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RESEARCH PAPER

Bilateral regional extracranial blood flow regulation to hypoxia and unilateral duplex ultrasound measurement error

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[Correction made on 1 July 2021, after first online publication: The article has been updated to rectify inconsistencies in the notation of some of the units of measurement.]

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Abstract

Whether blood flow regulation to hypoxia is similar between left and right internal carotid arteries (ICAs) and vertebral arteries (VAs) is unclear. Extracranial blood flow is regularly calculated by doubling a unilateral assessment; however, lateral artery differences may lead to measurement error. This study aimed to determine extracranial blood flow regulation to hypoxia when factoring for vessel type (ICAs or VAs) and vessel side (left or right) effects, and to investigate unilateral assessment measurement error compared to bilateral assessment. In a repeated-measures cross-over design, extracranial arteries of 44 participants were assessed bilaterally by duplex ultrasound during 90 min of normoxic and poikilocapnic hypoxic (12.0% fraction of inspired oxygen) conditions. Linear mixed model analyses revealed no Condition × Vessel Type × Vessel Side interaction for blood flow, vessel diameter and flow velocity (all $P > 0.05$) indicating left and right ICA and VA blood flow regulation to hypoxia was similar. Bilateral hypoxic reactivity was comparable (ICAs, 1.4 (1.0) vs. VAs, 1.7 (1.1) $\Delta\%\cdot\Delta SpO_2^{-1}$; $P = 0.12$). Compared to bilateral assessment, unilateral mean measurement error of the relative blood flow response to hypoxia was up to 5%, but individual errors reached 37% and were greatest in ICAs and VAs with the smaller resting blood flow due to a ratio-scaling problem. In conclusion, left and right ICA and VA regulation to hypoxia is comparable when factoring for vessel type and vessel side. Assessing the ICA and VA vessels with the larger resting blood flow, not the left or right vessel, reduces unilateral measurement error.

KEYWORDS
bias, cerebrovascular, doppler, hypoxia, internal carotid artery, ultrasonography, vertebral artery

1 | INTRODUCTION

Cerebral blood flow regulation is critical to support oxygen delivery to match the high metabolic demand of the brain and to maintain normal neurovascular function (Ogoh, 2017; Willie et al., 2014). During acute hypoxia when arterial oxygen content is reduced, global cerebral blood flow increases (Hoiland et al., 2018). However, hypoxia-induced changes in blood flow are not uniform across the brain (Lawley et al., 2017; Rossetti et al., 2021), which may in part relate to different regulation of blood flow at the extracranial arteries (Lewis et al., 2014; Ogoh et al., 2013; Subudhi et al., 2014; Willie et al., 2012).

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Regional extracranial blood flow regulation can be measured non-invasively by duplex ultrasound of the vertebral arteries (VAs) that feed the homeostatic posterior brain regions and the internal carotid arteries (ICAs) that feed the more functional anterior brain regions. In response to hypoxia, absolute increases in blood flow within the ICAs are greater than in the VAs (Lafave et al., 2019). When indexed as a relative response some evidence indicates that the blood flow increase to hypoxia in the VAs is greater than in the ICAs (Lewis et al., 2014; Ogoh et al., 2013; Subudhi et al., 2014; Willie et al., 2012). Although the majority of studies reporting differences between ICA and VA blood flow regulation have been in response to hypoxia, the greater relative response in the VAs has also been observed in response to other stressors including carbon dioxide, orthostasis and exercise, and is proposed as a mechanism to preferentially maintain posterior blood flow to the homeostatic brain regions (Sato et al., 2016; Sato, Fisher, et al., 2012; Sato, Sadamoto, et al., 2012). Despite this compelling argument, a disparity exists within the literature with a similar number of studies failing to report differences between the ICA and VA blood flow regulation to hypoxia (Hoiland et al., 2017; Lafave et al., 2019; Willie, Smith, et al., 2014). It is also contentious whether the increase in blood flow to hypoxia at the extracranial arteries is regulated by a change in vessel diameter (Lewis et al., 2014) or not (Ogoh et al., 2013).

In contrast to the study of ICA (anterior) versus VA (posterior) extracranial regional blood flow response to hypoxia, the effect of vessel side has yet to be considered in studies using duplex ultrasound. Regulation of extracranial blood flow to hypoxia is typically assessed by doubling unilateral measurements of the ICAs and VAs. Reports suggest that at rest the left and right ICAs have equal blood flow, whereas the right VA has 20–30% less blood flow than the left VA as a consequence of its smaller resting vessel diameter (Khan et al., 2017; Schöning et al., 1994). Anatomical variations in the aortic branching that alter shear stress and vascular resistance between arteries have been proposed as a possible mechanism for the difference in lateral extracranial blood flow (Hu et al., 2013; van Campen et al., 2018). Moreover, intra- and extracranial cerebrovasculature have the capacity for compensatory collateral flow as demonstrated during and after vessel occlusion (Romero et al., 2010; Wang et al., 2019), and immediately after endarterectomy (Aleksic & Brunkwall, 2009; Wang et al., 2019). Therefore, the interplay between extracranial artery vessel type (ICA or VA) *and* vessel side (left or right) should be considered when investigating the global haemodynamic response to a stressor.

Another possible explanation for the equivocal findings in regional extracranial blood flow regulation to hypoxia is the method by which extracranial blood flow data are acquired and expressed. In assessments of brachial artery vascular function by flow-mediated dilatation (FMD), a negative correlation between resting brachial artery diameter and the percentage change in diameter suggests that the calculation of diameter percentage change in smaller brachial arteries overestimates the relative FMD response (Atkinson & Batterham, 2013a, 2013b; Atkinson et al., 2013). Further, brachial arteries with smaller diameters displayed more varied responses than those with large diameters. Consequently, the relatively larger blood flow increase to hypoxia in the VAs compared to ICAs previously reported (Lewis et al., 2014; Ogoh et al., 2013; Subudhi et al., 2014; Willie et al., 2012) may be a product of a ratio-scaling problem arising from random intra-individual lateral anatomical and resting blood flow differences, more prominent in the VAs than the ICAs, when unilateral rather than bilateral measurements are used.

The primary aim of this study was to determine the extracranial blood flow regulation to acute poikilocapnic hypoxia when factoring for vessel type (ICA or VA) and vessel side (left or right) effects. In this study, we assessed left and right ICA and VA blood flow, vessel diameter and flow velocity by duplex ultrasound. We hypothesised that when factoring for vessel type and vessel side, extracranial blood flow regulation to hypoxia would be similar in left and right ICAs and VAs. The secondary aim of this study was to investigate the measurement error in unilateral compared to bilateral calculations of extracranial blood flow. Although other stimuli might be used (e.g., carbon dioxide, exercise), we chose to examine the extracranial measurement error to acute poikilocapnic hypoxia as it has previously been shown to increase cerebral blood flow by approximately 30% for a 1–2 h period (Lewis et al., 2014; Morris et al., 2017). Based on the previously identified ratio-scaling problem with FMD, we hypothesised that a negative relationship would exist between extracranial artery resting normoxic blood flow and the relative blood flow response to hypoxia. We also expected that extracranial arteries with the smaller resting blood flow would have a more varied relative blood flow response to hypoxia than those arteries with larger resting blood flows. Consequently, the measurement error to the relative blood flow response to hypoxia was hypothesised to be greater when calculated from doubling a unilateral

New Findings

- **What is the central question of this study?**
  Is blood flow regulation to hypoxia different between the internal carotid arteries (ICAs) and vertebral arteries (VAs), and what is the measurement error in unilateral extracranial artery assessments compared to bilateral?
- **What is the main finding and its importance?**
  ICA and VA blood flow regulation to hypoxia is comparable when factoring for vessel type and vessel side. Compared to bilateral assessment, vessels assessed unilaterally had individual measurement errors of up to 37%. Assessing the vessel with the larger resting blood flow, not the left or right vessel, reduces unilateral measurement error.

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measurement of the lateral extracranial artery with the smaller resting blood flow.

2 | METHODS

2.1 | Ethical approval

Ethical approval for this study was obtained from Bangor University (proposal number 2019-16489, accepted 11 March 2019) and was conducted following the standards of the *Declaration of Helsinki 2013*, except for registration in a database, with written informed consent obtained from all study volunteers.

