affects each component of D_{LNO} and D_{LCO} in an opposite sense. The effects of heterogeneity are expected to be accentuated in abnormal lungs, although these effects have not undermined the clinical use of D_{LCO}.

Measurements of single-breath D_{LNO} in normal subjects and in cardiopulmonary diseases

By the late 1980s analysers could detect NO concentrations down to 1 ppb, allowing detection of back tension (endogenous respiratory tract production) of ~10 ppb NO and longer breath-hold times, up to the conventional 10 s. Now, rapidly responding analysers allow alveolar profile measurements by the intra-breath [50] and steady-state methods [58]. Commercial pulmonary function systems incorporating NO analysers also became available using a cheaper, but less sensitive NO electrochemical cell, requiring a shorter breath-hold time of 4–6 s. Studies appeared over the next 25 years measuring combined D_{LNO} and D_{LCO} in volunteers and in patients with different diseases.

D_{LNO} in the normal lung

In normal subjects, D_{LNO} decreases to a greater extent than D_{LCO} when lung volume declines [10, 59] (figure 3). Compared to 100% of V_A, D_{LNO} is decreased by ~40% when V_A is decreased by ~50% (figure 3). This is in opposition to D_{LCO}, which only decreases by ~25% for the same decrease in V_A (figure 3). Thus, for the same decrease in lung volume, the percentage increase in K_{CO} (D_{LCO}/V_A) is approximately double that of K_{NO} (D_{LNO}/V_A) (figure 3), reflecting greater D_{LNO} dependence on the D_{MNO}/V_A ratio than on the V_C/V_A ratio.

After adjusting for postural changes in V_A, both D_{LNO} and D_{LCO} increase ~5% from upright, sitting to supine [60], which may be explained by an ~13% increase in V_C in the supine position compared to sitting [60]. In contrast, changing from a supine to a prone position has yielded varying results [61].

D_{LNO} increases linearly with increasing exercise intensity, measured by the single-breath [19, 40, 41], steady-state [62] or rebreathing [35, 36, 42] methods (see figure 2c for an example using rebreathing data).

After 2–30 days at altitude (4400–5000 m), D_{LNO} and D_{LCO} (at rest) increases in healthy lowlanders [18, 63, 64]. But acutely (2–3 days' exposure), the D_{LNO}/D_{LCO} ratio falls (8%), and it returns towards baseline (along with D_{LNO} and D_{LCO}) after a week at altitude [63]. These increases in D_{LNO} and D_{LCO} on acute high altitude exposure may be explained by alveolar expansion (weighted by D_{LNO}) and capillary recruitment (weighted by D_{LCO}) due to hyperventilation and increased cardiac output.

In healthy high-altitude Quechuans in Peru [64], D_{LCO} and D_{LNO} are increased in relation to healthy lowlanders after 4 days at the same altitude, but the D_{LNO} increase was smaller and the D_{LNO}/D_{LCO} ratio fell by 5%. In a similar study involving Sherpas in Tibet, the relative increases in D_{LCO} and D_{LNO} were greater, but, again, there was a lower D_{LNO}/D_{LCO} ratio (by ~12%) [65]. In high-altitude Quechuans with chronic mountain sickness, D_{LCO} and D_{LNO} are increased further compared to healthy Quechuans, with a ~8% decrease in the D_{LNO}/D_{LCO} ratio [64].

Diving has biphasic effects. Both D_{LCO} and D_{LNO} increase transiently after short compressed air or maximal breath-hold dives due to pulmonary vasodilation and central blood volume shifts that increase V_C, followed later by parallel decreases in D_{LCO} and D_{LNO} reflecting the development of interstitial oedema and ventilation–perfusion mismatch [66–68]. Dives of longer durations are associated with reduced D_{LCO} due to oxygen toxicity [69, 70].

D_{LNO} in disease

When comparing D_{LNO} in disease to a control group, it is helpful to examine D_{LNO} and the simultaneously measured D_{LCO} and the D_{LNO}/D_{LCO} ratio [21]. As D_{LNO} is weighted by D_M and D_{LCO} is weighted by V_C, the D_{LNO}/D_{LCO} ratio (assuming D_{LNO} and D_{LCO} are reduced) reflects a relative change in the membrane-to-capillary components of uptake (D_{MCO}/V_C) [21]. An increase in D_{LNO}/D_{LCO} signifies a reduction in V_C greater than the reduction in D_M, meaning that there is greater microvascular disruption than membrane disruption (and *vice versa* for a decrease in D_{LNO}/D_{LCO}). Likewise, since D_{LNO} is insensitive to changes in haematocrit in the physiological range, the D_{LNO}/D_{LCO} ratio should rise in anaemia and decrease in polycythaemia. As predicted, increasing Hb concentration by 33% (from 7.8 to 10.4 $g \cdot dL^{-1}$) by transfusion caused a minimal increase in D_{LNO} (∼3%, $p>0.05$), while D_{LCO} increased by ∼20% ($p<0.05$), and the D_{LNO}/D_{LCO} ratio decreased from 5.7 to 4.8 [17].

Microvascular disease

In pulmonary arterial hypertension (PAH), studies [71–73] have shown a mainly microvascular component, with a reduction in V_C greater than the reduction in D_{MCO}, leading to a rise in D_{MCO}/V_C and D_{LNO}/D_{LCO} ratios. D_{MCO} falls as V_C falls because of their interdependence ("coupling"). Nevertheless, in patients with idiopathic PAH, there were equal reductions in D_{MCO} and V_C, but no change in the D_{LNO}/D_{LCO} ratio [73]. In liver cirrhosis with hepatopulmonary syndrome (HPS) [74], there was a greater reduction in V_C and D_{MCO} (and a lower arterial oxygen pressure) *versus* non-HPS patients, but both groups demonstrated a similar rise in D_{LNO}/D_{LCO} and D_{MCO}/V_C ratios compared to controls, consistent with microvascular disease. In heart failure, D_{LNO}/D_{LCO} and D_{MCO}/V_C ratios were reported to be increased [75], contrary to predictions, but there were methodological issues in the calculations of D_{MCO}/V_C [76]. As such, more studies are needed examining microvascular disease and its effects on diffusing capacity.

Interstitial lung disease

A greater reduction of D_{MCO} than V_C (with a fall in D_{LNO}/D_{LCO} ratio) was observed in patients with sarcoidosis using a rebreathing technique [35], whereas the opposite was found [72] using a single-breath technique in patients with diffuse parenchymal lung disease and PAH. The disparity could reflect the different pathophysiology and clinical stages of these diseases.

Airflow obstruction

In a lung cancer screening trial in asymptomatic smokers without airflow obstruction (Global Initiative for Chronic Obstructive Lung Disease stage 0) [77], D_{MCO} was preserved in relation to V_C, and the D_{LNO}/D_{LCO} and D_{MCO}/V_C ratios were increased compared to controls (Borland and Hughes, personal communication), suggesting that a reduction in V_C may be an early sign of chronic obstructive pulmonary disease (COPD). In established COPD, both D_M and V_C appear to be reduced [53].

Miscellaneous

In chronic renal failure [78], D_{LNO} and both D_{MCO}/V_C and D_{LNO}/D_{LCO} ratios are reduced (after adjusting for Hb). In morbid obesity [40, 79] there is a slight reduction in D_{MCO}/V_C. In cystic fibrosis D_{MCO}/V_C and D_{LNO}/D_{LCO} are reduced [80]. Following bone marrow transplant, both D_{LNO} and D_{LCO} are reduced [81].

Conclusion

Different pathologies will reduce the membrane (D_M) and microvascular ($\theta \cdot V_C$) components differently and, within a specific disease, affected and less- or non-affected areas may co-exist. Thus, heterogeneity of function within and between pathological entities means that disease-specific patterns of D_{LNO} and D_{LCO}, D_{LNO}/D_{LCO}, D_{MCO} and V_C will remain imprecise until more clinical studies are reported using a standardised technique.

