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Exhaled carbon monoxide: variations due to collection method and physiology

Shahriar Arbabi[1] , Eric P Smith[1] , Jacob J Fondriest[1], Nagako Akeno[2], Robert S Franco[3] and Robert M Cohen[1,*]

[1] Department of Internal Medicine, Division of Endocrinology Diabetes and Metabolism, Cincinnati College of Medicine and Cincinnati Veterans Affairs Medical Center, Cincinnati, OH, United States of America
[2] Division of Pathology and Laboratory Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States of America
[3] Department of Internal Medicine, Division of Hematology-Oncology, University of Cincinnati College of Medicine, Cincinnati, OH, United States of America
[*] Author to whom any correspondence should be addressed.

E-mail: cohenrm@ucmail.uc.edu

Abstract

The measurement of exhaled carbon monoxide (eCO) is relevant to understanding normal physiology and disease states but has been limited by deficiencies in valid sampling protocols, accurate and feasible measurement methods, and the understanding of normal physiological variation. The purposes of this study were (**1**) to compare the three collection methods for eCO and (**2**) to gain a better understanding of patterns of normal variation by obtaining repeated daily and weekly measurements. We compared three techniques to sample eCO: continuous breathing (**ConB**), breath-holding (**BrH**), and short rebreathing (**SrB**). We used a Carbolyzer mBA-2000 instrument that involves an electrochemical method to quantify CO, with the final value corrected for ambient CO. In **Phase I**, we compared **ConB** with **BrH** in 10 healthy non-smokers (5 male, five female). On day 1, the eCO was determined from 07:30 to 17:00 (11 samples), and the first four morning time points were repeated on days 7, 14, and 21. **ConB** had a lower eCO than **BrH,** and eCO_2 was frequently below the threshold of 4.6% compatible with inadequate alveolar sampling. The eCO measured by the **ConB** and **BrH** methods increased during the day and showed week-to-week variability. In **Phase II**, we compared the **BrH** and **SrB** techniques by collecting prebreakfast samples weekly for four weeks in 30 healthy non-smokers (15 male,15 female). Comparing the **SrB** vs. the **BrH** method, **SrB** was the easier for the participants to perform, generated higher eCO (~ 0.5 ppm), and produced higher eCO2 levels ($5.2\% \pm 0.3$ vs. $5.0\% \pm 0.2$); Importantly, **Phase II** study revealed that week-to-week changes in prebreakfast fasting eCO for individual participants were $\geqslant 1.0$ ppm in $\sim 37\%$. This variability complicates the interpretation of the relationship between small changes in eCO and the underlying physiological or disease states.

1. Introduction

In 400 BC, Hippocrates described the detection of odors on human breath, marking one of the earliest documented instances of 'analytical' health assessment [1]. Today, the constituents of breath currently considered to have potential relevance to disease fall into three categories: (1) organic and inorganic gases, (2) volatile compounds, and (3) condensates with aerosol particles [2]. The focus of this study was the measurement and interpretation of exhaled carbon monoxide(eCO) and how this relates to the use of eCO to assess health and disease [3].

Endogenous CO arises from the breakdown of heme proteins, primarily hemoglobin (75%–80%) and other hemoproteins such as myoglobin, cytochromes, peroxidases, and catalase [3]. Heme oxygenase (HO) enzymes (HO-1, HO-2, and HO-3) regulate this degradation process, producing biliverdin/bilirubin, ferrous ions, and CO [4]. Although these enzymes are found in most tissues, the reticuloendothelial system of the liver and spleen is the

primary site of heme catabolism, and consequently, the major producers of endogenous CO [5–8].

While endogenous CO can be converted to CO_2 by cytochrome C oxidase in the mitochondria, its primary elimination from the body occurs through exhalation, i.e., diffusion across the alveolar-capillary membrane [9, 10]. Therefore, assuming negligible exhaled CO originating from the environment [11], the concentration of eCO mirrors the endogenous carbon monoxide production. However, although other sources of CO are low, this tissue-specific HO enzyme activity can modulate localized CO cell signaling functions [4, 12], and in some disease states, such as asthma, leads to increased systemic and exhaled CO [13].

Much of the challenge in the interpretation of eCO, independent of source considerations, relates to the impact of the methods of collection and detection. Many original studies [14] in the 1950s and the 60's focused on labor-intensive and complex strategies in which the rate of endogenous production of CO (or blood COHb) was measured in a closed system [15]. Subsequent efforts to develop more practical and feasible measures of eCO have yielded a variety of alternative collection methods, most commonly quantification of end-tidal CO (ETCO) that correlates well with CO production approaches [16, 17]. These more feasible and practical methods have generally combined simplified and standardized collection with less cumbersome CO detection technology in which issues of specificity and sensitivity are addressed [18, 19]. In most studies, eCO has been measured with less expensive and more portable electrochemical devices than with infrared or gas spectrometry methods. The enhanced accessibility of eCO measurements has led to an impressive effort to relate measured CO to numerous clinical conditions, including smoking cessation [20], asthma [21], hemolysis [22, 23], and inflammatory diseases [24].

In this study, we compared three different methods for collecting eCO: a breath-holding technique (**BrH**) similar in principle to that used by Levitt [17, 25] and two others involving 'continuous breathing (**ConB**)' and 'short rebreathing' (**SrB**)' techniques [26, 27]. All three are suitable for routine use. As part of the comparison, we addressed aspects of eCO collection that could affect its interpretation. Specifically, we addressed the reproducibility of eCO over the course of the day and when repeated weekly over the course of four weeks. We found that (1) there was a significant diurnal eCO variation, (2) levels repeated weekly showed significant variation in a substantial subset of subjects, which was not explained by any intercurrent medical issues or changes in ambient environmental CO in the Clinical Research Unit (CRU), and (3) the **SrB** and **BrH** methods are likely to reflect end-tidal better than **ConB**. These results raise questions regarding the validity of a single eCO

measurement in applications where small changes have a putative physiological meaning.

2. Methods

2.1. Participants and study design

This study was conducted in two phases (figure 1) at the CRU of the Cincinnati VA Medical Center (CVAMC. After obtaining consent, we measured the height, weight, and resting vital signs. We collected blood and urine samples to establish baseline values and assessed the criteria outlined in table 1 for inclusion and exclusion.

