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RESEARCH ARTICLE

The reliability of a breath-hold protocol to determine cerebrovascular reactivity in adolescents

Jodie L. Koep[1,2] | Alan R. Barker[1] | Rhys Banks[1] | Rohit R. Banger[1] | Kate M. Sansum[1] | Max E. Weston[1,2] | Bert Bond[1]

[1]Children's Health and Exercise Research Centre, Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, Exeter, UK

[2]School of Human Movement and Nutrition Sciences, University of Queensland, Saint Lucia, Queensland, Australia

Correspondence
Bert Bond, Children's Health and Exercise Research Centre, College of Life and Environmental Sciences, University of Exeter, Exeter, EX1 2LU, UK.
Email: b.bond@exeter.ac.uk

Abstract

Purpose: Cerebrovascular reactivity (CVR) is impaired in adolescents with cardiovascular disease risk factors. A breath-hold test is a noninvasive method of assessing CVR, yet there are no reliability data of this outcome in youth. This study aimed to assess the reliability of a breath-hold protocol to measure CVR in adolescents.

Methods: Twenty-one 13 to 15 year old adolescents visited the laboratory on two separate occasions, to assess the within-test, within-day and between-day reliability of a breath-hold protocol, consisting of three breath-hold attempts. CVR was defined as the relative increase from baseline in middle cerebral artery mean blood velocity following a maximal breath-hold of up to 30 seconds, quantified via transcranial Doppler ultrasonography.

Results: Mean breath-hold duration and CVR were never significantly correlated ($r < .31$, $P > .08$). The within-test coefficient of variation for CVR was 15.2%, with no significant differences across breath-holds ($P = .88$), so the three breath-hold attempts were averaged for subsequent analyses. The within- and between-day coefficients of variation for CVR were 10.8% and 15.3%, respectively.

Conclusions: CVR assessed via a three breath-hold protocol can be reliably measured in adolescents, yielding similar within- and between-day reliability. Analyses revealed that breath-hold length and CVR were unrelated, indicating the commonly reported normalization of CVR to breath-hold duration (breath-hold index) may be unnecessary in youth.

KEYWORDS

cerebral blood flow, endothelial function, hypercapnic stimulus, reproducibility, transcranial Doppler ultrasound, youth

1 | INTRODUCTION

Cerebrovascular reactivity (CVR) refers to the ability of the human brain to modulate cerebral blood flow in response to changes in stimuli, such as the partial pressure of arterial carbon dioxide ($PaCO_2$). Impairments in CVR are an important hallmark for cerebrovascular disease (CVD) progression. Research highlights that impairments in CVR in adults is associated with Alzheimer's disease,[1] neurocognitive decline,[2] stroke,[3,4] and independently predicts future CVD events in patients with CVD risk factors.[5] Impairments in CVR are present in

youth with CVD risk factors, such as hypertension[6,7] and white coat hypertension,[8] supporting its sensitivity to CVD risk factor status in this population. Consequently, there is a growing interest in the non-invasive measurement of CVR in pediatric populations.[9]

Transcranial Doppler ultrasonography can be used to determine the reactivity of middle cerebral artery blood velocity (MCAv) to a hypercapnic stimulus, such as CO_2 breathing.[10] A breath-hold stimulus might provide a more convenient alternative to CO_2 breathing, as it is easier to administer, has minimal associated cost, and may be less intimidating, which may be of particular value when working with pediatric groups. During a breath-hold test, changes in $PaCO_2$ (reflected as end-tidal CO_2, $P_{ET}CO_2$) account for approximately two-thirds of the CVR response, with one quarter attributed to changes in mean arterial pressure (MAP).[11] Furthermore, CVR determined from transcranial Doppler ultrasonography using a breath-hold test is significantly positively correlated (r = .67) with CO_2 breathing induced CVR in adults presenting symptoms for cerebrovascular disease.[12] Given data support the breath-hold as an appropriate surrogate measure of CO_2 breathing tests, a breath-hold stimulus has merit in pediatric groups, when CO_2 breathing might not be feasible. However, despite the continued use of the breath-hold test in the literature,[8,13,14] the within- and between-day reliability of this approach in a pediatric population is unknown. Additionally, a precise methodological approach needs to be made clear, as current breath-hold protocols differ between existing adult studies.

Breath-hold induced CVR is commonly quantified using the breath-hold index (BHI), or the percentage increase in MCAv divided by breath-hold length.[12,15] The BHI has been reported to have appropriate within-day reliability (60 minutes) in a study of healthy adults, however its between-day reliability (24 hours) was poor.[16,17] This questions the appropriateness of the BHI as a measure of CVR for studies involving multiple visits on separate days. Furthermore, no studies have explored the relationship between breath-hold length and the increase in $MCAv_{mean}$, to determine the validity of normalizing increases in $MCAv_{mean}$ to breath-hold length.

