Lung diffusing capacities for nitric oxide and carbon monoxide at rest and post-walking in long COVID

Giovanni Barisione and Vito Brusasco

Abstract

**Background** Approximately one-third of long coronavirus disease 2019 (long COVID) patients report breathlessness and fatigue even during activities of daily living. We hypothesised that abnormalities of combined diffusing capacity of the lung for nitric oxide ($D_{LNO}$) and carbon monoxide ($D_{LCO}$) at rest or after mild exercise are associated with breathlessness in patients with long COVID.

**Methods** Single-breath combined $D_{LNO}$ and $D_{LCO}$ were measured at rest and immediately after a short bout of treadmill exercise simulating ordinary walking in 32 Caucasian patients with long COVID and dyspnoea at rest. 20 subjects served as a control group.

**Results** At rest, combined $D_{LNO}$, $D_{LCO}$ and alveolar volume ($V_A$) were significantly lower in long COVID than in controls, with $D_{LNO}$ and $D_{LCO}$ being below the limits of normal in 69% and 41% of cases, respectively. Mean values of $D_{LNO}/V_A$ and $D_{LCO}/V_A$ in long COVID patients were less than controls, yet, in only 22% and 12% of long COVID patients were the values of $D_{LNO}/V_A$ and $D_{LCO}/V_A$ below the limits of normal. After treadmill exercise, $D_{LNO}$, $D_{LNO}/D_{LCO}$, $V_A$ and heart rate increased significantly without differences between groups. $D_{LNO}$ remained below the limit of normal in 47% of long COVID patients.

**Conclusion** These data suggest localised discrete loss of lung units in approximately half of long COVID patients, not completely explained by loss of $V_A$ or of alveolar-capillary recruitment during exercise.

Introduction

Although the novel coronavirus disease 2019 (COVID-19) is often associated with relatively self-limiting upper airway syndrome, a substantial proportion of patients may develop interstitial pneumonia, which may ultimately progress to a severe hypoxaemic respiratory failure [1]. Besides the clinical burden of acute disease, it has been recognised that ~30% of hospitalised patients and outpatients may experience various persisting symptoms, including breathlessness and poor exercise tolerance, for ≥3 months after recovery from the acute phase, a condition referred to as long COVID [1]. Exercise studies showed reduced aerobic capacity after COVID-19 variably explained by ventilatory inefficiency [2], inappropriate hyperventilation [3], chronotropic and/or inotropic incompetence [4], reduced oxygen extraction by peripheral muscles [5], loss of mechanical efficiency [6] and deconditioning [7]. Moreover, approximately one-third of patients with long COVID complain of breathlessness and fatigue even during activities of daily living [1]. Although a decreased diffusing capacity of the lung for carbon monoxide ($D_{LCO}$) has been found at various time intervals ranging from zero [8] to 6 months [9, 10] after hospital discharge, only one recent study reported decreased $D_{LCO}$ associated with fatigue and dyspnoea in highly symptomatic long COVID patients [11]. Whether abnormalities of $D_{LCO}$ are mechanistically involved in poor tolerance to ordinary physical activities in long COVID is unclear. In a previous study of patients recovering from the acute phase of COVID-19, the diffusing capacity of the lung for nitric oxide ($D_{LNO}$) was reduced more than $D_{LCO}$, which was interpreted as an impairment of alveolar membrane diffusive conductance (DM) with relatively preserved pulmonary capillary blood volume ($V_c$) [12].
Both $D_{LNO}$ and $D_{LCO}$ are expected to increase from rest to exercise because of alveolar and microvascular recruitment [13]. Thus, we hypothesised that abnormalities of $D_{LNO}$ and $D_{LCO}$ at rest or after exercise might be associated with breathlessness in patients with long COVID. To test this hypothesis, we measured combined $D_{LNO}$ and $D_{LCO}$ at rest and immediately after a short bout of mild treadmill exercise in patients with long COVID referred to our pulmonary function laboratory because of dyspnoea during activities of daily living.

Methods

Study subjects

32 Caucasian patients, three of whom had participated in a previous investigation [12], with a history of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, confirmed by nasopharyngeal swab with real-time PCR, were included in the study. They were referred to our pulmonary function laboratory, between 98 and 686 days after being tested negative for SARS-CoV-2, because of dyspnoea, fatigue and exercise intolerance persisting or occurring $\geq 3$ months after the COVID-19 acute phase and lasting $\geq 2$ months [14]. None of them had history of diseases potentially causing dyspnoea or affecting pulmonary gas transport, i.e. bronchial asthma, COPD, pulmonary interstitial fibrosis or vasculitis, haematological diseases, systemic collagen diseases, congestive heart failure and liver or renal diseases.

