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The Influence of Sex Hormones on Cerebrovascular Function

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ABSTRACT

The disproportionate rise in cerebrovascular disease risk for females in the first 10 years post-menopause indicates a role for ovarian sex hormones (i.e., oestrogen and progesterone) in healthy cerebrovascular function and regulation. However, the exact influence of changing sex hormones across the lifespan on the cerebrovasculature is yet to be elucidated. The primary aim of this thesis was to determine the influence of sex hormones on cerebrovascular function.

A systematic review and meta-analysis established that hormone replacement therapy in post-menopausal females has the potential to improve cerebrovascular function, as reflected by a significant reduction in pulsatility index. Further, the effects of changing sex hormones in other hormone groups (e.g., menstrual cycle, menopause) remains unclear due to the substantial heterogeneity and limited evidence in the literature. Subsequently, three experimental studies examined measures of cerebrovascular function across the menstrual cycle, between males and females, and between pre- and post-menopausal females. Firstly, cerebrovascular responsiveness (indexed via measures of middle/posterior cerebral blood flow velocity (MCA*v*/PCA*v*) responses to altered arterial partial pressure of carbon dioxide (CO_2)) across the menstrual cycle revealed a greater MCA*v*-CO_2 responsiveness to hypocapnia during ovulation (O) when compared to the early follicular (EF) phase. Assessment of sex differences showed cerebrovascular-CO_2 responsiveness to hypo- and hypo-to-hypercapnia was greater in females during EF in the PCA, and females during O in the MCA, when compared to males. Use of passive heat stress as an additional perturbation did not change cerebrovascular-CO_2 responsiveness across the menstrual cycle, while a

diminished PCA responsiveness to hypercapnia during heat stress was only evident in males. Finally, pre-menopausal females were shown to have an improved MCA*v*-CO_2 responsiveness to hypercapnia compared to post-menopausal females, irrespective of menstrual phase. Preliminary data indicates possible differences between pre- and post-menopausal females in internal carotid artery (ICA) responsiveness to hypercapnia, and in cerebral autoregulation (indexed via MCA*v* responses to repeated squat-to-stand-induced changes in blood pressure).

Collectively, these findings indicate that 1) acute fluctuations in oestrogen across the menstrual cycle can alter the vasoconstrictive capacity of the MCA, while progesterone appears to counteract the effects of oestrogen. 2) Sex differences in cerebrovascular-CO_2 responsiveness are dependent on menstrual phase and the insonated vessel, and differences are present even when females are early follicular phase of the menstrual cycle. Further, cerebrovascular responses to passive heating are sex specific. Finally, 3) post-menopausal females have a blunted MCA*v* responsiveness to hypercapnia compared to pre-menopausal females. Overall, the findings that pre-menopausal females exhibit improved intracranial cerebrovascular-CO_2 responsiveness compared to both young males and post-menopausal females supports the premise that sex hormones play a protective role in cerebrovascular function.

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LIST OF ABBREVIATIONS

ABP	Arterial blood pressure
ACA	Anterior cerebral artery
ARI	Autoregulation index
BHI	Breath-hold index
BMI	Body mass index
BP	Blood pressure
CA	Cerebral autoregulation
CBF	Cerebral blood flow
CCA	Common carotid artery
CO_2	Carbon dioxide
CV	Coefficient of variation
CVC	Cerebrovascular conductance
CVR	Cerebrovascular responsiveness
ECA	External carotid artery
ECG	Electrocardiogram
EF	Early follicular
HRT	Hormone replacement therapy
ICA	Internal carotid artery
IVF	In vitro fertilisation
MAP	Mean arterial pressure
MCA	Middle cerebral artery

ML	Mid-luteal
MRI	Magnetic resonance imaging
O	Ovulatory
O_2	Oxygen
OC	Oral contraceptives
OHS	Ovarian hyperstimulation
PCA	Posterior cerebral artery
PET	Positron emission tomography
$P_{ET}CO_2$	End-tidal carbon dioxide
PI	Pulsatility index
PM	Post menopause
RI	Resistance Index
TCD	Transcranial Doppler
T_C	Body core temperature
TFA	Transfer function analysis
T_S	Skin temperature
VA	Vertebral artery

GLOSSARY

Terms and their definitions within the context of this thesis, unless otherwise stated in-text.

Cerebral autoregulation: The ability to maintain cerebral blood flow despite changes in blood pressure.

Cerebrovascular-CO_2 responsiveness: The cerebral blood flow response to a changing CO_2 stimulus.

Hormone replacement therapy: The treatment of menopausal symptoms with oestrogen and/or progesterone in post-menopausal females.

Sex: determined by the biological characteristics of a person; male or female.

Sex hormones: hormones produced primarily by the ovaries in females (i.e., oestrogen, progesterone).

1 GENERAL INTRODUCTION

1.1 Incidence and prevalence of cerebrovascular disease

Cerebrovascular disease arises when an area of the brain is temporarily or permanently affected by ischaemia or bleeding within the cerebrovasculature. Global mortality caused by cerebrovascular diseases (e.g. stroke, vascular dementia) increased between 2000 and 2019 (WHO, 2020). Stroke remains the second leading cause of global mortality, while dementia accounted for twice as many deaths in 2019 than in 2000. Indeed, in the UK dementia was the leading cause of death in 2019 (WHO, 2020). While dementia encompasses a range of morbidities, vascular dementia resulting from cerebrovascular pathology is the second most common cause behind Alzheimer's disease (Gannon et al., 2019), with over 50% of those with Alzheimer's disease believed to have co-morbid cerebrovascular pathology (Brenowitz et al., 2017). Cerebrovascular diseases are considered a significant public health burden due to the associated serious disabilities, functional limitations and compromised quality of life (Ramos-Lima et al., 2018). The aggregated societal costs associated with stroke and dementia in the UK were approximately £46 billion in 2015, with this expected to rise by up to 195% by 2035 (King et al., 2020; Wittenberg et al., 2020). Thus, the increasing incidence and prevalence of cerebrovascular diseases presents a substantial health problem both globally and within the UK. Understanding cerebrovascular function is vital to reducing the incidence of cerebrovascular disease, as well as improving the recovery and quality of life after diagnosis.

The risk of stroke and dementia are higher in ageing females than males. While pre-menopausal females are less likely to experience stroke compared to age-matched males, stroke risk increases disproportionately for females in the first 10 years after

menopause (Haast et al., 2012; Lisabeth & Bushnell, 2012). This rise in stroke and stroke-related risk factors in post-menopause, combined with the overall longer life expectancy of females, means females account for 60% of total stroke cases (Mozaffarian et al., 2016), suffer more severe strokes and have worse functional outcomes (Petrea et al., 2009). Subsequently, the abrupt and chronic decline in ovarian sex hormones (i.e., oestrogen, progesterone) during the menopausal transition has been identified as a potential cause for the disproportionate increase observed in stroke incidences in post-menopausal females. Further, prevalence of vascular dementia is shown to be greater in males compared to females, with this trend reversing with advancing age (Lobo et al., 2000). Although this may be reflective of differences in survival between males and females at older ages, evidence indicates a role for sex hormones in dementia-related risk factors (Szoeke et al., 2021). An understanding of the role of sex hormones in healthy cerebrovascular function and regulation may help identify the underlying cause of observed sex differences in cerebrovascular injury. This in turn may allow for more focused interventions in the prevention of, and recovery from, cerebrovascular injury such as stroke.

1.2 Cerebral blood flow regulation

The brain accounts for ~20% of the body's oxygen consumption despite being only 2-3% of total body weight (Willie et al., 2011). The brain's high energetic cost combined with its limited storage capacity means regulation of cerebral blood flow is tightly regulated to provide a sufficient supply of oxygen. Thus, the cerebrovasculature can rapidly adapt to changes in humoral or metabolic conditions, particularly changes in arterial blood gases, perfusion pressure, and regional metabolic demands (Willie et al.,

2011). Measurement of both resting cerebral blood flow and its regulatory components allow for assessment of cerebrovascular function and regulation. Resting cerebral blood flow has proven to be a valuable prognosis tool in stroke patients (Fiehler et al., 2002), and a useful pre-clinical marker of Alzheimer's disease (Wierenga et al., 2014). Pulsatility index (PI) is a frequently reported measure of vessel resistance derived from cerebral blood flow, with a greater PI indicative of greater downstream flow resistance and arterial stiffness. Increased PI is also associated with greater cognitive impairment (Harris et al., 2018) as well as the presence and severity of cerebral small-vessel disease (Ghorbani et al., 2015). Thus, while resting cerebral blood flow can provide valuable insight into cerebrovascular function and/or dysfunction, assessment of the cerebrovasculatures' regulatory components can give further information on underlying physiological mechanisms and subsequently, be a more dynamic and sensitive assessment tool.

Firstly, the cerebrovasculature is highly sensitive to changes in arterial carbon dioxide partial pressure (P_aCO_2), with increased P_aCO_2 resulting in vasodilation of downstream arterioles and increased cerebral blood flow, while decreased P_aCO_2 causes vasoconstriction and decreased cerebral blood flow (Kety & Schmidt, 1948). While the cerebrovasculature is also sensitive to changes in arterial oxygen partial pressure (P_aO_2), this only occurs during severe hypoxia (i.e. below a P_aO_2 of ~50 mmHg) and the cerebrovascular response is still dependent on the prevailing P_aCO_2 (Willie et al., 2014). Cerebrovascular-CO_2 responsiveness measures the cerebral blood flow response to a changing CO_2 stimulus, providing a functional measure of cerebrovascular regulation that is commonly applied in healthy and clinical populations

(note that this outcome measure is also referred to as cerebrovascular reactivity (CVR), with the terms used interchangeably in the literature). Indeed, impairment of cerebrovascular-CO_2 responsiveness has been shown to predict the risk of stroke in patients with carotid artery occlusion (Gupta et al., 2012; Markus & Cullinane, 2001; Webster et al., 1995), as well as an indication of the severity of vascular dementia (Keage et al., 2012). As such, a higher cerebrovascular-CO_2 responsiveness is interpreted as an improved cerebrovascular function and is utilised frequently throughout the literature (Ainslie & Duffin, 2009; Willie et al., 2011).

The ability to maintain cerebral blood flow despite changes in blood pressure is known as cerebral autoregulation (Lassen, 1959). Traditionally, “static” cerebral autoregulation (i.e., measurement of blood flow over gradual changes in blood pressure conditions) was believed to maintain a constant cerebral blood flow through a mean arterial pressure range of ~60-150 mmHg (Lassen, 1959). However, more recent data indicates that cerebral blood flow has a more linear relationship with changing blood pressure (Lucas et al., 2010; Numan et al., 2014). Dynamic cerebral autoregulation assesses the ability of the cerebral vasculature to respond to rapid changes in perfusion pressure, either from spontaneous fluctuations or stimulus-induced changes in blood pressure. Blunted cerebral autoregulation renders the brain more vulnerable to ischaemia or haemorrhage at low and high pressures, respectively, with an improved dynamic cerebral autoregulation associated with better functional outcomes post-stroke (Castro et al., 2017). Although less clear, the presence of Alzheimer’s disease appears to be associated with impaired cerebral autoregulation (Brickman et al., 2015; van Beek et al., 2012). In addition to cerebral autoregulation,

the close spatial and temporal relationship between neural activity and cerebral blood flow, termed neurovascular coupling, indicates that cerebral blood flow is altered to meet regional metabolic demands (Willie et al., 2014). The interaction between neural activity and the cerebrovasculature is disrupted in several brain pathologies, including ischaemic stroke and dementia (Girouard & Iadecola, 2006), and preserved neurovascular coupling is associated with improved functional outcomes post-stroke (Salinet et al., 2019).

1.3 Sex hormones and cerebrovascular function

There is substantial evidence from cellular and animal models that gonadal sex hormones directly affect brain vascular function. Reviews of this literature by Orshal and Khalil, (2004) and Krause and collegues (2006) have previously examined the effect of oestrogen, progesterone and testosterone on vascular tone and the cerebrovasculature, respectively. Oestrogen is reported to have a number of neuroprotective effects, including suppression of the inflammatory response and influence perfusion after ischaemic injury (Hurn et al., 1995; Santizo et al., 2002). Additionally, exposure to oestrogen is associated with enhanced production or activity of vasodilatory factors, such as endothelial NO synthase and prostacyclin pathways (Geary et al., 2000; Pelligrino et al., 2000), and may also suppress the effect of vasoconstrictors (Chrissobolis et al., 2004). The effects of progesterone on the cerebrovasculature are much less extensively studied. Evidence indicates that progesterone has a neuroprotective role and supresses the inflammatory response following cerebral injury (Gibson et al., 2005), yet diminishes the vasoprotective benefits of oestrogen observed in response to inflammation (Sunday et al., 2006).

Testosterone has been reported to also have both protective and detrimental effects on the cerebrovasculature (Abi-Ghanem et al., 2020). Furthermore, testosterone is believed to act in an opposing manner to oestrogen by enhancing thromboxane-mediated vasoconstriction (Gonzales et al., 2005) and suppressing endothelium-dependent dilation (Gonzales et al., 2004). While the literature provides substantial evidence that the cerebrovasculature can be influenced by sex hormones, these studies were performed in animal models, *in vitro*, or using supraphysiological levels of a sex hormone to examine the cerebrovascular response. Whether sex hormones induce similar responses in humans with natural hormonal fluctuations is less certain.

There remains no clear consensus on the role of acute and chronic changes in sex hormones on cerebrovascular function in humans. To date, the majority of evidence has examined the effect of hormone replacement therapy (HRT) on the cerebrovasculature in post-menopausal females due to the interest in using HRT to relieve menopausal symptoms (e.g., hot flushes). Early clinical trials of HRT in post-menopausal females revealed an elevated risk of stroke (Hendrix et al., 2006; Wassertheil-smoller et al., 2003). As such, this population have been more extensively studied to better understand the potential role oestrogen and progesterone treatment may have in increasing stroke risk. However, there appears to be considerable heterogeneity in the reported outcome measures of cerebrovascular function, making comparisons of studies to elucidate the exact role of sex hormones on the cerebrovasculature challenging.

The influence of oestrogen and progesterone on cerebrovascular function in other hormone groups (e.g., pregnancy, the menstrual cycle) have been less thoroughly

examined, and with those studies often reporting contradictory outcomes. For example, increased circulating oestrogen levels has been associated with both increased cerebral blood flow (Krejza et al., 2001) and no influence on cerebral blood flow (Diomedi et al., 2001; Favre & Serrador, 2019) across the menstrual cycle. Similarly, contradictory outcomes have been reported for cerebrovascular-CO_2 responsiveness, with some studies reporting similar responsiveness in pre- and post-menopausal females (Brislane et al., 2020), while others report greater responsiveness in pre-menopausal when compared to post-menopausal females (Matteis et al., 1998). Cerebral autoregulation is reported to be both similar between pregnant and non-pregnant females (Sherman et al., 2002), and enhanced during pregnancy when circulating sex hormones are elevated, compared to non-pregnant females (van Veen et al., 2016). These contradictory findings are likely due to methodological inconsistencies in outcome measures, as well as differences in the vessels insonated and stimuli used. Indeed, there is no agreement within the literature as to what the optimum method to assess cerebrovascular function should be, making it difficult to compare and collate data across studies. As such, how the interaction, absence or increases in oestrogen and progesterone affects cerebrovascular function in females is yet to be elucidated. Improved knowledge in this area will help inform and further our understanding of the role sex hormones have in both cerebrovascular health and disease.

1.4 Passive heat stress

Passive heat stress is an experimental model that acutely stresses the cerebrovasculature. Passive heating causes a redistribution of cardiac output to the

cutaneous circulation for heat dissipation (Nelson et al., 2011) as well as hyperthermia-induced hyperventilation (Brothers et al., 2009) which causes reductions in blood pressure and arterial P_aCO_2. Since cerebral blood flow is highly sensitive to P_aCO_2 and fluctuations in arterial blood pressure, passive heat stress presents a unique challenge to the cerebrovasculature.