Phase I

Phase I investigated the optimal time for eCO collection by repeated sampling throughout the day (before breakfast to before dinner) and repeated weekly prebreakfast sampling over 4 weeks. We compared two breath gas sampling methods, Continuous breathing (**ConB**) and breath holding (**BrH**). We enrolled ten healthy, non-smoking subjects aged 18–76 (5 males, 5 females) who met the criteria in table 1. Participants consumed a light, low-carbohydrate meal the night prior and fasted overnight. To reinforce the importance of exhaling slowly and steadily during eCO collection, the participants, prior to starting the study, were trained to exhale steadily into a filling bag with the goal of filling the bag over approximately 10 s. Before each collection session, they were then asked to sit quietly for approximately 30 min or until their vital signs approached baseline/resting values (within $\pm 10\%$ resting vital signs at the time of enrollment visit). Breath sample collection commenced at 08:00, with samples collected every 30 min before breakfast (totaling four collections). Breakfast was provided at approximately 10:00, after which breath samples were collected every hour from 11:00 to 17:00. (11 CO measurements). For each time point, we obtained one sample using **ConB** and one sample using **BrH** and alternated the technique used first. There was at least a five-minute interval between the two collections. The subjects also returned weekly for three more weeks for four eCO measurements before breakfast using both **ConB** and **BrH** methods.

Phase II

Phase I results suggested that the **BrH** method was superior to **ConB** and that a fasting sample before breakfast was the more stable baseline for CO measurement. However, because of apparent significant week-to-week variation even in the prebreakfast fasting sample, **Phase II** assessed the week-to-week reproducibility of the morning breath sampling by expanding the number of subjects to 30 and substituting the short rebreathing (**SrB**) technique for the **ConB** method in addition to retaining the **Phase I** participants [28]. We invited all **Phase I** participants
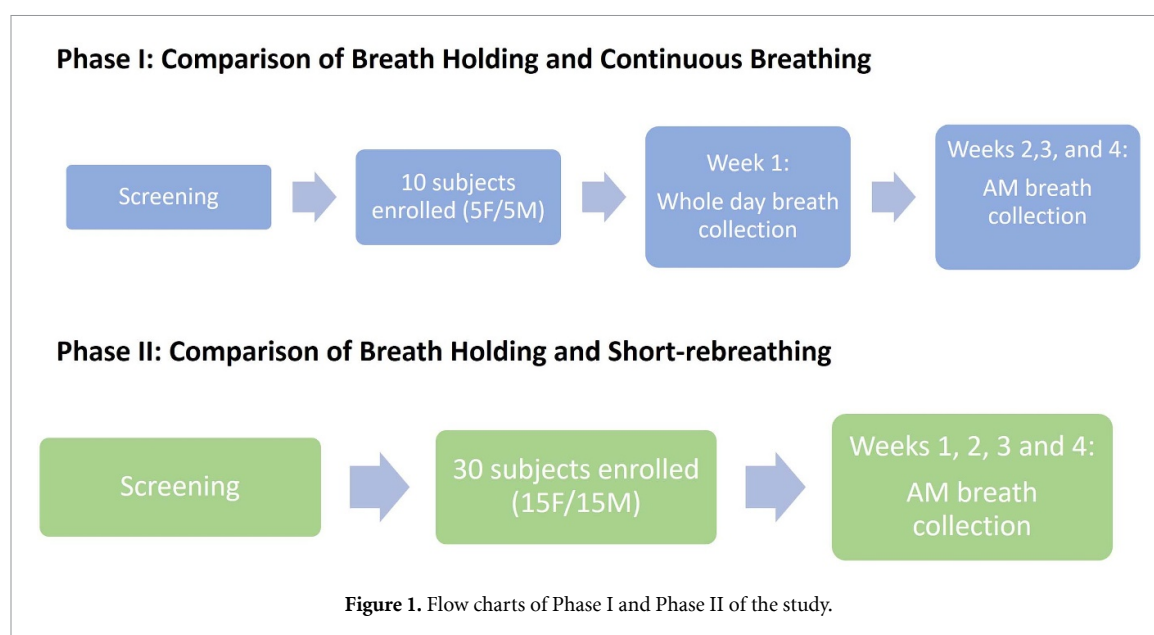
Figure 1. Flow charts of Phase I and Phase II of the study.

Table 1. Study participants' inclusion and exclusion criteria.

Inclusion	Medical history exclusion	Laboratory test exclusion
18–75 years old	**Hematologic:**	**Hematologic:**
Non-pregnant	Known hemoglobinopathy, red blood cell disorder, coagulopathy, or serum protein disorder such as multiple myeloma	Hemoglobin < 10 gm/dl; Reticulocyte count $> 2\%$
Non-smoker	Blood donation or surgery within the preceding three months, history of GI blood loss, or recent hematoma	MCV: outside 80–100 femtoliters range
		WBC: outside 3800–10800 μl^{-1} range
	Medications affecting heme-oxygenase activity	
		Platelet count outside 140–400 000 μl^{-1} range
	Pulmonary:	**Renal:**
	Asthma, COPD, or cystic fibrosis	Spot urine microalbumin > 30 mcg mg^{-1} creatinine
	Lung transplantation	
	Other:	**Hepatic:**
	Active infection	Transaminases > 3 X the upper limit of normal
	Known rheumatologic disease	
	Pregnant, nursing, or planning pregnancy	
	$\geqslant$ NYHA stage 3 heart failure	
	Irradiation	
	History of hypo- or hyperthyroidism	
	Environmental Exposure:	
	Open fire or wood stove at home	
	Job-related exposure to CO	
	Plans to move from area during study.	

MCV: mean corpuscular volume; WBC: white blood cell count.

to enroll in **Phase II**. Four male and four female participants agreed to participate and provided consent for **Phase II**. Additionally, we enrolled 22 new participants, resulting in a total of 30 participants (15 males and 15 females). Participants were instructed to consume a light, low-carb meal the night before the measurement session, fast overnight, arrive at the research clinic at around 07:30, and sit quietly for approximately 30 min. Similar to Phase 1, the importance of slow and steady exhalation was emphasized

for any new subjects and reinforced for those subjects previously enrolled in **Phase I**. Breath samples were collected before breakfast, starting at 08:00 and repeated every 30 min until 1000.

2.2. eCO and carbon dioxide measurement

The concentrations of CO and CO_2 in the exhaled breath were measured using a Carbolyzer (Carbolyzer mBA-2000 TAIYO Instruments INC, Osaka, Japan) [29]. This instrument was equipped with a sensitive

electrochemical detector for CO measurement with a range of 0–50 ppm and a sensitivity of $\pm$ 0.1 ppm. The Carbolyzer instrument measured the concentration of carbon dioxide (CO_2) at the same time as CO. The CO_2 concentration was used as an indicator that the collected gas represented accurate sampling of alveolar gas ($CO_2 \geqslant 4.62\%$) [30]. The analyzer was capable of continuous side-stream sampling with a flow rate of 0.2 l min^{-1}. With the objective of minimizing ambient CO variation, the selected well-ventilated study room was monitored for CO levels over several weeks to confirm that the CO concentration remained stable between 100–200 PPB (0.1–0.2 PPM) when the room was unoccupied and inactive. Additionally, the potential influence of at least two people being present in the room was assessed and confirmed to not affect the measured CO levels.

