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Novel volumetric capnography indices measure ventilation inhomogeneity in cystic fibrosis

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Take home message: Novel volumetric capnography indices are promising markers of ventilation inhomogeneity in patients with cystic fibrosis.
1. Introduction

Volumetric capnography (VCap) is a simple and non-invasive lung function technique that describes the dynamics of carbon dioxide (CO₂) exhalation breath-by-breath [1]. The volume-based capnogram is the plot of CO₂ concentration against the exhaled air volume, and consists of three phases: Phase I, represents the washout of the uppermost conductive airways that contain atmospheric -CO₂-free- air; Phase II, that is characterized by a steep CO₂ rise and reflects the mixing between atmospheric air and CO₂-rich gas from the alveolar compartment, and Phase III, the so-called “alveolar plateau”, that represents the expiration of alveolar gas. Volumetric capnography allows for the assessment of dead space ventilation and ventilation-perfusion abnormalities [2-7], while the slope of phase III (SIII) is considered an index of ventilation inhomogeneity (VI) in obstructive lung disorders, such as cystic fibrosis (CF) [7-11].

Since VCap does not require a complex measurement setup or extensive signal processing, it may be considered a simpler alternative to techniques aiming to detect VI, such as Multiple Breath Washout (MBW) [2, 7, 10]. However, classical VCap indices are considerably dependent on expiratory volume (VE) and respiration dynamics [12-14]. This is particularly important in children, where variable breathing patterns may significantly affect the diagnostic performance of the method [6, 7, 9, 15].

In this paper, we introduce novel VCap indices, called Capnographic Inhomogeneity Indices (CIIs) that may overcome the above limitations. Moreover, they may serve as promising VI indices in clinical settings where advanced MBW setups are not available, or as faster and more attainable markers in obstructive lung disorders that require continuous follow-up. We assess the feasibility and repeatability of these novel CIIs in clinical practice, and we explore their diagnostic performance in a cohort of CF patients.
2. Methods

2.1 Study design and population

In this pilot study, novel VCap indices were validated using existing Nitrogen (N₂) MBW data from 4-18-year-old CF patients and healthy controls. All measurements were obtained at the University Children’s Hospital of Bern, Switzerland, between 2013-2019 (see sample size estimation, below) [16-21]. All subjects were free from pulmonary exacerbations [16-21]. Only subjects with at least two good quality MBW trials were included. Local ethic committees approved all studies, and participants or caregivers provided informed written consent.

2.2 Lung function

MBW trials were performed with the children in sitting position while breathing normally, according to international guidelines [22]. N₂-MBW measurements were obtained as previously described [20], using the Exhalyzer D device (Ecomedics, Duernen, Switzerland) that incorporates an ultrasonic flowmeter and a main-stream CO₂ sensor (Capnostat, infrared single beam, dual-wavelength technology; rise time <60ms) with Spiroware version 3.1.6 or 3.2.1. All data were reloaded and reanalyzed using the updated software provided by the manufacturer Spiroware 3.3.1. MBW quality control was performed by experienced operators, based on established criteria [21, 22].

The following MBW parameters were investigated: functional residual capacity (FRC) lung clearance index (LCI), tidal volume, respiratory rate and minute ventilation [22]. Additional information on age, sex, weight and height were obtained from patient files.
2.3 Volumetric capnography

Volumetric capnograms were obtained from N$_2$-MBW trials after CO$_2$ signals were corrected for setup-dependent signal alignment and sensor-specific delays (Spiroware software). Breath-by-breath volumetric capnograms were obtained by plotting the CO$_2$ fraction against the corresponding VE. The expired CO$_2$ volume (VECO$_2$) was calculated by integrating the CO$_2$ signal over VE. The slope of phase II (S$_{II}$) and the S$_{III}$ were obtained by fitting a linear regression line (least squares method) over the capnogram, between 10 and 60% of the end-tidal CO$_2$ value and 65-95% of VE, respectively [6]. The capnographic index (KPIv) was calculated as the ratio S$_{III}$/S$_{II}$. The slopes were normalized by the corresponding mixed expired CO$_2$ fraction (FECO$_2$), which equals the VECO$_2$ divided by VE [12]. The airway dead space (VD) was calculated using the equal-area method proposed by Fowler [23].

To avoid inclusion of irregular capnograms, MBW breaths were automatically excluded from analysis if: 1. end-tidal CO$_2$ (ETCO$_2$) was <3.5%; 2. their corresponding VE deviated more than two SDs from trial average; 3. VD was <10% or >30% of VE; 4. intersection of S$_{II}$ and S$_{III}$ was not calculable, fell outside the capnogram range (i.e. intersection at VE<0, VE>VE at the end of expiration, CO2>CO2max), or below the CO$_2$ curve; 5. S$_{III}$ was steeper than S$_{II}$; 6. coefficient of determination ($R^2$) for S$_{III}$ fitting was <0.7. For all acceptable breaths of each MBW trial, we calculated average ETCO$_2$, S$_{II}$, S$_{III}$, normalized S$_{II}$ (nS$_{II}$), normalized S$_{III}$ (nS$_{III}$), KPIv, and VD values. Calculation of VCap indices and quality control were performed using a custom Python script (Python Software Foundation, https://www.python.org/).
2.4 Capnographic Inhomogeneity Indices