Previous work has also failed to identify the most reliable method of analysis of the $MCAv_{mean}$ response within a test protocol, with some reporting CVR as a BHI[15] and others as percentage change from baseline (CVR%).[7] In addition, the number of breath-holds performed is not standardized and averaging methods are unclear, or not reported.[12,18] Importantly, evidence suggests that the breath-hold response might only be sensitive to changes in cerebrovascular health when performed multiple times within a single assessment.[19] Finally, many studies have failed to report the time when peak $MCAv_{mean}$ is recorded following the breath-hold,[11,12] while others record the percentage increase during the breath-hold.[3] In order to determine how best to analyze and report CVR, measures of within-test reliability are needed. In addition, given that cerebral blood flow is sensitive to changes in $PaCO_2$, partial pressure of arterial oxygen (PaO_2) and MAP,[11] the simultaneous measurement of these variables has merit in determining the impact of a breath-hold challenge on these physiological parameters, which may contribute to altered CVR.[20]

The purpose of this study was to identify the within-test, and within- and between-day, reliability of a CVR breath-hold protocol in an adolescent population, and to identify methodological and analytical approaches to improve the reliability of the breath-hold test to determine CVR in youth.

2 | METHODS

2.1 | Participants

Twenty-one 13 to 15 year old adolescents volunteered to take part in this study. Participant assent was obtained alongside written informed parental consent prior to participation in the study, which was approved by the institutional ethics committee (171206/B/07). Exclusion criteria included any known cardiometabolic diseases, contraindications to exercise, or use of medication known to influence vascular function. One participant was removed from analyses due to an inability to regularly perform the breath-holds without a valsalva maneuver.

Participants were familiarized to all measures on a preliminary visit. During this visit, body mass (Hampel XWM-150K, Hampel Electronics Co., Taiwan) and stature (Seca stadiometer SEC-225, Seca, Hamburg, Germany) were recorded to the nearest 0.1 kg and 0.1 cm, respectively, using standard procedures. Body mass index (BMI) cut-off points were used to define lean, overweight and obesity status.[21] Pubertal status was determined through self-assessment of secondary sex characteristics according to the five stages of pubic hair development.[22]

2.2 | Study protocol

Participants completed two experimental visits to the laboratory, within a 3-week period. Participants were required to avoid vigorous exercise for 24 hours prior to testing. Following a 12-hour overnight fast, participants were transported to the laboratory for 8 AM and rested in a darkened and temperature controlled room (24°C) in the supine position for 30 minutes prior to CVR assessment. To assess within-day reliability, participants repeated these measures after 60 minutes, consuming only 300 mL of water and remained sedentary in the laboratory.

2.3 | Assessment of cerebrovascular function

CVR was determined as the percentage increase in MCA_{vmean} from baseline to peak following each breath-hold attempt via transcranial Doppler ultrasonography (Equation ((1))) (DWL, Doppler-BoxX, Compumedics, Germany). Peak MCAv was defined as the highest beat-to-beat $MCAv_{mean}$ following the breath hold. The time, measured in seconds, from exhalation to peak $MCAv_{mean}$ was defined as time to peak.

