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

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**Pulmonary diffusing capacity for nitric oxide is standardised by a panel of experts for use around the world** <http://ow.ly/TpV1306Yhji>

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**ABSTRACT** Diffusing capacity of the lung for nitric oxide ( $DLNO$ ), otherwise known as the transfer factor, was first measured in 1983. This document standardises the technique and application of single-breath  $DLNO$ . 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 ( $PAO_2$ ) should be measured by sampling the expired gas; 5) a finite specific conductance in the blood for NO ( $\theta_{NO}$ ) should be assumed as  $4.5 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot \text{mL}^{-1}$  of blood; 6) the equation for  $1/\theta_{CO}$  should be  $(0.0062 \cdot PAO_2 + 1.16) \cdot (\text{ideal haemoglobin}/\text{measured haemoglobin})$  based on breath-holding  $PAO_2$  and adjusted to an average haemoglobin concentration (male  $14.6 \text{ g} \cdot \text{dL}^{-1}$ , female  $13.4 \text{ g} \cdot \text{dL}^{-1}$ ); 7) a membrane diffusing capacity ratio ( $DMNO/DMCO$ ) 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 (*DLNO*) 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 *DLNO* 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”, “*DLNO*” and “*TLNO*” (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 *DLNO*. 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 *DLNO* or *TLNO*

### *Origins of DLNO*

Initially, interest in nitric oxide (NO) uptake was toxicological. High concentrations of nitrogen dioxide (NO<sub>2</sub>; 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 *DLNO* and the diffusing capacity of the lung for carbon monoxide (*DLCO*); these data were initially presented as abstracts in 1983–1984 [1, 2], and reported in BORLAND’s doctoral project [3]. Subsequently, these *DLCO* and *DLNO* observations on varying breath-hold time and back tension were published in 1989 [10], in which the differences in *DLCO* and *DLNO* were undetectable within the sensitivity of the analyser (1 ppm). However, there was greater volume dependence of *DLNO* compared to *DLCO*, and independence of *DLNO* (but not *DLCO*) from hyperoxia [10].

Independently, Daniel Bargeton and Hervé Guénard in Paris had speculated that the ROUGHTON and FORSTER equation ( $1/DLCO = 1/DM + 1/\theta CO \cdot VC$ ) [11] could be solved using a single manoeuvre with simultaneous measurement of carbon monoxide (CO) and NO uptake. *DM* 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), *VC* is the total volume of blood in the lung capillaries exposed to alveolar air in millilitres and  $\theta CO$  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 ( $1/DLNO$  or  $1/DLCO$ ,  $1/DM$  and  $1/\theta \cdot VC$ ) 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 *DM* and *VC* from simultaneous single-breath *DLNO* and *DLCO* in 1987.

### *Evolution of DLNO (1989–2016)*

Early work had shown that mean *DLNO* exceeded mean *DLCO* by 4.3–5.3-fold [10, 12]. In other words, the transfer resistance for NO ( $1/DLNO$ ) 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 ( $\theta NO$ ) 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  $\theta_{NO}$  was finite. In clinical studies, the fact that  $DL_{NO}$ , unlike  $DL_{CO}$ , 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  $\theta$  value for blood, seemed to support the original notion that  $\theta_{NO}$  was “effectively” infinite, and that  $DL_{NO}$  is a surrogate for the alveolar membrane diffusing capacity, *i.e.*  $DL_{NO}=DM_{NO}=1.97 \cdot DL_{CO}$ ; this view is still held by some [18, 19]. But the current consensus is that  $DL_{NO}$  is weighted, but not dominated, by the membrane gas conductance, while the  $DL_{CO}$  is dominated by  $\theta_{CO}$  [20]. The  $DL_{NO}/DL_{CO}$  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,  $\theta_{NO}$  cannot be “infinite”, as originally thought [10, 12] or more recently claimed [18, 19]. Further support for a “finite”  $\theta_{NO}$  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,  $DL_{NO}$  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  $DL_{NO}$  [14].

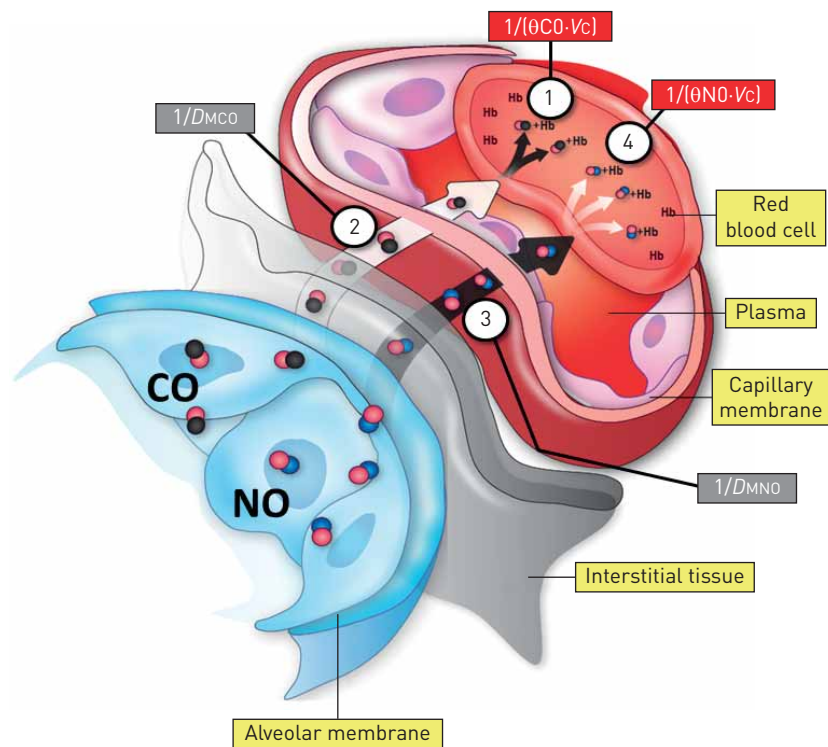


FIGURE 1 Diagram of the uptake pathways for inhaled nitric oxide (NO) and carbon monoxide (CO) from the alveolar membrane to their combination with haemoglobin (Hb) within the red blood cell, in terms of the Roughton–Forster equation,  $1/DL=1/DM+1/(\theta \cdot Vc)$ , where  $1/DL$  is the total resistance to NO or CO uptake,  $1/DM$  is the resistance from the alveolar membrane to the red cell membrane (membrane resistance) and  $1/(\theta \cdot Vc)$  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  $\theta_{CO}$* 

Due to competitive binding between CO and oxygen for Hb-accessible sites, there is a strong association between  $\theta_{CO}$  and mean pulmonary capillary oxygen tension ( $P_{O_2}$ ) ( $1/\theta_{CO}$ , 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  $1/\theta_{CO}$  and alveolar (capillary)  $P_{O_2}$  is usually expressed as:

$$1/\theta_{CO} = (a \cdot P_{O_2} + b) \cdot (\text{ideal Hb} \div \text{measured Hb}) \quad (1)$$

where the units for  $1/\theta_{CO}$  are  $\text{mL of CO} \cdot (\text{mL blood} \cdot \text{min} \cdot \text{mmHg})^{-1}$ ; “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  $\div$  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  $DMCO$  using the classical Roughton–Forster multistep alveolar  $P_{O_2}$  method. For example, upon exercise, and depending on the  $1/\theta_{CO}$  versus  $P_{O_2}$  equation used,  $DMCO$  may vary from 48 to  $128 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1}$  and for  $VC$  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  $DMCO$  and pulmonary capillary blood volume are inconsistent, although the directly measured  $DLNO$  and  $DLCO$  should be available for others to calculate  $DMCO$  and  $VC$  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  $1/\theta_{CO}$  versus  $P_{O_2}$  equations for a constant  $DMCO/VC$  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  $DMCO$  and  $VC$ , using the one-step NO–CO technique with a finite  $\theta_{NO}$ , 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]:

$$1/\theta_{CO} = (0.0062 \cdot P_{A_{O_2}} + 1.16) \cdot (\text{ideal Hb} \div \text{measured Hb}) \quad (2)$$

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  $1/\theta_{CO}$  versus  $P_{O_2}$  equations derived *in vitro*.

*An optimal value for  $\theta_{NO}$* 

Using the same continuous flow rapid mixing apparatus as the 1957  $\theta_{CO}$  measurements [11],  $\theta_{NO}$  can be calculated as  $4.5 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot \text{mL}^{-1}$  of blood [29]. Less direct estimates have ranged from  $3.0 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot \text{mL}^{-1}$  (humans, *in vivo*; GUÉNARD *et al.* [16]) to  $4.0 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot \text{mL}^{-1}$  (membrane oxygenator, *in vitro*; BORLAND *et al.* [30]) to  $<4.5 \text{ min}^{-1} \cdot \text{mmHg}^{-1}$  (dog, *in vivo*, exchange transfusion; BORLAND *et al.* [13]). The consensus is that  $\theta_{NO}$  should be taken as  $4.5 \text{ mL} \cdot \text{min}^{-1} \cdot \text{mmHg}^{-1} \cdot \text{mL}^{-1}$  of blood. The influence of inspired oxygen concentration (and thus alveolar  $P_{O_2}$ ) on  $DLNO$  (and therefore  $\theta_{NO}$ ) is small [15, 16], and for clinical purposes, can be ignored. Similarly, the influence of Hb concentration  $>5\text{--}7 \text{ g} \cdot \text{dL}^{-1}$  on  $DLNO$  is too small to matter [14, 17, 31].

*Alveolar–capillary membrane diffusing capacity for NO and the  $\alpha$ -ratio*

The alveolar–capillary membrane diffusing capacity ( $DM$ ) 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],  $DM$  is the y-axis intercept, at zero  $P_{O_2}$ , on a plot of  $1/DLCO$  versus  $1/\theta_{CO}$ ; this definition does not extend to NO, which is effectively  $P_{O_2}$ -independent [15]. An important determinant of  $DMNO$

and  $DMCO$  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  $DMNO$  and  $DMCO$  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  $DM$  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  $\text{g}\cdot\text{mol}^{-1}$ ), 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  $\alpha$ . Thus,  $DMNO = \alpha \cdot DMCO$ . 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  $DMNO/DMCO$  ratio.