Gas analysers and general equipment

System design

All commercially available D_{LNO} apparatus is based on the single-breath D_{LCO} measurement system with the addition of the NO transfer gas. The first requirement is that the inspiratory gas sample is prepared, mixed and stored for the subsequent inhalation. Because both inspiratory and expiratory gas concentrations have to be measured, gas analysers have to be connected with the inspiratory reservoir and the expiratory sampling bag. Increasingly, continuous high-speed gas analysers are used and recommended. With electrochemical (low sensitivity, low speed) analysers, the inspired gases should be sampled from the inspiratory reservoir. As such, in relation to the patient's mouth, the gas sampling port should be near the inspiratory–expiratory switching valve; for the combined one-step NO–CO manoeuvre, sampling of the inspired NO, CO, inert tracer gas and oxygen concentrations should be from the inspiratory reservoir itself. On expiration, continuous gas analysis defines the extent of the anatomical dead space, and allows different parts of the subsequent "alveolar plateau" to be examined. High-speed gas analysis, with continuous sampling, is required if the three-equation model (inhalation, breath holding and exhalation) of diffusing capacity is applied [82]. Finally, the inspired and expired volume must be measured using pneumotachometers or mass flow meters [83].

Performance standards for equipment

The standard D_{LNO} system is basically a single-breath D_{LCO} system with the addition of NO in the inspiratory gas mixture and the presence of an NO analyser. Two major subtypes can be defined: the first type is characterised by an inspiratory reservoir, such as a balloon, for the storing and measurement of the inspiratory gas mixture. The second type has a mixing chamber in which the inspired gases are mixed, from different sources, before each inspiration. The basic equipment for *D*LCO systems has been described elsewhere [84]. Importantly, NO is reactive with oxygen (O_2), to form NO_2 (O_2+2·NO→2·NO_2). NO_2 is formed at a rate of ~0.02 ppm·s^{-1} (~1.2 ppm NO_2·min^{-1}) in a gas mixture containing close to 21% oxygen and 60 ppm NO [85]; <3 ppm of NO_2 is produced in 2 min when ~60 ppm NO gas is mixed with ~21% oxygen [85]. Were that mixture to be left in the inspiratory bag for 2 min before testing, *D*LNO would be overestimated by ~1%. As such, NO gas (along with nitrogen (N_2)) is stored in a separate gas cylinder (apart from oxygen) containing NO in a high concentration in N_2, ranging from 400 to 1200 ppm NO in N_2. The greater the concentration of NO with N_2 in the cylinder, the less N_2 is injected into the inspiratory bag, with less dilution of the inspired oxygen concentration. Since NO reacts with certain plastics, polytetrafluoroethylene (Teflon) tubing should be used. The connections and regulators should be made of stainless steel in order to prevent reaction of the NO with metals. Two types of NO analysers are available: the highly sensitive but expensive chemiluminescence analysers, with a lower detection limit of 0.5 ppb, and linear to the upper detection limit of 500 ppm and with a reaction time of ~70 ms. Because the chemiluminescence analysers are expensive, commercial pulmonary function testing equipment that performs *D*LNO measurements is usually equipped with a less expensive, slower speed, less sensitive, NO electrochemical cell. These cells have lower sensitivity, with a detection range of 0–100 ppm, and a response time of <10 s (90% full scale), and so are suitable only for the standard single-breath test.

Typically, in the single-breath *D*LCO, a breath-hold time is 10±2 s calculated by the JONES and MEADE formula [86]. If an electrochemical cell is used for the *D*LNO test, a shorter breath-hold time of 4–6 s is necessary because of the lower sensitivity of the analyser. For this purpose, prediction equations for *D*LNO, *D*LCO, *D*MCO and *V*C have been developed by combining several studies using breath-hold times that varied between 4 s and 10 s (with a mean of ~6 s). Subject characteristics are presented in table 1 and prediction equations are presented in table 2. Supplementary appendix H allows patients' individual values to be inserted in relation to predicted values.

Nevertheless, there is a disadvantage of using shorter breath-hold times of 5 s instead of 10 s for combined *D*LNO and *D*LCO measurement. In adult subjects with ventilatory heterogeneity, the shorter breath-hold times can overestimate the diffusion capacity [54, 82] *versus* the conventional 10 s test. However, in healthy children the difference between 10 s and 5 s breath-hold times is small [87].

Inspiratory NO concentrations of 40–60 ppm should be used, leading to expiratory NO levels that are ~3–5 ppm after a ~5 s breath hold [49, 88]. Even after 22 consecutive *D*LNO tests on subjects that inspired ~55 ppm NO for each test, *D*LNO remained unchanged [48]. Furthermore, there is minimal interaction between NO and CO [10, 50], therefore the *D*LNO and *D*LCO can be measured simultaneously. The

TABLE 1 Subject characteristics from previously published studies from which prediction equations were made [57, 106, 107]

	Males	Females	Combined
Subjects	248	242	490
Age years	44±17 (18–93)	45±18 (18–87)	44±17 (18–93)
Weight kg	76.7±9.4 (55.0–105.0)	61.6±8.8 (44.0–95.0)	69.3±11.8 (44.0–105.0)
Height cm	176±8 (154–196)	164±7 (147–182)	170±10 (147–196)
Body mass index kg·m^{-2}	24.7±2.5 (18.9–29.9)	23.0±3.0 (17.2–29.8)	23.8±2.9 (17.2–29.9)
***D*LNO mL·min^{-1}·mmHg^{-1}**	164±31 (67–235)	119±25 (47–186)	142±36 (47–235)
***D*LCO mL·min^{-1}·mmHg^{-1}**	34.1±6.3 (11.9–49.9)	25.1±5.3 (11.3–38.6)	29.6±7.4 (11.3–49.9)
***D*MCO mL·min^{-1}·mmHg^{-1}**	161±39 (72–250)	104±26 (33–182)	133±44 (33–250)
***V*C mL**	78±16 (25–121)	65±15 (30–105)	72±17 (25–121)
***D*MCO/*V*C ratio min^{-1}·mmHg^{-1}**	2.11±0.57 (1.01–4.03)	1.63±0.40 (0.88–2.96)	1.90±0.57 (0.88–4.03)
***K*CO mL·min^{-1}·mmHg^{-1}·L^{-1}**	4.9±0.8 (2.7–7.1)	4.8±0.7 (3.0–6.8)	4.9±0.8 (2.7–7.1)
***K*NO mL·min^{-1}·mmHg^{-1}·L^{-1}**	23.8±3.9 (13.7–34.2)	22.8±3.2 (13.5–31.5)	23.3±3.6 (13.5–34.2)
***D*LNO/*D*LCO ratio**	4.83±0.40 (3.83–5.82)	4.74±0.39 (3.85–5.78)	4.79±0.40 (3.83–5.82)

Data are presented as n or mean±SD (range). The alveolar–capillary membrane diffusing capacity for carbon monoxide (*D*MCO) and total volume of blood in the lung capillaries exposed to alveolar air (*V*C) values reported in these studies [57, 106, 107] have been recalculated according to the parameters listed in table 4. *D*LNO: diffusing capacity of the lung for nitric oxide; *D*LCO: diffusing capacity of the lung for carbon monoxide; *K*CO: rate of uptake of carbon monoxide from alveolar gas; *K*NO: rate of uptake of nitric oxide from alveolar gas.