It is well established that passive heat stress reduces cerebral blood flow, with a 1 °C increase in core temperature reducing cerebral blood flow by 10-15% (Bain et al., 2015). Indeed, during supine passive heat stress of up to +2 °C in core temperature, data indicates that hyperventilation-induced hypocapnia is responsible for much of the observed reduction in cerebral blood flow (Bain et al., 2015; Brothers et al., 2009; Nelson et al., 2011). Whether heat stress alters cerebrovascular-CO_2 responsiveness is less clear. Previous studies have shown cerebrovascular-CO_2 responsiveness to increase (Lucas et al., 2008), decrease (Ogoh et al., 2014), or remain similar (Low et al., 2008) during passive heat stress when compared to normothermic conditions. Furthermore, the majority of studies investigating cerebrovascular function and passive heat stress have evaluated only males, making it difficult to determine the role, if any, of sex hormones on cerebrovascular function during passive heating (Barnes & Charkoudian, 2021). Since elevated oestrogen has been associated with increased cerebral blood flow (Krejza et al., 2004), and females have a lower end-tidal CO_2 compared to males (Minhas et al., 2018), the cerebrovascular response to heat stress, and the relationship between cerebral blood flow and P_aCO_2, may be differentially affected in high hormone compared to low hormone groups (e.g., pre- compared to post-menopausal females). As such, passive heat stress can be used as a

physiological perturbation to advance the understanding of the mechanisms underlying any observed differences in cerebral blood flow regulation with changing sex hormones.

1.5 Summary of individual chapters

The primary aim of this thesis is to assess the influence of sex hormones on cerebrovascular function and regulation. Therefore, a systematic review and meta-analysis (Chapter 2) will synthesise and identify knowledge gaps in the current literature regarding how sex hormone changes across the lifespan affect cerebrovascular function in females. Chapter 3 will present the methodology used in this thesis and assess its reliability and limitations. The experimental chapters aim to assess the influence of sex, the menstrual cycle and menopause on cerebrovascular function and regulation. Chapter 5 will examine the influence of menstrual cycle phase on cerebrovascular function during normothermia and passive heat stress, while Chapter 4 will assess cerebrovascular responsiveness during normothermic and passive heat stress conditions in females when compared to males. Chapter 6 will examine cerebrovascular responsiveness and dynamic cerebral autoregulation in post-menopausal females when compared to pre-menopausal females. The findings of this thesis will be summarised in Chapter 7 in addition to future recommendations.

2 A SYSTEMATIC REVIEW AND META-ANALYSIS EXAMINING WHETHER CHANGING OVARIAN SEX STEROID HORMONE LEVELS INFLUENCE CEREBROVASCULAR FUNCTION

This chapter was published in *Frontiers in Physiology* (Skinner, B.D., Davies, R.J., Weaver, S.R., Cable, N.T., Lucas, S.J.E. and Lucas, R.A.I. [2021]. A systematic review and meta-analysis examining whether changing ovarian sex steroid hormone levels influence cerebrovascular function. *Frontiers in physiology*, *12*) and has undergone minor modifications to adapt to the format of a thesis.

2.1 Abstract

Sex differences in cerebrovascular disease rates indicate a possible role for ovarian sex steroid hormones in cerebrovascular function. To synthesise and identify knowledge gaps a systematic review and meta-analysis was conducted to assess how ovarian sex steroid hormones changes across the lifespan affect cerebrovascular function in females. Three databases (EMBASE, MEDLINE and Web of Science) were systematically searched for studies on adult cerebrovascular function and ovarian sex steroid hormones. Forty-five studies met pre-defined inclusion criteria. Studied hormone groups included hormone replacement therapy (HRT; n = 17), pregnancy (n = 12), menstrual cycle (n = 7), menopause (n = 5), oral contraception (n = 2), and ovarian hyperstimulation (n = 2). Outcome measures included pulsatility index (PI), cerebral blood flow/velocity (CBF), resistance index (RI), cerebral autoregulation, and cerebrovascular reactivity. Meta-analyses were carried out on HRT studies. PI significantly decreased (-0.05, 95% CI: [-0.10, -0.01]; p=.01) in post-menopausal females undergoing HRT compared to post-menopausal females who were not, though there was considerable heterogeneity (I^2 = 96.8%). No effects of HRT were seen in CBF (p = .24) or RI (p = .77). This review indicates that HRT improves PI in post-menopausal females. However, there remains insufficient evidence to determine

how changing ovarian sex steroid hormone levels affects cerebrovascular function in females during other hormonal phases (e.g., pregnancy, menopause).

2.2 Introduction

Sex differences in the rate and occurrence of cerebrovascular diseases (i.e., stroke and vascular dementia) indicate a possible role for ovarian sex steroid hormones in brain vascular function and regulation. For example, despite females having a lower risk of stroke than males during midlife their risk doubles in the decade after menopause (Lisabeth & Bushnell, 2012), a time in which endogenous oestrogen and progesterone concentrations decline significantly.

Prolonged exposure to oestrogen has been shown to promote vasodilatory factors (Geary et al., 2000; Pelligrino et al., 2000; Skarsgard et al., 1997). Additionally, oestrogen has demonstrated a number of neuroprotective effects including suppression of the inflammatory response and influence perfusion after ischaemic injury (Hurn et al., 1995; Santizo et al., 2002). The effects of progesterone are far less clear, with the literature suggesting that it both promotes and reduces the inflammatory response to cerebrovascular injury (Gibson et al., 2005; Sunday et al., 2006).

There is substantial evidence for the direct effect of gonadal sex hormones on brain vascular function in cellular and animal experimental models. Indeed, the narrative reviews by Orshal and Khalil (2004) and Krause and collegues (2006) provide a detailed account of research investigating the effect of oestrogen, progesterone, and testosterone on vascular tone and the cerebrovasculature. To date, however, no review (systematic or otherwise) has examined the effects of changing ovarian sex

steroid hormones on cerebrovascular function in humans. Subsequently, it remains unclear how the interaction, absence or increase in oestrogen and progesterone affects cerebrovascular function in females

Therefore, to synthesise and identify knowledge gaps a systematic review and meta-analysis was conducted to assess how changes in ovarian sex steroid hormones across the lifespan affect cerebrovascular function in females. This will help to inform future research directions relating to sex hormones and cerebrovascular function, as well as sex differences in cerebrovascular diseases.

2.3 Methods

This review utilised a systematic search strategy to provide a succinct yet thorough overview of the available literature. The protocol used was informed by the Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA-P) guidelines (Shamseer et al., 2015).

2.3.1 Search Strategy

A formal literature search, using bibliographic search databases, was the primary method of identifying relevant texts. The electronic databases MEDLINE, Web of Science and EMBASE were searched for publications relating to ovarian sex steroid hormones and cerebrovascular function using MeSH terms and free-text terms to capture relevant research. Core keywords used in the search included for example, ‘cerebrovascular circulation’ OR ‘middle cerebral artery’ OR ‘brain blood flow’ AND ‘gonadal steroid hormones’ OR ‘menstrual cycle’. The complete search strategy can

be found in Appendix A. A manual search for work on ovarian sex steroid hormones and cerebrovascular function, including article reference lists, was conducted to ensure all relevant texts were identified. Database and manual searches included texts from the first available date to March 2021.

2.3.2 Inclusion and Exclusion Criteria

Inclusion and exclusion criteria were developed through researcher discussion and guided by the Population, Intervention, Comparison/Control Group, Outcome, and Time (PICOT) framework (Higgins & Green, 2011). The criteria covered population (e.g., postmenopausal females), intervention/domain studied (e.g., hormone replacement therapy), study type (e.g., randomised control trials), and relevance/outcome measures (e.g., brain blood flow). Research articles needed to address both cerebrovascular function and changing ovarian sex steroid hormones within a healthy adult population (>18 years) to be suitable for inclusion. Exclusion criteria included clinical populations (i.e., cerebrovascular injury, pre-eclampsia), synthetic hormone treatment, animal studies, as well as sources of information such as book chapters, conference abstracts or poster formats.

2.3.3 Study Selection

Two reviewers (BDS, RJD) independently searched the literature using three databases and the defined keywords. All citations identified in the search were independently screened by the reviewers on the basis of the title and the abstract to assess their match with inclusion criteria. To ensure the inclusion/exclusion criteria were applied consistently, 20% of identified citations were screened by both reviewers

and the results compared. Disagreements regarding eligibility of studies were resolved by discussion and consensus with a third reviewer (RAIL). The reviewers checked the references of included studies to identify any relevant papers not captured in the search. Included title and abstracts were then screened for their full texts.

2.3.4 Data Extraction and Quality Assessment

Full texts deemed eligible for inclusion underwent data extraction using a data extraction form. This included participant demographics (e.g., number, age, population), study characteristics (e.g., design, protocol), outcome measures (e.g., hormones reported, brain blood flow measures) and results. Where possible, results were expressed as absolute values (mean ± SD) in order to determine percentage change across time points/experimental groups. To minimise bias, and ensure the accuracy of the study selection procedure, a random sample of 20% of included studies were extracted by both reviewers and results compared. The two independent reviewers graded all identified studies as either a high (level 1; e.g., randomised control trial), moderate (level 2; e.g., cohort, case-control studies) or low (level 3; e.g., cross-sectional study) level of evidence. The criterion for each level of evidence can be found in Appendix B.

Reviewers applied a modified version of the National Heart, Lung, and Blood Institute quality assessment tool for observational cohort and cross-sectional studies (2014) to assess the internal validity and risk of bias for each study. They independently evaluated the 15 components of the tool as “Yes”, “No”, “Not Applicable” or “Not Recorded” to achieve a rating of “High”, “Moderate” or “Low” to assess the quality of

included studies. In case of disagreement the third reviewer was consulted and a consensus opinion reached.

2.3.5 Data Synthesis and Analysis

A ‘low hormone phase’ and ‘high hormone phase’ were identified (defined in Table 2.1) to allow for comparisons between studies within the same hormone group. The absolute and relative change in cerebrovascular function was calculated from a low to high hormone phase and summarised separately within each hormone group (e.g., menopause, pregnancy). Specifically, the markers of cerebrovascular function were pulsatility index (PI; marker of downstream flow resistance), cerebral blood flow/velocity (CBF; marker of cerebral perfusion), resistance index (RI; marker of downstream flow resistance), cerebrovascular reactivity to CO_2 (CVR; the vasodilatory reserve capacity to a CO_2 stimulus), and cerebral autoregulation (CA; the capacity to maintain cerebral blood flow despite changes in perfusion pressure). If measures were presented as mean difference with 95% confidence intervals, then standard deviation was calculated using the method described in the Cochrane Handbook (7.7.7.2; Higgins & Green, 2011). Due to the wide heterogeneity of included studies in terms of study design, population and outcome measures, only some hormone groups were deemed to have sufficient data to be included in the meta-analysis. This was determined by there being 4 or more studies reporting the outcome measure in a comparable manner, reporting data as mean and standard deviation, and the low and high hormone group being clearly defined. As such, only HRT studies reporting pulsatility index, cerebral blood flow/velocity, and resistance index were included in the meta-analysis to quantify the differences between high and low hormone groups.

Table 2.1. Definition of 'low hormone phase' and 'high hormone phase' for each hormone group.

Hormone Group	Low Hormone Phase	High Hormone Phase
Pregnancy	Non-pregnant	2^{nd}-3^{rd} Trimester
Menstrual Cycle	Follicular Phase	Ovulation or Luteal Phase
Menopause	Post-menopause	Pre-menopause
Hormone Replacement Therapy (HRT)	Pre-HRT or control group	HRT
Ovarian Hyperstimulation	Pituitary suppression	Human menopausal gonadotropin (hMG) stimulation
Oral Contraception (OC)	Non-OC users	OC Users

Hormone group differences were investigated using a random effect model for outcome measures of PI, CBF, and RI. Mean differences between a low and high hormone phase were calculated, and overall effect estimates were calculated using random effect models, and reported alongside estimate significance (p-value) and heterogeneity (I^2). The I^2 statistic was examined to evaluate heterogeneity, with I^2 >50% and I^2 >75% indicative of substantial and considerable heterogeneity, respectively (Higgins et al., 2003).

Moderator analysis was carried out for studies reporting PI in order to identify possible sources of heterogeneity, by comparison of overall and subgroup estimated effects based on the artery in which measures were taken and the type/types of medication

used. There were deemed to be an insufficient number of studies to perform sub-group analyses for CBF and RI. Meta-regression were conducted using mixed effects models with each of these factors included as a moderator, to determine their impact on both effect size and heterogeneity in each outcome measure. The impact of moderators on the effects of hormone level was evaluated by the proportion of heterogeneity accounted for, with the significance of this assessed by omnibus tests for the overall model effect and Wald-type Chi-Squared tests for each moderator within the model.

Potential outliers were identified and evaluated by externally standardised studentized deleted residuals, based on the size of each study's individual residual, with values <-2 or >2 considered outlying. The impact of each study on the overall effect was then assessed using model fit impact analysis (DFFITS and Cook's distance); covariance from the mean; residual heterogeneity test statistics; overall result influence (hat values), and study weight (Viechtbauer, 2010). If a study was deemed to be outlying, the random effects model in which it was included was refitted excluding the outlier and both model fits reported in the final results. All statistical analyses were carried out in RStudio, (R Core Team, 2019) with meta-analysis conducted using the Metafor package (Viechtbauer, 2010). All effect estimates are reported as mean difference with 95% confidence intervals (MD, [95% CI]), unless otherwise stated, with statistical significance of p-values being assessed at $\alpha = .05$.

2.4 Results

2.4.1 Included Studies

A total of 2,770 citations were identified through the electronic database search, and an additional 9 records were identified through manual searches. Once duplicates were removed, the title and abstracts of 1,858 articles were screened, leaving 151 full texts to be assessed for eligibility. Overall, 45 articles met the inclusion criteria for the qualitative synthesis. Of the 45 articles, 15 articles were suitable for inclusion in the meta-analyses (Figure 2.1).

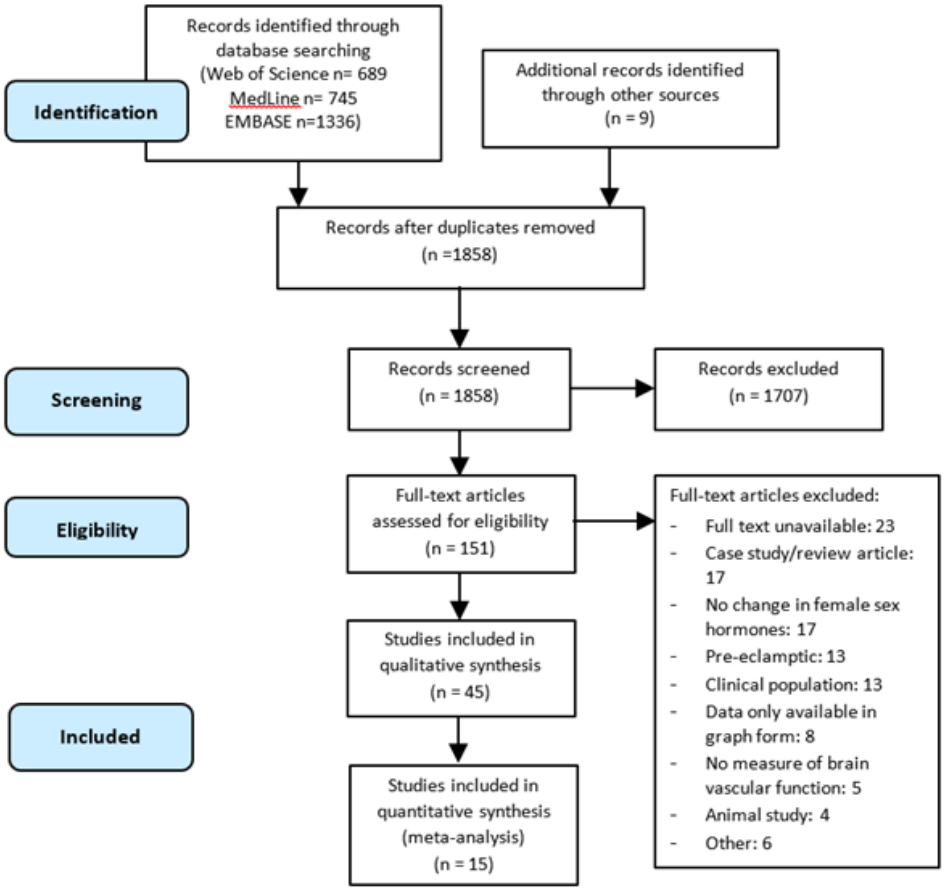

Figure 2.1 PRISMA-P flow diagram of the literature search and selection process for articles included in the systematic review and meta-analysis.