The group included six patients who had mild COVID-19 treated at home with antipyretics (paracetamol or ibuprofen) and 26 patients who had been hospitalised with moderate-to-severe COVID-19 pneumonia and arterial hypoxaemia treated with oxygen supplementation only (n=8), helmet continuous positive airway pressure support (n=10) or invasive mechanical ventilation via tracheal intubation (n=8). During hospitalisation, they had received corticosteroids (n=26), antibiotics (n=22), enoxaparin (n=21), oral hydroxychloroquine (n=4), tocilizumab or anakinra (n=4) and various antiviral drugs. As a control group, we selected 20 healthy volunteers among health professionals and their relatives without history of COVID-19 and vaccinated against SARS-CoV-2 infection who best matched our long COVID patients for anthropometric characteristics.

Standard lung function measurements at rest

The modified Medical Research Council (mMRC) dyspnoea scale was used to score breathlessness (from 0 to 4) before starting lung function measurements. Digital pulse oximetry (Oxy-3 Pulse Oximeter; GIMA, Gessate, Italy) was measured after a resting period of $>5$ min.

Lung volumes [15], spirometry [16] and standard single-breath $D_{LCO}$, with actual breath-hold time of 11±0.5 s [17], were sequentially measured with subjects sitting in a whole-body plethysmograph (Vyaire Vyntus Body; Vyaire Medical, Höchberg, Germany). Smokers were asked to refrain from smoking for 24 h prior to the study. Results were compared with the predicted values from HALL et al. [18] for lung volumes, QUANIER et al. [19] for spirometry and STANOJEVIC et al. [20] for $D_{LCO}$ after adjustment for effective haemoglobin concentration measured from available arterial or venous blood samples [21].

$D_{LNO}$-$D_{LCO}$ measurements at rest and post-walk

$\geq 5$–10 min after standard $D_{LCO}$, combined single-breath $D_{LNO}$ and $D_{LCO}$, with actual breath-hold time of 5.3±0.3 s, were measured simultaneously (MasterScreen PFT System; Jaeger, Vyaire Medical) twice at 5-min intervals at rest with subjects in a sitting posture and wearing a nose clip, as detailed elsewhere [22]. The values retained for analysis were the average of two repeatable measurements, i.e. within 17 and 3.2 mL·min$^{-1}$·mmHg$^{-1}$ for $D_{LNO}$ and $D_{LCO}$, respectively, obtained during the same testing session [23].

5 min later, subjects wearing a heart rate thoracic belt (Polar T31; Polar, Kempele, Finland) started walking on a treadmill (MTC climb e motion; Runner, Cavezzo, Italy) at a speed of 4 km·h$^{-1}$ with 5% incline, which were increased by 2 km·h$^{-1}$ and 2%, respectively, every minute until the achievement of a target exertional heart rate ($HR_{exer}$), calculated from maximal predicted heart rate ($HR_{max}$=208–0.7×age) [24] and resting heart rate ($HR_{rest}$) as follows [25]:

$$HR_{exer} = \frac{HR_{max} - HR_{rest}}{3} + HR_{rest}$$

Then, within 5–10 s of stopping exercise, combined single-breath $D_{LNO}$ and $D_{LCO}$, with actual 5.1±0.4 s breath-hold time, were measured once in a sitting position. Predicted values for combined $D_{LNO}$-$D_{LCO}$, $V_A$, $D_{LNO}/V_A$ and $D_{LCO}/V_A$ were from ZAVORSKY et al. [23]

Chest computed tomography

In 10 long COVID patients who had been hospitalised during the acute phase, a thin-section computed tomography (CT) scan obtained between $-10$ and 88 days after pulmonary function measurements were
available. Scans of the entire chest were obtained at 1.25-mm slice thickness while supine, during breath-holding at full inspiration, by a multi-detector row-spiral scanner (SOMATOM Emotion 6; Siemens AG Medical, Forchheim, Germany) [22]. Only scans with lung volume determined by CT $\geq 80\%$ of plethysmographic total lung capacity (TLC) (n=9) were retained for automatic quantitative three-dimensional analysis to obtain mean lung attenuation, coefficient of variation (itk-SNAP 3.8.0; itk-SNAP, Philadelphia, PA, US) [26], kurtosis and leftward skewness of density histograms (Horos OsiriX 3.3.6; Pixmeo, Geneva, Switzerland).

**Statistical analysis**

For each lung function measure, the percentage of predicted and z-score were calculated. As lower limits of normal (LLN) for combined $D_{LNO}$-$D_{LCO}$ measures, both the 5th (LLN$_{5}\%$ = 1.645 z-score) and the 2.5th (LLN$_{2.5}\%$ = 1.96 z-score) percentiles of the reference population were considered. Unpaired t-test and two-factor (between/within groups) repeated-measures ANOVA, with the Holm–Sidak method for pairwise comparison testing, were used for significance testing of continuous variables, while Fisher’s exact or McNemar’s tests were used for categorical variables (SigmaPlot 11; 2008 Systat Software, Germany). Associations between variables were tested for significance by the coefficient of determination ($R^2$) (GraphPad Prism 8.4.2; GraphPad Software, San Diego, CA, USA). Data are presented as mean±SD. In all analyses, the acceptable Type I error was set at $p<0.05$.