TABLE 2 Predictive equations for white adults at a breath-hold time of ~6 s, inspired nitric oxide (NO) of ~35 ppm and inspired oxygen of ~19.5%, from three studies [57, 106, 107]

	Height cm	Age^2	Sex	Constant	Adjusted R^2	SEE	LLN and ULN
D_{LCO} mL·min^{-1}·mmHg^{-1}	0.23	−0.002	6.0	−8.5	0.68	4.2	±8.2
D_{LNO} mL·min^{-1}·mmHg^{-1}	0.81	−0.010	34.4	9.7	0.69	20.0	±39.2
D_{MCO} mL·min^{-1}·mmHg^{-1}		−0.011	56.4	129.6	0.61	27.3	±53.5
V_C mL	0.84	−0.004		−59.9	0.49	12.0	±23.5
V_A L	0.079		0.73	−7.7	0.67	0.72	±1.4
V_C/V_A mL·L^{-1}		−0.0006	−1.25	13.9	0.27	1.89	±3.70
D_{MCO}/V_A mL·min^{-1}·mmHg^{-1}·L^{-1}	−0.200	−0.002	5.9	56.6	0.41	3.81	±7.47
K_{CO} mL·min^{-1}·mmHg^{-1}·L^{-1}		−0.00027		5.5	0.34	0.6	±1.2
K_{NO} mL·min^{-1}·mmHg^{-1}·L^{-1}		−0.00137		26.4	0.39	2.8	±5.5

Alveolar–capillary membrane diffusing capacity for carbon monoxide (D_{MCO}) and total volume of blood in the lung capillaries exposed to alveolar air (V_C) values in these studies [57, 106, 107] have been recalculated according to the formulas and constants in table 4 and then re-analysed for the regression. A predictive model was not found for the ratio of diffusing capacities of the lung for nitric oxide and carbon monoxide (D_{LNO}/D_{LCO}). Sex: 1 for male, 0 for female; SEE: standard error of the estimate. To convert D_{LNO}, D_{LCO} and D_{MCO} to mmol·min^{-1}·kPa^{-1}, divide by 3. Lower limit of normal (LLN)=2.5th percentile; upper limit of normal (ULN)=97.5th percentile. n=490. V_A: alveolar lung volume; K_{CO}: rate of uptake of carbon monoxide from alveolar gas; K_{NO}: rate of uptake of nitric oxide from alveolar gas. Supplementary appendix H allows the patient's individual values to be inserted in relation to the predicted values.

preferred inspired test gas concentrations for D_{LCO} measurement are close to 0.30% CO and 21% O_2 [84]. For measurement of V_A, either 10% He or 0.3% CH_4 can be used.

Linearity and accuracy
Since D_{LCO} and D_{LNO} are very sensitive to errors in relative gas concentration, nonlinearity for CO, NO and tracer gas analysers should not exceed 1.0% of full scale for discrete systems. That is, any nonlinearity must not exceed 1.0% of full scale once zero and full-scale values have been set [84]. The CO, NO and tracer gas analysers should be accurate to within 1.0% of full scale [84].

Drift
The gas analysers should have minimal drift in zero and gain so that the output is stable over the test interval. Drift is determined by comparing the CO, NO and tracer values measured in room air immediately prior to and immediately following the single-breath manoeuvre. The CO analyser drift should be ⩽10 ppm (or ⩽0.33% drift) when inhaling 3000 ppm CO, ⩽1 ppm when inhaling 40–60 ppm NO and ⩽0.5% for the tracer gas over 30 s. It would be preferable to have a display of the measured gas concentrations so that stability is confirmed. If significant drift is present over the 30-s time period (*i.e.* >10 ppm CO, >1 ppm NO (since the resolution of a typical NO electrochemical cell is 0.5–1 ppm) and >0.5% for tracer gas), then adjustment algorithms should be devised to compensate for the analyser drift from measured data.

Interference and noise
Carbon dioxide and water can interfere with the gas analyser performance, for which corrections should be made [84] (refer to supplementary appendix D). Circuit resistance should be <1.5 cmH_2O·L^{-1}·s^{-1} at 6 L·s^{-1} flow [84]. Anatomical dead space volume should be measured; the dead space volume of valve, filter and mouthpiece should be <200 mL [84]. The system, including all tubing, should be leak free.

Flow and volume
Flow measurement accuracy over a range of ±10 L·s^{-1} must be within 2% [84]. For calibration with a 3-L syringe, a 2.5% volume accuracy (±75 mL), including 0.5% for testing syringe error, is recommended [84].

Equipment quality control
Research shows that 36–70% of the variation in D_{LCO} can be due to instrument choice [89]. We assume that the same variation exists for D_{LNO}. Therefore, calibration and standardisation of equipment specifications are necessary [90].

1) Gas analysers should be zeroed before each test, and the zero level should be measured after each test, preferably *via* an automated procedure. If there is a difference between the zero level before and after each test, adjustment algorithms should be devised to compensate for the analyser drift from

measured data. If using a discrete system, the inspired NO concentration should be checked after its injection into inspiratory reservoir, just prior to testing.

2) Volume calibration should be performed on a daily basis with the aid of a validated 3-L syringe.
3) Once a week or whenever problems are suspected, leak testing on the syringe should be performed. This is achieved by filling the 3-L syringe fully with air and then placing a stopper at the syringe input. Push the syringe in by 50 mL and hold for 10 s and release. If the syringe does not return to within 10 mL of the full position, it should be sent for repair. The procedure is then repeated with the syringe at 50 mL below full, applying the stopper and pulling the syringe to the full position [84].
4) Every week, standard subject testing (biological control) should be performed on healthy nonsmokers. Attention should be paid whenever the D_{LCO} varies by ⩾5.0 mL·min^{-1}·mmHg^{-1} or D_{LNO} varies by ⩾20 mL·min^{-1}·mmHg^{-1}, from the mean of previously obtained values (table 3). A biological control whose D_{LNO} and D_{LCO} values measured week to week on the same pulmonary function system should be within 20 and 5 mL·min^{-1}·mmHg^{-1}, respectively, 95% of the time. If there are week-to-week changes in diffusing capacity beyond those limits, then this would indicate that there is only a 5% chance that the diffusing capacity value obtained in the present week is not a real change and is due to machine error or some other factor. The D_{LNO} and D_{LCO} should be recorded in a laboratory log book so that slowly drifting values are noticed. Standard subject testing should be performed every time gas cylinders are changed.
5) Linearity of gas analysers should be tested every month, for He/CH_4, CO and NO, by using serial dilutions of known test gas concentrations. Most importantly, laboratory staff should review the D_{LCO} and D_{LNO}, inspiratory vital capacity and V_A values in every test, not only to observe the week-to-week variability (table 3), but also to identify aberrations of the expected values due to technical matters.

Using a 3-L syringe at ambient temperature and pressure (ATP), linearity issues may also be identified by performing the following test: with ∼1 L of air in the syringe, the remaining 2 L is filled with the test gases. The syringe is then emptied following the 4–6 s breath hold. The calculation of V_A must be within

TABLE 3 Intra-session and inter-session variability of single-breath measurements of the diffusing capacities of the lung for nitric oxide (D_{LNO}) and carbon monoxide (D_{LCO}) (5 s breath hold) at rest

	Test-to-test measurement error (within the same testing session)	Repeatability# (within the same testing session)	Reproducibility¶ (week-to-week or month-to-month change) More stringent	Smallest measurable change+ (week-to-week or month-to-month change) Less stringent
D_{LNO}				
mL·min^{-1}·mmHg^{-1}	6.2 (4)	17 (10)	20 (13)	10 (7)
mmol·min^{-1}·kPa^{-1}	2.1 (4)	5.8 (10)	6.5 (13)	3.3 (7)
D_{LCO}				
mL·min^{-1}·mmHg^{-1}	1.2 (4)	3.2 (10)	4.9 (16)	2.5 (8)
mmol·min^{-1}·kPa^{-1}	0.4 (4)	1.1 (10)	1.6 (16)	0.8 (8)
D_{LNO}/D_{LCO} ratio	0.12 (2)	0.36 (7)	0.23 (5)	0.13 (3)
D_{MCO}				
mL·min^{-1}·mmHg^{-1}	12 (7)	34 (19)	47 (28)	24 (28)
mmol·min^{-1}·kPa^{-1}	4.1 (7)	11.2 (19)	15.8 (28)	8 (14)
V_C mL	4 (5)	10 (13)	16 (24)	8 (12)

Numbers are presented as the value with the percentages in parentheses. Within-session data [49] and reproducibility data (between sessions) [88] were obtained from healthy subjects. The diffusing capacity of the membrane for carbon monoxide (D_{MCO}), nitric oxide (D_{MNO}) and total volume of blood in the lung capillaries exposed to alveolar air (V_C) values are recalculations from the original dataset using the formulas and constants in table 4 as well as the supplementary appendices. The D_{LCO} repeatability and reproducibility in subjects with pulmonary pathophysiology (mean D_{LCO} 11–18 mL·min^{-1}·mmHg^{-1}) are 2.7 and 4 mL·min^{-1}·mmHg^{-1}, respectively [113, 114]. The repeatability was calculated as follows: the mean within-subject standard deviation (which is the average standard deviation between several diffusing capacity tests performed in one session) multiplied by 2.77. The reproducibility is performed the same way, except the mean week-to-week standard deviation is used (which is the average standard deviation between diffusing capacity measured over several weeks multiplied by 2.77). Refer to supplementary appendix G for in-depth statistical methodology of the calculation. #: the difference between two trials for D_{LNO}, D_{LCO}, D_{MCO} and V_C measured on the same subject in the same testing session is expected to be <17, <3.2 and <34 mL·min^{-1}·mmHg^{-1} and <10 mL, respectively, for 95% of observations; ¶: the difference in D_{LNO}, D_{LCO}, D_{MCO} and V_C measured on the same subject over two different weeks is expected to be less than 20, 4.9, and 47 mL·min^{-1}·mmHg^{-1} and 16 mL, respectively, 95% of the time. Any diffusing capacity parameter that has a week-to-week or month-to-month change that is equal to or greater than the reproducibility has only a 5% chance that it is not a real change; +: half the reproducibility and thus less stringent than the reproducibility. Any week-to-week or month-to-month change that is equal to the smallest meaningful change has a 20% chance that it is not a real change. The reproducibility and smallest meaningful change are most correct when using the same equipment for the duration of the assessment.