#### *An “empirical” value ( $\alpha$ ) for $DMNO/DMCO$*

Several groups have measured  $DMCO$  using the Roughton–Forster multistep alveolar  $PO_2$  method and related it to  $DMNO$  (assuming an “infinite”  $\theta_{NO}$ , so that  $DLNO = DMNO$ ). This “ $DMNO/DMCO$ ” ratio was significantly greater than the 1.97 predicted from the tissue diffusivity ratio ( $\alpha$ ), and varied from 2.06 to 4.4, depending on the equation used [19, 25, 35, 36]. Even higher values of  $\alpha$  would have been obtained if a finite value for  $\theta_{NO}$  had been used. Since  $\theta_{NO}$  has a finite value (and the evidence is overwhelming) this empirical  $DMNO/DMCO$  ratio ( $\alpha$ ) merely states the fact that  $DMCO$  calculated from the simultaneous one-step NO–CO method (with or without a finite  $\theta_{NO}$  value) is significantly greater than  $DMCO$  calculated by the classical Roughton–Forster multistep alveolar  $PO_2$  method. When recalculating data from a study that used a rebreathing technique [36], with a finite  $\theta_{NO}$  and GUENARD’s  $1/\theta_{CO}$  equation (equation 2) [16], the results show that the  $DMCO$  from the simultaneous one-step NO–CO method was 1.25 times greater than the  $DMCO$  calculated by the classical Roughton–Forster multistep alveolar  $PO_2$  method, at rest and upon exercise. With other equations, with or without an infinite  $\theta_{NO}$ , the discrepancy was even greater.

With the simultaneous one-step NO–CO method,  $DMNO$  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,  $\theta_{NO}$  would have to double (to  $9.0 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}\cdot\text{mL}^{-1}$ ) to reduce  $DMNO$  sufficiently in the simultaneous one-step NO–CO method. One probable reason for the  $DMCO$  discrepancy lies in the methods of calculation. Many of the measurements are common to both methods ( $DLNO$ ,  $DLCO$ ,  $\theta_{NO}$  and  $\theta_{CO}$  at a nominal  $PO_2$  (100 mmHg)), but the simultaneous one-step NO–CO method uses the diffusivity ratio constant,  $\alpha$  (1.97) whereas the Roughton–Forster multistep alveolar  $PO_2$  method extrapolates the  $1/\theta_{CO}-PO_2$  equation to zero  $PO_2$  to obtain the intercept ( $1/DMCO$ ). Experimentally, the  $1/\theta_{CO}-O_2$  relationship appears to be linear (see figure 3 of FORSTER’s article [4]), but REEVES and PARK [26] found that  $\theta_{CO}$  doubled at  $PO_2 < 40$  mmHg, possibly due to CO binding of unliganded Hb sites *versus* the  $HbO_2$  replacement reaction at higher  $PO_2$ . Nonlinearity of the  $1/\theta_{CO}-PO_2$  relationship could lead to overestimation of the zero  $PO_2$  intercept and underestimation of  $DMCO$  with the Roughton–Forster multistep alveolar  $PO_2$  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 ( $\sim 0.02\%$ ) (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  $DLNO$  was observed [37]. There was no consistent effect with  $DLCO$  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  $DLNO$  will be small ( $\sim 5\%$  of total  $1/DLNO$ ) [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  $DL$  is the diffusing capacity,  $\beta$  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  $\beta=2.5 \text{ mmHg}^{-1}$  and hence  $DL/\beta$ , or rather  $DL_{NO}/B\dot{Q}$  at rest =  $150/(2.5 \cdot 5000) = 0.012$ . With exercise, the ratio is even lower since the increase in  $\dot{Q}$  is much greater than the increase in  $DL_{NO}$ . This low value of  $\sim 0.012$  (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  $DL_{NO}$  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  $DL_{NO}$  of  $\sim 16\text{--}22 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  for every  $1.0 \text{ L}\cdot\text{min}^{-1}$  increase in oxygen uptake [40–42], or  $\sim 5\text{--}7 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  for every  $1.0 \text{ L}\cdot\text{min}^{-1}$  increase in cardiac output [35, 36] (figure 2c). Pulmonary sarcoidosis reduces the slope to  $\sim 2.2 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  per  $1.0 \text{ L}\cdot\text{min}^{-1}$  increase in cardiac output [35]. The increase in  $DL_{NO}$  (and  $DL_{CO}$ ) 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  $DL_{CO}$  with pulmonary blood flow is tighter than that of  $DL_{NO}$  with blood flow (figure 2c), suggesting that  $DL_{CO}$  is more sensitive than  $DL_{NO}$  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  $DL_{CO}$  or pulmonary blood flow [36].

### Back tension

The endogenous alveolar NO concentration is  $\sim 8\text{--}20$  ppb during tidal breathing [45] and  $\sim 100\text{--}140$  ppb in the nose [46]. The mean $\pm$ SD fraction of NO from a single-breath exhalation at  $50 \text{ mL}\cdot\text{s}^{-1}$  is significantly higher in asthmatics ( $73\pm 11$  ppb) compared with healthy subjects ( $35\pm 4$  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  $DL_{NO}$  [10, 48, 49] or  $DL_{CO}$  [10, 50].

### Heterogeneity

A drawback of the single-breath  $DL_{NO}$  (and  $DL_{CO}$ ) 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 ( $\text{CH}_4$ ), *etc.*) exist within the alveolar sample, as shown by sloping alveolar plateaus of concentrations *versus* time or

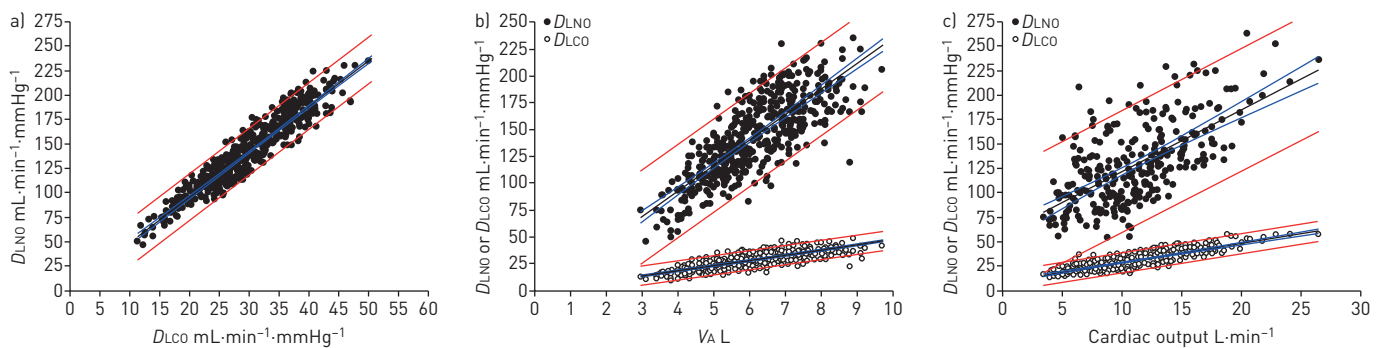


FIGURE 2 a) The association between diffusing capacities of the lung for nitric oxide ( $DL_{NO}$ ) and carbon monoxide ( $DL_{CO}$ ) measured at rest (single-breath; average breath-hold time was  $\sim 6$  s). Several published studies were used [57, 106, 107].  $DL_{NO}=4.65\cdot(DL_{CO})+3.8$ ,  $R^2$  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 (VA) measured at rest (single-breath; average breath-hold time was  $\sim 6$  s). Several published studies were used [57, 106, 107].  $DL_{NO}=23.0\cdot(VA)+2.4$ ,  $R^2$  0.64, SEE 21.9,  $p<0.001$ , 95% CI of the slope 21.4–24.5;  $n=493$ .  $DL_{CO}=4.63\cdot(VA)+1.55$ ,  $R^2$  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  $\sim 45\%$  of previously unpublished data.  $DL_{NO}=6.3\cdot(Q)+58.2$ ,  $R^2$  0.42, SEE 31.3,  $p<0.001$ , 95% CI of the slope 5.5–7.2;  $n=76$ , four data points per subject.  $DL_{CO}=2.0\cdot(Q)+9.0$ ,  $R^2$  0.71, SEE 5.3,  $p<0.001$ , 95% CI of the slope 1.8–2.1;  $n=76$ , four data points per subject. When using rebreathing manoeuvres,  $DL_{CO}$  is more tightly associated with cardiac output than  $DL_{NO}$  [comparison of correlation coefficients z-statistic 5.52,  $p<0.01$ ]; however,  $DL_{NO}$  is more tightly related to alveolar volume compared to  $DL_{CO}$  [comparison of correlation coefficients z-statistic 2.27,  $p=0.023$ ]. The association between  $DL_{NO}$  and  $DL_{CO}$  in relation to VA during rebreathing manoeuvres ( $r=0.73$  between  $DL_{NO}$  versus VA, and  $r=0.63$  between  $DL_{CO}$  versus VA) is not shown here.

