Title: The within and between-day reliability of cerebrovascular reactivity using traditional and novel analytical approaches

Running title: Reliability of traditional and novel cerebrovascular reactivity analyses

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Abstract: Cerebrovascular reactivity of the middle cerebral artery velocity (CVR MCAv) to carbon dioxide (CO_2) is a common method to assess cerebrovascular function. Yet, the approaches used to calculate CVR outcomes vary. The aim of this study was to explore the within and between-day reliability of traditional CVR outcomes. The second aim was to explore the reliability of novel kinetic-based analyses. Healthy adults (n=10, 22.3±3.4 years) completed assessments of CVR over four minutes using a fixed fraction of inspired CO_2 (6%). This was repeated across four separate visits (between-day), and on one visit measures were repeated 2.5 hours later (within-day). No mean biases were present between assessments for traditional CVR metrics, expressed as absolute (cm/s/mmHg) or relative (%/mmHg) outcomes (minute-3, minute-4, peak 1 second, peak 30 second) (between-day: $P>0.14$, $\eta p^2<0.20$, within-day: $P>0.22$, $d>0.27$). Absolute, rather than relative CVR, yielded the most reproducible parameters (coefficient of variation: 8.1-13.2% versus 14-83% respectively). There were significant differences between CVR outcomes ($P<0.001$, $\eta p^2>0.89$) dependent on the time point used to determine CVR, as a steady state MCAv response was rarely observed. Furthermore, the MCAv response was not reproducible within an individual (kappa=0.15, $P=0.09$). No mean differences were present for novel kinetic outcomes (amplitude, time-delay, time constant) (between-day: $P>0.05$, $d<0.33$, within-day: $P>0.38$, $d<0.25$). The results support the need for standardisation and indicate CVR should be defined as a dynamic peak, rather than a set time point for increased reliability. For novel kinetic outcomes variability was greater (CV: 8.7-120.9%) due to the nature of time-based metrics.

Key words: Carbon dioxide breathing, middle cerebral artery, transcranial Doppler, kinetics

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Subject area: cardiovascular control

New findings:

- *What is the central question of the study?*
  What is the reliability of middle cerebral artery velocity cerebrovascular reactivity (CVR) when using traditional and novel outcomes, as measured by transcranial doppler?
- *What is the main finding and its importance?*
  Traditional CVR approaches presented acceptable reproducibility but should be expressed as an absolute CVR. Large within- and between-individual differences in the MCAv response profile support using a dynamic peak, rather than a set time point, for the most reliable interpretation.
  Our study highlights the utility of novel kinetic CVR outcomes, however, due to increased variability in time-based metrics, this analysis requires larger sample sizes then traditional methods.

Introduction:

The partial pressure of arterial carbon dioxide ($PaCO_2$) plays a primary role in the regulation of cerebral blood flow (CBF) in adults (Hoiland et *al.*, 2019). Elevations in $PaCO_2$ cause increased CBF and decreased cerebrovascular resistance within regions of the cerebrovasculature, from the extra- and intracranial arteries to the pial arteries (Kety & Schmidt, 1948; Wasserman & Patterson, 1961). Researchers can exploit this phenomenon by describing the change in CBF for a given CO_2 stimulus as an index of cerebrovascular reactivity (CVR). A diminished CVR response is associated with elevated risk of stroke (Yonas *et al.*, 1993; Markus & Cullinane, 2001; Reinhard *et al.*, 2014), and cardiovascular mortality (Portegies *et al.*, 2014). CVR is therefore an important outcome for understanding the influence of disease and interventions using both cross-sectional and longitudinal study designs.

A number of methodological variations in the measurement of CVR exist, and this lack of standardisation make comparisons between studies challenging. For example, estimates of CVR differ when determined by magnetic resonance imaging (MRI) or transcranial Doppler ultrasound (TCD) (Burley *et al.*, 2021). The latter approach is popular as it is more convenient, cheaper and can provide valuable information on the time course of the change in MCAv. However, studies adopting TCD also demonstrate important differences in the CO_2 concentrations administered, test duration, and CO_2 breathing techniques, which can alter the interpretation of CVR.

A commonly used method to assess CVR is CO_2 breathing via an open-circuit system. This is administered by inhalation of a fixed concentration of 5-7% CO_2 (Fierstra *et al.*, 2013), for a

duration typically ranging between 2-6 minutes (Kastrup *et al.*, 1998; Murrell *et al.*, 2013; Favre *et al.*, 2020; Tallon *et al.*, 2020). The CVR outcome is often reported as the change in middle cerebral artery blood velocity (MCAv) from baseline levels, relative to the change in end-tidal CO_2 ($P_{ET}CO_2$) (Murrell *et al.*, 2013; Reinhard *et al.*, 2014), used as a surrogate of $PaCO_2$ (McSwain *et al.*, 2010). Forced end-tidal clamping is sometimes used as a method of obtaining a standardised end-tidal O_2 and CO_2 stimulus (Fisher, 2016; Howe *et al.*, 2020). Using this approach, MCAv increases and then attains a steady-state following 2-3 minutes (Fisher, 2016). Thus, one advantage of end-tidal clamping is that the CVR outcome may be less influenced by the duration of the test provided that it was taken following 2 minutes and $P_{ET}CO_2$ was constant. However, this requires specialised equipment and involves expertise in monitoring and controlling the precise regulation of inhaled gasses. Given this, open-circuit CO_2 breathing is more typically employed. However, $P_{ET}CO_2$ concentrations may fluctuate as a result (Lu *et al.*, 2014) and a steady-state MCAv response may not be consistently attained, with some individuals reaching and maintaining steady-state, whilst others have an MCAv response that varies depending on the duration of the stimulus used or analysed (Burley *et al.*, 2020). Specifically, Burley et al (2020) reported CVR calculated from minute 2 of the CO_2 stimulus was ~22% greater than CVR values calculated from the fifth minute. Therefore, if a steady state MCAv profile is not achieved consistently within and between individuals, using a set time point for analysis (e.g. 4^{th} minute) of CVR may be inappropriate.

Inconsistencies in how open-circuit CO_2 breathing data are expressed include differences in MCAv time point e.g 3^{rd} vs 4^{th} minute, whether CVR is reported as a relative (percentage increase in MCAv per mmHg change in $P_{ET}CO_2$) or absolute (cm/s change in MCAv per mmHg change in $P_{ET}CO_2$) change in MCAv, and the time period of data used for calculating average MCAv. Despite its common uses, current knowledge on the within- and between-day reliability of CO_2 breathing CVR as assessed using TCD is limited. There are limited data regarding the reliability of open-circuit CO_2 breathing (Totaro *et al.*, 1999; McDonnell *et al.*, 2013). McDonnell *et al.* (2013) reported 'acceptable' reproducibility between measures (Coefficient of variation: 36.7 ± 8.1%), but the authors utilised a test duration of two minutes and reported CVR as the percent increase in MCAv only, which does not take into account the $P_{ET}CO_2$ response. Totaro *et al.* (1999) also reported 'acceptable' reproducibility of CVR when presented as relative CVR using a short ~2 minute CO_2 breathing stimulus (Intraclass correlation coefficient: 0.55 and 0.45 for short-term (1 hour) and long-term (24 hours) reproducibility, respectively).