2.4.1 Modeling ventilation inhomogeneity

To understand the calculation of novel CIIs, we consider a lung model (Figure 1) in which we define $V_A$, the volume of the alveolar compartment that contributes to expiration, $V_D$ the volume of the dead space compartment, and $V_E$ the expired volume of air. Let us assume that the alveolar compartment comprises of three equal sub-compartments connected in series, which empty sequentially without air mixing among them (Figure 1). At end-inspiration, the dead space compartment is filled with atmospheric air (with a CO$_2$ concentration of zero). All CO$_2$ is contained in $V_A$, at a mixed alveolar concentration of $F_A$CO$_2$, which is the average CO$_2$ concentration of alveolar sub-compartments (i.e., $F_{A1}$CO$_2$, $F_{A2}$CO$_2$ and $F_{A3}$CO$_2$) (Figure 1). As expiration commences, dead space air emerges first, followed by gas from the three alveolar sub-compartments. At end-expiration, the concentration of CO$_2$ in the expired air ($F_E$CO$_2$) results from the mixing between the CO$_2$-free dead space air and the CO$_2$-rich alveolar air; an amount of CO$_2$ remains in the dead space compartment at a concentration $F_D$CO$_2$ (Figure 1).

In an ideal lung, $F_{A1}$CO$_2$, $F_{A2}$CO$_2$ and $F_{A3}$CO$_2$ are equal (Figure 1A). Thus, time-dependent inhomogeneities do not exist, the rate of CO$_2$ exhalation is constant, and the phase III of the capnogram is horizontal (Figure 1A). In this case, $F_D$CO$_2$ at end-expiration equals $F_A$CO$_2$ (Figure 1A). When time-dependent inhomogeneities exist, due to stratified VI distal to the airway-alveolar interface and delayed emptying of respiratory units with low ventilation-perfusion (V/Q) ratios [24, 25], it applies that $F_{A1}$CO$_2$ < $F_{A2}$CO$_2$ < $F_{A3}$CO$_2$ (Figure 1B). In this case, the rate of exhaled CO$_2$ increases as expiration commences, and the S$_{III}$ rises (Figure 1B). At end-expiration, $F_D$CO$_2$ equals $F_{A3}$CO$_2$ (Figure 1B). The magnitude of sequential inhomogeneity in this model is reflected by the difference among $F_{A1}$CO$_2$, $F_{A2}$CO$_2$ and $F_D$CO$_2$
(Figure 1B). However, since neither FACO₂ nor FdCO₂ can be measured \textit{in vivo}, these differences cannot be computed.

2.4.2 Estimating FdCO₂

Let us consider the volumetric capnogram of Figure 2A and analyze it according to the concept of Aitken and Clark-Kennedy [26], as follows: a) The volume of CO₂ that remains in the dead space compartment at end-expiration (VdCO₂) can be obtained by extending the line of phase III to the right (line be) using linear regression until the distance cf becomes equal to Vd, and calculating the area of the trapezoid befc; the FdCO₂ is the area befc divided by Vd. b) The total volume of CO₂ leaving the alveolar compartment (VACO₂) is the area of the trapezoid aefd; FACO₂ can be obtained by dividing this area by Ve (Figure 2A).

The above concept was applied to volumetric capnograms of our study (Figure 2B). After the Vd was calculated by Fowler’s method [23], each capnogram was extended to the “right” (linear extension according to SIII) by Vd. The VeCO₂ was computed as the integral of the CO₂ signal over Ve, and the VdCO₂ as the integral of the extended part of the capnogram over Vd (Figure 1B); the VACO₂ was computed as the sum VeCO₂ and VdCO₂. Then, by dividing VeCO₂ by Ve, VdCO₂ by Vd, and VACO₂ by Ve, the FdCO₂, FACO₂ and FACO₂, respectively, were calculated. In addition, the concentration of CO₂ in the exhaled air coming exclusively from the alveolar compartment (FexCO₂) was calculated by dividing VeCO₂ by the difference Ve – Vd.

2.4.3 Capnographic inhomogeneity indices

Two novel CIIs were calculated:

A. CII₁, which is the relative difference between FdCO₂ and FACO₂ or \(\frac{(FdCO₂ - FACO₂)}{FACO₂}\) and represents a raw estimate of sequential inhomogeneity.
B. CII2, which is the relative difference between FDCO2 and FexCO2 or (FDCO2 – FexCO2) / FexCO2.

Since CII2 reflects the difference of CO2 concentration between the alveolar sub-compartments A1+A2 and A3 (Figure 1B), it should theoretically represent a more precise estimate of sequential VI.

2.5 Statistics

2.5.1 Sample size estimation

Since CIIIs were introduced for the first time in clinical practice, no data were available for a priori sample size estimation. Based on the hypothesis that CIIIs (as markers of VI) would be correlated with the LCI, we estimated that MBW measurements from at least 112 subjects would be required to reveal a significant correlation (P <0.05), with a Pearson’s r ≥0.3 and at least 90% power. By reviewing the database of our laboratory (different independent studies [16-21]), we found that such a sample with at least two acceptable MBW trials could be obtained by including the children measured between January 2013 and December 2019 (N=115; 50 CF and 65 healthy controls). We preferred to include all 115 children (instead of 112) for reasons of consistency. Post-hoc effect size calculation was also performed.

