Appendix A-G

A. Airway NO Retention

The original crude uptake experiments suggested that there was no uptake of NO until it reached the alveoli [1]. The NO diffusing capacity of the airway has now been estimated in healthy volunteers [2] as 6.8 $nL \cdot s^{-1}$ $(ppb \times 10^{-3})^{-1} = 0.006\ L \cdot s^{-1} \cdot atm^{-1}$. In a single breath manoeuvre, the total volume of NO retained in the lung = ViFi – FEVA = $10^{-6} \cdot (35 \times 4.95 - 2.2 \times 7.8) = 1.56 \times 10^{-4}$ L, where ViFi is inhaled volume multiplied by fractional inhaled NO concentration and FEVA is exhaled fractional NO concentration multiplied by alveolar volume (VA) using data from Borland [3]. By integration, volume retained in the airways = $Vair \cdot (Cair_0 - Cair_0 \cdot e^{-D/VP}) = 0.2 \cdot (0.00004 - 0.00004 \cdot e^{-0.00422}) = 3.37 \times 10^{-8}$ L, where Vair is airways volume = 0.2 L, D is NO diffusing capacity of the airway, $Cair_0$ is airway NO concentration at the start of the breath hold (40 ppm), and V is rate of inhalation = 1.5 $L \cdot s^{-1}$, and P is pressure in standard atmospheres (atm) = 1. So $D / V \cdot P = 0.00633 / (1.5 \cdot 1) = 0.00422$. Percent airway retention = $[(3.37 \times 10^{-8}) / (1.56 \times 10^{-4})] \cdot 100 = 0.02\%$. These calculations confirm that airway NO retention is negligible.

B. Derivation and calculation for *D*L,NO

The units of *D*L,NO are quantity (volume)·$time^{-1}$·$pressure^{-1}$ (SI: $mmol \cdot min^{-1} \cdot kPa^{-1}$, or traditional units: $mL \cdot min^{-1} \cdot mmHg^{-1}$]. The units and derivation for *D*L,CO are the same as for *D*L,NO . Thus,

$$DL,NO = KNO \cdot VA \qquad \textbf{Equation 1, Appendix B}$$

$$DL,NO/VA = KNO \qquad \textbf{Equation 2, Appendix B}$$

where KNO is the rate of change of NO concentration (*k*NO) per unit barometric minus water vapour pressure ($P_B - P_{H_2O}$), with units $min^{-1} \cdot mmHg^{-1}$, and *K*NO in the form of *D*L,NO/VA (See **Equation 2**, Appendix) is NO diffusing capacity per unit alveolar volume with units mL

(STPD)·min^{-1}·mmHg^{-1}·L^{-1} (BTPS). In spite of the difference in units and values *D*L,NO/VA and *K*NO are physiologically the same. *D*L,NO/VA (equivalent to *K*NO) is itself volume dependent, and more so than *D*L,CO/VA (**Figure 3**). It is incorrect and misleading to think of *DL,*NO/VA despite its name and its units, as the NO diffusing capacity "corrected" for alveolar volume, because it is no more than the rate of change of alveolar NO concentration (kCO) per unit barometric minus water vapour pressure ($P_B - P_{H_2O}$), and thus equivalent to KNO. In laboratory reports, KNO is preferred to *D*L,NO/VA.

As with *D*L,CO and *kCO*, derivation of *k*NO is calculated from the monoexponential decay of alveolar NO concentration:

$$kNO = \frac{\ln(NO_0/NO_t)}{BHT} \qquad \textbf{Equation 3, Appendix B}$$

where NO_0 and NO_t are the alveolar NO concentrations at the beginning and end of the breath hold, respectively, breath-hold time (BHT) is the "effective" breath hold time according to Jones and Meade [4], and *ln* is the natural logarithm. The initial alveolar NO concentration is calculated from the inspired NO concentration (NO_i) on the assumption that before NO uptake starts, NO will be diluted by the residual gas in the lung in the same proportion as the inert tracer gas (Tr), which mixes "instantaneously", so that Tr_t (at the end of the breath-hold) is the same as Tr_0 (at the beginning of the breath hold):

$$NO_0 = \frac{NO_i \,.\, Tr_t}{Tr_i} \qquad \textbf{Equation 4, Appendix B}$$

$$kNO/[P_B - P_{H_2O}] = KNO \qquad \textbf{Equation 5, Appendix B}$$

C. Calculating alveolar volume

From gas dilution principles:

$$VA = (V_{insp} - VD_{sum})\cdot(Tr_i \div Tr_t) \qquad \textbf{Equation 6, Appendix C}$$

where V_{insp} is the inspired volume (inspiratory vital capacity or IVC), VD_{sum} is the sum of the anatomic and instrumental dead spaces. Anatomic dead space (mL) is estimated from *2.2·(body mass in kg)* as per the 2017 Standardization document for *D*L,CO [5]. In more obese subjects (body mass index ≥ 30 kg/m^2), or if the weight of the subject is unknown, the anatomic dead space can be calculated by *height^2/189.4* [5]. The instrumental dead space is usually given by the manufacturers but should include the volume of any filters attached to the mouthpiece (estimated by water displacement). The shared instrumental dead space (for inspiration and expiration) should be flushed with room air to remove expiratory gas from the immediately preceding measurement. The calculation of alveolar volume is common to *D*L,NO and *D*L,CO. Thus, the *DL,NO*/*DL,CO* ratio = *K*NO/*K*CO.

D. Adjustment for CO_2, H_2O and temperature

These corrections are applied to the calculation of VA if CO_2 and water are absorbed before the inert gas tracer (Tr) is analyzed because the expired alveolar concentration of tracer gas (FAtr) will be falsely raised. In the calculation of the rate of alveolar NO uptake, $ln(NO_0/NO_t)/BHT$, NO_t (and CO_t) do not need "correction" since NO_0/NO_t is a ratio where the correction factors cancel out, i.e. a "corrected" expired tracer concentration Tr_t is used in the calculation of $NO_0 = [NO_i \cdot (Tr_t/Tr_i)]$ — see **Equation 4, Appendix B**.

For greater accuracy in the calculation of VA, the temperature at which exhaled and inhaled gasses are analyzed should be controlled or measured, and adjustments made [5]. Gasses that are measured with rapid response analyzers, measure the exhaled gas close to the mouth, at body temperature and pressure, saturated with water vapor (BTPS). In most systems, expired gas concentrations are measured in an expiratory reservoir (expiratory bag) where the gasses cool to around 30 to 33^{0}C. Manufacturers should specify the corrections made to the calculation algorithms.

E. Calculation of *D*M,CO and pulmonary capillary blood volume

The calculation is from reference [6]. Also, see on-line supplementary excel spreadsheet

$kNO = \log_e \cdot (NO_0/No_t)$ / BHT

$KNO = kNO / (P_B - P_{H_2O})$ where P_B is barometric pressure and P_{H_2O} is water vapor pressure at body temperature (37°C).

*D*L,NO (or *T*L,NO) = *K*NO·VA

(*D*L,CO is calculated in an identical way)

Defining α as *D*M,NO/*D*M,CO = 1.97

and psi (ψ) as θNO/θCO

Vc = [(1/θCO)·(1-α/ψ)] / (1/*D*L,CO- α/*D*L,NO)

*D*M,CO = (1/α-1/ψ) / (1/*D*L,NO-1/(ψ·*D*L,CO))

Using Guénard's value for 1/θCO as $(0.0062 \cdot P_AO_2 + 1.16) \cdot$(ideal Hb ÷ measured Hb) and θNO = 4.5 mL NO·(mL blood·min·mmHg)$^{-1}$,ψ = θNO/θCO = θNO/(a+b·PO_2) = 4.5 ÷ [1/(1.16 + 0.0062·100) = 8.01. Note that ψ is a function of alveolar PO_2 (P_AO_2) and only equals 8.01 at P_AO_2 of 100 mmHg and an ideal and measured Hb of 14.6 g·dL^{-1}.

F. Inspired gas preparation.

Gasses coming from two different cylinders are injected into one inspiratory bag just prior to use; the gas concentrations are measured directly from this bag before the patient inhales to total lung capacity. For example, one cylinder would have 80 ppm NO, balance N_2 from which the NO electrochemical cell is calibrated. From a second tank with a concentration of 1000 ppm NO, balance N_2, about 150 mL is injected into a 7 to 9 L inspiratory bag when the patient's vital capacity is 3 L, and about 350 mL when a patient's vital capacity is 7 L, resulting in about 50 ppm of NO in the inspiratory bag. Thus, the final desired concentration of NO in the inspiratory bag is determined by the concentration of NO/N_2 in the second tank and the patient's vital capacity. From there, the appropriate volume of NO/N_2 gas that is injected into the bag can be calculated. As an example, if a patient's vital capacity is 5 L,

then we would fill up the bag with 5.5 L of the total gas mixture (the extra 0.5 L is just to make sure the patient has enough gas mixture to inhale). About 5.23 L of *D*L,CO gas mixture [21% O_2, 0.3% CO, 10% He (or < 1% CH_4), Bal N_2] from a third tank would be injected into the inspiratory bag first, followed by 0.275 L of 1000 ppm NO, balance N_2 injected from the second tank. This would result in approximately 50 ppm NO in the inspiratory bag (0.275 ÷ 5.5 = 0.05, then 0.05 x 1000).