2.2 | Participants

Forty-four young healthy participants were recruited in this study (17 females, 24 (5) years, 177 (9) cm, 72 (9) kg, haemoglobin 15 (1) $g \cdot dl^{-1}$, haematocrit 44 (4)%). Participants were non-smokers, free from cardiovascular, haematological and neurological disease and had not resided overnight at an altitude of >2500 m within the past 6 months. Participants were screened for vascular abnormalities to ensure reliable ICA and VA ultrasound images could be acquired. To minimise the impact of fluctuations in sex hormones on blood flow measurements (Krejza et al., 2001), female participants were included if they had contraceptive-induced amenorrhoea or a regular menstrual cycle. Participants with a regular menstrual cycle were tested during the early follicular phase (day 1–7) or the placebo phase of the oral contraceptive. Participants were instructed to refrain from consuming alcohol and from undertaking exhaustive exercise within 24 h of experimental trials. Experimental trials were completed at the same time of day and participants were encouraged to match their diet and supplement intake, including caffeinated beverages, before arrival at the laboratory.

2.3 | Study design

A repeated-measures, crossover design was used in which each participant completed two experimental trials separated by a minimum of 48 h. Experimental trials consisted of a 90 min exposure to either normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) or poikilocapnic hypoxia (F_{iO_2} = 12.0%) in a temperature (26 (2)°C) and humidity (30 (4)%) controlled environmental chamber (Hypoxico Inc., New York, USA).

2.3.1 | Experimental procedures

On entry to the chamber, participants were instrumented with a three-lead electrocardiogram, pulse oximeter and blood-pressure cuff to measure heart rate, peripheral arterial oxygen saturation (S_{pO_2}; Model 9590 Oximeter; Nonin Medical Inc., Plymouth, MN, USA) and mean arterial blood pressure (MAP; Model M6 AC ME, Omron Healthcare Co., Ltd, Kyoto, Japan), respectively. After 20 min participants lay supine for 10 min before a facemask was attached to measure the partial pressure of end-tidal carbon dioxide (P_{ETCO_2}) and minute ventilation ($\dot{V}_E$) for 5 min (Metalyzer 3B, Cortex Biophysik, GmbH, Leipzig, Germany). Following this, blood flow measurements of the left and right ICA and VA were completed by duplex ultrasound. Cardiovascular measurements were obtained at 30 min intervals and respiratory measurements were obtained at 30 and 90 min.

2.3.2 | Duplex ultrasound acquisition and analysis

All extracranial blood flow measurements were collected by the same operator (A.T.F.), using duplex ultrasound with a 12 MHz linear transducer (Acuson X300, Siemens Healthcare, GMbH, Erlanden, Germany) at 30 $frames \cdot s^{-1}$, as per recommended technical guidelines (Thomas et al., 2015). Bilateral ICA and VA blood flow was calculated from consecutive left and right measurements. To improve the accuracy of the blood flow measurements and minimise the trade-off between B-mode and pulsed-wave Doppler mode (Thomas et al., 2015), vessel diameter and flow velocity measurements were collected in consecutive 30 s recordings with care taken to maintain the same position within the vessel. High-resolution B-mode images were used to measure vessel diameter. Flow velocity was measured using Doppler velocity spectrum with the cursor set in the centre of the vessel with a 60° angle of insonation with the Doppler gate adjusted to fill the size of the vessel. ICAs were measured 1.0–1.5 cm distal to the carotid bifurcation and VAs were measured between C3 and the subclavian artery. The order of imaging was (1) VA right, (2) ICA right, (3) VA left and (4) ICA left. In a separate day-to-day reproducibility study ($n = 10$) completed by the same operator (A.T.F.), the coefficient of variation of this technique for blood flow, vessel diameter and flow velocity of the ICA (11%, 4% and 7%) and VA (9%, 2% and 7%) were comparable with recommended guidelines (Thomas et al., 2015).

All data were captured and stored for subsequent offline analysis by an investigator blinded to the condition of the experimental trials. Following a conservative image quality check, data and statistical analysis were completed on 33 ICA pairs (24 males, 9 females) and 43 VA pairs (26 males, 17 females). The 11 ICA exclusions were due to lack of clear insonation and poor image quality whilst the one VA exclusion was due to the presence of an unidentified branching vessel. Offline analysis was adapted from standardised procedures described elsewhere (Hoiland et al., 2017; Ogoh et al., 2013). Specifically, mean flow velocity was calculated using half the time-averaged maximum velocity (TAMx) and was averaged from 10 cardiac cycles to minimise the impact of respiration. Mean vessel diameter was measured using automated edge-detection tracking software (Brachial Analyser, Medical Imaging Applications, Coralville, IA, USA) and was calculated from a weighted average of the peak systolic and diastolic diameters

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across 10 cardiac cycles [(systolic diameter × ⅓) + (diastolic diameter × ⅔)]. Subsequently, blood flow was calculated using the following equation:

$$\text{Blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right) = [\text{TAMx}\ (\text{cm}\cdot\text{s}^{-1})/2] \times [\pi \times (\text{mean artery diameter}\ (\text{mm})/2^2)] \times 60$$

Absolute and relative change in blood flow was calculated as the change in blood flow from normoxia to hypoxia at the same time point using the following equations:

$$\text{Absolute change in blood flow to hypoxia}\ \left(\text{ml}\cdot\text{min}^{-1}\right) = \text{hypoxic blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right) - \text{normoxic blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right)$$

$$\text{Relative change in blood flow to hypoxia}\ (\%) = \left[\left(\text{hypoxic blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right) - \text{normoxic blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right)\right) / \text{normoxic blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right)\right] \times 100$$

To control for differences between individual responses to poikilocapnic hypoxia, an index of absolute and relative hypoxic blood flow reactivity was calculated by normalising these values to the absolute change in S_{pO_2} (ΔS_{pO_2}).

To calculate the difference in resting normoxic blood flow between lateral arteries of the ICAs and VAs, the following equation was used:

$$\text{Lateral artery difference in resting normoxic blood flow}\ (\%) = \left[\left(\text{larger blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right) - \text{smaller blood flow}\ (\text{ml}\cdot\text{min}^{-1})\right)/\text{smaller blood flow}\ \left(\text{ml}\cdot\text{min}^{-1}\right)\right] \times 100$$

Extracranial arteries were also identified and grouped by the lateral vessel (left or right) with the larger resting normoxic blood flow.

2.4 | Statistical analysis

Statistical analysis was conducted using SPSS Statistics v25 (IBM Corp., Armonk, NY, USA). Values are the mean (SD) unless otherwise stated and statistical significance was set at $P < 0.05$.

To determine any differences in cardiorespiratory variables during normoxia and hypoxia, a linear mixed model (LMM) was used. Fixed effects of interest were Condition (normoxia or hypoxia), Time (30, 60 or 90 min) and the interaction (Condition × Time), with Participant ID added as a random effect. Baseline data were not included as these measurements were collected during seated rest before entry to the environmental chamber.

To determine whether there are resting normoxic blood flow differences between left and right ICA and VA, a LMM was used with fixed effects of interest Vessel Type (ICA or VA), and Vessel Side (left or right), as well as the interaction (Vessel Type × Vessel Side), adding Participant ID as a random effect.

To determine whether there are differences in blood flow regulation to hypoxia between the four extracranial arteries, a LMM was used to examine left and right ICA and VA absolute blood flow, vessel diameter and flow velocity in normoxia and hypoxia. Specifically, fixed effects of interest were Condition, Vessel Type and Vessel Side, with Participant ID added as a random effect. The primary outcome of interest was the interaction (Condition × Vessel Type × Vessel Side). In addition, for conventional purposes, a LMM was used to examine the absolute change and relative change in blood flow, vessel diameter and flow velocity regulation to hypoxia (i.e., change from normoxia) between the ICAs and VAs with Vessel Type, and Vessel Side and the interaction (Vessel Type × Vessel Side) as fixed effects of interest, adding Participant ID as a random effect. Values from LMM analysis are reported as estimated marginal means and an estimated SD that was derived from the standard error (SE), where n is the sample size (estimated SD = SE × $\sqrt{n}$) (Shenouda et al., 2017).

