Title: The relationships between age, sex and cerebrovascular reactivity to hypercapnia using traditional and kinetic-based analyses in healthy adults

Running head: Age, sex and cerebrovascular reactivity

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Abstract

The effect of age and sex on intracranial and extracranial cerebrovascular function is poorly understood. We investigated the relationships between age, sex and cerebrovascular reactivity (CVR) to hypercapnia in 73 healthy adults (18-80 years, N=39 female). CVR to hypercapnia was assessed in the middle cerebral artery (MCA) using transcranial Doppler ultrasound and at the internal carotid artery (ICA) using duplex ultrasound. MCA CVR was characterised by peak MCA velocity (MCAv) response per mmHg increase in end-tidal CO_2, and by using a mono-exponential model to characterize the kinetics (time-constant) of the MCAv response. ICA reactivity was assessed as the relative peak increase in artery diameter. Hierarchical multiple regression determined the relationships between age, sex, and the age by sex interaction on all baseline and CVR outcomes. There was no relationship between ICA reactivity (%) with age (P=0.07), sex (P=0.56) or a moderator effect of sex on the age effect (P=0.24). MCAv CVR showed no relationship with age (P=0.59), sex (P=0.09), or an age by sex moderator effect (P=0.90). We observed a positive relationship of MCAv CVR time-constant with age (P=0.013), such that the speed of the MCA response was slower with advancing age. The present study provides comprehensive data on age and sex specific relationships with intracranial and extracranial cerebrovascular responses to hypercapnia. Despite similar MCAv CVR and ICA reactivity between sexes, kinetic responses of the MCA revealed a slower rate of adjustment with advancing age.

New and noteworthy

We observed similar MCA CVR and ICA reactivity in males and females. However, kinetic responses of the MCA to hypercapnia suggest that advancing age slows down the rate at which MCA velocity increases in response to hypercapnia. These data indicate distinct regulatory differences, and an impaired vasomotor control of the cerebrovasculature with advancing age, not detected by traditional methods.

Keywords: internal carotid artery, carbon dioxide, ageing, lifespan, cerebral blood flow

Introduction

Cerebral blood flow (CBF) responsiveness to hypercapnia, termed cerebrovascular reactivity (CVR), is vital in stabilising pH levels and maintaining delivery of oxygen and nutrients to the brain [1]. Previous research has evidenced the clinical importance of this outcome, with a lower CVR later in life (60+ years of age) associated with increased risk of age- associated disease including Alzheimer's disease, cognitive decline [2], and all cause-mortality [3]. Thus, it is important to understand the physiological changes in hypercapnia induced CVR associated with advancing age.

Despite numerous studies investigating the effect of advancing age on CVR of the anterior circulation, conclusions remain unclear [4]. Some studies demonstrate CVR to decline in older compared to young adults [5-8], however, other studies show CVR to remain unchanged in older adults [9-12], or even increase [13, 14]. To date, research has largely ignored comparisons to middle aged groups (~40-60 years), where regulatory, functional, and structural alterations to the vasculature may manifest, and the rate of decline in CVR might be greatest [5, 15]. These disparate findings between studies may be underpinned by several factors including the method of assessment, nature of hypercapnia administration, or differences in the sample population; such as age, sex and physical fitness, which may all independently influence CVR [16-19].

Burley *et al* (2021) showed that when utilising transcranial Doppler (TCD) ultrasound, older adults had significantly greater CVR compared to younger adults. However, with blood-oxygen-level-dependent magnetic resonance imaging (BOLD-MRI) measures, no differences between groups were observed. Most previous studies utilise TCD and are limited by the reliance on velocity measures of a single intracranial vessel (middle cerebral artery (MCA)). Previous findings utilising extracranial internal carotid artery (ICA) measures have shown a decreased ICA reactivity in older (68±1 years) compared to young adults (23±11 years) [20]. However, given the small sample size of 20 participants, sex differences and the sex-dependent effect of age have yet to be adequately addressed.

Few studies investigate the effect of sex on CVR in ageing adults [13, 14, 20]. This is a vital consideration given the potential effects of estrogen, and evidence that the effects of ageing

on the vasculature are sex dependent [21]. Carter *et al* (2016) demonstrated that in young females, MCAv CVR was greater compared to males, however, this sex difference was not evident in the ICA. This is consistent across some [18, 23], but not all studies [24, 25], possibly due to the different methodologies and the use of TCD measures alone, as TCD relies on the assumption that the MCA diameter does not change [26]. This, however, may not hold true during hypercapnia [27], with the magnitude of changes in dilation potentially influenced by age [6, 28] and sex [24]. In contrast, Miller *et al* (2019), using MRI, demonstrated that decreases in intracranial artery responses to hypercapnia were evident with advancing age in males, but not females. This highlights the need to study the effects in males and females separately as not to confound interpretations on the effect of advancing age.

Recent research has highlighted the importance of investigating dynamic kinetic-based analyses on cerebrovascular regulation, in addition to traditional amplitude based inferences [29]. This can be achieved using a mono exponential model, where the time delay, time constant and mean response time can provide additional information on the speed of the response [29, 30]. These outcomes have been shown to be indicative of regulatory responses during exercise stressors [31, 32], but have yet to be applied to examine the effect of age and sex on cerebrovascular responses to hypercapnia.

The first aim of this study is to determine intracranial and extracranial CVR to hypercapnia across the healthy adult lifespan in males and females, exploring traditional CVR in both the MCA and the ICA and the kinetic response to hypercapnia. The second aim is to investigate if any alterations in CVR with age are sex dependent. We hypothesised that a) cerebrovascular reactivity in the intra- and extracranial vessels would show a negative relationship with age, and b) the rate of decline would be sex dependent, with higher CVR in both the MCA and ICA in younger females and a greater rate of decline with advancing age compared to males.

Methods

Ethical approval

All experimental procedures and protocols were approved by the University of Queensland ethics committee (2019001863), and the study conformed to the standards set by the

114 Declaration of Helsinki. Written informed consent was obtained prior to participation in the
115 study.

Participants

Participant recruitment was based off an a priori power calculation to detect differences between age groups for ICA dilation (%) in response to hypercapnia, set for a large effect size (F=0.4), power (0.8) and alpha (0.05) [20] (G* Power 3.1 Kiel, Germany). This resulted in a target recruitment of 20 participants per age group (young=18-39 years; middle=40-64 years; older=40-64 years). Assuming a 20% data loss due to image capture/analysis problems, a target of ~70-75 participants was set. Seventy-three adults between the ages of 18-80 years volunteered to take part in this study.

Exclusion criteria included diagnosed arterial hypertension, smoking, any known cardiometabolic or respiratory diseases, the use of any prescribed medications known to influence cardiovascular function (e.g. statins, thyroid medication) and a body mass index (BMI) >35 kg/m^2. Any pre-menopausal females with an irregular menstrual cycle or the use of progesterone only contraceptive pill were excluded from the study. In addition, naturally menstruating pre-menopausal females (N=8) were tested in the follicular phase *(1-14 days)* to allow better comparisons between sexes [33]. Females on the combined contraceptive pill (N=10) were tested during the inactive pill phase (days 1-7). Post-menopausal females on hormone replacement therapy were also excluded from the study.

Study design

Following baseline screening, participants completed one visit to the laboratory. They were required to fast for a minimum of three hours, and refrain from nitrate rich foods for 12 hours, prior to testing. In addition, participants were required to avoid vigorous physical activity, caffeine and alcohol consumption for 24 hours prior to testing. Body mass and stature were measured according to standard procedures to the nearest 0.1 kg and 0.1 cm, respectively. BMI classifications were used to determine weight status of participants [34]. Physical activity levels were assessed via the Active Australia survey [35], and reported as METmin/week, which accounts for time spent in different intensities of aerobic activity [36]. This survey has been validated against pedometer and accelerometery data in healthy middle-aged adults (R=0.52) [37], and compares favourably to other self-reported physical activity surveys (ICC=0.64) [38]. Female participants self-reported menopausal status via a questionnaire and were categorised into either pre-menopausal (regular periods), peri-menopausal (irregular cycles), early post-menopausal (1-3 years following last menstrual period (LMP)) or post-menopausal (6+ years LMP) [39]. Following initial screening and

questionnaires, participants were required to rest in a darkened temperature-controlled laboratory (~23ºC) for 15 minutes in the supine position prior to instrumentation and the commencement of the protocol.

Experimental measures

The CVR protocol was conducted in the supine position in line with recommendations [40] and to replicate existing studies, given the potential effects of body position on CBF outcomes [41]. It consisted of a two-minute baseline breathing ambient room air, followed by five minutes of hypercapnia. During hypercapnia, 5% CO_2 was administered with 21% O_2 (balanced nitrogen). This replicates the protocol from other laboratories which have investigated the effects of hypercapnia on advancing age [8], and within the normal vasodilatory stimuli ranges of 5-7% CO_2 [42]. A three-way valve (Hans Rudolph Inc, Shawne, USA) allowed inspiratory gases to be switched from ambient air to the 5% CO_2 mixture (using a 170 L Douglas bag, Hans Rudolph Inc, Shawne, USA). Participants were instructed to breathe normally during hypercapnia and the baseline periods. Cardiorespiratory measures were determined simultaneously throughout the protocol, as described below. Hypercapnia was chosen as the stimulus due to its sensitivity to disease risk [42], and the availability of reliability data on this outcome [30].

Cardiorespiratory measures

During the protocol, beat-by-beat blood pressure was measured continuously by finger volume-clamp method (Finapress, NOVA, Netherlands). Participants wore a snorkel mouthpiece and nose-clip (Hans Rudolph, Kansas) to measure end-tidal carbon dioxide ($P_{ET}CO_2$) and end-tidal oxygen concentrations ($P_{ET}O_2$) using a gas analyser (ADInstruments, ML206, Colorado, USA), which was calibrated prior to each participant via known concentrations of O_2 and CO_2. $\dot{V}_E$ was measured using a spirometer (ADInstruments, Colorado, USA), calibrated with a 3 L syringe. Heart rate was assessed using a three lead ECG (Finapress, NOVA, Netherlands). 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, ADI instruments) for offline analysis.