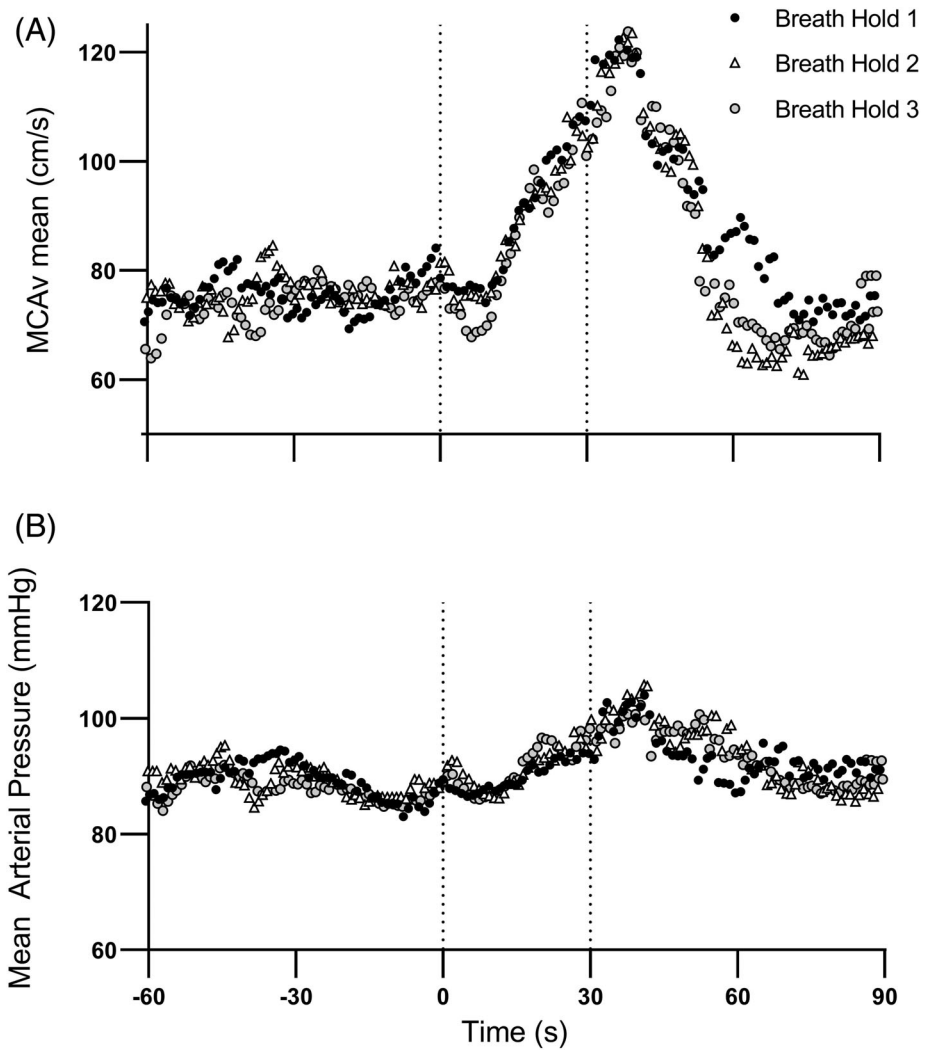

FIGURE 1 Beat-by-beat responses of middle cerebral artery mean blood velocity, A, and mean arterial pressure, B, for a representative participant across three breath-hold attempts

TABLE 1 Within-test reliability

Variable	Breath-hold			Change in mean		*P*	Typical error	CV (%)	*r*
	1	2	3	(1, 2)	(2, 3)				
Baseline MCA_{vmean} (cm/s)	89.6 ± 14.9	86.9 ± 11.4	84.8 ± 12.5*	−2.7	−2.1	**.002**	0.3	4.6	**.92**
Peak MCA_{vmean} (cm/s)	130.5 ± 19.1	128.2 ± 17.6	124.5 ± 19.8*	−2.3	−3.7	**.003**	0.3	3.2	**.95**
Recovery MCA_{vmean} (cm/s)	82.7 ± 13.2	81.1 ± 12.2	80.4 ± 11.2	−1.6	−0.8	.14	0.3	3.9	**.93**
BH length (s)	25.5 ± 4.8	26.0 ± 4.4	25.0 ± 5.3	0.5	−1.9	.42	0.5	13.5	**.73**
Time to peak (s)	4.7 ± 2.6	4.2 ± 2.9	3.9 ± 2.9	−0.5	−0.3	.67	0.7	40.7	.43
CVR (%)	46.7 ± 12.0	47.5 ± 11.5	47.4 ± 14.5	0.8	−0.1	.88	0.5	15.2	**.77**
BHI (% s^{-1})	1.88 ± 0.48	1.85 ± 0.43	1.94 ± 0.60	−0.1	0.1	.62	0.6	16.2	**.64**
MAP baseline (mm Hg)	82 ± 14	82 ± 15	82 ± 15	−0.01	−0.02	.99	0.2	3.8	**.97**
MAP Δ during BH (mm Hg)	9 ± 9	10 ± 8	9 ± 9	1.2	−1.1	.53	0.5	119.7	**.78**
MAP peak (mm Hg)	97 ± 11	98 ± 11	99 ± 11	0.45	1.42	.46	0.4	4.4	**.84**
CVCi (cm/s mm Hg)	0.94 ± 0.2	1.10 ± 0.2	1.07 ± 0.2)	0.14	−0.03	.10	0.2	28.5	**.91**
Δ End-Tidal CO_2	4 ± 3	4 ± 3	4 ± 3	−0.08	0.15	.95	0.5	22.4	**.75**
Stimulus index (CO_2/O_2) break point	0.43 ± 0.1	0.43 ± 0.1	0.42 ± 0.1	−0.01	−0.01	.48	0.4	8.1	**.83**

Note: Data presented as mean ± SD. *P*-values indicate ANOVA main effect, with significant effects highlighted in bold.

Abbreviations: ANOVA, analysis of variance; BH, breath-hold; BHI, breath-hold index; CO_2, carbon dioxide; CV, coefficient of variation; CVR, cerebrovascular reactivity; MAP, mean arterial pressure; MCA_{Vmean}, mean middle cerebral artery velocity; O_2, oxygen.