**Results**

The mMRC dyspnoea scale score was 0 in all control subjects, $\geq 2$ in 24 long COVID patients and 1 in eight long COVID patients. Body mass index was significantly higher in long COVID patients than in the control group ($p=0.001$), with 10 and three patients having obesity of class I and class II, respectively.

**Standard lung function at rest**

Pulse oximetry values were within the normal range in all patients without significant difference between the control and long COVID groups (97.6±0.7% versus 97.3±0.9%, $p=0.226$). TLC, standard $D_{LCO}$ and $V_A$, either as percentage of predicted or z-score, were significantly lower ($p<0.001$) in the long COVID group than in the control group. Nine long COVID patients had a restrictive abnormality; in four of them this was associated with decreased standard $D_{LCO}$ while four showed an isolated reduction of the latter. None of the six long COVID patients who had been treated at home showed any standard lung function measures outside the normal range (table 1).

**Combined $D_{LNO}$-$D_{LCO}$ at rest and post-walk**

At rest, both absolute values (table 2) and z-scores (figure 1) of combined $D_{LNO}$-$D_{LCO}$, $V_A$, $D_{LNO}/V_A$ and $D_{LCO}/V_A$ were significantly lower in the long COVID than in the control group ($p<0.001$ for all comparisons). The $D_{LNO}/D_{LCO}$ ratio did not differ significantly between groups (p=0.411) and heart rate was significantly higher (p=0.005) in long COVID than in the control group. $D_{LNO}$, as opposed to combined $D_{LCO}$, was decreased in a greater number of long COVID using both LLN$_5$ (n=22 (~69%)) versus n=13 (~41%), p=0.008) and LLN$_{2.5}$ (19 (~59%) versus 10 (~31%), p=0.004) as a threshold. By contrast, $D_{LNO}/V_A$ and $D_{LCO}/V_A$ were <LLN$_5$ in seven and four patients, respectively, and <LLN$_{2.5}$ in four patients and one patient, respectively, without significant differences (p=0.371 with LLN$_5$ and p=0.248 with LLN$_{2.5}$). The CT scans, obtained between ~10 and 88 days from lung function studies in nine patients who had been hospitalised during the acute phase of COVID-19, showed normal mean lung attenuation (~809±50 HU), coefficient of variation (18±2%), kurtosis (5.57±1.63) and leftward skewness (2.15±0.32) of CT histogram without high- (1±2%) or low-attenuation (<1% in all cases) areas. Yet, seven of them had $D_{LNO}$ <LLN$_{2.5}$. There was no significant relationship between $D_{LNO}$ and time elapsed from the acute phase of COVID-19 (figure 2).

Post-walk, heart rate significantly increased within groups (p<0.001), without significant interactions between groups, while Borg scale ratings of breathlessness were 0 in controls and 1–4 in long COVID patients. There were significant increments in $D_{LNO}$ (p<0.002), $D_{LNO}/D_{LCO}$ (p<0.001) and $V_A$ (p=0.020) within groups, with no significant interactions between groups. By contrast, there were no significant changes within groups in combined $D_{LCO}$ (p=0.626), $D_{LNO}/V_A$ (p=0.144) and $D_{LCO}/V_A$ (p=0.097). In the long COVID group, the number of patients with $D_{LNO}$ <LLN$_5$ was reduced from 22 at rest to 15 post-walk (p=0.023) and those with $D_{LNO}$ <LLN$_{2.5}$ from 19 to 13 (p=0.041). Of the six patients who had mild COVID-19 treated at home, one had $D_{LNO}$ slightly less than LLN$_5$ and one was less than LLN$_{2.5}$ at rest, but both had it increased above LLN$_5$ post-walk, without other lung function abnormalities (table 3). The mean rates of rise (slope) in $D_{LNO}$ with heart rate were remarkably similar between controls and long COVID patients (0.439 versus 0.387 mL·min$^{-1}$·mmHg$^{-1}$·beats·min$^{-1}$, respectively) whereas the mean y-intercept was lower in the latter (69 mL·min$^{-1}$·mmHg$^{-1}$ versus 115 mL·min$^{-1}$·mmHg$^{-1}$, respectively) (figure 3).
Discussion

The main findings of this study are that patients with long COVID and dyspnoea on activities of daily living had 1) combined $D_{LNO}$–$D_{LCO}$ and $\bar{V}A$ significantly lower than anthropometrically matched healthy controls; 2) resting $D_{LNO}$ below the normal ranges in approximately two-thirds of cases, but combined