Cerebrovascular measures

Intracranial

A 2-MHz transcranial Doppler ultrasound probe (Spencer Technologies, ST3, Redmond, WA, USA) was used to insonate the right MCA at an initial depth of ~50 mm through the trans-temporal window using previously described guidelines [43]. The Doppler signals were acquired, optimised and secured using an adjustable headset (adult M600 bilateral head frame; Spencer Technologies, WA, USA). 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).

Extracranial

Diameter and mean blood velocity were measured in the right ICA using a 12 MHz linear array Doppler probe through a high-resolution ultrasound machine (Terason, 3300, U-smart, Burlington, MA, USA). The ICA was identified, and the image and waveform were optimised in accordance with extracranial carotid artery guidelines [40]. Doppler velocity assessments were obtained using pulse wave mode with an insonation angle ≤60 degrees. Following optimisation of the longitudinal B-mode image of the arterial walls, images of the artery and associated velocity waveforms were simultaneously recorded during the baseline and hypercapnic periods.

Data processing

Steady state response

Baseline values were averaged over 120 seconds of supine rest. All data from LabChart were exported as 1-second averages into Excel (Microsoft, Seattle, WA). Analysis of ICA diameter and flow were performed using custom-designed, edge-detection and wall-tracking software (BloodFlow Analysis, version 5.1). This approach is independent of investigator bias with automated wall tracking and has previously been comprehensively described and validated [44, 45]. Analysis was performed blinded to the participant age and sex. From synchronized diameter and velocity data, blood flow (the product of lumen cross-sectional area and Doppler velocity) and shear rate (4×mean blood velocity/vessel diameter) were calculated at 30 Hz [46]. ICA data were then interpolated from 30 Hz to 1 Hz and exported into an Excel spreadsheet. The LabChart and ICA vascular data were time aligned to the start of the hypercapnic protocol in Labchart and re-exported to Excel for subsequent analysis using in-house, carotid shear–mediated dilation software. This automated software calculated indices of baseline (median value of the baseline), peak response following the onset of CO_2, and the

relative change (%) from baseline to peak, for all ICA variables (diameter, peak systolic velocity, mean blood flow, shear rate).

Flow pulsatility index (PI) was calculated as the difference between diastolic and systolic MCAv divided by the mean MCAv ($MCAv_{systolic} - MCAv_{diastolic} / MCAv_{mean}$) [47]. The PI response was obtained at baseline and the final 30 seconds of each minute of hypercapnia. Cerebrovascular resistance index (CVRi) was calculated as mean arterial pressure (MAP) divided by MCAv ($mmHg/cms^{-1}$) at baseline and the last 30 seconds of hypercapnia to appropriately capture the hypercapnic response, given that CVRi decreases throughout the response. The responses to hypercapnia were obtained in the final 30 seconds of each minute, and differences from baseline to peak during hypercapnia were calculated for HR, MAP, ICA blood flow, MCAv, VE, $P_{ET}CO_2$ and $P_{ET}O_2$.

Calculation of CVR to hypercapnia was expressed as the absolute change from baseline MCAv per unit increase (mmHg) in $P_{ET}CO_2$. This response was quantified as the peak rolling 30 second average during hypercapnia, wherever this occurred [30]. CVR was calculated in this way, as the most reliable analysis method, to address recent concerns on the variability of changes in MCAv during open circuit breathing [30, 48].

Kinetic response

Data were baseline-corrected for the 120 seconds preceding hypercapnia and analysed using a mono-exponential model with time delay using GraphPad Prism (**Figure 1**) (GraphPad Software, San Diego, CA, USA) as follows: $MCAv(t) = \Delta MCAvA\ (1 - e^{-(t-TD/\tau)})$, where MCAv(t) is the MCAv at a given time (t), ΔMCAvA 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 [29, 30]. Mean response time (MRT) was calculated as the sum of the model derived τ and the TD. The model was fitted from the start of the exponential rise until a deviation from a subjective visual steady-state was observed. All models were then checked by two independent researchers for consistency, and any disagreements discussed until a consensus was reached. Acceptability of appropriate fit was determined as; goodness of fit $R^2>0.50$, and normality of residuals. The precision of the derived τ was quantified using 95% confidence intervals.

[Insert Figure 1 near here]

Internal carotid artery dilation

All ICA data were passed through a 2-stage filtering process; a median filter (with a rank of 7) was applied to the parameter data, followed by passage through a Savitzky–Golay finite impulse response smoothing filter with a window size of 13 data points and a polynomial order of 1 [22]. These filters removed high-frequency noise to reveal the underlying lower frequency physiological response profiles. All subsequent analyses were performed using this graphed, filtered data of variables including ICA shear rate, diameter, mean blood flow, peak blood flow, mean velocity and peak systolic velocity. The following variables were automatically detected and calculated by the software: (1) baseline: the median value of the 2-minute baseline period preceding hypercapnia; (2) peak response: the autodetected maximum value of the filtered data identified after the onset of CO_2; and (3) relative change: change from baseline to peak, calculated as ((peak−baseline)/baseline) ×100). The total and initial stimulus for subsequent dilation were quantified as the shear rate (SR) area under the curve (AUC) from the time of CO_2 onset to the time of peak diameter (SR_{AUC}), and to the first 50 seconds after CO_2 onset ($SR_{AUC\text{-}initial}$). $SR_{AUC\text{-}initial}$ was chosen, as an attempt to account for the initial shear stress stimulus, driving the potential changes in diameter. SR_{AUC} and $SR_{AUC\text{-}50s}$ were calculated using the trapezoid rule (GraphPad, Prism, version 9) . In addition, a thresholding algorithm was applied to each data array (e.g., ICA shear, ICA diameter), which identified threshold points. These thresholds were defined as the point at which each variable began to systematically increase, above the baseline level, after the application of the CO_2 stimulus. The threshold point was calculated as follows: threshold point = (maximum value – minimum value) x variation factor %) + baseline median value [20, 22]. This variation factor was chosen to ensure that the variable had increased to a point that represented a definitive deviation from baseline, which also exceeded fluctuations associated with cardiac and respiratory cycles. Once the software had automatically detected the threshold points, they were depicted on the raw data array and visually inspected to ensure they met the following criteria: (1) the algorithm-detected threshold point occurred before the peak value and (2) the variable did not decrease below the algorithm-detected threshold point before the peak value occurring [22]. In cases where they did not meet the criteria (~22%), the threshold points were manually adjusted independently to a point where it was deemed there was a clear deviation from baseline values that met the above criteria. This was then checked by two independent researchers.

ICA dilation (%) was allometrically scaled to account for differences in baseline ICA diameter as previously described [49, 50].

Statistical analyses

Statistical analyses were conducted using SPSS (version 25; IBM, Armonk, New York). All data are presented as mean ± SD. Differences in participant characteristics were explored using a two-way analysis of variance (ANOVA) with sex (male, female) and age (young, middle-aged, older) as the independent variables. For aim 1), a hierarchical multiple regression was used to determine the relationships between age (years) (model 1) and sex (model 2) with all baseline and hypercapnic variables of interest. For aim 2, an interaction term of age x sex (model 3) was added to address whether sex moderated the effects of age by assessing the differences in regression slope coefficients between males and females on all variables of interest. The outputs for model 1 and 2 included the slope coefficient (unstandardised β), the explained variance of the full model (R^2) and the significance of the relationship (P value). For model 3 the output included slope coefficients (unstandardised β) of the interaction terms for males by age, and for females by age. The P value describes whether there was a significant difference in slope coefficients between males and females with advancing age (significant interaction), and the R^2 denotes the degree of explained variance of the entire regression model with the interaction term included.

In order to adjust for any variance explained by body mass and physical activity levels; BMI (kg/m^2) and self-reported physical activity (METs/week) were added to the model. Lastly, MAP was added to the model to adjust for any variance on cerebrovascular and ICA outcomes explained by changes in perfusion pressure. In instances where these factors (BMI, physical activity, MAP) significantly explained any variance in the overall model response, the results are presented for the full model, and whether the addition of this variable altered the effects of age, sex and the age by sex interaction term. A simple linear regression was run to investigate the influence of menopause on variables of interest in a female only model. The model investigated the relationship between early post-menopause (1-3 years LMP), and late post-menopause (6+ years LMP) to a reference group of pre-menopausal females on variables of interest. All data were normally distributed as assessed by visual inspection of Q-Q plots and homoscedasticity of the studentized residuals plotted against the predicted values. Linearity was established by visual inspection of a scatterplot. There was no evidence of multicollinearity, as evidenced by no tolerance values less than 0.24. Although some data points were identified as above 2-3 standard deviations from the mean, none were deemed implausible and removed. Statistical significance was accepted at an alpha of $P<0.05$.

Results:

Participants were recruited into young (N=25, 12 female, age 27.0±2.6 years, range=22-32 years), middle aged (N=30, 17 female, age 52.9±7.5 years, range=35-63 years) and older groups (N=18, 10 female, age 69.8±3.5 years, range=65-77 years). Participant characteristics of the cohort can be seen **in Table 1**. Intracranial kinetic analyses are presented for 71 adults (38 females), due to unacceptable model fit in 2 individuals. Extracranial analyses are included for 58 individuals (31 females). Reasons for data loss of ICA analyses were the inability to obtain a sufficiently clear ultrasound image in 15 individuals.

The main effects of age and sex on participant characteristics are highlighted in Table 1. A significant age by sex interaction was present for PI (P=0.006). Post-hoc pairwise comparisons revealed significant differences between young males compared to females (P=0.004). In male participants, significant differences between young compared to middle-aged adults was present (P=0.008). In female participants, significant differences between older adults compared to young (P=0.001) and middle-aged adults was observed (P=0.01).