*$P < .05$ compared to other breath-holds.

$$\text{CVR}\% = \frac{\text{Peak MCAvmean} - \text{Baseline MCAvmean}}{\text{Baseline MCAvmean}} \times 100 \quad (1)$$

A 2-MHz probe was used to insonate the right MCA at an initial depth of ~50 mm. The Doppler signal was then acquired and secured using an adjustable headset (DWL, DiaMon, Compumedics, Germany, GmbH). Efforts were made to replicate the position of the probe and depth of the scan, and on within- and between-day scans, the baseline $MCAv_{mean}$ was recorded in an attempt to replicate the same position and minimize any error. Beat-by-beat MCA_{vmean} was calculated as the mean across each cardiac cycle and exported for analysis. End-Tidal CO_2 ($P_{ET}CO_2$) and End-Tidal O_2 ($P_{ET}O_2$) were measured throughout the protocol, as a surrogate of $PaCO_2$ and PaO_2 (McSwain et al.[23]). Participants wore a leak-free facemask (Hans Rudolph, Shawnee, Kansas) during the protocol to sample $P_{ET}CO_2$ and $P_{ET}O_2$ through a gas analyzer (ADInstruments, Gas analyzer, ML206, Colorado Springs, Colorado), which was calibrated via known concentrations of oxygen and CO_2. During the protocol, beat-by-beat blood pressure was non-invasively measured by finger plethysmography (Finometer PRO, Netherlands). All data were collected (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).

Baseline readings were averaged over 1 minute. Participants then performed a maximal breath-hold for up to 30 seconds following a normal inspiration while avoiding a valsalva maneuver, which was coached on the preliminary visit. This protocol, consisting of a baseline, breath-hold, and 1-minute recovery phase, was repeated three times. Figure 1 shows representative $MCAv_{mean}$ and MAP responses to this protocol.

2.4 | Data analyses

MAP was calculated from the raw blood pressure trace as one-third systolic blood pressure + two-third diastolic blood pressure. The change from baseline during the last 5 seconds of the breath-hold was calculated, to determine the presence of a substantial increase in blood pressure, defined as a valsalva maneuver. This increase was analyzed visually by two researchers, and if MAP was substantially elevated (>15 mm Hg) following the breath-hold, this breath-hold was removed.

Given that $P_{ET}CO_2$ and $P_{ET}O_2$ change simultaneously during the breath-hold protocol, a stimulus index, defined as the ratio between $P_{ET}CO_2$ and $P_{ET}O_2$ ($P_{ET}CO_2/P_{ET}O_2$) was calculated following previously used methods.[24] This was calculated to quantify the magnitude of the stimulus provided by the breath-hold assessment.

Data from the three breath-holds within a single assessment of CVR were subsequently averaged and used to identify within-test reproducibility, and how to reliably analyze CVR. This informed the analysis of the within- and between-day CVR measures, in terms of whether it is appropriate to take an average of the three breath-holds, when the peak $MCAv_{mean}$ occurs, and whether reporting CVR as a BHI is appropriate.

To explore changes in the ratio between MAP and $MCAv_{mean}$, the cerebrovascular conductance index (CVCi) was calculated as described in Equation ((2)):

$$\text{CVCi} = \text{MCAv}_{\text{mean}}/\text{MAP} \quad (2)$$

where $MCAv_{mean}$ and MAP are taken as the average during the baseline preceding each breath-hold attempt.

2.5 | Statistical analyses

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 .05. Baseline and peak $MCAv_{mean}$, CVR, BHI, breath-hold duration, time to peak $MCAv_{mean}$, MAP, CVCi, and $P_{ET}CO_2$ and $P_{ET}O_2$ were analyzed using a mixed model analysis of variance (ANOVA) with assessment (within-test and

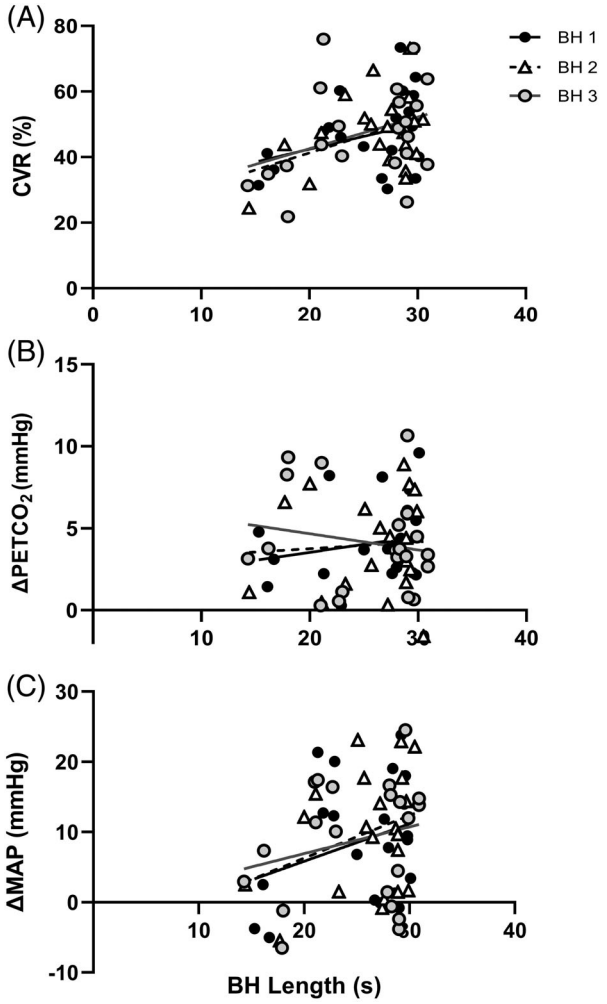

FIGURE 2 Within-test correlation between breath-hold length, A, cerebrovascular reactivity (CVR%), B, change in $P_{ET}CO_2$ from baseline to break point, and C, change in mean arterial pressure (MAP) from baseline to break point