Critical scrutiny of the methodological considerations for CVR are important, as they may account for the equivocal nature of findings in the literature, such as the effect of ageing on CVR (Murrell *et al.*, 2013; Coverdale *et al.*, 2016; Hoiland *et al.*, 2019; Burley *et al.*, 2021). This is evident in a recent study in which Tallon et al (2020) showed that CVR expressed using traditional approaches (the absolute and relative MCAv response to 6% CO_2 breathing after 4 minutes) demonstrated no difference between healthy adults and children. However, the authors also utilised a novel approach in which a mono-exponential model with a time delay was used to characterise the amplitude and time-based parameters of the MCAv response and found that the MCAv response to increases in CO_2 was slower in children compared to adults. This highlights how traditional CVR metrics may provide limited insight and fail to account for non-steady state trajectories of MCAv. By utilising novel kinetic analyses, additional outcomes of interest such as the amplitude, time delay (TD) and time constant (τ) can be investigated, which may provide unique insights into changes in age and disease status. Kinetic analyses have been utilised to characterise cerebrovascular responses to exercise, demonstrating unique regulatory profiles in younger and older adults (Billinger *et al.*, 2017; Ward *et al.*, 2018). In order to advance the uses of these novel kinetic outcomes, reliability data on the amplitude and time-based characteristics of the MCAv response is essential.

The aim of this study was to establish the between and within-day reliability of traditional CVR outcomes to CO_2 breathing in healthy adults. The second aim was to explore the utility and reliability of novel kinetic-based analyses to CO_2 breathing.

Methods:

Ethical approval: All experimental procedures and protocols were approved by the University of Exeter ethics committee (180613/A/07), and the study conformed to the standards set by the Declaration of Helsinki, except for registration in a database. Written informed consent was obtained prior to participation in the study.

Participants:

Eleven healthy participants volunteered to take part in this study. One participant was removed from data analysis due to inability to obtain a clear MCAv signal, therefore data are presented for n=10 (7 female) (*age:* 22.3±3.4 years, *stature:* 170±8 cm, *body weight:* 67±10 kg). These data were collected as part of a wider experimental design. Specifically, the present study scrutinizes the resting data from baseline assessments performed on four separate days (between-day), which included one seated control trial (within-day). Participants were

familiarised to all measures before the first day of data collection. Exclusion criteria included diagnosed hypertension, smoking, any known cardiometabolic or respiratory diseases, contraindications to maximal exercise, the use of any prescribed medications known to influence cardiovascular function, and individuals not between the ages of 18-35 years.

Study design:

Participants were required to fast for 12 hours prior to all testing sessions, and refrain from vigorous physical activity, caffeine and alcohol consumption for 24 hours prior to testing. For each of the four visits, participants arrived at the laboratory at 8:00 am to control for any potential circadian effect (Otto *et al.*, 2004). Participants were then provided with a standardised breakfast of 50 g cornflakes (Kellogg's, UK) and 150 mL semi skimmed milk (providing approximately: 47 g of carbohydrate, 9 g of protein, 3 g of fat and 262 kcal). The macronutrient contribution of this is unlikely to have influenced endothelial function (Vogel *et al.*, 1997; Koep *et al.*, 2021). Following this, participants rested in a darkened, temperature-controlled room (~23ºC) for 15 minutes in the supine position prior to measurement of CVR, which commenced 30 minutes after breakfast consumption. For within-day reliability, the CVR measure was repeated on one of these visits (randomised), 2.5 hours after the completion of the first assessment, with the participant remaining seated in the laboratory throughout, and only permitted to drink water.

Experimental measures

The CVR assessment protocol consisted of a one-minute resting baseline followed by four minutes of hypercapnia in the supine position. During hypercapnia, 6% CO_2 was administered with 21% oxygen (balance nitrogen). A three-way valve (Hans Rudolph) allowed inspiratory gases to be switched from ambient air to the 6% CO_2 mixture (using a 1,000 L Douglas bag). This replicates other laboratories which have performed traditional and kinetic assessments of CVR (Tallon *et al.*, 2020). Participants were instructed to breathe normally during the hypercapnia period. Throughout the protocol, MCA_V, $P_{ET}CO_2$, mean arterial pressure (MAP), and minute ventilation ($\dot{V}_E$) were measured simultaneously (described below).

Cardiorespiratory measures

During the protocol, beat-by-beat blood pressure was measured continuously by finger plethysmography (NIBP, ADInstruments, Colorado). Participants wore a facemask (Hans Rudolph, Kansas) to measure $P_{ET}CO_2$ using a gas analyser (ADInstruments, ML206, Colorado), which was calibrated prior to each participant via known concentrations of oxygen and carbon dioxide. $\dot{V}_E$ was measured with a spirometer (ADI instruments, Colorado), calibrated with a 3 L syringe. All data were sampled continuously (Powerlab; model - 8/30, ADInstruments) and stored at 200 Hz using an analogue-to-digital converter interfaced with a laptop computer (Lab Chart version 8, ADInstruments) for offline analysis.

Cerebrovascular measures

A 2-MHz Transcranial Doppler ultrasound probe was used to insonate the right MCA at an initial depth of ~50 mm. The Doppler signal was then acquired, optimised, and secured using an adjustable headset (DWL, DiaMon, Compumedics, Germany). The probe position and depth and gain of the TCD signal were replicated for each scan for each participant. Beat-by-beat MCAv was calculated as the mean across each cardiac cycle and exported from LabChart as second by second data for analysis (Version 8, ADI instruments). To account for the influence of MAP on MCAv, the ratio between MCAv and mean arterial pressure was expressed as the cerebrovascular resistance index (CVRi) (CVRi = MAP/MCAv).

Traditional steady-state response to hypercapnia

Baseline values were averaged over one minute of supine rest. Steady-state CVR was calculated as both absolute and relative (percentage) change from baseline in MCAv per unit increase (mmHg) in $P_{ET}CO_2$. This response was taken in the final 30 seconds of minute three and minute four, as well as the peak response (1 s) and peak response as a rolling 30 second average. These approaches allow the comparison between typical standardised time points and addresses concerns about the time course of the MCAv response by taking this as a peak wherever it occurs rather than a set time point (Burley *et al.,* 2020).

Novel Kinetic response to hypercapnia

MCAv and $P_{ET}CO_2$ data were exported as 1-s averages. Data were baseline-corrected for the 60-s preceding hypercapnia and analysed using a mono-exponential model with time delay (equation 1) using GraphPad Prism (Graphpad Software, San Diego, CA).

$$MCAv(t) = \Delta MCAv_A (1 - e^{-(t-TD/\tau)}) \quad \text{(equation 1)}$$

where MCAv(t) is the MCAv at a given time (t), $\Delta MCAv_A$ is the amplitude change of MCAv from baseline to its asymptote, TD is the time delay and τ is the time constant, in accordance with kinetic modelling in previous work (Poole & Jones, 2012; Tallon *et al.*, 2020). The exponential model was fitted from the start of the exponential rise in MCAv or $P_{ET}CO_2$ until a deviation from a visual steady-state was observed. The start and end of each exponential fit was blindly verified by two researchers, and any disagreements discussed with the research team until a consensus was reached. All models were then checked for consistency of approach, and to determine model acceptability (goodness of fit $r^2>0.50$, standard error of the τ, and normality of residuals).

Responses were classified as: 1) steady-state; 2) increase following steady-state, or 3) decrease following steady-state for each assessment for MCAv and $P_{ET}CO_2$. Steady-state was identified by an exponential that could be fit from the onset of the response to the end of the test, or as a change in $MCAv/P_{ET}CO_2$ from baseline to the last 30 seconds of a test that fell within the 95% confidence intervals of the amplitude of the exponential rise. Any response above or below this 95% confidence interval of the amplitude was identified as an increase or a decrease, respectively.

Statistical analyses

The reproducibility of cerebrovascular and respiratory variables was analysed using repeated measures analysis of variance (ANOVA) with assessment (within-day) or visit (between-day) as the main effects. For within-day analyses where two assessments were analysed, follow up pairwise comparisons were interpreted as standardised effect sizes (*d)* to document the magnitude of the effect using the following thresholds: ≥0.2 <0.5 = small, ≥0.5 <0.8 = moderate and ≥0.8 = large (Cohen, 1988). The main effect of visit for the between-day ANOVA are displayed as partial eta squared (ηp^2), and interpreted as ≤0.06 = small, 0.06 to 0.14 = moderate and >0.14 = large (Cohen, 1988). The reproducibility of these outcomes were further explored using the typical error, also expressed as a coefficient of variation (CV) and intraclass correlation coefficient (r) for within-day, and between-day analyses respectively (Hopkins, 2000). For all variables, 95% confidence intervals (CI) were calculated for the CV, to compare reliability between outcomes. Statistical analyses were conducted using SPSS (version 25; IBM, Armonk, New York) and data are presented as mean ± SD. Statistical significance was accepted at an alpha of 0.05.