[Insert Table 1 near here]

Baseline responses

The relationships between age and baseline cerebrovascular and cardiorespiratory variables of interest can be seen in **Table 2.** These data are presented for age (model 1), age and sex (model 2) and the moderator effect of sex on the relationship between age and variables of interest (model 3).

[Insert Table 2 near here]

There was a negative relationship between MCAv and age (model 1). With the addition of sex to the model (model 2) this showed a relationship between MCAv and sex explaining 21% of variance (P=0.003) and MCAv higher in females (β=10.2 ± 3.3 cm/s) (**Figure 2A, Table 2**). There was no age by sex interaction (model 3) for baseline MCAv (P=0.41). There was a positive relationship between CVRi and age (P<0.001) (R^2=0.30) (Figure 2B), with the addition of sex in model 2 this did not lead to an increase in explained variance for CVRi (P=0.09). Sex did not moderate the effect of age on baseline CVRi, (P=0.49).

Baseline ICA diameter showed no relationship with age (P=0.09), but there was an effect of sex, with the ICA diameter greater in males and explaining 23% of variance (P<0.001). Sex

did not moderate the effect of age on baseline ICA diameter such that the slope coefficients were not different in males and females with age (P=0.73).

Baseline ICA shear rate, peak systolic velocity, and mean blood flow all showed no relationship with age (P≥0.14). With the addition of sex to the model there was a relationship for shear rate (P=0.007). All other ICA variables (peak systolic velocity, and mean blood flow) showed no relationship with the addition of sex to the model (P≥0.07). Sex did not moderate the effects of age on baseline shear rate, peak systolic velocity, and mean blood flow (P≥0.24) (**Table 2**).

[Insert Figure 2 near here]

For baseline $P_{ET}CO_2$, with the addition of physical activity (weekly METminutes) to the model, this led to an increase in explained variance of the full model (R^2=16, P=0.04) (β=-0.005±0.002). The addition of physical activity to the model did not significantly influence the effects of age (R^2=0.052, P=0.05), sex (R^2=0.052, P=0.97) or the interaction of age and sex on baseline $P_{ET}CO_2$ (R^2=0.095, P=0.17). For all other baseline cardiovascular and cerebrovascular outcomes, the addition of physical activity and BMI did not lead to an increase in explained variance and therefore was not included in the model (R^2≤0.41, P≥0.057). With the addition of MAP to the model, this led to an increase in explained variance for baseline MCAv (R^2=0.28, P=0.03) (β=0.25±0.11). The addition of MAP to the model did not influence any of the relationships between baseline MCAv and age (R^2=0.11, P=0.005), sex (R^2=0.21, P=0.003) or the interaction of age and sex (R^2=0.22, P=0.41). There was no relationship between MAP and any ICA baseline responses (R^2≤0.02, P≥0.26).

Peak hypercapnia responses

The relationship between age and sex and the peak hypercapnic cerebrovascular and cardiorespiratory variables of interest can be seen in **Table 3.** These data are presented for age (model 1), age and sex (model 2) and the moderator effect of sex on the relationship between age and variables of interest (model 3). Data for cardiovascular and cerebrovascular responses to hypercapnia are presented in **supplementary table 1.**

[Insert Table 3 near here]

There was a negative relationship between age and peak MCAv (P=0.03) (**Table 3**). With the addition of sex to the model there was a significant relationship, explaining an additional 11% of variance (P=0.002), with peak MCAv greater in females. Sex did not moderate the

effect of age on peak MCAv (P=0.28). With the addition of MAP to the model, this led to an increase in explained variance for peak MCAv (R^2=0.26, P=0.02). The addition of MAP to the model did not alter the relationship with age (R^2=0.07, P=0.03), sex (R^2=0.18, P=0.002) and the interaction of age and sex (R^2=0.20, P=0.28).

MCAv CVR (cms/mmHg) showed no relationship with age (P=0.59), sex (P=0.09) or any moderator effect (P=0.90) **(Table 3, Figure 3A).** With the addition of physical activity (weekly METminutes) to the model this did not lead to an increase in explained variance (R^2= 0.05, P=0.05) and did not influence the relationships with age (P=0.84, R^2=0.001), sex (P=0.08, R^2=0.05) or the moderator effect (P=0.94, R^2=0.05). With the addition of MAP to the model, an increase in explained variance for MCAv CVR was observed (R^2=0.13, P=0.01). This did not alter the relationships between MCAv CVR with age (P=0.59, R^2=0.004), sex (P=0.09, R^2=0.05), or any moderator effect (P=0.90, R^2=0.05). For peak $P_{ET}CO_2$ there was no increase in explained variance with the addition of physical activity (R^2=0.07, P=0.05).

ICA dilation was not explained by the magnitude of the SR_{AUC} (P=0.60) (R^2=-0.005) or $SR_{AUC\text{-}initial}$ (P=0.25) (R^2=0.02), therefore ICA dilation (%) was not normalised to shear rate. This held true at all age groups when analyses were investigated in young (P>0.11), middle (P>0.69) and older groups (P>0.23) separately for SR_{AUC} and $SR_{AUC\text{-}initial}$.

Hypercapnic peak responses for the ICA are shown in **Table 3.** For percent dilation of the ICA (allometrically scaled), there was no relationship with age (P=0.07) (**Figure 3B**). With the addition of sex to the model there was no relationship (P=0.56), and no significant moderator effect was observed for ICA dilation (%) (P=0.24).

[Insert Figure 3 near here]

Mean ICA blood flow, and the percent change in mean blood flow all showed a positive relationship with age (P≤0.03). However, no sex (P≥0.20), or moderator effects were observed (P≥0.10).

The percent change in peak systolic ICA velocity showed no relationship with age (P=0.21). With the addition of sex to the model there was no relationship (P=0.43), however, sex moderated the effect of age on percent change ICA velocity (P=0.02). Simple slopes analyses revealed that there was a positive relationship between age and peak ICA velocity in males (β=0.78 ± 0.29) (P=0.02), but not in female participants (β=-0.20 ± 0.29) (P=0.10).

Peak shear rate showed no relationship with age (P=0.97) (R=-0.005), however, with the addition of sex to the model this showed a relationship with shear rate higher in female participants and the model explaining 13% of variance (P=0.008). No significant moderator effect of age was observed for peak shear rate (P=0.11). As a percent change in shear rate no relationship with age (P=0.10) or sex (P=0.57) was observed, however, sex was shown to moderate the relationship with age (P=0.01). Simple slopes analyses revealed that there was a positive linear relationship in males with age (P=0.003) (β=0.96 ± 0.31), and a negative relationship with age in females for shear rate as a percent change (P=0.01) (β=-1.91 ± 0.31) **(Figure 4**).

[Insert Figure 4 near here]

Intracranial kinetics analyses

Dynamic onset response data are shown in **Table 4**. The MCAv response was well fitted by an exponential model (standard error of the τ: 2.46 ± 1.43). For all kinetic analysis outcomes of interest there was no increase in explained variance with the addition of physical activity and BMI to the regression model (R^2<0.023, P>0.23), and therefore were not included in the full model.

For the MCA time constant there was a positive relationship with age, such that the speed of the MCA response was slower with advancing age (P=0.013). With the addition of sex to the model this did not explain any variance in the response (P=0.13), and no moderator effect of sex was observed for the MCAv time constant (P=0.77) **(Table 4, Figure 5A).** For the $P_{ET}CO_2$ time constant there was no relationship with age (P=0.81) **(Figure 5B).** With the addition of sex to the model no relationship was observed (P=0.84), and no moderator effect (P=0.52).

[Insert Figure 5 near here]

For the amplitude of the MCA response expressed as an absolute change there was no relationship between the response and age (P=0.49) **(Figure 6B)**. With the addition of sex to the model this showed a relationship with MCAv amplitude higher in female participants and the model explaining 8% the variance (P=0.03). However, no moderator effect was observed (P=0.31). The amplitude of the $P_{ET}CO_2$ response showed a positive relationship with age (P=0.004). There was no relationship with sex (P=0.41), and no moderator effect (P=0.64) **(Figure 6A)**.

[Insert figure 6 near here]

For the MCAv MRT there was a positive relationship with age, such that the speed of the response was slower with advancing age (P=0.002). With the addition of sex to the model this did not explain any additional variance (P=0.26), and no moderator effect of sex was observed for the MCAv MRT (P=0.37). For the $P_{ET}CO_2$ MRT there was no relationship with age (P=0.69). With the addition of sex to the model no relationship was observed (P=0.97), and no moderator relationship was observed for the $P_{ET}CO_2$ MRT (P=0.76)

[Insert Table 4 near here]

As a pooled data set there was a positive relationship between the MCAv and $P_{ET}CO_2$ time constants (R^2=0.29, P=0.013). When split into age categories there was a positive relationship present in young adults (R^2=0.62, P=0.001), however, this relationship was lost in middle (R^2=0.25, P=0.18) and older aged adults (R^2=0.13, P=0.39).

Relationships between baseline and hypercapnic variables and menopausal status

The relationships between menopausal status and variables of interest are shown in **Table 5.** There was a negative relationship between MCAv and menopausal stage (P=0.03, R^2=0.18). Simple slopes showed the decrease in MCAv was different in late-postmenopausal females compared to pre-menopausal females (P=0.02), however, early postmenopausal females showed no difference in slope coefficients to pre-menopausal females (P=0.78).

[Insert Table 5 near here]

Discussion:

This is the first study to document the cross-sectional relationships of age and sex on kinetic responses to hypercapnia. The primary findings were that; 1) despite a negative relationship between age and absolute peak MCAv to hypercapnia, the relative amplitude-based responses to hypercapnia in the MCA and ICA reactivity were preserved with age, 2) a positive relationship between age and the MCAv τ kinetic response suggesting that age slows down the speed at which MCAv increases in response to a hypercapnic challenge; and 3) reduced baseline and peak MCAv in late post-menopausal females, but no differences in ICA blood flow or CVR compared with pre-menopausal females.