The Carbolyzer continuously aspirated air from the sampling tube and displayed ambient baseline CO values. The CO value was adjusted by subtracting the ambient CO concentration measured by the Carbolyzer before each breath sample collection. The specificity of CO detection by the Carbolyzer is affected by cross-interference from high concentrations of H_2 (1 ppm H_2 = 0.0035 ppm CO) that can be present, in particular, after meals. Therefore, in **Phase I**, to correct for potential interference, the concentration of H_2 in the same breath samples was analyzed using a QuinTron BreathTracker SC Analyzer (QuinTron, New Berlin, WI, USA). The eCO measured by the Carbolyzer was corrected for H_2 by subtracting 0.0035 ppm for each detected ppm of H_2. Because the **Phase I** results indicated insignificant levels of H_2 in a fasting state, H_2 concentrations were not measured in **Phase II** samples.

The Carbolyzer instrument was calibrated for the zero point and the sensitivity (span point) of the CO/CO_2 detector. Calibration was performed at least once every week with three known mixtures: purified air without CO or CO_2, 1.8 ppm CO and 5% CO_2 balanced air, and 6 ppm CO and 10% CO_2 balanced air (Praxair, Cincinnati, Ohio). In addition, to perform an assessment of the Carbolyzer precision before initiating the study, we collected eCO using the BrH technique on 12 individuals. The collection was repeated three times with a brief minute interval between each collection. This was repeated on a separate day. The average SD and CV for both days were 0.05 and 3%.

2.3. eCO collection techniques

2.3.1. Breath-holding technique (figure 2, Panel (B)):
A nose clip was placed with the participant in a standing position and at rest. A deep breath was held for 30 s and then exhaled into the QuinTron AlveoSampler breath collection kit (QuinTron, New Berlin, WI), consisting of a mouthpiece, T-piece flutter valve, accessory discard bag, and sample bag. The exhaled gas first fills the accessory bag with dead-space gas, and then a valve opens, directing the alveolar expiratory gas to the sample bag. The sample bag was connected to the Carbolyzer machine to measure CO and CO_2 concentrations. A CO_2 concentration of 4.6% (equivalent to CO_2 35 mmHg) or higher was considered to indicate an adequate alveolar gas sample [27, 31, 32].

2.3.2. Continuous breathing technique (figure 2, Panel (A))
A nose clip was placed with the participant in a standing position and at rest. Exhaled air was directed into a T-tube with a one-way flutter valve that allowed ambient room air to be inhaled but prevented mixing of exhaled gas with ambient air. A side port connected the collected gas directly to the Carbolyzer machine [31].
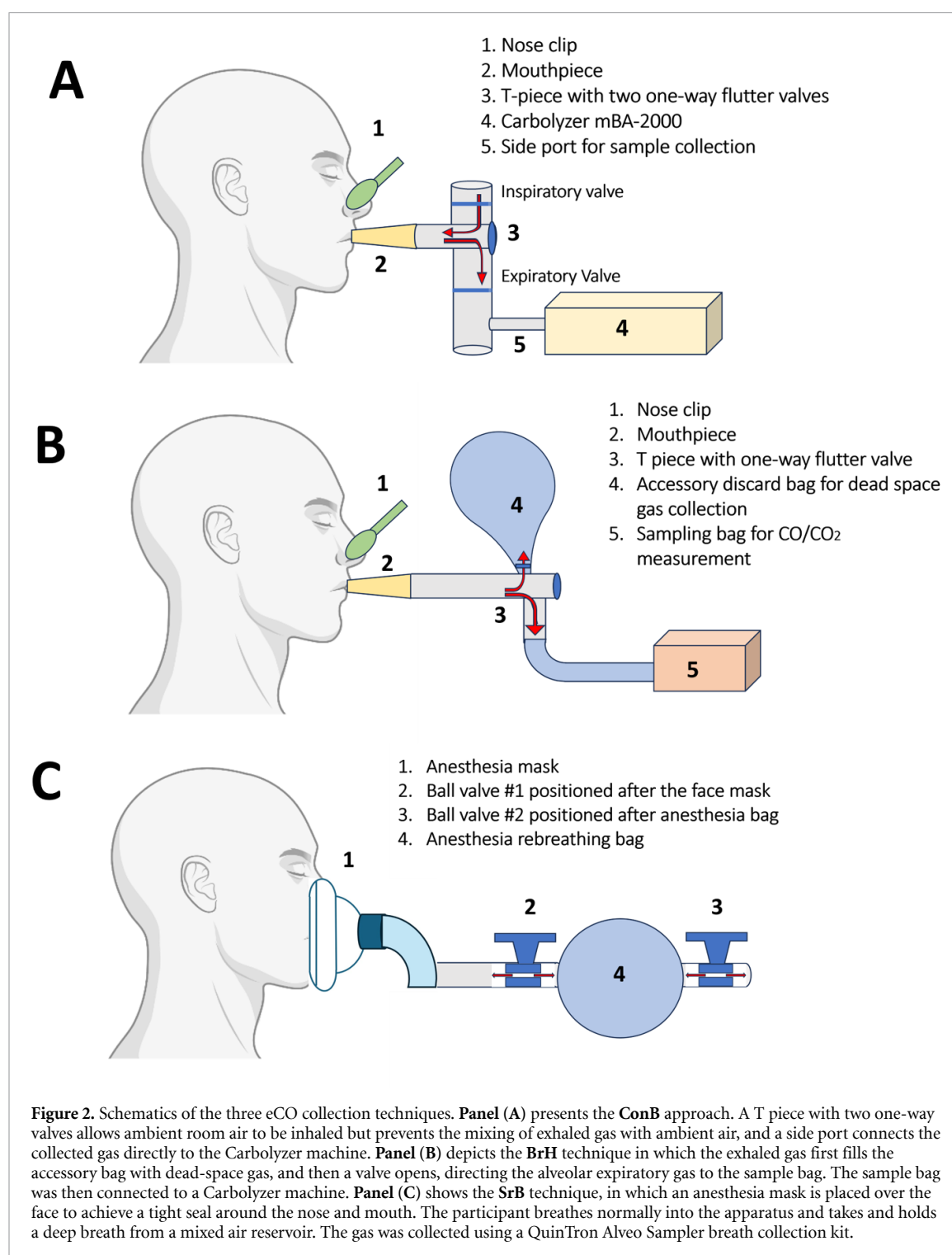
2.3.3. Short rebreathing technique: (figure 2, Panel (C))
A closed breathing circuit was a modification of previously described approaches for measuring carbon monoxide lung diffusing capacity [33, 34]. The circuit was simplified to include a non-latex 3.0 l anesthesia bag with two valves and a face mask with a sealing cushion to fit securely on the participant's face. At the beginning of the sampling, the anesthesia bag was filled with a mixture of room air and oxygen (4 ml kg^{-1} body weight, consisting of 2/3rd room air and 1/3 oxygen from the hospital clinic's oxygen outlet) to minimize potential discomfort from rebreathing in a closed system with a low oxygen concentration. The anesthesia bag served two purposes: as a reservoir for the air/oxygen mixture and as a container for exhaled mixed alveolar gas during rebreathing.