Cohen's kappa was used to determine the agreement between the MCAv and $P_{ET}CO_2$ categorical classification of response profiles across the assessments (within-day), and Fleiss' kappa was used to characterise the between-day responses. The strength of the agreement between responses was determined as; kappa <0.2 = poor, 0.21-0.40 = fair, 0.41-0.60 = moderate, 0.61-0.80 = good, 0.81-1.00 = very good (Landis & Koch, 1977).

In order to compare between CVR outcomes, separate 2-way repeated measures ANOVA were performed, with outcome (peak 1 s, peak 30 s, minute 3, minute 4) and visit (between-day) or assessment (within-day) as the main effects. Post hoc comparisons (LSD) were performed to identify which outcomes were significantly different and are discussed in the text.

Results

Within-day reliability

Traditional cerebrovascular reactivity

The within-day reliability for CVR parameters can be seen in Table 1. There were no significant differences between assessment 1 and 2 for any of the CVR outcomes (all $P>0.22$, $d<0.27$).

CVR was significantly different dependent on the time point used to calculate the CVR outcome (main effect of outcome $P<0.001$, $\eta p^2=0.52$), except between minute-3 and minute-4 ($P=0.56$).

MCAv

The within-day reliability for MCAv and $P_{ET}CO_2$ parameters of interest are presented in Table 2. No mean differences were present for baseline MCAv, peak MCAv during hypercapnia as a 1 s or 30 s peak, and the time taken to achieve peak MCAv as a 1 s or as a 30 s rolling average ($P>0.17$, $d<0.35$ for all). Likewise, at both the three- and four-minute time points, MCAv was not significantly different between assessments ($P>0.31$, $d>0.21$ for all).

Comparisons between MCAv parameters showed there were significant differences between outcomes dependent on the time point analysed ($P<0.001$, $\eta p^2=0.72$). Pairwise comparisons

revealed significant differences between all MCAv outcomes (P<0.002), except between minute-3 and minute-4 (P=0.78).

$P_{ET}CO_2$

No mean differences were present for baseline $P_{ET}CO_2$, peak $P_{ET}CO_2$ during hypercapnia as a 1 s or 30 s peak, and the time taken to achieve peak $P_{ET}CO_2$ as both a 1 second peak or 30 second rolling average (P>0.29, d<0.29 for all). Likewise, at both the minute-3 and minute-4 time point $P_{ET}CO_2$ was not significantly different between assessments (P>0.83, d<0.10 for all).

Comparisons between $P_{ET}CO_2$ parameters showed there were significant differences between outcomes dependent on the time point analysed (P<0.001, ηp^2=0.71). Pairwise comparisons revealed significant differences between all $P_{ET}CO_2$ outcomes (P<0.005), except between minute-3 and minute-4 (P=0.24).

Novel kinetic analyses

For the MCAv mono-exponential kinetic parameters, no mean biases were present between assessments for the τ, TD or amplitude (P>0.38, d<0.25 for all). Three different MCAv profiles to hypercapnia were observed. Across the 20 within-day assessments, 4 were classified as steady-state, 9 decreased and 7 increased throughout the test. Cohen's kappa analysis, showed no within participant agreement between the three response profiles for MCAv across the two assessments (kappa=0.27; 95% CI=0.19–0.14; P=0.17). Representative profiles from 3 participants are provided in Figure 1.

For the $P_{ET}CO_2$ mono-exponential kinetic parameters, no mean biases were present between visits for the TD and amplitude (P>0.24, d<0.48 for all). The τ showed significant differences between assessments (P=0.04, d=0.89). Cohen's kappa analysis, showed no agreement between the three steady-state classifications of response profiles across the two assessments within an individual (kappa=0.14 95% CI, 0.24 – 0.57; P=0.57).

Mean arterial pressure and minute ventilation

The within-day reliability for MAP, $\dot{V}_E$ and CVRi parameters of interest are provided in supplementary Table 1. There were no mean differences for any of the MAP (P>0.22, d<0.46 for all), $\dot{V}_E$ (P>0.21, d<0.38 for all) or CVRi parameters (P>0.084, d<0.29) between assessment 1 and assessment 2.

Comparisons between $\dot{V}_E$ and MAP parameters showed there were significant differences between outcomes dependent on the time point analysed (P<0.001, ηp^2<0.78 for all). Pairwise comparisons revealed significant differences between all $\dot{V}_E$ outcomes (P<0.001). For MAP outcomes, pairwise comparisons revealed significant differences between outcomes (P<0.008), except between minute-3 and minute-4 (P=0.18).

Between-day reliability

Traditional cerebrovascular reactivity

The between-day reliability for CVR parameters of interest is presented in Table 3. There were no mean differences present between assessments for any of the CVR parameters (P>0.14, ηp^2<0.20 for all).

For both absolute and relative CVR metrics there were significant differences between outcomes dependent on the time point used to calculate CVR (P<0.001, ηp^2>0.89 for all). Pairwise comparisons revealed significant differences between all CVR outcomes (P<0.05), except between minute-3 and minute-4 (P=0.184).

MCAv

The between-day reliability for MCAv and $P_{ET}CO_2$ parameters of interest are presented in Table 4. No differences between visits were present for baseline MCAv, and time to peak MCAv as a 30 s rolling average (P>0.27, ηp^2<0.15 for all). There were mean differences present for peak MCAv as a 1 s peak or 30 s rolling average, and time taken to achieve peak MCAv as a 1 s peak between visits (P<0.001, ηp^2 <0.45 for all). Mean differences were present between visits for both the 3 and 4 minute time points (P<0.001, ηp^2 <0.47). Post-hoc pairwise comparisons identifying the location of significant differences between visits are provided in Table 4.

Comparisons between MCAv parameters showed there were significant differences between outcomes dependent on the time point analysed (1s peak, 30 s peak, minute 3, minute 4) (P<0.001, ηp^2=0.72). Pairwise comparisons revealed significant differences between all MCAv outcomes (P<0.008), except between minute-3 and minute-4 (P=0.18).

$P_{ET}CO_2$

For the $P_{ET}CO_2$ outcomes no mean differences were present between visits for baseline $P_{ET}CO_2$ and time taken to achieve peak $P_{ET}CO_2$ as a 1 s peak or 30 s rolling average (P>0.24, ηp^2<0.17

for all). Peak $P_{ET}CO_2$ during hypercapnia as a 1 second peak or as a 30 s rolling average, and $P_{ET}CO_2$ at the 3 and 4 minute time points was significantly different between visits ($P<0.01$, $\eta p^2<0.36$ for all). Post-hoc pairwise comparisons identifying the location of significant differences between visits can be seen in Table 4.

Comparisons between $P_{ET}CO_2$ parameters showed there were significant differences between outcomes dependent on the time point analysed (1s peak, 30 s peak, minute 3, minute 4) ($P<0.001$, $\eta p^2=0.79$). Pairwise comparisons revealed significant differences between all $P_{ET}CO_2$ outcomes ($P<0.008$), except between minute 3 and minute 4 ($P=0.317$).

Novel kinetic analyses

For the MCAv mono-exponential kinetic parameters, no mean differences were observed between visits for the τ, TD and amplitude ($P>0.05$, $\eta p^2<0.33$ for all). Of the 40 between-day assessments, the MCAv profile was classified as steady-state in 6, decreasing in 16 and increasing in 17 assessments. Fleiss' kappa showed no agreement within an individual between the three different profiles of the response between the four assessments (Fleiss kappa=0.15, 95% CI, -0.026 – 0.330, $P=0.09$).