0.3 L of 3 L with the syringe dead space being used for the anatomical dead space in the V_A calculation. The absolute value for D_{LCO} must be <0.5 mL·min^{-1}·mmHg^{-1} (<0.167 mmoL·min^{-1}·kPa^{-1}) and for D_{LNO} <3 mL·min^{-1}·mmHg^{-1} (<1 mmoL·min^{-1}·kPa^{-1}). Manufacturers should provide this test option, which is the same as the usual testing procedure for a patient, with the exception that V_A will be reported at ATP rather than body temperature, ambient pressure, saturated with water vapour (BTPS) [84].

Infection

Transmission of infection from patients to other patients or staff must be prevented. The spirometry guidelines also apply to D_{LCO} and D_{LNO}, as is described in detail elsewhere [91].

Testing technique

Subject preparation

Since a D_{LCO} measurement is often performed in conjunction with a D_{LNO} test, the carboxyhaemoglobin (COHb) concentration should be minimised, as COHb reduces D_{LCO}. Since it takes up to 6 h to remove half the CO from blood at rest breathing room air [92], subjects should refrain from smoking for 12 h prior to testing, and any deviation should be indicated in the report. As urban pollution can also increase COHb levels, where possible the COHb or an exhaled breath sample should be measured so that the predicted D_{LCO} can be adjusted.

Subjects should refrain from wearing clothing that substantially restricts full chest and abdominal expansion, and from eating a large meal within 2 h of testing. Also, evidence indicates that D_{LNO} [93, 94] and D_{LCO} [93, 95, 96] remain impaired for several hours after strenuous exercise. Thus, while D_{LNO} and D_{LCO} increase during exercise, and the increase parallels exercise intensity (*i.e.* cardiac output), both D_{LNO} and D_{LCO} are reduced 1–2 h post-exercise [93–96], and can last several hours post-exercise [95, 96]. The mechanisms for this reduction could be a combination of several factors: alveolar-membrane thickening due to mild interstitial pulmonary oedema [93, 94, 97] or reduced pulmonary capillary blood volume due to active pulmonary vasoconstriction and/or peripheral vasodilation [95, 96]. As such, diffusing capacity testing should be avoided ⩽12 h after vigorous exercise.

The subject's demographic information, body position, Hb concentration and the ambient room temperature and atmospheric pressure should be recorded. Any special conditions, *e.g.* exercise or altered inspired O_2 fraction, or medication that affects lung function or vasomotor tone, *e.g.* bronchodilators or β-blockers, should be noted. Baseline lung function parameters measured by spirometry should be obtained. Subjects should be comfortably seated. Prior to testing, each subject should be familiarised with the testing equipment and instructed on the breathing manoeuvres, first *via* demonstration then by asking the subjects to perform practice manoeuvres with the mouthpiece and nose clip in place.

Performing the manoeuvre

In both clinical and laboratory practice, should the D_{LNO} be performed simultaneously with D_{LCO}, the current D_{LCO} guidelines should be followed [84]. Following a period of quiet tidal breathing to stabilise respiratory pattern, the single-breath technique for D_{LNO}–D_{LCO} involves rapid inspiration from residual volume to total lung capacity of a bolus of a test gas mixture containing a known quantity of NO (usually with CO and an inert tracer gas such as He, CH_4 or neon); achieving an inspired volume of at ⩾90% of inspiratory vital capacity in <2.5 s is preferred. At full inspiration, the subject will hold the breath for a prescribed period (5–10 s) at near atmospheric intrapulmonary pressure. A subject that relaxes on the shutter during apnoea (in effect, increasing intrathoracic pressure) will decrease D_{LCO} by ∼3% (1 mL·min^{-1}·mmHg^{-1}) [98]. As such, subjects should refrain from making Valsalva (forced positive pressure against a closed glottis) and Müller manoeuvres (increased negative pressure in the thorax), because these will alter thoracic and pulmonary capillary blood volume. Following breath hold, the subject exhales smoothly and rapidly to residual volume within 4 s. The actual duration of exhalation should be measured and recorded. If continuous monitoring of expired gas concentrations is available, the washout of tracer gas from the previous test may be confirmed by observing end-tidal gas concentrations before beginning the next test. Secondly, if continuous monitoring of expired gas concentrations is available, the timing of the alveolar gas sample should be determined as the point of dead space washout rather than using a fixed washout volume of 0.75–1 L [84, 99].

Between successive tests, an interval of ⩾4–5 min should be allowed to ensure complete elimination of prior test gases from the lungs. A longer interval between tests may be necessary in subjects with poor gas mixing due to intrapulmonary airflow obstruction. For systems using continuous monitoring, verification of washout rather than using an arbitrary 4–5-min washout interval is preferable. Should the tests be repeated on separate days, they should be performed around the same time of the day to minimise potential variability in the D_{LCO} due to diurnal fluctuations in Hb and COHb [100, 101]. The D_{LCO}

decreases by 0.4% [101] to 1.2% [100] per hour from 09:30 h to 17:30 h. There is no reason to suggest that D_{LNO} alters throughout the day, since small changes in Hb and COHb do not affect it [17, 48].

Sample collection

The initial volume of gas expired from the anatomical dead space is routinely discarded before collecting the alveolar gas sample. This "washout volume" may be arbitrarily set (0.75–1.0 L at BTPS for most adults, or 0.50 L BTPS for subjects with a small vital capacity <2.0 L), or individually determined in cases where exhaled gas concentrations are monitored continuously throughout expiration with rapid gas analysers.

Following dead space washout, which includes instrument, mouthpiece, valve, filter and anatomical and physiological dead spaces, an alveolar sample of 0.5–1.0 L is collected for analysis. In subjects with small vital capacities, a dead space washout volume <0.5 L may be acceptable as long as all the dead spaces have been cleared. The actual parameters used in sample collection and any customised adjustments should be reported.

In subjects with poor gas mixing or marked sequential emptying of various lung regions, the gas sample collected will only reflect the properties of the regions contributing to that sample.

Inspired gases

The test gases used to calculate D_{LCO}, include CO (usually close to 0.3%) and a tracer gas such as He (usually ~10%), CH_4 or neon (both usually ~0.3%) for measuring V_A. The remainder of the test gas mixture includes close to 21% oxygen with nitrogen as balance so that the average alveolar oxygen pressure of ~100 mmHg is reached during a maximal inspiration to total lung capacity with a 6-s breath-hold. As the D_{LCO} increases by ~1.5% for every 1% decrease in inspired oxygen concentration [16, 102, 103], the D_{LNO}/D_{LCO} ratio should decrease by ~3% when the inspired oxygen concentration is lowered from 21% to 19% (due to the increase in D_{LCO} only). In fact, studies show that for every 1% decrease in inspired oxygen concentration, the measured D_{LNO}/D_{LCO} ratio decreases by ~2% [16, 102]. It is important to note that while the traditional diffusion gas mixtures report 21% oxygen in their gas tanks, by the time it reaches the inspiratory bag and gets slightly diluted with the NO/N_2 mixture, the inspired oxygen concentration may be closer to 20% (supplementary appendix F).