With participants standing and at rest, maximal inspiration was performed, followed by breath-holding. An anesthesia mask was then placed over the face to ensure a tight seal around the nose and mouth. Valve 1 was opened, and the participant was instructed to breathe normally into the apparatus, while valve 2 remained closed, and the mask was sealed. After 30 s, the participants took and held a deep breath from the gas mixture inside the bag. A nose clip was applied, and a Quintron-alveolar sampler bag was filled with expiratory gas. Like the **BrH** method, CO/CO_2 levels were measured using a Carbolyzer machine.

2.4. Statistical analysis
Demographic and clinical laboratory characteristics were summarized and compared among the groups.

Figure 2. Schematics of the three eCO collection techniques. **Panel (A)** presents the **ConB** approach. A T piece with two one-way valves allows ambient room air to be inhaled but prevents the mixing of exhaled gas with ambient air, and a side port connects the collected gas directly to the Carbolyzer machine. **Panel (B)** depicts the **BrH** technique in which the exhaled gas first fills the accessory bag with dead-space gas, and then a valve opens, directing the alveolar expiratory gas to the sample bag. The sample bag was then connected to a Carbolyzer machine. **Panel (C)** shows the **SrB** technique, in which an anesthesia mask is placed over the face to achieve a tight seal around the nose and mouth. The participant breathes normally into the apparatus and takes and holds a deep breath from a mixed air reservoir. The gas was collected using a QuinTron Alveo Sampler breath collection kit.

Mean (M), Coefficient of variation (CV) and standard deviation (SD) were used to quantify and compare the variation measurements. Student's *t*-test was used to compare two groups. When more than two groups were compared, analysis of variance (ANOVA) for repeated measures, followed by Tukey's *post hoc* test, was used. $P \leqslant 0.05$ was considered statistically significant.

3. Results

3.1. Inclusion/exclusion criteria, demographics, and screening laboratory tests

The inclusion and exclusion criteria are summarized in table 1. Particular emphasis was placed on excluding any known respiratory or hematologic conditions that might affect eCO and thorough documentation

Table 2. Demographics and CBC data of participants enrolled in **Phase I** and **Phase II** of the study.

	Phase I			Phase II		
	($n = 10$)			($n = 30$)		
	Male (5)	Female (5)	Total (10)	Male (15)	Female (15)	Total (30)
Age (yr)	36.0 (15.5)	41.4 (12.6)	38.7 (13.6)	42.3 (16.3)	36.3 (14.5)	39.3 (15.4)
Height (cm)	181.0 (12.4)	165.2 (4.8)	173.3 (12.3)	174.3 (5.6)	163.0 (5.5)	168.7 (7.9)
Weight (kg)	105.4 (21.3)	81.0 (12.5)	93.2 (20.9)	89.0 (15.4)	76.1 (16.1)	82.6 (16.9)
Waist (cm)	99.2 (9.9)	90.4 (14.9)	94.8 (12.8)	97.1 (9.61)	85.6 (15.5)	91.3 (14.0)
BMI (kg m^{-2})	31.4 (3.5)	29.7 (4.2)	30.6 (3.7)	29.3 (5.0)	28.6 (5.7)	28.9 (5.3)
Hemoglobin (g/dl^{-1})	14.9 (1.0)	13.8 (1.4)	14.4 (1.3)	14.9 (.5)	13.0 (1.3)	13.9 (1.7)
Hematocrit (%)	43.8 (3.1)	41.6 (3.7)	42.7 (3.4)	42.9 (3.1)	39.1 (3.6)	41.0 (3.9)
RBC (10^9 μl^{-1})	5.0 (0.3)	4.7 (0.5)	4.8 (0.4)	4.9 (0.5)	4.3 (0.4)	4.6 (0.5)
RDW (%) (normal range: 12–15)	13.12 (1.7)	14.5 (87)	13.8 (1.4)	13.6 (1.1)	13.3 (0.7)	13.4 (0.9)
Reticulocyte count (%) (normal range: 0.6–2.4)	1.5 (0.4)	1.1 (0.3)	1.3 (0.4)	1.4 (0.6)	1.4 (0.6)	1.4 (0.6)

RBC: red blood cell; RDW: red cell distribution width; Hemoglobin normal range: adult male: 13.5 0 17.5/ adult female: 12.0–16.0; RBC normal range: adult male: 4.5–6.0/adult female: $4.0-5.2$; numbers represent means with $\pm$ 1SD in parentheses.

of smoking history and possible exposure to unusual levels of environmental CO. Demographic characteristics and the results of hematology screening parameters of the study participants in **Phase I** (10 subjects) and **Phase II** (30 subjects) are shown in table 2.

3.2. Phase I

3.2.1. eCO measurements between 07:30 and 17:00 using BrH and ConB methods

Figure 3**, Panels (A)** and **(B),** respectively, show the **ConB** and **BrH** eCO diurnal variation for the individual participants. Figure 3(**Panel C**) compares the diurnal profile of both techniques by averaging each time point and includes the simultaneous CO_2 measurements. eCO, corrected for H_2, appeared to be lower in the morning and increased gradually throughout the afternoon for both methods. Using repeated measures one-way ANOVA with Tukey's *post hoc* correction, the **BrH** eCO concentration on average at 07:30 (2.3 ppm $\pm$ 0.5) was significantly different ($P \leqslant 0.05$) from all time points except at 09:00, 11:00, and 12:00. Pre-prandial and fasting H_2 concentrations ranged from 3 to 8 ppm and, therefore, did not significantly affect the accuracy of eCO. However, postprandial H_2 concentrations were as high as 17 ppm in some participants. CO_2 was rarely equal to or higher than 4% using the **ConB** method. In contrast, the measured concentration of CO_2 with the **BrH** technique consistently equaled or exceeded 4.6%, a level considered to reflect a valid ETCO$_2$ sampling [33]. The correlation between the two methods was substantial but suggested room for improvement. ($R^2 = 0.86$ (supplemental figure 1).

Days 1, 8, 15, and 22: Weekly repeated eCO fasting measurements between 07:30 at 10:00 using BrH and ConB methods. Following the day 1 diurnal variation assessment, the week-to-week variation was determined. Prebreakfast samples from 0800 to 1000 using the **ConB** (figure 4, **Panel (A)**) and **BrH**

(figure 4, **Panel (B)**) methods were collected. eCO levels varied from week to week for both methods. Figure 4**, Panel (C)** shows the average results for both methods. There was a statistically significant difference between weeks 1 and 2 with a standard *t*-test ($P = 0.044$) for the **BrH** method, but repeated measures one-way ANOVA with Tukey's correction did not achieve statistical significance because of the high overall variance. Table 3 further summarizes the results of **Phase 1**. The CV was 10% or greater in all participants over the course of the 4-week testing.