For the $P_{ET}CO_2$ mono-exponential kinetic parameters, no mean differences were observed between visits for the τ, TD and amplitude ($P>0.21$, $\eta p^2>0.039$ for all). Fleiss' kappa showed a fair agreement between the three different profiles of the response between the four assessments within an individual, (Fleiss kappa=0.21, 95% CI, 0.03-0.39, $P=0.02$).

Mean arterial pressure and minute ventilation

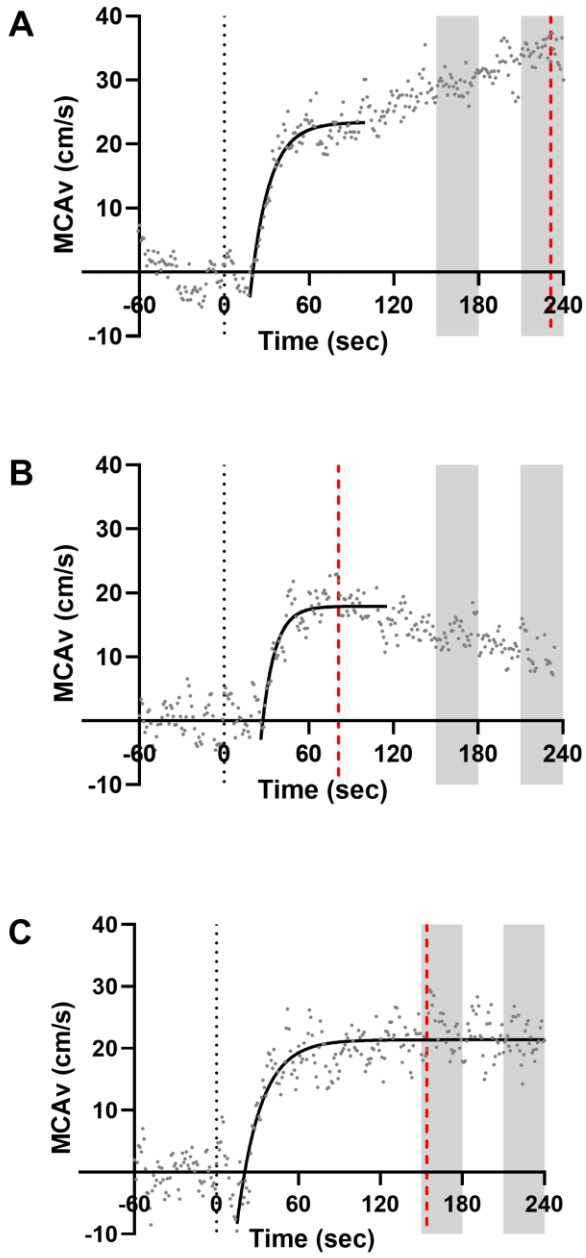
The between-day reliability for MAP, $\dot{V}_E$ and CVRi outcomes is provided in supplementary Table 2. There were no mean differences present between visits for peak MAP (as a 1 s peak or 30 s rolling average), or when expressed as an amplitude as a 1 s or 30 s peak ($P>0.07$, $\eta p^2<0.16$ for all). Differences were present for baseline MAP, as well as MAP at both minute three and minute four time points ($P<0.03$, $\eta p^2<0.30$ for all). Post-hoc pairwise comparisons identifying the location of significant differences between visits can be seen in Supplementary Table 2.

Comparisons between MAP parameters showed there were significant differences between MAP outcomes dependent on the time point taken (1s peak, 30 s peak, minute 3, minute 4)

(*P*<0.001, ηp^2=0.75). Pairwise comparisons revealed significant differences between all MAP outcomes (*P*<0.002), except between minute-3 and minute-4 (*P*=0.08).

There were no mean differences between assessments for any of the $\dot{V}_E$ parameters of interest (*P*>0.15, ηp^2>0.61 for all), and no significant differences between $\dot{V}_E$ outcomes dependent on the time point analysed (1s peak, 30 s peak, minute 3, minute 4) (*P*=0.13, ηp^2=0.75).

There were no mean differences present between visits for baseline CVRi (*P*=0.07, ηp^2=0.25) and peak CVRi as a 1 second peak (*P*=0.05, ηp^2=0.31). Differences were present for peak CVRi expressed as a 30 second peak, as well as CVRi at both the minute three and minute four time points (P<0.04, ηp2<0.35 for all).



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355 *Figure 1.* Representative traces illustrating the individual responses observed during hypercapnia in
356 three participants. The grey boxes show where the three minute (150-180 seconds) and four minute
357 (210-240) set time points occur. The black dotted line represents the onset of CO_2 breathing (0 seconds).
358 The red dashed line represents where the peak MCAv occurs during the test as a 1 second peak. A)
359 Continual increase in MCAv following steady-state; B) MCAv decreases following steady-state; and
360 C) Steady-state MCAv response. MCAv, middle cerebral artery velocity.

Table 1. Within-day traditional cerebrovascular reactivity reliability

Variable	Assessment 1	Assessment 2	Change in mean	*P* value	*d*	Typical error	Typical error as CV % (95% CI)	r
Absolute CVR (1s peak) (cm/s/mmHg)	2.5 ± 0.5	2.4 ± 0.6	0.1	0.38	0.18	0.2	11.7 (8.3-18.7)	0.84
Absolute CVR (30s average) (cm/s/mmHg)	2.3 ± 0.4	2.2 ± 0.6	0.1	0.32	0.22	0.2	12.3 (8.6-19.4)	0.82
Absolute CVR (minute 3) (cm/s/mmHg)	2.3 ± 0.4	2.2 ± 0.5	0.1	0.22	0.23	0.2	10.7 (7.4-16.6)	0.88
Absolute CVR (minute 4) (cm/s/mmHg)	2.2 ± 0.5	2.2 ± 0.6	0.1	0.60	0.11	0.3	13.2 (9.0-21.5)	0.84
Relative CVR (1s peak) (%/mmHg)	4.4 ± 0.8	4.8 ± 1.8	0.4	0.35	0.27	1.2	28.0 (21.6-52.3)	0.35
Relative CVR (30s peak) (%/mmHg)	3.5 ± 0.9	3.5 ± 1.2	0.1	0.90	0.04	0.8	29.7 (21.7-52.7)	0.49
Relative CVR (minute 3) (%/mmHg)	3.1 ± 1.0	3.4 ± 2.2	0.2	0.78	0.14	1.8	77.8 (49.9-139.3)	0.17
Relative CVR (minute 4) (%/mmHg)	2.9 ± 1.7	2.8 ± 1.4	0.2	0.74	0.11	1.1	83.0 (55.0-168.7)	0.53

Data presented as mean ± SD (n=10). CVR, cerebrovascular reactivity.