Resting cerebral blood flow with age

Declines in resting CBF with advancing age have been well documented [51-54]. The current data corroborates this, documenting decreases in baseline MCAv with advancing age by ~3cm/s per decade alongside an increased CVRi. Despite a higher baseline MCAv in females than males, the relationship between age and MCAv was similar between males and females. This implies that the declines observed with ageing were similar in both males and females with no age by sex interaction evident. Previous research has documented higher MCAv in young females, however a greater rate of decline with advancing age [55, 56]. This is contrary to the current study, where we found preserved MCAv and CVRi in early postmenopausal females, with 13 cm/s lower MCAv in late menopausal females; thus, declines in females in the current sample were driven by post-menopausal females. Differences between studies may be due to a younger average female sample in the current data, with prior research comparing young vs older adults at extremes of the ageing spectrum.

Declines in resting MCAv with advancing age may be due to numerous factors, inclusive of; decreased brain volume, cerebral metabolism, increases in arterial stiffness, oxidative stress and decreased nitric oxide (NO) bioavailability [57-60]. The greater declines in females are likely related to the loss of estrogen with the onset of menopause, which plays a pivotal role in mediating vascular function and blood pressure via the production of NO [61-63]. However, the effects of the menopausal transition and loss of estrogen are less well documented in the cerebral vessels [64]. Future research whereby blood markers for female sex hormones and NO concentrations are sampled is required to further understand the effects of menopause disentangled from the effects of age on the cerebrovascular circulation. Additionally, in premenopausal females large inter- and intra-individual differences in estrogen concentrations prevail; even when controlling for cycle phase [65]. Thus, direct measures of hormone concentrations in larger sample sizes are required in future investigations to account for this potential added variability in female responses, and to fully understand the effects of female sex hormones on the cerebral circulation.

Despite observing a negative relationship between MCAv and age, baseline ICA mean blood flow showed no relationship with advancing age. This is in line with previous findings [66-68]. It is proposed that declines in the posterior circulation with advancing age are more marked compared with the anterior circulation [68, 69]. A potential explanation for the preserved mean ICA blood flow response may be due to the high physical activity levels in the current sample (~2182 METmins/week), with regular physical activity shown to attenuate the age-related decline in CBF [70, 71], and endurance exercise training shown to increase

CBF and CVR responses [72]. When accounted for as a covariate, physical activity did not show a relationship with ICA mean flow, however, physical activity levels in the current sample were all relatively high across the sample (89% met or exceeded the PA guidelines) and therefore comparisons on the influence of physical activity levels are limited and does not negate the potential for physical activity to influence the current results. Aerobic fitness levels were not measured in the current study, which may be a better predictor of both endothelial function, and cognitive function [73, 74]. In particular, a recent study has documented a positive relationship between fitness and resting MCAv in females but not in males, however, this relationship was no longer significant when adjusted for age [75]. Further research investigating the relationships of age and sex on cerebral responses should therefore include direct measures of cardiorespiratory fitness to fully understand the effects of ageing and sex on the cerebral circulation, and any age and sex specific interactions with cardiorespiratory fitness.

Cerebrovascular reactivity and age

Contrary to our hypotheses, MCAv CVR and ICA % reactivity showed no association with age. Interestingly however, when included as a covariate, MAP showed a significant relationship with CVR. With the addition of MAP this did not alter the relationships between CVR with age and sex and therefore indicates the effect of MAP are independent from the effects of age and sex. However, when blood pressure was factored into the hypercapnic response, presented as MCAv CVC, this showed a reduced response with age. It therefore seems that, in older adults, there is a reliance on increased perfusion pressure to increase CBF to hypercapnia, consistent with recent data [6, 14, 28, 76]. Despite similar absolute responses, current and previous data highlights regulatory differences with advancing age; inclusive of increased cerebrovascular resistance during the vasodilatory CO_2 stimulus. Our findings are in line with Oudegeest-Sander *et al* (2014), Ito *et al* (2002), Murrell *et al* (2013), and Stefanidis *et al* (2019) who all observed preserved responses to hypercapnia with advancing age. However, research is conflicting with others reporting declines in CVR, in both the MCA and ICA [6, 20, 77]. The study by Miller *et al* (2019) was able to account for MCA diameter changes and highlighted the ability of the MCA to dilate in young adults during hypercapnia, but not in older adults. This again indicates distinct regulatory differences with age. It suggests that utilising TCD based measures of the MCA may underestimate flow and thus MCAv CVR in our younger adults, masking any potential alterations with age.

The current findings of no relationship between ICA reactivity and age, are observed despite an increased mean blood flow and velocity in the ICA. Therefore, despite a potentially greater blood flow stimulus and perfusion pressure, no alterations in dilation of the ICA were observed with age. This may be indicative of an impaired response; with a greater stimulus required to elicit similar responses. Shear rate has previously been shown as the driving stimulus for changes in ICA dilation during hypercapnia [20]. However, in the current study, there was no relationship between ICA dilation and shear stress, irrespective of age. This is in line with emerging evidence, highlighting that CVR assessed via steady state CO_2 does not reflect endothelial NO dependent dilation [78]. Given that this metric was not solely an endothelial mediated measure of cerebrovascular function, this may explain why no alterations were found with advancing age.

Endothelial shear stress is an important regulator of vessel tone, mediating alterations in vessel structure and function [79]. Additionally, declines in shear stress have been highlighted as an independent predictor of cognitive decline in older adults [80]. In the present study, baseline shear rate was higher in females then males with no effect of age. We did observe a trend for a lower shear stress with older age, however this was not significant. This is contrary to previous research which shows decreases in shear stress in cerebral arteries with age [81, 82]. These decreases in shear rate with ageing occur alongside increases in baseline diameter [82]. However, the present study did not find increases in diameter with age, and therefore may explain the preserved shear rate with age. This is in line with Iwamoto *et al* (2018) who observed no changes in baseline shear rate or diameter in older vs younger adults. It therefore seems that in the present sample population, vascular remodelling and increases in arterial diameter have not yet manifested, potentially due to the good health status and high physical activity levels reported. For shear rate responses to hypercapnia, no ageing effect was seen, similar to previous findings [20]. However, we did observe a sex dependent effect of ageing on shear rate responses, with an increase in males and a blunted response in females.

A notable difference with advancing age was the slowed MCAv speed of response (τ, MRT), observed in both male and female participants. These data indicates that, despite a maintained capacity to obtain the same relative increase in MCAv, advancing age slows down the rate at which this response occurs. Reasons for this blunted MCAv τ and MRT with ageing are largely speculative, however it seems that in a healthy sample where cerebrovascular function still remains intact, the time course of the response may reveal impairments in vasomotor

control of the cerebrovasculature. Given the cerebrovaculature is comprised of numerous integrative mechanisms governing cerebrovascular control to ensure adequate CBF is maintained [83], this delayed response may be reflective of a compensatory response and reliance on differential mechanistic pathways to meet the demands of the brain. The MCAv τ was shown to be related to the $P_{ET}CO_2$ τ in young adults, however, this relationship was lost with advancing age. This highlights distinct regulatory differences with advancing age and indicates the blunted response with age was not due to the ventilatory response and may be due to other cerebral factors; inclusive of decreased cerebral metabolism, increased arterial stiffness, reduced compliance, slower autoregulatory responses, and greater reliance on perfusion pressure.

This study provides a comprehensive assessment of the age and sex dependent cerebrovascular responses to hypercapnia, utilising intra- and extra-cranial assessments in an adequately powered study design. Despite the novelties, the limitations of the current study should be acknowledged. Firstly, TCD measures the velocity of the MCA and not absolute flow [4]. Although the two are highly correlated [84], and prior work has shown the MCA diameter to remain constant during moderate elevations in MAP and $P_{ET}CO_2$ as seen in the current study [26, 85, 86], more recent evidence suggests this may not be the case [27, 87], particularly in the context of ageing [6]. Despite the draw backs of TCD, the higher temporal resolution provides possibilities for novel assessments of the dynamic responses to hypercapnia which the current study employs. Further, the current study was unable to utilise prospective end-tidal targeting to standardise the end-tidal to arterial CO_2 gradient [4]. Therefore, the potential for differences in MCA and ICA reactivity between individuals may have been due to differing $P_{ET}CO_2$ stimuli and ventilatory responses. However, we found no differences in $P_{ET}CO_2$ concentrations with age, or sex in the current sample and therefore believe that the lack of end-tidal targeting did not have a marked effect. Also, by not utilising end-tidal targeting we were able to explore the kinetic relationships between $P_{ET}CO_2$ and MCAv responses with age. A final consideration of the current study is that we did not include measures of hypocapnia induced CVR, which may have offered additional insights into the ageing response [88]. Future research should therefore employ both hypo- and hyper-capnia induced CVR to discern the effects of age and sex on CVR across the entire ventilatory range. However, given there is a lack of standardisation of hypocapnia measures of CVR, this should first be addressed. Future research should also include measures of the

posterior circulation, given the presence of regional differences in cerebrovascular regulation [23, 89].

In conclusion, this study provides insight into the relationships between age and sex on MCA and ICA reactivity to hypercapnia. Additionally, this is the first study to investigate the kinetic responses of the MCA to hypercapnia. Our findings demonstrated that despite similar MCAv CVR and ICA reactivity, dynamic responses of MCAv were significantly blunted with ageing. These novel findings highlight the need for further investigation into the effects of age and sex on CVR responses. In particular, longitudinal study designs in larger sample sizes, with direct measures of sex hormones in females and cardiorespiratory fitness across the cohort. This will aid in accounting for any individual variability in the age-related responses, and advance current understanding on the influence of sex hormones and menopause on cerebrovascular responses.

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

Author Contributions

J.L.K, B.B, A.R.B, J.S.C, T.G.B conceived the study design. J.L.K, S.L.R and F.K.P were involved with data collection. J.L.K analysed all data which was checked for accuracy by S.L.R and F.K.P. J.L.K drafted the work. All authors contributed to revising of the draft 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.

