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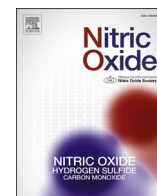
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Morning-to-evening decline in pulmonary diffusing capacity and spirometry

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ABSTRACT

Pulmonary function tests are performed throughout the day, yet time-of-day effects on spirometry and pulmonary diffusing capacities for nitric oxide (DLNO) and carbon monoxide (DLCO) are poorly characterized, and DLNO's diurnal behavior is unknown. We examined whether these measurements vary in healthy men and derived within-day limits of change. Thirty-eight men (median age, 29.8 years) underwent up to six testing sessions during one waking day. Each session included spirometry (FVC, FEV₁, SVC, and FEV₁/FVC), followed by single-breath DLNO/DLCO testing to obtain DLCO, DLNO, alveolar volume (V_A), transfer coefficients KCO and KNO, and the DLNO/DLCO ratio. Time-of-day effects were assessed using linear mixed-effects models with subject-specific random intercepts; within-subject variability was quantified using the within-subject standard deviation. After quality filtering and multiplicity adjustment, FVC, FEV₁, SVC, DLNO, DLCO, and V_A decreased across the day ($q \leq 0.014$). KCO declined before but not after false-discovery-rate adjustment ($p = 0.041$, $q = 0.058$); FEV₁/FVC, KNO, and DLNO/DLCO did not change significantly. Within-day variability ranged from 1.5% for SVC to 3.5% for DLCO. One-sided 95% within-day change limits were approximately 11 mL/min/mmHg for DLNO (z-score, 0.59), 2.8 mL/min/mmHg for DLCO (z-score, 0.59), 0.28 L for V_A (z-score, 0.35), and 0.27 for DLNO/DLCO. Spirometric volumes and absolute diffusing capacities declined from morning to evening, consistent with decreasing V_A, whereas ratio and transfer-coefficient indices were largely stable. Serial measurements should be performed at consistent times. These limits reflect measurement error plus short-term physiological or procedural variation and are specific to this device, protocol, and population.

1. Introduction

Pulmonary function tests (PFTs) are fundamental to the diagnosis and longitudinal monitoring of respiratory diseases, including asthma, chronic obstructive pulmonary disease (COPD), and interstitial lung diseases. Reliable serial measurement depends not only on instrument precision and patient effort but also on identifying sources of biological variability that may be mistaken for clinically meaningful change. Time of day is one such source: airway calibre and lung volumes exhibit diurnal (time-of-day) variation, with peak forced expiratory volume in 1 s (FEV₁) and forced vital capacity (FVC) typically occurring in the morning and nadir values in the evening [1–3], but not always [4]. A recent meta-analysis confirms this temporal pattern and that FEV₁ and FVC fluctuate by ~3% throughout the waking day [5]. These findings informed recommendations that serial spirometry be performed at the

same time of day to minimise within-person variability. However, few studies have examined all these variables simultaneously in the same individuals across multiple time points within a single day, and none have included DLNO [4].

Pulmonary diffusing capacity for carbon monoxide (DLCO) shows a similar pattern. Some studies report higher values in the morning and lower values in the afternoon/evening [6–9]. The decline in DLCO of 0.4–0.6% per hour can be explained by rising carboxyhaemoglobin concentration [COHb] and falling haemoglobin concentration [Hb], and when these are adjusted, there is no true diurnal variation in DLCO [7].

On the other hand, pulmonary diffusing capacity for nitric oxide (DLNO) is much less affected by haemoglobin or carboxyhaemoglobin concentration compared to DLCO [10,11]; thus the variation of DLNO within a day may prove to be smaller than that of DLCO. One model suggests that DLNO represents surface absorption related to the plasma

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Table 1
Subject characteristics.

Variable	n	Distribution	Summary
Age (years)	38	Non-normal	29.8 (26.1, 34.8) (8.6)
Weight (kg)	38	Normal	87.7 (8.9) [69.0, 105.0]
Height (cm)	38	Normal	184 (6) [172, 196]
BMI (kg/m ²)	38	Normal	25.8 (2.2) [20.4, 31.1]
Hb (g/dL)	38	Normal	14.7 (1.2) [11.9, 16.4]
Pack-years	38	Non-normal	0.9 (0.0, 4.4) (4.4)
FEV ₁ z-score	38	Normal	−0.09 (0.93) [−1.93, 2.63]
FVC z-score	38	Normal	0.33 (0.93) [−1.36, 2.83]
FEV ₁ /FVC z-score	38	Normal	−0.91 (0.76) [−2.17, 0.80]
DLNO z-score	38	Normal	−0.58 (0.85) [−2.19, 1.35]
DLCO z-score	38	Normal	−0.14 (0.61) [−1.42, 0.87]
VA z-score	38	Normal	0.11 (0.81) [−1.29, 2.39]
KCO z-score	38	Normal	−0.24 (0.88) [−1.81, 1.35]

One value per participant, taken from their earliest session of the day ($n = 38$, before quality filtering). Distribution assessed by Shapiro-Wilk. Normal ($p \geq 0.05$): mean (SD) [min, max]. Non-normal ($p < 0.05$): median (Q1, Q3) (IQR). BMI calculated as weight (kg) / height (m)². z-scores computed on each subject's mean of all within-day timepoints: FEV₁, FVC, and FEV₁/FVC from GLI-2012 [30]; DLCO, VA, and KCO from GLI-2017 [31,32]; DLNO from GAMLSS reference equations [33]. DLCO, KCO and DLNO/DLCO are standardised to each participant's baseline haemoglobin rather than the session-specific value.

boundary layer's morphology, while DLCO corresponds to volume or bulk absorption, indicating haematocrit levels [12]. Thus, DLNO and DLCO provide complementary but related measures: they are obtained from the same manoeuvre, share VA, and reflect common gas-transfer resistances as described by the Roughton-Forster equation [13,14].