2.5.2 Statistical analyses

VCap indices were assessed both per subject (average of at least two acceptable trials) and per MBW trial (analysis per trial) to allow better physiological appraisal. Per subject data are presented throughout the manuscript, while per trial analyses are presented in the Online supplement (OLS). Between-group comparisons were performed using student’s t test. The percentage of breaths acceptable for VCap analysis was calculated as percentage of all washout breaths. Linear and non-linear regression was applied to assess the relationship between SIII
and $V_ε$, and $S_{III}$ and CIs. Pearson correlation analysis was used to assess the relationship between LCI and VCap outcomes. The intra-trial (i.e. between breaths of the same trial) and inter-trial (between different trials of the same subject) variability was calculated using the coefficient of variation (CV). In presence of only two acceptable MBW, the inter-trial variability was calculated as relative difference between the two trials. Receiver Operation Characteristics analysis was used to estimate the overall diagnostic ability of LCI and VCap indices by means of Area Under the Curve (AUC). Optimal cutoff values for each index were determined using Youden Index analysis. All analyses were performed in Stata (StataCorp, College Station, TX).

3. Results

All 320 MBW-trials from 50 patients with CF (137 trials) and 65 HCs (183 trials) were analyzed. The clinical characteristics and lung function parameters of the two study groups are presented in Table 1. The LCI was significantly higher in CF patients compared to healthy children (mean±SD LCI 7.8±1.7 vs 6.2±0.4, P<0.001).

3.1 VCap indices

Calculation of VCap indices was feasible in all trials of all study participants, independent of disease status. The percentage of breaths within each trial that were acceptable for VCap analysis, was higher in CF patients compared with healthy children (72.4±17.8 (42.7-98.3)% vs 63.5±19.3 (19.7-96.7)%). Further information regarding non-accepted breaths (percentages, stratification per exclusion criteria) is presented in Table S2. Slope III and KPIv were higher in CF patients compared with healthy children ($S_{III}$ 2.3±1.0%/L vs 1.9±0.7%/L, P=0.013; KPIv 3.9±1.3% vs 3.5±1.2%, P=0.07), but only $S_{III}$ was significantly higher. The CIs were also significantly higher in CF patients compared with healthy controls ($CII_{1}$ 5.9±1.4% vs 5.1±1.0%,...
Post-hoc effect-size analysis revealed that these differences yield a study power of 71% and 68%, respectively, for the given level of statistical significance, or 96% and 89.5%, respectively, for a P-value <0.05. VCap parameters and indices are presented in Table 2, Table 3, and Figure 3. AUC values and the corresponding optimal cutoff values of LCI and VCap indices are presented in the OLS (Table S5). Above the cutoff value of LCI were classified 76% (n=38) of patients with CF and 10.8% (n=7) of controls, above the cutoff value of SIII 34% (n=17) of patients with CF and 10.8% (n=7) of controls, above the cutoff value of KPIv 76% (n=38) of patients with CF and 47.7% (n=31) controls, above the cutoff value of CII1 44% (n=22) of patients with CF and 20% (n=13) of controls, and above the cutoff value of CII2 48% (n=24) of patients with CF and 18.5% (n=12) of controls.

3.2 Correlations of VCap indices

There was a strong inverse curvilinear relationship between SIII and VE and a weak linear relationship between CII1 and SIII and CII2 and SIII (Figure 4, Figure S3). The correlations between classical VCap indices and CIIs are shown in the OLS (Table S6). The correlation SIII-LCI and KPIv-LCI was weak (SIII-LCI $R^2=0.03$; KPIv-LCI $R^2=0.08$ in CF patients), while the correlation between CIIs and LCI was stronger (CII1-LCI $R^2=0.47$ and CII2-LCI $R^2=0.44$ in CF patients). Overall (all participants), the Pearson’s correlation coefficient between CII1 and LCI was 0.572 and between CII2 and LCI 0.557; both values yield a study power of 100% (p-value < 0.001). More correlations between VCap parameters and LCI are presented in Table 4 and Table S7. Both CII1 and CII2 were significantly correlated with age ($R^2=0.162$ and $R^2=0.118$, respectively, while age was also significantly correlated with the LCI ($R^2=0.09$). There was no relationship between CIIs and sex in our cohort (data not shown).
3.3 Intra- and inter-trial variability

The intra-trial variability CVs of SIII and KPIv were higher compared with CII1 and CII2 (CV of SIII 37.5±19.2%, KPIv 35.3±15.5% vs CII1 31.1±8.9% and CII2 31.7±9.4%, in all trials). For all these VCap indices, the intra-trial variability was lower in CF patients compared with controls (Table S8). Similarly, the inter-trial (intra-subject) variability of SIII and KPIv was higher compared with CII1 and CII2 in all trials (SIII 16.3±13.5%, KPIv 15.9±12.8% vs CII1 11.1±8.2% and CII2 11.0±8.0%). However, the inter-trial variability was comparable between CF patients and controls (Table S9). The LCI showed lowest inter-trial variability (LCI 5.9±4.2%) (Table S9, Figure S2).

4. Discussion

In this pilot study, we introduced novel capnographic indices of VI and we assessed their diagnostic performance in comparison with classical VCap parameters (i.e. SIII and KPIv) and the LCI. We found that SIII, and the novel CIIIs were higher in CF patients than in controls. However, the novel capnographic indices CII showed better correlation with the LCI and lower intra-trial and inter-trial variability than SIII and KPIv, although their overall diagnostic performance was inferior to the LCI.