To determine the measurement error in unilateral compared to bilateral calculations of relative extracranial blood flow response to hypoxia, we investigated whether a ratio-scaling problem exists and quantified the measurement error by Bland–Altman analysis (Bland & Altman, 1986). Disproportionate ratio-scaling in the calculation of relative change ratios has been described extensively elsewhere (Atkinson & Batterham, 2013a, 2013b; Atkinson et al., 2013). For clarity, the analysis used in the present study is described. Pearson's correlation was used to determine whether a negative relationship between resting normoxic blood flow and the relative blood flow response to hypoxia existed in the left and right ICAs and VAs. To confirm that the relationships between resting normoxic blood flow and the relative blood flow response to hypoxia were statistically different from the relationships between resting normoxic blood flow and the absolute blood flow response to hypoxia, correlation coefficients were compared using Fisher's Z transformation using the cocor online software (Diedenhofen & Musch, 2015). Then, appropriate ratio-scaling was applied to the ICAs and VAs to calculate the 'corrected' ICA and VA blood flow response to hypoxia. The calculation of the regression slope between logarithmically transformed normoxic blood flow and hypoxic blood flow was used to determine whether hypoxic blood flow scales disproportionately for the range of values of normoxic blood flow, with an upper confidence limit (95% CI) being less than 1.0 indicating this to be true. An analysis of covariance (ANCOVA) model was used to determine group differences between unilateral and bilateral calculations of logged-scale change in blood flow (Δblood flow), with logarithmically transformed normoxic blood flow as a covariate. Back-transformation of covariate-adjusted Δblood flow was converted to Δ%blood flow as the final corrected, and more conventional, calculation of the relative blood flow response to hypoxia. Bland–Altman analysis was used to

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TABLE 1 Cardiorespiratory responses to normoxia and acute poikilocapnic hypoxia

	Normoxia				Hypoxia				*P*		
	Baseline	30 min	60 min	90 min	Baseline	30 min	60 min	90 min	Condition	Time	Interaction
S_{pO_2} (%)	98 (1)	98 (1)	99 (1)	99 (1)	99 (1)	80 (5)	78 (6)	78 (6)	<0.001	0.70	0.13
Heart rate (bpm)	71 (14)	63 (10)	60 (9)	60 (10)	68 (12)	73 (14)	71 (12)	72 (12)	<0.001	0.30	0.84
MAP (mmHg)	90 (7)	84 (7)	83 (7)	85 (7)	91 (6)	85 (9)	85 (8)	86 (8)	<0.05	<0.01	0.71
P_{ETCO_2} (mmHg)	—	37.5 (3.6)	—	37.4 (3.6)	—	34.2 (3.2)	—	32.6 (3.7)	<0.001	0.12	0.06
$\dot{V}_E$ (l·min^{-1})	—	9.5 (1.7)	—	8.6 (1.3)	—	10.3 (1.9)	—	9.6 (1.9)	<0.001	<0.05	0.45

Values are means (SD). Data were collected during a seated baseline and during supine rest between 30 and 90 min in a temperature (26 (2)°C) and humidity (30 (4)%) controlled environmental chamber during normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) and acute poikilocapnic hypoxia (F_{iO_2} = 12.0%). Linear mixed model analysis was completed for the period of supine rest. Abbreviations: MAP, mean arterial pressure; P_{ETCO_2}, partial pressure of end-tidal carbon dioxide; S_{pO_2}, peripheral arterial oxygen saturation; $\dot{V}_E$, minute ventilation.

determine the level of agreement between unilateral and bilateral calculations of the relative blood flow response to hypoxia for the ICAs and VAs. Unilateral measurements were determined by resting normoxic blood flow (smaller or larger) or vessel side (left or right) and doubled before calculating the relative blood flow response to hypoxia. The mean difference between unilateral and bilateral calculations of the relative blood flow response to hypoxia was determined as the measurement bias (error), with the respective 95% CI, and the 95% limits of agreement.

3 | RESULTS

3.1 | Cardiorespiratory responses to hypoxia

There were no interactions for cardiorespiratory responses during the period of supine rest (Table 1, all $P > 0.05$). Compared to normoxia, acute poikilocapnic hypoxia increased heart rate (main effect of Condition: +11 (6) bpm; $P < 0.001$), MAP (+1 (4) mmHg; $P < 0.05$) and $\dot{V}_E$ (+0.9 (1.4) l·min^{-1}; $P < 0.001$), and decreased P_{ETCO_2} (−3.9 (2.1) mmHg; $P < 0.001$) and S_{pO_2} (−20 (3)%; $P < 0.001$) during supine rest. Cardiorespiratory responses were stable between 30 and 90 min with the exception of an increase in MAP (main effect of Time: +2 (4) mmHg, 90 vs. 60 min; $P < 0.01$) and a decline in $\dot{V}_E$ (−0.5 (1.5) l·min^{-1}, 90 vs. 30 min; $P < 0.05$) in both conditions.

3.2 | Resting extracranial artery characteristics

There was no Vessel Type × Vessel Side interaction ($P = 0.17$), nor a main effect of Vessel Side ($P = 0.74$), in resting normoxic blood flow between the left and right ICA (299 (43) and 288 (43) ml·min^{-1}, 4%) and left and right VA (103 (43) and 95 (43) ml·min^{-1}, 8%), but there was a main effect of Vessel Type ($P < 0.001$). The difference in resting normoxic blood flow between lateral arteries ranged from 1 to 91% for the ICAs and 0 to 400% for the VAs. More participants had a larger resting normoxic blood flow in the right ICA (14 left, 19 right) and VA (21 left, 22 right) than left.

3.3 | Extracranial artery blood flow regulation to hypoxia

LMM analyses revealed no Condition × Vessel Type × Vessel Side interaction for blood flow (Figure 1a; $P = 0.62$), vessel diameter (Figure 1b; $P = 0.70$) and flow velocity (Figure 1c; $P = 0.64$), indicating that ICA and VA blood flow regulation to acute poikilocapnic hypoxia did not differ as a function of vessel type and vessel side. There was also no Condition × Vessel Side interaction for vessel diameter ($P = 0.32$), flow velocity ($P = 0.18$), blood flow ($P = 0.86$), and the volume of left and right extracranial blood flow were similar in hypoxia (left ICA + VA, 495 (93) ml·min^{-1} vs. right ICA + VA, 501 (93) ml·min^{-1}; $P = 0.89$).

Due to the ICA and VA differences at normoxic baseline, there was a Condition × Vessel Type interaction for blood flow ($P < 0.001$). Subsequently, to account for the large discrepancy between ICA and VA blood flow at normoxic baseline, this variable was analysed using the conventionally reported change scores from normoxia. LMM analysis revealed no Vessel Type × Vessel Side interaction for the absolute and relative blood flow response to hypoxia between the left and right ICA and VA (Figure 2a,c,e,g, all $P > 0.05$) and reaffirmed that there was no main effect of Vessel Side for these blood flow variables (all $P > 0.05$). As expected, a main effect of Vessel Type (i.e., when data were pooled as the bilateral value) revealed that the ICAs had a greater absolute blood flow response to hypoxia (Figure 2b; 155 (63) vs. 57 (65) Δml·min^{-1}, $P < 0.001$) and absolute hypoxic reactivity (Figure 2d, 8.0 (3.3) vs. 3.0 (3.4) Δml·min^{-1}·ΔSpO_2^{-1}; $P < 0.001$) than the VAs, whereas, there was no main effect of Vessel Type for the relative blood flow response to hypoxia (Figure 2f, 26.6 (21.6) vs. 33.2 (22.6) Δ%; $P = 0.053$) and relative hypoxic reactivity (Figure 2h, 1.4 (1.0) vs. 1.7 (1.1) Δ%·ΔSpO_2^{-1}; $P = 0.12$). When calculated bilaterally, acute poikilocapnic hypoxia increased global blood flow by 29.1 (18.1)% (776 (124) vs. 995 (124) ml·min^{-1}; $P < 0.001$). There was no Condition × Vessel Type interaction for vessel diameter ($P = 0.29$) and flow velocity ($P = 0.37$), which had a similar relative vessel diameter response to hypoxia (ICAs 6.9 (3.7) vs. VAs 7.0 (3.8)%; $P = 0.83$) and relative flow velocity response to hypoxia (ICAs 11.2 (15.7) vs. VAs 15.6 (16.1)%; $P = 0.09$) between bilateral calculations of the ICAs and VAs. Raw means(SD) data are presented in Table 2.