The within-session repeatability of DLNO [11,15–17], week-to-week-reproducibility of DLNO [16–20], and test-retest reliability of DLNO during exercise [21–23] have been previously established. However, the variation of DLNO throughout a waking day (diurnal variation) has not been determined. Understanding diurnal variation in DLNO is clinically important because it has been shown that combined DLNO + DLCO z-scores or DLNO-based models by themselves improve prediction and classification of disease (compared to controls) when pitted against DLCO-based models [24–26]. Thus, DLNO may add complementary information to DLCO when monitoring disease progression and response to therapy, and the test-to-test variation attributable to time of day could obscure or mimic a true clinical change.

Thus, the primary aim of this study was to determine whether time of day significantly affects spirometry and simultaneous DLNO + DLCO results in healthy adults tested repeatedly across a single waking day. The secondary aims were to quantify total observed within-subject variability across the study day and to derive empirical one-sided change limits specific to this device and protocol.

2. Materials and methods

2.1. Study design and participants

This was a single-day repeated-measures study conducted in 38 white, healthy, adult male military divers of the Royal Netherlands Navy between 2010 and 2012. Both datasets were acquired using the same equipment and testing procedures at the same facility. Approximately 23% of the data were initially presented as an abstract at the 2014 European Respiratory Society (ERS) international conference in 2014 [27], and then only some of those data were eventually published as a full-length article elsewhere [28]. Then about 66% data were obtained through a separate study [29], and 11% of the data remained unpublished. See online supplement in which the data is provided. All measurements analysed here were obtained under resting, non-intervention control conditions—before any dive or hyperoxic exposure on the study day—using identical equipment, gas mixtures, and testing procedures across the source studies; no post-dive or post-hyperoxia measurements

were included.

All participants were men; women were not recruited in the original studies as the divers in the Royal Netherlands Navy were mostly men. Eligibility criteria included age ≥ 18 years, no diagnosed respiratory or cardiovascular disease, no acute illness, a light or absent smoking history, and ability to perform technically acceptable spirometry and diffusing capacity manoeuvres. Subject characteristics are presented in Table 1. The study was conducted in accordance with the Declaration of Helsinki, and written informed consent was obtained from all participants. The Institutional Review Boards of University Medical Center Groningen (IRB # METC 2010/089) and Academic Medical Center Amsterdam (IRB # NL35768.018.11) approved the aforementioned studies.

2.2. Study protocol

Each participant attended a single laboratory visit and performed six repeated pulmonary function test sessions throughout the day. The median start time of the first session was 09:05 h (range: 07:56–11:34 h) and the median start time of the final session was 20:44 h (range: 19:54–21:38 h). The mean $\pm$ SD time span between the first and last sessions was 11.6 ± 0.4 h (range: 10.2–12.8 h). After retaining only good quality-filtered observations, the mean number of testing sessions retained per participant was 5.4 ± 1.2 (range: 3–6), and the mean time span between the first and last sessions was 10.9 ± 1.9 h. Between testing sessions, participants remained in the laboratory and were permitted to engage in routine sedentary activities (e.g., reading, sitting) but were instructed to avoid strenuous physical exertion or napping. Meals and fluid intake were not standardised but were permitted ad libitum. Participants were requested to refrain from strenuous exercise, alcohol, and bronchodilator use for 12 h before and during the study day.

2.3. Pulmonary function measurements

Within each timepoint throughout the day, each subject performed about three spirometry manoeuvres and three diffusing capacity tests before obtaining the reported values. At each timepoint, subjects first performed a slow vital capacity (SVC) manoeuvre followed by forced spirometry (FVC, FEV₁, FEV₁/FVC) in accordance with 2005 ERS/ATS standards [34]. Then single-breath DLNO/DLCO was performed using the Jaeger MasterScreen™ PFT Pro lung function testing device (Vyaire Medical). Each subject inspired from residual volume – a mixture of two tanks – Tank 1 = 500 ppm NO, Balance N₂; Tank 2 = 0.25% CO, 9% He, 19% O₂, and Balance of N₂. As such, the inspired mixture was ~ 70 ppm NO, $\sim 0.25\%$ CO, $\sim 9\%$ He, $\sim 19\%$ O₂, and Balance of N₂. Breath-hold time was ~ 8 s. This yielded DLCO, DLNO, alveolar volume (V_A), the transfer coefficient for CO (KCO = DLCO/V_A), the transfer coefficient for NO (KNO = DLNO/V_A), and the DLNO/DLCO ratio. Gas analysers and volume transducers were calibrated before each testing session in accordance with manufacturer and ATS/ERS specifications. Spirometry manoeuvres that met ATS/ERS acceptability and repeatability criteria [34], and single-breath DLNO/DLCO manoeuvres that met the acceptability criteria of the ERS DLNO technical standard were kept [35]. At least 4 min between successive diffusing-capacity manoeuvres to allow test gases to clear. The mean of the two technically acceptable single-breath DLNO/DLCO manoeuvres at each session was used for analysis. Haemoglobin was measured once in 13 subjects, and at multiple times in the remaining subjects. The measurement system standardised DLCO — and hence KCO and the DLNO/DLCO ratio — to the haemoglobin value entered at each session, as $DLCO_{std} = DLCO \times (10.22 + [Hb]) / (1.7 \times [Hb])$, with [Hb] in g/dL. Among the participants with repeated measurements, the entered haemoglobin varied within the day by more than short-term physiology or the assay can account for (within-participant SD 1.0 g/dL), so session-specific standardisation transferred that variation into DLCO, where it accounted for 23% of the

Table 2

Effect of time of day on pulmonary function and diffusing capacity variables.