3.3. Phase II

3.3.1. Phase II rationale

The diurnal variation observed was consistent with previous studies in the literature [27, 34], but the apparent week-to-week variability was unanticipated, in part because this aspect has not been explored comprehensively in the published literature to our knowledge [3, 18, 34]. This prompted a **Phase II** study to further assess week-to-week variation in a healthy population. In addition, the **ConB** approach was replaced by a third collection method, **SrB**. This technique is a simplified version (See **Methods**) of an approach demonstrated by other investigators to provide potentially more accurate alveolar air [34, 35]. **Phase II** was performed approximately 12 months after **Phase I. Phase II**, in addition to the week-to-week variability of the eCO, also included the evaluation of the reproducibility of the two techniques by performing repeated fasting samples from 08:00 to 10:00.

3.3.2. Prebreakfast morning weekly eCO variation

Figure 5**, Panels (A)** and **(B),** show the **BrH** and **SrB** weekly changes in eCO for each of the 30 subjects. **Panels (C)** and **(D)** show the respective distribution of weekly differences for each the thirty **Phase II** participants calculated as follows: week 2 minus week 1,

Table 3. Phase 1 study: fasting prebreakfast eCO (ppm) measured with **ConB** and **BrH** techniques every week for four weeks.

ConB									
	Female					Male			
Subjects	F1	F2	F3	F4	F5	M1	M2	M3	M4
WK1	1.1	1.4	3.1	1.6	2.4	1.7	1.7	3	1.7
WK2	2.2	1.5	3.6	1.6	1.8	1.7	1.9	2.6	1.9
WK 3	1.9	1.3	3.4	1.8	1.7	1.6	1.6	2.6	2.1
WK4	1.6	1.2	3.5	1.5	1.7	1.4	1.2	1.9	1.6
Mean	1.7	1.4	3.4	1.6	1.9	1.6	1.6	2.5	1.8
SD	0.5	0.1	0.2	0.1	0.3	0.1	0.3	0.5	0.2
CV	28%	10%	6%	8%	18%	9%	18%	18%	12%

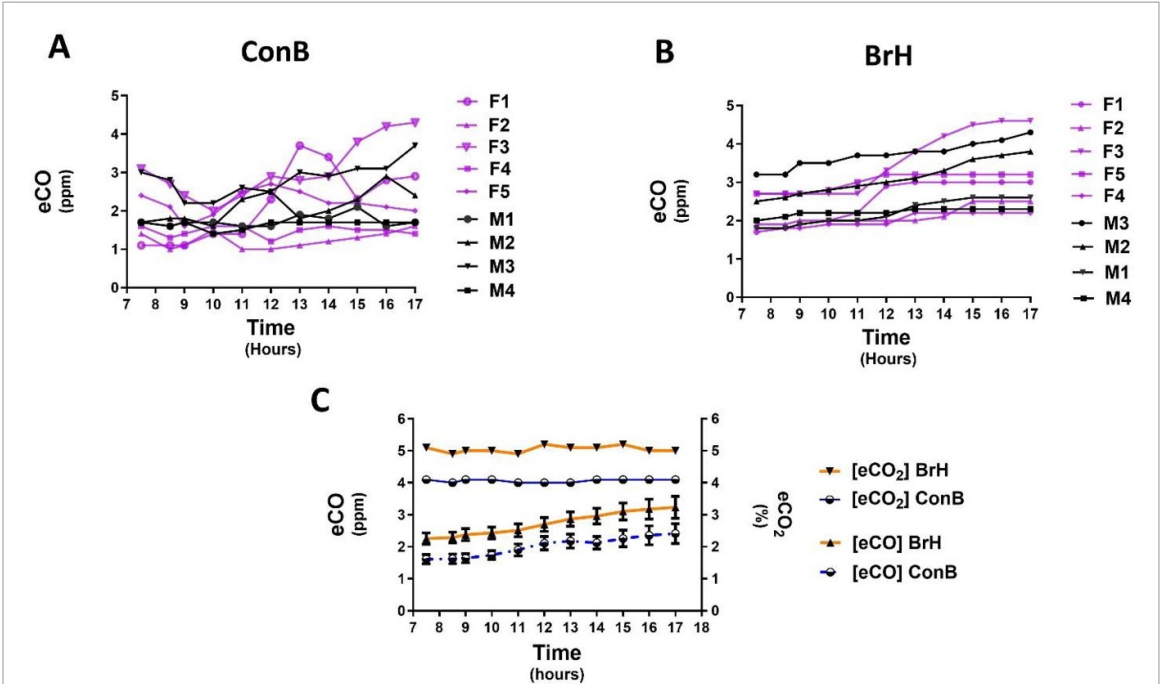| BrH									
	Female					Male			
Subjects	F1	F2	F3	F4	F5	M1	M2	M3	M4
WK1	1.7	1.9	2.7	1.8	2.7	1.8	2.5	3.2	2
WK2	3.4	2.2	4.1	1.8	2.9	1.9	2.8	3.1	2
WK 3	2.9	2.5	3.9	2.4	2.5	2.1	2.4	4	2.9
WK4	2.6	2.1	4.1	2.1	3	1.7	2.2	2.8	1.8
Mean	2.7	2.2	3.7	2	2.8	1.9	2.5	3.3	2.2
SD	0.7	0.3	0.7	0.3	0.2	0.2	0.3	0.5	0.5
CV	27%	11%	18%	14%	8%	9%	10%	16%	23%

Figure 3. Phase I comparison of BrH and ConB techniques. **Panel (A)** eCO concentration variation using **ConB** method during a day (07:30–17:00) for nine subjects (**5F purple**/**4 M black**). **Panel (B)** eCO concentration variation using **BrH** method during a day (07:30–17:00) for nine subjects (**5F purple**/**4 M black**). **Panel (C)** Average eCO and eCO_2 concentration variations between 07:30 and 17:30 using the two techniques. Breath-holding, **BrH**; Continuous breathing, **ConB**.

week 3 minus week 2, and week 3 minus week 3. The range of BrH differences was 0–2.7 ppm, 36% of the differences were $\geqslant$ 1.0 ppm, and 13% were $\geqslant$ 2 ppm. Similarly, for **SrB**, the range of differences was 0–2.95, 40% were $\geqslant$ 2 ppm, and 11% $\geqslant$ 2 ppm. **Panel E** depicts the average weekly values for the two methods. Although there appeared to be a trend towards

an increase in the average values, this was not statistically significant.