4.1 Performance of VCap indices

As expected, SIII and KPIv were increased in CF patients. These findings are in line with previous studies, suggesting that classical VCap indices may be useful VI markers in adults and children with CF [8-11, 27]. In CF, VI results in delayed CO₂ mixing within the conductive airways and, eventually, to non-homogeneous CO₂ exhalation (i.e. steeper phase III) [1, 3, 8] (Figure 1); the KPIv (i.e. the SIII to SII ratio) increases respectively [8]. Thus, increased SIII and KPIv are consistent findings in CF [9-11, 27], albeit their discriminative ability is moderate [11] and, in any case,
inferior to that of the LCI [10]. Of note, the correlation between $S_{III}$ and LCI or KPIv and LCI was rather weak. The latter contrasts the findings of Fuchs et al. [10] who showed a stronger correlation between those indices and LCI. The calculation of $S_{II}$ and $S_{III}$ on an “averaged”, user-defined capnogram in their study [10], as opposed to breath-by-breath calculation of VCap indices using well-defined criteria in ours, might explain these differences.

Conversely, CII1 and CII2 presented better discriminative characteristics than the classical VCap indices. In addition, both CIIs correlated significantly with the LCI - a robust index of VI in CF patients [28, 29], and did so better than the classical VCap parameters (Table 4). Thus, CIIs may be considered valid measures of VI, which may also relate to the severity of the disease.

4.2 Theoretical advantages of CIIs over classical VCap indices

The $S_{III}$ reflects the rate of CO$_2$ exhalation beyond mid-expiration and is an unstandardized index that depends on the dynamics of expiration, especially on VE [12-14]. However, normalization of $S_{III}$ by VE [11] is not justified, because as shown in previous studies [12-14] and confirmed in ours (Figure 4A, Figure S3A), the $S_{III}$-VE relationship is not linear. $S_{III}$ normalization by the FeCO$_2$ (i.e. the normalized $S_{III}$) may allow for intra- or inter-subject adjustment for different CO$_2$ concentrations, but does not eliminate the dependency from VE. Therefore, in subjects with variable respiratory patterns and/or changing lung volumes, as it typically is in children, the utility of $S_{III}$ is limited [14]. In our study, this disadvantage is also reflected by the unexpected negative correlation between $S_{III}$ and LCI (Table 4). Higher LCI values are typically seen in older CF patients due to the progression of the disease [21]; but since older children also have larger lung volumes, their $S_{III}$ is lower due to the strong $S_{III}$-VE relationship (Figure 4A, Figure S3A).
The theoretical background for calculation of CIIs is different: in lung disease, variable gas mixing within the respiratory units (serial inhomogeneities) and/or regional $\dot{V}/Q$ variations (parallel inhomogeneities) result in sequential variations of CO$_2$ concentration distal to the airway-alveolar interface, which are exacerbated further by the delayed emptying of respiratory units with altered mechanical properties and low $\dot{V}/Q$ [23, 24]. Overall, these phenomena result in time-dependent inhomogeneities of CO$_2$ concentration that can be detected at the airway opening [1, 12]. The CIIs, which according to the proposed model (Figure 1) are calculated as differences of CO$_2$ concentrations, reflect these time-dependent inhomogeneities. The CO$_2$ concentrations (i.e. VCO$_2$ - $V_E$ fractions) also include an inherent normalization for $V_E$ and their use may thus overcome the limitations of classical VCap indices. The significant positive correlation between CIIs and LCI and the weak correlation between CIIs and $S_{III}$ further support this hypothesis.

4.3 Feasibility and repeatability of CIIs

CIIs calculation was feasible in all MBW trials, independent of disease status. The percentage of acceptable breaths for VCap analysis was approximately 75% in CF and 65% in control trials. We found lower variability in CIIs compared with $S_{III}$ and KPIv, but higher compared with LCI. The higher percentage of acceptable breaths together with a lower intra-trial variability found in CF patients may be because they were familiar with the MBW procedure and, thus, able to maintain more stable breath patterns during MBW measurement.

4.4 Strengths and limitations

This study presents an innovative method for VI assessment that in principle requires only flow and CO$_2$ signals, thus being potentially more attainable in clinical settings. In our large cohort
of both healthy children and children with CF, with a wide age range and diversity in disease severity, these novel CIIs showed a good association with the more complicated LCI, thus confirming their clinical potential. The use of N₂-MBW files facilitated direct comparison of MBW and VCap outcomes derived from the same files, limiting the effect of possible influencing factors (e.g. related to breathing pattern or testing circumstances). All MBW trials were analyzed with the newest Spiroware version (i.e., 3.3.1), so that our results are not affected by the recently-revealed sensor crosstalk error in the Exhalyzer D device [30]. Overall, Posteriori effect size calculations yielded study power in the range of 68-100% for the differences between CIIs in CF and controls and the Pearson's correlation coefficients between CIIs and LCI.

Inevitably, our study also has some limitations. First, VCap analysis was performed retrospectively using previously collected N₂-MBW data from one center. This implies that the baseline quality control criteria (e.g. those related to breathing pattern and variability) were specific to MBW [21, 22] and not to capnography. This may have affected the intra- and inter-trial variability of CIIs (which was larger than the inter-trial variability of the LCI) and influenced their discriminative ability. Prospective data acquisition for capnographic analysis, including instructions and/or incentives to reduce breathing variability and VCap-specific quality control, may improve the quality of capnograms, decrease the variability of CIIs and increase their diagnostic performance. Second, the effect of 100% O₂ concentration (N₂-MBW technique) on VCap parameters is unknown. However, the effect, if any, would not be different between CF and controls.

Finally, CIIs calculation was based on the extension of capnograms “to the right”. Arguably, the evolution of events that determine phase III cannot be predicted [25]. Yet, if we assume that these events remain stable during the end of expiration, a forward extension of
capnograms may be justified [26]. In fact, this assumption is the basis of the universally accepted Fowler's method to calculate VD [23], where the capnogram is extended “to the left”.