Variable	All Data						Quality-Filtered Data					
	N obs	N subjects	Mean (SD)	F (df1, df2)	p-value	q-value	N obs	N subjects	Mean (SD)	F (df1, df2)	p-value	q-value
Spirometry Variables												
FVC (L)	228	38	6.14 (0.81)	14.2 (1, 189.0)	< 0.001	< 0.001	194	36	6.17 (0.79)	9.9 (1, 157.1)	0.002	0.005
FEV ₁ (L)	228	38	4.78 (0.68)	10.6 (1, 189.0)	0.001	0.003	194	36	4.78 (0.67)	7.1 (1, 157.1)	0.008	0.014
FEV ₁ /FVC	228	38	0.76 (0.06)	1.0 (1, 189.3)	0.318	0.318	194	36	0.76 (0.05)	0.7 (1, 157.7)	0.403	0.403
SVC (L)	228	38	6.26 (0.79)	15.6 (1, 189.0)	< 0.001	< 0.001	194	36	6.30 (0.76)	14.3 (1, 157.0)	< 0.001	0.001
Diffusing Capacity Variables												
DLNO (mL/min/mmHg)	228	38	158 (21)	17.1 (1, 189.1)	< 0.001	< 0.001	194	36	157 (21)	9.2 (1, 157.3)	0.003	0.006
DLCO (mL/min/mmHg)	228	38	34.4 (3.8)	19.9 (1, 189.1)	< 0.001	< 0.001	194	36	34.3 (3.8)	13.8 (1, 157.4)	< 0.001	0.001
V _A (L)	228	38	7.29 (0.83)	8.2 (1, 189.1)	0.005	0.008	194	36	7.25 (0.81)	11.8 (1, 157.1)	< 0.001	0.002
KCO (mL/min/mmHg/L)	228	38	4.75 (0.57)	4.0 (1, 189.1)	0.046	0.066	194	36	4.77 (0.56)	4.3 (1, 157.4)	0.041	0.058
KNO (mL/min/mmHg/L)	228	38	21.8 (2.5)	2.1 (1, 189.1)	0.145	0.181	194	36	21.8 (2.5)	2.0 (1, 157.3)	0.162	0.199
DLNO/DLCO	228	38	4.60 (0.36)	2.0 (1, 189.1)	0.164	0.182	194	36	4.58 (0.37)	1.8 (1, 157.6)	0.179	0.199

Linear mixed-effects model: Variable ~ Time (decimal hours) + (1 | Subject). *F*-test with Satterthwaite degrees of freedom.Quality filter: removed rows where V_A-SVC > 1.500 (n = 22), IV/SVC > 1.030 (n = 2), or IV/SVC < 0.845 (n = 12). Total removed: 34 of 228 rows.DLCO and KCO (and hence the DLNO/DLCO ratio) are standardised to haemoglobin using each participant's baseline value, not the session-specific value applied by the measurement system. Haemoglobin was measured at every session in 25 of 38 participants and once in the remainder, and its session-to-session variation exceeded what within-day physiology and the assay can resolve. The spirometry variables, DLNO and V_A do not carry this correction and are unchanged.*q*-value = Benjamini-Hochberg false-discovery-rate-adjusted *p*-value across the ten outcomes tested (m = 10), applied separately within each dataset.Bold *p*-values and *q*-values indicate statistical significance (*p* < 0.05; *q* < 0.05).

within-day variance. The device correction was therefore reversed and re-applied once per participant, using the earliest-session haemoglobin reported in Table 1. Because the resulting factor is constant within a participant, it rescales each participant's whole trajectory by a fixed proportion and cannot itself create or remove a within-day trend; the values remain on the haemoglobin-standardised scale and stay comparable with the GLI-2017 reference equations. Spirometry, DLNO and V_A carry no haemoglobin correction and are unchanged. As carboxyhaemoglobin was not remeasured at each time point, DLCO values were not corrected for carboxyhaemoglobin, and no smoking-specific correction was applied. Across all participants and sessions, 228 observations were reported.

2.3.1. Quality filtering

A pre-specified quality filter was applied to all pulmonary function data: manoeuvres were excluded if V_A exceeded SVC by more than 1.50 L (indicating a supra-vital-capacity inspiration; n = 22), if the inspired volume to SVC ratio (IV/SVC) exceeded 1.03 (n = 2), or if IV/SVC was below 0.845 (indicating incomplete inspiration; n = 12). These criteria, derived from the ERS DLNO technical standard [35], were applied to all variables in each session because spirometry and diffusing capacity were measured simultaneously in the same manoeuvre. A total of 34 observations were removed from analyses, leaving 194 quality-filtered observations from 36 subjects. The criteria for removal were not mutually exclusive; some observations met more than one criterion. Excluded manoeuvres were evenly distributed across the day (11–21% per session; chi-square test of exclusion by session order, *p* = 0.86), so the quality filter did not preferentially remove observations at particular time points and is therefore unlikely to have biased the temporal trend.

2.4. Statistical analysis

The effect of time of day on each pulmonary function variable was

assessed using a linear mixed-effects model with time (expressed as decimal hours from midnight) as a fixed-effect predictor and subject as a random intercept. *F*-statistics and *p*-values were computed using Satterthwaite degrees of freedom. We conducted analyses on both the full dataset and the quality-filtered dataset.

The association between the within-day change in alveolar volume and the concurrent changes in DLNO, DLCO, and the DLNO/DLCO ratio was quantified with a repeated-measures correlation using the *rmcorr* package (version 0.7.0) for R [36,37]. This method fits an analysis of covariance with participant as a factor, yielding a single common within-participant slope with participant-specific intercepts; it therefore accounts for the non-independence of the repeated sessions contributed by each participant, rather than pooling observations as if they were independent. Both variables were expressed as percent change from the participant's first (morning) session. Because that reference session is zero for both variables by definition, it was excluded from the correlations, leaving 158 observations from 36 participants (121 degrees of freedom).