Figure 6 further compares the two techniques. Correlation analysis of the two methods showed a high R^2 value of 0.91. The tabular data summarizes the average eCO values for the **BrH** and **SrB** collection methods for the 4 weeks of **Phase II**. The **SrB** eCO
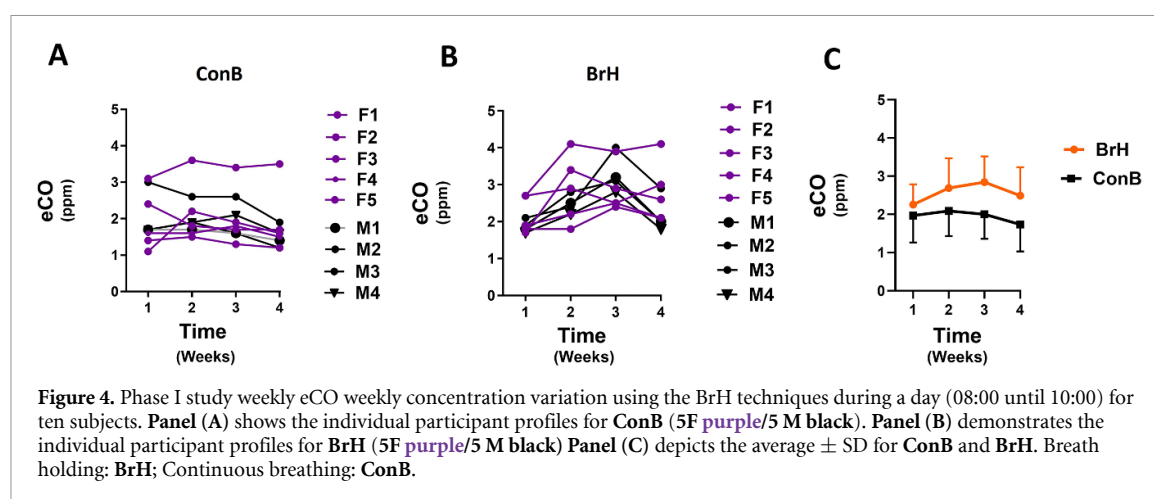
Figure 4. Phase I study weekly eCO weekly concentration variation using the BrH techniques during a day (08:00 until 10:00) for ten subjects. **Panel (A)** shows the individual participant profiles for **ConB** (**5F purple/5 M black**). **Panel (B)** demonstrates the individual participant profiles for **BrH** (**5F purple/5 M black**) **Panel (C)** depicts the average ± SD for **ConB** and **BrH**. Breath holding: **BrH**; Continuous breathing: **ConB**.
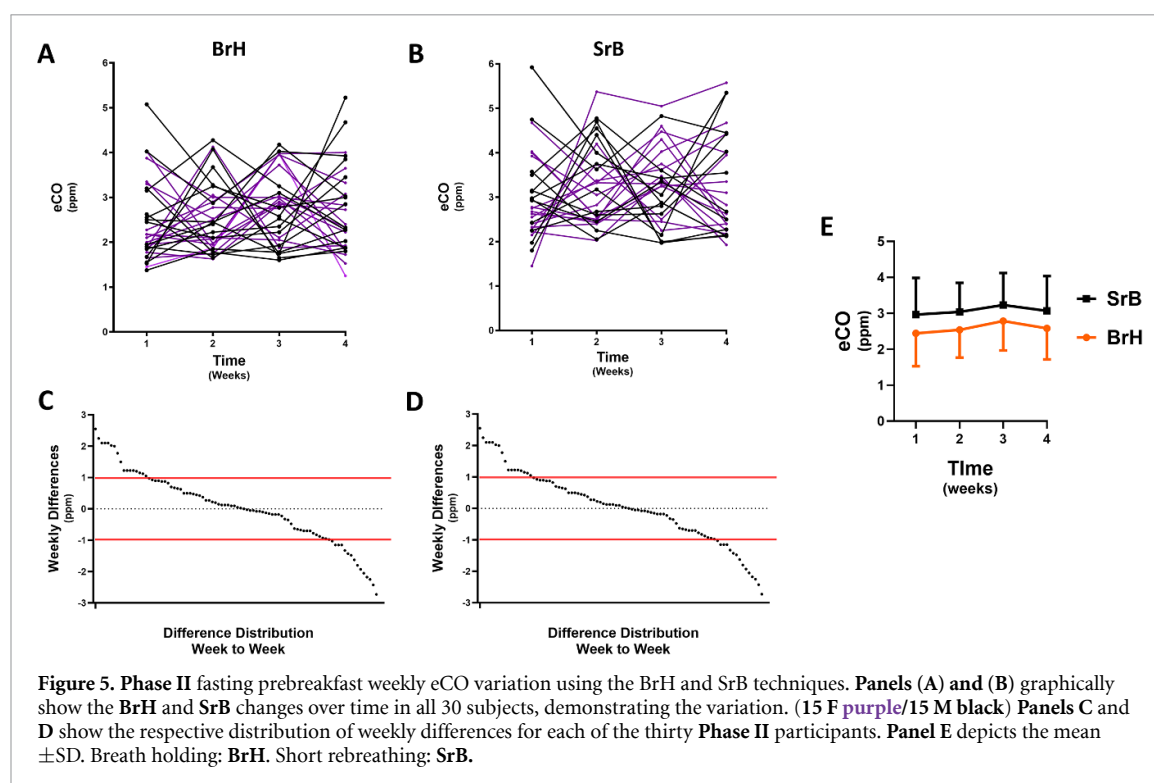

Figure 5. Phase II fasting prebreakfast weekly eCO variation using the BrH and SrB techniques. **Panels (A) and (B)** graphically show the **BrH** and **SrB** changes over time in all 30 subjects, demonstrating the variation. (**15 F purple/15 M black**) **Panels C** and **D** show the respective distribution of weekly differences for each of the thirty **Phase II** participants. **Panel E** depicts the mean ±SD. Breath holding: **BrH**. Short rebreathing: **SrB**.

results were consistently higher than **BrH** (0.5 ppm) and was highly significant. The SD for the eCO collections repeated four times in succession was low for both methods with an average CV of approximately 4%. There was no statistically significant difference between males and females. Importantly, both methods generated CO_2 levels greater than 4.6% and the CO_2 values by **SrB** method were significantly greater than the **BrH** (0.2%).