4.5 Clinical implications

Volumetric capnography is a simple non-invasive technique that does not require complex respiratory maneuvers, exogenous gases, extensive signal processing or operator’s expertise [2,5,7]. Current evidence suggests that classical VCap indices, such as $S_{III}$ and $KPI_v$, may be sensitive markers of early lung changes in patients with CF [11], however their diagnostic performance is limited due to the strong dependence on breathing dynamics [12-14]. Our results indicate that novel CIIs have lower variability compared to classical VCap indices, thus suggesting that they may be less influenced by the breathing pattern; however, further studies are needed to clarify this important issue. Future research should also focus on defining the methodological requirements and the proper quality-control criteria to obtain high-quality capnograms that might reduce variability and increase the performance of these novel indices. The novel CIIs should be assessed using simpler (i.e., non-MBW) setups, ideally on multiple occasions to allow for repeatability assessment, and in large cohorts with a range of obstructive lung disorders (e.g., asthma). Finally, further multicenter research is required to assess the external validity and the potential clinical value of this method.

5. Conclusions

In conclusion, the herein introduced CIIs performed better than the classical VCap parameters in detecting VI in CF patients. The CIIs also correlated well with the LCI and had lower variability compared to classical VCap indices. Their calculation was feasible in all study participants, independently of disease status. Thus, although their overall diagnostic performance was
inferior to the LCI, they may be considered as promising and simpler markers of VI. Further research is required to define the exact methodological requirements, improve the diagnostic performance, and assess the true clinical value of CIIs, especially at the bedside.

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**Conflicts of interest:**

Sotirios Fouzas has nothing to disclose.

Anne-Christianne Kentgens repots no other conflicts of interest.

Olga Lagiou has nothing to disclose.

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Table 1. Characteristics and lung function of the study groups

|                               | Cystic Fibrosis (n=50) | Healthy Controls (n=65) |
|-------------------------------|------------------------|-------------------------|
| **General characteristics**   |                        |                         |
| Male sex, n (%)               | 54.0 (50.3)            | 53.8 (50.2)             |
| Age (years)                   | 9.8 (4.1)              | 10.1 (3.9)              |
| Weight (kg)                   | 32.3 (14.3)            | 37.8 (17.6)             |
| Weight (z-score)*             | -0.1 (0.8)             | 0.4 (0.8)               |
| Height (cm)                   | 134.4 (19.7)           | 140.7 (23.0)            |
| Height (z-score)*             | -0.1 (0.8)             | 0.4 (1.0)               |
| Body Mass Index (kg/m²)       | 17.0 (2.6)             | 17.9 (3.1)              |
| Body Mass Index (z-score)*    | 0.0 (0.8)              | 0.2 (0.8)               |
| **N₂-MBW**                    |                        |                         |
| Number of trials              | 137                    | 183                     |
| Tidal volume (mL)             | 402.9 (175.8)          | 437.4 (199.0)           |
| Tidal volume per kg (mL/kg)   | 12.7 (2.8)             | 12.0 (2.7)              |
| Respiratory rate (per minute) | 18.7 (4.1)             | 18.2 (4.8)              |
| Minute ventilation (mL/kg x min) | 231.2 (46.0)    | 215.3 (67.6)            |
| FRC (ml/kg)                   | 40.2 (7.8)             | 41.4 (8.9)              |
| LCI 2.5%                      | 7.8 (1.7)              | 6.2 (0.4)*              |

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student’s T test)

# Weight, height and BMI z-scores were calculating according WHO growth charts. Weight z-scores for children older than 10 years of age were calculated according to CDC growth charts.

N₂-MBW: nitrogen multiple breath washout, FRC: functional residual capacity, LCI: lung clearance index, CDC: Centers for Disease Control and Prevention (CDC)
Table 2. VCap parameters and indices

| VCap Parameters and Indices | Cystic Fibrosis (n= 50) | Healthy Controls (n=65) | Mean difference (95% confidence interval) |
|-----------------------------|-------------------------|-------------------------|------------------------------------------|
| VE (mL)                     | 402.9 (174.6)           | 438.7 (201.6)           | 35.7 (-35.2 to 106.7)                    |
| VE per kg (mL/kg)           | 12.7 (3.1)              | 12.1 (2.7)              | -0.7 (-1.8 to 0.4)                       |
| VD (mL)                     | 88.6 (27.5)             | 98.3 (34.3)             | 9.8 (-2.0 to 21.5)                       |
| VD per kg (mL/kg)           | 2.9 (0.5)               | 2.8 (0.5)               | -0.1 (-0.3 to 0.1)                       |
| VD % of VE (%)              | 23.7 (4.9)              | 23.9 (4.3)              | 0.2 (-1.5 to 1.9)                        |
| ETCO₂ (%)                   | 5.3 (0.5)               | 5.3 (0.5)               | 0.0 (-0.1 to 0.2)                        |
| VCO₂ (mL)                   | 16.3 (5.9)              | 18.2 (7.9)              | 1.9 (-0.7 to 4.6)                        |
| FECO₂ (%)                   | 3.9 (0.4)               | 4.0 (0.4)               | 0.0 (-0.1 to 0.2)                        |
| SII (%/L)                   | 59.3 (19.2)             | 54.5 (16.8)             | -4.8 (-11.5 to 1.9)                      |
| Normalized SII (1/L)        | 15.0 (4.2)              | 13.7 (3.9)              | -1.2 (-2.7 to 0.3)                       |
| SIII (%/L)                  | 2.3 (1.0)               | 1.9 (0.7)               | -0.4 (-0.7 to -0.1)*                     |
| Normalized SIII (1/L)       | 0.6 (0.3)               | 0.5 (0.2)               | -0.1 (-0.2 to 0.0)*                      |
| KPIv (%)                    | 3.9 (1.3)               | 3.5 (1.2)               | -0.4 (-0.9 to 0.0)                       |