If D_{MCO} and V_C are to be calculated from the one-step NO–CO technique (supplementary appendix E), the expired "alveolar" oxygen concentration should be measured so that θCO can be calculated. The oxygen concentration in the expired sample is a good approximation of the alveolar oxygen pressure. If the expired sampled oxygen concentration is 15% then the estimated alveolar oxygen pressure at sea level would be the current atmospheric pressure minus the water vapour pressure (~47 mmHg at 37°C) multiplied by 0.15=107 mmHg. In a 5 s breath-hold test in normal subjects where the mean inspired oxygen concentration was 19–20%, the mean expired oxygen concentration sampled from the expiratory reservoir ranged from 15% to 17% [49, 88].

The gases in the inspiratory reservoir are at ambient temperature and pressure, dry conditions (ATPD). The inspired volume (the subject's inspired vital capacity), and the V_A calculated from it needs to be converted from ATPD to BTPS conditions for calculation of D_{LNO}/V_A (equivalent to K_{NO}), and standard temperature and pressure, dry (760 mmHg, 0°C, 0% humidity) conditions for the calculation of D_{LNO} (equals $K_{NO} \times V_A$). Manufacturers should specify these conversion factors in the software.

Calculations for D_{LNO}, D_{LCO} and V_A

The derivation and calculation of D_{LNO} and D_{LCO} are identical except for the difference in gas species. The formulation (supplementary appendix B) given for D_{LNO} stems from a recent review [104] and emphasises an important concept, that the D_{LNO} (and D_{LCO}) are each the product of two components, the rate of change of alveolar concentration (k_{NO} and k_{CO}) per unit total gas pressure ($P_B - P_{H_2O}$) and the volume of distribution of that gas in the alveolar region of the lung (V_A). This concept derives from Marie Krogh, who was the originator of the D_{LCO} measurement in 1915 [105]. It is important that the total dead space (anatomical dead space and the instrumental dead space) are taken into consideration in the calculation of V_A, otherwise errors in the calculation of alveolar volume will occur (supplementary appendix C).

Calculating breath-hold time

Subjects are encouraged, from the start, to breathe in "as rapidly as possible", from residual volume to TLC, otherwise known as an inspiratory vital capacity. At TLC, the usual breath-hold time is ~4–10 s for D_{LNO} measurements. The shorter breath-hold time is permitted if NO is measured using the less sensitive electrochemical cell. At the end of the breath hold, the expiration for the collection of an alveolar sample need not be "forced", as the combined recoil of the chest wall and the lung ensures that it will be "rapid" (unless there is severe, usually extrathoracic airflow obstruction).

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Ideally, in the single-breath test, all contact of NO and CO with the alveolar surface should be at a breath-hold volume close to TLC. Since neither the preceding inspiration nor the subsequent expiration is "instantaneous", that ideal cannot be fulfilled. JONES and MEADE [86] addressed the problem of an "effective breath-hold time" in some depth, and their recommendations for its calculation have been accepted [99]. Breath-hold time starts after the first 30% of inspiratory time and finishes halfway through the collection of the expired sample (after an initial expiration of 750–1000 mL). Thus, this task force agrees that the Jones–Meade formula be used.

Use of breath-hold times <10 s

Because the alveolar uptake of NO is five times faster than the uptake of CO (figure 2a), alveolar NO concentration is ∼5% of the inspired concentration after 5 s of breath holding, and ∼1% after 10 s. To maximise the expired NO signal, investigators in epidemiological studies have reduced breath-hold times to 4 s [106] or 5.5 s [107], although others, with more sensitive analysers, have kept to 10 s [57].

Implications for breath-hold times <10 s

For physiological reasons, partly gravitational and part due to the intrinsic structure of the lung, neither ventilation nor D_{LNO} is uniformly distributed. In a theoretical study of a two-compartment lung, PIIPER and SIKAND [51] showed that uneven distribution of inspired volume and D_{LCO}/V_A (equivalent to K_{CO}) always lead to an underestimation of D_{LCO} (and, by extension D_{LNO}) compared to the homogeneous situation.

DRESSEL *et al.* [54] systematically studied the dependence of D_{LNO}, D_{LCO} and their components V_A, K_{NO} and K_{CO} in normal subjects and patients with airflow obstruction due to cystic fibrosis. In normal subjects, the "accessible" V_A was 3% greater at a breath-hold time of 10 s than at 4 s (more time for gases to penetrate the alveoli if a 10 s breath-hold time is used), but that the K_{NO} and D_{LNO} were ∼14% less. The probable reason for the decrease in K_{NO} and K_{CO} with longer breath-hold times is that more weight is given, at longer breath-hold times, to more slowly filling and emptying units, whose D_L/V_A (equivalent to K) is less than the faster units. In the normal subjects, there was a 9% decrease in D_{LCO} from a 10 s breath-hold time compared to 4 s breath hold, so the D_{LNO}/D_{LCO} ratio was relatively unaffected. In airflow obstruction (cystic fibrosis), V_A at 10 s exceeded V_A at 4 s by 8%, compared to the 3% increase in normal subjects. When comparing the 4–10 s breath-hold time, the ~14% decrease in D_{LNO} and 18% decrease in K_{NO} were similar in normal subjects and those with cystic fibrosis. But since the D_{LCO} and K_{CO} were less affected over the same time periods, the D_{LNO}/D_{LCO} ratio decreased by 15% in those with cystic fibrosis. These findings suggest that ventilation distribution, or inspired gas penetration, is heterogeneous, even in normal subjects, because a greater V_A occurs at 10 s *versus* 4 s breath hold; in contrast, heterogeneity increases K_{NO} and K_{CO} at 4 s *versus* 10 s by more than the change in V_A, and this overcomes the smaller decrease in the 4-s V_A. Thus, the net effects on D_{LNO} and D_{LCO} at 4 s *versus* 10 s breath-hold depend on the combination of opposing changes in V_A and K_{NO} and K_{CO}, since $D_L = K \times V_A$.

Some studies show a different pattern. Studies in the early 1990s did not find a decrease in D_{LNO} [53] or D_{LCO} [53, 108] as breath-hold time increased, but the breath-hold times were short (down to 3 s breath hold) and the K and V_A values were not reported, so no conclusion about the mechanism can be reached. In normal, healthy children, THOMAS *et al.* [87] found that D_{LCO} and V_A were about equally increased at 10 s *versus* 5 s breath-hold time, and that K_{CO} did not change significantly (D_{LNO} was only studied at 5 s). From the modelling studies of PIIPER and SIKAND [51], independence of breath-hold time implies homogeneous distribution of D_{LCO}/V_A (equivalent to K_{CO}), which may be related to the smaller lung size (and less gravitational and iso-gravitational influences) in children.

Evaluating the measurement of D_{LNO}

Repeatability, reproducibility and number of tests

It is necessary to report the intra- and inter-session variability of D_{LCO} and D_{LNO} measurements so that a distinction can be made between normal biological variability/technical variability of the measurement and a clinically measureable change in diffusing capacity. Table 3 presents both acceptable intra-session (within a given testing session) and inter-session (between sessions, or between days) variability for the 5 s breath-hold manoeuvre for D_{LNO} and D_{LCO} in absolute numbers [48, 49, 88]. An average value of two trials performed within 4–10 min of each other whose differences in D_{LNO} and D_{LCO} is within 17 and 3 $mL \cdot min^{-1} \cdot mmHg^{-1}$, respectively, is acceptable in healthy subjects and those with pulmonary pathophysiology. The reproducibility in D_{LCO} and D_{LNO} that occurs week to week or month to month is 5 and 20 $mL \cdot min^{-1} \cdot mmHg^{-1}$, respectively, in healthy subjects and those with pulmonary pathophysiology (table 3). That is, any diffusing capacity parameter that has a week-to-week change that is equal to or greater than the reproducibility has only a 5% chance that it is not a real change. For less stringent reproducibility criteria, where there's a 20% chance that the change in D_{LCO} and D_{LNO} that occurs week to week or month to month is not a real change, look at the "smallest measureable change" column in table 3. It is half the reproducibility. Refer to supplementary appendix G for the statistical calculations of repeatability and reproducibility.