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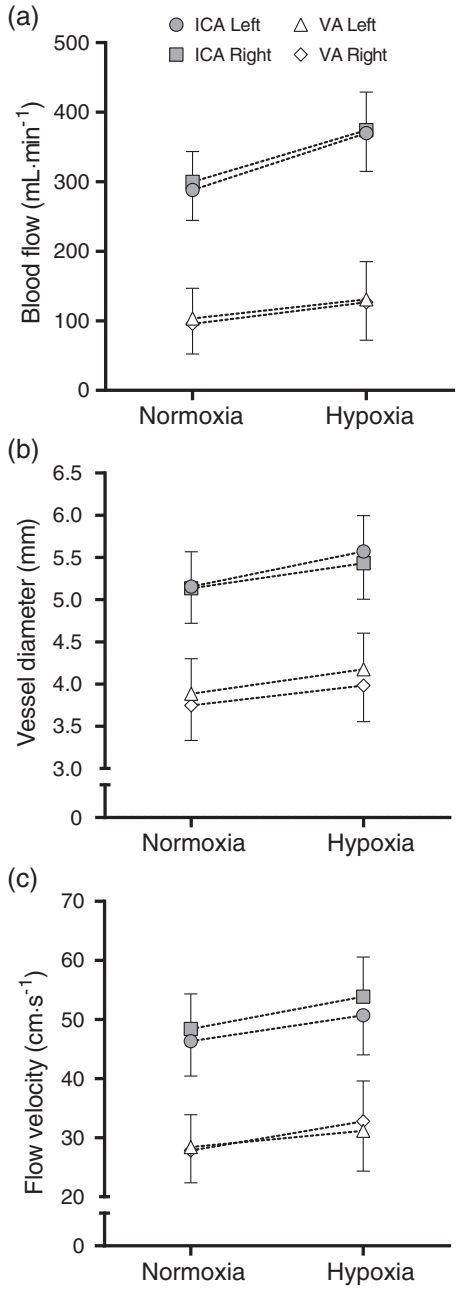

FIGURE 1 Extracranial artery blood flow regulation in normoxia and acute poikilocapnic hypoxia. Blood flow regulation of left and right internal carotid artery (ICA) and vertebral artery (VA) was measured from normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) to acute poikilocapnic hypoxia (F_{iO_2} = 12.0%). Linear mixed model analysis revealed no Condition (normoxia or hypoxia) × Vessel Type (ICA or VA) × Vessel Side (left or right) interaction for blood flow (a, ml·min^{-1}; $P = 0.62$), vessel diameter (b, mm; $P = 0.70$) and flow velocity (cm·s^{-1}; $P = 0.64$), adding Participant ID as a random effect. Data points are estimated marginal means (estimated SD) from LMM analysis. Raw means (SD) data are presented in Table 2

3.4 | The relationship between resting normoxic blood flow and the blood flow response to hypoxia

No relationship was observed between resting normoxic blood flow and the absolute blood flow response to hypoxia (Figure 3a,b). However, negative relationships were identified between resting normoxic blood flow and the relative ICA and VA blood flow response to hypoxia (Figure 3c,d, $r = -0.33$ and -0.37, respectively; $P < 0.001$). These relationships were statistically different from the relationships observed between resting normoxic blood flow and the absolute blood flow response to hypoxia (both $P < 0.001$). These relationships indicated that vessels with smaller resting blood flow were associated with a greater and more varied relative blood flow change to hypoxia. As negative relationships were identified between normoxic blood flow and the relative blood flow response to hypoxia, ratio-scaling was conducted. The regression slopes between logarithmically transformed resting normoxic blood flow and hypoxic blood flow were 0.70 (95% CI: 0.49–0.91) for the ICA and 0.79 (95% CI: 0.67–0.91) for the VA with each upper confidence limit of less than 1.0, indicating that vessels with smaller resting blood flow were associated with a disproportionately large relative change in hypoxic blood flow. The covariate-adjusted group means for the relative blood flow response to hypoxia after ANCOVA indicated smaller differences between left and right calculations of the ICAs (27.5 (16.8) and 25.1 (16.8)%) and VAs (28.3 (22.5) and 32.4 (22.5)%) compared with non-corrected values of the relative blood flow response to hypoxia (Table 2).

3.5 | Measurement bias between unilateral and bilateral calculations of the relative blood flow response to hypoxia

Bland–Altman analysis revealed doubling unilateral ICA measurements from the vessel with the Bilateral blood flow was calculated as the sum of left and right unilateral measurements and, for comparison with bilateral, blood flow for left and right vessel only was the double of unilateral measurements smaller resting normoxic blood flow overestimated the relative ICA blood flow response to hypoxia by 5% (95% CI: 1–9; limits of agreement: −19 to +29%) compared to bilateral calculations (Figure 4d).

Bland–Altman analysis revealed no significant bias in the relative ICA blood flow response to hypoxia from doubling left or right unilateral ICA measurements compared to bilateral calculations (Figure 5a,b). Bland–Altman analysis revealed doubling unilateral right VA measurements overestimated the relative VA blood flow response to hypoxia by 4% (95% CI: 0–7; limits of agreement: −21 to +28%) compared to bilateral calculations (Figure 5d). In contrast, there was no significant bias in the relative VA blood flow response to hypoxia from doubling unilateral left VA measurements compared to bilateral calculations (Figure 5c).

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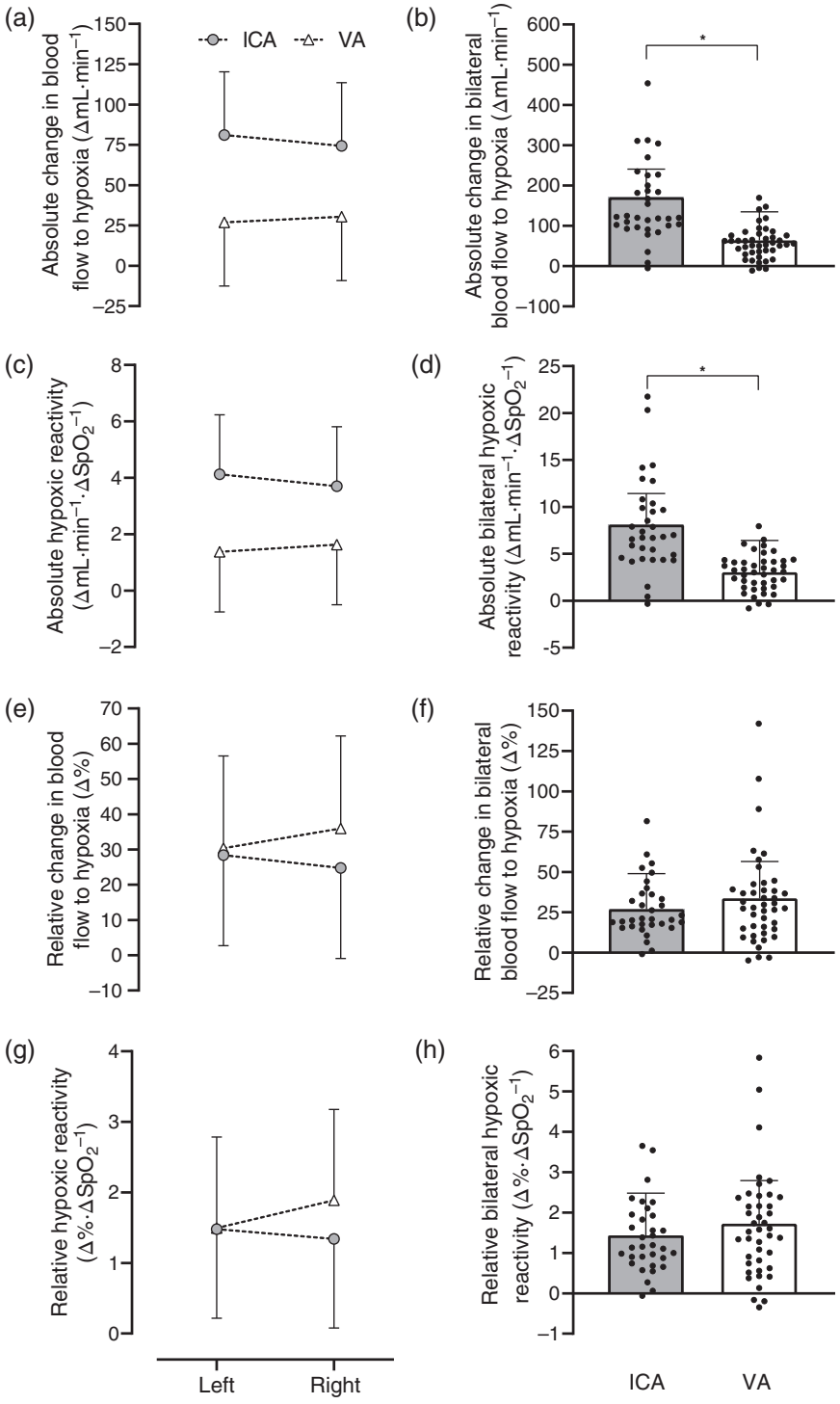