within-day) or visit (between-day) as the main effects. For within-test data, the relationship between mean breath-hold length and CVR was explored using Pearson's correlation. Effect sizes for the ANOVA model were displayed as partial eta squared (η_p^2), and interpreted as <0.06 = small, 0.06 to 0.14 = moderate and >0.14 = large.[25] For within-test analyses where three breath-holds were analyzed, significant difference between breath-hold attempts were located using pairwise comparisons and interpreted using the *P*-value and standardized effect sizes (*d*) to document the magnitude of the effect using the following thresholds: ≥0.2 < 0.5 = small, <0.8 = moderate and ≥0.8 = large.[25] The reproducibility of these outcomes was explored using the typical error, expressed as a coefficient of variation (CV) and intraclass correlation coefficient (*r*) for within-test, within-day, and between-day analyses.[26] Within-test outcomes of interest were also analyzed for sex differences using an independent samples *t* test, with effect sizes (*d*) calculated for these comparisons.

3 | RESULTS

Ten boys and ten girls were included in the study. The mean (SD) age of the group was 14.3 (0.4) years, body mass: 55.1 (11) kg, stature: 154.5 (8.2) cm. Three participants were defined as overweight according to BMI centile cut points.[21] The maturity status was as follows: stage 2, n = 2 (one male); stage 3, n = 2 (one male); stage 4, n = 14 (seven males); and stage 5, n = 2 (one male).

3.1 | Within-test reliability

The within-test reproducibility for parameters of interest is presented in Table 1. Baseline MCA_{Vmean} significantly declined across the three breath-holds (η_p^2 = 0.29), with a significantly lower baseline MCA_{Vmean} in breath-hold 3 than 1 (P = .001, d = 0.4) and 2 (P = .034, d = 0.2). Peak MCA_{Vmean} systematically declined across the three breath-holds, with breath-hold 3 lower than 1 and 2 (P < .001, η_p^2 = 0.27; 1 vs 3: P = .003, d = 0.3, 2 vs 3: P = .02, d = 0.2). Baseline $P_{ET}O_2$ systematically increased across the three breath-holds (n_p^2 = 0.24), with a significantly lower baseline $P_{ET}O_2$ in breath-hold 1 than 2 (P = .034, d = 0.3) and 3 (P = .006, d = 0.5). No significant mean differences were apparent between breath-holds for all other outcomes, including CVR and BHI ($\eta_p^2 \leq 0.19$). Significant intraclass correlations were observed between breath-holds for all outcomes of CVR ($.64 \leq r \leq .95$) (P < .01), except time to peak (r = .43, P = .67). Mean breath-hold duration was not significantly correlated with CVR for breath-hold one (r = .31, P = .17); two (r = .39, P = .08); or three (r = .35, P = .13), as shown in Figure 2A. Mean breath-hold duration was not significantly correlated with the change in $P_{ET}CO_2$ from baseline to break point for breath-hold one (r = .17, P = .44); two (r = .06, P = .79); or three (r = .17, P = .47), as shown in Figure 2B. In addition, mean breath-hold duration was not significantly correlated with the change in MAP for breath-hold one (r = .30, P = .19); two (r = .32, P = .16); or three (r = .24, P = .31), as shown in Figure 2C. The typical error expressed as a CV for all other outcomes ranged from 2.0% (baseline $P_{ET}CO_2$) to 119.7% (MAP Δ during BH). The within-test outcomes informed the analysis of breath-hold data for within- and between-day analysis, with it deemed appropriate to take an average of the three breath-hold attempts within the protocol. Within-test analyses also revealed that there were no significant sex differences for baseline $MCAv_{mean}$ (boys 84.1 ± 10.2 vs girls 90.3 ± 14.4 cm/s, P = .30, d = 0.5); peak $MCAv_{mean}$ (boys 125.4 ± 17.7 vs girls 130.2 ± 19.8 cm/s, P = .56, d = 0.3); and CVR (boys 48.9 ± 13.3 vs girls 45.3 ± 9.0%, P = .48, d = 0.3).