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student's T test)

VCap: volumetric capnography, VE: expiratory volume, VD: dead space volume, ETCO₂: end-tidal CO₂ fraction, VCO₂: expired CO₂ volume, FECO₂: mixed expired CO₂ fraction, SII: slope of phase II, SIII: slope of phase III, KPIv: capnographic index
Table 3. Novel VCap parameters and CII s

|                     | Cystic Fibrosis (n=50) | Healthy Controls (n=65) | Mean difference (95% confidence interval) |
|---------------------|------------------------|-------------------------|------------------------------------------|
| FACO₂ (%)           | 5.1 (0.5)              | 5.2 (0.5)               | 0.1 (-0.1 to 0.2)                        |
| FDCO₂ (%)           | 5.4 (0.5)              | 5.5 (0.5)               | 0.0 (-0.1 to 0.2)                        |
| FexCO₂ (%)          | 5.1 (0.5)              | 5.1 (0.5)               | 0.1 (-0.1 to 0.2)                        |
| CII1 (%)            | 5.9 (1.4)              | 5.1 (1.0)               | -0.7 (-1.2 to -0.3)*                     |
| CII2 (%)            | 7.7 (1.8)              | 6.8 (1.4)               | -1.0 (-1.5 to -0.3)*                     |

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student’s T test)

VCap: volumetric capnography, FACO₂: CO₂ fraction in alveolar compartment, FDCO₂: CO₂ fraction in dead space compartment, FexCO₂: CO₂ fraction in the air expired from the alveolar compartment, CII: capnographic inhomogeneity index (see text for details)
Table 4. Correlations between VCap parameters and the Lung Clearance Index

|                  | Cystic Fibrosis | Healthy Controls |
|------------------|-----------------|------------------|
|                  | r    | R^2  | P-value*    | r    | R^2  | P-value*    |
| SII              | -0.52 | 0.27 | <0.001      | -0.04 | 0.00 | 0.764      |
| Normalized SII   | -0.44 | 0.20 | 0.002       | -0.01 | 0.00 | 0.912      |
| SIII             | -0.18 | 0.03 | 0.209       | 0.04  | 0.00 | 0.771      |
| Normalized SIII  | -0.07 | 0.01 | 0.623       | 0.06  | 0.00 | 0.629      |
| KPIv             | 0.29  | 0.08 | 0.04        | 0.124 | 0.02 | 0.325      |
| CII1             | 0.68  | 0.47 | <0.001      | 0.06  | 0.00 | 0.620      |
| CII2             | 0.66  | 0.44 | <0.001      | 0.11  | 0.01 | 0.389      |

Data are presented as Pearson correlation coefficients (r) and coefficients of determination (R^2).

* Student's T test

VCap: volumetric capnography, LCI: lung clearance index, SII: slope of phase II, SIII: slope of phase III, KPIv: capnographic index, CII: capnographic inhomogeneity index
Novel volumetric capnography indices measure ventilation inhomogeneity in cystic fibrosis

ONLINE SUPPLEMENT

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|                                | Cystic Fibrosis (n=137) | Healthy Control (n=183) | Mean differences (95% confidence interval) |
|--------------------------------|-------------------------|--------------------------|-------------------------------------------|
| Tidal volume, mL               | 390.8 (173.9)           | 424.8 (203.6)            | 33.9 (-8.6 to 76.5)                       |
| Tidal volume per kg, ml/kg     | 12.5 (2.8)              | 12.0 (2.9)               | -0.5 (-1.1 to 0.1)                        |
| Respiratory rate, per minute   | 18.9 (4.2)              | 18.6 (5.1)               | -0.4 (-1.4 to 0.7)                        |
| Minute ventilation, ml/kg x min| 232.0 (46.1)            | 219.8 (68.7)             | -12.1 (-25.5 to 1.2)                      |
| FRC, ml/kg                     | 39.9 (8.0)              | 40.9 (8.8)               | 1.0 (-0.9 to 2.9)                         |
| Mean LCI2.5%                   | 7.8 (1.7)               | 6.2 (0.5)                | -1.6 (-1.8 to -1.3)*                      |

**Table S1.** Basic Lung function characteristics and the Lung Clearance Index 2.5% in Cystic Fibrosis and healthy control (analysis per trial)

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student’s T test)

FRC: functional residual capacity, LCI: lung clearance index
|                          | Low ETCO₂ outlier | VE outlier | VD outlier | Intersection was not calculable | SII-SIII intersection outside the capnogram range | SII-SIII intersection below CO₂ curve | SIII steeper than SII | R² fit SIII <0.7 | All error |
|--------------------------|-------------------|------------|------------|---------------------------------|-----------------------------------------------|----------------------------------------|----------------------|-----------------|-----------|
| Cystic Fibrosis          | 1.0 (1.7)         | 4.3 (2.0)  | 13.5 (14.2)| 0.6 (2.0)                       | 0.0 (0.3)                                     | 0.3 (1.1)                              | 0.1 (0.5)            | 4.5 (7.5)       | 24.4 (16.5) |
| Healthy Control          | 1.2 (2.6)         | 4.4 (2.1)  | 15.3 (16.3)| 0.5 (1.4)                       | 0.1 (0.5)                                     | 0.3 (1.2)                              | 0.1 (0.3)            | 13.0 (17.6)     | 34.8 (19.2) |
| All trials               | 1.1 (2.3)         | 4.4 (2.0)  | 14.5 (15.4)| 0.5 (1.7)                       | 0.1 (0.4)                                     | 0.3 (1.1)                              | 0.1 (0.4)            | 9.3 (14.8)      | 30.4 (18.8) |

**Table S2.** N₂-MBW breaths that met one of the exclusion criteria as a proportion of the total breaths within a trial (%) (analysis per trial).