FIGURE 2 Extracranial artery blood flow response and reactivity to acute poikilocapnic hypoxia. Blood flow response to acute poikilocapnic hypoxia (fraction of inspired oxygen (F_{iO_2}) = 12.0%) of left and right internal carotid artery (ICA; grey circles or bars) and vertebral artery (VA; white triangles or bars). Linear mixed model (LMM) analysis revealed no Vessel Type (ICA or VA) × Vessel Side (left or right) interaction for the absolute change in blood flow to hypoxia (a, $\Delta ml \cdot min^{-1}$; $P = 0.32$), absolute hypoxic reactivity (c, $\Delta ml \cdot min^{-1} \cdot \Delta SpO_2{}^{-1}$; $P = 0.37$), the relative change in blood flow to hypoxia (e, Δ%; $P = 0.15$) or relative hypoxic reactivity (g, $\Delta\% \cdot \Delta SpO_2{}^{-1}$; $P = 0.13$). There were no main effects of Vessel Side for these blood flow variables (all $P > 0.05$). Main effects of Vessel Type were revealed for the absolute change in bilateral blood flow to hypoxia (b; $P < 0.001$) and absolute bilateral hypoxic reactivity (d; $P < 0.001$), but not for the relative change in bilateral blood flow to hypoxia (f; $P = 0.053$), or relative bilateral hypoxic reactivity (h; $P = 0.12$). $^{*}P < 0.001$ between ICAs and VAs. Data points represent individuals' ICA and VA blood flow responses to acute hypoxia. Bars are estimated marginal means (estimated SD) from LMM analysis

4 | DISCUSSION

4.1 | Main findings

The principal finding of this study was that extracranial blood flow regulation to hypoxia is comparable (Figure 1) when factoring for vessel type (ICA or VA) and vessel side (left or right). The increase in blood flow to hypoxia was regulated by an increase in vessel diameter and flow velocity in all extracranial vessels. Global extracranial blood flow to hypoxia increased from 776 (124) to 995 (124) $ml \cdot min^{-1}$ (29.1%, $P < 0.001$), which was equally distributed between the left and right sides (left ICA + VA, 495 (93) vs. right ICA + VA, 501 (93) $\Delta ml \cdot min^{-1}$); $P = 0.89$). When conventionally reported as the change score from normoxia, the bilateral absolute blood flow response to hypoxia was greater in the ICA than the VA (Figure 2b,d), whereas the bilateral relative blood flow response to hypoxia was comparable between the ICA and VA (Figure 2f,h). We are unaware of previous duplex ultrasound investigations that have assessed bilateral extracranial

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TABLE 2 Extracranial artery blood flow, vessel diameter and flow velocity in normoxia and acute poikilocapnic hypoxia

	Normoxia	Hypoxia	Δ	Δ%
Blood flow (ml·min^{-1})*				
Internal carotid artery				
Left only	575 (94)	738 (130)	163 (108)	29.7 (21.6)
Right only	598 (117)	747 (155)	149 (117)	26.1 (21.7)
Bilateral	587 (79)	743 (117)	156 (97)	27.3 (17.8)
Vertebral artery				
Left only	206 (64)	261 (83)	55 (53)	30.6 (33.0)
Right only	190 (76)	252 (86)	62 (40)	36.2 (28.3)
Bilateral	198 (44)	256 (50)	58 (40)	32.5 (28.4)
Vessel diameter (mm)				
Internal carotid artery				
Left only	5.16 (0.37)	5.58 (0.39)	0.42 (0.26)	8.24 (5.24)
Right only	5.14 (0.40)	5.43 (0.43)	0.30 (0.23)	5.86 (4.62)
Mean	5.15 (0.31)	5.50 (0.34)	0.36 (0.21)	7.00 (4.14)
Vertebral artery				
Left only	3.88 (0.42)	4.17 (0.44)	0.29 (0.17)	7.57 (4.38)
Right only	3.75 (0.49)	3.98 (0.47)	0.23 (0.16)	6.50 (4.69)
Mean	3.82 (0.28)	4.08 (0.27)	0.26 (0.13)	6.98 (3.66)
Flow velocity (cm· s^{-1})				
Internal carotid artery				
Left only	46.0 (7.2)	50.4 (7.3)	4.4 (7.3)	10.8 (17.9)
Right only	48.1 (7.9)	53.5 (8.6)	5.4 (8.5)	12.7 (18.9)
Mean	47.1 (7.0)	52.0 (6.9)	4.9 (6.8)	11.6 (15.5)
Vertebral artery				
Left only	28.3 (5.4)	31.1 (5.6)	2.8 (5.5)	11.9 (21.8)
Right only	27.7 (5.6)	32.7 (5.9)	4.9 (4.8)	19.7 (20.2)
Mean	28.0 (4.6)	31.9 (4.6)	3.8 (4.5)	15.4 (19.0)

Data are means (SD) of the raw values, with the absolute (Δ) and relative change (Δ%) from normoxia. Blood flow (ml· min^{-1}), vessel diameter (mm), and flow velocity (time-averaged maximum velocity; cm· s^{-1}) were assessed in the left and right internal carotid arteries (ICA) and vertebral arteries (VA) during normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) and acute poikilocapnic hypoxia (F_{iO_2} = 12.0%). Bilateral blood flow was calculated as the sum of left and right unilateral measurements and, for comparison with bilateral, blood flow for left and right vessel only was the double of unilateral measurements. Linear mixed model analysis revealed no Condition (normoxia or hypoxia) × Vessel Type (ICA or VA) × Vessel Side (left or right) interaction for blood flow ($P = 0.62$), vessel diameter ($P = 0.70$), and flow velocity ($P = 0.64$), with Participant ID as a random effect. LMM revealed no Condition × Vessel Side interactions.
*Interaction effect of Condition × Vessel Type ($P < 0.001$).

blood flow regulation to hypoxia, nor considered the effect of vessel type and vessel side.

This study also identified negative relationships between extracranial artery resting normoxic blood flow and the relative blood flow response to hypoxia for the ICAs and VAs (Figure 3c,d), which illustrated a ratio-scaling problem akin to that previously described with FMD assessment of brachial artery vascular function (Atkinson & Batterham, 2013a, 2013b; Atkinson et al., 2013). Compared with bilateral measurement of relative blood flow change to hypoxia, the common practice of doubling unilateral measurements led to average errors of up to 5%, and individual errors of up to 37%, which were greatest and more varied in the extracranial arteries with smaller resting normoxic blood flow (Figures 4a, 5d).