363 **Table 2**. Within-day MCAv and $P_{ET}CO_2$ reliability, and novel kinetic analyses

Variable		Assessment 1	Assessment 2	Change in mean	*P* value	*d*	Typical error	Typical error as CV (%) (95%CI)	r
Baseline	*MCA_v (cm/s)*	84.1 ± 11.1	81.6 ± 12.7	-2.5	0.17	0.21	4.1	5.3 (3.8–8.8)	0.91
	$P_{ET}CO_2$ (mmHg)	39.1 ± 4.0	40.4 ± 4.7	1.3	0.29	0.29	2.5	6.3 (4.5-10.5)	0.72
Peak 1s	*MCA_v (cm/s)*	119.6 ± 21.1	116.0 ± 26.9	-3.6	0.62	0.15	15.5	14.3 (10.2-24.6)	0.65
	$P_{ET}CO_2$ (mmHg)	48.7 ± 5.4	49.4 ± 7.1	0.8	0.60	0.12	3.1	6.9 (5.0-11.6)	0.81
Peak 30s average	*$MCA_{v\ mean}$ (cm/s)*	111.7 ± 21.2	107.3 ± 25.6	-4.4	0.54	0.19	15.7	15.1 (10.8-26.0)	0.61
	$P_{ET}CO_2$ (mmHg)	48.2 ± 5.6	49.0 ± 7.2	0.8	0.60	0.12	3.2	7.2 (5.2-12.1)	0.80
Time to 1s peak (s)	*MCAv*	157.2 ± 60.8	134.7 ± 68.9	-22.5	0.33	0.35	49.0	39.7 (27.6-73.3)	0.48
	$P_{ET}CO_2$	151.5 ± 58.2	145.4 ± 77.6	-6.1	0.72	0.09	37.3	46.4 (32.1-87.3)	0.76
Time to 30s peak (s)	*MCAv*	149.9 ± 54.7	140.4 ± 67.0	-9.5	0.55	0.16	33.9	27.4 (19.3-48.9)	0.74
	$P_{ET}CO_2$	168.0 ± 60.2	160.2 ± 75.9	-7.8	0.71	0.11	44.7	40.7 (28.3-75.4)	0.62
Minute 3	*MCAv (cm/s)*	107.8 ± 20.9	101.1 ± 25.2	-6.8	0.31	0.21	14.1	13.9 (9.9-23.8)	0.69
	$P_{ET}CO_2$ (mmHg)	47.8 ± 5.6	47.6 ± 7.9	-0.2	0.92	0.02	3.4	7.8 (5.7-13.2)	0.80
Minute 4	*MCAv (cm/s)*	105.0 ± 21.5	102.6 ± 28.0	-2.4	0.75	0.10	16.0	16.2 (11.6-28.0)	0.64
	$P_{ET}CO_2$ (mmHg)	47.6 ± 5.8	48.0 ± 7.6	0.3	0.83	0.05	3.3	7.6 (5.5-12.8)	0.81

τ (s)	*MCAv*	17.3 ± 8.5	20.2 ± 8.0	2.9	0.38	0.25	7.1	51.9 (35.6-98.9)	0.29
	$P_{ET}CO_2$	16.6 ± 8.1	11.1 ± 3.1	-5.5	**0.04**	0.89	4.9	33.6 (23.5-61.0)	0.40
TD (s)	*MCAv*	21.7 ± 7.3	20.4 ± 5.9	-1.3	0.70	0.19	7.0	37.3 (26.0-68.4)	0.12
	$P_{ET}CO_2$	8.8 ± 6.7	11.6 ± 4.9	2.8	0.24	0.48	4.9	154.5 (95.6-394.6)	0.34
Amplitude	*MCAv (cm/s)*	107.1 ± 18.5	106.2 ± 21.7	-0.9	0.87	0.14	12.5	12.6 (9.1-21.6)	0.67
	$P_{ET}CO_2$ (mmHg)	47.1 ± 5.8	48.5 ± 6.6	1.4	0.36	0.05	3.2	7.1 (5.1-11.9)	0.78
Amplitude delta	*MCAv (cm/s)*	23.0 ± 10.2	24.6 ± 12.5	1.6	0.72	0.14	9.9	75.1 (50.4-151.3)	0.29
	$P_{ET}CO_2$ (mmHg)	8.0 ± 2.3	8.1 ± 2.7	0.1	0.87	0.04	1.8	31.9 (22.4-57.7)	0.57
Amplitude delta (%)	*MCAv*	27.2 ± 10.8	29.9 ± 12.8	2.7	0.61	0.23	11.6	71.9 (48.5-143.9)	0.40
	$P_{ET}CO_2$	20.3 ± 4.7	19.9 ± 6.2	-0.3	0.88	0.07	4.8	31.1 (21.8-56.1)	0.27

Data presented as mean ± SD (n=10). Significant main effect P values are shown in bold. $P_{ET}CO_2$**,** end-tidal carbon dioxide; MCAv, middle cerebral artery velocity. MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; τ, Tau; TD, time-delay.

366 **Table 3**. Between-day traditional cerebrovascular reactivity reliability

Variable	Visit 1	Visit 2	Visit 3	Visit 4	*P* value	ηp^2	Typical error	Typical error as CV % (95% CI)	r
Absolute CVR (1s peak) (cm/s/mmHg)	2.6 ± 0.6	2.4 ± 0.5	2.5 ± 0.5	2.5 ± 0.4	0.23	0.16	0.2	8.1 (6.5-11.4)	0.90
Absolute CVR (30s peak) (cm/s/mmHg)	2.4 ± 0.6	2.3 ± 0.5	2.3 ± 0.5	2.4 ± 0.4	0.24	0.16	0.2	8.7 (7.0-12.3)	0.88
Absolute CVR (minute 3) (cm/s/mmHg)	2.3 ± 0.5	2.2 ± 0.5	2.3 ± 0.5	2.3 ± 0.4	0.42	0.11	0.2	9.4 (7.5-13.3)	0.87
Absolute CVR (minute 4) (cm/s/mmHg)	2.4 ± 0.6	2.2 ± 0.5	2.3 ± 0.5	2.3 ± 0.4	0.15	0.20	0.2	9.3 (7.4-13.1)	0.88
Relative CVR (1s peak) (%/mmHg)	4.7 ± 1.2	4.3 ± 1.0	4.8 ± 0.8	4.6 ± 0.8	0.70	0.06	0.6	14.0 (11-20)	0.71
Relative CVR (30s peak) (%/mmHg)	3.9 ± 1.2	3.3 ± 0.8	3.6 ± 0.9	3.9 ± 0.7	0.32	0.06	0.6	18.1 (14.4-26.1)	0.67
Relative CVR (minute 3) (%/mmHg)	3.4 ± 1.2	3.0 ± 1.1	3.3 ± 1.2	3.6 ± 0.8	0.60	0.07	0.8	35.0 (27.0-51.0)	0.50
Relative CVR (minute 4) (%/mmHg)	3.7 ± 1.3	3.0 ± 1.1	3.2 ± 1.4	3.8 ± 0.8	0.14	0.2	0.8	29.7 (23.4-29.7)	0.65

367 **Data presented as mean ± SD (n=10)**. CVR, cerebrovascular reactivity.

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369 **Table 4**. Between-day MCAv and $P_{ET}CO_2$, and novel kinetic analyses

Variable		Visit 1	Visit 2	Visit 3	Visit 4	*P* value	ηp^2	Typical error	Typical error as CV (%) (95%CI)	r
Baseline	*MCA_v (cm/s)*	83.7 ± 8.5	84.2 ± 17.7	87.9 ± 14.0	85.4 ± 10.5	0.71	0.03	8.5	9.3 (7.4-13.2)	0.64
	$P_{ET}CO_2$ (cm/s)	39.3 ± 5.0	38.7 ± 5.6	41.8 ± 3.7	41.0 ± 4.1	0.24	0.17	2.0	5.4 (4.3-7.5)	0.86
Peak 1s	*MCAv (cm/s)*	128.7 ± 26.8[a]	113.7 ± 20.9[a,d,e]	127.2 ± 22.3[d]	129.3 ± 20.3[e]	**0.01**	0.45	9.8	8.2 (6.6-11.6)	0.85
	$P_{ET}CO_2$ (mmHg)	50.5 ± 5.2[a]	48.0 ± 5.4[a,d,e]	51.1 ± 4.7[d]	52.2 ± 4.2[e]	**0.01**	0.36	2.4	5.1 (4.1-7.2)	0.81
Peak 30s (cm/s)	*MCAv (cm/s)*	120.1 ± 24.6[a]	106.0 ± 19.7[a,e]	117.5 ± 22.8	121.3 ± 19.9[e]	**0.003**	0.44	10.2	9.2 (7.4-13.1)	0.82
	$P_{ET}CO_2$ (mmHg)	50.1 ± 5.4[a]	47.7 ± 5.6[a,d,e]	50.8 ± 4.7[d]	51.9 ± 4.2[e]	**0.01**	0.35	2.4	5.2 (4.2-7.3)	0.82
Time to 1s peak (s)	*MCAv*	177.3 ± 68.0[b]	139.8 ± 53.7[e]	133.4 ± 49.8[b,f]	195.5 ± 48.2[e,f]	**0.01**	0.45	42.7	37.3 (29.2-55.5)	0.46
	$P_{ET}CO_2$	159.4 ± 69.9	154.4 ± 48.0	151.2 ± 61.5	186.2 ± 47.6	0.38	0.12	46.6	45.5 (35.3-68.6)	0.38
Time to 30s peak (s)	*MCAv*	173.9 ± 70.7	149.2 ± 58.0	164.0 ± 63.0	198.9 ± 52.9	0.27	0.15	47.1	37.7 (29.5-56.2)	0.46
	$P_{ET}CO_2$	180.1 ± 65.7	176.6 ± 47.2	180.2 ± 66.6	192.6 ± 51.3	0.75	0.05	42.6	37.4 (29.2-55.7)	0.51
Minute 3	*MCAv (cm/s)*	114.4 ± 23.7[a]	102.0 ± 19.4[a,e]	113.6 ± 22.5	116.9 ± 19.3[e]	**0.01**	0.39	10.4	9.9 (8.0-14.1)	0.81