2.4.2 Study Characteristics

Of the studies included in the qualitative synthesis, 29 were based in Europe, 8 in North America, 5 in Asia, and 3 in South America. Seven of these were randomised controlled trials, 15 were non-randomised controlled trials or cohort studies, 16 were cross-sectional, 10 were longitudinal and 1 was an observational study. Hormone replacement therapy (HRT) was the most frequently studied hormone group (n = 21), followed by pregnancy (n = 12), menstrual cycle (n = 7), menopause (n = 5), oral contraception (n = 2), and ovarian hyperstimulation (n = 2). Eleven studies were assessed to be of high quality and therefore low risk of bias. Thirty-two studies were deemed to be of moderate quality and 2 of low quality. Figure 2.2 summarises both the level and quality of evidence of included studies, both overall and within hormone groups.

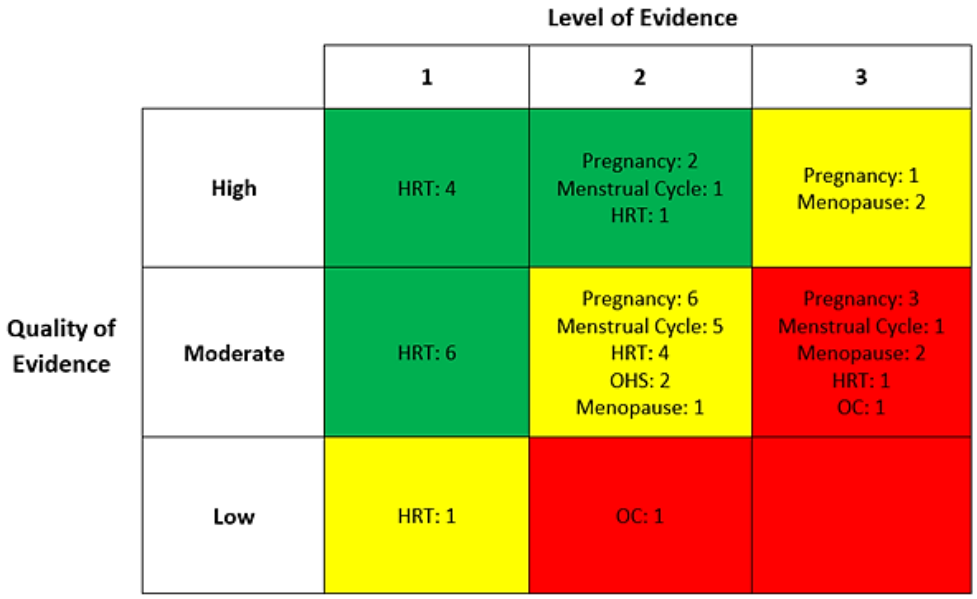

Figure 2.2 The level and quality of evidence of each included study within individual hormone groups. HRT, hormone replacement therapy; OHS, ovarian hyperstimulation; OC, oral contraception.

2.4.3 Effect of changing ovarian sex steroid hormones on pulsatility index (PI)

Twenty-six studies were identified that reported PI in low and high hormone phases (Table 2.2). PI was most frequently reported using the internal carotid artery (ICA; n = 22), with fewer studies reporting PI of the middle cerebral artery (MCA; n =13), external carotid artery (ECA; n = 4), common carotid artery (CCA; n = 4) and posterior cerebral artery (PCA; n = 1).

Fourteen studies investigated changes with HRT and showed that PI was 4.6 ± 10.2% lower in the high hormone phase as compared to the low phase. Twelve studies reporting PI changes with HRT were suitable for inclusion in the meta-analyses. Within these 12 studies, three reported outcomes in two experimental groups (i.e., different HRT treatments), and six reported outcomes in two or more vessels, resulting in a total of 25 cohorts. The combined effects of the 25 cohorts showed an estimated mean difference of -0.05 [-0.10, -0.01] from a low to high hormone phase, which was significant and showed considerable levels of heterogeneity (p = .01; I^2 = 96.8%; Figure 2.3). Mixed effect modelling was used to evaluate the effects of variation in HRT type and vessel of insonation, however little-to-no heterogeneity was accounted for by either moderator (5.96%, p = .303; and 0%, p = .952, respectively).

Four studies examined changes in PI with pregnancy, showing a 22 ± 8% higher PI in a high hormone phase compared to a low hormone phase. Five studies examining changes across the menstrual cycle, showed PI was 3 ± 6% higher in the high hormone phase compared to the low hormone phase, while two studies showed PI to be 8 ± 12% lower post-menopause compared to pre-menopause. PI increased by 14 ± 2% in

the high hormone phase compared to the low hormone phase in oral contraceptive use (n = 2), and increased by 14% in the one study observing changes in PI from a low to high hormone phase in ovarian hyperstimulation.

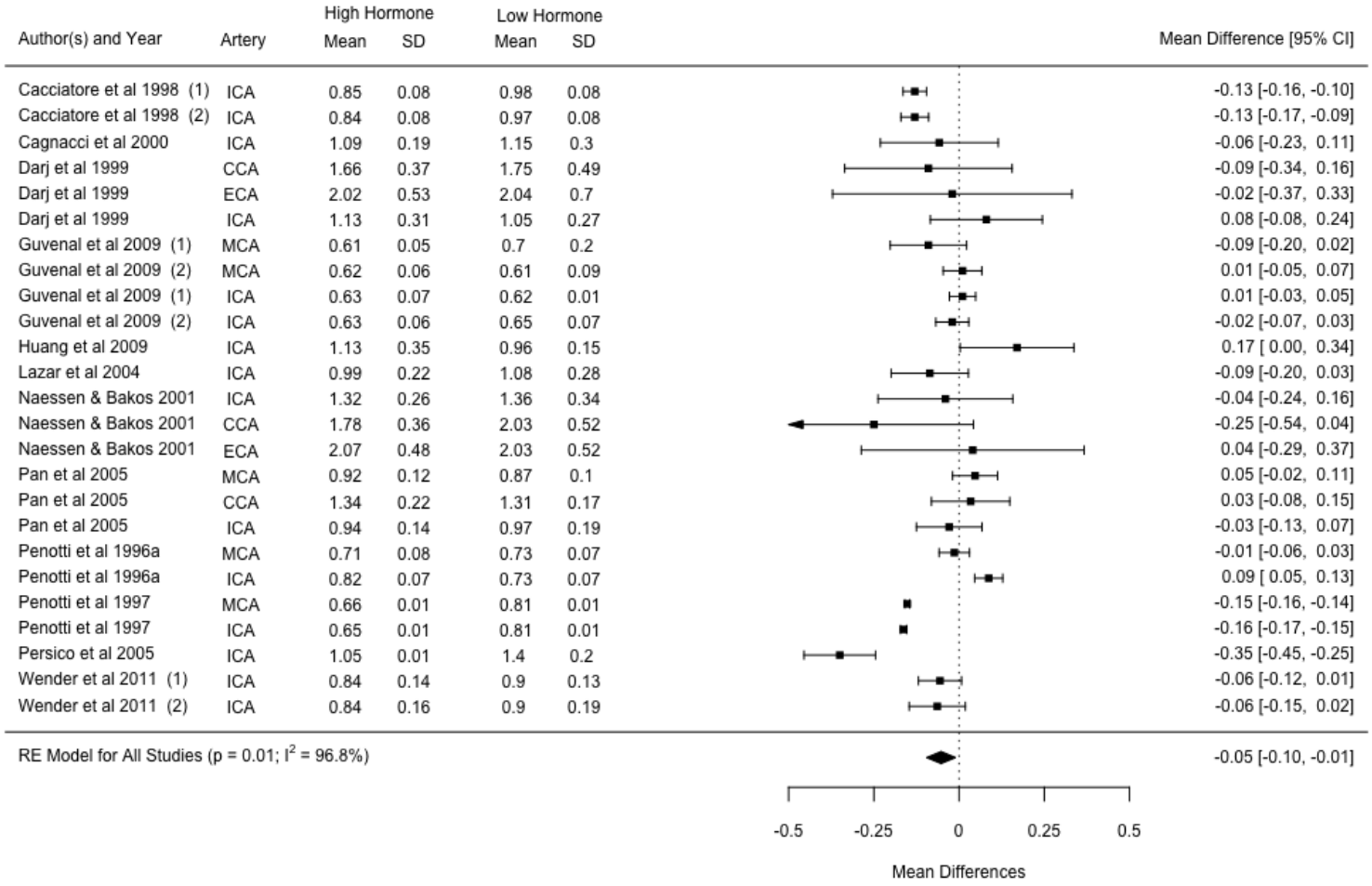

Figure 2.3 Forest plot showing mean difference and 95% confidence intervals for the impact of HRT administration in post-menopausal females compared to a post-menopausal control group on the pulsatility index. Numbers indicate different cohorts within a study (i.e., different HRT types). Abbreviations: ICA (internal carotid artery), CCA (common carotid artery), ECA (external carotid artery), MCA (middle cerebral artery).

2.4.4 Effect of changing ovarian sex steroid hormones on cerebral blood flow/velocity (CBF)

A total of 25 included studies reported an index of CBF, the characteristics of which are summarised in Table 2.3. All but two studies presented CBF as indexed by blood flow velocity through a vessel. CBF was most frequently reported using the MCA (n = 14), with fewer studies reporting CBF in the ICA (n = 12), PCA (n = 2), CCA (n = 2), ECA (n = 1) and vertebral artery (VA; n = 1).

HRT studies (n = 5) showed CBF was 3 ± 12% lower during HRT compared to pre-HRT or control groups. All five studies were included in the meta-analyses, with one study reporting outcomes in two groups using different HRT and two studies reporting outcomes in two vessels (total cohorts = 9). No effects were found in CBF between the high and low hormone phase (-2 cm/s [-6.16, 1.54]; p = .24, I^2 = 75.5%).

Eight studies reported CBF was 10 ± 15% lower in the high hormone phase during pregnancy as compared to the low. Four studies examining CBF across the menstrual cycle showed CBF was 3 ± 10% lower in a high hormone phase compared to a low hormone phase. Across different stages of ovarian hyperstimulation (n = 2) CBF was 12 ± 7% higher in the high hormone phase compared to the low. Four studies investigating menopause showed CBF was 23 ± 25% higher in the high hormone phase, while oral contraceptive studies (n = 2) reported that CBF was 15 ± 4% lower in the high hormone phase as compared to the low.

2.4.5 Effect of changing ovarian sex steroid hormones on resistance index (RI)

Eleven included studies reported RI in a low and high hormone phase (Table 2.4). RI was most frequently reported using the ICA (n = 7), with fewer studies reporting the MCA (n = 5), ECA (n = 4), CCA (n = 4), PCA (n = 1), and VA (n = 1).

Five studies reported RI with HRT was 1 ± 3% lower in the high hormone phase compared to the low hormone phase. Of these studies four were suitable for inclusion in the meta-analyses with two studies reporting outcomes in three vessels, resulting in eight cohorts in total. No effects were found in RI between a high and low hormone phase (-0.00 [-0.02, 0.02]; p = .77, I^2 = 57.5%).

RI was 2 ± 3% and 12 ± 13% higher in the high hormone phase when looking across the menstrual cycle (n = 3) and pregnancy (n = 2), respectively. One study investigated changes in RI with ovarian hyperstimulation, reported a 29% lower RI during high hormone phases compared to low.

2.4.6 Effect of changing ovarian sex steroid hormones on cerebrovascular reactivity (CVR)

Four studies report a measure of CVR within the MCA (Table 2.5). Two studies investigated CVR using the breath-holding index, with one study reporting CVR was 79% higher pre-menopause compared to post-menopause, and the other reporting CVR was 32% higher in the high hormone phase of the menstrual cycle compared to the low.

Two studies examined CVR using CO_2 inhalation. In pregnancy, CVR (using a target end-tidal CO_2 1kPa above baseline with O_2 enriched air) was 1% lower in the high hormone phase as compared to the low. Comparing pre- to post-menopausal females, CVR (using a 5% CO_2, 21% O_2, balance N_2 gas mix) was 7% higher in pre-menopausal females.

2.4.7 Effect of changing ovarian sex steroid hormones on cerebral autoregulation (CA)

Four studies report measures of CA (Table 2.6). Two studies investigated changes in CA of the MCA in pregnancy, with one study reporting 'strength of autoregulation' from the transient hyperaemic response was 7% higher in the high hormone phase compared to the low. The second study in pregnancy reported the autoregulation index at rest as 25% higher in the high hormone phase. One study reported changes in CA across the menstrual cycle in the MCA and ACA, showing the autoregulation index during repeated sit-to-stand manoeuvres was 12 ± 3% lower in the high hormone phase compared to low. Finally, one study reported CA using transfer function analysis of repeated squat-to-stand manoeuvres. Comparing pre- to post-menopausal females, phase was 5% lower and normalised gain was 4% higher in pre-menopausal females, indicating marginally less efficient CA in the high hormone phase.

Table 2.2 Characteristics for included studies reporting Pulsatility Index (PI; arbitrary units). ‡ Median (Inter-quartile range) • Mean (median). Studies in ***bold*** indicate those included in the meta-analyses. Abbreviations: HRT (hormone replacement therapy), ICA (internal carotid artery), CCA (common carotid artery), ECA (external carotid artery), MCA (middle cerebral artery), PCA (posterior cerebral artery).