3.4. Discussion

This study aimed to examine factors that may cause variations in eCO, including the measurement protocol, diurnal changes, and week-to-week differences. Understanding these factors would allow for the development of standard protocols that minimize variation and enhance the application of eCO to study

physiological and disease states. The study was performed in two **Phases**. In **Phase I**, two breathing protocols, **ConB** and **BrH,** were evaluated. While both methods gave consistent diurnal variation, the **ConB** technique was judged inadequate because it not only gave lower eCO values but also did not achieve CO_2 concentrations reflecting sufficient alveolar sampling. In **Phase II** we compared **BrH** with a third technique, **SrB**, and evaluated both reproducibility and week-to-week changes. Both **BrH** and **SrB** produced CO_2 concentrations that reached a threshold that was consistent with adequate alveolar sampling. For both methods, the eCO values were essentially identical within the method of repeated sampling over 20–30 min. However, eCO for **SrB** were consistently about 0.5 ppm higher than for **BrH**. Similarly, the CO_2 measurements were approximately 0.2% higher. These results support the possibility of using
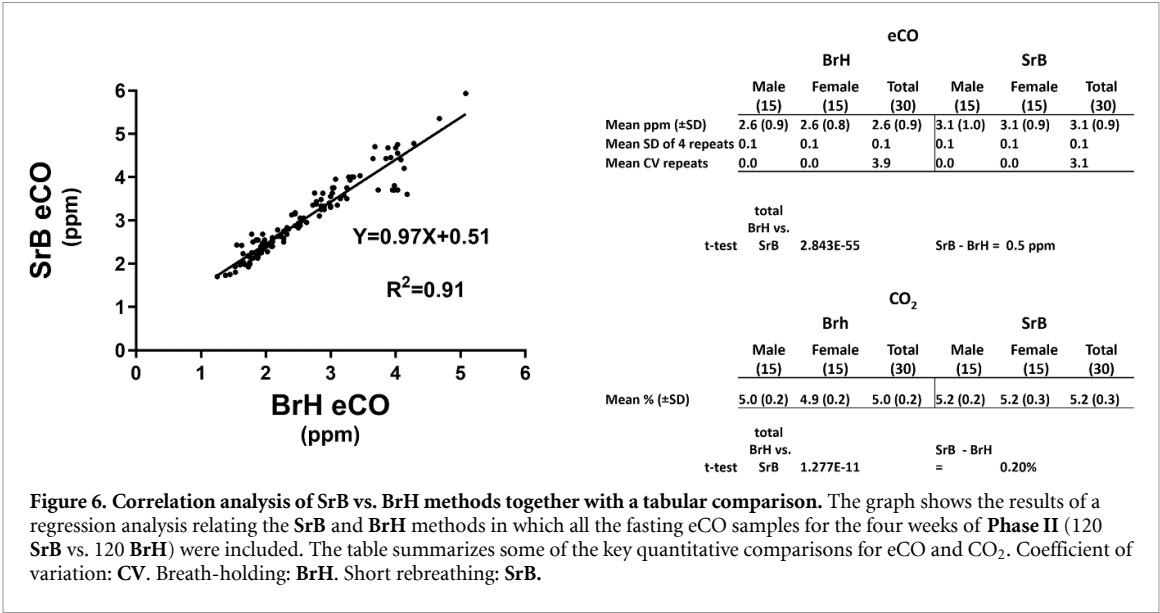
Figure 6. Correlation analysis of SrB vs. BrH methods together with a tabular comparison. The graph shows the results of a regression analysis relating the **SrB** and **BrH** methods in which all the fasting eCO samples for the four weeks of **Phase II** (120 **SrB** vs. 120 **BrH**) were included. The table summarizes some of the key quantitative comparisons for eCO and CO_2. Coefficient of variation: **CV**. Breath-holding: **BrH**. Short rebreathing: **SrB**.

breath-holding and/or short rebreathing methods to measure the value of exhaled CO that reflects the alveolar level and is reproducible over a 30-to-60 min time frame [17, 36, 37]. However, the eCO measurements for both **SrB** and **BrH** varied weekly in many participants. This variation was observed even though the studied population had been carefully screened for health conditions and environmental exposures known to affect circulating CO [10, 26, 34]. We conclude that factors distinct from collection methodology and environmental conditions within the CRU contribute to eCO and cause variation, which makes it difficult to interpret the contribution of modest changes in physiological and/or disease states.

Early methods using closed rebreathing circuits to assess CO production rate by measuring the linear increase in circulating CO were complex and not suited for routine measurements [15, 34]. In 1992, Strocchi *et al* [32] showed that eCO collected using a breath-holding method, in which the initial dead space of exhaled air was discarded, correlated well with a simultaneously performed 'gold standard' CO production rate measurements. CO_2 was quantified to indicate adequate alveolar air collection. Other investigators have utilized airtight face masks and one-way valves to prevent mixing with ambient air [36] and factoring ventilation status [35] during sampling, while providing a collection that, based on CO_2 concentrations, implied adequate collection. Alternative approaches include rebreathing strategies in which each inhalation consists of previously exhaled gases [28]. Through repeated cycles of inhalation and exhalation, eCO progressively increases to achieve maximal equilibrium with blood CO concentration and may represent a value higher than the actual ETCO [31].

In our study, the ranges of eCO values for both **BrH** and **SrB** (∼1–4 ppm) are consistent with concentrations published using similar methods in

the smoking cessation literature [38, 39], hemolysis assessment in the newborn [18, 19, 40], and physiological variation in red cell lifespan calculations, as it may be related to diseases such as diabetes [25, 41–43] and inflammatory conditions often involving the pulmonary system [21, 44–47]. Additionally, consistent with prior studies, we detected significant hourly differences in eCO with an increase in eCO values throughout the day. Specifically, Coburn reported a variable CO production rate in one male patient tested for a 48 h period, peaking (∼ 3–4 fold greater than nadir) at around midnight on the second day [14]. Levitt *et al* [27], using a novel technique of measuring ETCO corrected for ambient CO, observed in seven healthy subjects a 26% increase in CO with a nadir at about midnight [2, 27]; in our study, we collected samples after a period of sitting at rest with stable vital signs to minimize the impact of acute changes in ventilation [26]. Finally, Sandberg *et al*, using a breath-holding approach and the Smokerlyzer Micro with a sensitivity of about one ppm and with less specificity than gas chromatography and infrared devices, performed a 1 h interval time course during the day on seven nonsmoker controls; they showed an increase at lunchtime, although they did not test for possible effects of H_2 [20]. A consensus from the literature and our data strongly indicates that the time of day is a method-independent contributor to eCO and needs to be accounted for when interpreting the implications of a single eCO value.

To our knowledge, this study is the first to systematically assess week-to-week eCO variations. Other investigators have reported evidence of variation over days to weeks, but typically as a peripheral observation in a study focused on other aspects. For example, Yamaya *et al*, using a breath-holding technique, showed that eCO levels during an upper respiratory infection averaged ∼ 5.8 ppm but decreased

to approximately 1.2 ppm three weeks after the resolution of symptoms [48]. A more recent study by Ye *et al*, which focused on comparing the variation of the Levitt method for eCO collection with ^{15}N-glycine labeling technique for assessing red cell lifespan, showed a clear week-to-week variation with the eCO method [22]. In this small-scale study involving only five control subjects and three hemolysis patients, a typical participant had eighteen eCO measurements over the course of 180 d, approximately at week intervals coinciding with blood draws for ^{15}N-heme analysis. The eCO results were in the 1–3 ppm range with a SD as high as 0.60, with corresponding lifespan calculations ranging from approximately 80 to 110 d.