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Supplementary data

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Figure Captions:

Figure 1. An example MCAv trace for one participant at rest (120 seconds) and following onset of hypercapnia (time 0 – denoted by dotted line). The residual plot is included below. Hypercapnic response characterised by a mono-exponential model with a delay term shown by the solid black line. The time delay (TD) presents the time from the start of hypercapnia (0 seconds) to the onset of the exponential rise. The time constant (τ) presents the time taken to reach 63% of the response amplitude and it reflects the rate of increase in MCAv. The amplitude (Amp) presents the change from baseline to the peak of the exponential increase in MCAv.

Figure 2. Linear regression analysis demonstrating the relationships between age and baseline (A) Middle cerebral artery velocity (MCAv) and (B) Cerebrovascular resistance index (CVRi) (N=73, females=39). The solid line represents the regression fit for males ( ) and the dotted line represents the regression fit for female participants ( ). Hierarchical regression models (P value and R^2) are presented for the relationship with age (model 1), the relationship with sex (model 2) and the moderator relationship of the sex dependent relationship with age (model 3). Slope coefficients (β) of the relationship with age are presented for males and females separately (model 3).

Figure 3. Linear regression analysis demonstrating the relationships between age and (A) CVR (N=73, females=39) (B) % ICA dilation and age (N=58, females=31). The solid line represents the regression fit for males ( ) and the dotted line represents the regression fit for female participants ( ). Hierarchical regression models (P value and R^2) are presented for the relationship with age (model 1), the relationship with sex (model 2) and the moderator relationship of the sex dependent relationship with age (model 3). Slope coefficients (β) of the relationship with age are presented for males and females separately (model 3).

Figure 4. Linear regression analysis demonstrating the relationships between age and (A) ICA percent change in mean blood flow (B) ICA percent change in shear rate (N=58, females=31). The solid line represents the regression fit for males ( ) and the dotted line represents the regression fit for female participants ( ). Hierarchical regression models (P value and R^2) are presented for the relationship with age (model 1), the relationship with sex (model 2) and the moderator relationship of the sex dependent relationship with age (model 3). Slope coefficients (β) of the relationship with age are presented for males and females separately (model 3).

Figure 5. Linear regression analysis demonstrating the relationships between age and (A) MCAv time constant (τ) (B) $P_{ET}CO_2$ time constant (N=71, females=38). The solid line represents the regression fit for males ( ) and the dotted line represents the regression fit for female participants ( ). Hierarchical regression models (P value and R^2) are presented for the relationship with age (model 1), the relationship with sex (model 2) and the moderator relationship of the sex dependent relationship with age (model 3). Slope coefficients (β) of the relationship with age are presented for males and females separately (model 3).

Figure 6. Linear regression analysis demonstrating the relationships between age and (A) $P_{ET}CO_2$ amplitude (B) MCAv amplitude (N=71, females=38). The solid line represents the regression fit for males ( ) and the dotted line represents the regression fit for female participants ( ). Hierarchical regression models (P value and R^2) are presented for the relationship with age (model 1), the relationship with sex (model 2) and the moderator relationship of the sex dependent relationship with age (model 3). Slope coefficients (β) of the relationship with age are presented for males and females separately (model 3).

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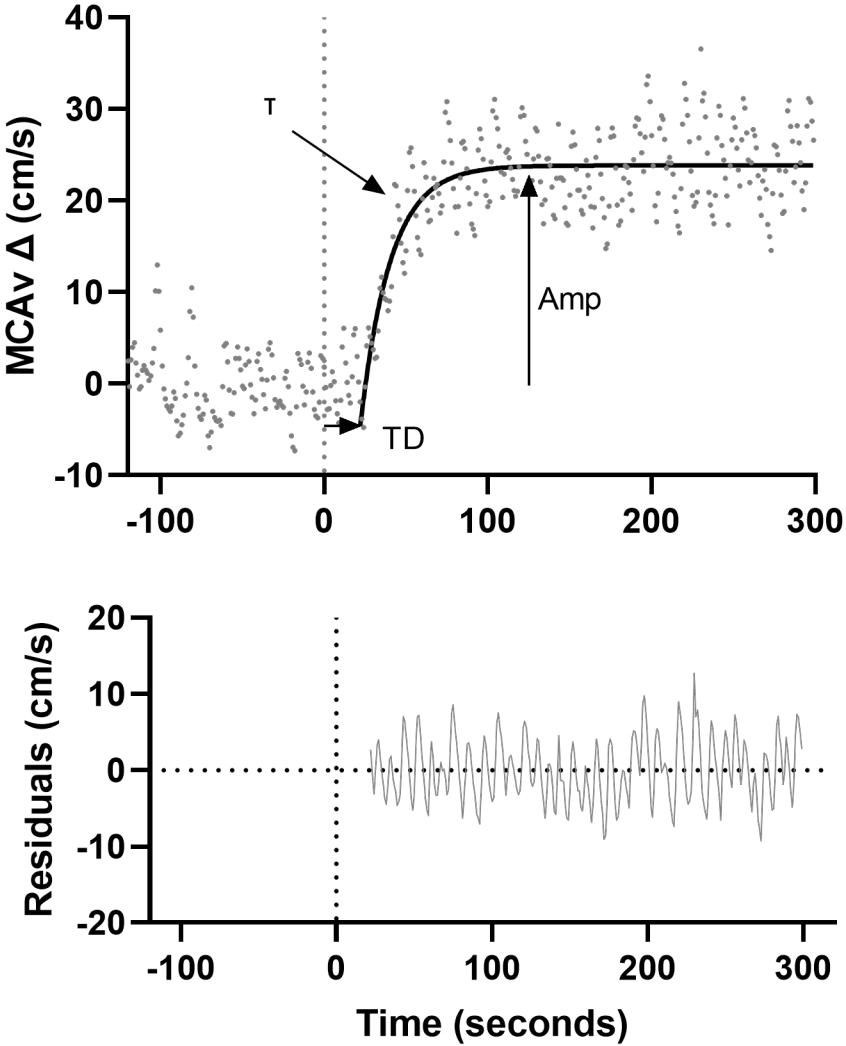