Study	Technique	Hormone Group	Sample size (n; low hormone phase, high hormone phase)	Vessel Insonated	Outcome measure	Low hormone phase; mean (SD)	High hormone phase; mean (SD)	% change in PI from low to high hormone phase
Cagnacci et al. (2000)	Vascular Doppler Ultrasound	HRT	13, 18	ICA	PI	1.15 (0.3)	1.09 (0.19)	-5
Cacciatore et al. (1998)	Vascular Doppler Ultrasound	HRT	58	ICA	PI	0.98 (0.08)	0.85 (0.08)	-13
Crook et al. (1991) ‡	Vascular Doppler Ultrasound	HRT	12	ICA	PI	0.92 (0.77-1.18)	0.82 (0.70-0.90)	-11
Darj et al. (1999)	Vascular Doppler Ultrasound	HRT	20	CCA	PI	1.75 (0.49)	1.66 (0.37)	-5
				ECA	PI	2.04 (0.7)	2.02 (0.53)	-1
				ICA	PI	1.05 (0.27)	1.13 (0.31)	8
Guvenal et al. (2009)	Transcranial Doppler Ultrasound	HRT	47	MCA	PI	0.65 (0.08)	0.62 (0.05)	-5
				ICA	PI	0.64 (0.05)	0.63 (0.06)	-2
Huang et al. (2009)	Vascular Doppler Ultrasound	HRT	20	ICA	PI	0.96 (0.15)	1.13 (0.35)	18

Jackson & Vyas (1998)	Vascular Doppler Ultrasound	HRT	15	ICA	PI	1.07 (0.28)	0.96 (0.17)	-11
Lazar et al. (2004)	Vascular Doppler Ultrasound	HRT	38	ICA	PI	1.08 (0.28)	0.99 (0.22)	-7
Naessen & Bakos (2001)	Vascular Doppler Ultrasound	HRT	18	ICA	PI	1.36 (0.34)	1.32 (0.26)	-3
				CCA	PI	2.03 (0.52)	1.78 (0.36)	-12
				ECA	PI	2.26 (0.83)	2.07 (0.48)	-8
Pan et al. (2002)	Vascular Doppler Ultrasound	HRT	40	MCA	PI	0.87 (0.1)	0.92 (0.1)	6
				CCA	PI	1.30 (0.17)	1.35 (0.22)	3
				ICA	PI	0.97 (0.19)	0.94 (0.14)	-3
Penotti et al. (1996)a	Vascular Doppler Ultrasound	HRT	23	MCA	PI	0.73 (0.07)	0.71 (0.08)	-2
				ICA	PI	0.73 (0.07)	0.82 (0.07)	12
Penotti et al. (1998)	Vascular Doppler Ultrasound	HRT	30	MCA	PI	0.81 (0.01)	0.66 (0.01)	-19
				ICA	PI	0.81 (0.01)	0.65 (0.02)	-20
Persico et al. (2005)	Vascular Doppler Ultrasound	HRT	14	ICA	PI	1.40 (0.20)	1.05 (0.01)	-25
Wender et al. (2011)	Vascular Doppler Ultrasound	HRT	75	ICA	PI	0.90 (0.16)	0.84 (0.15)	-7

Brackley et al. (1998)‡	Transcranial Doppler Ultrasound	Pregnancy	17	MCA	PI	0.73 (0.64 – 0.78)	0.85 (0.81 – 0.96)	16
	Vascular Doppler Ultrasound	Pregnancy	17	ICA	PI	0.83 (0.80 – 0.96)	0.98 (0.87 – 1.08)	18
Janzarik et al. (2014)	Transcranial Doppler Ultrasound	Pregnancy	26, 61	MCA	PI	0.66 (0.10)	0.84 (0.14)	27
			26, 59	PCA	PI	0.59 (0.08)	0.78 (0.14)	32
Lindqvist et al. (2006)	Transcranial Doppler Ultrasound	Pregnancy	14	MCA	PI	0.79 (0.16)	0.99 (0.22)	25
Serra-Serra et al. (1997)	Transcranial Doppler Ultrasound	Pregnancy	25, 22	MCA	PI	0.82 (0.14)	0.91 (0.14)	11
Brackley et al. (1999) ‡	Transcranial Doppler Ultrasound	Menstrual Cycle	19	MCA	PI	0.72 (0.70 – 0.82)	0.81 (0.71 – 0.94)	13
	Vascular Doppler Ultrasound	Menstrual Cycle	19	ICA	PI	0.87 (0.76 – 0.98)	0.90 (0.81 – 1.01)	4
				ECA	PI	2.52 (2.31 – 3.30)	2.48 (2.31 – 2.99)	-2
Hazlett & Edgell (2018)	Transcranial Doppler Ultrasound	Menstrual Cycle	14	MCA	PI	0.76 (0.04)	0.77 (0.03)	1
Krejza et al. (2004)•	Vascular Doppler Ultrasound	Menstrual Cycle	14	ICA	PI	0.99 (1.05)	1.03 (0.99)	4
				CCA	PI	1.79 (1.63)	1.84 (1.84)	3
				ECA	PI	2.13 (2.06)	2.34 (2.29)	10

Arangino et al. (1998)	Vascular Doppler Ultrasound	Oral Contraception	22, 22	ICA	PI	1.30 (0.12)	1.46 (0.19)	12
		Menstrual Cycle	10, 12	ICA	PI	1.32 (0.13)	1.27 (0.19)	-4
Cagnacci et al. (1999)	Vascular Doppler Ultrasound	Oral Contraception	17, 17	ICA	PI	1.34 (0.12)	1.54 (0.17)	15
		Menstrual Cycle	18	ICA	PI	1.34 (0.12)	1.30 (0.16)	-3
Penotti et al. (1996)b	Vascular Doppler Ultrasound	Menopause	18, 18	MCA	PI	0.80 (0.11)	0.68 (0.15)	-15
				ICA	PI	0.79 (0.1)	0.68 (0.15)	-14
Robertson et al. (2008)	Transcranial Doppler Ultrasound	Menopause	12, 12	MCA	PI	0.81 (0.12)	0.86 (0.18)	6
Shamma et al. (1992)	Transcranial Doppler Ultrasound	Ovarian Hyper-stimulation	9	MCA	PI	0.72 (0.08)	0.82 (0.04)	14

Table 2.3 Characteristics for included studies reporting cerebral blood flow/velocity (CBF). *Average of two or more non-significant groups. † Mean (95% confidence intervals). Studies in ***bold*** indicate those included in the meta-analyses. Abbreviations: HRT (hormone replacement therapy), ICA (internal carotid artery), CCA (common carotid artery), ECA (external carotid artery), MCA (middle cerebral artery), PCA (posterior cerebral artery), VA (vertebral artery), ACA (anterior cerebral artery).

Study	Technique	Hormone Group	Sample size (n; low hormone phase, high hormone phase)	Vessel Insonated	Outcome measure (units)	Low hormone phase	High hormone phase	% change in CBF from low to high hormone phase
Bergersen et al. (2006)†	Vascular Doppler Ultrasound	Pregnancy	14	ICA	Mean flow velocity (cm/s)	27 (5)	19 (6)	-30
Janzarik et al. (2014)	Transcranial Doppler	Pregnancy	26, 61	MCA	Mean flow velocity (cm/s)	61 (9)	57 (10)	-7
				PCA	Mean flow velocity (cm/s)	41 (5)	38 (13)	-8
Lindqvist et al. (2006)	Transcranial Doppler	Pregnancy	13, 12	MCA	Mean flow velocity (cm/s)	57 (15)	57 (16)	0
Nevo et al. (2010)	Dual-beam, angle-independent, Doppler	Pregnancy	15, 129	ICA	Cerebral blood flow (mL/min)	294 (53)	382 (50)	23
Serra-Serra et al. (1997)	Transcranial Doppler	Pregnancy	25, 22	MCA	Mean flow velocity (cm/s)	70 (9)	60 (9)	-15

Sherman et al. (2002)	Transcranial Doppler	Pregnancy	30, 30	MCA	Mean flow velocity (cm/s)	69 (14)	57 (12)	-18
van Veen et al. (2016)	Transcranial Doppler	Pregnancy	18, 50	MCA	Mean flow velocity (cm/s)	72 (11)	66 (9)	-9
Zeeman et al. (2003)	Phase contrast magnetic resonance imaging	Pregnancy	9	MCA	Cerebral blood flow (mL/min)	148 (5)	118 (5)	-20
				PCA	Cerebral blood flow (mL/min)	56 (3)	44 (2)	-21
Acar et al. (2005)	Vascular Doppler Ultrasound	HRT	18	ICA	Peak systolic velocity (cm/s)	59 (17)	62 (16)	5
				VA	Peak systolic velocity (cm/s)	46 (14)	40 (8)	-13
Cagnacci et al. (2000)	Vascular Doppler Ultrasound	HRT	18, 13	ICA	Peak systolic velocity (cm/s)	34 (6)	40 (9)	18
Clapauch et al. (2007)	Vascular Doppler Ultrasound	HRT	9	ICA	Systolic flow velocity (cm/s)	52 (3)	54 (2)	4

Guvenal et al. (2009)*	Transcranial Doppler	HRT	47	MCA	Peak systolic velocity (cm/s)	58 (14)	53 (13)	-8
				ICA	Peak systolic velocity (cm/s)	39 (7)	36 (5)	-8
Vidović et al. (2001)	Vascular Doppler Ultrasound	HRT	32	CCA	Peak systolic velocity (cm/s)	85 (17)	72 (15)	-15
Diomedi et al. (2001)	Transcranial Doppler	Menstrual Cycle	20	MCA	Mean flow velocity (cm/s)	68 (13)	67 (12)	-1
Favre & Serrador, (2019)	Transcranial Doppler	Menstrual Cycle	13	MCA	Mean flow velocity (cm/s)	82 (16)	78 (19)	-5
				ACA	Mean flow velocity (cm/s)	58 (15)	54 (10)	-6
Hazlett & Edgell, (2018)	Transcranial Doppler	Menstrual Cycle	14	MCA	Mean flow velocity (cm/s)	69 (4)	71 (3)	4
Krejza et al. (2001)	Vascular Doppler Ultrasound	Menstrual Cycle	14	ICA	Mean flow velocity (cm/s)	42 (5)	47 (6)	12
				CCA	Mean flow velocity (cm/s)	42 (4)	42 (5)	0

				ECA	Mean flow velocity (cm/s)	28 (5)	25 (5)	-11
Brislane et al. (2020)	Transcranial Doppler	Menopause	50, 50	MCA	Mean flow velocity (cm/s)	61 (15)	72 (15)	18
Iwamoto et al. (2021)	Vascular Doppler Ultrasound	Menopause	11, 10	ICA	Mean flow velocity (cm/min)	28 (8)	43 (10)	54
					Blood flow (mL/min)	281 (106)	404 (87)	44
Matteis et al. (1998)	Transcranial Doppler	Menopause	40, 45	MCA	Mean flow velocity (cm/s)	60 (9)	62 (12)	4
Robertson et al. (2008)	Transcranial Doppler	Menopause	12, 12	MCA	Mean flow velocity (cm/s)	70 (10)	67 (11)	-4
Nevo et al. (2007)	Vascular Doppler Ultrasound	Ovarian Hyperstimulation	12	ICA	Mean flow velocity (cm/s)	40 (2)	47 (2)	17
Shamma et al. (1992)	Transcranial Doppler	Ovarian Hyperstimulation	9	MCA	Peak systolic velocity (cm/s)	98 (12)	105 (12)	7
Arangino et al. (1998)	Vascular Doppler Ultrasound	Oral Contraceptive	22, 22	ICA	Peak flow velocity (cm/s)	51 (4)	45 (4)	-13

Cagnacci et al. (1999)	Vascular Doppler Ultrasound	Oral Contraceptive	18, 17	ICA	Peak flow velocity (cm/s)	61 (7)	50 (2)	-18
		Menstrual Cycle	18	ICA	Peak flow velocity (cm/s)	61 (7)	48 (3)	-21

Table 2.4 Characteristics for included studies reporting Resistance Index (RI). ‡ Median (IQR). • Mean (median). Studies in ***bold*** indicate those included in the meta-analyses. Abbreviations: HRT (hormone replacement therapy), ICA (internal carotid artery), CCA (common carotid artery), ECA (external carotid artery), MCA (middle cerebral artery), PCA (posterior cerebral artery), VA (vertebral artery).

Study	Technique	Hormone Group	Sample size (n; low hormone phase, high hormone phase)	Vessel Insonated	Outcome measure	Low hormone phase; mean (SD)	High hormone phase; mean (SD)	% change in RI from low to high hormone phase
Cagnacci et al. (2000)	Vascular Doppler Ultrasound	HRT	18, 13	ICA	RI	0.66 (0.09)	0.64 (0.05)	-3
Clapauch et al. (2007)	Vascular Doppler Ultrasound	HRT	9	ICA	RI	0.60 (0.02)	0.56 (0.02)	-7
Darj et al. (1999)	Vascular Doppler Ultrasound	HRT	20	CCA	RI	0.75 (0.08)	0.74 (0.06)	-1
				ECA	RI	0.81 (0.98)	0.80 (0.06)	-1
				ICA	RI	0.59 (0.09)	0.63 (0.10)	7
Pan et al. (2002)	Vascular Doppler Ultrasound	HRT	40	MCA	RI	0.56 (0.04)	0.57 (0.05)	2
				CCA	RI	0.69 (0.06)	0.70 (0.07)	1
				ICA	RI	0.58 (0.07)	0.60 (0.08)	3
Brackley et al. (1999) ‡	Transcranial Doppler Ultrasound	Menstrual Cycle	19	MCA	RI	0.55 (0.51 – 0.59)	0.56 (0.53 – 0.59)	8

	Vascular Doppler Ultrasound	Menstrual Cycle	19	ICA	RI	0.51 (0.49 – 0.55)	0.54 (0.49 – 0.58)	2
				ECA	RI	0.87 (0.83 – 0.90)	0.86 (0.84 – 0.90)	-1
Krejza et al. (2003) •	Vascular Doppler Ultrasound	Menstrual Cycle	14	ECA	RI	0.81 (0.81)	0.84 (0.84)	4
				ICA	RI	0.60 (0.62)	0.59 (0.58)	-2
				CCA	RI	0.75 (0.74)	0.75 (0.76)	0
Hazlett & Edgell, (2018)	Transcranial Doppler	Menstrual Cycle	14	MCA	RI	0.51 (0.02)	0.52 (0.01)	2
Janzarik et al. (2014)	Transcranial Doppler	Pregnancy	26, 61	MCA	RI	0.46 (0.04)	0.54 (0.06)	18
				PCA	RI	0.43 (0.04)	0.52 (0.06)	21
van Veen et al. (2016)	Transcranial Doppler	Pregnancy	18, 50	MCA	RI	0.43 (0.05)	0.42 (0.04)	-2
Nevo et al. (2007)	Vascular Doppler Ultrasound	Ovarian Hyperstimulation	12	ICA	RI	0.14 (0.01)	0.10 (0.01)	-29

Table 2.5 Characteristics for included studies reporting cerebrovascular reactivity (CVR). Abbreviations: MCA (middle cerebral artery), AU (arbitrary units).

Study	Technique	Hormone Group	Sample size (n; low hormone phase, high hormone phase)	Vessel Insonated	Outcome measure (units)	Low hormone phase; mean (SD)	High hormone phase; mean (SD)	% change in CVR from low to high hormone phase
Brislane et al. (2020)	Transcranial Doppler / CO_2 inhalation	Menopause	50, 50	MCA	Absolute change in MCA*v* per mmHg (cm/s/mmHg)	3.5 (1.9)	3.8 (1.5)	7
Matteis et al. (1998)	Transcranial Doppler / Breath-holding	Menopause	40, 45	MCA	BHI (AU)	0.9 (0.3)	1.6 (0.3)	79
Sherman et al. (2002)	Transcranial Doppler / CO_2 inhalation	Pregnancy	30, 30	MCA	% change MCA*v* per kPa (%)	28.0 (6.4)	27.7 (7.7)	-1
Diomedi et al. (2001)	Transcranial Doppler / Breath-holding	Menstrual Cycle	20	MCA	BHI (AU)	1.3 (0.3)	1.7 (0.3)	32

Table 2.6 Characteristics for included studies reporting cerebral autoregulation. Abbreviations: MCA (middle cerebral artery), ACA (anterior cerebral artery), ARI (autoregulation index), AU (arbitrary index).

Study	Technique	Hormone Group	Sample size (n; low hormone phase, high hormone phase)	Vessel Insonated	Outcome measure (units)	Low hormone phase; mean (SD)	High hormone phase; mean (SD)	% change in autoregulation from low to high hormone phase
Favre & Serrador, (2019)	Transcranial Doppler / Sit-to-Stand	Menstrual Cycle	13	MCA	ARI (AU)	3 (1.4)	2.8 (0.8)	-10
				ACA	ARI (AU)	3.0 (1.2)	2.6 (1.1)	-13
Sherman et al. (2002)	Transcranial Doppler / Transient Hyperaemic Response	Pregnancy	30, 30	MCA	Strength of Autoregulation (AU)	1.1 (0.2)	1.2 (0.2)	7
van Veen et al. (2016)	Transcranial Doppler / Resting	Pregnancy	18, 50	MCA	ARI (AU)	5.3 (1.4)	6.6 (0.9)	25
Brislane et al. 2020	Transcranial Doppler / Squat-to-Stand	Menopause	50, 50	MCA	Normalised gain (%)	1.3 (0.4)	1.4 (0.4)	4
					Phase (degrees)	23.8 (13.2)	22.6 (14.8)	-5
					Coherence (AU)	0.7 (0.1)	0.6 (0.1)	-8

2.5 Discussion

2.5.1 General Findings

To the best of our knowledge, this is the first systematic review and meta-analysis to examine the interaction between brain vascular function and changing ovarian sex steroid hormones. This review identifies the current gaps in the literature and provides a clear basis from which future research can be established. Most commonly, included studies examined the effects of HRT or pregnancy using either PI or CBF as a measure of brain vascular function. The main outcome from the meta-analyses was that females undergoing HRT had a significant reduction in PI compared to post-menopausal females not on HRT. However, CBF and RI were found to have no changes with HRT.