4.2 | Bilateral extracranial blood flow regulation to hypoxia

When assessed bilaterally, acute poikilocapnic hypoxia caused the same vasodilatation (ICAs 6.9 (3.7) vs. VAs 7.0 (3.8)%; $P = 0.87$) and comparable relative increases in blood flow and blood flow reactivity in the ICAs and VAs (Figure 2f,h). These regional blood flow responses to acute hypoxia are similar to those previously reported from studies employing the typical method of doubling unilateral measurements (Lewis et al., 2014; Morris et al., 2017; Willie et al., 2012). There are as many studies reporting that the increase in blood flow to hypoxia is mediated by vasodilatation in both ICAs and VAs to extreme (<80% S_{pO_2}) poikilocapnic hypoxia (Lewis et al., 2014; Morris et al., 2017) or

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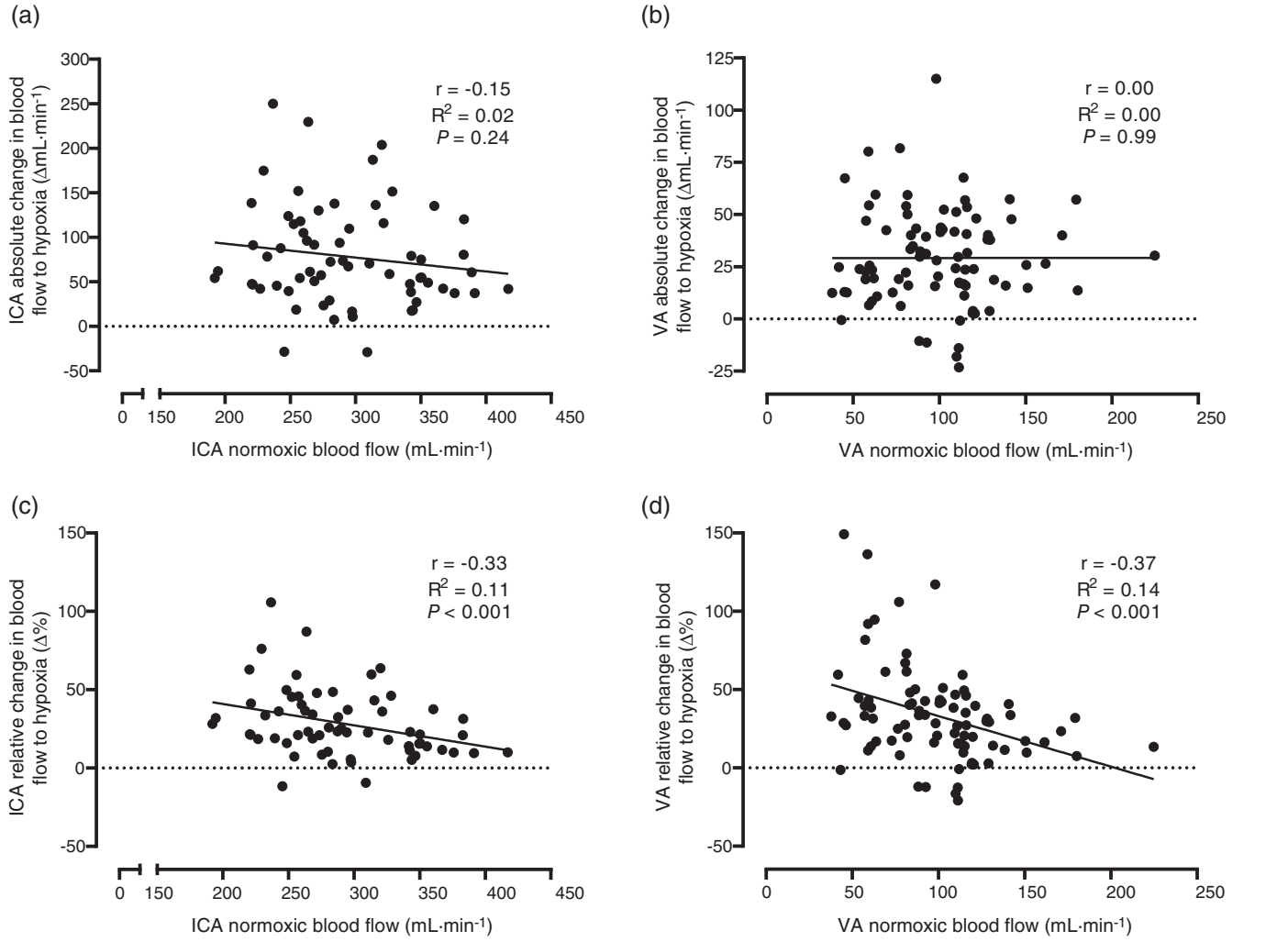

FIGURE 3 Relationships between resting normoxic blood flow and the absolute or relative blood flow response to acute poikilocapnic hypoxia in the extracranial arteries. Blood flow response of internal carotid arteries (ICAs) and vertebral arteries (VAs) was assessed from normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) to acute poikilocapnic hypoxia (F_{iO_2} = 12.0%). The blood flow response to hypoxia is presented as the absolute change (Δml· min^{-1}; a,b) and the relative change (Δ%; c,d) from resting normoxic blood flow (ml· min^{-1}). Data plots include the left and right vessels of the ICAs and the VAs

isocapnic hypoxia (Fernandes et al., 2018; Hoiland et al., 2017) as there are reporting no vasodilatation (Lafave et al., 2019; Ogoh et al., 2013; Willie et al., 2012; Willie, Smith, et al., 2014), with others suggesting regionally specific vasodilatation (Kellawan et al., 2017; Subudhi et al., 2014). Notwithstanding the methodological differences of inducing hypoxia that is known to affect the cerebrovascular response, such as the clamping of carbon dioxide (Kellawan et al., 2017; Ogoh et al., 2013; Willie et al., 2012), exposure to high-altitude hypobaric hypoxia (Hoiland et al., 2017; Lafave et al., 2019; Subudhi et al., 2014; Willie, Smith, et al., 2014) and length of exposure (Lewis et al., 2014), the aforementioned studies are often limited by their sample size and therefore sensitivity to detect small differences where high inter-individual variability with exposure to acute severe hypoxia is notable (Willie, Smith, et al., 2014). Compared to previous literature, the present study was conducted in a relatively large cohort and is strengthened by bilateral measurement of the blood flow response to hypoxia, which provides more certainty that blood flow is similarly regulated in ICAs and VAs in response to acute poikilocapnic hypoxia.

4.3 | Extracranial artery blood flow measurement error

The absolute increase in blood flow to hypoxia was comparable within ICAs (Figure 3a) and VAs (Figure 3b) irrespective of the resting blood flow. In contrast, significant negative relationships were identified between resting blood flow and the relative blood flow response to hypoxia in both the ICAs and the VAs, where vessels with smaller resting blood flow had greater relative blood flow responses to hypoxia (Figure 3c,d). This indicated the same ratio-scaling problem in extracranial arteries as has previously been described with FMD assessment of the brachial artery vascular