| TABLE 1 | Subjects’ anthropometric characteristics and standard lung function data at rest |
|---------|---------------------------------|
|         | Controls | Long COVID | p-value |
| **Female/male** | 1/19 | 7/25 | 0.132 |
| **Age (years)** | 50.4±9.81 | 56.3±11.2 | 0.058 |
| **Stature (cm)** | 175±6 | 172±7 | 0.060 |
| **Weight (kg)** | 81±10 | 87±13 | 0.078 |
| **BMI (kg·m$^{-2}$)** | 26±3 | 30±4 | 0.001 |
| **Smokers (current or former/never)** | 10/10 | 16/16 | 1.000 |
| **[Hb] (g·dL$^{-1}$)** | 14.6±0.34 | 14.2±1.44 | 0.219 |
| **$\bar{S}pO_2$ (%)** | 97.6±0.71 | 97.3±0.86 | 0.226 |
| **FVC (L)** | 4.96±0.69 | 4.06±0.79 | $<0.001$ |
| % predicted | 105±14 | 97±16 | 0.057 |
| z-score | 0.29±1.00 | −0.26±1.07 | 0.052 |
| **FEV$_1$ (L)** | 3.95±0.46 | 3.29±0.62 | $<0.001$ |
| % predicted | 106±11 | 100±16 | 0.168 |
| z-score | 0.41±0.83 | −0.02±1.09 | 0.137 |
| **TLC (L)** | 7.00±0.93 | 5.63±1.04 | $<0.001$ |
| % predicted | 101±9 | 87±13 | $<0.001$ |
| z-score | 0.07±0.77 | −1.06±1.08 | $<0.001$ |
| **$D_{LCO}$ (mL·min$^{-1}$·mmHg$^{-1}$)** | 30.8±3.82 | 22.5±4.58 | $<0.001$ |
| % predicted | 110±13 | 89±16 | $<0.001$ |
| z-score | 0.56±0.77 | −0.77±1.05 | $<0.001$ |
| **$V_A$ (L)** | 6.88±0.91 | 5.61±0.94 | $<0.001$ |
| % predicted | 108±12 | 95±12 | $<0.001$ |
| z-score | 0.65±0.93 | −0.43±1.01 | $<0.001$ |
| **$D_{LNO}$/$D_{LCO}$** | 4.18±0.29 | 4.57±0.36 | $<0.001$ |
| % predicted | 4.22±0.41 | 4.67±0.46 | $<0.001$ |
| z-score | 5.22±0.76 | 5.09±0.76 | $<0.001$ |
| **Heart rate (beats·min$^{-1}$)** | 66±11 | 102±9 | $<0.001$ |

Data are presented as n or mean±SD, unless otherwise stated. COVID: coronavirus disease 2019; BMI: body mass index; Hb: haemoglobin; $\bar{S}pO_2$: pulse oximetry (at room air); FVC: forced vital capacity; FEV$_1$: forced expiratory volume in 1 s; TLC: total lung capacity; $D_{LCO}$: standard single-breath (11±0.5 breath-hold time) diffusing capacity of the lung for carbon monoxide; $V_A$: alveolar volume.

TABLE 2 | Combined diffusing capacities of the lung for nitric oxide ($D_{LNO}$) and carbon monoxide ($D_{LCO}$) at rest and post-walk |
|---------|---------------------------------|
|         | Controls | Long COVID | p-values (two-way ANOVA) |
|         | Rest | Post-walk | Rest | Post-walk | Within-group | Between-groups | Interaction |
| **$D_{LNO}$ (mL·min$^{-1}$·mmHg$^{-1}$)** | 34.7±4.76 | 35.2±24.9 | 23.4±5.33 | 23.9±6.01 | 0.026 | >0.050 | <0.050 | 0.335 |
| **$D_{LCO}$ (mL·min$^{-1}$·mmHg$^{-1}$)** | 4.18±0.29 | 4.57±0.36 | 2.22±0.41 | 2.67±0.46 | <0.001 | >0.050 | <0.050 | 0.335 |
| **$V_A$ (L)** | 5.01±0.72 | 5.01±0.76 | 3.99±0.76 | 4.09±0.85 | 0.714 | >0.050 | <0.050 | 0.335 |
| **$D_{LNO}/V_A$ (mL·min$^{-1}$·mmHg$^{-1}$·L$^{-1}$)** | 21.1±1.89 | 21.9±2.33 | 18.3±2.84 | 19.1±2.98 | 0.144 | >0.050 | <0.050 | 0.335 |
| **$D_{LCO}/V_A$ (mL·min$^{-1}$·mmHg$^{-1}$·L$^{-1}$)** | 5.06±0.56 | 4.82±0.64 | 4.38±0.75 | 4.13±0.82 | 0.097 | >0.050 | <0.050 | 0.335 |
| **Heart rate (beats·min$^{-1}$)** | 66±11 | 102±9 | 74±12 | 106±9 | <0.001 | >0.050 | <0.050 | 0.335 |

Data are presented as mean±SD, unless otherwise stated. COVID: coronavirus disease 2019; $V_A$: inspired volume of test gas; $V_A$: alveolar volume. **: combined single-breath (with actual 5.3±0.3 s and 5.1±0.4 s breath-hold times at rest and post-walk, respectively).
DLNO only in a minority of them; 3) DLNO/V_A and DLCO/V_A significantly lower than control subjects, but within the ranges of normality in the vast majority of cases; and 4) significant increments of DLNO and V_A after walking, like control subjects, although DLNO normalised in a minority of cases only.