The following discussion will assess the outcome measures examined in this review in the context of cerebrovascular health and function across the different hormone groups reported.

2.5.2 Brain vascular function and changing ovarian sex steroid hormones

The HRT-PI meta-analysis indicated that administration of HRT improved (i.e., lowered) PI in post-menopausal females when compared to post-menopausal females not receiving HRT. This is consistent with previous studies that have shown increases in PI positively correlate with time elapsed since menopause in females not on HRT (Crook et al., 1991). Further, timing of HRT initiation post-menopause has been shown to reduce adverse cardiac events and mortality (Nudy et al., 2019). The majority of included HRT studies in this review report the effects of HRT initiation within 5-10 years

of menopause onset (Appendix C). This “early initiation” may contribute to the beneficial effect of HRT on PI reported herein. However, the considerable heterogeneity in this data set could not be accounted for with meta-regression analysis of potential moderators (type of HRT and insonated vessels), indicating there are unaccounted moderators causing this large variability (discussed below). Limited PI data for the other female hormone groups included in this review determined that a meta-analysis could not be performed. Data from the wider cohort included within this review reported both an increase and decrease in PI from a low to high hormone phase, highlighting the inconsistencies within the literature on this measure. PI is used as an indication of downstream resistance to flow, with a higher PI indicative of greater downstream resistance and therefore, reduced cerebral tissue perfusion. A higher PI has been associated with a diagnosis of Alzheimer’s Disease (Roher et al., 2011), as well as correlated with the presence of small-vessel ischaemic disease (Kidwell et al., 2001), and shown to predict poorer functional outcomes post-stroke (Aoki et al., 2013). Thus, it appears that administration of HRT has the potential to alleviate the risk of cerebrovascular disease in post-menopausal females. It could be expected that PI would also be improved in other high hormone phases (i.e., 3rd trimester of pregnancy) but there is insufficient evidence to support this.

The HRT-CBF meta-analysis indicated no overall effects for CBF in post-menopausal females receiving HRT compared to those who were not. As with PI, all hormone groups showed no clear directional change in CBF with changing ovarian sex steroid hormone levels. Since CBF is used as a functional index of cerebral perfusion, CBF could be expected to be greater during high hormone phases. Given that a decline in

CBF is believed to precede and contribute to the onset of clinical dementia (Ruitenberg et al., 2005), it is important to identify if HRT during menopause might reduce/delay disease onset particularly for females with other risk factors.

RI was most commonly reported in studies investigating HRT, with four of the five studies in this review suitable for inclusion in the meta-analyses. No significant overall effect was found in RI in post-menopausal females receiving HRT. The small study number and small sample sizes within these studies likely contributed to this outcome. As an index of downstream flow impedance, RI could be expected to be greater in females not receiving HRT as observed with PI. RI reported in other hormone groups showed no clear change in RI from a low to high hormone phase.

CVR was on average greater during high hormone phases compared to low hormone phases, though a formal meta-analysis comparison was not possible due to the limited data available. Of the four included studies, three different hormone groups and three different CVR assessment methods were used. These differences likely explain the wide-ranging changes observed in CVR from the low to high hormone phases (range: -1% to 79%). CVR is an indication of the vasodilatory reserve capacity (Hoiland et al., 2011), with a higher CVR associated with improved cerebrovascular function while CVR impairment can predict the risk of stroke in patients with carotid artery occlusion (Markus & Cullinane, 2001; Webster et al., 1995). Therefore, it could be predicted that high hormone phases would elicit greater CVR, however there is insufficient evidence to conclude this.

The four studies that reported CA showed no clear consensus on the effect with changing ovarian sex steroid hormones. Again, this is most likely due to the range of methods employed to assess CA, the different outcome measures used to index CA, and the low number of included studies. CA has previously been shown to be impaired in stroke (Eames et al., 2002), with a more severe impairment associated with poorer functional outcomes post-stroke (Castro et al., 2017). As such, better CA may be expected during high hormone phases (e.g., pre-menopause compared to post-menopause) but the limited literature available to date provides insufficient evidence to support this.

2.5.3 Causes of heterogeneity

This meta-analysis showed that there was considerable heterogeneity within HRT studies. However, while both the composition and routine of HRT interventions varied across included studies (detailed in Appendix C), meta-regression results showed that HRT type did not account for this heterogeneity and nor did the insonated vessel. While age is known to influence cerebrovascular function (Peng et al., 2018), studies investigating HRT effects typically controlled for age, therefore age is unlikely to have caused this significant heterogeneity.

Overall, given the limitations in the available data, including the limited number of studies and different methodologies used to assess a broad range of cerebrovascular function measures, the considerable heterogeneity for ovarian sex steroid hormone effects might be expected.

2.5.4 Recommendations for future studies

The 48 included studies in this review illustrates the growing body of knowledge in the area of sex hormones and cerebrovascular function. However, the effect of ovarian sex steroid hormones on brain vascular function remains largely unknown due to the heterogeneity of the available data and/or the limited number of studies. The following recommendations address these concerns. For the true effect of changing ovarian sex steroid hormones on cerebrovascular function to be better understood future studies must report hormone levels as standard. Of the studies included within this review, only 36% (16 out of the 45) reported hormone levels. Thus, it was not possible to formally compare changes in cerebrovascular function to changes in absolute hormone levels, levels which covered a wide range. For example, plasma progesterone levels during the “high hormone phase” of pregnancy (i.e. 2nd-3rd trimester) is 150 ng/ml as compared to the high hormone phase of the menstrual cycle (i.e. luteal phase), which is 25 ng/ml (Elliott, 2001). Reporting of oestrogen and progesterone could help verify the hormonal status of a group (e.g., phase of menstrual cycle) but also account for individual differences in circulating hormones. In turn, this may help understand some of the variation seen in reported cerebrovascular outcome measures and aid comparisons between studies.

In order to help address the considerable heterogeneity observed in HRT studies, future studies should consider time since menopause and/or time on HRT when recruiting participants. Recording and reporting of this information will allow for assessment of its impact as a moderating factor for the effects of HRT on cerebrovascular function. Additionally, further research should be done to determine

the importance of timing, and whether benefits exist for the cerebrovasculature if HRT is initiated closer to the onset of menopause.

This systematic review highlights the major gaps in the current literature. Only two studies in this review looked at the effect of controlled ovarian hyperstimulation. This has implications for females undergoing in-vitro fertilisation (IVF) and the effect of supraphysiological changes in ovarian sex steroid hormones on cerebrovascular function. Furthermore, only two studies investigated changes with oral contraceptive use. A recent NHS England report (2017) found 42% of females in England used oral contraceptives, with the total number presumably being higher when including all types of hormonal contraception (e.g., coil, implant). This high rate of hormonal contraceptive use indicates that it is currently vastly under-represented in research and its effects on cerebrovascular function remain largely unknown.

The variance in cerebrovascular measure and/or methodology used to date likely contributed to data heterogeneity. There remains no consensus on the optimal methodology to assess cerebrovascular function, a problem across the literature assessing brain vascular health. Additionally, even within a single outcome measure (e.g., CVR) there can be multiple modes of assessment (e.g., CO_2 inhalation, breath-holding index). In fact, both duration of the CO_2 stimulus and the timepoint used for analysis has recently been shown to significantly alter CVR values (Burley et al., 2020). The consistent implementation of both methodology and data extraction is vital to producing robust cerebrovascular function measures and improving the subsequent conclusions regarding brain vascular health. For CVR, Burley and colleagues recommended using a CO_2 inhalation stimulus duration of 3 minutes and extracting

data from the final 30-60 s. Future research should look to consistently implement methodological approaches across all cerebrovascular outcome measures in order for studies to be comparable.

2.5.5 Limitations

Due to the low number of studies for most hormone groups, statistical analysis of the data was inappropriate. As such, the relative changes from low to high hormone groups reported here should be used to help identify the knowledge gaps in the literature and aid the direction of future research, rather than to infer the specific effect of ovarian sex steroid hormones on cerebrovascular function at this stage.

The majority of included studies in this review were deemed to be of moderate quality and either a moderate or low level of evidence (i.e., cohort or cross-sectional study design) and therefore a moderate risk of bias. In fact, only some HRT studies were categorised as being of a high level of evidence due to the often clinical-based setting of these studies and the research question being frequently suited to a randomised controlled trial design. It is important to consider the differing quality of evidence across hormone groups when interpreting the results of this review.

The substantial heterogeneity reported within hormone groups should also be considered. The meta-regression performed on HRT studies reporting PI and included in the meta-analysis were unable to identify the moderators driving this heterogeneity. Meta-regression could not be performed in those studies reporting CBF and RI due to the low number of included studies.

Of note, this review primarily identified and included studies using TCD-based measures of cerebral blood flow, with few using neuroimaging techniques (n=2). Since TCD is unable to image the vessel, and thus vessel diameter, TCD has been shown to result in over- and under-estimations of CBF when CBF*v* is used as an index of absolute flow, particularly with changing CO_2 levels (Coverdale et al., 2014; Verbree et al., 2014). As such, results from this review should be interpreted with this in mind, and that outcomes may differ if neuroimaging techniques (e.g., MRI) are used to determine CBF.

This review did not exclude studies based on country or location, and as a result confounding factors such as dietary behaviours or cultural lifestyle differences may account for some of the heterogeneity seen. For example, phytoestrogens, evidenced to cause increases in CBF (Kennedy et al., 2010), are of much greater prevalence in the soy-based foods found in Asian diets compared to Western diets (Rietjens et al., 2017).

2.5.6 Conclusion

This review has shown that HRT has the capacity to improve PI in post-menopausal females, and therefore the potential to improve cerebrovascular function. There remains insufficient evidence to determine if this effect of HRT is reflected in other cerebrovascular outcome measures. The effect of changes in ovarian sex steroid hormones in hormone groups other than HRT remains largely unclear, at least in part due to the substantial heterogeneity in the current literature and considerable under-representation of certain hormone groups in research (e.g., oral contraception).

Despite this, this review provides a foundation for future research through the clear identification of gaps in the current literature. Future research in ovarian sex steroid hormones and cerebrovascular function should aim to improve the consistency and generalisability of findings through reporting of hormone levels and implementation of standardised methodology to assess cerebrovascular function.

3 GENERAL METHODS

3.1 Participants

All participants were healthy and free of any known cardiovascular, neurological or metabolic diseases. Participants were informed to abstain from alcohol consumption and vigorous exercise for 24 hours, in addition to caffeine consumption for 12 hours and food consumption for 2 hours prior to the experimental testing sessions.

Inclusion criteria for males (Chapter 4) and pre-menopausal females (Chapters 4, 5, and 6) were to be 18-50 years old, and for pre-menopausal females to have a regular menstrual cycle (<35 days in length) and not taking any hormonal contraceptive medication. Inclusion criteria for post-menopausal participants (Chapter 6) were to be 45-60 years old, and at least 2 years post-menopausal (i.e., >2 years since last period). Post-menopausal females were included irrespective of hormone replacement therapy (HRT) status. Participant characteristics of the studied populations can be found in Table 3.1.

Table 3.1 Participant characteristics for the studied populations in the three experimental chapters. *The female cohorts presented in Chapters 4 and 5 are comprised of the same participants. BMI, body mass index.

	Chapter 4	**Chapters 4 & 5**	**Chapter 6**	
	Males	Females*	Pre-Menopausal	Post-Menopausal
N	9	13	9	6
Age (yrs)	22 ± 3	25 ± 5	20 ± 3	57 ± 2
Height (m)	1.79 ± 0.06	1.70 ± 0.06	1.72 ± 0.09	1.66 ± 0.03
Weight (kg)	72.6 ± 5.3	66.3 ± 3.7	67.3 ± 9.8	65.7 ± 3.4
BMI (kg/m^2)	23.1 ± 1.5	23.2 ± 1.4	22.7 ± 1.8	23.9 ± 0.9

3.2 Screening and Familiarisation

All participants were familiarised to the equipment and measures prior to experimental testing sessions. The transcranial Doppler (TCD) was fitted and acceptable signals were obtained, with baseline parameters recorded to aid standardisation of TCD values between experimental sessions. Participants underwent cerebrovascular-CO_2 responsiveness challenges to familiarise breathing with a mouthpiece and nose peg, and to ensure there were no adverse reactions to the hyper- and hypocapnic stimuli. Where appropriate, participants practised the squat-to-stand manoeuvres, moving to a metronome at the required frequencies (Chapter 6 only).

Ethical approval was obtained for all experimental protocols and procedures by the University of Birmingham Ethics Committee (project code: ERN_15-1179). All testing took place in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Prior to participation, a detailed verbal and written explanation of the study procedure was provided, and written informed consent obtained.

3.3 Measures

3.3.1 Middle and posterior cerebral artery blood flow velocity

Transcranial doppler (TCD) is a non-invasive technique using ultrasound technology to measure cerebral blood flow velocity (CBF*v*), an index of cerebral blood flow. TCD is frequently used in research to assess cerebrovascular function due to its cost-effective nature, portability, and capacity to provide a continuous ‘real-time’ measure

of CBF compared to other techniques. Neuroimaging techniques such as Magnetic Resonance Imaging (MRI) or positron emission tomography (PET) also provide accurate, quantitative measures of CBF. However, these techniques have a fixed location, are more expensive and less accessible compared to TCD (Willie et al., 2011). Additionally, in the case of PET, there is a high radiation exposure because of the tracer used to quantify CBF. Of course, TCD has its own disadvantages, specifically its operator dependent nature and capacity to only provide a single CBF value per cerebral hemisphere (Willie et al., 2011). Moreover, a primary assumption of TCD is that the insonated vessel diameter does not change and therefore, blood velocity is representative of blood flow. TCD does not image the vessel itself leaving the vessel diameter unknown and thus providing only a measure of CBF*v* and not absolute volumetric flow. While initial validation studies supported this assumption (Serrador et al., 2000; Valdueza et al., 1997), more recent MRI studies have highlighted the potential for changing vessel diameters in response to changes in CO_2 (Coverdale et al., 2014; Verbree et al., 2014) and exercise (Verbree et al., 2017), resulting in possible over- and under-estimations of CBF when CBF*v* is used as a direct index of absolute flow (discussed further below; Ainslie & Hoiland, 2014). Nevertheless, primarily due to its ease of use, TCD-based examination of cerebrovascular function in response to a range of physiological perturbations has become commonplace in research, as well as in clinical settings to assess the impact of a number of pathologies (e.g. vasospasm, hypertension, ischaemic events; Willie et al., 2011).

Doppler ultrasound technology emits sound waves that are reflected off moving red blood cells and subsequently detected by the transducer. The frequency difference between the transmitted and received signal is proportional to the velocity of the blood and called the Doppler Shift (Aaslid, 1986). This is expressed by the formula:

$$Doppler\ frequency\ shift = 2\ x\ V\ x\ Ft\ x\ cos\theta / C$$

where *V* is the velocity of the reflector (red cells), *Ft* is the transmitted frequency (2 MHz), *C* is the speed of sound in soft tissue (1540 m/s), and cosθ is the correction factor based on the angle of insonation (θ) (Moppett & Mahajan, 2004).