	$P_{ET}CO_2$ *(mmHg)*	49.5 ± 5.2[a]	47.2 ± 5.9[a,d,e]	50.3 ± 4.9[d]	51.3 ± 4.2[e]	**0.02**	0.34	2.4	5.4 (4.3-7.6)	0.81
Minute 4	*MCAv (cm/s)*	117.8 ± 26.[5a,b]	101.9 ± 20.5[a,e]	113.4 ± 24.0[b]	119.2 ± 20.3[e]	**0.001**	0.47	11.1	10.2 (8.1-14.5)	0.81
	$P_{ET}CO_2$ *(mmHg)*	49.7 ± 5.4[a]	47.2 ± 5.9[a,d,e]	50.4 ± 4.7[d]	51.4 ± 4.2[e]	**0.02**	0.34	2.6	5.7 (4.5-8.0)	0.79
τ (s)	*MCAv*	16.6 ± 6.5	21.0 ± 8.4	18.5 ± 6.1	17.6 ± 6.8	0.46	0.10	6.8	42.1 (32.8-63.10	0.08
	$P_{ET}CO_2$	13.4 ± 7.6	13.2 ± 4.8	11.7 ± 3.7	10.6 ± 1.9	0.34	0.13	4.2	33.0 (25.9-48.8)	0.34
TD (s)	*MCAv*	21.0 ± 5.7	20.1 ± 6.4	17.7 ± 8.7	18.0 ± 5.1	0.75	0.05	4.9	120.9 (90.0-202.8)	0.48
	$P_{ET}CO_2$	10.2 ± 5.4	10.2 ± 5.4	10.1 ± 5.9	8.6 ± 5.3	0.81	0.04	3.7	102.1 (76.9-171.9)	0.60
Amplitude	*MCAv (cm/s)*	113.1 ± 19.3[a]	103.8 ± 19.0[a,e]	113.7 ± 22.9	115.3 ± 17.8[e]	**0.02**	0.33	9.0	8.7 (7.0-12.4)	0.84
	$P_{ET}CO_2$ *(mmHg)*	48.7 ± 5.7	46.8 ± 5.9	50.6 ± 5.7	49.8 ± 4.5	0.21	0.19	2.9	6.0 (4.8-8.4)	0.77
Amplitude delta	*MCAv (cm/s)*	29.4 ± 12.0	22.6 ± 11.5	25.8 ± 13.2	30.0 ± 9.5	0.05	0.30	7.7	40.6 (31.6-60.7)	0.62
	$P_{ET}CO_2$ *(mmHg)*	9.4 ± 2.0	8.1 ± 2.6	8.8 ± 3.8	8.8 ± 1.7	0.16	0.19	2.3	30.8 (24.2-45.4)	0.26
Amplitude delta (%)	*MCAv (cm/s)*	34.4 ± 11.7	27.7 ± 12.7	29.0 ± 13.3	34.9 ± 9.5	0.11	0.25	9.5	41.9 (32.7-62.9)	0.40
	$P_{ET}CO_2$ *(mmHg)*	24.2 ± 5.7	21.5 ± 9.2	21.2 ± 9.4	21.6 ± 4.7	0.14	0.20	6.7	32.9 (25.8-48.6)	0.23

Data presented as mean ± SD (n=10). Significant main effect P values are shown in bold. Significant pairwise comparisons (P<0.05) between the data collected on each of the four days are as follows: a: 1v2. b: 1v3. c: 1v4. d: 2v3.e: 2v4. f: 3v4. MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$**,** end-tidal carbon dioxide; τ, Tau; TD, time-delay.

Discussion:

This study examined the within and between-day reliability of traditional and novel approaches for quantifying CVR to CO_2 breathing in healthy adults. These data have important implications with regard to selecting the most appropriate analysis methods to express CVR, as well as providing key data to inform future studies. The main finding was that traditional CVR outcomes yielded adequate reliability within- and between-days. Absolute CVR (cm/s/mmHg) provided the most reliable estimate compared to relative CVR. The results highlight the differential profiles of the MCAv response within and between individuals, indicating that traditional CVR should be determined as a dynamic 1 s or 30 s peak MCAv response wherever this occurs, rather than using a set time point. The significant differences between CVR outcomes dependent on the time point used to calculate CVR, highlight the importance of standardisation. For novel kinetic outcomes, variability was higher due to the nature of time-based metrics.

Traditional CVR analyses

It is evident from the current study and previous research (Burley *et al.*, 2020), that CVR is altered dependent on the time point analysed for some individuals, whilst the MCAv response in others remain relatively stable. The adoption of a set time-point to assess CVR assumes that individuals present a steady-state response to CO_2 breathing. Our data demonstrate that such a MCAv steady-state response is not common within-day (4 of 20 assessments) or between days (6 of 40 assessments). Furthermore, the classification of the CVR response profile was not reliable within a participant. Thus, determining CVR using a pre-defined time point introduces variability, and may under- or over-estimate CVR in an unpredictable manner. The variability of this MCAv response is paralleled by the high within (37.3%) and between (39.7%) day typical error for the time when the MCAv peak was observed. This can be accounted for by using either a 1 second or 30 second rolling average at peak MCAv which always captures the peak response, and in doing so provides a more reliable CVR outcome (Table 1 and Table 3).

Interpreting CVR as the relative change in MCAv (%/mmHg) resulted in poorer reliability than using absolute change (cm/s/mmHg), due to variations in baseline. Reporting CVR as the relative change resulted in increased typical errors ranging from 28 to 83% and 14 to 35% for within and between-day, respectively. By contrast, CVR was most reliable within and between-days when expressed as an absolute CVR (cm/s/mmHg). When analysed in this way, the results of the current study demonstrated acceptable within and between-day reliability (8.1-11.7%)

using the MCAv peak response. This compares favourably to similar variables investigating cerebrovascular regulation via TCD using breath hold induced CVR or as the percentage increase in MCAv without normalising to the $P_{ET}CO_2$ stimulus (McDonnell *et al.*, 2013; Koep *et al.*, 2020). It therefore seems that expressed as an absolute CVR using the peak MCAv is the most reliable method to assess CVR.