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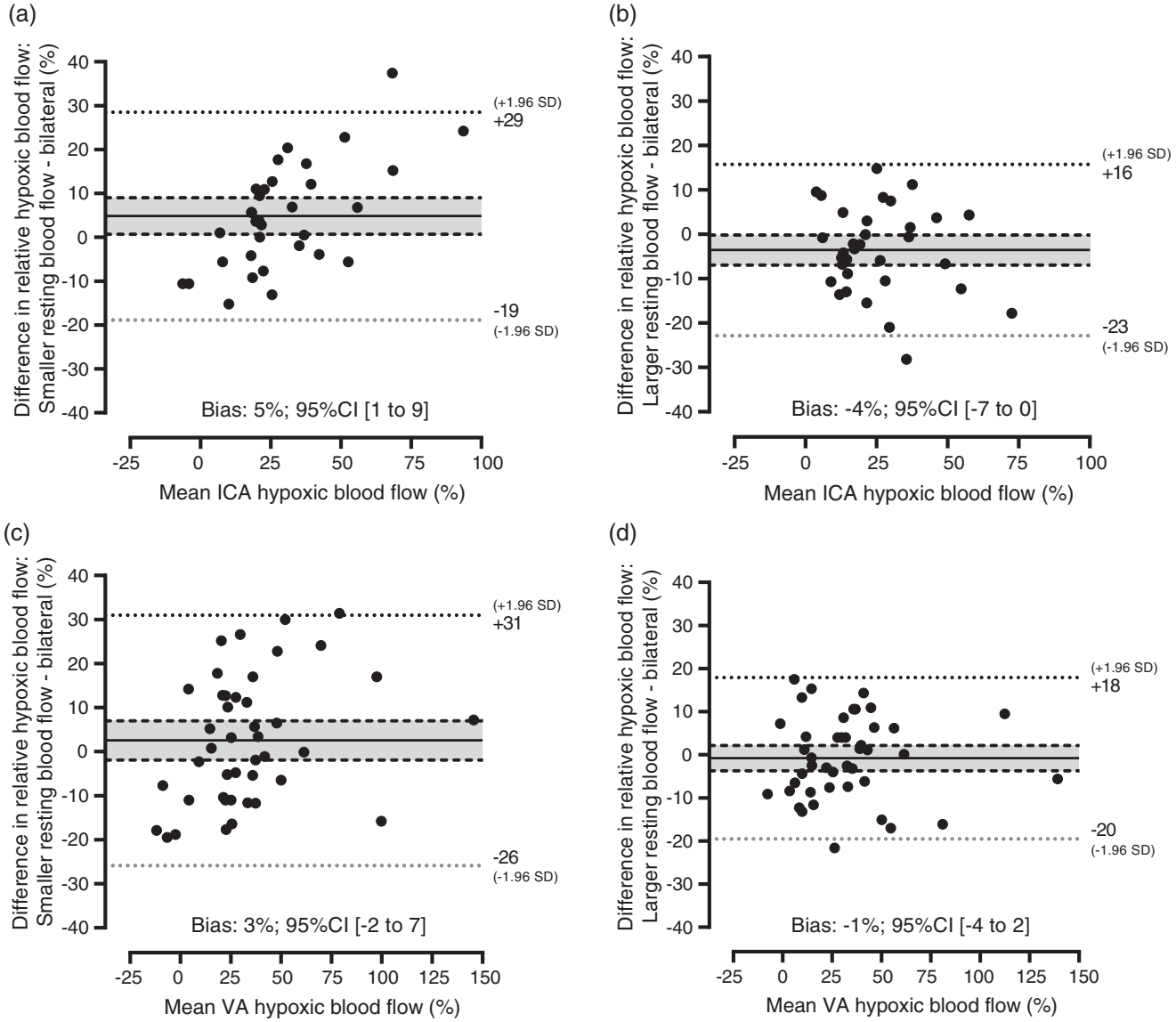

FIGURE 4 Bland–Altman plots of the measurement bias between a unilateral assessment of the vessel with the smaller or larger resting normoxic blood flow and the bilateral calculation of the relative change in blood flow from normoxia to acute poikilocapnic hypoxia of the extracranial arteries. Relative blood flow response from normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) to acute poikilocapnic hypoxia (F_{iO_2} = 12.0%) of internal carotid arteries (ICAs) and vertebral arteries (VAs) was calculated from doubling unilateral measurements of the vessel with the smaller (a,c) or larger (b,d) resting normoxic blood flow and compared to the bilateral calculation of the relative blood flow response to hypoxia. Average bias (solid black line) is reported with respective 95% confidence intervals (dashed black lines), and ±1.96 SD limits of agreement (dotted black lines)

function (Atkinson & Batterham, 2013a, 2013b; Atkinson et al., 2013). In brief, Atkinson and Batterham describe this relationship to be a fundamental ratio-scaling problem when using relative change ratios (i.e., Δ%FMD = (peak diameter − resting diameter)/resting diameter × 100) where the numerator (i.e., difference in diameter) does not scale proportionately for the range of denominator values (i.e., resting diameter). The negative relationships also indicated that the relative change in blood flow to hypoxia were more varied in vessels with smaller resting normoxic blood flow. This skewness towards the group with the smaller scores (i.e., smaller resting normoxic blood flow) is common with ratio indices since ratios cause the outcome data to be non-normally distributed even when the two ratios are normally distributed (Atkinson & Batterham, 2013b; Vickers, 2001). This relationship highlights a mathematical, rather than physiological, source of measurement error when adopting a unilateral rather than bilateral assessment.

When compared to the bilateral calculation, doubling of a unilateral extracranial measurement of the relative blood flow response to hypoxia from the vessel with the smaller resting blood flow led to a greater mean measurement bias (5%) and wider limits of agreement (up to 31%) than from the vessel with the larger resting blood flow (Figure 4). Despite the mean bias of a unilateral measurement compared to the

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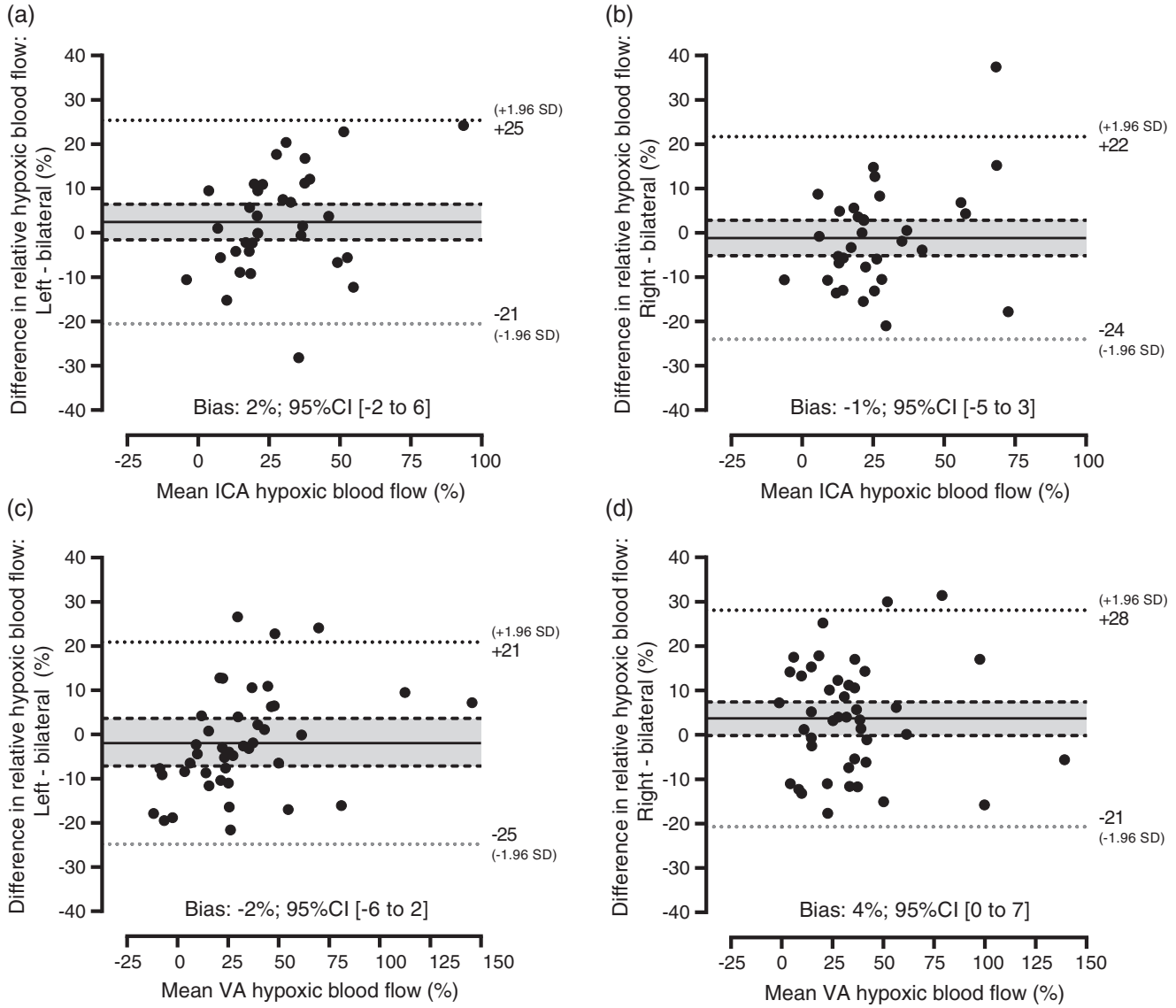