Total observed within-subject variability across the study day was expressed as the within-subject standard deviation (SDw), estimated from an intercept-only mixed-effects model applied to the quality-filtered data (N = 194 from 36 subjects). The coefficient of repeatability for the difference between two session means was calculated as $1.96 \times \sqrt{2} \times \text{SDw} = 2.77 \times \text{SDw}$. Ninety-five percent confidence intervals for SDw were derived from the chi-squared distribution. One-sided empirical within-day change limits were calculated as $\sqrt{2} \times \text{SDw} \times z$, where *z* = 0.84 (80% level), 1.28 (90%), or 1.64 (95%). Under a no-change model, a change beyond these limits in the specified direction would occur with probability 20%, 10%, or 5%, respectively, from the total variation represented by SDw; exceeding a limit does not by itself prove that a physiological change occurred. The observed session mean includes systematic time-of-day change, unsystematic short-term physiological and manoeuvre-related variation, and random measurement error arising from the instrument, calibration, gas

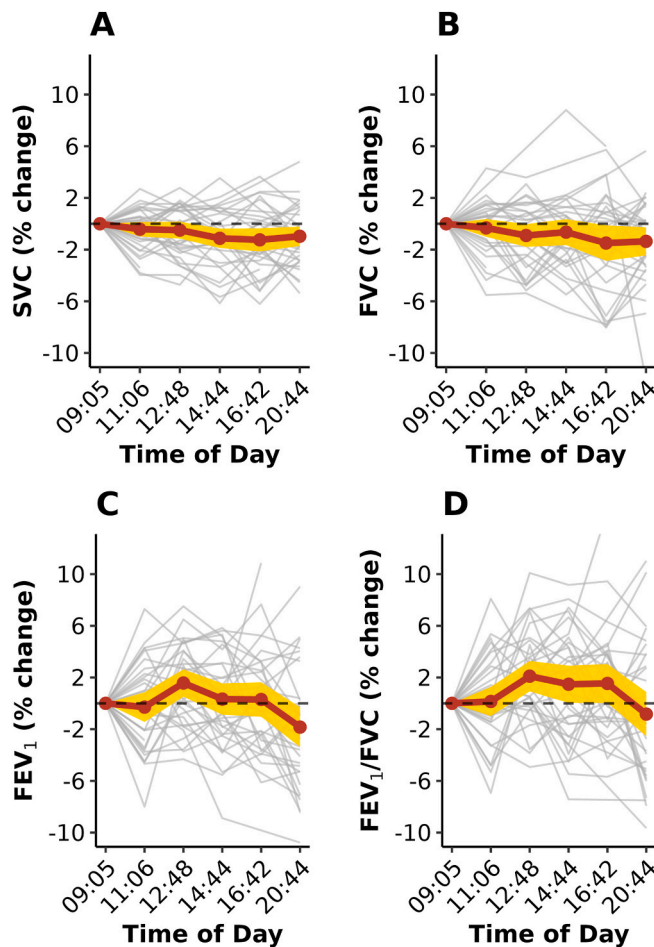


Fig. 1. Individual longitudinal trajectories of spirometry across the waking day using median clock times (09:05 to 20:44 h). Only quality filtered data was used. The data is expressed as a percent change from each participant's first (morning) session. Thin grey lines are individual participants; the bold red line and yellow band are the mean and its 95% confidence interval. (A) Slow vital capacity (SVC); (B) forced vital capacity (FVC); (C) forced expiratory volume in 1 s (FEV₁); (D) FEV₁/FVC ratio. SVC, FVC, and FEV₁ decline from the morning baseline across the day, whereas FEV₁/FVC shows no significant time-of-day effect (Table 2).

analysis, and processing algorithm [38]. Because only session means were available, these components could not be estimated separately. SDw was therefore estimated both from the intercept-only model and from a sensitivity model that additionally included time of day as a fixed effect. The latter removes the fitted linear time trend, but its residual SDw remains a mixture of random measurement error and unsystematic physiological or procedural variation. All analyses were performed in RStudio (version 2026.06.0, Build 242, R Foundation for Statistical Computing, Vienna, Austria) using the lme4, lmerTest, and rmcorr packages. Because ten pulmonary function outcomes were tested, the false discovery rate across outcomes was controlled using the Benjamini-Hochberg procedure, and adjusted p-values (q-values) are reported alongside unadjusted values for all the data and the quality-filtered data. Spirometry volumes, DLNO, and VA were pre-specified as the primary outcomes; DLCO, the transfer coefficients (KCO, KNO), and the ratios were considered secondary. For the trajectory plots, all six session positions per subject were plotted in chronological order. Within-person associations between relative change in VA and relative changes in DLNO, DLCO, and DLNO/DLCO were estimated after subject centering; 95% confidence intervals were obtained from the Fisher z transformation, as implemented in rmcorr. In addition, z-scores were calculated for descriptive purposes from the GLI-2012

spirometry equations (FEV₁, FVC, FEV₁/FVC) [30], the GLI-2017 equations (DLCO, VA, KCO) [31,32], and published GAMLSS equations for DLNO [33]. For Table 1, these equations were applied to each participant's mean of all within-day timepoints. For the change-in-z-score thresholds in Table 4, z-scores were instead computed for each quality-filtered session, and the thresholds were derived from the within-subject SD of those session-level z-scores, using the same formulae as the native-unit thresholds.

3. Results

3.1. Subject characteristics

Thirty-eight healthy adult men (military divers) participated (Table 1). Participants had a median age of 29.8 years (IQR 26.1–34.8), with a mean weight of 87.7 ± 8.9 kg, mean height of 184 ± 6 cm, and mean BMI of 25.8 ± 2.2 kg/m². Mean haemoglobin was 14.7 ± 1.2 g/dL. At the time of measurement, 17 participants were never-smokers, 6 were former smokers, and 15 were current smokers; the smoking burden was nonetheless light overall (median 0.9 pack-years, IQR 0.0–4.4, maximum 16).

3.2. Effect of time of day on spirometry

In both the full ($n = 228$) and quality-filtered ($n = 194$) datasets, FVC, FEV₁, and SVC decreased significantly across the day (all $p \leq 0.01$; Table 2, Fig. 1). In the full dataset, the linear mixed-effects model yielded F-statistics of 14.2 for FVC ($p < 0.001$), 10.6 for FEV₁ ($p = 0.001$), and 15.6 for SVC ($p < 0.001$). The FEV₁/FVC ratio did not change significantly ($F = 1.0$, $p = 0.318$), indicating that the decreases in FVC and FEV₁ were proportional. Results were consistent after quality filtering. Across the ~11-h test day these correspond to declines of roughly 1–2% (FVC -0.10 L, 95% CI -0.15 to -0.05 ; FEV₁ -0.09 L, -0.14 to -0.03 ; SVC -0.07 L, -0.11 to -0.04 ; slopes -0.009 , -0.008 , and -0.007 L/h, respectively), and individual longitudinal trajectories are shown in Fig. 1.