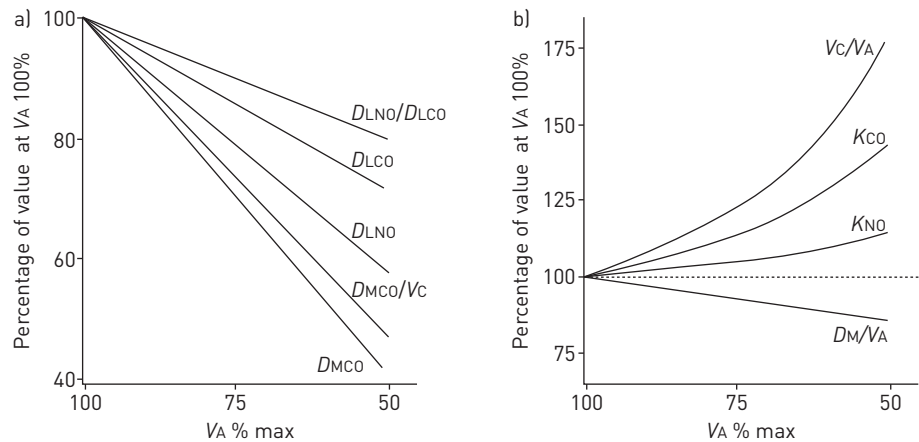


FIGURE 3 Plots of a) pulmonary diffusing capacity for nitric oxide ( $DLNO$ ) and pulmonary diffusing capacity for carbon monoxide ( $DLCO$ ), their ratio ( $DLNO/DLCO$ ), alveolar–capillary membrane diffusing capacity for carbon monoxide ( $DMCO$ ) and the  $DMCO$  to pulmonary capillary blood volume ( $Vc$ ) ratio ( $DMCO/Vc$ ), 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,  $KNO$  and  $KCO$  (mathematically equivalent to  $DLNO/V_A$  and  $DLCO/V_A$ , respectively), and the membrane diffusing capacity ( $DM$ ) and pulmonary capillary volume ( $Vc$ ), both per unit alveolar volume ( $V_A$ ) ( $DM/V_A$  and  $Vc/V_A$ ), 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 ( $\Delta V_A$ ),  $\Delta DLNO$  is better related to membrane diffusing capacity ( $\Delta DMCO$ ) and  $\Delta DMCO/Vc$  change than the  $DLCO$  change. In b),  $\Delta KCO$  is a better reflection of changes in the pulmonary microcirculation (capillary volume per unit alveolar volume,  $Vc/V_A$ ) than the  $KNO$ ; decrease of  $DM/V_A$  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  $DLNO$  (and  $DLCO$ ) 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  $DLNO$  and  $DLCO$  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  $DLNO$ . 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. PIPER and SIKAND [51] used the classical two-compartment parallel model in which  $DLCO$  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  $DLNO$  is the product of  $V_A$  during breath holding and  $KNO$  (rate of change of NO from alveolar gas, per unit pressure of NO, and equivalent to  $DLNO/V_A$ ). If  $KNO$  was uniform but alveolar volume was uneven between the two compartments,  $DLNO$  was less than if alveolar volume had been evenly distributed, but this underestimation was independent of breath-hold time [51]. If both  $KNO$  and alveolar volume were unevenly distributed,  $DLNO$  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  $DLNO$  and  $DLCO$  measurements in normal subjects and patients with airflow obstruction.  $DLNO$  and  $DLCO$  decrease as breath-hold time is prolonged [54] because the decrease in  $KNO$  (and  $KCO$ ) at a longer breath-hold time (more weight being given to the low  $KNO$  and  $KCO$  compartments) outweighs the increase in alveolar volume (more time for inert gas equilibration at 10 s breath-hold).  $DLNO$  and  $DLCO$  are affected similarly, so the effect of heterogeneity on the  $DLNO/DLCO$  ratio is small in normal subjects.

There is no recognised method that “corrects” the  $DLNO$  and  $DLCO$  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 $\pm$ SD  $V_A$  to TLC ratio is 0.94 $\pm$ 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  $DLNO$  as  $KNO \times TLC$ , where TLC is measured separately. Nonetheless, this has not found favour as it presumes that the  $KNO$  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  $DLNO$  and  $DLCO$  at 10 s breath hold is compared to 5 s breath hold. There is a tendency for a trade-off between an increase in  $VA$  and a decrease in  $KNO$  and  $KCO$  (or *vice versa*) at 10 s *versus* 5 s, so that dispersion of  $VA$  and  $DL$  affects each component of  $DLNO$  and  $DLCO$  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  $DLCO$ .

### Measurements of single-breath $DLNO$ 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  $DLNO$  and  $DLCO$  in volunteers and in patients with different diseases.

#### *DLNO in the normal lung*

In normal subjects,  $DLNO$  decreases to a greater extent than  $DLCO$  when lung volume declines [10, 59] (figure 3). Compared to 100% of  $VA$ ,  $DLNO$  is decreased by ~40% when  $VA$  is decreased by ~50% (figure 3). This is in opposition to  $DLCO$ , which only decreases by ~25% for the same decrease in  $VA$  (figure 3). Thus, for the same decrease in lung volume, the percentage increase in  $KCO$  ( $DLCO/VA$ ) is approximately double that of  $KNO$  ( $DLNO/VA$ ) (figure 3), reflecting greater  $DLNO$  dependence on the  $DMNO/VA$  ratio than on the  $VC/VA$  ratio.

After adjusting for postural changes in  $VA$ , both  $DLNO$  and  $DLCO$  increase ~5% from upright, sitting to supine [60], which may be explained by an ~13% increase in  $VC$  in the supine position compared to sitting [60]. In contrast, changing from a supine to a prone position has yielded varying results [61].

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

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

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

#### *DLNO in disease*

When comparing  $DLNO$  in disease to a control group, it is helpful to examine  $DLNO$  and the simultaneously measured  $DLCO$  and the  $DLNO/DLCO$  ratio [21]. As  $DLNO$  is weighted by  $DM$  and  $DLCO$  is weighted by  $VC$ , the  $DLNO/DLCO$  ratio (assuming  $DLNO$  and  $DLCO$  are reduced) reflects a relative change in the membrane-to-capillary components of uptake ( $DMCO/VC$ ) [21]. An increase in  $DLNO/DLCO$  signifies a reduction in  $VC$  greater than the reduction in  $DM$ , meaning that there is greater microvascular disruption than membrane disruption (and *vice versa* for a decrease in  $DLNO/DLCO$ ). Likewise, since  $DLNO$  is

insensitive to changes in haematocrit in the physiological range, the  $DLNO/DLCO$  ratio should rise in anaemia and decrease in polycythaemia. As predicted, increasing Hb concentration by 33% (from 7.8 to 10.4 g·dL<sup>-1</sup>) by transfusion caused a minimal increase in  $DLNO$  (~3%,  $p>0.05$ ), while  $DLCO$  increased by ~20% ( $p<0.05$ ), and the  $DLNO/DLCO$  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  $VC$  greater than the reduction in  $DMCO$ , leading to a rise in  $DMCO/VC$  and  $DLNO/DLCO$  ratios.  $DMCO$  falls as  $VC$  falls because of their interdependence (“coupling”). Nevertheless, in patients with idiopathic PAH, there were equal reductions in  $DMCO$  and  $VC$ , but no change in the  $DLNO/DLCO$  ratio [73]. In liver cirrhosis with hepatopulmonary syndrome (HPS) [74], there was a greater reduction in  $VC$  and  $DMCO$  (and a lower arterial oxygen pressure) *versus* non-HPS patients, but both groups demonstrated a similar rise in  $DLNO/DLCO$  and  $DMCO/VC$  ratios compared to controls, consistent with microvascular disease. In heart failure,  $DLNO/DLCO$  and  $DMCO/VC$  ratios were reported to be increased [75], contrary to predictions, but there were methodological issues in the calculations of  $DMCO/VC$  [76]. As such, more studies are needed examining microvascular disease and its effects on diffusing capacity.

#### *Interstitial lung disease*

A greater reduction of  $DMCO$  than  $VC$  (with a fall in  $DLNO/DLCO$  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],  $DMCO$  was preserved in relation to  $VC$ , and the  $DLNO/DLCO$  and  $DMCO/VC$  ratios were increased compared to controls (Borland and Hughes, personal communication), suggesting that a reduction in  $VC$  may be an early sign of chronic obstructive pulmonary disease (COPD). In established COPD, both  $DM$  and  $VC$  appear to be reduced [53].

#### *Miscellaneous*

In chronic renal failure [78],  $DLNO$  and both  $DMCO/VC$  and  $DLNO/DLCO$  ratios are reduced (after adjusting for Hb). In morbid obesity [40, 79] there is a slight reduction in  $DMCO/VC$ . In cystic fibrosis  $DMCO/VC$  and  $DLNO/DLCO$  are reduced [80]. Following bone marrow transplant, both  $DLNO$  and  $DLCO$  are reduced [81].

#### **Conclusion**

Different pathologies will reduce the membrane ( $DM$ ) and microvascular ( $\theta \cdot VC$ ) 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  $DLNO$  and  $DLCO$ ,  $DLNO/DLCO$ ,  $DMCO$  and  $VC$  will remain imprecise until more clinical studies are reported using a standardised technique.