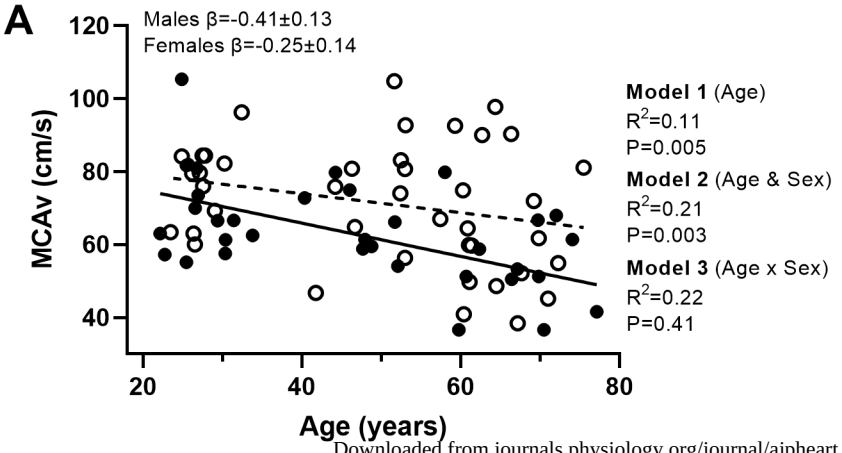

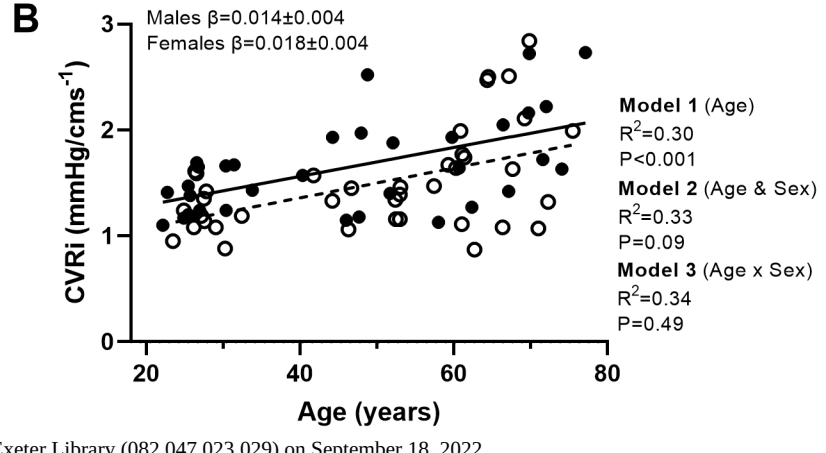

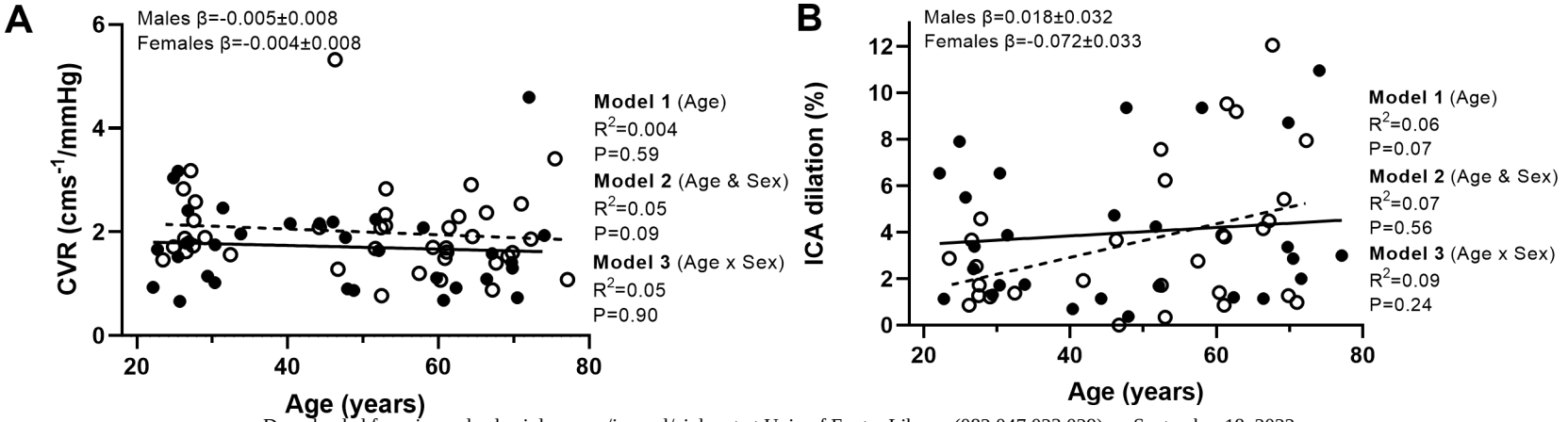

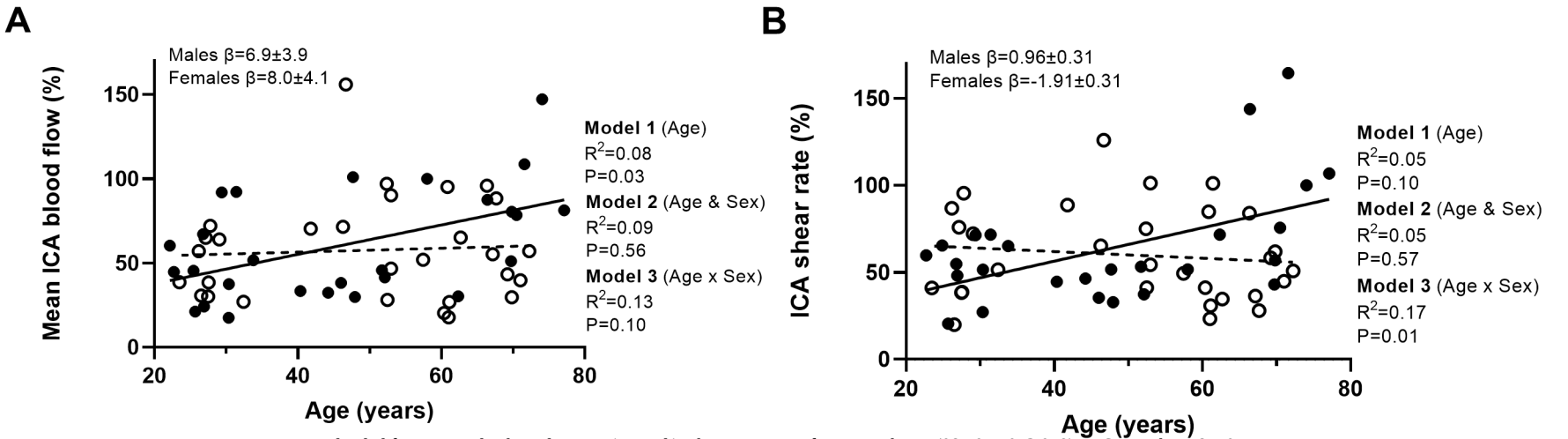

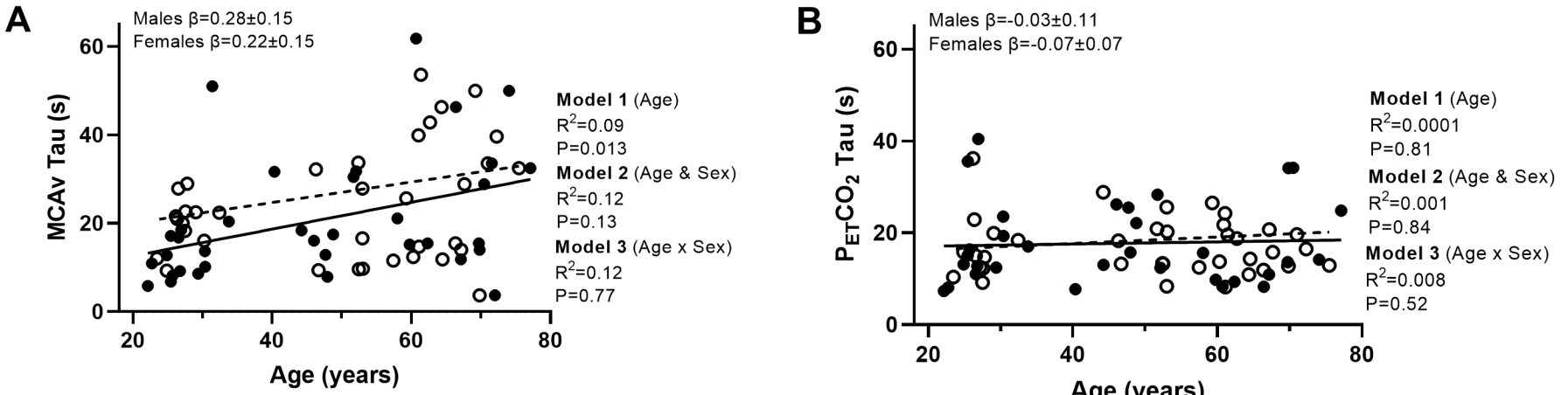

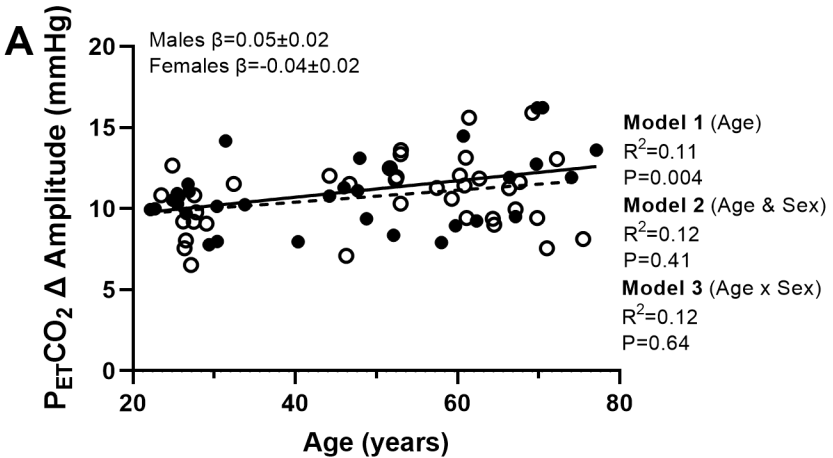

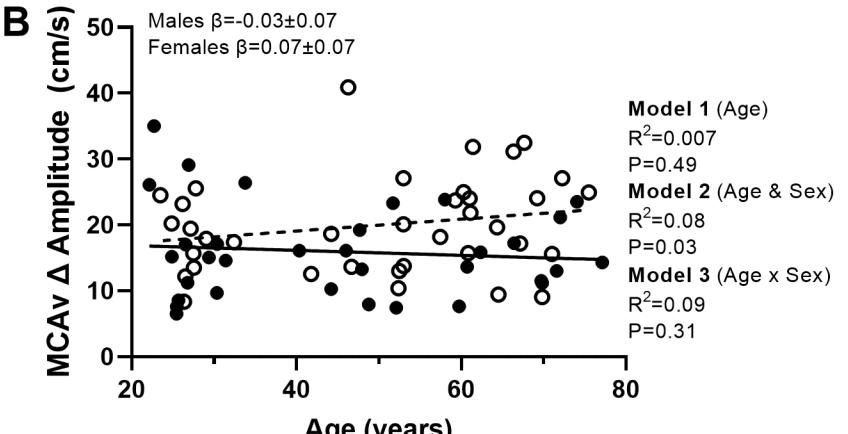

Table 1. Participant characteristics and baseline cerebrovascular parameters

Characteristics	Total		Young		Middle		Older	
	Males (N=34)	Females (N=39)	Males (N=13)	Females (N=12)	Males (N=13)	Females (N=17)	Males (N=8)	Females (N=10)
Age	46.9 ±18.5	50.6 ±16.5	26.8±2.9[a,b]	27.2±2.3[a,b]	50.3±8.4[a,c]	54.7±6.5[a,c]	70.9 ±3.3[b,c]	70.0±3.5[b,c]
Stature (cm)	175.7±9.4*	171.5±7.9	179.4±6.7	172.0±7.9	170.2±9.9	174.4±7.5	178.4±8.8	165.7±5.5
Weight (kg)	77.0±13.7	73.7±13.5	77.7±13.0	79.0±17.7	70.3±11.1	72.1±11.5	85.8±14.0	70.2±10.0
BMI ($kg \cdot m^2$)	21.8±3.2	21.4 ±3.3	21.6±3.4	22.9±4.3	20.6±2.5	20.6±2.7	24.0±3.1	21.2±2.9
Normal weight (%) Ω	75%	74%	62%	67%	85%	76%	75%	80%
PA ($METmins \cdot week^{-1}$)	2358±1590	2029±1071	2172±829	2309±1159	1867±1076	1944±992	3373±2579	1859±1151
MAP (mmHg)	96±19	103±17	87±26[a,b]	93±11[a,b]	97±10[a,c]	103±15[a,c]	108±7[b,c]	111±13[b,c]
Intracranial cerebrovascular parameters (N=73, Females=39)								
MCAv (cm/s)	63.7±14.0*	72.7±15.9	70.8±14.0[b]	76.9±10.9[b]	62.8±12.2[c]	74.8±15.0[c]	53.7±11.3[b,c]	64.2±20.2[b,c]
CVCi ($cm.s^{-1}$/mmHg)	0.65±0.19*	0.74±0.21	0.77±0.18[a,b]	0.88±0.16[a,b]	0.64±0.15[a,c]	0.73±0.16[a,c]	0.48±0.14[b,c]	0.58±0.23[b,c]
CVRi (mmHg/ $cm.s^{-1}$)	1.6±0.5	1.5±0.5	1.3±0.4[a,b]	1.2±0.2[a,b]	1.6±0.4[a,c]	1.4±0.3[a,c]	2.1±0.5[b,c]	2.0±0.6[b,c]
PI (a.u.)	0.76±0.16†	0.74±0.12	0.82±0.22	0.67±0.10	0.69±0.10[c]	0.72±0.10[c]	0.78±0.08[c]	0.85±0.11[c]
$P_{ET}CO_2$ (mmHg)	41.7±6.2	41.4±8.8	44.6±5.4	42.4±4.6	42.9±4.4	41.2±4.6	36.7±6.0	40.5±4.9
V_E (L/min)	11.7±4.2	10.8±4.2	11.9±4.1	11.4±4.3	11.6±5.2	11.4±4.8	11.3±2.7	9.2±2.8
Extracranial Internal Carotid Artery parameters (N=58, females=31)								
ICA diameter (cm)	0.61±0.11*	0.52±0.08	0.60±0.10	0.49±0.05	0.56±0.03	0.54±0.08	0.68±0.16	0.53±0.10
Peak systolic Velocity (cm/s)	30.9±7.7	36.2±12.5	29.8±7.5	34.4±9.9	31.4±8.1	35.2±10.6	31.9±8.4	40.2±18.5
Mean blood flow (ml/min)	470.8±235.8	417.1±270.0	476.4±267.2	266.4±98.5	395.3±221.0	535.7±388.9	566.3±200.8	412.3±199.5
Shear rate (s^{-1})	213.1±69.2*	268.2±86.5	207.7±57.4	287.7±104.0	222.4±50.9	256.4±80.6	206.9±106.9	263.8±78.0