There is a 15% difference between the reproducibility value for D_{LNO} (20 mL·min^{-1}·mmHg^{-1}) and the repeatability value for D_{LNO} (17.2 mL·min^{-1}·mmHg^{-1}). There is a 35% difference between the reproducibility value for D_{LCO} (4.9 mL·min^{-1}·mmHg^{-1}) and the repeatability value for D_{LCO} (3.2 mL·min^{-1}·mmHg^{-1}). However, the percentage difference is increased to 34% for D_{LCO} (table 3). This suggests that D_{LNO} is a more stable measure over months compared to D_{LCO} and that the majority of the variability in D_{LNO} is within-session and not between sessions [88].

Repeated tests do not affect D_{LNO} within a given session, irrespective of COHb concentration [48, 49]. Even after 22 consecutive D_{LNO} measurements, D_{LNO} is unaffected, and the rise in methaemoglobin is minimal [48]. Since the largest slopes of the decrease in D_{LCO} observed with rising COHb was ~0.4–0.5 mL·min^{-1}·mmHg^{-1} decrease in D_{LCO} per 1% increase in COHb (males and females combined) [48], the minimum number of repeated tests that would elicit a decrease in D_{LCO} larger than its repeatability (*i.e.* ⩾3.2 mL·min^{-1}·mmHg^{-1}) would be eight for a 5 s breath-hold manoeuvre and six for a 10 s breath-hold manoeuvre. Thus, not more than eight 5 s breath-hold manoeuvres, or six 10 s breath-hold manoeuvres should be performed in a single session.

Calculating D_{MCO} and V_C

Using the simultaneous one-step NO–CO technique, measurements are made at a single alveolar P_{O_2} level. Values for θNO and θCO are required in the calculations (table 4 and supplementary appendix E). The literature in relation to published values for θNO and θCO is reviewed in the earlier section Origins of D_{LNO}. There is general consensus for using a finite θNO of 4.5 mL NO·(mL blood·min^{-1}·mmHg^{-1}) from CARLSEN and COMROE [29], which for clinical purposes is independent of alveolar P_{O_2} or Hb concentration. Conversely, the whole-blood transfer conductance for carbon monoxide is dependent on mean capillary P_{O_2} (approximately alveolar P_{O_2}) and Hb concentration (reflected in the haematocrit). Many equations for the 1/θCO relationship exist (*i.e.* table 5). We selected the 1/θCO from GUÉNARD *et al.* [16] (equation 2 and table 5) as the most representative. Negative values for D_{MCO} cannot occur unless the D_{LNO}/D_{LCO} ratio is >7.5, which is very unlikely, since normal values for D_{LNO}/D_{LCO} range from 3.8 to 5.8 (table 1).

In the literature, several versions of the 1/θCO–P_{O_2} relationship (table 5) have been used in the calculation of D_{MCO} and V_C. The ROUGHTON and FORSTER formula [11] yielded strong correlations between D_{LNO} (as a surrogate for D_{MNO}) and D_{MCO} for experimental data at rest and at exercise [35, 36, 109]. Others (for example [18, 62, 63, 94]) preferred the later formula given by FORSTER [4], but negative or excessively high D_{MCO} values have been observed with its use [19]; thus, some [19, 25] favoured the formula given by REEVES and PARK [26] and "best fit" α-ratios (all >2.0) for getting the best agreement for D_{MCO} between

TABLE 4 Summary consensus statement for simultaneous single-breath measurement of diffusing capacities of the lung for nitric oxide (D_{LNO}) and carbon monoxide (D_{LCO}) in healthy adults

Issue	Agreement
Breath-hold time	10 s is desired for better gas mixing 4–6 s is acceptable if using a single electrochemical NO cell that measures in the ppm range
Measured inspired NO concentration	40–60 ppm, placed in the inspiratory bag ⩽2 min before use
Measured inspired O_2 concentration	Close to 21%
Measured expired O_2 concentration	Used to calculate P_{AO_2} and θCO
1/θCO [16]	$(0.0062 \cdot P_{AO_2}+1.16)\cdot$(ideal Hb÷measured Hb)
θNO [14, 29]	4.5 mL NO·(mL blood·min·mmHg)$^{-1}$ (1/θNO=0.222)#
θNO/θCO ratio	Average 8.01 (male Hb 14.6 g·dL^{-1}), 8.73 (female Hb 13.4 g·dL^{-1}) at P_{AO_2} of 100 mmHg#
D_{MCO}	D_{MNO} divided by 1.97#
Presentation of results	Report D_{LNO}, D_{LCO}, K_{NO} and K_{CO} in absolute numbers and as % predicted from regression equations (at the appropriate breath-hold time), with the corresponding LLN, ULN and z-score (standardised residuals: number of standard deviations above or below the reference value) Report alveolar volume in L BTPS and as TLC % pred

NO: nitric oxide; O_2: oxygen; θCO (NO): specific conductance in the blood for carbon monoxide (NO) in mL·(mL blood·min·mmHg)$^{-1}$; D_{MCO}: alveolar–capillary membrane diffusing capacity for CO; P_{O_2}: oxygen tension; P_{AO_2}: alveolar oxygen tension; Hb: haemoglobin; D_{MNO}: alveolar–capillary membrane diffusing capacity for NO; K_{NO}: rate of change of NO from alveolar gas; K_{CO}: rate of change of CO from alveolar gas; LLN: lower limit of normal; ULN: upper limit of normal; BTPS: body temperature and pressure, saturated (760 mmHg, 37°C, 100% humidity); TLC: total lung capacity. #: used in tables 1, 2 and 3 and the supplementary appendices.

TABLE 5 1/θCO equations that show reasonable agreement

	Formula for 1/θCO	1/θCO		θNO/θCO	
		Ideal Hb ($14.6\ g\cdot dL^{-1}$)	Ideal Hb ($13.4\ g\cdot dL^{-1}$)	Ideal Hb ($14.6\ g\cdot dL^{-1}$)	Ideal Hb ($13.4\ g\cdot dL^{-1}$)
Derived *in vivo*					
Guénard *et al.* [16]	$(0.0062\cdot P_{AO_2}+1.16)\cdot$(ideal Hb÷measured Hb)	1.780	1.939	8.010	8.727
Derived *in vitro*					
Forster [4] α=∞, pH=7.4	$(0.0041\cdot P_{AO_2}+1.3)\cdot$(ideal Hb÷measured Hb)	1.710	1.863	7.695	8.384
Roughton and Forster [11] α=1.5, pH=8.0	$(0.0058\cdot P_{AO_2}+1.0)\cdot$(ideal Hb÷measured Hb)	1.580	1.721	7.110	7.747
Holland [28] α=1.5	$(0.0065\cdot P_{AO_2}+1.08)\cdot$(ideal Hb÷measured Hb)	1.730	1.885	7.785	8.482

Numbers given are for the following standards: alveolar oxygen tension (P_{AO_2}) 100 mmHg and specific conductance in the blood for nitric oxide (θNO) 4.5 mL NO· (mL blood·min·mmHg)$^{-1}$ and thus 1/θNO=0.222, from [14, 29]. θCO: specific conductance in the blood for carbon monoxide; Hb: haemoglobin; α: the ratio of permeability of the red cell membrane to that of the red cell interior.

the one-step NO–CO technique, and the classical Roughton–Forster multistep alveolar P_{O_2} method. Guénard *et al.* [16] proposed a new 1/θCO–P_{O_2} formula empirically derived from single-breath measurements of *D*LNO and *D*LCO at two P_{AO_2} levels while maintaining θNO at 4.5 mL NO·(mL blood·min^{-1}·mmHg^{-1}). This formula potentially incorporates some of the physiological complexities lacking in earlier formulas derived using *in vitro* apparatus, but the agreement between calculations from the new and old formulas is reasonably close (table 5 [4, 11, 28]). Compared to the Forster formula [4] listed in table 5, the Guenard *et al.* 1/θCO formula (also in table 5) yields an average *V*C of 7% (5 mL) greater (95% limits of agreement −5 –11 mL) and an average *D*MCO ~6% lower (95% limits of agreement −23–7 mL·min·mmHg^{-1}). Figure 4 demonstrates this graphically.

While *in vivo* factors could explain some of the differences between formulae, and an "optimal" θCO value under exercise or pathological conditions still remain to be determined, there are several formulae that show reasonable agreement (table 5). As θCO varies with P_{AO_2}, and there is a range of P_{AO_2} even among normal subjects, expired oxygen concentration should be estimated wherever possible.