The final O_2 concentration in the inspiratory bag should be close to 21% but the dilution of NO/N_2 into the bag may render the inspired O_2 concentration closer to 20%. If using a discrete system, the inspired NO concentration should be checked after its injection into the inspiratory reservoir just prior to testing.

G. Repeatability, reproducibility and the smallest meaningful change.

The variability of the parameters *D*L,NO and *D*L,CO can be independent of the magnitude of the measurement [7], thus using a percentage value to describe intra-session variability may not be appropriate. Using a percentage may lead to underestimation of variability in low values and overestimation for high values. Others studies have also suggested using an absolute value rather than a percentage [8, 9], since the diffusing capacity was also independent of the magnitude of the measurement. As such, **Table 3** in the manuscript presents both acceptable intra and intersession variability values for the 5 s breath-hold manoeuvre for *D*L,NO and *D*L,CO in absolute numbers, but percentages are also provided for an easier interpretation of the variability. The measurement error (otherwise known as the typical error) is the square root of the mean square error obtained from a repeated measures analysis of variance from subjects performing five repeated tests over a single session performed over an hour or so [7]. The square root of the mean square error is the common within-subject standard deviation (SD_w). The repeatability is then reported as $2.77 \cdot SD_W$ [10]. That is, the difference between two measurements obtained within the same testing session done on the same day for the same subject is expected to be less than 2.77

times the within-subject standard deviation for 95% of pairs of observations [10]. Reproducibility was calculated the same way as the repeatability data: by obtaining the square root of the mean square error obtained from a repeated measures analysis of variance obtained from diffusing capacity tests that were performed over several weeks [11]. The square root of the mean square error is the common week-to-week within subject standard deviation (SD_w). Reproducibility was defined as $2.77 \cdot SD_w$ [10]. That is, the difference between the *D*L,NO or *D*L,CO value obtained on different weeks or days for the same subject is expected to be less than 2.77 times the within-subject standard deviation for 95% of pairs of observations [10].

As such, the difference between two trials for *D*L,NO, *D*L,CO, *D*M,CO, and Vc measured on the same subject in the same testing session is expected to be less than 17, 3.2, and 34 $mL \cdot min^{-1} \cdot mmHg^{-1}$ and 10 mL, respectively, for 95% of observations (repeatability, **Table 3**). The American Thoracic Society and European Respiratory Society suggests that the two highest acceptable GRADE A trials whose differences in *D*L,CO is within 2 $mL \cdot min^{-1} \cdot mmHg^{-1}$ is averaged and reported [5]. As such, it is great to strive for a repeatability of 2 $mL \cdot min^{-1} \cdot mmHg^{-1}$ for *D*L,CO, but it is acceptable if the repeatability is about 3 $mL \cdot min^{-1} \cdot mmHg^{-1}$ between two properly performed manoeuvres.

The difference in *D*L,NO, *D*L,CO, *D*M,CO, and Vc measured on the same subject over two different weeks is expected to be less than 20, 4.9, and 47 $mL \cdot min^{-1} \cdot mmHg^{-1}$ and 16 mL, respectively, 95% of the time (reproducibility, **Table 3**). Any diffusing capacity parameter that has a week-to-week change that is equal to or more than the reproducibility has only a 5% chance that it is not a real change.

The smallest meaningful change is half the reproducibility and thus less stringent than the reproducibility [12]. Any week-to-week change in any diffusing capacity parameter listed in **Table 3** that is equal to the smallest meaningful change has an approximate 20% chance that it is not a real change, and an approximate 80% chance that the change is real. It is up to the physician, researcher, or technologist to decide how stringent the week-to-

week or month-to-month changes in diffusing capacity (and components) need to be before it is considered a meaningful change. If one needs to be more stringent, with only a 5% chance that the differences between one month to the next is not real, then use the reproducibility column in **Table 3**, otherwise, use the smallest meaningful change column in **Table 3**.

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