3.3. Effect of time of day on diffusing capacity

DLNO, DLCO, and V_A all decreased significantly across the day in both the full and quality-filtered datasets (Table 2, Fig. 2). In magnitude, DLNO fell by about 4.0 mL/min/mmHg (95% CI 2.1 to 5.9), DLCO by 1.1 mL/min/mmHg (0.6 to 1.6), and V_A by 0.13 L (0.04 to 0.22) across the test day, corresponding to declines of roughly 2–3%. Individual trajectories (Figs. 1 and 2) show that the decline was broadly consistent across participants rather than driven by a subset; DLCO showed an early fall with a partial mid-day recovery before declining again, whereas DLNO and the volumes declined more steadily from their morning peak. After controlling for the false discovery rate across the ten outcomes, the declines in SVC, FVC, FEV₁, DLNO, DLCO, and V_A all remained significant ($q \leq 0.014$). KCO declined before but not after adjustment ($p = 0.041$, $q = 0.058$) and is therefore interpreted with caution as a secondary outcome. Furthermore, successive single-breath DLCO maneuvers can raise carboxyhaemoglobin (COHb) levels, progressively lowering measured DLCO [11]; however, this effect requires approximately 12 consecutive 10-s maneuvers performed in rapid succession within a single session [11]. As the ~2-h intervals between our six daily sessions allowed COHb to dissipate, CO back-pressure is therefore unlikely to explain the observed diurnal decline in DLCO. DLNO, in contrast, is unaffected by COHb, even after 22 consecutive 5-s breath-hold maneuvers [11]. Accordingly, the parallel decline in DLNO, which is unaffected by COHb, supports a genuine, volume-driven fall in diffusing capacity rather than a reduction from the build-up of carboxyhaemoglobin.

Relative change in V_A from the morning baseline was positively associated with relative changes in DLNO (repeated-measures $r = 0.64$;

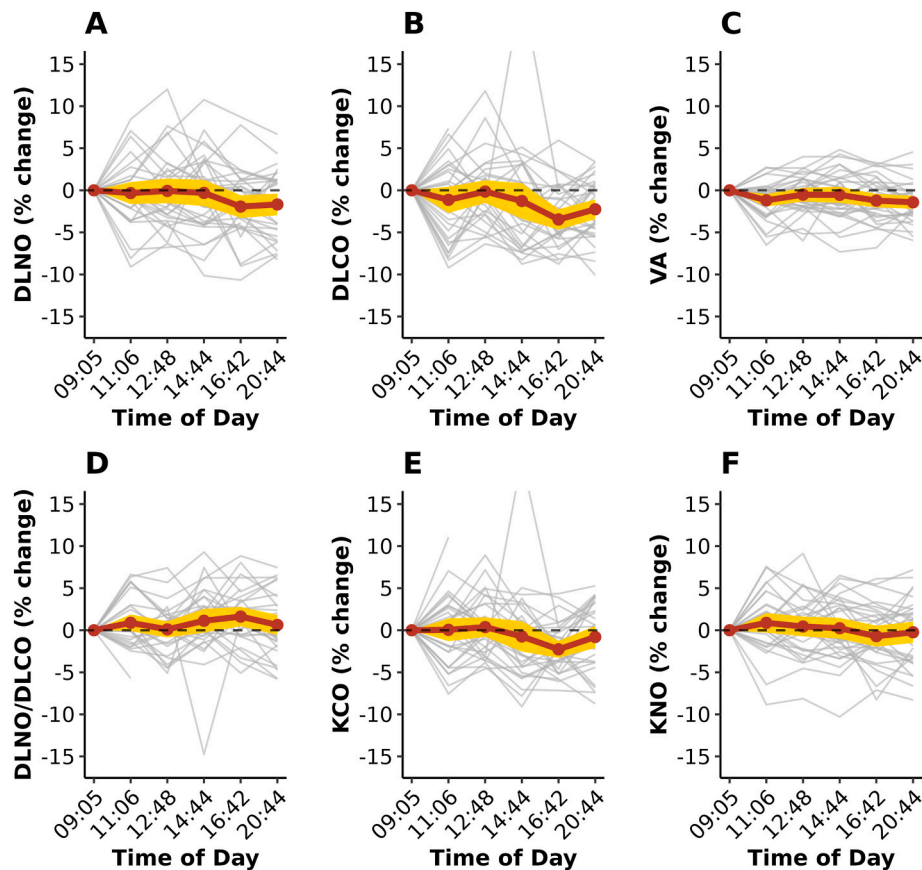


Fig. 2. Individual longitudinal trajectories of pulmonary diffusing capacity across the waking day using quality filtered data. The data are expressed as a percent change from each participant's first morning session. (A) Diffusing capacity of the lung for nitric oxide (DLNO); (B) diffusing capacity of the lung for carbon monoxide (DLCO); (C) alveolar volume (V_A); (D) DLNO/DLCO ratio; (E) transfer coefficient for carbon monoxide (KCO); (F) transfer coefficient for nitric oxide (KNO). DLNO, DLCO, and V_A decline from the morning peak, and DLCO shows an early fall with a partial mid-day recovery; the DLNO/DLCO ratio, and KNO show no significant time-of-day effect. KCO is significant before but not after controlling for the false discovery rate (Table 2).

95% CI 0.52 to 0.73; $p < 0.001$) and DLCO ($r = 0.47$; 95% CI 0.32 to 0.60; $p < 0.001$), but not with the DLNO/DLCO ratio ($r = 0.11$; 95% CI -0.07 to 0.28; $p = 0.212$); each correlation used 158 observations from 36 participants with 121 degrees of freedom (Fig. 3).