The middle cerebral artery (MCA) is most commonly insonated because of the ease of access through the temporal window, the quality of the signal, and the vital role the MCA has in CBF perfusion. The MCA arises from the internal carotid artery (ICA) and runs laterally and slightly anteriorly after bifurcation at the ICA (Willie et al., 2011; Figure 3.1). The MCA carries 50-60% of ipsilateral carotid artery blood flow and can be taken as a representation of blood flow to the hemisphere (Moppett & Mahajan, 2004). The posterior cerebral artery (PCA) originates from the basilar artery and supplies blood to the occipital lobe (Willie et al., 2011; Figure 3.1). The PCA is best insonated from the anterior temporal window, often seen simultaneously with the basal vein of Rosenthal running parallel, providing confirmation that the PCA, not MCA, is being insonated (Valdueza et al., 1996). The PCA will also exhibit a smaller signal than the MCA. In ~15% of the population the PCA is supplied by the ICA via the posterior communicating arteries, not the basilar artery (known as a 'fetal-type' PCA), and in these cases can be difficult to insonate and correctly identify (Aaslid, 1986).

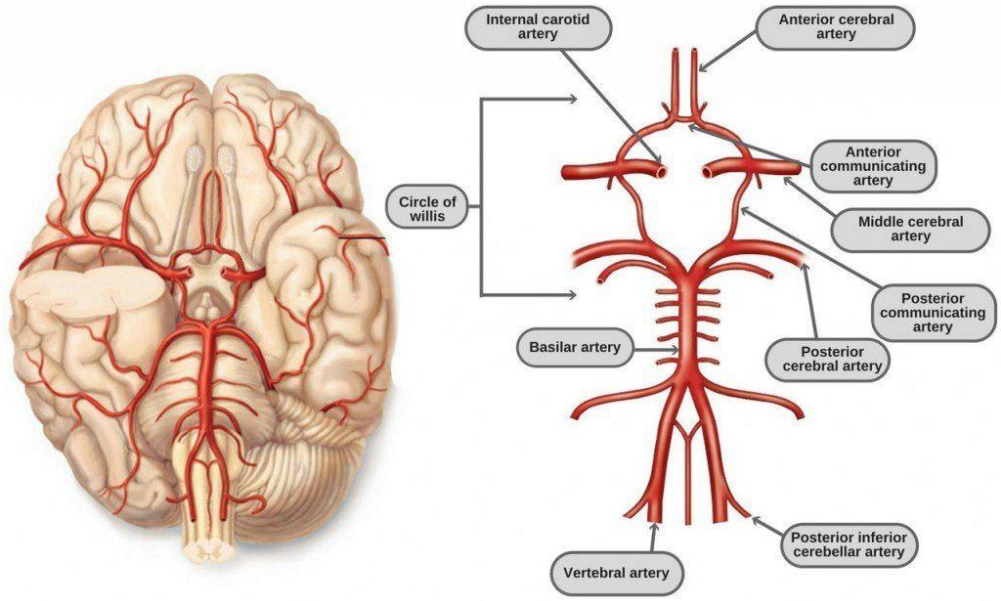

Figure 3.1 The main components of the cerebral circulation and the Circle of Willis (taken from www.biostasis.com).

In this thesis, blood flow velocity in the right middle cerebral artery (MCA*v*) and left posterior cerebral artery (PCA*v*) was measured continuously using a 2-MHz pulsed Doppler ultrasound system (Dopplerbox, DWL Compumedics 156 Ltd, Singen, Germany). Search and positioning techniques described by Aaslid et al. (1982) and Willie et al. (2011) were used to isolate the MCA*v* and PCA*v*. The Doppler probe was adjusted over the temporal window, in the region above the zygomatic arch and 1 to 5 cm in front of the ear. Ultrasound gel was applied to the end of the Doppler probe to improve the signal. During the initial search, Doppler ultrasound parameters were set at a gain of 38 and an insonation depth of 50 mm for the MCA and 65 mm for the PCA. If a faint signal was found, then slight adjustments to the depth, and angle between the probe and skull allowed an optimal signal to be obtained. The MCA was typically found at a depth of ~45-55 mm, and the PCA at a depth of ~60-70 mm, with the direction of

flow towards the transducer. Where possible, the MCA was insonated from the anterior window due to the insonation angle being near-zero, making absolute velocity measurements viable. The PCA was insonated from a posterior direction through the anterior temporal window. It was determined that the anterior cerebral artery was not being measured as it lies deeper than the MCA (~60-75 mm) and its direction of flow is the opposite to that of the MCA and PCA (away from the transducer) (Moppett & Mahajan, 2004). Since the PCA supplies blood flow to the occipital lobe, further positive identification of PCA insonation was obtained from observing the response to a visual stimulus. Briefly, confirmation of the PCA signal was done via checking for a corresponding decrease (eyes closed) and increase (eyes open) in PCA blood flow velocity. Once an appropriate signal was obtained, the Doppler probe was held in place using a headpiece to maintain insonation angle throughout the protocol. In this thesis, PCA insonation was not possible in a number of participants and in this case both left and right MCA*v* was obtained. Example traces for the MCA*v* and PCA*v* can be seen in Figure 3.2.

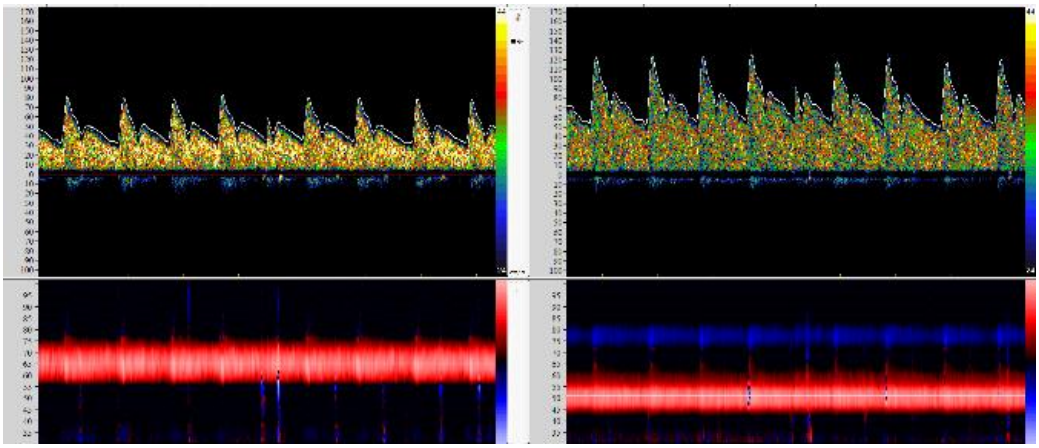

Figure 3.2 A typical trace of the transcranial Doppler signal for one participant, showing the posterior cerebral artery (PCA; left) and middle cerebral artery (MCA; right).

3.3.1.1 Reliability of MCA and PCA measurements

Measurement of MCA*v* and PCA*v* by TCD are highly operator dependent and require practice in order to produce reliable and accurate outcome measures (McMahon et al., 2007). Therefore, this section aims to assess the inter-day repeatability of the MCA*v* and PCA*v* measurements reported in this thesis.

Measurements were performed in 13 healthy young individuals (4 female; age: 23 ± 4 years) on two separate occasions. Measurements were performed at the same time of day at least 24 hours apart, and female participants were tested during the early follicular phase of the menstrual cycle. During each study visit, participants were asked to rest supine for a minimum of 20 minutes in a quiet, temperature-controlled room before measurements began. Where possible the right MCA and left PCA were insonated. If the PCA could not be reliably detected the MCA was insonated on both the left- and right-hand side. Agreement between the inter-day measurements were assessed using the coefficient of variation (CV) and intraclass correlation coefficient (ICC). CV was calculated as:

$$CV = \left(\frac{SD}{\bar{x}}\right) * 100$$

where SD is the standard deviation of the sample and $\bar{x}$ is the sample mean. ICC estimates and their 95% confidence intervals were calculated based on a mean-rating (two measurements), absolute agreement, two-way mixed-effects model (Koo & Li, 2016). ICC values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9, and greater than 0.90 are indicative of poor, moderate, good, and excellent reliability, respectively.

TCD assessment of the MCA (n = 16) and PCA (n= 8) were performed on two separate occasions as described above and stored for offline analysis. MCA*v* and PCA*v* were calculated using LabChart software (ADInstruments, Dunedin, New Zealand).

Inter-day repeatability for MCA*v* had a mean difference of 0.9 cm/s with limits of agreement ranging from -4.7 to 6.6 cm/s, and a CV of 2.65 (95%CI: 1.89-3.41; Figure 3.3). ICC estimates for MCA*v* was 0.98 (95% CI: 0.95-0.99) indicating excellent inter-day reliability across the two days.

Inter-day repeatability for PCA*v* had a mean difference of 1.3 cm/s with limits of agreement ranging from -5.7 to 8.4 cm/s, and a CV of 4.36 (95%CI: 2.10-6.61; Figure 3.3). ICC estimates for PCA*v* was 0.95 (95% CI: 0.81-0.99) indicating excellent inter-day reliability.

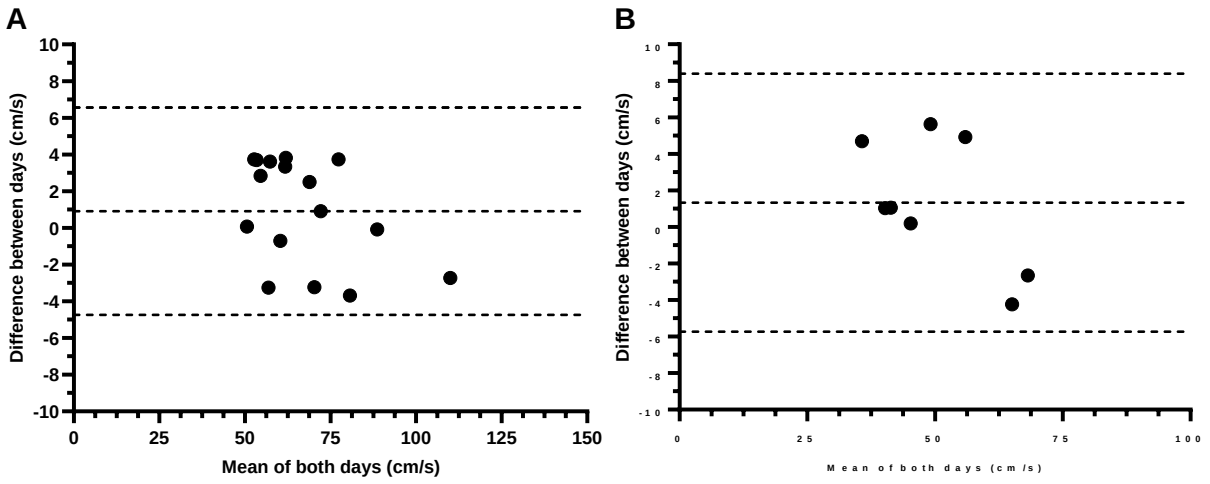

Figure 3.3 Bland-Altman plots of the inter-day repeatability of blood flow velocity measurements in the MCA (panel A) and PCA (panel B). Dashed lines indicate the mean of the differences and the limits of agreement.

In this thesis, assessment of the MCA and PCA blood flow velocity are shown to be reliable, with sufficient measurement agreement between days. Average inter-day CV for both MCA and PCA were <5% and indicates that data are reproducible. End-tidal CO_2 was not measured during this assessment of repeatability and may account for some of the measurement variability.

3.3.2 Cerebrovascular conductance

An index of cerebrovascular conductance (CVC) was calculated from the ratio of mean MCA*v* to mean arterial pressure (MAP), allowing examination of the cerebrovascular response without changes in MAP influencing cerebral blood flow velocity. MAP measurements from beat-to-beat arterial blood pressure were used to calculate CVC where possible, otherwise brachial artery blood pressure was used.

3.3.3 Internal and common carotid artery blood flow

Duplex Doppler techniques can be used to simultaneously measure blood flow velocity and vessel diameter of the extracranial vessels. Such measurements can provide a measure of volumetric cerebral blood flow that TCD cannot, albeit in different vessels (Thomas et al., 2015). There is currently no direct evidence that changes in blood flow or diameter within the extracranial arteries (e.g., ICA, common carotid artery (CCA)) are indicative of similar responses within the intracranial vessels (e.g., MCA, PCA). However, a combination of intra- and extracranial ultrasound can provide complementary information and provide a more global picture of blood flow regulation, as long as such results are interpreted with caution (Thomas et al., 2015).

Assessment of the carotid arteries using duplex ultrasound provides a measure of absolute blood flow, as opposed to the blood flow index provided by TCD (i.e., velocity). Nevertheless, there are a number of limitations that should be considered. Duplex ultrasound assumes parabolic flow, and estimation of the insonation angle with the assumption that this is consistent for all red blood cells in the sample. As noted in the technical recommendations published by Thomas and colleagues (2015), duplex ultrasound requires skilled and practised operators to obtain acceptable and consistent images.

ICA blood flow was assessed in Chapter 6 of this thesis, using a Terason3300 and a 15L4 linear transducer (10 Hz), with ultrasound gel applied between the transducer and skin for image acquisition. At a point usually 1-3 cm below the angle of the jaw the ICA and /or ECA can be seen. Care was taken to correctly differentiate between these vessels, which can be determined in a number of ways, primarily by observing the diameter at the bifurcation (the ICA is usually larger than the ECA; ~6 mm vs. ~3-4 mm), the presence or absence of extracranial branches (ECA and ICA, respectively), and observing the low-resistance spectral Doppler waveform with a gradual upstroke and broad systolic peak indicative of the ICA (Thomas et al., 2015). Once the participant was supine and instrumented, they were instructed to turn their head and neck slightly to the left side. Using Cardiovascular Suite analysis software (Quipu, Pisa, Italy), a two-minute recording of the ICA was obtained, saved and stored offline for analysis.

3.3.3.1 Reliability of ICA and CCA measurements

Although duplex Doppler reproducibility is reported to be good at rest (Schöning & Scheel, 1996), this is dependent on the training and experience of the investigator. Therefore, this section aims to assess the intra- and inter-day repeatability of the ICA and CCA blood flow measurements used in the study described in Chapter 6.

Participants (age: 25 ± 5 years) attended experimental sessions on three separate occasions to assess inter-day reliability (day 1, 2 and 3), with measures performed at the same time of day and at least 24 hours apart. Within each experimental session, ultrasound assessment was performed twice to assess intra-day reliability (measurement a and b), at least an hour apart. Female participants were tested during the same phase of the menstrual cycle. During each study visit, participants were asked to rest supine for a minimum of 20 minutes in a quiet, temperature-controlled room before measurements began. Agreement between the intra- and inter-day measurements was assessed using the coefficient of variation (CV). CV was calculated as:

$$CV = \left(\frac{SD}{\bar{x}}\right) * 100$$

where SD is the standard deviation of the sample and $\bar{x}$ is the sample mean. An inter-day CV of <10% was deemed to be evidence of reproducible data (Thomas et al., 2015). The intraclass correlation coefficient (ICC) was calculated to assess inter-day reliability. ICC estimates and their 95% confidence intervals (CI: lower limit, upper limit) were calculated based on a mean-rating (three measurements), absolute agreement, two-way mixed-effects model (Koo & Li, 2016). ICC values less than 0.50, between

0.50 and 0.75, between 0.75 and 0.90, and greater than 0.90 are indicative of poor, moderate, good, and excellent reliability, respectively.

Doppler ultrasound assessments in the common carotid artery (CCA; n = 11) and internal carotid artery (ICA; n= 8) were performed on three separate occasions as described above and stored for offline analysis. Arterial diameter and blood flow velocity were calculated using Cardiovascular Suite analysis software (Quipu, Pisa, Italy). Blood flow was calculated in each artery using the following equation:

$$Flow = \pi \times \left(\frac{diameter}{2}\right)^2 \times velocity \times 60$$

Table 3.2 Coefficients of variation (mean values and 95% confidence intervals) for resting blood flow in the common carotid artery (CCA) and internal carotid artery (ICA).