Novel kinetic analyses

Analysis of the kinetic onset response of MCAv to CO_2 breathing may avoid any confounding influences of $\dot{V}_E$ variability typically observed in the latter half of the hypercapnic stimulus (Weston, 2020), and to identify group differences not detected by traditional CVR metrics (Tallon *et al.*, 2020). In the current study, the MCAv amplitude (cm/s) showed acceptable levels of reliability both within and between days (12.6% and 8.7% respectively). These levels of reproducibility were also observed in the $P_{ET}CO_2$ response (7.1% and 6.0% respectively). The TD of the MCAv and $P_{ET}CO_2$ responses were more variable (ranging from 37.3% to 154.4%). Although the coefficient of variation was large, no significant differences were observed between trials, and on closer observation the differences were only reflective of a few seconds (mean difference of -1.3 seconds for within-day and ranging from 0.6 seconds to 1.7 seconds for between-day). Similar observations were made for the τ, which showed a coefficient of variation of 51.9 and 42.1% for within- and between-day, respectively. The small absolute nature of the mean difference in τ (mean difference of 2.9 seconds for within-day and ranging from 0.3 seconds to 4.4 seconds for between-day) should be interpreted within the context of differences which a study may detect between groups or by follow-up measures. For example, using these methods Tallon *et al.* (2020) showed the difference in τ between adults and children was 40 seconds, thus ~10 times greater than the variability of kinetics analysis in this study. The time course of the $P_{ET}CO_2$ stimulus also displayed considerable variability, showing a CV% of 33.6% and 33.0% for within- and between-day respectively. Similarly, mean differences in the τ between trials were small and were not statistically significant.

Given the above, the kinetic analysis metrics have merit in future research, although larger sample sizes may be required to detect meaningful differences outside the range of the variability of the measurement. However, this appears to be a worthwhile approach, given that kinetic analyses may be able to better elucidate regulatory differences in the MCAv response, and thus provide insight regarding how mechanisms of regulation may be altered by disease, age, and sex (Weston, 2020). The variation in MCAv could be largely influenced by the

variation in $P_{ET}CO_2$ stimulus responses, with this variability potentially much lower if end-tidal forcing were used. The kinetics of the MCAv response to CVR via end-tidal forcing have not yet been investigated and would add insight into this growing area. An additional factor potentially contributing to the variation in MCAv responses could be changes in baseline CVRi between days. In the current analysis, baseline differences in CVRi were not statistically significant (P=0.068, np^2=0.25), however, it is recommended that future research presents CVRi data or includes MAP as a covariate to account for any influence this may have on the subsequent response.

Implications and considerations

This study provides valuable insight into the reliability of CVR data, which is necessary in order to establish the best method to express CVR by traditional and novel methods. Further work is required to fully develop our understanding of open circuit CVR approaches, preferably through international collaboration in order to pool data across laboratories. This would provide compelling evidence into the sensitivity and clinical use of these methodologies, to create normative data to aid in the development of clinical prediction tools. The goal of this endeavour is to establish a measure in which we are confident in both its reliability and sensitivity to changes in risk factor status, whereby established cut points can be utilised. This has been done previously with methods of peripheral blood vessel function such as brachial artery flow-mediated dilation (Peretz *et al.*, 2007; Black *et al.*, 2008; Thijssen *et al.*, 2019), enabling its use as a clinical and research tool in which confident conclusions can be drawn (Holder *et al.*, 2021). To this end, the data presented here can be used to drive future power calculations for a variety of study designs, and will aid researchers establish the smallest meaningful change that denotes a true effect beyond the error of the measurement (Atkinson & Nevill, 1998; Hopkins, 2000; de Vet *et al.*, 2006).

Limitations and future directions

Whilst the current study provides foundations for establishing a standardised CVR assessment, further research is required in order to establish validity and clinical significance of these methods. However, CVR analysed in any of the ways utilised in the current study has been used as a measure to predict current health status and disease risk and shown to be sensitive to disease (Markus & Cullinane, 2001; den Abeelen *et al.*, 2014), age (Tallon *et al.*, 2020) and fitness levels (Barnes *et al.*, 2013; Smith *et al.*, 2021). Methods of hyperventilation induced hypocapnia are also utilised as a measure of CVR and cerebrovascular health (Ringelstein *et*

al., 1988). Future research should assess the reliability of MCAv responses to hypocapnia, to move towards improvement and standardisation of CVR methods across the entire $P_{ET}CO_2$ range.

A consideration of the current work is the use of TCD as a surrogate measure of cerebral blood flow. Limitations of utilising TCD include the inability to measure changes in vessel diameter in response to the changes in $P_{ET}CO_2$. Research demonstrates MCAv diameter to remain constant to modest increases in CO_2, however this remains debated (Brothers & Zhang, 2016; Hoiland & Ainslie, 2016). Changes in vessel diameter of the MCAv and downstream extracranial arteries could potentially account for some of the differences in MCAv responses. A recent study by (Al-Khazraji *et al.*, 2019) using 7 T MRI, demonstrated all large intracranial arteries dilate with hypercapnia and hence underestimate MCAv velocity and flow. The MCAv cross sectional area was shown to change by 10±11%, however, large inter-individual variability was observed. This could account for the difference response profiles observed in the current study (Figure 1B). Future research should explore whether changes in vessel diameter account for the variability in minute 3 - 4 of the MCAv response. Although speculative, the response profile in Figure 1A could be indicative of poorer ventilatory responses and hence greater CO_2 build up in the cerebral circulation.

The current study only investigated CO_2 breathing via TCD. Previous results however, have shown that TCD based measures do not correlate with MRI (Burley *et al.*, 2021) or Doppler based extracranial measures of CVR, which is likely due to regional differences along the vascular tree, and vessel specific reactivity (Al-Khazraji *et al.*, 2019). Given this, using a combination of intracranial and extracranial measures may further our understanding of CVR. Finally, the use of voluntary breathing to acquire changes in $P_{ET}CO_2$, rather than utilising an end-tidal forcing system to reach targeted $P_{ET}CO_2$ levels (Fisher, 2016), is a key part of the current design and reflects the approach taken by many laboratories. Therefore, the current findings cannot be extrapolated for approaches where $P_{ET}CO_2$ is fixed.

Conclusions

The present study demonstrated that the method of CVR analysis utilised can alter CVR outcomes substantially. We highlight that the most reliable interpretation of CVR data is an absolute CVR, calculated as either a 30 second or 1 second peak, and therefore recommend this approach to be utilised for future studies to help standardise CVR methods. This study also investigated the use of a mono-exponential model to interpret CVR kinetics. Given the

variability of time course responses, reliability of CVR kinetics were poorer than traditional CVR methods. However, this method may still allow for additional insights into individual responses, and further investigation into control processes underpinning these responses. Given this, future research should continue to investigate the use of mono-exponential analyses to characterise the hypercapnic response, although this approach will require larger sample sizes than traditional CVR methods.

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Competing interests:

The authors have no conflicts of interest, financial or otherwise.

Author contributions:

B.B and A.R.B conceived the study design. J.L.K, M.E.W and A.L were involved with data collection. J.L.K, M.E.W, A.R.B and B.B were involved with data analysis and interpretation. J.L.K, M.E.W, B.B, J.S.C, T.G.B, A.L, and A.R.B contributed to drafting of the work or revising it critically for important intellectual content. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Supporting Information:

Data Availability Statement:

All data supporting the results of this paper is reported in the manuscript (Table 1, Table 2, Table 3 and Table 4). Data not included in this manuscript can be found on the online repository as supporting information:

File name: [link]

Title: **Table S1**. Within-day MAP, CVRi and $\dot{V}_E$ reliability

File name: [link]

Title: **Table S2.** Between-day MAP, CVRi and $\dot{V}_E$ reliability

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Supplementary table 1. Within-day MAP, $\dot{V}_E$ and CVRi reliability