Data are presented as mean ± SD. Data were compared using a two-way ANOVA with main effects of age (males, females) and sex (young, middle, older). *Asterix symbol denotes significant main effects of sex. When main effect of age is present post-hoc pairwise comparisons reveal where significant differences lie: a=young vs middle, b=young vs older, c=middle vs older. † Symbol denotes significant age by sex interaction effect. BMI, body mass index; PA, physical activity; MAP, mean arterial pressure; MCAv, middle cerebral artery velocity; CVCi, cerebrovascular conductance index; CVRi; cerebrovascular resistance index; PI, pulsatility index; MAP, mean arterial pressure; $P_{ET}CO_2$; end-tidal carbon dioxide; V_E; minute ventilation. Ω According to BMI classifications (Weir & Jan, 2022), proportion of participants classified as normal weight are presented as a percentage.

Table 2. Baseline cardiovascular and cerebrovascular variables

	Model 1: Age			Model 2: Age and Sex					Model 3: Interaction (Age x Sex)			
				Age		Sex						
	p-value	R^2	β	p-value	β	p-value	β	Combined R^2	p-value	R^2	β Males	β Females
Intracranial cerebrovascular parameters (N=73, females=39)												
MCAv (cm/s)	**0.005**	0.11	-0.29 ± 0.10	**0.001**	-0.33 ± 0.09	**0.003**	10.2 ± 3.3	0.21	0.41	0.22	-0.41 ± 0.13	-0.25 ± 0.14
CVCi ($cm.s^{-1}$/mmHg)	**<0.001**	0.33	-0.006 ± 0.001	**<0.001**	-0.007 ± 0.001	**0.006**	0.11 ± 0.04	0.37	0.80	0.37	-0.007 ± 0.002	-0.006 ± 0.002
CVRi (mmHg/ $cm.s^{-1}$)	**<0.001**	0.30	0.016 ± 0.003	**<0.001**	0.016 ± 0.003	0.09	0.17 ± 0.10	0.33	0.49	0.34	0.014 ± 0.004	0.018 ± 0.004
PI (a.u.)	0.29	0.016	0.001 ± 0.001	0.25	0.001 ± 0.001	0.42	-0.027 ± 0.033	0.025	**0.002**	0.14	-0.001 ± 0.001	0.004 ± 0.001
Cardiovascular parameters (N=73, females=39)												
MAP (mmHg)	**<0.001**	0.25	0.49 ± 0.10	**<0.001**	0.47 ± 0.10	0.15	5.20 ± 3.50	0.27	0.92	0.27	0.48 ± 0.14	0.46 ± 0.15
$P_{ET}CO_2$ (mmHg)	0.06	0.048	-0.09 ± 0.05	0.07	-0.10 ± 0.05	0.93	-0.15 ± 1.7	0.048	0.16	0.075	-0.16 ± 0.07	-0.02 ± 0.07
V_E (L/min)	0.28	0.02	-0.031 ± 0.03	0.31	-0.029 ± 0.03	0.57	-0.57 ± 0.9	0.02	0.45	0.03	-0.009 ± 0.04	-0.05 ± 0.04
Extracranial Internal Carotid Artery parameters (N=58, females=31)												
ICA diameter (cm)	0.09	0.05	0.001 ± 0.001	0.08	0.002 ± 0.001	**0.001**	-0.09 ± 0.02	0.23	0.73	0.23	0.002 ± 0.001	0.001 ± 0.001
Peak systolic velocity (cm/s)	0.31	0.02	0.08 ± 0.08	0.44	0.06 ± 0.06	0.07	5.4 ± 2.8	0.08	0.70	0.08	0.094 ± 0.12	0.03 ± 0.12
Mean blood flow (ml/min)	0.14	0.04	2.9 ± 1.9	0.12	3.0 ± 1.9	0.38	-60.5 ± 67.9	0.06	0.24	0.08	0.91 ± 2.7	5.5 ± 2.8
Shear rate (s^{-1})	0.48	0.009	-0.47 ± 0.65	0.34	-0.60 ± 0.62	**0.007**	59.2 ± 21.3	0.14	0.49	0.14	-0.17 ± 0.8	-1.0 ±0.9

Data presented as mean ± SD. Bold indicates significant relationship ($P<0.05$). Model 1 presents the relationship between age and the indicated variable. Model 2 presents the addition of sex to the model and the relationship between age and sex with the indicated variable. Model 3 indicates if sex moderates the relationship between age and the indicated variable, with individual unstandardised beta-coefficients shown for males and females. In models 1,2 and 3 the R^2 value reflects the full model. In model 1 and 3 β represents the unstandardised beta coefficient representing the change in variable units for every year increase in age. In model 2 the β coefficient provides the difference in females vs males in the variable units. MCAv, middle cerebral artery velocity; CVCi, cerebrovascular conductance index; CVRi; cerebrovascular resistance index; PI, pulsatility index; MAP, mean arterial pressure; $P_{ET}CO_2$; end-tidal carbon dioxide; V_E; minute ventilation; ICA, internal carotid artery.

Table 3. Peak cardiovascular and cerebrovascular responses to hypercapnia

	Model 1: Age			Model 2: Age and Sex					Model 3: Interaction (Age x Sex)			
				Age		Sex						
	p-value	R^2	β	p-value	β	p-value	β	Combined R^2	p-value	R^2	β Males	β Females
Intracranial cerebrovascular parameters												
MCAv (cm/s)	**0.03**	0.07	-0.30 ± 0.13	**0.007**	-0.35 ± 0.13	**0.002**	14.0±4.4	0.18	0.28	0.20	-0.49 ± 0.18	-0.21 ± 0.18
CVR (cm^{s-1}/mmHg)	0.59	0.004	-0.003 ± 0.006	0.45	-0.004 ± 0.006	0.09	0.34 ± 0.20	0.05	0.90	0.05	-0.005 ±0.008	-0.004 ± 0.008
PI (a.u.)	0.45	0.008	0.003 ± 0.004	0.47	0.003 ± 0.004	0.74	0.046 ± 0.14	0.01	0.72	0.01	0.001 ± 0.005	0.004 ± 0.006
CVCi (cms^{-1}/mmHg)	**<0.001**	0.26	-0.007 ± 0.001	**<0.001**	-0.007 ± 0.001	**0.01**	0.11 ± 0.05	0.32	0.83	0.32	-0.007 ± 0.002	-0.007 ± 0.002
CVRi (mmHg/ cms^{-1})	**<0.001**	0.24	0.012 ± 0.003	**<0.001**	0.013 ± 0.003	0.07	0.16 ± 0.09	0.27	0.43	0.28	0.015 ± 0.004	0.011 ± 0.004
Cardiovascular parameters												
MAP (mmHg)	**<0.001**	0.31	0.48 ± 0.10	**<0.001**	0.47 ± 0.12	0.34	3.24 ± 3.32	0.32	0.30	0.33	0.36 ± 0.14	0.57 ± 0.13
$P_{ET}CO_2$ (mmHg)	0.32	0.01	-0.14 ± 0.13	0.28	-0.15 ± 0.14	0.43	-3.9 ± 4.8	0.02	0.77	0.02	-0.12 ± 0.20	-0.19 ± 0.19
V_E (L/min)	0.74	0.002	-0.017 ± 0.05	0.93	-0.005 ± 0.05	**0.04**	-3.8 ± 1.8	0.06	0.99	0.06	-0.005 ± 0.05	-0.004 ± 0.08
Extracranial Internal Carotid Artery parameters												
ICA diameter dilation (%)	0.07	0.06	0.043 ± 0.02	0.06	0.044 ± 0.02	0.56	-0.47 ± 0.79	0.07	0.24	0.09	0.018 ± 0.032	0.072 ± 0.033
Peak Velocity (cm/s)	0.09	0.05	0.21 ± 0.12	0.13	0.19 ± 0.12	0.14	6.43 ± 4.29	0.09	0.41	0.10	0.29 ± 0.17	0.09 ± 0.17
Peak Velocity (Δ%)	0.21	0.03	0.27 ± 0.21	0.18	0.27 ± 0.21	0.43	-6.0 ± 7.5	0.04	**0.02**	0.13	0.78 ± 0.29	-0.20 ± 0.29
Mean blood flow (ml/min)	**0.02**	0.11	7.1 ± 2.8	**0.01**	7.4 ± 2.8	0.20	-128.7 ± 98.0	0.13	0.86	0.13	6.9 ± 3.9	8.0 ± 4.1
Mean blood flow (Δ%)	**0.03**	0.08	0.50 ± 0.23	**0.03**	0.51 ± 0.23	0.56	-4.8 ± 8.2	0.09	0.10	0.13	0.87 ± 0.32	0.11 ± 0.34
Shear rate (s^{-1})	0.97	0.00	-0.036 ± 1.02	0.80	-0.24 ± 0.96	**0.008**	91.82 ± 33.17	0.13	0.11	0.17	1.30 ± 1.33	-1.82 ± 1.35
Shear rate (Δ%)	0.10	0.05	0.38 ± 0.23	0.10	0.39 ± 0.23	0.57	-4.5 ± 7.9	0.05	**0.01**	0.17	0.96 ± 0.31	-1.91 ± 0.31
SR_{AUC} (s^{-1})	0.17	0.03	-140.7 ± 101.5	0.14	-152.5 ± 101.7	0.25	4136.7 ± 3563.9	0.06	0.53	0.07	-90.37 ± 141.33	-220.80 ± 148.18
$SR_{AUC\ initial}$ (s^{-1})	0.83	0.001	-2.74 ± 12.93	0.68	-5.29 ± 12.63	0.05	894.89 ± 442.92	0.07	0.07	0.13	16.34 ± 17.09	-29.07 ± 17.92