	Intra-day (1a to 1b)	**Intra-day (2a to 2b)**	**Intra-day (3a to 3b)**	**Inter-day (1a to 2a)**	**Inter-day (2a to 3a)**
CCA	7.50 (4.48 - 10.53)	10.85 (6.57 - 15.12)	7.00 (4.13 - 9.86)	6.11 (3.63 - 8.59)	3.85 (1.69 - 8.01)
ICA	7.75 (5.31 - 10.19)	10.65 (7.63 - 13.66)	5.00 (1.85 - 8.15)	7.09 (4.50 - 9.67)	8.03 (4.58 -11.49)

Intra-day repeatability (3a-3b) for CCA blood flow had a mean difference of 54.7 mL/min with limits of agreement ranging from -81.3 to 190.7 mL/min and a CV of 7.00 (95%CI: 4.13 – 9.86) (Table 3.2, Figure 3.4). CCA blood flow inter-day repeatability (2a-3a) had a mean difference of 22.8 mL/min with limits of agreement ranging from -

103.5 to 149.1 mL/min and a CV of 3.85 (95% CI: 1.69 – 8.01) (Table 3.2; Figure 3.5). ICC estimates for CCA blood flow was 0.88 (95% CI: 0.69 – 0.97) indicating good inter-day reliability across the three days.

Intra-day repeatability (3a-3b) for ICA blood flow had a mean difference of -7.0 mL/min with limits of agreement ranging from -86.6 to 72.6 mL/min and a CV of 5.00 (95% CI: 1.85-8.15; Table 3.2, Figure 3.4). ICA blood flow inter-day repeatability (2a-3a) had a mean difference of 4.3 mL/min with limits of agreement ranging from -123.6 to 132.3 mL/min and a CV of 8.03 (95% CI: 4.58-11.49) (Table 3.2; Figure 3.5). ICC estimates for ICA blood flow was 0.90 (95% CI: 0.71 – 0.98) indicating good inter-day reliability across the three days.

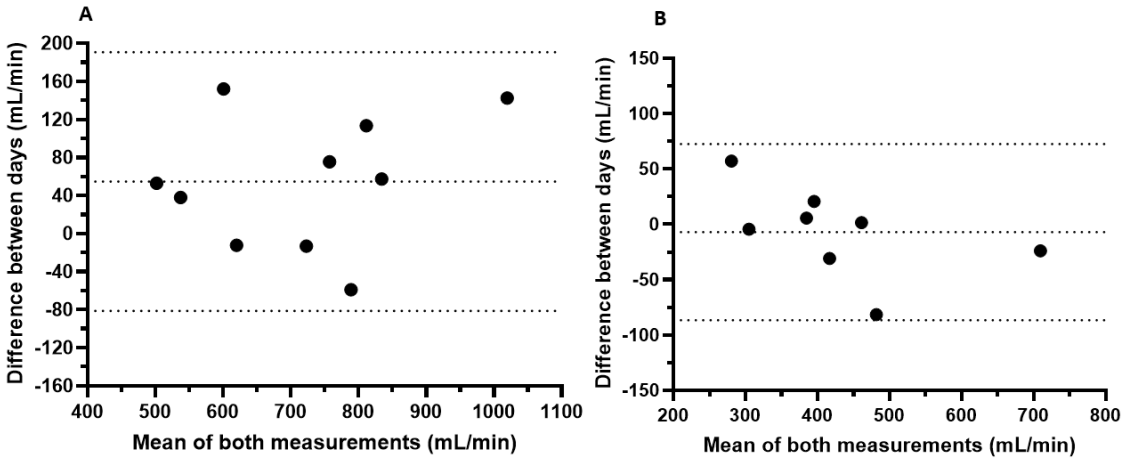

Figure 3.4 Bland-Altman plots of the intra-day (3a-3b) repeatability of blood flow measurements in the CCA (panel A) and ICA (panel B). Dashed lines indicate the mean of the differences and the limits of agreement.

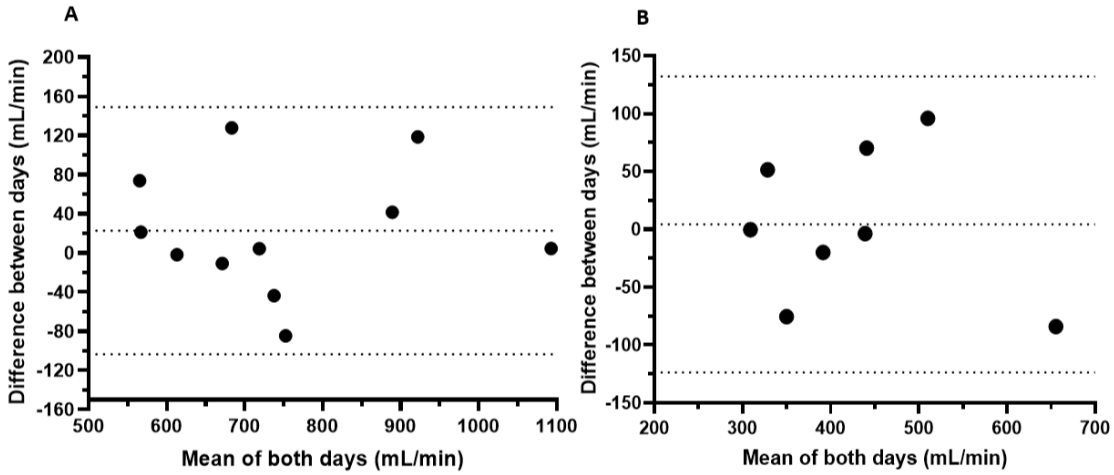

Figure 3.5 Bland-Altman plots of the inter-day (2a-3a) repeatability of blood flow measurements in the CCA (panel A) and ICA (panel B). Dashed lines indicate the mean of the differences and the limits of agreement.

In this thesis, assessment of blood flow of the CCA and ICA are shown to be reliable, with sufficient measurement agreement within and between days. Average intra- and inter-day CV for both CCA and ICA were <10% and indicates that data was reproducible. $P_{ET}CO_2$ was not measured during this assessment of repeatability and may account for some of the measurement variability.

3.3.4 Core Body Temperature

Measurement of core body temperature (T_C) is fundamental to thermal stress research in humans (Byrne & Lim, 2007). However, since 'core temperature' does not refer to one specific location within the body but the temperature of the internal organs, measurements can vary depending on the reference site used.

Rectal temperature is considered the most practical and accurate location to measure T_C, having been shown to provide reliable readings in core temperature at rest in

normothermic conditions (Edwards et al., 2002). However, a disadvantage associated with rectal temperature is the slow response time to rapid changes in T_C compared to other techniques (e.g., oesophageal or pulmonary artery temperature) (Lim et al., 2008). Despite this, rectal temperature is regarded as a 'gold standard' substitute for T_C in thermal illness and research settings due to the practicality and accuracy of the measurement (Moran & Mendal, 2002). It causes little discomfort as opposed to oesophageal temperature measurement, which can cause irritation to nasal passages and participant discomfort throughout an experiment (Moran & Mendal, 2002). Measurement of gut temperature has also proven to be an accurate substitute for T_C, although telemetry pills are expensive and generally non-retrievable (Waterhouse et al., 2005). As such, rectal temperature has proven to be a cost-effective and accurate tool for diagnosis and treatment of conditions such as exertional heat stroke (Casa et al., 2007).

In this thesis, rectal temperature was used because of the balance of measurement accuracy and participant comfort. Rectal temperature was measured at 1-min intervals using a disposable thermistor (General Purpose Temperature Probe 400TM, 150 Mon-a-therm®, Covidien, Mansfield, MA, USA). Participants were instructed to insert the thermistor to a depth of 10 cm past the external anal sphincter. The passive heating protocol used in this thesis (Chapters 5 and 4) was observing a relative 1°C increase in T_C rather than use of absolute values. Careful monitoring of T_C was employed throughout the experiments and reduction of the heat stimulus was enforced before the +1°C target was reached, accounting for the slower response of the rectal measurement and to ensure T_C plateaued at the target value.

3.3.5 Skin temperature

Mean skin temperature (T_S) was measured by the weighted average of four thermistors attached to the skin of the calf, thigh, bicep and chest (Grant EUS-U, Grant 152 Instruments Ltd., Cambridge, United Kingdom). Mean skin temperature was calculated from the standard area weightings (Ramanathan, 1964):

$$T_S = 0.3\,(chest) + 0.3\,(bicep) + 0.2\,(thigh) + 0.20\,(calf)$$

Temperatures were logged at 1-min intervals (Grant 2020 Series Squirrel Data Logger, Grant Instruments, Cambridge, United Kingdom) and subsequently downloaded to a computer.

3.3.6 Respiratory measures

Participants wore a nose peg and breathed through a mouthpiece connected to a two-way, non-rebreathing T-shaped valve. A heated pneumotachograph (3818 Series, Hans-Rudolph Inc, Kansas, MO, USA) was fitted to measure ventilation and respiratory rate. The partial pressure of end-tidal O_2 ($P_{ET}O_2$) and CO_2 ($P_{ET}CO_2$) were sampled from the mouthpiece and measured by a gas analyser (ML240, ADInstruments, Oxford, UK) on a breath-by-breath basis. Prior to testing, the gas analyser was calibrated with known oxygen (O_2) and carbon dioxide (CO_2) gas concentrations. The heated pneumotachometer was calibrated using a 3-L syringe (Hans-Rudolph 5530).

Variables were measured continuously and displayed in real time using an analogue-to-digital convertor (Powerlab, ADInstruments, New Zealand) and interfaced with a computer, which were then stored for subsequent offline analysis.

3.3.7 Arterial blood pressure

Beat-to-beat blood pressure was measured using a finger cuff placed on the middle finger on the left hand (Portapres, Finapres Medical Systems BV, The Netherlands, Chapters 4 and 5; Finapres NOVA, Finapres Medical Systems BV, The Netherlands, Chapter 6). Care was taken to position the light source and detector sensors symmetrically and to wrap the cuff securely around the finger. Once fitted, hand height was corrected to heart level via a levelling mechanism. In normothermic conditions the hand was warmed if necessary, to ensure adequate peripheral blood flow.

The Finapres measures finger arterial pressure using a volume clamp method via a finger cuff and an inflatable bladder, in combination with an infrared plethysmograph consisting of an infrared light source and detector. Infrared light is absorbed by the blood, and the pulsation of arterial diameter during a heartbeat causes a pulsation in the light detector signal. This method determines the point at which finger cuff pressure and intra-arterial pressure are equal, at which point the transmural pressure across the finger arterial walls is zero (the 'unloaded' diameter of the finger arteries; Imholz et al., 1998). Artery diameter is then clamped at this unloaded diameter by modulating the finger cuff pressure. Identifying the correct unloaded diameter of finger arteries is vital for the accuracy of measurement. However, changes in smooth muscle tone, stress and haematocrit can affect the unloaded diameter (Bogert & Van Lieshout, 2005). As such, the unloaded diameter is usually not held constant during a measurement and was intermittently verified via the inbuilt Physiocal algorithm in Finapres devices prior to each testing period. This automated calibration derives the unloaded finger arterial

diameter during periods of constant cuff pressure and adjusts the finger cuff accordingly.

Intermittent blood pressure at the brachial artery using an automated blood pressure cuff (Omron, Kyoto, Japan) was recorded alongside beat-to-beat blood pressure to ensure accurate measurements. Mean arterial blood pressure (ABP) was calculated using the following equation:

$$Mean\ ABP = \frac{1}{3}\ systolic\ ABP + \frac{2}{3}\ diastolic\ ABP$$

Where systolic ABP is systolic arterial blood pressure, and diastolic ABP is diastolic arterial blood pressure.

3.3.8 Heart rate

A 3-lead electrocardiogram (ECG) was used to continuously measure heart rhythm and electrical activity. The positive and negative electrodes were placed on the right and left clavicle respectively, and the earth electrode placed between the seventh and eighth intercostal space.

3.4 Experimental procedures

3.4.1 Cerebrovascular responsiveness

Cerebrovascular-CO_2 responsiveness tests were performed to determine the responsiveness of the MCA (Chapters 4, 5 and 6), PCA (Chapters 5 and 4) and ICA (Chapter 6) to a CO_2 stimulus (see Figure 3.6). While the participant lay supine, a period of baseline data was collected before undergoing two stages of normoxic

hypercapnia, followed by two stages of normoxic hypocapnia (Brothers et al., 2014). Hypercapnia was achieved by inhalation of pre-mixed gas mixtures, firstly containing 2% CO_2, 21% O_2 and balance N_2 and secondly containing 5% CO_2, 21% O_2 and balance N_2, for a period of 4 minutes each. A recovery period with inhalation of room air was allowed to permit respiratory and cerebrovascular variables to return to baseline. Participants were verbally guided to increase the rate and depth of their breathing to reach two target $P_{ET}CO_2$ levels of 30 mmHg and 24 mmHg for a period of 2 minutes each.

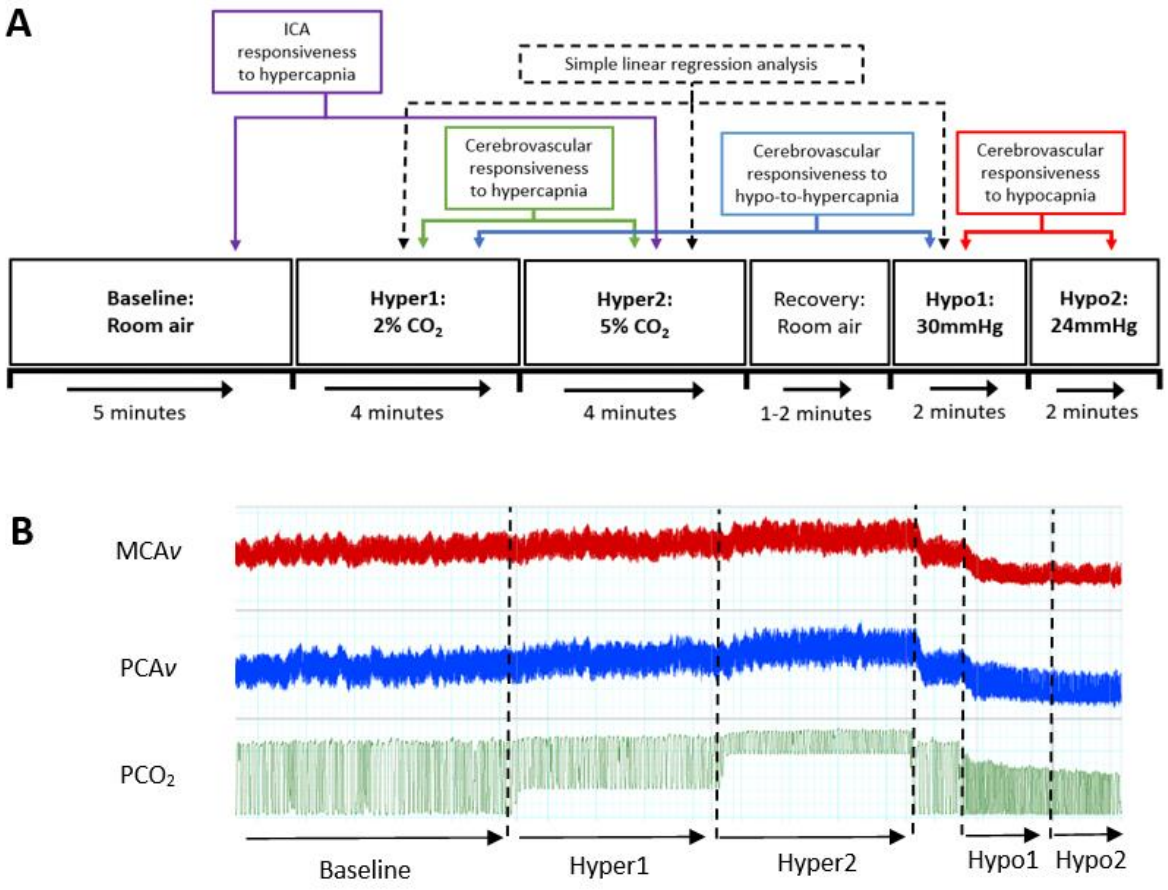

Figure 3.6 Schematic of the cerebrovascular-CO_2 responsiveness tests protocol (panel A) and an example of a raw data trace of the middle cerebral artery velocity (MCA*v*), posterior cerebral artery velocity (PCA*v*) and CO_2 (PCO_2) response across the protocol (panel B). Green, blue, and red arrows indicate points of data extraction to calculate the cerebrovascular responsiveness to hypercapnia (Hyper1 to Hyper2), hypo-to-hypercapnia (Hypo1 to Hyper1), and hypocapnia (Hypo1 to Hypo2), respectively. Purple arrows indicate points of data extraction to calculate the internal carotid artery (ICA) responsiveness to hypercapnia.