Variable		Assessment 1	Assessment 2	Change in mean	*P* value	*d*	Typical error	Typical error as CV (%) (95%CI)	r
Baseline	*MAP (mmHg)*	66 ± 13	71 ± 8	4.4	0.22	0.46	7.4	12.4 (8.9-21.2)	0.57
	$\dot{V}_E$ *(L/min)*	9.3 ± 3.5	8.0 ± 3.4	-1.3	0.21	3.8	2.1	41.2 (28.6-76.3)	0.68
	CVRi	1.3 ± 0.3	1.2 ± 0.2	-0.15	0.08	0.30	0.17	12.9 (9.2-22.0)	0.69
Peak 1s	*MAP (mmHg)*	82 ± 10	85 ± 10	3.2	0.31	0.30	6.8	8.2 (6.0-13.9)	0.58
	$\dot{V}_E$ *(L/min)*	20.8 ± 9.1	18.5 ± 9.0	-2.2	0.42	0.25	5.9	60.9 (41.5-118.7)	0.63
	CVRi	1.5 ± 0.4	1.4 ± 0.3	-0.12	0.21	0.17	0.20	14.2 (10.1-24.4)	0.73
Peak 30s	*MAP (mmHg)*	74 ± 10	77 ± 11	2.5	0.47	0.29	7.5	10.0 (7.2-16.9)	0.53
	$\dot{V}_E$ *(L/min)*	18.5 ± 7.5	16.4 ± 7.9	-2.1	0.37	0.27	4.9	62.8 (42.7-123.0)	0.66
	CVRi	1.5 ± 0.5	1.4 ± 0.3	-0.14	0.21	0.17	0.24	15.0 (10.7-25.8)	0.70
Minute 3	*MAP (mmHg)*	72 ± 11	74 ± 12	2.4	0.55	0.17	8.4	12.0 (8.6-20.5)	0.55
	$\dot{V}_E$ *(L/min)*	16.3 ± 6.6	13.7 ± 7.2	-2.6	0.23	0.38	4.5	82.7 (55.2-169.5)	0.64
	CVRi	1.6 ± 0.5	1.4 ± 0.4	-0.18	0.21	0.16	0.26	15.6 (11.2-27.0)	0.71
Minute 4	*MAP (mmHg)*	71 ± 11	74 ± 11	2.1	0.56	0.27	7.9	10.9 (7.9-18.6)	0.54

	$\dot{V}_E$ *(L/min)*	17.5 ± 8.0	15.2 ± 7.9	-2.4	0.31	0.29	4.9	78.8 (52.8-160.0)	0.67
	CVRi	1.5 ± 0.5	1.4 ± 0.4	-0.11	0.29	0.12	0.23	14.3 (10.3-14.3)	0.79
Amplitude (1s peak) (delta)	*MAP (mmHg)*	16 ± 7	15 ± 6	-1.2	0.43	0.15	3.2	27.3 (19.3-48.8)	0.81
	$\dot{V}_E$ *(L/min)*	27.2 ± 10.8	29.9 ± 12.8	2.7	0.59	0.23	11.6	71.9 (48.5-143.9)	0.04
Amplitude (30s peak) (delta)	*MAP (mmHg)*	8 ± 7	6 ± 5	-1.9	0.34	0.33	4.2	130.9 (82.4-318.5)	0.57
	$\dot{V}_E$ *(L/min)*	23.0 ± 10.2	24.6 ± 12.2	1.6	0.56	0.14	9.9	75.1 (50.4-151.3)	0.29

Data presented as mean ± SD. MAP, mean arterial pressure; $\dot{V}_E$, minute ventilation; CVRi, cerebrovascular resistance index.

Supplementary table 2. Between-day MAP, $\dot{V}_E$ and CVRi reliability

Variable		Visit 1	Visit 2	Visit 3	Visit 4	*P* value	ηp^2	Typical error	Typical error as CV (%) (95%CI)	r
Baseline	*MAP (mmHg)*	71 ± 13[c]	71 ± 13[e]	65 ± 8	61 ± 9[c,e]	**0.01**	0.30	5.6	8.2 (6.6-11.3)	0.78
	$\dot{V}_E$ *(L/min)*	12.0 ± 4.2	10.6 ± 4.3	12.8 ± 4.2	13.8 ± 4.2	0.63	0.61	3.2	33.7 (26.5-49.9)	0.47
	CVRi	1.2 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	1.4 0.3 0.3	0.07	0.25	0.17	10.4 (7.5-17.7	0.73
Peak 1s	*MAP (mmHg)*	98 ± 27	89 ± 24	82 ± 7	79 ± 7	0.17	0.16	16.0	15.7 (12.5-21.8)	0.30
	$\dot{V}_E$ *(L/min)*	26.0 ± 8.7	22.1 ± 7.8	26.7 ± 7.1	28.3 ± 6.3	0.28	0.13	5.7	28.1 (22.2-41.3)	0.48

	CVRi	1.4 ± 0.3	1.4 ±0.4	1.6 ± 0.3	1.6 ± 0.3	0.052	0.32	0.18	17.2 (12.3-29.9)	0.76
Peak 30s	*MAP (mmHg)*	85 ± 15	81 ± 20	75 ± 8	72 ± 8	0.07	0.16	10.2	12.1 (9.6-16.6)	0.48
	$\dot{V}_E$ *(L/min)*	23.1 ± 7.4	19.8 ± 6.7	24.2 ± 6.0	25.9 ± 5.4	0.22	0.15	5.3	29.6 (23.3-43.5)	0.36
	CVRi	1.4 ± 0.3 [c]	1.4 ±0.5 [e]	1.4 ± 0.6	1.7 ± 0.4 [c,e]	**0.04**	0.27	0.27	11.9 (8.5-20.3)	0.69
Minute 3	*MAP (mmHg)*	80 ± 13[c]	74 ± 13	72 ± 8	69 ± 9[c]	**0.03**	0.26	6.2	8.5 (6.8-12.0)	0.74
	$\dot{V}_E$ *(L/min)*	20.0 ± 6.0	17.9 ± 6.6	29.5 ± 21.3	23.0 ± 4.6	0.27	0.13	13.0	48.2 (37.4-73.0)	0.35
	CVRi	1.5 ± 0.4	1.4 ± 0.4	1.6 ± 0.3	1.7 ± 0.4	**0.01**	0.35	0.20	14.8 (10.6-25.5)	0.79
Minute 4	*MAP (mmHg)*	82 ± 15[a,b,c]	74 ± 13[a]	72 ± 10[b]	71 ± 8[c]	**0.01**	0.30	6.6	9.1 (7.2-12.4)	0.74
	$\dot{V}_E$ *(L/min)*	22.2 ± 7.5	18.9 ± 7.5	24.0 ± 6.0	24.9 ± 5.3	0.24	0.14	6.6	33.2 (26.0-49.0)	0.36
	CVRi	1.5 ± 0.4[c]	1.4 ±0.4[e]	1.6 ± 0.4	1.7 ± 0.3[c,e]	**0.01**	0.35	0.19	14.4 (10.3-24.7)	0.81
Amplitude (1s peak) (delta)	*MAP (mmHg)*	27 ± 27	18 ± 15	17 ± 3	18 ± 8	0.51	0.06	15.0	59.9 (46.1-92.4)	0.15
	$\dot{V}_E$ *(L/min)*	14.0 ± 6.7	11.5 ± 4.3	13.9 ± 4.3	14.5 ± 3.8	0.20	0.14	3.2	28.7 (22.6-42.1)	0.62
Amplitude (30 s peak) delta	*MAP (mmHg)*	14 ± 10	11 ± 10	10 ± 3	11 ± 7	0.80	0.03	8.3	78.4 (59.6-124.0)	0.3
	$\dot{V}_E$ *(L/min)*	11.1 ± 5.2	9.2 ± 2.9	11.4 ± 3.7	12.2 ± 2.6	0.14	0.18	2.9	31.9 (25.0-47.1)	0.42

Data presented as mean ± SD. Significant main effect P values are shown in bold. Significant pairwise comparisons between the data collected on each of the four days are as follows: a, $P<0.05$ 1v2. b, $P<0.05$ 1v3. c, $P<0.05$ 1v4. d, $P<0.05$ 2v3.e, $P<0.05$ 2v4. f, $P<0.05$ 3v4. MAP, mean arterial pressure; $\dot{V}_E$, minute ventilation; CVRi, cerebrovascular resistance index.