Meeting one exclusion criteria lead to direct exclusion and no further evaluation of other exclusion criteria.

Data are presented as mean (SD), unless stated otherwise.

ETCO₂: end-tidal CO₂ fraction, VE: expiratory volume, VD: dead space volume, SII: slope of phase II, SIII: slope of phase III
|                         | Cystic Fibrosis (n=137) | Healthy Control (n=183) | Mean differences (95% confidence interval) |
|-------------------------|-------------------------|--------------------------|---------------------------------------------|
| VE (mL)                 | 390.6 (173.1)           | 426.0 (206.5)            | 35.4 (-7.5 to 78.3)                         |
| VE per kg (mL/kg)       | 12.6 (3.0)              | 12.1 (3.0)               | -0.50 (-1.2 to 0.2)                         |
| VD (mL)                 | 86.9 (26.0)             | 95.9 (33.5)              | 9.0 (2.2 to 15.8)*                          |
| VD per kg (mL/kg)       | 2.9 (0.5)               | 2.8 (0.5)                | -0.1 (-0.2 to 0.0)                          |
| VD % of VE (%)          | 23.9 (5.2)              | 24.0 (4.9)               | 0.10 (-1.0 to 1.2)                          |
| ETCO\(_2\) (%)         | 5.3 (0.5)               | 5.3 (0.5)                | 0.0 (-0.1 to 0.1)                           |
| VCO\(_2\) (mL)         | 15.9 (5.8)              | 17.7 (8.1)               | 1.8 (0.2 to 3.4)*                           |
| FECO\(_2\) (%)         | 3.9 (0.4)               | 3.9 (0.4)                | 0.0 (-0.1 to 0.1)                           |
| SII (%/L)               | 60.3 (19.7)             | 55.4 (16.9)              | -4.9 (-8.9 to -0.8)*                        |
| Normalized SII (1/L)    | 15.2 (4.2)              | 14.1 (3.9)               | -1.1 (-2.0 to -0.2)*                        |
| SIII (%/L)              | 2.3 (1.0)               | 1.9 (0.8)                | -0.4 (-0.6 to -0.2)*                        |
| Normalized SIII (1/L)   | 0.6 (0.3)               | 0.5 (0.2)                | -0.1 (-0.2 to 0.0)*                         |
| KPI\(_v\) (%)          | 3.9 (1.4)               | 3.5 (1.3)                | -0.4 (-0.7 to -0.1)                         |

**Table S3.** Basic classical capnographic lung function characteristics in Cystic Fibrosis and healthy control (analysis per trial)

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student’s T test)

VE: expiratory volume, VD: dead space volume, ETCO\(_2\): end-tidal CO\(_2\) fraction, VCO\(_2\): expired CO\(_2\) volume, FECO\(_2\): mixed expired CO\(_2\) fraction, SII: slope of phase II, SIII: slope of phase III, KPI\(_v\): capnographic index
|                  | Cystic Fibrosis (n=137) | Healthy Control (n=183) | Mean difference (95% confidence interval) |
|------------------|-------------------------|-------------------------|------------------------------------------|
| FACO2 (%)        | 5.2 (0.5)               | 5.2 (0.5)               | 0.0 (-0.1 to 0.1)                        |
| FDCO2 (%)        | 5.4 (0.5)               | 5.4 (0.5)               | 0.0 (-0.1 to 0.1)                        |
| FexCO2 (%)       | 5.1 (0.5)               | 5.1 (0.5)               | 0.0 (-0.1 to 0.1)                        |
| CII1 (%)         | 5.8 (1.5)               | 5.1 (1.1)               | -0.7 (-1.0 to -0.5)*                     |
| CII2 (%)         | 7.7 (1.9)               | 6.7 (1.5)               | -1.0 (-1.3 to -0.6)*                     |

**Table S4.** Novel capnographic inhomogeneity indices in Cystic Fibrosis and healthy control (analysis per trial)

Data are presented as mean (SD), unless stated otherwise

* Statistically significant difference (P<0.05 using the Student’s T test)

FACO2: CO2 fraction in alveolar compartment, FDCO2: CO2 fraction in dead space compartment,
FexCO2: CO2 fraction in the alveolar expirate, CII: capnographic inhomogeneity index (see text for details)
|                | AUC (95% confidence Interval) | Optimal Cutoff | Sensitivity at optimal cutoff | Specificity at optimal cutoff |
|----------------|------------------------------|----------------|-------------------------------|------------------------------|
| LCI            | 0.87 (0.80 to 0.94)         | 6.68           | 0.76                          | 0.89                         |
| SIII           | 0.62 (0.51 to 0.73)         | 2.75           | 0.36                          | 0.89                         |
| KPIv           | 0.61 (0.50 to 0.71)         | 3.16           | 0.76                          | 0.52                         |
| CII1           | 0.65 (0.55 to 0.75)         | 6.02           | 0.46                          | 0.80                         |
| CII2           | 0.65 (0.55 to 0.75)         | 7.98           | 0.48                          | 0.82                         |