### **Gas analysers and general equipment**

#### **System design**

All commercially available  $DLNO$  apparatus is based on the single-breath  $DLCO$  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  $DLNO$  system is basically a single-breath  $DLCO$  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 DLCO systems has been described elsewhere [84]. Importantly, NO is reactive with oxygen (O<sub>2</sub>), to form NO<sub>2</sub> (O<sub>2</sub>+2·NO→2·NO<sub>2</sub>). NO<sub>2</sub> is formed at a rate of ~0.02 ppm·s<sup>-1</sup> (~1.2 ppm NO<sub>2</sub>·min<sup>-1</sup>) in a gas mixture containing close to 21% oxygen and 60 ppm NO [85]; <3 ppm of NO<sub>2</sub> 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, DLNO would be overestimated by ~1%. As such, NO gas (along with nitrogen (N<sub>2</sub>)) is stored in a separate gas cylinder (apart from oxygen) containing NO in a high concentration in N<sub>2</sub>, ranging from 400 to 1200 ppm NO in N<sub>2</sub>. The greater the concentration of NO with N<sub>2</sub> in the cylinder, the less N<sub>2</sub> 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 DLNO 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 DLCO, a breath-hold time is 10±2 s calculated by the JONES and MEADE formula [86]. If an electrochemical cell is used for the DLNO 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 DLNO, DLCO, DMCO and VC 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 DLNO and DLCO 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 DLNO tests on subjects that inspired ~55 ppm NO for each test, DLNO remained unchanged [48]. Furthermore, there is minimal interaction between NO and CO [10, 50], therefore the DLNO and DLCO 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
<b>Subjects</b>	248	242	490
<b>Age years</b>	44±17 [18–93]	45±18 [18–87]	44±17 [18–93]
<b>Weight kg</b>	76.7±9.4 [55.0–105.0]	61.6±8.8 [44.0–95.0]	69.3±11.8 [44.0–105.0]
<b>Height cm</b>	176±8 [154–196]	164±7 [147–182]	170±10 [147–196]
<b>Body mass index kg·m<sup>-2</sup></b>	24.7±2.5 [18.9–29.9]	23.0±3.0 [17.2–29.8]	23.8±2.9 [17.2–29.9]
<b>DLNO mL·min<sup>-1</sup>·mmHg<sup>-1</sup></b>	164±31 [67–235]	119±25 [47–186]	142±36 [47–235]
<b>DLCO mL·min<sup>-1</sup>·mmHg<sup>-1</sup></b>	34.1±6.3 [11.9–49.9]	25.1±5.3 [11.3–38.6]	29.6±7.4 [11.3–49.9]
<b>DMCO mL·min<sup>-1</sup>·mmHg<sup>-1</sup></b>	161±39 [72–250]	104±26 [33–182]	133±44 [33–250]
<b>VC mL</b>	78±16 [25–121]	65±15 [30–105]	72±17 [25–121]
<b>DMCO/VC ratio min<sup>-1</sup>·mmHg<sup>-1</sup></b>	2.11±0.57 [1.01–4.03]	1.63±0.40 [0.88–2.96]	1.90±0.57 [0.88–4.03]
<b>KCO mL·min<sup>-1</sup>·mmHg<sup>-1</sup>·L<sup>-1</sup></b>	4.9±0.8 [2.7–7.1]	4.8±0.7 [3.0–6.8]	4.9±0.8 [2.7–7.1]
<b>KNO mL·min<sup>-1</sup>·mmHg<sup>-1</sup>·L<sup>-1</sup></b>	23.8±3.9 [13.7–34.2]	22.8±3.2 [13.5–31.5]	23.3±3.6 [13.5–34.2]
<b>DLNO/DLCO ratio</b>	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 (DMCO) and total volume of blood in the lung capillaries exposed to alveolar air (VC) values reported in these studies [57, 106, 107] have been recalculated according to the parameters listed in table 4. DLNO: diffusing capacity of the lung for nitric oxide; DLCO: diffusing capacity of the lung for carbon monoxide; KCO: rate of uptake of carbon monoxide from alveolar gas; KNO: 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 <sup>2</sup>	Sex	Constant	Adjusted R <sup>2</sup>	SEE	LLN and ULN
$DL_{CO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup>	0.23	-0.002	6.0	-8.5	0.68	4.2	±8.2
$DL_{NO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup>	0.81	-0.010	34.4	9.7	0.69	20.0	±39.2
$DM_{CO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup>		-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 <sup>-1</sup>		-0.0006	-1.25	13.9	0.27	1.89	±3.70
$DM_{CO}/V_A$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup> ·L <sup>-1</sup>	-0.200	-0.002	5.9	56.6	0.41	3.81	±7.47
$K_{CO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup> ·L <sup>-1</sup>		-0.00027		5.5	0.34	0.6	±1.2
$K_{NO}$ mL·min <sup>-1</sup> ·mmHg <sup>-1</sup> ·L <sup>-1</sup>		-0.00137		26.4	0.39	2.8	±5.5

Alveolar-capillary membrane diffusing capacity for carbon monoxide ( $DM_{CO}$ ) 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 ( $DL_{NO}/DL_{CO}$ ). Sex: 1 for male, 0 for female; SEE: standard error of the estimate. To convert  $DL_{NO}$ ,  $DL_{CO}$  and  $DM_{CO}$  to mmol·min<sup>-1</sup>·kPa<sup>-1</sup>, 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  $DL_{CO}$  measurement are close to 0.30% CO and 21% O<sub>2</sub> [84]. For measurement of  $V_A$ , either 10% He or 0.3% CH<sub>4</sub> can be used.

#### Linearity and accuracy

Since  $DL_{CO}$  and  $DL_{NO}$  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<sub>2</sub>O·L<sup>-1</sup>·s<sup>-1</sup> at 6 L·s<sup>-1</sup> 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<sup>-1</sup> 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  $DL_{CO}$  can be due to instrument choice [89]. We assume that the same variation exists for  $DL_{NO}$ . 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  $DLCO$  varies by  $\geq 5.0 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  or  $DLNO$  varies by  $\geq 20 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ , from the mean of previously obtained values (table 3). A biological control whose  $DLNO$  and  $DLCO$  values measured week to week on the same pulmonary function system should be within 20 and  $5 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ , 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  $DLNO$  and  $DLCO$  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<sub>4</sub>, CO and NO, by using serial dilutions of known test gas concentrations. Most importantly, laboratory staff should review the  $DLCO$  and  $DLNO$ , 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  $\sim 1$  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 ( $DLNO$ ) and carbon monoxide ( $DLCO$ ) (5 s breath hold) at rest

	Test-to-test measurement error (within the same testing session)	Repeatability <sup>#</sup> (within the same testing session)	Reproducibility <sup>¶</sup> (week-to-week or month-to-month change) More stringent	Smallest measurable change* (week-to-week or month-to-month change) Less stringent
<b><math>DLNO</math></b>				
$\text{mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$	6.2 (4)	17 (10)	20 (13)	10 (7)
$\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$	2.1 (4)	5.8 (10)	6.5 (13)	3.3 (7)
<b><math>DLCO</math></b>				
$\text{mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$	1.2 (4)	3.2 (10)	4.9 (16)	2.5 (8)
$\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$	0.4 (4)	1.1 (10)	1.6 (16)	0.8 (8)
<b><math>DLNO/DLCO</math> ratio</b>	0.12 (2)	0.36 (7)	0.23 (5)	0.13 (3)
<b><math>DMCO</math></b>				
$\text{mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$	12 (7)	34 (19)	47 (28)	24 (28)
$\text{mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$	4.1 (7)	11.2 (19)	15.8 (28)	8 (14)
<b><math>V_C</math> mL</b>	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 ( $DMCO$ ), nitric oxide ( $DMNO$ ) 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  $DLCO$  repeatability and reproducibility in subjects with pulmonary pathophysiology (mean  $DLCO$  11–18  $\text{mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ) are 2.7 and  $4 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ , 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. <sup>#</sup>: the difference between two trials for  $DLNO$ ,  $DLCO$ ,  $DMCO$  and  $V_C$  measured on the same subject in the same testing session is expected to be  $<17$ ,  $<3.2$  and  $<34 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  and  $<10$  mL, respectively, for 95% of observations; <sup>¶</sup>: the difference in  $DLNO$ ,  $DLCO$ ,  $DMCO$  and  $V_C$  measured on the same subject over two different weeks is expected to be less than 20, 4.9, and  $47 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  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 VA calculation. The absolute value for  $DLCO$  must be  $<0.5 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  ( $<0.167 \text{ mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$ ) and for  $DLNO$   $<3 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  ( $<1 \text{ mmol}\cdot\text{min}^{-1}\cdot\text{kPa}^{-1}$ ). Manufacturers should provide this test option, which is the same as the usual testing procedure for a patient, with the exception that VA 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  $DLCO$  and  $DLNO$ , as is described in detail elsewhere [91].

### **Testing technique**

#### ***Subject preparation***

Since a  $DLCO$  measurement is often performed in conjunction with a  $DLNO$  test, the carboxyhaemoglobin (COHb) concentration should be minimised, as COHb reduces  $DLCO$ . 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  $DLCO$  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  $DLNO$  [93, 94] and  $DLCO$  [93, 95, 96] remain impaired for several hours after strenuous exercise. Thus, while  $DLNO$  and  $DLCO$  increase during exercise, and the increase parallels exercise intensity (*i.e.* cardiac output), both  $DLNO$  and  $DLCO$  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  $\leq 12$  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  $\beta$ -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  $DLNO$  be performed simultaneously with  $DLCO$ , the current  $DLCO$  guidelines should be followed [84]. Following a period of quiet tidal breathing to stabilise respiratory pattern, the single-breath technique for  $DLNO$ – $DLCO$  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  $\geq 90\%$  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  $DLCO$  by  $\sim 3\%$  ( $1 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ) [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  $\geq 4$ –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  $DLCO$  due to diurnal fluctuations in Hb and COHb [100, 101]. The  $DLCO$

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  $DLNO$  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  $DLCO$ , 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  $DLCO$  increases by ~1.5% for every 1% decrease in inspired oxygen concentration [16, 102, 103], the  $DLNO/DLCO$  ratio should decrease by ~3% when the inspired oxygen concentration is lowered from 21% to 19% (due to the increase in  $DLCO$  only). In fact, studies show that for every 1% decrease in inspired oxygen concentration, the measured  $DLNO/DLCO$  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  $DMCO$  and  $VC$  are to be calculated from the one-step  $NO-CO$  technique (supplementary appendix E), the expired “alveolar” oxygen concentration should be measured so that  $\theta CO$  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  $DLNO/V_A$  (equivalent to  $KNO$ ), and standard temperature and pressure, dry (760 mmHg, 0°C, 0% humidity) conditions for the calculation of  $DLNO$  (equals  $KNO \times V_A$ ). Manufacturers should specify these conversion factors in the software.

### Calculations for $DLNO$ , $DLCO$ and $V_A$

The derivation and calculation of  $DLNO$  and  $DLCO$  are identical except for the difference in gas species. The formulation (supplementary appendix B) given for  $DLNO$  stems from a recent review [104] and emphasises an important concept, that the  $DLNO$  (and  $DLCO$ ) are each the product of two components, the rate of change of alveolar concentration ( $kNO$  and  $kCO$ ) 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  $DLCO$  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  $DLNO$  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).

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  $DLNO$  is uniformly distributed. In a theoretical study of a two-compartment lung, PIPPER and SIKAND [51] showed that uneven distribution of inspired volume and  $DLCO/VA$  (equivalent to  $KCO$ ) always lead to an underestimation of  $DLCO$  (and, by extension  $DLNO$ ) compared to the homogeneous situation.