3.4. Total observed within-subject variability

Table 3 presents total observed within-subject variability across the study day, estimated from the quality-filtered data. For spirometry, variability ranged from 1.5% (SVC) to 3.2% (FEV_1/FVC). For diffusing-capacity variables, variability ranged from 1.7% (V_A) to 3.5% (DLCO). In absolute units, DLNO had an SDw of 4.8 mL/min/mmHg [95% CI 4.4, 5.4] and DLCO had an SDw of 1.2 mL/min/mmHg [1.1, 1.3]. Adjustment for the fitted linear time-of-day trend reduced residual variance by 5.0% for DLNO, 7.4% for DLCO, and 6.4% for V_A ; SDw changed from 4.84 to 4.72 mL/min/mmHg, 1.19 to 1.15 mL/min/mmHg, and 0.121 to 0.117 L, respectively.

3.5. Empirical within-day change limits

Table 4 presents empirical within-day change limits. Under the stated no-change model, an FEV_1 difference of 0.16 L corresponds to the one-sided 80% limit and 0.31 L to the one-sided 95% limit; for FVC, the corresponding limits are 0.16 and 0.32 L. For diffusing capacity, the one-sided 80% limits are 5.7 mL/min/mmHg for DLNO and 1.4 mL/min/mmHg for DLCO. The 95% repeatability limits for the absolute difference between two session means are 13.4 mL/min/mmHg for DLNO and 3.3 mL/min/mmHg for DLCO.

4. Discussion

This study demonstrates that FVC, FEV_1 , SVC, DLNO, DLCO, and V_A decrease significantly from morning to late afternoon in healthy young men, while the FEV_1/FVC ratio, the transfer coefficient KNO and the DLNO/DLCO ratio remain stable throughout the day. After correcting for the false discovery rate, KCO did not show significant change throughout the day. To our knowledge, this is the first study to characterise the diurnal variation of simultaneous DLNO/DLCO across multiple time points within a single day.

The direction of diurnal change in spirometry observed here—a decline from a morning peak toward lower afternoon and evening values—is consistent with the classical pattern of peak lung function in the morning [1–3] and with a prior meta-analysis reporting that the magnitude of diurnal spirometric variation is not uniform across populations [5]. The absence of a diurnal change in FEV_1/FVC indicates that both FVC and FEV_1 decline proportionally, suggesting that the primary mechanism is a change in lung volume rather than selective changes in airway calibre. Several factors may contribute to a progressive fall in lung volume across the waking day, including cumulative postural fluid shifts, diurnal variation in bronchomotor tone, and mild respiratory-muscle fatigue with repeated maximal manoeuvres [39].

The significant time-of-day decreases in DLNO and DLCO paralleled the decline in V_A , whereas KNO and the DLNO/DLCO ratio showed no significant systematic change, and the decline in KCO did not survive false-discovery-rate adjustment (Table 2). Because $DLNO = KNO \times V_A$ and $DLCO = KCO \times V_A$, these quantities are algebraically coupled and cannot be considered fully independent. The common V_A term cancels in

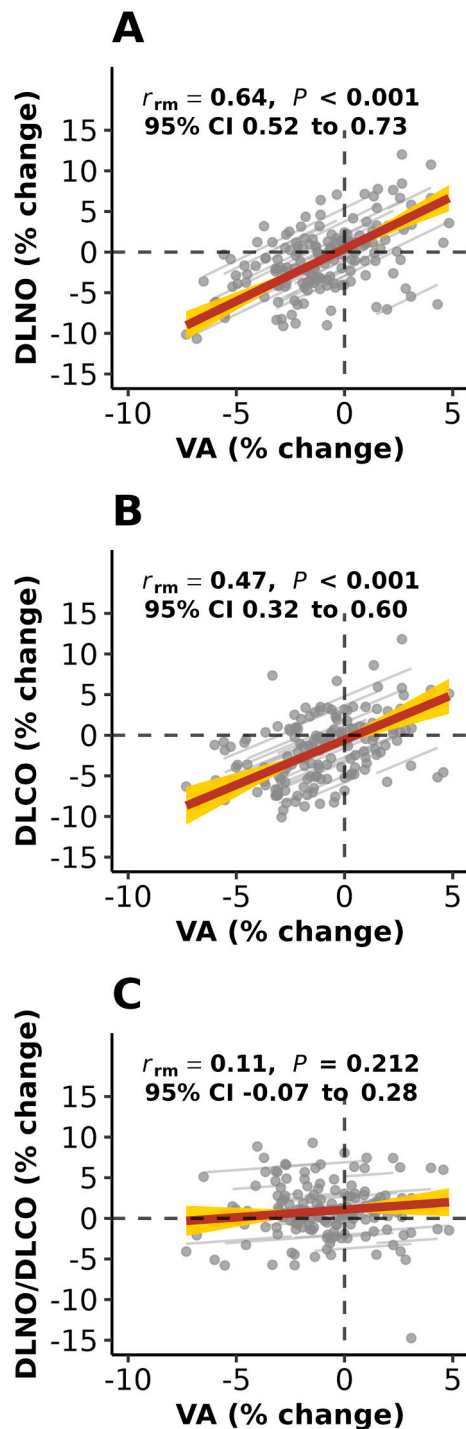


Fig. 3. Associations between within-day relative change in alveolar volume (VA) and relative changes in (A) diffusing capacity for nitric oxide (DLNO), (B) diffusing capacity for carbon monoxide (DLCO), and (C) the DLNO/DLCO ratio in 158 quality-filtered observations from 36 participants. Values are expressed as percent change from each participant's first morning session; that reference session is zero for both variables by definition and is therefore excluded from the plots and from the correlations. Grey points represent individual sessions and thin grey lines the participant-specific parallel fits; the bold line shows the common within-participant association with its 95% confidence band. Dashed lines indicate no change in VA (vertical) and no change in the diffusing-capacity variable (horizontal). Repeated-measures correlations (rmcorr) were $r = 0.64$ (95% CI 0.52 to 0.73; $p < 0.001$) for DLNO, $r = 0.47$ (0.32 to 0.60; $p < 0.001$) for DLCO, and $r = 0.11$ (−0.0724 to 0.28; $p = 0.212$) for DLNO/DLCO, each with 121 degrees of freedom.