TABLE 2 Within-day reliability

Variable	Assessment 1	Assessment 2	Change in mean	*P* value	Typical error	CV (%)	*r*
Baseline MCA_{vmean} (cm/s)	85.9 ± 11.9	**82.9 ± 13.7**	−2.9	**.02**	0.3	4.5	**.92**
Peak MCA_{vmean} (cm/s)	126.8 ± 15.5	**122.3 ± 21.0**	−5.5	**.02**	0.4	5.8	**.87**
Recovery MCA_{vmean} (cm/s)	81.0 ± 11.6	**77.7 ± 13.6**	−3.3	**.03**	0.4	5.7	**.89**
BH length (s)	25.2 ± 4.3	26.0 ± 4.2	0.8	.21	0.5	8.1	**.81**
CVR (%)	47.3 ± 11.7	46.2 ± 10.4	−1.1	.48	0.5	10.8	**.79**
BHI (% s^{-1})	1.9 ± 0.5	1.8 ± 0.4	−0.1	.12	0.7	14.0	**.70**
MAP baseline (mm Hg)	82 ± 14	79 ± 12	−1.5	.64	0.7	13.1	**.49**
MAP Δ during BH (mm Hg)	10 ± 7	9 ± 8	0.5	.77	1.1	150.7	**.46**
MAP peak (mm Hg)	93 ± 16	91 ± 17	−1.9	.58	0.9	14.8	**.57**
CVCi (cm/s mm Hg)	1.08 ± 0.2	1.06 ± 0.2	−0.1	.65	0.3	30.6	**.72**
Δ End-Tidal CO_2	5 ± 3	4 ± 3	0.33	.42	0.4	30.2	**.86**
Stimulus index (CO_2/O_2) break point	0.4 ± 0.1	0.4 ± 0.1	0.00	.56	0.3	3.9	**.91**

Note: Data presented as mean ± SD. Bold indicates significant mean difference between assessments 1 and 2.

Abbreviations: BH, breath-hold; BHI, breath-hold index; CO_2, carbon dioxide; CV, coefficient of variation; CVR, cerebrovascular reactivity; MAP, mean arterial pressure; MCA_{Vmean}, mean middle cerebral artery velocity; O_2, oxygen.

TABLE 3 Between-day reliability

Variable	Assessment 1	Assessment 2	Change in mean	*P* value	Typical error	Typical error as CV (%)	*r*
Baseline MCA_{vmean} (cm/s)	84.1 ± 14.7	87.1 ± 12.0	3.0	.11	0.4	6.6	**.83**
Peak MCA_{vmean} (cm/s)	125.9 ± 22.4	127.0 ± 17.5	1.1	.73	0.5	7.6	**.78**
Recovery MCA_{vmean} (cm/s)	78.2 ± 14.8	80.8 ± 11.6	2.7	.16	5.98	7.5	**.82**
BH length (s)	24.2 ± 5.0	25.5 ± 4.6	1.3	.11	0.5	11.5	**.74**
CVR (%)	49.4 ± 12.0	46.3 ± 12.0	−3.1	.17	0.7	15.3	**.64**
BHI (% s^{-1})	2.1 ± 0.5	**1.9 ± 0.4**	−0.2	**.005**	0.6	12.5	**.74**
MAP baseline (mm Hg)	82 ± 14	85 ± 7	3.4	.30	1.0	15.2	.11
MAP Δ during BH (mm Hg)	9 ± 9	8 ± 7	−1.5	.45	6.2	100.2	**.48**
MAP peak (mm Hg)	96 ± 4	98 ± 10	1.7	.59	1.12	12.9	**.46**
CVCi (cm/s mm Hg)	1.0 ± 0.2	1.0 ± 0.2	0.0	.72	0.2	16	.39
Δ End-Tidal CO_2	4 ± 3	4 ± 3	0.3	.52	0.5	25.9	**.76**
Stimulus index (CO_2/O_2) break point	0.4 ± 0.0	0.4 ± 0.0	0.02	.84	0.03	8.1	**.60**

Note: Bold indicates significant mean difference between assessments 1 and 2. Data presented as mean ± SD.
Abbreviations: BH, breath-hold; BHI, breath-hold index; CO_2, carbon dioxide; CV, coefficient of variation; CVR, cerebrovascular reactivity; MAP, mean arterial pressure; MCA_{Vmean}, mean middle cerebral artery velocity; O_2, oxygen.

3.2 | Within-day reliability

The within-day reliability for parameters of interest is presented in Table 2. Between assessments 1 and 2, a significant decline in baseline (η_p^2 = 0.24), peak (η_p^2 = 0.24), and recovery (η_p^2 = 0.22) MCA_{Vmean} was observed. No significant mean differences were apparent between assessments 1 and 2 for all other outcomes ($\eta_p^2 \leq 0.12$). Significant correlations were observed between assessments 1 and 2 for all outcomes (.46 < *r* < .91, *P* < .01).