DRESSEL *et al.* [54] systematically studied the dependence of  $DLNO$ ,  $DLCO$  and their components  $VA$ ,  $KNO$  and  $KCO$  in normal subjects and patients with airflow obstruction due to cystic fibrosis. In normal subjects, the “accessible”  $VA$  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  $KNO$  and  $DLNO$  were ~14% less. The probable reason for the decrease in  $KNO$  and  $KCO$  with longer breath-hold times is that more weight is given, at longer breath-hold times, to more slowly filling and emptying units, whose  $DL/VA$  (equivalent to  $K$ ) is less than the faster units. In the normal subjects, there was a 9% decrease in  $DLCO$  from a 10 s breath-hold time compared to 4 s breath hold, so the  $DLNO/DLCO$  ratio was relatively unaffected. In airflow obstruction (cystic fibrosis),  $VA$  at 10 s exceeded  $VA$  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  $DLNO$  and 18% decrease in  $KNO$  were similar in normal subjects and those with cystic fibrosis. But since the  $DLCO$  and  $KCO$  were less affected over the same time periods, the  $DLNO/DLCO$  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  $VA$  occurs at 10 s *versus* 4 s breath hold; in contrast, heterogeneity increases  $KNO$  and  $KCO$  at 4 s *versus* 10 s by more than the change in  $VA$ , and this overcomes the smaller decrease in the 4-s  $VA$ . Thus, the net effects on  $DLNO$  and  $DLCO$  at 4 s *versus* 10 s breath-hold depend on the combination of opposing changes in  $VA$  and  $KNO$  and  $KCO$ , since  $DL=K \times VA$ .

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

## **Evaluating the measurement of $DLNO$**

### ***Repeatability, reproducibility and number of tests***

It is necessary to report the intra- and inter-session variability of  $DLCO$  and  $DLNO$  measurements so that a distinction can be made between normal biological variability/technical variability of the measurement and a clinically measurable 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  $DLNO$  and  $DLCO$  in absolute numbers [48, 49, 88]. An average value of two trials performed within 4–10 min of each other whose differences in  $DLNO$  and  $DLCO$  is within 17 and 3 mL·min<sup>-1</sup>·mmHg<sup>-1</sup>, respectively, is acceptable in healthy subjects and those with pulmonary pathophysiology. The reproducibility in  $DLCO$  and  $DLNO$  that occurs week to week or month to month is 5 and 20 mL·min<sup>-1</sup>·mmHg<sup>-1</sup>, 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  $DLCO$  and  $DLNO$  that occurs week to week or month to month is not a real

change, look at the “smallest measurable 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  $DLNO$  ( $20 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ) and the repeatability value for  $DLNO$  ( $17.2 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ). There is a 35% difference between the reproducibility value for  $DLCO$  ( $4.9 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ) and the repeatability value for  $DLCO$  ( $3.2 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ). However, the percentage difference is increased to 34% for  $DLCO$  (table 3). This suggests that  $DLNO$  is a more stable measure over months compared to  $DLCO$  and that the majority of the variability in  $DLNO$  is within-session and not between sessions [88].

Repeated tests do not affect  $DLNO$  within a given session, irrespective of COHb concentration [48, 49]. Even after 22 consecutive  $DLNO$  measurements,  $DLNO$  is unaffected, and the rise in methaemoglobin is minimal [48]. Since the largest slopes of the decrease in  $DLCO$  observed with rising COHb was  $\sim 0.4\text{--}0.5 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$  decrease in  $DLCO$  per 1% increase in COHb (males and females combined) [48], the minimum number of repeated tests that would elicit a decrease in  $DLCO$  larger than its repeatability (*i.e.*  $\geq 3.2 \text{ mL}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1}$ ) 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 $DMCO$ and $Vc$

Using the simultaneous one-step NO–CO technique, measurements are made at a single alveolar  $PO_2$  level. Values for  $\theta NO$  and  $\theta CO$  are required in the calculations (table 4 and supplementary appendix E). The literature in relation to published values for  $\theta NO$  and  $\theta CO$  is reviewed in the earlier section Origins of  $DLNO$ . There is general consensus for using a finite  $\theta NO$  of  $4.5 \text{ mL NO}\cdot(\text{mL blood}\cdot\text{min}^{-1}\cdot\text{mmHg}^{-1})$  from CARLSEN and COMROE [29], which for clinical purposes is independent of alveolar  $PO_2$  or Hb concentration. Conversely, the whole-blood transfer conductance for carbon monoxide is dependent on mean capillary  $PO_2$  (approximately alveolar  $PO_2$ ) and Hb concentration (reflected in the haematocrit). Many equations for the  $1/\theta CO$  relationship exist (*i.e.* table 5). We selected the  $1/\theta CO$  from GUENARD *et al.* [16] (equation 2 and table 5) as the most representative. Negative values for  $DMCO$  cannot occur unless the  $DLNO/DLCO$  ratio is  $>7.5$ , which is very unlikely, since normal values for  $DLNO/DLCO$  range from 3.8 to 5.8 (table 1).

In the literature, several versions of the  $1/\theta CO\text{--}PO_2$  relationship (table 5) have been used in the calculation of  $DMCO$  and  $Vc$ . The ROUGHTON and FORSTER formula [11] yielded strong correlations between  $DLNO$  (as a surrogate for  $DMNO$ ) and  $DMCO$  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  $DMCO$  values have been observed with its use [19]; thus, some [19, 25] favoured the formula given by REEVES and PARK [26] and “best fit”  $\alpha$ -ratios (all  $>2.0$ ) for getting the best agreement for  $DMCO$  between

TABLE 4 Summary consensus statement for simultaneous single-breath measurement of diffusing capacities of the lung for nitric oxide ( $DLNO$ ) and carbon monoxide ( $DLCO$ ) in healthy adults

Issue	Agreement
<b>Breath-hold time</b>	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
<b>Measured inspired NO concentration</b>	40–60 ppm, placed in the inspiratory bag $\leq 2$ min before use
<b>Measured inspired <math>O_2</math> concentration</b>	Close to 21%
<b>Measured expired <math>O_2</math> concentration</b>	Used to calculate $PAO_2$ and $\theta CO$
<b><math>1/\theta CO</math> [16]</b>	$(0.0062\cdot PAO_2 + 1.16)\cdot(\text{ideal Hb} \div \text{measured Hb})$
<b><math>\theta NO</math> [14, 29]</b>	$4.5 \text{ mL NO}\cdot(\text{mL blood}\cdot\text{min}^{-1}\cdot\text{mmHg})^{-1}$ $[1/\theta NO = 0.222]^\#$
<b><math>\theta NO/\theta CO</math> ratio</b>	Average 8.01 (male Hb $14.6 \text{ g}\cdot\text{dL}^{-1}$ ), 8.73 (female Hb $13.4 \text{ g}\cdot\text{dL}^{-1}$ ) at $PAO_2$ of $100 \text{ mmHg}^\#$
<b><math>DMCO</math></b>	$DMNO$ divided by 1.97 <sup>#</sup>
<b>Presentation of results</b>	Report $DLNO$ , $DLCO$ , $KNO$ and $KCO$ 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;  $\theta CO$  (NO): specific conductance in the blood for carbon monoxide (NO) in  $\text{mL}\cdot(\text{mL blood}\cdot\text{min}^{-1}\cdot\text{mmHg})^{-1}$ ;  $DMCO$ : alveolar–capillary membrane diffusing capacity for CO;  $PO_2$ : oxygen tension;  $PAO_2$ : alveolar oxygen tension; Hb: haemoglobin;  $DMNO$ : alveolar–capillary membrane diffusing capacity for NO;  $KNO$ : rate of change of NO from alveolar gas;  $KCO$ : 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. <sup>#</sup>: 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·dL <sup>-1</sup> )	Ideal Hb (13.4 g·dL <sup>-1</sup> )	Ideal Hb (14.6 g·dL <sup>-1</sup> )	Ideal Hb (13.4 g·dL <sup>-1</sup> )
<b>Derived <i>in vivo</i></b>					
GUÉNARD <i>et al.</i> [16]	$(0.0062 \cdot P_{AO_2} + 1.16) \cdot (\text{ideal Hb} \div \text{measured Hb})$	1.780	1.939	8.010	8.727
<b>Derived <i>in vitro</i></b>					
FORSTER [4] α=∞, pH=7.4	$(0.0041 \cdot P_{AO_2} + 1.3) \cdot (\text{ideal Hb} \div \text{measured Hb})$	1.710	1.863	7.695	8.384
ROUGHTON and FORSTER [11] α=1.5, pH=8.0	$(0.0058 \cdot P_{AO_2} + 1.0) \cdot (\text{ideal Hb} \div \text{measured Hb})$	1.580	1.721	7.110	7.747
HOLLAND [28] α=1.5	$(0.0065 \cdot P_{AO_2} + 1.08) \cdot (\text{ideal Hb} \div \text{measured Hb})$	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 ( $\theta_{NO}$ ) 4.5 mL NO · (mL blood · min · mmHg)<sup>-1</sup> 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  $DLNO$  and  $DLCO$  at two  $P_{AO_2}$  levels while maintaining θNO at 4.5 mL NO · (mL blood · min<sup>-1</sup> · mmHg<sup>-1</sup>). 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 GUÉNARD *et al.* 1/θCO formula (also in table 5) yields an average  $VC$  of 7% (5 mL) greater (95% limits of agreement –5 –11 mL) and an average  $DMCO$  ~6% lower (95% limits of agreement –23–7 mL · min · mmHg<sup>-1</sup>). 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  $DLNO$  over the range of haematocrits encountered clinically [31]. However, for  $DLCO$ , adjustments should be made for COHb levels >2%, and for Hb levels that differ from the standard Hb concentration (14.6 g·dL<sup>-1</sup> for adult males and 13.4 g·dL<sup>-1</sup> for adult females) [84].