Table 3

Total observed within-subject variability across the study day.

	Total observed within-day variability (%) [95% CI]	Total observed within-day variability (units) [95% CI]
Spirometry Variables		
SVC	1.5% [1.4, 1.7]	0.10 L [0.09, 0.11]
FVC	2.2% [2.0, 2.5]	0.14 L [0.12, 0.15]
FEV ₁	2.8% [2.5, 3.2]	0.13 L [0.12, 0.15]
FEV ₁ /FVC	3.2% [2.9, 3.6]	0.02 [0.02, 0.03]
Diffusing Capacity Variables		
DLNO	3.1% [2.8, 3.5]	4.8 mL/min/mmHg [4.4, 5.4]
DLCO	3.5% [3.1, 3.9]	1.2 mL/min/mmHg [1.1, 1.3]
V _A	1.7% [1.5, 1.9]	0.12 L [0.11, 0.14]
DLNO/DLCO	2.5% [2.3, 2.8]	0.12 [0.10, 0.13]
KNO	2.5% [2.2, 2.8]	0.5 mL/min/mmHg/L [0.5, 0.6]
KCO	3.1% [2.8, 3.5]	0.15 mL/min/mmHg/L [0.14, 0.17]

The number of hours between the first and last testing sessions averaged 10.9 h (SD 1.9 h) within the day; the average number of testing sessions for each subject was 5.4 (SD 1.2) sessions within the day. Quality filter applied: removed rows where V_A -SVC > 1.50, IV/SVC > 1.03, or IV/SVC < 0.845. N = 194 observations from 36 subjects after filtering. Within-subject SD (SD_w) from intercept-only mixed model; 95% CI from chi-squared distribution. SD_w represents total observed within-subject variability and includes random instrument measurement error, manoeuvre and operator variation, unsystematic short-term physiological variation, and any systematic time-of-day trend not removed by the intercept-only model; these components cannot be separated from the available session means. DLCO, KCO and DLNO/DLCO are standardised to each participant's baseline haemoglobin rather than the session-specific value.

the ratio, so $DLNO/DLCO = KNO/KCO$; however, this algebraic cancellation does not guarantee physiological independence from operating lung volume because KNO and KCO may respond differently to changes in lung volume. The observed pattern is consistent with, but does not prove, a volume-mediated explanation. This interpretation is consistent with observations that body posture and lung volume influence DLNO and DLCO [21,22] and aligns with the physiological principle that changes in alveolar volume as a percent of maximum affect DLNO more than DLCO [40,41].

The DLNO/DLCO ratio can help identify various pathologies compared to control groups. Carbon monoxide poisoning, pulmonary arterial hypertension and pulmonary embolism can increase the ratio [11,42–46], while bronchopulmonary dysplasia, cystic fibrosis, and chronic mountain sickness can decrease the ratio [47–49]. Thus, knowing the diurnal variation is clinically important. This study found no significant systematic time-of-day change in the DLNO/DLCO ratio. Under the study's no-change model, a directional ratio change of 0.27 units is the one-sided 95% empirical limit (Table 4); exceeding this limit does not prove a physiological change. Because KNO and KCO are calculated by normalizing DLNO and DLCO to the same VA, their stability should not be interpreted as complete independence from VA or operating lung volume.

The total observed within-subject variability and empirical within-day change limits in Tables 3 and 4 may inform clinical and research interpretation, but they should be considered preliminary and specific to the device, protocol, population, and averaging scheme. Current guidelines do not specify time-of-day thresholds for DLCO monitoring. The one-sided 95% limit for DLCO was 2.8 mL/min/mmHg; this is similar in magnitude to within-session repeatability reported elsewhere [15]. For DLNO, the one-sided 95% within-day change limit was 11.2 mL/min/mmHg; published estimates of repeatability vary across studies and protocols, with smaller limits in some [11,15] but not all studies [17]. Furthermore, practicing the single-breath manoeuvre over three study visits can reduce the repeatability of DLNO from ~15 to ~9 mL/min/mmHg [16]. The 13.4 mL/min/mmHg coefficient of repeatability for DLNO is the 95% limit for the absolute difference

Table 4

Empirical change limits based on total observed within-day within-subject variability.

		One-sided empirical change limits (specified direction)		
95% repeatability limit for the absolute difference between two session means ($2.77 \times \text{SDw}$)		80% one-sided limit (4/5) $z = 0.84$	90% one-sided limit (9/10) $z = 1.28$	95% one-sided limit (19/20) $z = 1.64$
Spirometry Variables				
FEV ₁ (L)	0.37	0.16	0.24	0.31
FVC (L)	0.38	0.16	0.25	0.32
FEV ₁ /FVC	0.07	0.03	0.04	0.06
SVC (L)	0.27	0.11	0.17	0.22
Diffusing Capacity Variables				
DLNO (mL/min/mmHg)	13.4	5.7	8.8	11.2
DLCO (mL/min/mmHg)	3.3	1.4	2.2	2.8
V _A (L)	0.33	0.14	0.22	0.28
DLNO/DLCO	0.32	0.14	0.21	0.27
KCO (mL/min/mmHg/L)	0.42	0.18	0.27	0.35
KNO (mL/min/mmHg/L)	1.5	0.6	1.0	1.3
Change in z-scores				
FEV ₁ (z-score)	0.65	0.28	0.42	0.54
FVC (z-score)	0.53	0.23	0.34	0.44
FEV ₁ /FVC (z-score)	0.99	0.43	0.65	0.83
DLNO (z-score)	0.70	0.30	0.46	0.59
DLCO (z-score)	0.70	0.30	0.46	0.59
VA (z-score)	0.41	0.18	0.27	0.35
KCO (z-score)	0.66	0.28	0.43	0.56