Adjustment for Hb and COHb

In vitro [14] and *in vivo* [13, 17] work indicates that no adjustment for Hb is needed for *D*LNO over the range of haematocrits encountered clinically [31]. However, for *D*LCO, adjustments should be made for COHb levels >2%, and for Hb levels that differ from the standard Hb concentration (14.6 g·dL^{-1} for adult males and 13.4 g·dL^{-1} for adult females) [84].

Prediction equations

Methods

Currently, there are several prediction equations for single-breath *D*LNO in adults: one from North America [107], one from North Africa [110] and two from Europe [57, 106]. Prediction equations were created for white, European or North American adults, since there were few Asian, black African and Indian subjects (all <15 cases) in these studies [57, 106, 107]. We obtained de-identified data from two of these studies which used a 5 s breath hold [106, 107]. Data from van der Lee *et al.* [57] using a 10 s breath-hold time were also included in the analyses. We added 10 s breath-hold data from van der Lee *et al.* since there is only a small ~1 mL·min^{-1}·mmHg^{-1} absolute change in *D*LCO between 5- and 10-s breath-hold times in healthy subjects at rest in those with low *D*LCO values, and a ~3 mL·min^{-1}·mmHg^{-1} difference in those with high *D*LCO values [89]. These studies used a discrete sample of alveolar gas as opposed to continuous monitoring of exhaled gas concentrations.

From these datasets [57, 106, 107], the *D*MCO and *V*C values were first re-calculated according to the formulas in supplementary appendices A–E, with the selected values for θNO and θCO. Then a stepwise multiple linear regression procedure was used to determine which independent variable(s) best predicted nine dependent variables: *D*LCO and *D*LNO, *D*MCO and *D*MNO, *V*C, *D*LNO/*D*LCO ratio, *D*MCO/*V*A, *V*A, *V*C/*V*A, *D*MCO/*V*C, *K*CO and *K*NO. The independent variables entered into the model were age (years), age^2, weight (kg), height (cm), sex (male=1, female=0). An independent variable with an R^2 change that accounted for <5% of the total variance was eliminated from the model. When the full model accounted for <25% of the total variance, it was not included in table 2 or the supplementary material.

Data were screened to identify outliers. Any data point that exceeded a standard deviation of the residuals ⩾3.0 on the first and second screening for each dependent variable were eliminated. The first screening verified that the standardised residuals had a constant variance by visualising a plot between the standardised residuals (y-axis) and standardised predicted values (x-axis) to see if the values were consistently spread out, which would indicate normality and homoscedasticity. Linearity was analysed by creating a scatterplot matrix of the variables age, age^2, weight and height. To examine multicolinearity, the variance inflation factor (VIF) was used to see whether there was a strong association between *D*LCO or *D*LNO and all the predictors in the model. All independent variables in the model must have a VIF <10. To examine whether the errors were autocorrelated, a Durbin–Watson test was performed. The range is 0–4; a value of nearly 2 indicates non-autocorrelation, a value towards zero indicates a positive autocorrelation and a value close to 4 indicates a negative autocorrelation. To assess the prediction accuracy of the linear model, we randomly selected 90% of the subjects to fit a linear equation and then use the fitted linear equation to do the prediction for the remaining 10% of the subjects. This process was implemented for 1000 replicates, and we then reported the average correlation coefficient between each of the predicted values and the actual values obtained for 10% of the test subjects. In order to further check the accuracy of the measurement of alveolar volume from all three studies, we examined the predicted total lung capacity (using previous prediction equations [111]) and compared that to the predicted alveolar volume that was determined from the data obtained from three studies [57, 106, 107].

Given that 5% of the population is defined to be outside of "normal", the lower limit of normal (LLN, 2.5th percentile) and upper limit of normal (ULN, 97.5th percentile) were calculated for each prediction equation (two-tailed criteria, z-score ±1.96). The association between *D*LNO and *D*LCO, and their relationship to *V*A was examined from this dataset. A type I probability level of 0.05 was used. Statistical analysis used SPSS (version 21.0; IBM SPSS Statistics, Chicago, IL, USA), verified using R version 3.2.0. (www.r-project.org/).

Results

535 healthy white subjects with a body mass index (BMI) <30 $kg \cdot m^{-2}$ from three published studies [57, 106, 107] were used. Barometric pressure varied slightly between studies, but was not a meaningful predictor. There were 45 outliers (standardised residuals >3.0 in the prediction models), so 490 subjects were used in the final analyses (table 1). Overall, the *D*LNO and *D*LCO z-scores for the 490 subjects were both 0.0±1.0 with a skewness of 0.17 (*D*LNO) and 0.23 (*D*LCO). Mean±SD breath-hold time was 6.5±1.9 s

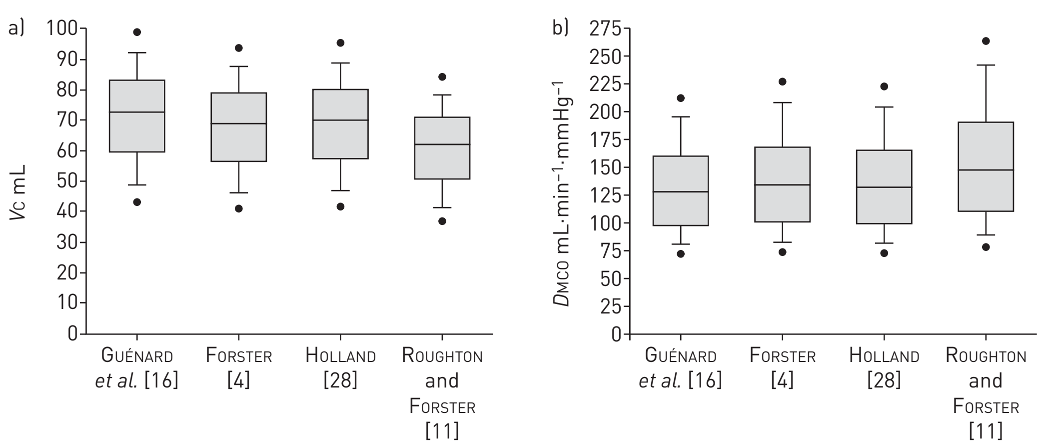

FIGURE 4 a) Pulmonary capillary blood volume (*V*C) and b) alveolar–capillary membrane diffusing capacity for carbon monoxide (*D*MCO), measured using four different formulas for specific conductance in the blood for carbon monoxide (θCO). Based on the subject data from table 1, *D*MCO and *V*C were calculated from the four formulas/constants listed in table 5. The mean values for both *V*C and *D*MCO were statistically significant between all four formulas (p<0.01). Each box represents the 25th (bottom border), 50th (middle) and 75th (top border) percentiles. The error bars above and below each box represent the 90th and 10th percentiles, respectively. The 5th and 95th percentiles are represented by solid black circles below and above the error bars, respectively. The formula from Guénard *et al.* [16] provided the highest *V*C and lowest overall *D*MCO, while the formula from Roughton and Forster [11] provided the highest *D*MCO and lowest *V*C. The three formulas developed by Guénard *et al.* [16], Forster [4] and Holland [28] provided the closest mean values with one another. Taken overall, these formulas show reasonable agreement with one another.