### Prediction equations

#### Methods

Currently, there are several prediction equations for single-breath  $DLNO$  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<sup>-1</sup> · mmHg<sup>-1</sup> absolute change in  $DLCO$  between 5- and 10-s breath-hold times in healthy subjects at rest in those with low  $DLCO$  values, and a ~3 mL · min<sup>-1</sup> · mmHg<sup>-1</sup> difference in those with high  $DLCO$  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  $DMCO$  and  $VC$  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:  $DLCO$  and  $DLNO$ ,  $DMCO$  and  $DMNO$ ,  $VC$ ,  $DLNO/DLCO$  ratio,  $DMCO/VA$ ,  $VA$ ,  $VC/VA$ ,  $DMCO/VC$ ,  $KCO$  and  $KNO$ . The independent variables entered into the model were age (years), age<sup>2</sup>, 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  $\geq 3.0$  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<sup>2</sup>, weight and height. To examine multicollinearity, the variance inflation factor (VIF) was used to see whether there was a strong association between DLCO or DLNO 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  $\pm 1.96$ ). The association between DLNO and DLCO, and their relationship to VA 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·m<sup>-2</sup> 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 DLNO and DLCO z-scores for the 490 subjects were both  $0.0 \pm 1.0$  with a skewness of 0.17 (DLNO) and 0.23 (DLCO). Mean  $\pm$ SD breath-hold time was  $6.5 \pm 1.9$  s

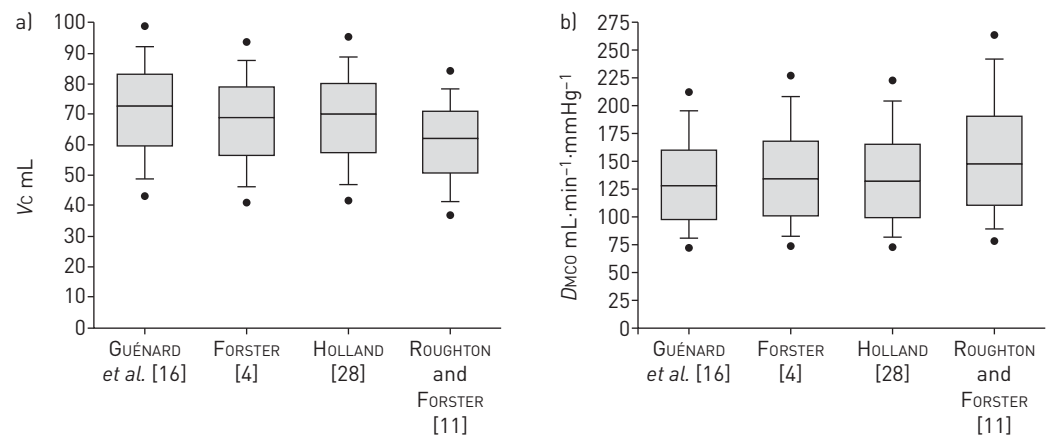


FIGURE 4 a) Pulmonary capillary blood volume ( $V_c$ ) and b) alveolar–capillary membrane diffusing capacity for carbon monoxide [ $DMCO$ ], measured using four different formulas for specific conductance in the blood for carbon monoxide ( $\theta_{CO}$ ). Based on the subject data from table 1,  $DMCO$  and  $V_c$  were calculated from the four formulas/constants listed in table 5. The mean values for both  $V_c$  and  $DMCO$  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  $DMCO$ , while the formula from ROUGHTON and FORSTER [11] provided the highest  $DMCO$  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 $\pm$ SD inspired oxygen concentration and inspired NO concentration measured in the inspiratory bag was 19.5 $\pm$ 0.7%, (range 18.1–20.5%) and 35 $\pm$ 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 ( $\leq$ 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.  $\sim$ 33% of the subjects were classified as overweight (BMI 25.0–29.9 kg·m<sup>-2</sup>). 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<sup>2</sup> and sex explained 45%, 13% and 11% of the model, respectively. For *DMCO* and *DMNO*, sex and age<sup>2</sup> explained 41% and 19% of the model, respectively. For *VC*, height and age<sup>2</sup> 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<sup>2</sup>, sex and height explained 22%, 12% and 8% of the model, respectively. For *KCO* and *KNO*, age<sup>2</sup> 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 $\pm$ SD 4.79 $\pm$ 0.40. For *VC/VA* ratio, age<sup>2</sup> 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·min<sup>-1</sup> increase in cardiac output. Regression equation: *DLNO* to *DLCO* ratio  $-0.061 \cdot (\text{cardiac output in L} \cdot \text{min}^{-1}) + 4.71$ ,  $R^2=0.16$ , 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  $1/\theta\text{CO}$  formula derived from *in vivo* data by GUÉNARD *et al.* [16]. There are several other formulas for  $1/\theta\text{CO}$  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  $1/\theta\text{CO}$  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·min<sup>-1</sup>·mmHg<sup>-1</sup> or 15%,  $p<0.01$ ; figure 4). In contrast, the lowest overall *VC* and highest overall *DMCO* was found with the  $1/\theta\text{CO}$  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  $DMCO$ ,  $DMNO/DMCO$  ratio and  $VC$  from simultaneously measured  $DLNO$  and  $DLCO$  remain controversial. Considerable research supports CARLSEN and COMROE's [29] data that  $\theta_{NO}$  is finite at 4.5 mL NO·(mL blood·min·mmHg)<sup>-1</sup>. GUÉNARD *et al.*'s [16]  $1/\theta_{CO}$  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  $\theta_{NO}$  and  $\theta_{CO}$  using innovative techniques would be welcome. Little is known about physiological variation in  $\theta_{NO}$  or  $\theta_{CO}$  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 ( $DMNO/DMCO$ ) 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  $DLNO$  and  $DLCO$ , and hence  $DLNO/DLCO$  and  $DMCO/VC$  needs to be examined. Further studies are needed to define the relative response between  $DLNO$  and  $DLCO$  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  $DLNO$  and  $DLNO/DLCO$  are lacking in non-Caucasian populations, and in relation to age. The relative impairment of  $DLCO$  and  $DLNO$  in disorders of the thorax, airway, parenchyma, vasculature and secondary to cardiac failure needs to be assessed. Whether the combination of  $DLNO$ ,  $DLCO$ ,  $DLNO/DLCO$  ratio,  $DMCO$  and  $VC$  will improve the management of cardiopulmonary diseases compared to the conventional use of  $DLCO$  remains to be determined.

### Summary and conclusions

- 1) Recommendations for the standard single-breath  $DLCO$  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  $DLNO$ – $DLCO$  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<sub>2</sub> should be as follows: NO 40–60 ppm, CO 0.3% and O<sub>2</sub> close to 21%. NO should be injected into the inspired bag  $\leq 2$  min before use, and the inspired concentrations of all these gases plus the inert tracer gas (He, CH<sub>4</sub> and neon) must be recorded. After 120 s of non-use, the NO concentration will be reduced by  $\sim 2.5$  ppm due to its conversion to NO<sub>2</sub> [85].
- 5) Expired concentration of O<sub>2</sub>: the exhaled “alveolar” O<sub>2</sub> concentration should be measured so that  $1/\theta_{CO}$  can be estimated from the measurement.
- 6) Presentation of results: the  $DLNO$  and  $DLCO$  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  $KNO$  and  $KCO$ . Alveolar volume should be recorded in L BTPS and as TLC % pred. The  $DLNO/DLCO$  ratio is a useful parameter, because it does not require choosing a physical constant ( $\theta$  or  $\alpha$ ) in its calculation, and is relatively independent of breath-hold time, age, height and sex.
- 7) The calculations for  $DMCO$  and  $VC$  are provided in supplementary appendix E with a sample algorithm provided in appendix H.

### References

- 1 Borland C, Chamberlain A, Higenbottam T. The fate of inhaled nitric oxide. *Clin Sci* 1983; 65: 37P.
- 2 Borland C, Cracknell N, Higenbottam T. Is the measurement of “DLNO” a true measure of membrane diffusing capacity? *Clin Sci* 1984; 67: 41P.
- 3 Borland CDR. Nitric Oxide and Carbon Monoxide in Cigarette Smoke in the Development of Cardiorespiratory Disease in Smokers. Cambridge, University of Cambridge, 1988.
- 4 Forster RE. Diffusion of gases across the alveolar membrane. In: Fishman AP, Farhi LE, Tenney SM, eds. Handbook of Physiology, Section 3, The Respiratory System IV, Gas Exchange. Washington, DC, American Physiological Society, 1987; pp. 71–88.
- 5 Clutton-Brock J. Two cases of poisoning by contamination of nitrous oxide with higher oxides of nitrogen during anaesthesia. *Br J Anaesth* 1967; 39: 388–392.
- 6 Roe FJ. Certain aspects of the responses of laboratory rats to exposure to (a) nitrogen dioxide and (b) tobacco smoke. *Tokai J Exp Clin Med* 1985; 10: 363–369.