Technical considerations

Substantial differences in DLNO and V_A have been reported between commercially available devices [27], and different predicting equations have been proposed [23, 28]. We estimated the suitability of the aforementioned predicting equations to our population by comparing the z-score standard deviations [29] of our database of 104 healthy subjects and found no substantial differences. Therefore, the choice of reference equations does not appear to be a major source of bias in our present study.

We did not derive DM and V_c subcomponents from combined DLNO–DLCO because the validity of their calculations is critically dependent on the values chosen for the rate of haemoglobin uptake (θ) and the diffusivity ratio of nitric oxide and carbon monoxide. Although the values of DMNO/DMCO (~tissue/plasma diffusivity) and θ_NO/θ_CO are deemed to be 1.97 and 8.1 in normoxia, respectively [30], controversies on these ratios remain and their values are currently being reassessed.

Comments on results

To our knowledge, this is the first study investigating combined DLNO–DLCO at rest and after a relatively short (~4–5 min) bout of treadmill exercise simulating ordinary walking, in patients with long COVID and...
dyspnoea on activities of daily living. Previous studies have reported a decrement of standard $D_{LCO}$ [8–10] and $D_{LNO}$ [12] after hospital discharge in ∼20–60% and >50%, respectively. Previous incremental symptom-limited exercise studies have documented a reduced aerobic capacity after COVID-19, suggesting ventilatory inefficiency [2], inappropriate hyperventilation [3], chronotropic and/or inotropic incompetence [4], reduced oxygen extraction by peripheral muscles [5], loss of mechanical efficiency [6] and muscle deconditioning [7] as possible responsible mechanisms. However, although the assessment of maximal aerobic capacity during an incremental test has a substantial clinical utility, its relevance to activities of daily living is limited. Moreover, none of these studies considered a possible association between breathlessness and decreased pulmonary gas exchange in long COVID.

Consistent with our previous study over shorter time intervals after the acute phase of COVID-19 [12], we have found that most patients with long COVID had resting $D_{LNO}$, expressed as z-score values, below the limits of normal, while combined $D_{LCO}$ was reduced in a significantly lower number of cases. Since $D_{LNO}$ is deemed to be more sensitive to changes in DM than $V_A$, while the opposite is the case for $D_{LCO}$ [30], the findings of this study suggest that a prevailing impairment of DM persists for 1–2 years in most patients with long COVID. A reduction of DM could be simply due to loss of $V_A$ because of obesity, which was indeed present in 41% of our long COVID patients [31]. However, loss of $V_A$ due to incomplete alveolar expansion is expected to cause large increments of $D_{LCO}/V_A$ [32] and, to a lesser extent, $D_{LNO}/V_A$ as alveolar dimensions reduce, with concomitant decrease of $D_{LNO}/D_{LCO}$ ratio [33, 34]. Thus, the apparently normal $D_{LNO}/V_A$ and $D_{LCO}/V_A$ z-scores, with $D_{LNO}/D_{LCO}$ ratio within the normal range, in the majority of our patients with long COVID suggest that 1) loss of $V_A$ was not the only cause

**FIGURE 2** Relationships between diffusing capacity of the lung for nitric oxide ($D_{LNO}$) z-scores and time from the end of the acute phase of coronavirus disease 2019. CT: computed tomography; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2. Horizontal dashed and dotted lines correspond to the 5th (z-score −1.645) and 2.5th (z-score −1.96) percentiles, respectively.

**TABLE 3** Lung function z-scores in six long COVID (coronavirus disease 2019) patients recovering from mild COVID-19

| Sex   | Age (years) | BMI (kg·m$^{-2}$) | Smoker | mMRC | Heart rate (beats·min$^{-1}$) | FVC (z-score) | FEV$_1$ (z-score) | FEV$_1$/FVC (z-score) | TLC (z-score) | $D_{LNO}$ (z-score) | $D_{LCO}$ (combined) (z-score) |
|-------|-------------|-------------------|--------|------|-------------------------------|---------------|-------------------|---------------------|---------------|----------------|-----------------------------|
| Male  | 61          | 30                | Former | 2    | 2                             | −0.17         | 0.00              | −0.34               | −1.34         | −2.30          | −1.59                      |
| Male  | 50          | 27                | Never  | 1    | 112                           | 0.00          | −0.34             | −1.19               | −1.71         | −0.30          | −0.15                      |
| Male  | 52          | 29                | Never  | 0    | 144                           | −0.93         | −0.30             | −1.19               | −1.16         | −0.59          | 0.13                       |
| Female| 50          | 27                | Never  | 2    | 103                           | −0.52         | −0.08             | −1.34               | −1.42         | −1.21          | −1.08                      |
| Female| 33          | 20                | Never  | 2    | 96                            | 1.32          | 0.42              | −1.31               | 0.55          | −0.77          | −0.34                      |
| Female| 54          | 29                | Never  | 1    | 117                           | −0.80         | −0.71             | −0.23               | 0.59          | −1.37          | −1.13                      |