FIGURE 5 Bland–Altman plots of the measurement bias between a unilateral assessment of the left or right vessel and the bilateral calculation of the relative change in blood flow from normoxia to acute poikilocapnic hypoxia of the extracranial arteries. Relative blood flow response from normoxia (fraction of inspired oxygen (F_{iO_2}) = 20.9%) to acute poikilocapnic hypoxia (F_{iO_2} = 12.0%) of internal carotid arteries (ICAs) and vertebral arteries (VAs) was calculated from doubling unilateral measurements of the left (a,c) or right (b,d) side and compared to the bilateral calculation of the relative blood flow response to hypoxia. Average bias (continuous black line) is reported with respective 95% confidence intervals (dashed black lines), and ±1.96 SD limits of agreement (dotted black lines)

bilateral measurement being small (3–5%), it is misleading to judge the measurement error of a unilateral assessment from this metric alone. To fully examine measurement error, mean bias, the width of the limits of agreement and visual inspection of the Bland–Altman plots for a constant or proportional bias should be completed (Giavarina, 2015). Here, the limits of agreement of a unilateral measurement were −26 to 31% (Figure 4), which can be considered significant when considering the magnitude is similar to the mean extracranial relative blood flow response to hypoxia. These wide limits of agreement indicate a low level of precision in unilateral measurements, compared to bilateral measurements, which may lead to erroneous interpretation of data particularly in small sample cohorts. Moreover, the Bland–Altman analysis revealed a proportional measurement bias that was more prominent in the vessels with the smaller blood flow (i.e., Figure 4a). Therefore, doubling a unilateral extracranial measurement from the vessel with the smaller resting normoxic blood flow is the least comparable, and causes the greatest measurement error, to the true bilateral relative blood flow response to hypoxia.

In investigations of extracranial blood flow regulation to hypoxia, a unilateral measurement of the right VA is overwhelmingly favoured (Fernandes et al., 2018; Hoiland et al., 2017; Lafave et al., 2019; Lewis et al., 2014; Morris et al., 2017; Ogoh et al., 2013; Willie et al., 2012) compared to the left VA (Subudhi et al., 2014; Willie, Smith, et al., 2014). The rationale often stated for the right side being chosen is to account

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for the 20–30% smaller blood flow in the right VA compared to the left VA such that absolute calculations of regional and global blood flow are an underestimation (Lewis et al., 2014; Ogoh et al., 2013). However, due to the stark differences in resting blood flow between the ICAs and VAs, regional blood flow response to stressors such as hypoxia are commonly reported relative to resting blood flow (Willie et al., 2012). Disproportionate scaling in the calculation of blood flow relative change may, in part, have contributed to conclusions of preferential blood flow regulation to the posterior circulation compared to anterior circulation in previous research (Lewis et al., 2014; Ogoh et al., 2013; Subudhi et al., 2014; Willie et al., 2012). In these studies, the relative blood flow response to hypoxia was greater in the VAs (posterior) than ICAs (anterior) based on unilateral measures from the right VA (Lewis et al., 2014; Ogoh et al., 2013; Willie et al., 2012). In the current study, when unilateral extracranial measurements were selected on the vessel side, right VA measurements overestimated the relative blood flow response to the greatest degree (4%; Figure 5d) and had the widest limits of agreement (−21 to 28%) compared to the bilateral calculation. This finding is particularly noteworthy given that many investigators choose to scan the right rather than the left VA presuming that the right VA is the conservative option when doubling a unilateral measurement. But, as detailed in this study, vessels with the smaller resting blood flow are more susceptible to greater and more varied measurement errors due to the ratio-scaling problem when describing a relative blood flow response.

4.4 | Perspectives and application

In this study, there was no statistical difference in resting blood flow between the left and right vessels of the ICA (4%) and VA (8%). Therefore, we may have underestimated the group mean measurement error of unilateral compared to bilateral assessment that may be found in future studies. This is particularly likely for the VAs as the left-to-right blood flow difference in the VAs is typically reported in the range of 20–30% (Khan et al., 2017; Schöning et al., 1994). The heterogeneity between individuals in the magnitude of difference between left and right extracranial arteries' blood flow (ICA: 1–91% and VA: 0–400%) means that without examining the contralateral vessel there is an increased likelihood of substantial measurement error in the calculation of the relative blood flow response to hypoxia.

To eliminate measurement error in the relative blood flow response to hypoxia, bilateral measurements should be used. However, if this is infeasible, we advise the ICA and VA vessel with the larger resting normoxic blood flow be measured for each participant following prescreening of both the left and right vessels based on two inferences. Firstly, vessels with the smaller resting normoxic blood flow were associated with a greater and more varied relative blood flow response to hypoxia (Figure 3). This is also supported by the Bland–Altman analysis that identified the greatest measurement bias and limits of agreement, and the presence of a proportional bias is caused by unilateral measurements of the extracranial vessel with the smaller blood flow (Figure 4). Secondly, from a practical aspect, successfully imaging a vessel, maintaining consistent flow velocity with a centrally positioned Doppler gate and accurately measuring vessel diameter (whether manual or automated) are all easier in the vessel containing the larger blood flow. Moreover, to improve efficiency and feasibility of identifying the vessel with the larger resting normoxic blood flow before experimental trials, the left and right vessel diameter could be measured using the standard built-in caliper method available in ultrasound devices as a strong index of vessel blood flow (Cipolla, 2009). Where simultaneous insonation of two extracranial arteries is necessary, this is normally achieved by contralateral measurements due to ultrasound interference and physical probe space limitations (Sato et al., 2016). In this instance, the extracranial artery (ICA or VA) with the widest difference in resting normoxic blood flow between the left and right vessels should be prioritised in imaging to minimise measurement error. We advise these methods to be applied when measuring extracranial blood flow to other vasoactive stimuli such as carbon dioxide, orthostasis and exercise.

4.5 | Methodological considerations

The interaction of oxygen and carbon dioxide tensions are key factors in the overall change in cerebral blood flow during exposure to hypoxia (Bruce et al., 2016; Friend et al., 2019; Lucas et al., 2011). The single bout of poikilocapnic hypoxia used here resulted in a range of S_{pO_2}, $\dot{V}_E$ and P_{ETCO_2} between participants, and therefore not all individuals had similar systemic hypoxia. However, when the relative blood flow response to hypoxia was corrected for the differences in S_{pO_2} as an index of relative hypoxic reactivity, the ICAs and VAs were found to remain similar (Figure 2h). Future research should use stepwise gas manipulations to investigate the regulation of extracranial arteries through the range of hypoxic severities typically experienced. Bilateral calculations of blood flow were derived from consecutive rather than simultaneous measurements of the left and right arteries as we only had access to a single ultrasound. However, the short time difference introduced by using consecutive left and right measurements likely had limited influence on the interpretation of our findings as the ICA and VA measurements were obtained whilst participants were rested. A wide range of methodological techniques are currently employed to measure cerebral blood flow at rest and in response to stressors, each with its advantages and disadvantages (Tymko et al., 2018). Duplex ultrasound offers a non-invasive, volumetric measurement of intravascular blood flow with excellent temporal resolution important for the assessment of cerebral blood flow to dynamic stressors (e.g., hypoxia, carbon dioxide, orthostasis and exercise). However, to obtain accurate and reliable (~10% day-to-day coefficient of variation) measurements considerable ultrasound training is required (Thomas et al., 2015). Notwithstanding this, the results presented here reveal a source of previously under-recognised measurement error in the assessments of unilateral extracranial relative blood flow response to vasoactive stimuli, and provide a systematically approached consensus for the selection of unilateral extracranial measurements to minimise this measurement error when bilateral measurement is infeasible.

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4.6 | Conclusions

ICA and VA blood flow regulation to hypoxia is comparable when factoring for vessel type (ICA or VA) and vessel side (left or right) effects. Bilateral calculations of the ICAs and VAs indicated the same degree of vasodilatation and comparable increases in relative blood flow to acute poikilocapnic hypoxia. Compared to bilateral assessment of the relative blood flow response to hypoxia, individual unilateral measurement errors reached 37%, and were greatest in ICAs and VAs with the smaller resting blood flow due to a ratio-scaling problem. Where bilateral assessment is infeasible, assessing the ICA and VA vessels with the larger resting blood flow, not the left or right vessel, reduces unilateral measurement error.

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COMPETING INTERESTS

There authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.T.F. and S.J.O. conceived and designed the study. All authors contributed to the acquisition, analysis or interpretation of data for the work. A.T.F. and S.J.O. drafted the manuscript, with all remaining authors reviewing and providing critical feedback important for intellectual content. All authors have approved the final version of the paper 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.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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