- 7 Clough PN, Thrush BA. Mechanism of chemiluminescent reaction between nitric oxide and ozone. *Trans Faraday Soc* 1967; 63: 915–925.
- 8 Borland CDR, Chamberlain AT, Higenbottam TW, *et al.* Comparison between the rate of reaction of nitric oxide in the gas phase and in whole cigarette smoke. *Beitr Tabakforsch* 1985; 13: 67–73.
- 9 Borland C, Higenbottam T. Do the oxides of nitrogen in cigarette smoke cause emphysema in smokers? *Tokai J Exp Clin Med* 1985; 10: 371–373.
- 10 Borland CD, Higenbottam TW. A simultaneous single breath measurement of pulmonary diffusing capacity with nitric oxide and carbon monoxide. *Eur Respir J* 1989; 2: 56–63.
- 11 Roughton FJ, Forster RE. Relative importance of diffusion and chemical reaction rates in determining rate of exchange of gases in the human lung, with special reference to true diffusing capacity of pulmonary membrane and volume of blood in the lung capillaries. *J Appl Physiol* 1957; 11: 290–302.
- 12 Guénard H, Varene N, Vaida P. Determination of lung capillary blood volume and membrane diffusing capacity in man by the measurements of NO and CO transfer. *Respir Physiol* 1987; 70: 113–120.
- 13 Borland CD, Dunningham H, Bottrill F, *et al.* Significant blood resistance to nitric oxide transfer in the lung. *J Appl Physiol* 2010; 108: 1052–1060.
- 14 Borland C, Bottrill F, Jones A, *et al.* The significant blood resistance to lung nitric oxide transfer lies within the red cell. *J Appl Physiol* 2014; 116: 32–41.
- 15 Borland CD, Cox Y. Effect of varying alveolar oxygen partial pressure on diffusing capacity for nitric oxide and carbon monoxide, membrane diffusing capacity and lung capillary blood volume. *Clin Sci* 1991; 81: 759–765.
- 16 Guénard HJ, Martinot JB, Martin S, *et al.* *In vivo* estimates of NO and CO conductance for haemoglobin and for lung transfer in humans. *Respir Physiol Neurobiol* 2016; 228: 1–8.
- 17 van der Lee I, Zanen P, Biesma DH, *et al.* The effect of red cell transfusion on nitric oxide diffusing capacity. *Respiration* 2005; 72: 512–516.
- 18 Taylor BJ, Coffman KE, Summerfield DT, *et al.* Pulmonary capillary reserve and exercise capacity at high altitude in healthy humans. *Eur J Appl Physiol* 2016; 116: 427–437.
- 19 Coffman KE, Taylor BJ, Carlson AR, *et al.* Optimizing the calculation of  $DM_{CO}$  and  $V_C$  via the single breath single oxygen tension  $DL_{CO}/NO$  method. *Respir Physiol Neurobiol* 2016; 221: 19–29.
- 20 Martinot JB, Guénard H, Dinh-Xuan AT, *et al.* Nitrogen monoxide and carbon monoxide transfer interpretation: state of the art. *Clin Physiol Funct Imaging* 2015 [In press DOI: 10.1111/cpf.12316].
- 21 Hughes JM, van der Lee I. The  $TL_{NO}/TL_{CO}$  ratio in pulmonary function test interpretation. *Eur Respir J* 2013; 41: 453–461.
- 22 Gibson QH, Roughton FJ. The kinetics and equilibria of the reactions of nitric oxide with sheep haemoglobin. *J Physiol* 1957; 136: 507–524.
- 23 Vaughn MW, Huang KT, Kuo L, *et al.* Erythrocytes possess an intrinsic barrier to nitric oxide consumption. *J Biol Chem* 2000; 275: 2342–2348.
- 24 Azarov I, Liu C, Reynolds H, *et al.* Mechanisms of slower nitric oxide uptake by red blood cells and other hemoglobin-containing vesicles. *J Biol Chem* 2011; 286: 33567–33579.
- 25 Ceridon ML, Beck KC, Olson TP, *et al.* Calculating alveolar capillary conductance and pulmonary capillary blood volume: comparing the multiple- and single-inspired oxygen tension methods. *J Appl Physiol* 2010; 109: 643–653.
- 26 Reeves RB, Park HK. CO uptake kinetics of red cells and CO diffusing capacity. *Respir Physiol* 1992; 88: 1–21.
- 27 Hughes JM, Bates DV. Historical review: the carbon monoxide diffusing capacity ( $DL_{CO}$ ) and its membrane ( $DM$ ) and red cell ( $\theta \cdot V_C$ ) components. *Respir Physiol Neurobiol* 2003; 138: 115–142.
- 28 Holland RA. Rate at which CO replaces  $O_2$  from  $O_2Hb$  in red cells of different species. *Respir Physiol* 1969; 7: 43–63.
- 29 Carlsen E, Comroe JH Jr. The rate of uptake of carbon monoxide and of nitric oxide by normal human erythrocytes and experimentally produced spherocytes. *J Gen Physiol* 1958; 42: 83–107.
- 30 Borland C, Dunningham H, Bottrill F, *et al.* Can a membrane oxygenator be a model for lung NO and CO transfer? *J Appl Physiol* 2006; 100: 1527–1538.
- 31 Zavorsky GS. No red cell resistance to NO? I think not! *J Appl Physiol* 2010; 108: 1027–1029.
- 32 Hsia CC, Chuong CJ, Johnson RL Jr. Critique of conceptual basis of diffusing capacity estimates: a finite element analysis. *J Appl Physiol* 1995; 79: 1039–1047.
- 33 Hsia CC, Johnson RL Jr, Shah D. Red cell distribution and the recruitment of pulmonary diffusing capacity. *J Appl Physiol* 1999; 86: 1460–1467.
- 34 Wilhelm E, Battino R, Wilcock RJ. Low-pressure solubility of gases in liquid water. *Chem Rev* 1977; 77: 219–262.
- 35 Phansalkar AR, Hanson CM, Shakir AR, *et al.* Nitric oxide diffusing capacity and alveolar microvascular recruitment in sarcoidosis. *Am J Respir Crit Care Med* 2004; 169: 1034–1040.
- 36 Tamhane RM, Johnson RL Jr, Hsia CC. Pulmonary membrane diffusing capacity and capillary blood volume measured during exercise from nitric oxide uptake. *Chest* 2001; 120: 1850–1856.
- 37 Hsia CC, Yan X, Dane DM, *et al.* Density-dependent reduction of nitric oxide diffusing capacity after pneumonectomy. *J Appl Physiol* 2003; 94: 1926–1932.
- 38 Paiva M, Engel LA. Pulmonary interdependence of gas transport. *J Appl Physiol Respir Environ Exerc Physiol* 1979; 47: 296–305.
- 39 Linnarsson D, Hemmingsson TE, Frostell C, *et al.* Lung diffusing capacity for nitric oxide at lowered and raised ambient pressures. *Respir Physiol Neurobiol* 2013; 189: 552–557.
- 40 Zavorsky GS, Kim DJ, McGregor ER, *et al.* Pulmonary diffusing capacity for nitric oxide during exercise in morbid obesity. *Obesity* 2008; 16: 2431–2438.
- 41 Zavorsky GS, Quiron KB, Massarelli PS, *et al.* The relationship between single-breath diffusion capacity of the lung for nitric oxide and carbon monoxide during various exercise intensities. *Chest* 2004; 125: 1019–1027.
- 42 Zavorsky GS, Beck KC, Cass LM, *et al.* Dynamic vs. fixed bag filling: impact on cardiac output rebreathing protocol. *Respir Physiol Neurobiol* 2010; 171: 22–30.
- 43 Asadi AK, Sá RC, Kim NH, *et al.* Inhaled nitric oxide alters the distribution of blood flow in the healthy human lung, suggesting active hypoxic pulmonary vasoconstriction in normoxia. *J Appl Physiol* 2015; 118: 331–343.
- 44 Sheel AW, Edwards MR, Hunte GS, *et al.* Influence of inhaled nitric oxide on gas exchange during normoxic and hypoxic exercise in highly trained cyclists. *J Appl Physiol* 2001; 90: 926–932.



- 45 Borland C, Cox Y, Higenbottam T. Measurement of exhaled nitric oxide in man. *Thorax* 1993; 48: 1160–1162.
- 46 Alexanderson C, Olin AC, Dahlman-Höglund A, *et al.* Nasal nitric oxide in a random sample of adults and its relationship to sensitization, cat allergen, rhinitis, and ambient nitric oxide. *Am J Rhinol Allergy* 2012; 26: e99–e103.
- 47 Paredi P, Kharitonov SA, Meah S, *et al.* A novel approach to partition central and peripheral airway nitric oxide. *Chest* 2014; 145: 113–119.
- 48 Zavorsky GS. The rise in carboxyhemoglobin from repeated pulmonary diffusing capacity tests. *Respir Physiol Neurobiol* 2013; 186: 103–108.
- 49 Zavorsky GS, Murias JM. A small amount of inhaled nitric oxide does not increase lung diffusing capacity. *Eur Respir J* 2006; 27: 1251–1257.
- 50 Tsoukias NM, Dabdub D, Wilson AF, *et al.* Effect of alveolar volume and sequential filling on the diffusing capacity of the lungs: II. Experiment. *Respir Physiol* 2000; 120: 251–271.
- 51 Piiper J, Sikand RS. Determination of D-CO by the single breath method in inhomogeneous lungs: theory. *Respir Physiol* 1966; 1: 75–87.
- 52 Tsoukias NM, Wilson AF, George SC. Effect of alveolar volume and sequential filling on the diffusing capacity of the lungs: I. Theory. *Respir Physiol* 2000; 120: 231–249.
- 53 Moinard J, Guenard H. Determination of lung capillary blood volume and membrane diffusing capacity in patients with COPD using the NO-CO method. *Eur Respir J* 1990; 3: 318–322.
- 54 Dressel H, Filser L, Fischer R, *et al.* Lung diffusing capacity for nitric oxide and carbon monoxide: dependence on breath-hold time. *Chest* 2008; 133: 1149–1154.
- 55 Cotton DJ, Newth CJ, Portner PM, *et al.* Measurement of single-breath CO diffusing capacity by continuous rapid CO analysis in man. *J Appl Physiol Respir Environ Exerc Physiol* 1979; 46: 1149–1156.
- 56 Roberts CM, MacRae KD, Seed WA. Multi-breath and single breath helium dilution lung volumes as a test of airway obstruction. *Eur Respir J* 1990; 3: 515–520.
- 57 van der Lee I, Zanen P, Stigter N, *et al.* Diffusing capacity for nitric oxide: reference values and dependence on alveolar volume. *Respir Med* 2007; 101: 1579–1584.
- 58 Hsia CC. Recruitment of lung diffusing capacity: update of concept and application. *Chest* 2002; 122: 1774–1783.
- 59 Glénet SN, De Bisschop C, Vargas F, *et al.* Deciphering the nitric oxide to carbon monoxide lung transfer ratio: physiological implications. *J Physiol* 2007; 582: 767–775.
- 60 Verbanck S, Kerckx Y, Schuermans D, *et al.* The effect of posture-induced changes in peripheral nitric oxide uptake on exhaled nitric oxide. *J Appl Physiol* 2009; 106: 1494–1498.
- 61 Rohdin M, Petersson J, Sundblad P, *et al.* Effects of gravity on lung diffusing capacity and cardiac output in prone and supine humans. *J Appl Physiol* 2003; 95: 3–10.
- 62 Borland C, Mist B, Zammit M, *et al.* Steady-state measurement of NO and CO lung diffusing capacity on moderate exercise in men. *J Appl Physiol* 2001; 90: 538–544.
- 63 Martinot JB, Mulé M, de Bisschop C, *et al.* Lung membrane conductance and capillary volume derived from the NO and CO transfer in high-altitude newcomers. *J Appl Physiol* 2013; 115: 157–166.
- 64 Groepenhoff H, Overbeek MJ, Mulé M, *et al.* Exercise pathophysiology in patients with chronic mountain sickness exercise in chronic mountain sickness. *Chest* 2012; 142: 877–884.
- 65 Faoro V, Huez S, Vanderpool R, *et al.* Pulmonary circulation and gas exchange at exercise in Sherpas at high altitude. *J Appl Physiol* 2014; 116: 919–926.
- 66 van Ooij PJ, van Hulst RA, Houtkooper A, *et al.* Nitric oxide and carbon monoxide diffusing capacity after a 1-h oxygen dive to 9 m of sea water. *Clin Physiol Funct Imaging* 2014; 34: 199–208.
- 67 Dujčić Z, Eterović D, Denoble P, *et al.* Lung diffusing capacity in a hyperbaric environment: assessment by a rebreathing technique. *Br J Ind Med* 1992; 49: 254–259.
- 68 Garbella E, Piarulli A, Fornai E, *et al.* Preliminary observations on the effect of hypoxic and hyperbaric stress on pulmonary gas exchange in breath-hold divers. *Diving Hyperb Med* 2011; 41: 97–100.
- 69 Shykoff BE. Pulmonary effects of submerged oxygen breathing: 4-, 6-, and 8-hour dives at 140 kPa. *Undersea Hyperb Med* 2005; 32: 351–361.
- 70 Suzuki S. Probable lung injury by long-term exposure to oxygen close to 50 kilopascals. *Undersea Hyperb Med* 1994; 21: 235–243.
- 71 Borland C, Cox Y, Higenbottam T. Reduction of pulmonary capillary blood volume in patients with severe unexplained pulmonary hypertension. *Thorax* 1996; 51: 855–856.
- 72 van der Lee I, Zanen P, Grutters JC, *et al.* Diffusing capacity for nitric oxide and carbon monoxide in patients with diffuse parenchymal lung disease and pulmonary arterial hypertension. *Chest* 2006; 129: 378–383.
- 73 Farha S, Laskowski D, George D, *et al.* Loss of alveolar membrane diffusing capacity and pulmonary capillary blood volume in pulmonary arterial hypertension. *Respir Res* 2013; 14: 6.
- 74 Degano B, Mittaine M, Guénard H, *et al.* Nitric oxide and carbon monoxide lung transfer in patients with advanced liver cirrhosis. *J Appl Physiol* 2009; 107: 139–143.
- 75 Magini A, Apostolo A, Salvioni E, *et al.* Alveolar-capillary membrane diffusion measurement by nitric oxide inhalation in heart failure. *Eur J Prev Cardiol* 2015; 22: 206–212.
- 76 Zavorsky GS, Borland C. Confusion in reporting pulmonary diffusing capacity for nitric oxide and the alveolar-capillary membrane conductance for nitric oxide. *Eur J Prev Cardiol* 2015; 22: 312–313.
- 77 van der Lee I, Gietema HA, Zanen P, *et al.* Nitric oxide diffusing capacity versus spirometry in the early diagnosis of emphysema in smokers. *Respir Med* 2009; 103: 1892–1897.
- 78 Moinard J, Guenard H. Membrane diffusion of the lungs in patients with chronic renal failure. *Eur Respir J* 1993; 6: 225–230.
- 79 Zavorsky GS, Kim DJ, Sylvestre JL, *et al.* Alveolar-membrane diffusing capacity improves in the morbidly obese after bariatric surgery. *Obes Surg* 2008; 18: 256–263.
- 80 Dressel H, Filser L, Fischer R, *et al.* Lung diffusing capacity for nitric oxide and carbon monoxide in relation to morphological changes as assessed by computed tomography in patients with cystic fibrosis. *BMC Pulm Med* 2009; 9: 30.
- 81 Barisione G, Bacigalupo A, Brusasco C, *et al.* Mechanisms for reduced pulmonary diffusing capacity in haematopoietic stem-cell transplantation recipients. *Respir Physiol Neurobiol* 2014; 194: 54–61.