BMI: body mass index; mMRC: modified Medical Research Council questionnaire score; FVC: forced vital capacity; FEV$_1$: forced expiratory volume in 1 s; TLC: total lung capacity; $D_{LNO}$: diffusing capacity of the lung for nitric oxide; $D_{LCO}$: diffusing capacity of the lung for carbon monoxide.
of reduced $D_{LNO}$ and $D_{LCO}$; 2) reduced $D_{LNO}$ and $D_{LCO}$ are compatible with “localised” discrete loss of lung units; and 3) normal $D_{LNO}/V_A$ and $D_{LCO}/V_A$ may be due to diversion of capillary blood volume from the lost to remaining alveolar units [32]. The combined $D_{LNO}$ and $D_{LCO}$ measurements of patients with long COVID were similarly reduced both at rest and post-walk in comparison with control subjects, thus leaving $D_{LNO}/D_{LCO}$ unchanged. This suggests that long COVID could affect DM and $V_c$ to a similar extent [35]. Indeed, concomitant changes of alveolar surface area and capillary volume are likely to occur in a complex parenchymal disease such as COVID-19. In our previous study, a reduced $D_{LNO}$ was observed even in patients with absent or minimal CT abnormalities, which suggests that mechanisms other than alveolar membrane thickening may contribute to diffusion abnormality after COVID-19 [12]. Another explanation might be that functional abnormalities of alveolar–capillary diffusion occurred, which were too small to be seen on CT. In the present study, none of the patients with available CT scans had fibrotic or ground-glass abnormalities, although the interpretation of this data in terms of structure-to-function is hindered by the time interval between pulmonary function tests and CT. But this was beyond the scope of the present study.

After walk, $D_{LNO}$ significantly increased in both groups while combined $D_{LCO}$ did not change, thus resulting in an increased $D_{LNO}/D_{LCO}$ ratio. These changes were associated with a significant increase in heart rate and $V_A$, without significant differences between groups. We have no data to explain the increase in $V_A$ post-walk. Although studies on lung volume responses to exercise in healthy subjects have consistently reported no changes of TLC at high intensities of exercise [36, 37], a slight increase of TLC [36] and a compatible decrement of pleural pressure suggestive of a reduction of lung elastic recoil [37] were observed at low intensities of exercise. The increment in $V_A$ in the present study was substantially higher than the increase in TLC observed by HANSON et al. [36], but the difference might have been related to methods and times of measurements. However, the increment of $D_{LNO}$ with insignificant change in $D_{LNO}/V_A$ in the present work can be explained not just by a post-exercise unfolding of the alveolar membranes, but also by capillary blood recruitment within the alveolar septa allowing more nitric oxide binding with red cell haemoglobin. The similarity of rate of $D_{LNO}$ rise with exercise between long COVID patients and control subjects with persistent reduction in the former suggest a residual decrease of DM and possibly $V_c$ despite a preserved capacity for alveolar–capillary recruitment.

The lack of post-exercise increase of $D_{LCO}$ of the combined manoeuvre in both groups is rather surprising, considering the expected $V_c$ recruitment, and at odds with studies using rebreathing technique during exercise either in health [38, 39] or disease [40]. Physiological and methodological reasons may explain the inconsistent changes of combined $D_{LNO}$ and $D_{LCO}$ and the increased $D_{LNO}/D_{LCO}$ ratio found 5–10 s after cessation of mild exercise. The $D_{LNO}$-$D_{LCO}$ single-breath technique requires a breath-hold of 4–6 s.
duration at full lung inflation following a rapid (<2.5 s) inhalation from residual volume [23]. This imposes large pressure swings on the pulmonary capillary wall with the effects of surface forces being negative in the alveoli, but strongly positive on the free edge of the alveolar septa [41]. Such squeezing of interalveolar vessels with erythrocyte deformation [42] could be accentuated by decreasing thoracic blood volume during an inadvertent Valsalva manoeuvre [43]. Thus, owing to the greater impact of \( V_c \) on carbon monoxide than nitric oxide uptake [30], the single-breath manoeuvre may blunt the signal of enhanced carbon monoxide uptake due to expected recruitment of \( V_c \) with increased cardiac output, depending on whether the subject actively maintains lung volume or relaxes against the closed airway during breath-holding [44]. Thus, unlike the rebreathing method, the breath-hold technique may underestimate the exercise-related increment of gas transfer relatively more for \( D_{LCO} \) than \( D_{LNO} \). Thus, we cannot exclude that a microvascular impairment may go undetected by this method.

All participating patients had been referred to our laboratory because of dyspnoea, but in a number of them, particularly those who had mild COVID-19, we found no abnormalities in lung function either at rest or post-walk. Other factors not investigated in this study (e.g. chronotropic incompetence, muscle deconditioning, obesity, anxiety) might have contributed to dyspnoea in these subjects.