(range 4.6–10.0 s). While all DLCO gas mixtures reported in the studies displayed 21% oxygen in the tanks, the mean±SD inspired oxygen concentration and inspired NO concentration measured in the inspiratory bag was 19.5±0.7%, (range 18.1–20.5%) and 35±12 ppm (range 6–65 ppm). The differences in breath-hold time between studies had a minimal influence in predicting any of the variables in table 2 (⩽4% of the total variance). 117 (24%) subjects were aged 18–29 years, 193 (39%) subjects were aged 30–49 years, 132 (27%) subjects were aged 50–69 years and 48 (10%) subjects were aged 70–93 years. ∼33% of the subjects were classified as overweight (BMI 25.0–29.9 $kg \cdot m^{-2}$). 109 subjects were from the study by VAN DER LEE *et al.* [57], 115 subjects were from the study by ZAVORSKY *et al.* [107] and 266 subjects were from the dataset provided by AGUILANIU *et al.* [106]. The equations for DLNO and DLCO are presented in table 2. For DLCO and DLNO, height, age^2 and sex explained 45%, 13% and 11% of the model, respectively. For DMCO and DMNO, sex and age^2 explained 41% and 19% of the model, respectively. For VC, height and age^2 explained 36% and 14% of the model, respectively. For VA, height and sex explained 62% and 5% of the model, respectively. For DMCO/VA, age^2, sex and height explained 22%, 12% and 8% of the model, respectively. For KCO and KNO, age^2 explained 34% and 39% of the model, respectively. The DLNO/DLCO ratio was 2% larger in males compared to females (p=0.013), which was not clinically or physiologically different (95% CI of the difference 0.02–0.16 units larger in males) with an overall mean±SD 4.79±0.40. For VC/VA ratio, age^2 and sex explained 19% and 8% of the model, respectively. As the predictive models for DMCO/VC and the DLNO/DLCO ratio each explained <25% of the total variance, prediction equations were not developed for these parameters.

In terms of the prediction accuracy of the linear model, we found the following. For DLCO, DLNO, DMCO, VC, VA, DMCO/VA ratio, KCO, KNO and VC/VA ratio, the average correlation coefficients of the predicted values associated with the actual values were 0.82, 0.83, 0.78, 0.69, 0.82, 0.64, 0.57, 0.63 and 0.51, respectively.

The mean predicted TLC was within 0.6% of the mean predicted alveolar volume for males (range 0.4–0.8%), and within 4% of the mean predicted alveolar volume for females (range 0–7%). This suggests that DLNO, DLCO, DLNO/DLCO, VA, KCO, KNO and DMCO/VA were less likely to be over- or underestimated, and the prediction equations are probably satisfactory.

DLNO and DLCO are strongly correlated (figure 2a, single breath). DLNO and DLCO correlate with VA (R^2 0.64 and 0.62, respectively) (figure 2b, single breath). These data are consistent with published [35, 109] and unpublished rebreathing data from Connie Hsia (personal communication) that DLCO is more tightly correlated with cardiac output compared to DLNO (figure 2c, rebreathing). The DLNO/DLCO ratio decreased by 0.05–0.08 units for every 1.0 $L \cdot min^{-1}$ increase in cardiac output. Regression equation: DLNO to DLCO ratio −0.061·(cardiac output in $L \cdot min^{-1}$)+4.71, R^2=0.16, standard error of the estimate (SEE) 0.57, p<0.001).

The DMCO and VC calculations in tables 1–3 were performed according to the values prescribed in table 4 using the 1/θCO formula derived from *in vivo* data by GUÉNARD *et al.* [16]. There are several other formulas for 1/θCO which could change the predicted values for VC and DMCO (table 5). This ERS task force agrees that there may be other suitable formulas based on *in vitro* data (table 5). Nevertheless, we agree that using equations and constants provided in table 4 allow for clinical comparisons across studies. Based on human subject data from table 1, the 1/θCO formula from GUÉNARD *et al.* [16] provided the highest overall VC (by as much as +11 mL or 17%, p<0.01) and lowest overall DMCO (by as low as 24 $mL \cdot min^{-1} \cdot mmHg^{-1}$ or 15%, p<0.01; figure 4). In contrast, the lowest overall VC and highest overall DMCO was found with the 1/θCO formula from ROUGHTON and FORSTER [11]. HOLLAND [28], FORSTER [4] and GUÉNARD *et al.* [16] provided mean DMCO and VC data that were within 5% of each other (figure 4 and table 5). As such, the formulas presented in table 5 show reasonable agreement with one another.

Contraindications to DLNO and DLCO assessments

There are no contraindications for DLNO and DLCO measurements other than patients who are unable to understand or collaborate to the procedure or unwilling to provide consent. Children aged <18 years are allowed to undergo DLNO and DLCO measurements, as are pregnant subjects [112].

Future investigations

There are three broad categories of research priorities in further development of the single-breath DLNO–DLCO technique: technology, physiology and clinical application.

Technology

Development of affordable, rapid-response chemiluminescence analysers with a resolution range from <100 ppb to 100 ppm would be welcome. If electrochemical cells are used, the target resolution should be in the same range.

Physiology

The calculations of D_{MCO}, D_{MNO}/D_{MCO} ratio and V_C from simultaneously measured D_{LNO} and D_{LCO} remain controversial. Considerable research supports CARLSEN and COMROE's [29] data that θNO is finite at 4.5 mL NO·(mL blood·min·mmHg)$^{-1}$. GUÉNARD *et al.*'s [16] 1/θCO equation is the only one that is derived from actual physiological measurements and the results agree reasonably well with several equations derived *in vitro*. More measurements of θNO and θCO using innovative techniques would be welcome. Little is known about physiological variation in θNO or θCO due to changes in pH, the oxygen tension corresponding to 50% oxyhaemoglobin saturation P_{50}, temperature and 2,3-bisphosphoglycerate levels. Similarly, measurements of the NO/CO diffusivity ratio (D_{MNO}/D_{MCO}) in lung tissue would be helpful. Whether the surface area relative to thickness of the diffusion barrier in the bronchial wall, or the lower than systemic pulmonary capillary haematocrit or the heterogeneous capillary erythrocyte distribution differentially alter D_{LNO} and D_{LCO}, and hence D_{LNO}/D_{LCO} and D_{MCO}/V_C needs to be examined. Further studies are needed to define the relative response between D_{LNO} and D_{LCO} under a range of perturbations such as exercise and high-altitude exposure; these comparisons could yield mechanistic insight into alveolar microvascular recruitment. Comparison of single breath and rebreathing methods could offer insight into ventilatory heterogeneity.

Clinical application

Reference values of D_{LNO} and D_{LNO}/D_{LCO} are lacking in non-Caucasian populations, and in relation to age. The relative impairment of D_{LCO} and D_{LNO} in disorders of the thorax, airway, parenchyma, vasculature and secondary to cardiac failure needs to be assessed. Whether the combination of D_{LNO}, D_{LCO}, D_{LNO}/D_{LCO} ratio, D_{MCO} and V_C will improve the management of cardiopulmonary diseases compared to the conventional use of D_{LCO} remains to be determined.

Summary and conclusions

1) Recommendations for the standard single-breath D_{LCO} technique [84] should be followed with exceptions for breath-hold time, inspiratory time, expiratory time and repeatability criteria.
2) NO analysers: the sensitivity and performance of NO electrochemical cells are less than ideal compared to the much more expensive chemiluminescence analyser. A lack of sensitivity has meant that breath-holding time has had to be reduced. Electrochemical NO cell analysers could continue to be used for the combined D_{LNO}–D_{LCO} measurement until more sensitive analysers at a more affordable price become available.
3) Breath-hold time: for users who have the less-sensitive electrochemical NO analysers, we agree on a breath-hold time of 4–6 s.
4) Inspired concentrations of NO, CO and O_2 should be as follows: NO 40–60 ppm, CO 0.3% and O_2 close to 21%. NO should be injected into the inspired bag ⩽2 min before use, and the inspired concentrations of all these gases plus the inert tracer gas (He, CH_4 and neon) must be recorded. After 120 s of non-use, the NO concentration will be reduced by ~2.5 ppm due to its conversion to NO_2 [85].
5) Expired concentration of O_2: the exhaled "alveolar" O_2 concentration should be measured so that 1/θCO can be estimated from the measurement.
6) Presentation of results: the D_{LNO} and D_{LCO} should be given in absolute numbers, as % predicted from regression equations (at the appropriate breath-hold time) and with the LLN (mean −1.96·SEE) and the ULN (mean +1.96·SEE). In addition, the z-score (standardised residuals: number of standard deviations above or below the reference value) should be presented. The same applies for K_{NO} and K_{CO}. Alveolar volume should be recorded in L BTPS and as TLC % pred. The D_{LNO}/D_{LCO} ratio is a useful parameter, because it does not require choosing a physical constant (θ or α) in its calculation, and is relatively independent of breath-hold time, age, height and sex.
7) The calculations for D_{MCO} and V_C are provided in supplementary appendix E with a sample algorithm provided in appendix H.

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