- 82 Graham BL, Dosman JA, Cotton DJ. A theoretical analysis of the single breath diffusing capacity for carbon monoxide. *IEEE Trans Biomed Eng* 1980; 27: 221–227.
- 83 Cotes JE, Chinn DJ, Quanjer PH, *et al.* Standardization of the measurement of transfer factor (diffusing capacity). *Eur Respir J* 1993; 6: Suppl. 16, 41–52.
- 84 Graham BL, Brusasco V, Burgos F, *et al.* 2017 ERS/ATS standards for single breath carbon monoxide uptake in the lung. *Eur Respir J* 2017; 49: 1600016.
- 85 Fine DH. Critical evaluation of the Saltzman technique for NO<sub>x</sub> analysis in the 0–100 ppm range. *Environ Sci Technol* 1972; 6: 348–350.
- 86 Jones RS, Meade F. A theoretical and experimental analysis of anomalies in the estimation of pulmonary diffusing capacity by the single breath method. *Q J Exp Physiol Cogn Med Sci* 1961; 46: 131–143.
- 87 Thomas A, Hanel B, Marott JL, *et al.* The single-breath diffusing capacity of CO and NO in healthy children of European descent. *PLoS One* 2014; 9: e113177.
- 88 Murias JM, Zavorsky GS. Short-term variability of nitric oxide diffusing capacity and its components. *Respir Physiol Neurobiol* 2007; 157: 316–325.
- 89 Jensen RL, Teeter JG, England RD, *et al.* Sources of long-term variability in measurements of lung function: implications for interpretation and clinical trial design. *Chest* 2007; 132: 396–402.
- 90 McCormack MC. Facing the noise: addressing the endemic variability in DL<sub>CO</sub> testing. *Respir Care* 2012; 57: 17–23.
- 91 Miller MR, Crapo R, Hankinson J, *et al.* General considerations for lung function testing. *Eur Respir J* 2005; 26: 153–161.
- 92 Zavorsky GS, Smoliga JM, Longo LD, *et al.* Increased carbon monoxide clearance during exercise in humans. *Med Sci Sports Exerc* 2012; 44: 2118–2124.
- 93 Zavorsky GS, Lands LC. Lung diffusion capacity for nitric oxide and carbon monoxide is impaired similarly following short-term graded exercise. *Nitric Oxide* 2005; 12: 31–38.
- 94 Zavorsky GS, Milne EN, Lavorini F, *et al.* Small changes in lung function in runners with marathon-induced interstitial lung edema. *Physiol Rep* 2014; 2: e12056.
- 95 Sheel AW, Coutts KD, Potts JE, *et al.* The time course of pulmonary diffusing capacity for carbon monoxide following short duration high intensity exercise. *Respir Physiol* 1998; 111: 271–281.
- 96 Stewart IB, Potts JE, McKenzie DC, *et al.* Effect of body position on measurements of diffusion capacity after exercise. *Br J Sports Med* 2000; 34: 440–444.
- 97 Zavorsky GS, Milne EN, Lavorini F, *et al.* Interstitial lung edema triggered by marathon running. *Respir Physiol Neurobiol* 2014; 190: 137–141.
- 98 Normand H, Lavigne F, Mouadil A. Performing the apnea of the single-breath carbon monoxide diffusing capacity: relaxation on the shutter or full inspiration with near atmospheric intrapulmonary pressure? *Chest* 2006; 130: 207–213.
- 99 Macintyre N, Crapo RO, Viegi G, *et al.* Standardisation of the single-breath determination of carbon monoxide uptake in the lung. *Eur Respir J* 2005; 26: 720–735.
- 100 Cinkotai FF, Thomson ML. Diurnal variation in pulmonary diffusing capacity for carbon monoxide. *J Appl Physiol* 1966; 21: 539–542.
- 101 Frey TM, Crapo RO, Jensen RL, *et al.* Diurnal variation of the diffusing capacity of the lung: is it real? *Am Rev Respir Dis* 1987; 136: 1381–1384.
- 102 Borland C, Cox Y. NO and CO transfer. *Eur Respir J* 1991; 4: 766.
- 103 Crapo RO, Kanner RE, Jensen RL, *et al.* Variability of the single-breath carbon monoxide transfer factor as a function of inspired oxygen pressure. *Eur Respir J* 1988; 1: 573–574.
- 104 Hughes JM, Pride NB. Examination of the carbon monoxide diffusing capacity (DL<sub>CO</sub>) in relation to its K<sub>CO</sub> and V<sub>A</sub> components. *Am J Respir Crit Care Med* 2012; 186: 132–139.
- 105 Krogh M. The diffusion of gases through the lungs of man. *J Physiol* 1915; 49: 271–300.
- 106 Aguilaniu B, Maitre J, Glénet S, *et al.* European reference equations for CO and NO lung transfer. *Eur Respir J* 2008; 31: 1091–1097.
- 107 Zavorsky GS, Cao J, Murias JM. Reference values of pulmonary diffusing capacity for nitric oxide in an adult population. *Nitric Oxide* 2008; 18: 70–79.
- 108 Turcotte RA, Perrault H, Marcotte JE, *et al.* A test for the measurement of pulmonary diffusion capacity during high-intensity exercise. *J Sports Sci* 1992; 10: 229–235.
- 109 Chance WW, Rhee C, Yilmaz C, *et al.* Diminished alveolar microvascular reserves in type 2 diabetes reflect systemic microangiopathy. *Diabetes Care* 2008; 31: 1596–1601.
- 110 Rouatbi S, Ben Saad H, Latiri I, *et al.* North-African reference values of alveolar membrane diffusion capacity and pulmonary capillary blood volume. *Respiration* 2010; 80: 301–312.
- 111 Quanjer PH, Tammeling GJ, Cotes JE, *et al.* Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J* 1993; 16: Suppl. 5–40.
- 112 Zavorsky GS, Blood AB, Power GG, *et al.* CO and NO pulmonary diffusing capacity during pregnancy: safety and diagnostic potential. *Respir Physiol Neurobiol* 2010; 170: 215–225.
- 113 Punjabi NM, Shade D, Wise RA. Correction of single-breath helium lung volumes in patients with airflow obstruction. *Chest* 1998; 114: 907–918.
- 114 Robson AG, Innes JA. Short term variability of single breath carbon monoxide transfer factor. *Thorax* 2001; 56: 358–361.