**Study limitations**

The present study has limitations. First, the long COVID and control groups were not perfectly matched for anthropometric characteristics. There was a tendency, although statistically insignificant, for female-to-male ratio, age and body weight to be higher in long COVID than the control group. Although the \( D_{LCO} \) responses to exercise may be greater in men than women and decreases with age [45], these differences would have blunted the response to exercise in long COVID more than control group, which was not the case (insignificant between- and within-group interaction terms). Conversely, greater body weight might have caused tachycardia to occur earlier in long COVID patients than in controls. However, the heart rate difference between rest and post-walk was the same in the two groups. Second, we did not measure oxygen uptake, carbon dioxide output, exercise ventilation or ventilation equivalents, and this may, at least in part, limit the interpretation of our findings. Third, combined \( D_{LNO} - D_{LCO} \) were measured in duplicate at rest, but only once post-walk. This was necessary because the required 5-min interval between measurements would have allowed complete heart-rate recovery post-walk. Moreover, we did not attempt to measure combined \( D_{LNO} - D_{LCO} \) during walking, because the inspiratory vital capacity manoeuvre necessary to inhale test gases would have been difficult during walking in most subjects and measurement in standing upright posture would have been not comparable with reference values obtained in sitting posture. Thus, it cannot be excluded that the relationships between variables might have been influenced by variability in recovery time after walk. Fourth, the study was cross-sectional without a control group of patients with prior COVID-19, but no symptoms of long COVID.

**Conclusions**

The results of this study show that “localised” discrete loss of lung units, not completely explained by loss of \( V_a \) or of alveolar–capillary recruitment during exercise, may persist in about half of patients with long COVID. Moreover, even though abnormalities of \( D_{LNO} \) and \( D_{LCO} \) at rest or after exercise could be associated with breathlessness and poor tolerance to activities of daily living in patients with long COVID, no definitive causal inference between gas exchange abnormalities and respiratory symptoms can be made.
References

1. Serviente C, Decker ST, Layec G. From heart to muscle: pathophysiological mechanisms underlying long-term physical sequelae from SARS-CoV-2 infection. J Appl Physiol 2022; 132: 581–592.

2. Skjørtken I, Ankerstjerne OAW, Trebinjac D, et al. Cardiopulmonary exercise capacity and limitations 3 months after COVID-19 hospitalisation. Eur Respir J 2021; 58: 2100996.

3. Motiejunaite J, Balagny P, Arnoult F, et al. Hyperventilation as one of the mechanisms of persistent dyspnoea in SARS-CoV-2 survivors. Eur Respir J 2021; 58: 2101578.

4. Szekely Y, Lichter Y, Sadon S, et al. Cardiorespiratory abnormalities in patients recovering from coronavirus disease 2019. J Am Soc Echocardiogr 2021; 34: 1273–1284.

5. Baratto C, Caravita S, Faini A, et al. Impact of COVID-19 on exercise pathophysiology: a combined cardiopulmonary and echocardiographic exercise study. J Appl Physiol 2021; 130: 1470–1478.

6. Pleguezuelos E, Del Carmen A, Llorensi G, et al. Severe loss of mechanical efficiency in COVID-19 patients. J Cachexia Sarcopenia Muscle 2021; 12: 1056–1063.

7. Debeaumont D, Boujibar F, Ferrand-Devouge E, et al. Cardiopulmonary exercise testing to assess persistent symptoms at 6 months in people with COVID-19 who survived hospitalization: a pilot study. Phys Ther 2021; 101: pzab099.

8. Mo X, Jian W, Su Z, et al. Abnormal pulmonary function in COVID-19 patients at time of hospital discharge. Eur Respir J 2020; 55: 2000127.

9. van den Borst B, Peters JB, Brink M, et al. Comprehensive health assessment 3 months after recovery from acute coronavirus disease 2019 (COVID-19). Clin Infect Dis 2021; 73: e1089–e1098.

10. Huang C, Huang L, Wang Y, et al. 6-month consequences of COVID-19 in patients discharged from hospital: a cohort study. Lancet 2021; 397: 220–232.

11. Kersten J, Wolf A, Hoyo L, et al. Symptom burden correlates to impairment of diffusion capacity and exercise tolerance in long COVID patients. Sci Rep 2022; 12: 8801.

12. Barisone G, Brusasco V. Lung diffusing capacity for nitric oxide and carbon monoxide following mild-to-severe COVID-19. Physiol Rep 2021; 9: e14748.

13. Hsia CCW. Recruitment of lung diffusing capacity: update of concept and application. Chest 2002; 122: 1774–1783.

14. World Health Organization (WHO). A Clinical Case Definition of post COVID-19 condition by a Delphi consensus, 6 October 2021. www.who.int/publications/i/item/WHO-2019-nCoV-Post_COVID-19_condition-Clinical_case_definition-2021.1 Date last updated: 6 October 2021.

15. Wanger J, Clausen JL, Coates A, et al. Standardisation of the measurement of lung volumes. Eur Respir J 2005; 26: 511–522.

16. Graham BL, Steenbruggen I, Miller MR, et al. Standardization of spirometry 2019 update. An Official American Thoracic Society and European Respiratory Society Technical Statement. Am J Respir Crit Care Med 2019; 200: e70–e88.

17. 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.