Our results regarding diurnal and week-to-week variation using three different methods suggest that a one-time measurement of eCO may need to be interpreted with caution. These findings have implications for the application of eCO to assess normal physiology and disease states. For example, since the earliest CO production rate studies, there has been a focus on the estimation of red cell lifespan [49]. A formula for this was derived by Strocchi *et al* [32], which incorporated a number of significant assumptions. Since then, many authors have used the Strocchi formula to estimate hemoglobin turnover and red cell lifespan from eCO measured in a variety of ways [22]. Perhaps the most established clinical application for using eCO as a measure of hemolysis is in the setting of neonatal jaundice [8]. eCO is well-documented to have predictive value to assess the degree of pathologic hemolysis as an abnormally high eCO level can be distinguished from normal when subjected to receiver operating characteristic (ROC) curve analysis [50]. However, if the goal is to use eCO to determine the impact of normal variation of red cell lifespan on the relationship between blood glucose and HbA1c (which depends on both blood glucose and red cell lifespan [51]) and then adjust the final value to reflect the impact, this degree of week-to-week variation is unlikely to be informative. More specifically, applying the Strocchi equation with the assumption that eCO reflects the range of normal for red cell lifespan [52, 53], eCO would vary only from about 1.4–2.3 ppm. The current study demonstrating weekly variation often exceeds 1 ppm precludes applying eCO to adjust HbA1c for red cell life span

Our results suggest that improving the understanding of the physiology underlying CO production in normal and disease states will be a focus of future research. Efforts to refine techniques of collection and the sensitivity and specificity of measurement remain important. For example, Non-Dispersive Infrared sensors could be an option for measuring eCO due to their precision, non-invasive nature, and ability to provide real-time measurements [54]. A representative example that reflects these challenges is the interpretation of eCO and the need for further research is asthma. Most studies on asthma

demonstrate an increased level of systemic and exhaled eCO, and the correlation between increased eCO and asthma is well-documented [55–57]. The increased eCO is thought to originate from the induction of airway epithelial cell HO as a part of the underlying inflammatory process [58]. However, how to apply this in a clinically useful manner is not clear. For example, a study by Ohara *et al* [21] assessed eCO in asthmatic infants and toddlers. The study showed that patients with an established diagnosis of asthma, eCO a cut-off $\geqslant 2$ ppm discriminated an active asthma episode from asymptomatic state with a sensitivity of 95.6%. However, the specificity was only 43.3%. Moreover, even with a cutoff level of $\geqslant 3$ ppm, eCO established the diagnosis of asthma with a sensitivity of only 38.9% and a specificity of 74.1%. Improving the value of eCO collection in asthma and other disease states is likely to be dependent on advances in understanding the precise role of HO, how it varies in individuals with the apparent similar underlying pathology, and how this affects eCO. The variation in eCO in our study, although presumably explained to some extent by differences in exhalation flow rate and other collection technique issues, may also be ultimately explained by poorly understood physiologic factors, including subclinical changes in HO.

Our study has several limitations. The first limitation is the specificity of the Carbolyzer instrument measurements. Although the instrument is sensitive and measures CO and CO_2 in real time, electrochemical detector technology is not as specific as gold-standard methods, such as infrared spectroscopy [54] or gas chromatography. However, we accounted for the lack of specificity at high H_2 concentrations by subtracting these values. Regardless, this adds some possibility of additional inaccuracy to the final numbers. The second is the degree to which our eCO levels reflect ETCO. Samples that exceeded the 4.6% threshold of eCO_2 were considered adequate alveolar samples, but this may not have been a valid assumption. Third, although participants were screened for environmental factors that might influence eCO, and the Carbolyzer eCO was corrected for ambient CO concentrations, it is possible that environmental factors, particularly distinct from the controlled environment of our CRU, may have significantly contributed to the results. Fourth, our approach could have been enhanced by accounting for the impact of factors such as the expiratory flow rate, as summarized in a recent comprehensive study by Ghorbani *et al* [26] Finally, the difference in the absolute values of the **BrH** and **SrB** techniques raises a question about which reflects the true alveolar concentration of CO. The difference is relatively small but is consistently observed in most of the samples obtained. Moreover, because the CO_2 concentrations were also significantly higher with the **SrB** technique, our conclusion, within the limitations cited, is that the **SrB** approach is likely to reflect ETCO better than

Table 4. Comparison of the eCO collection methods.

Parameter	Continuous breathing	Breath holding	Short rebreathing
Average fasting eCO (ppm)	~ 1.9	~ 2.6	~ 3.1
Average fasting CO2 (%)	~ 4.1	~ 5	~ 5.2
Consistency/reproducibility (CV)	$\sim 9\%$	$\sim 4\%–5\%$	$\sim 4\%–5\%$
Comfort for subject	Good	Can be a challenge to hold breath	Good
Ease of Execution	Good	acceptable	Requires supplemental Oxygen source
eCO measurement	Continuous	One sample	One sample

BrH. (**see** table 4 **summarizing the three methods**) In addition, though not specifically surveyed, it was anecdotally observed that the **SrB** method, similar to reports by other investigators [28] applying similar techniques, was noted to be easier for participants to complete.

4. Conclusions

This study examined the collection of eCO using three techniques with an electrochemical detector. Given the consistent minute-to-minute concentrations and careful screening of the study participants, the diurnal and week-to-week variations in eCO levels were unlikely to be due to technical issues of measurement and collection. Therefore, we conclude that there is a significant physiological variation in the production of CO within the body, a phenomenon that is not yet fully understood. This presents a limitation in using CO as a reliable marker for assessing the physiological and disease states linked to changes in ETCO.

Data availability statement

The data cannot be made publicly available upon publication because the cost of preparing, depositing and hosting would be prohibitive with the terms of this research project. The data supporting the findings of this study are available upon reasonable request from the authors.

Conflict of interest

The authors declare that they have no affiliations with or involvement in any organization or entity with any financial interest in the subject matter or materials discussed in this manuscript.

Institutional Review Board Statement

The study was approved by the Institutional Review Board of the University of Cincinnati (protocol #2015-1070) and CVAMC Research and Development Committee. In both study phases, each participant received a written informed consent form for review, reading, and signing during the screening visit.

ORCID iDs

Shahriar Arbabi ● https://orcid.org/0009-0003-2395-0121
Eric P Smith ● https://orcid.org/0000-0002-9606-0109
Robert M Cohen ● https://orcid.org/0000-0002-3164-5843

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