Limits were calculated from the total observed within-subject SD (SDw). The 95% repeatability limit for the absolute difference between two independent session means is $1.96 \times \sqrt{2} \times \text{SDw} = 2.77 \times \text{SDw}$. One-sided empirical change limits are $\sqrt{2} \times \text{SDw} \times z$. For example, if SDw for FEV₁ is 0.135 L, then $\sqrt{2} \times \text{SDw}$ is 0.191 L and the one-sided 80% limit is $0.191 \times 0.84 = 0.160$ L. Under a no-change model, a decline larger than 0.160 L would occur from the total variation represented by SDw with probability 20% in that direction; it does not prove a physiological change. Change-in-z-score limits were derived identically from the within-subject SD of the corresponding z-scores: FEV₁, FVC, and FEV₁/FVC (GLI-2012); DLCO, VA, and KCO (GLI-2017); and DLNO (GAMLSS-Zavorsky & Cao, 2022). DLCO, KCO and DLNO/DLCO are standardised to each participant's baseline haemoglobin rather than the session-specific value.

between two session means, not a confidence interval for a single measurement. It was smaller than week-to-week reproducibility estimates of approximately 20 mL/min/mmHg reported elsewhere [17,18,20], and similar to the week-to-week reproducibility found in patients with cystic fibrosis [16]. Thus, exceeding these empirical limits makes the total random variation represented by this study less likely, but does

not by itself prove that a physiological change occurred.

Although DLNO possesses clear technical strengths and offers clinically relevant complementarity with DLCO, the test remains underused in routine pulmonary function testing [50]. Many laboratories do not currently measure DLNO, showing a thin commercial ecosystem, regulatory inertia (including the absence of FDA clearance for combined NO-CO systems in some regions), device-specific differences within sensor technology, response characteristics, and calculation algorithms, and the lack of globally harmonized reference equations that adequately account for altitude and genetic ancestry [50]. The modern single-breath DLNO—an ERS Task Force has standardized DLNO technique [35], and is ready for clinical use, yet acceptance has lagged. Recent individual-participant data meta-analyses nevertheless demonstrate that adding DLNO to DLCO improves diagnostic classification and prediction in heart failure [26], emphysema [25], and post-COVID [24] patients, compared to controls, supporting the pragmatic recommendation to measure both gases together whenever equipment permits.

Several limitations of this study should be acknowledged. In particular, the interpretive thresholds reported here should be regarded as preliminary estimates derived from a small, healthy, young, male military-diver cohort; they require validation before being applied to women, older adults, patients with cardiopulmonary disease, or routine clinical pulmonary function populations. First, only men were studied. Hormonal influences on pulmonary vasculature and haemoglobin binding—including menstrual cycle phase and oestrogen effects—may alter diurnal patterns and MDC thresholds in women, and future studies should include female participants. Second, participants were all healthy, relatively young (median age ~30 years), physically active adults with high lung function values. Older individuals may exhibit greater biological variability due to subclinical disease or vascular stiffness, meaning that the MDC thresholds derived here may be too narrow for older or diseased populations. Third, the sample size of 36 subjects, while sufficient to detect the reported effects, limits our ability to characterise individual variation in diurnal profiles or to perform subgroup analyses by age or baseline lung function. Fourth, we did not standardise meals, caffeine, hydration, or posture between testing sessions, all of which may influence pulmonary function variables—particularly DLCO and VA. Ambient temperature and humidity were also not recorded. Fifth, because testing occurred over a single waking day without control of sleep timing, chronotype, light exposure, or posture, our findings reflect a within-day (time-of-day) effect rather than an endogenous circadian rhythm. A multi-day protocol would have allowed assessment of reproducibility and true circadian patterns. Finally, the results and empirical change limits were obtained with a single Jaeger MasterScreen™ PFT Pro using an approximately 8-s breath-hold protocol and session means of about three technically acceptable manoeuvres. They therefore incorporate device- and algorithm-specific effects, calibration, operator and participant performance, and short-term biological variation, and may not generalise to other systems or protocols. Individual-manoeuve or phantom replicate data were not available, so random instrument measurement error could not be separated from the other components of the observed within-subject variability.

In conclusion, spirometry volumes and absolute DLNO, DLCO, and VA decreased from morning to late afternoon in healthy young men, whereas the volume-normalized indices and DLNO/DLCO ratio showed no detectable systematic time-of-day effect. This pattern is consistent with approximately proportional, volume-related changes, but the variables KNO, KCO and the DLNO/DLCO ratio are algebraically coupled and are not fully independent. The empirical change limits are specific to the device, protocol, population, and averaging scheme used here and do not by themselves prove that a physiological change has occurred. Serial measurements should be performed at consistent times of day.

CRedit authorship contribution statement

Pieter-Jan van Ooij: Writing – review & editing, Project administration, Methodology, Data curation. **Antoinette Houtkooper:** Writing – review & editing, Supervision, Methodology, Data curation. **Gerald S. Zavorsky:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics declaration

Written informed consent to take part in the study and to publish the article has been obtained from all participants or their legal representatives. The privacy rights of participants have been observed.

This study was performed in compliance with relevant laws, regulatory frameworks and guidelines where the research took place. This study was approved by the Institutional Review Boards of University Medical Center Groningen (IRB # METC 2010/089) and Academic Medical Center Amsterdam (IRB # NL35768.018.11). (Approval No. METC 2010/089 and NL35768.018.11)

Use of generative artificial intelligence

During the revision of this manuscript, the authors used Claude (Anthropic, San Francisco, CA, USA), a generative AI assistant, to improve the clarity and readability of selected passages in the Discussion and to check statistical code written in R. The authors reviewed and edited all output and take full responsibility for the content of the manuscript. No generative AI tools were used for data analysis, interpretation of results, or generation of scientific conclusions.

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Declaration of competing interest

Gerald S. Zavorsky is a member of the Global Lung Function Initiative (GLI) Network, which has published reference equations for spirometry, diffusing capacity, and static lung volumes, and is a member of the American Thoracic Society Pulmonary Function Testing Committee. The authors report there are no other competing interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.niox.2026.08.002>.

Data availability

The data are attached as an online supplement.

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