18. Hall GL, Filipow N, Ruppel G, et al. Official ERS technical standard: Global Lung Function Initiative reference values for static lung volumes in individuals of European ancestry. Eur Respir J 2021; 57: 2000289.

19. Quanjer PH, Stanojevic S, Cole TJ, et al. Multi-ethnic reference values for spirometry for the 3–95-yr age range: the global lung function 2012 equations. Eur Respir J 2012; 40: 1324–1343.

20. Stanojevic S, Graham BL, Cooper BG, et al. Official ERS technical standards: Global Lung Function Initiative reference values for the carbon monoxide transfer factor for Caucasians. Eur Respir J 2017; 50: 1700010.

21. Cotes JE, Dabbs JM, Elwood PC, et al. Iron-deficiency anaemia: its effect on transfer factor for the lung (diffusing capacity) and ventilation and cardiac frequency during sub-maximal exercise. Clin Sci 1972; 42: 325–335.

22. Barisone G, Garlaschi A, Occhipinti M, et al. Value of lung diffusing capacity for nitric oxide in systemic sclerosis. Physiol Rep 2019; 7: e14149.

23. Zavorsky GS, Hsia CC, Hughes JM, et al. Standardisation and application of the single-breath determination of nitric oxide uptake in the lung. Eur Respir J 2017; 49: 1600962.

24. Tanaka H, Monahan KD, Seals DR. Age-predicted maximal heart rate revisited. J Am Coll Cardiol 2001; 37: 153–156.

25. Huang YC, Helms MJ, MacIntyre NR. Normal values for single exhalation diffusing capacity and pulmonary capillary blood flow in sitting, supine positions, and during mild exercise. Chest 1994; 105: 501–508.

26. Yushkevich PA, Piven J, Hazlett HC, et al. User-guided 3D active contour segmentation of anatomical structures: significantly improved efficiency and reliability. Neuroimage 2006; 31: 1116–1128.

27. Radtke T, de Groot Q, Haile SR, et al. Lung diffusing capacity for nitric oxide measured by two commercial devices: a randomised crossover comparison in healthy adults. ERJ Open Res 2021; 7: 00193.

28. Munkholm M, Marott JL, Bjerrum-Kristensen L, et al. Reference equations for pulmonary diffusing capacity of carbon monoxide and nitric oxide in adult Caucasians. Eur Respir J 2018; 52: 1500677.
29 Stocks J, Quanjer PH. Reference values for residual volume, functional residual capacity and total lung capacity. ATS Workshop on Lung Volume Measurements. Official Statement of The European Respiratory Society. *Eur Respir J* 1995; 8: 492–506.

30 Borland CDR, Hughes JMB. Lung diffusing capacities ($D_L$) for nitric oxide (NO) and carbon monoxide (CO): the evolving story. *Compr Physiol* 2019; 10: 73–97.

31 Jones RL, Nzekwu MM. The effects of body mass index on lung volumes. *Chest* 2006; 130: 827–833.

32 Hughes JMB, Pride NB. Examination of the carbon monoxide diffusing capacity ($D_L CO$) in relation to its $K CO$ and $V_d$ components. *Am J Respir Crit Care Med* 2012; 186: 132–139.

33 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.

34 Hughes JM, van der Lee I. The $T L NO / T L CO$ ratio in pulmonary function test interpretation. *Eur Respir J* 2013; 41: 453–461.

35 Kang MY, Sapoval B. Time-based understanding of $D L CO$ and $D L NO$. *Respir Physiol Neurobiol* 2016; 225: 48–59.

36 Hanson JS, Tabakin BS, Caldwell EJ. Response of lung volumes and ventilation to posture change and upright exercise. *J Appl Physiol* 1962; 17: 783–786.

37 Younes M, Kivinen G. Respiratory mechanics and breathing pattern during and following maximal exercise. *J Appl Physiol Respir Environ Exerc Physiol* 1984; 57: 1773–1782.

38 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: 1850–1860.

39 Coffman KE, Carlson AR, Miller AD, et al. The effect of aging and cardiorespiratory fitness on the lung diffusing capacity response to exercise in healthy humans. *J Appl Physiol* 2017; 122: 1425–1434.

40 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.

41 Weibel ER. The Pathway for Oxygen. Structure and Function in the Mammalian Respiratory System. Cambridge, MA, Harvard University Press, 1984.

42 Nabors KL, Baumgartner WA Jr, Janke SJ, et al. Red blood cell orientation in pulmonary capillaries and its effect on gas diffusion. *J Appl Physiol* 2003; 94: 1634–1640.

43 Smith TC, Rankin J. Pulmonary diffusing capacity and the capillary bed during Valsalva and Müller maneuvers. *J Appl Physiol* 1969; 27: 826–833.

44 Johns DP, Berry D, Maskrey M, et al. Decreased lung capillary blood volume post-exercise is compensated by increased membrane diffusing capacity. *Eur J Appl Physiol* 2004; 93: 96–101.

45 Kendrick AH, Laszlo G. CO transfer factor on exercise: age and sex differences. *Eur Respir J* 1990; 3: 323–328.