Data presented as mean ± SD. Bold indicated significant relationship ($P<0.05$). Model 1 presents the relationship between age and the indicated variable. Model 2 presents the addition of sex to the model and the relationship between age and sex with the indicated variable. Model 3 indicates if sex moderates the relationship between age and the indicated variable, with individual beta-coefficients shown for males and females. In models 1,2 and 3 the R^2 value reflects the full model. In model 1 and 3 β represents the unstandardised beta coefficient representing the change in variable units for every year increase in age. In model 2 the delta beta coefficient provides the difference in females vs males in the variable units. MCAv, middle cerebral artery velocity; CVR, cerebrovascular reactivity; CVCi, cerebrovascular conductance index; CVRi;

cerebrovascular resistance index; PI, pulsatility index; MAP, mean arterial pressure; $P_{ET}CO_2$; end-tidal carbon dioxide; V_E; minute ventilation; ICA, internal carotid artery; SR_{AUC}, shear rate area under the curve.

Table 4. Intracranial responses to hypercapnia

	Model 1: Age			Model 2: Age and Sex					Model 3: Interaction (Age x Sex)			
				Age		Sex						
Kinetic parameters (N=71, females=38)	**p-value**	**R^2**	**β**	**p-value**	**β**	**p-value**	**β**	**Combined R^2**	**p-value**	**R^2**	**β Males**	**β Females**
MCAv τ (s)	**0.013**	0.09	0.28 ± 0.11	**0.023**	0.25 ± 0.11	0.13	5.79 ± 3.77	0.12	0.77	0.12	0.28 ± 0.15	0.22 ± 0.15
MCAv Amp Δ (cm/s)	0.49	0.007	0.035 ± 0.05	0.72	0.018 ± 0.05	**0.03**	3.87 ± 1.74	0.08	0.31	0.09	-0.033 ± 0.071	0.07 ± 0.07
MCAv TD (s)	0.05	0.05	0.095 ± 0.048	0.05	0.10 ± 0.049	0.52	-1.12 ± 1.71	0.06	0.13	0.09	0.18 ± 0.07	0.026 ± 0.07
MCAv MRT (s)	**0.002**	0.13	0.37 ± 0.12	**0.004**	0.35 ± 0.12	0.26	4.67 ± 4.10	0.15	0.37	0.16	0.46 ± 0.17	0.25 ± 0.17
$P_{ET}CO_2$ τ (s)	0.81	0.001	0.018 ± 0.08	0.79	0.021 ± 0.08	0.84	-0.54 ± 2.63	0.001	0.52	0.008	-0.03 ± 0.11	0.07 ± 0.11
$P_{ET}CO_2$ Amp Δ (mmHg)	**0.004**	0.11	0.042 ± 0.01	**0.003**	0.044 ± 0.01	0.41	-0.42 ± 0.50	0.12	0.64	0.12	0.05 ± 0.02	0.04 ± 0.02
$P_{ET}CO_2$ TD (s)	0.77	0.001	0.014 ± 0.05	0.81	0.012 ± 0.05	0.71	0.63 ± 1.69	0.003	0.13	0.04	0.086 ± 0.07	-0.062 ± 0.07
$P_{ET}CO_2$ MRT (s)	0.69	0.002	0.033 ± 0.08	0.70	0.032 ± 0.05	0.97	-0.09 ± 2.80	0.002	0.76	0.004	0.06 ± 0.12	0.007 ± 0.12

Data presented as mean ± SD. Bold indicates significant relationship ($P<0.05$). Model 1 presents the relationship between age and the indicated variable. Model 2 presents the addition of sex to the model and the relationship between age and sex with the indicated variable. Model 3 indicates if sex moderates the relationship between age and the indicated variable, with individual β coefficients shown for males and females. In models 1,2 and 3 the R^2 value reflects the full model. In model 2 the β coefficient provides the difference in females vs males in the variable units. MCAv, middle cerebral artery velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; τ, time constant; Amp, amplitude; TD, time delay; MRT, mean response time.

Table 5. Relationships between menopausal status and cerebrovascular variables

	Model		Coefficients			
	p-value	R^2	Early post-menopausal (N=7)		Late post-menopausal (N=15)	
			β	p	β	p
Baseline intracranial parameters						
MCAv (cm/s)	**0.03**	0.18	2.0 ± 7.0	0.78	-13.0 ± 5.2	**0.02**
MAP (mmHg)	**0.01**	0.22	5.2 ± 6.4	0.42	15.1 ± 4.7	**0.003**
CVCi (cms-1/mmHg)	**0.002**	0.30	-0.05 ± 0.09	0.57	-0.24 ± 0.06	**<0.001**
CVRi (mmHg/ cms-1)	**<0.001**	0.35	0.07 ± 0.19	0.73	0.59 ± 0.14	**<0.001**
Baseline cardiovascular parameters						
$P_{ET}CO_2$ (mmHg)	0.72	0.018	-3.2 ± 4.2	0.45	-1.6 ± 3.1	0.60
PI (a.u.)	**0.001**	0.31	-0.004 ± 0.05	0.94	0.13 ± 0.04	**0.001**
Baseline extracranial Internal Carotid Artery parameters						
Diameter (cm)	0.08	0.16	0.08 ± 0.05	0.09	0.05 ± 0.03	0.07
Shear rate (s-1)	0.49	0.05	-29.4 ± 54.9	0.60	-39.6 ± 33.3	0.24
Mean blood flow (ml/min)	0.21	0.11	289.6 ± 166.3	0.09	94.8 ± 103.5	0.37
Peak velocity (cm/s)	0.83	0.013	4.2 ± 7.2	0.57	1.8 ± 4.8	0.70
Peak intracranial parameters						
MCAv (cm/s)	0.11	0.12	-1.1±9.6	0.89	-15.0 ± 7.1	**0.04**
MAP (mmHg)	**0.009**	0.23	5.4±6.7	0.43	16.4 ± 5.0	**0.002**
CVCi (cm.s-1/mmHg)	**0.003**	0.28	-0.07±0.10	0.48	-0.27 ± 0.07	**0.001**
CVRi (mmHg/ cm.s-1)	**0.005**	0.21	0.06±0.17	0.73	0.43 ± 0.13	**0.002**
Peak extracranial Internal Carotid Artery parameters						
ICA Dilation (%)	0.17	0.12	1.2±1.8	0.51	2.2 ± 1.1	0.06
ICA Mean blood flow (ml/min)	0.16	0.13	447.8±227.1	0.06	111.1 ± 141.4	0.79
ICA Mean blood flow (%)	0.71	0.03	12.5±19.5	0.53	-4.3 ± 12.1	0.73
ICA Shear rate (s^{-1})	0.31	0.02	-22.2±83.9	0.79	-79.6 ± 50.9	0.13
Peak systolic velocity (cm/s)	0.59	0.04	9.5±10.4	0.37	-1.4 ± 7.0	0.84
Peak systolic velocity (%)	0.22	0.04	9.6±15.2	0.53	-14.4 ± 10.2	0.17
SR (%)	0.41	0.06	10.7±17.8	0.55	-11.0 ± 10.8	0.32
SR_{AUC} (AU)	0.18	0.12	-11279.7 ± 11174.3	0.32	-10353.1 ± 5689.7	0.08
Kinetic parameters						
MCAv τ (s)	0.78	0.014	0.74 ± 9.5	0.94	4.6 ± 6.6	0.12
MCA Amp Δ (cm/s)	0.25	0.08	-3.7 ± 3.6	0.32	2.4 ± 2.5	0.34
$P_{ET}CO_2$ τ (s)	0.99	0.001	0.8 ± 5.1	0.87	0.2 ± 3.8	0.96
$P_{ET}CO_2$ Amp Δ (mmHg)	0.25	0.08	-3.7 ± 3.6	0.32	2.4 ± 2.5	0.34

Data presented as mean ± standard deviations. Bold indicated significant relationship ($P<0.05$). β-coefficients for early post-menopausal and late post-menopausal females are presented compared to reference group of premenopausal females. MCAv, middle cerebral artery velocity; CVR, cerebrovascular reactivity; CVRi; cerebrovascular resistance index; PI, pulsatility index; MAP, mean arterial pressure; $P_{ET}CO_2$; end-tidal carbon dioxide; V_E; minute ventilation; SR_{AUC}, shear rate area under the curve; τ, time constant; Amp, amplitude; TD, time delay.