**Table S5.** ROC analysis (analysis per subject)

ROC: Receiver operator characteristic, AUC: area under the curve, LCI: lung clearance index, SIII: slope of phase III, KPIv: capnographic index, CII: capnographic inhomogeneity index. Optimal Cutoffs were determined using the Youden Index analysis.
Table S6. Correlations between classical VCap indices and CII values (analysis per trial)

|       | Cystic Fibrosis | Healthy Controls |
|-------|-----------------|------------------|
|       | SII  | SIII | KPIv | SII    | SIII   | KPIv   |
| CII1  | -0.429 (-0.184) | 0.142 (0.020) | 0.595 (0.354) | -0.515 (-0.265) | 0.120 (0.014) | 0.555 (0.308) |
| CII2  | -0.385 (0.148) | 0.284 (0.081) | 0.747 (0.558) | -0.465 (-0.217) | 0.282 (0.079) | 0.718 (0.516) |

Data are Pearson correlation coefficients with $R^2$ (in parentheses)

SII: slope of phase II, SIII: slope of phase III, KPIv: capnographic index, CII: capnographic inhomogeneity index
### Table S7. Correlation between VCap indices and LCI (analysis per trial)

Data are presented as Pearson correlation coefficients ($r$) and coefficients of determination ($R^2$).

*Student's T-test

VCap: volumetric capnography, LCI: lung clearance index, SII: slope of phase II, SIII: slope of phase III,

KPIv: capnographic index, CII: capnographic inhomogeneity index
### Table S8. Intra-trial variability of VCap indices (analysis per trial)

Data are presented as mean coefficient of variation (SD)%

* Statistically significant difference between Cystic Fibrosis and Healthy Control Trials

(P<0.05 using the Student's T test)

VCap: volumetric capnography, SII: slope of phase II, SIII: slope of phase III, KPIv: capnographic index, CII: capnographic inhomogeneity index

|                  | Cystic Fibrosis Trials (n=137) | Healthy Control Trials (n=183) | All Trials (n=320) |
|------------------|--------------------------------|--------------------------------|--------------------|
| SII              | 12.6 (16.4)                    | 10.5 (5.5)                     | 11.4 (11.5)        |
| Normalized SII   | 12.0 (14.0)                    | 10.9 (4.9)                     | 11.4 (9.9)         |
| SIII             | 34.4 (19.0)*                   | 39.9 (19.0)*                   | 37.5 (19.2)        |
| Normalized SIII  | 37.0 (21.7)*                   | 43.2 (22.0)*                   | 40.6 (22.0)        |
| KPIv             | 31.6 (13.0)*                   | 38.1 (16.6)*                   | 35.3 (15.5)        |
| CII1             | 29.4 (8.4)*                    | 32.4 (9.1)*                    | 31.1 (8.9)         |
| CII2             | 29.7 (8.7)*                    | 33.2 (9.7)*                    | 31.7 (9.4)         |
|                  | Cystic Fibrosis Trials (n=137) | Healthy Control Trials (n=183) | All Trials (n=320) |
|------------------|--------------------------------|--------------------------------|-------------------|
| SII              | 8.4 (7.6)                      | 8.6 (6.7)                      | 8.5 (7.1)         |
| Normalized SII   | 7.0 (7.4)                      | 6.4 (5.3)                      | 6.6 (6.2)         |
| SIII             | 14.0 (12.3)                    | 18.1 (14.2)                    | 16.3 (13.5)       |
| Normalized SIII  | 14.7 (12.7)                    | 19.0 (14.8)                    | 17.2 (14.0)       |
| KPIv             | 14.8 (11.7)                    | 16.8 (13.7)                    | 15.9 (12.8)       |
| CII1             | 10.5 (7.9)                     | 11.6 (8.5)                     | 11.1 (8.2)        |
| CII2             | 10.7 (7.4)                     | 11.3 (8.4)                     | 11.0 (8.0)        |

**Table S9.** Inter-trial (intra-subject) variability of VCap indices

Data are presented as mean coefficient of variation (SD)%

No statistically significant difference between Cystic Fibrosis and Healthy Control Trials

(P<0.05 using the Student's T test)

VCap: volumetric capnography, SII: slope of phase II, SIII: slope of phase III, KPIv: capnographic index, CII: capnographic inhomogeneity index
**Figure S1.** Boxplots showing the difference between Cystic Fibrosis and healthy control in mean values of CII I and II, LCI, SIII and KPIv (analysis per trial).

The individual black dots represent mean values per trial.

CII: capnographic inhomogeneity index, LCI: lung clearance index, SIII: slope of phase III, KPIv: capnographic index.
Figure S2. Inter-trial (intra-subject) variability of LCI, CII I and CII II in patients with CF (black dots) and healthy controls (white dots).

The inter-trial (intra-subject) variability was calculated as the coefficient of variation in presence of three MBW trials, or as the relative difference in presence of two MBW trials. The red and green bars symbolize the mean inter-trial (intra-subject) variability for patients with CF and healthy controls respectively.

LCI: lung clearance index, CII: capnographic inhomogeneity index, CF: cystic fibrosis, MBW: Multiple Breath Washout.
Figure S3. Relationship between SIII and VE (A), CII1 and SIII (B) and CII2 and SIII (C) in CF (black dots) and healthy control trials (open dots) (analysis per trial).

CF: cystic fibrosis, SIII: slope of phase III, VE: expiratory volume, CII: capnographic inhomogeneity index.