3.3 | Between-day reliability

The between-day reliability for parameters of interest is presented in Table 3. Significant mean differences were observed for BHI with a decline between assessments 1 and 2 (η_p^2 = 0.34). No significant mean differences were apparent between assessments 1 and 2 for all other outcomes ($\eta_p^2 \leq 0.14$). Significant correlations were observed between assessments 1 and 2 for all variables (.46 < *r* < .83; *P* < .01), except CVCi, and $P_{ET}O_2$.

4 | DISCUSSION

The main findings of this study were twofold. Within-test analyses demonstrated that that there were no significant differences across the three breath-holds performed in the protocol, deeming it appropriate to average the CVR from the three breath-hold attempts. Within- and between-day analyses for CVR using the percentage increase in $MCAv_{mean}$ following a breath-hold stimulus, yielded similar and encouraging levels of reliability (typical error expressed as a CV% of 10.8% and 15.3%, respectively).

4.1 | Within-test

The commonly used BHI outcome yielded a typical error expressed as a CV% of 16.2% for within-test reliability, in line with previously reported data (11.4%) in healthy adults.[27] Nevertheless, there are concerns with the application of the BHI[28] as the relationship between breath-hold length and the $PaCO_2$ stimulus remains unclear, with these data often not reported.[29,30] The BHI method was first employed to account for differences in breath-hold length and its possible influence on CVR, as it is thought to reflect the $PaCO_2$ stimulus,[12] considered to have merit in elderly patients who could not hold their breath for longer than 15 seconds.[7] The present study found that breath-hold length was not significantly associated with the increase in $MCAv_{mean}$ (*r* > .31, *P* > .08), nor the magnitude of the $P_{ET}CO_2$ stimulus index (*r* > .04, *P* > .44), and therefore the normalization of CVR to breath-hold length holds limited statistical support, at least within a sample of healthy adolescents. This is in line with adult data demonstrating breath-hold length is not strongly correlated with changes in $PaCO_2$.[31] Collectively, these data indicate that it is not appropriate to normalize the $MCAv_{mean}$ response to breath-hold length in healthy adolescents. One consideration that may have biased this analysis is that the present study used a 30-second maximal breath-hold length; therefore, it is impossible to determine if the BHI has merit with larger variations in breath-hold length. However, the present study indicates that when comparing between participants using a 30-second stimulus cut-off, breath-hold length did not alter the CVR.

There is a lack of consistency in the determination of CVR from a breath-hold stimulus, and it is evident that protocol standardization is needed, with no consensus for protocol and analysis methods of CVR. Some studies determine peak $MCAv_{mean}$ in the 4 seconds following the breath-hold,[12] while others analyze the peak during the breath-

hold.[27] In addition, some studies take the increase in MCA_{Vmean} "immediately following" the breath-hold, though when this occurs is not stated.[7] In the present study, the time taken to peak $MCAv_{mean}$ following the breath-hold was variable between (4.1 ± 1.8 seconds) and within (CV = 65.3%) individuals. This may be due to the contingency of the time to peak outcome on the heart rate of the participant, as $MCAv_{mean}$ is determined on a beat-to-beat basis. This indicates that using a predefined point of 4 seconds following the breath-hold, such as in previous studies[12] is unlikely to always capture the peak increase in $MCAv_{mean}$. In the present study, peak $MCAv_{mean}$ always occurred in the 10 seconds following the breath-hold, in line with previous literature.[32] This informed subsequent analyses for within- and between-day outcomes. Similarly to the widely used approach for interpretation of peripheral endothelial function using flow mediated dilation,[33] it is recommended that researchers use peak $MCAv_{mean}$, whenever it occurs following the breath-hold.

It has not been made clear in previous studies whether breath-hold data are reported as an average across several attempts, or whether the highest or lowest attempts are removed. In addition, it is not clear or consistent how many breath-holds are performed, with some studies reporting six,[18] three,[34] or two,[12] while others fail to report this.[27] From the three breath-hold protocol used in the current study, baseline and peak $MCAv_{mean}$ systematically declined from breath-hold one, with no difference between breath-holds two and three. However, there were no significant differences across breath-holds for CVR, with breath-hold one to three being strongly correlated (r = .77) with a within participant CV of 15.2%. It therefore seems appropriate to take an average of the three breath-holds for analysis, and also suggests that a single breath-hold may be sufficient for calculation of CVR if required for a time sensitive protocol.

4.2 | Within- and between-day reliability

Evidence of within- and between-day reliability of breath-hold induced CVR protocols is essential when conducting interventional and observational studies. In the present study, within-day data demonstrated a systematic decline in baseline and peak $MCAv_{mean}$ after 60 minutes from assessments 1 to 2. Previous literature has reported diurnal variation in $MCAv_{mean}$ due to variations in MAP.[35] However, in the present study, measures of CVCi demonstrated no differences between assessments both within- and between-days. This suggests that, although there was a high individual variation in MAP, when baseline MAP was accounted for, the CVR response was seemingly not influenced by this variation in MAP. This lends supports to the use of a breath-hold protocol as a measure of CO_2-induced vessel reactivity. The 1-hour within-day variation of baseline $MCAv_{mean}$ highlights the time sensitivity of this measure and the importance of conducting measures at the same time of day to minimize variation. Despite this, CVR was not significantly different within-day and evidenced a CV of 10.8%. This indicates that the responsiveness of the vessel is not altered through the day despite different baseline $MCAv_{mean}$. The reliability of CVR may be considered as acceptable when compared to the within-day CV following CO_2 breathing tests in adults ranging from 4.8% to 40.6%.[36]

In the present study, between-day CVR assessments were correlated (r = .64) and elicited a CV of 15.3% (r = .64, P = .002). This is consistent with CVR data from CO_2 breathing in adults, with a between-day intraclass correlation coefficient of 0.73.[17] The magnitude of the relative change in $MCAv_{mean}$ following the breath-hold stimulus (34%-62%) is in line with previous reports of normal variation in a pediatric population of between 40% and 69%.[7]

The reported variability in CVR between and within-days in the current study could be attributed to a number of potential sources of error in the breath-hold protocol. It is important that the breath-hold is completed following a normal inspiration, to avoid substantial alterations in $PaCO_2$ concentrations,[7] and avoid alterations in MAP during and after a Valsalva maneuver, which may result in misinterpretation of CVR.[12] However, in this study and previous literature,[15] it is evident that this protocol is well tolerated and appropriately performed in most adolescents. In the present study, MAP baseline and peak were reliable within a participant, both within (CV: baseline = 13.1% and peak = 14.8%) and between-day (CV: baseline = 15.2% and peak = 12.9%). However, the change in MAP during the breath-hold was highly variable with both within-day (CV = 150.7%) and between-day (CV = 100.2%). Although this variation is large, this is summative of the variation of MAP at both baseline and peak, and when expressed as a percentage this variability becomes amplified. Despite this seemingly large variation, there were no resultant changes in CVR, supporting these changes in MAP as being acceptable ranges and not having a substantial influence on the subsequent $MCAv_{mean}$ response. Measurement of both MAP and $P_{ET}CO_2$ are of importance to ensure that any changes in CVR are attributable to changes in responsiveness in the blood vessel, and not breath-hold execution. In the current study, $P_{ET}CO_2$ at break point was reliable within a participant, both within-day (CV = 2.9%) and between-day (CV = 3.2%), and therefore any influence on the variability on outcomes of CVR is unlikely to be from variability in $P_{ET}CO_2$ following breath-hold execution.

4.3 | Considerations

In the present study, there were no sex differences between outcomes of interest. However, effect sizes demonstrated a moderate effect of sex on baseline $MCAv_{mean}$, with girls displaying a higher baseline $MCAv_{mean}$ on average than boys. This is in line with arterial spin labeling data on the impact of puberty on evolution of cerebral perfusion during adolescence.[37] This highlights the need for future research to continue to explore the influence of sex on markers of cerebrovascular health. It should be noted that although the breath-hold is a commonly used noninvasive technique for accessing CVR to a CO_2 stimulus, the $PaCO_2$ levels cannot be standardized and are constantly changing, with the time course of $PaCO_2$ changes and peak responses unknown. Furthermore, the breath-hold protocol is accompanied by hypoxia and blood pressure changes which may confound

the stimulus effect.[38] Despite this, it is a commonly used technique, particularly in pediatric studies,[7,8,39] and therefore knowledge on the reliability and analysis of this outcome is imperative. In the present study, a valsalva criteria cut off of a 15 mm Hg increase in blood pressure following the breath-hold. Although there is no study which directly informs this, the present study introduced this standardization upon laboratory observations, and data highlighting that a valsalva maneuver increases in MCAv with a 12 mm Hg increase in MAP.[40]

5 | CONCLUSIONS

Analyses revealed that breath-hold length and CVR were unrelated, and provided no statistical justification for the commonly reported BHI, at least in healthy youth. Within-test analyses demonstrated that CVR was reproducible within a protocol, indicating that it was appropriate to take an average of the three breath-holds. Using these methods, this study addressed the within- and between-day reliability of a single protocol to noninvasively measure CVR of the MCA. The present study demonstrated that the breath-hold protocol was a reliable method of assessing CVR in adolescents. Importantly, this supports its use in future studies investigating changes in CVR that utilizes measures between and within visits. Future analyses, however, need to be conducted to establish whether CVR assessed by this method is valid, and correlates with direct measures of CO_2 breathing techniques. Furthermore, associations with clinical outcomes to support this as a valuable predictor of future health outcomes warrants investigation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ORCID

Jodie L. Koep https://orcid.org/0000-0003-4137-6840
Alan R. Barker https://orcid.org/0000-0001-8610-5417
Kate M. Sansum https://orcid.org/0000-0003-3392-6750
Max E. Weston https://orcid.org/0000-0003-4808-6512
Bert Bond https://orcid.org/0000-0003-3597-8562

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