As mentioned previously, cerebral blood flow velocity measured by TCD is widely cited as an accurate surrogate for cerebral blood flow, as vessel diameter has been reported to remain constant during CO_2 challenges (Serrador et al., 2000). However, more recent data using MRI has illustrated that this primary assumption of TCD may not be true and that MCA diameter may increase by ~8% and decrease by ~4% in hyper- (+9

mmHg $P_{ET}CO_2$ from baseline) and hypocapnia (-13 mmHg $P_{ET}CO_2$), respectively (Coverdale et al., 2014). Conversely, another MRI study by Verbree and colleagues (2014) reported no significant changes in vessel diameter during hypercapnia (+7.5 mmHg) and hypocapnia (-7.5 mmHg), although they did report a significant ~7% diameter increase during a greater level of hypercapnia (+15 mmHg). Thus, CBF*v* derived from TCD may underestimate CBF by as much as 25% in hypercapnic ranges and overestimate by up to 10% in hypocapnic ranges (Ainslie & Hoiland, 2014). However, it is important to note that these studies used a greater hypercapnic CO_2 stimulus and therefore elicited greater changes in $P_{ET}CO_2$ than those produced in this thesis (~5 mmHg $P_{ET}CO_2$ increase from baseline). The two stages of hypocapnia in this thesis produced a ~7 mmHg and ~13 mmHg decrease in $P_{ET}CO_2$ from baseline, and as such the possible confounding influence of changes in vessel diameter should be considered when interpreting CBF*v* during more extreme hypocapnia. Nonetheless, impaired cerebrovascular-CO_2 responsiveness assessed by TCD has still been found to be associated with numerous pathologies (Willie et al., 2011). Therefore, TCD measures of cerebrovascular-CO_2 responsiveness (aka cerebrovascular reactivity) can still provide valuable information on cerebrovascular health and function, provided data is interpreted with these limitations in mind.

Investigations into cerebrovascular-CO_2 responsiveness have utilised a number of different methods to manipulate P_aCO_2. Firstly, breath-holding to prevent CO_2 elimination allows for a progressive increase in P_aCO_2 with time. The duration of the breath-hold is used as an indication of the strength of the P_aCO_2 stimulus and thus, used to normalise changes in cerebral blood flow, establishing a ‘breath-hold index’

(Kastrup et al., 1999). However, the relationship between breath-holding time and P_aCO_2 has since been shown to be non-linear and highly variable between participants (Totaro et al., 1999). Therefore, breath-holding cannot produce a standardised hypercapnic stimulus or be compared to other vasoactive stimuli (Fierstra et al., 2013). Rebreathing of exhaled gas allows for examination of the ventilatory response (i.e. without the influence of the cerebrovascular response to CO_2), since the PCO_2 gradient between end-tidal and arterial concentrations is abolished (Ainslie & Duffin, 2009). This thesis employed a steady-state CO_2 technique to measure cerebrovascular responsiveness to hypercapnia as it incorporates both the ventilatory and cerebrovascular response to a CO_2 stimulus, and thus is more reflective of real-life breathing conditions. Although inspiration of a fixed fractional concentration of CO_2 has been shown to produce some variation in the cerebrovascular response both within and between participants (Prisman et al., 2008), it is an accessible and easily administrable stimulus frequently employed in the literature.

3.4.2 Cerebral Autoregulation

Cerebral autoregulation is the intrinsic ability of the brain to maintain an adequate cerebral perfusion in the presence of changes in blood pressure (Lassen, 1964). The ability of the cerebrovasculature to maintain adequate perfusion despite changes in blood pressure is vital, and reduced effectiveness of cerebral autoregulation renders the brain more sensitive to both hypo- and hyper-perfusion injury. In Chapter 6 of this thesis, repeated squat-to-stand manoeuvres at two different frequencies were used to manipulate blood pressure for the assessment of dynamic cerebral autoregulation. Participants, once instrumented, were asked to stand and then squat to the sound of

a metronome, holding the squat position until the metronome sounded again and return to a standing position. Repeated squat-to-stand manoeuvres were performed at a frequency of 0.05 Hz (10 s squat, 10 s stand) and 0.10 Hz (5 s squat, 5 s stand) for a period of 5 minutes each (Smirl et al., 2015), and the order of which were randomised between participants. During the squat phase, contraction of the leg muscles increases venous return alongside an increase in resistance caused by contraction of the surrounding vessels. Conversely, when standing there is a reduction in blood pressure caused by the reduction in venous pressure in the legs and subsequent pooling of blood in the lower extremities. Consequently, these manoeuvres evoked oscillations in blood pressure alongside cerebral blood flow velocity in a frequency-dependent manner. Example raw data traces elicited from the squat-to-stand procedures are shown in Figure 3.7.

Cerebral autoregulation was assessed in the low frequency range since higher frequency oscillations in blood pressure (>0.2 Hz) are transferred to the cerebrovasculature unimpeded while lower frequency oscillations (<0.2Hz) are dampened by the cerebral arterioles (Smirl et al., 2015). This is likely because the response of the cerebral arterioles is not fast enough to counteract higher frequency oscillations, and as such characterises cerebral autoregulation as a 'high-pass filter' (Claassen et al., 2009). This pressure-flow relationship can be assessed using transfer function analysis (TFA). TFA is used to describe the relationship between a system's input (blood pressure) and output (CBF*v*) signals, via three outcomes: 1) The amplitude modulation (gain) between blood pressure and CBF*v* indicates the transfer of blood pressure changes into CBF, thus representing how efficiently the cerebral

vessels buffer blood pressure manipulations. A lower gain represents less transfer of blood pressure changes in CBF. 2) The timing of waveforms (phase), where a positive phase shift is interpreted as active vasodilation and constriction in response to changes in pressure. Finally, 3) coherence, which gives an indication of the reliability of the TFA, where a high value (e.g., close to 1) indicates a linear relationship between input and output and a low value (e.g., <0.5) could be a result of multiple factors (e.g., no relationship between input and output, poor signal-to-noise-ratio).

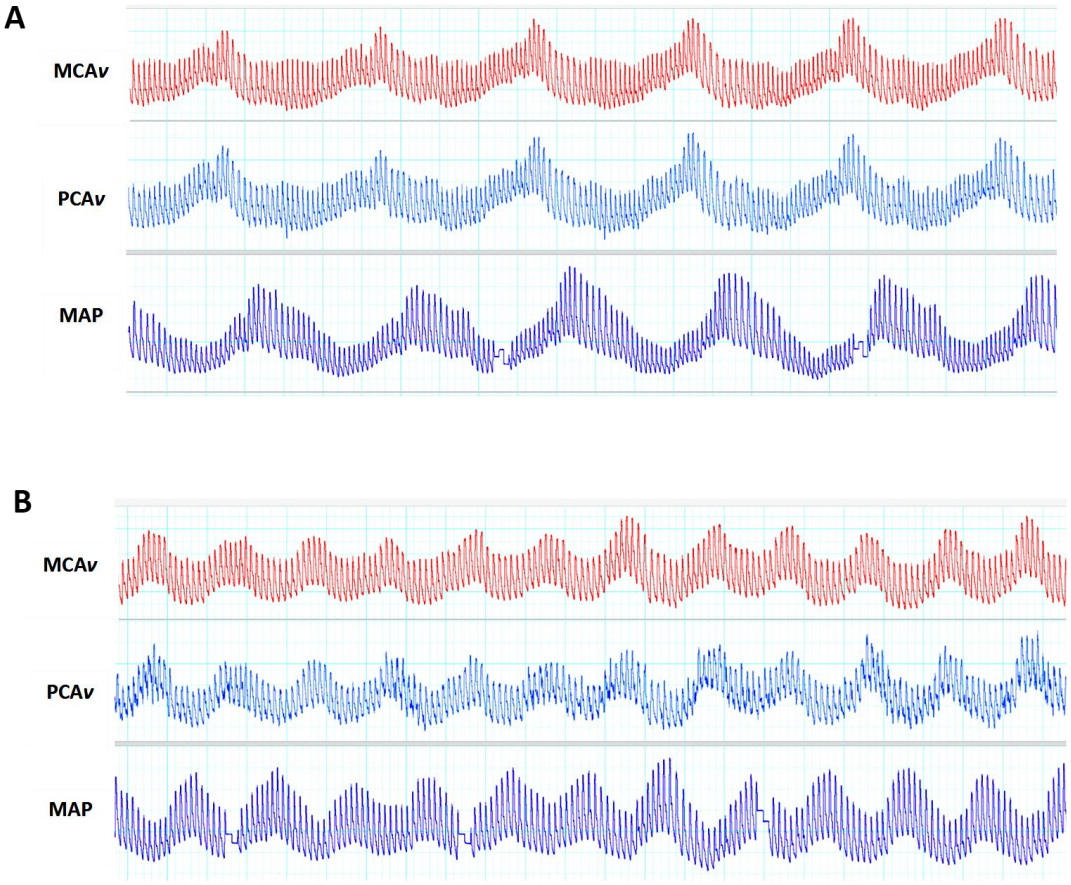

Figure 3.7 Example raw data traces from one participant performing 0.05 Hz (panel A) and 0.10 Hz (panel B) frequency squat-to-stand manoeuvres. MCA*v*, middle cerebral artery velocity; PCA*v*, posterior cerebral artery velocity; MAP, mean arterial pressure.

TFA were performed using Ensemble-R software (Elucimed Ltd, New Zealand). In brief, beat-to-beat blood pressure and flow velocity values are spline interpolated and re-sampled for spectral and transfer function analyses based on the Welch algorithm. This approach subdivides each selected recording period into successive overlapping windows. Data within each window are then linearly detrended and passed through a Hanning window before fast Fourier transform analysis. TFA coherence, gain and phase of the forced MAP oscillations were sampled at the point estimate of the driven frequency (0.05 Hz and 0.10 Hz). All procedures were performed in accordance with the recommendations of the Cerebral Autoregulation Research Network (CARNet; Claassen et al., 2015).

3.4.3 Thermal Stress (water perfusion suit)

To create a thermal stress challenge (Chapters 5 and 4) participants were dressed in a water-perfused tube-lined suit (Med-Eng, Ottawa, Canada) covering the entire body, excluding the head, face, hands and feet. The water perfusion suit allowed control of skin and core temperature by the alteration of the temperature of the circulating water. Participants lay supine with thermo-neutral water (34°C) perfusing the suit to create a constant normothermic environment. Passive heat stress was induced with ~49°C water circulating, for a duration sufficient to cause a 1°C increase in T_C. Participants were also covered in a foil blanket to minimise evaporative heat loss. Once a 0.8°C increase in T_C was achieved, the foil blanket was removed to slow the increase in temperature. Upon reaching the targeted 1°C increase in Tc, water temperature was lowered by ~2°C to maintain T_C for the duration of the cerebrovascular responsiveness tests.

4 THE INFLUENCE OF SEX ON CEREBROVASCULAR FUNCTION

4.1 Introduction

Sex differences in the rate and occurrence of cerebrovascular diseases (e.g., stroke and vascular dementia) indicates a possible sex hormone specific role in brain vascular function and regulation. For example, females have a lower risk of stroke than males during midlife, however their risk doubles in the decade after menopause (Lisabeth & Bushnell, 2012), a time in which endogenous oestrogen and progesterone concentrations decline significantly. Oestrogen has been reported to have a number of neuroprotective effects, including suppression of the inflammatory response and influence perfusion after ischaemic injury (Hurn et al., 1995; Santizo et al., 2002). These oestrogen-related effects are thought to result from the enhanced production and activity of vasodilatory factors associated with prolonged oestrogen exposure (e.g., endothelial NO synthase, prostacyclin pathways; Krause et al., 2006). Androgens (e.g., testosterone) have been reported to have both protective and detrimental effects on the cerebrovasculature (Abi-Ghanem et al., 2020). Specifically, testosterone is believed to act in an opposing manner to oestrogen by enhancing thromboxane-mediated vasoconstriction (Gonzales et al., 2005) and suppressing endothelium-dependent dilation in the cerebrovasculature (Gonzales et al., 2004).

Cerebrovascular responsiveness to CO_2 is an indication of the vasodilatory reserve capacity (Hoiland et al., 2011), with a higher cerebrovascular-CO_2 responsiveness (when measured via TCD) associated with improved cerebrovascular function (Ainslie & Duffin, 2009; Willie et al., 2011). Meanwhile, impairment has been shown to predict the risk of stroke in patients with carotid artery occlusion (Markus & Cullinane, 2001; Webster et al., 1995). Previous research examining sex differences in

cerebrovascular-CO_2 responsiveness has found it to be both greater in females compared to males (Kastrup et al., 1997; Oláh et al., 2000), similar between the sexes (Favre et al., 2020; Peltonen et al., 2015), or greater in males compared to females (when using MRI-derived CBF outcomes; Miller et al., 2019). However, these studies either did not control for the menstrual cycle (Kastrup et al., 1997; Oláh et al., 2000), studied females during the early follicular phase of the menstrual cycle or non-active pill phase of oral contraceptives (Miller et al., 2019; Peltonen et al., 2015), or included females during different menstrual phases within the same cohort (i.e., follicular and luteal phases; Favre et al., 2020). As such, comparison of sex differences in cerebrovascular-CO_2 responsiveness between studies has proved challenging. Additionally, to the best of our knowledge only one study has directly compared males and females' cerebrovascular-CO_2 responsiveness to hypocapnia (Favre et al., 2020). Therefore, how cerebrovascular responsiveness across the entire CO_2 range differs in males and females in different phases of the menstrual cycle remains uncertain.

Potential sex differences in cerebrovascular-CO_2 responsiveness may be further differentiated when the cerebrovasculature is acutely stressed, such as during passive heat stress. Indeed, passive heat stress reduces cerebral blood flow and challenges cerebrovascular regulatory mechanisms. Since elevated oestrogen has been shown to be associated with increases in cerebral blood flow (Krejza et al., 2004), the cerebrovascular response to heat stress may be differentially affected in males and females. At present, the majority of studies investigating passive heat stress and cerebrovascular control consider only males. Studies that include females either do not control for the menstrual cycle or examine females in the early follicular phase

when circulating oestrogen and progesterone are lowest. Thus, whether cerebrovascular-CO_2 responsiveness during passive heat stress is differently affected in males and females is unknown. The addition of this environmental stressor may advance our understanding of the mechanisms underlying any observed differences in cerebrovascular regulation between the sexes.

Subsequently, this study aimed to compare cerebrovascular-CO_2 responsiveness during normothermia and passive heat stress conditions in: 1) males and females in the early follicular (EF) phase of the menstrual cycle; as well as 2) males and females in the ovulatory (O) phase of the menstrual cycle. Previous studies have shown oestrogen stimulates vasodilatory pathways, whereas testosterone promotes vasoconstriction. Subsequently, it was hypothesised that cerebrovascular-CO_2 responsiveness would be: 1) similar between males and females in the early follicular phase, 2) lower in males compared to females in the ovulatory phase, and 3) greater in females during O compared to males during passive heat stress.

4.2 Methods

Ethical approval was obtained for all experimental protocols and procedures by the University of Birmingham Ethics Committee (project code: ERN_15-1179). All testing took place in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Prior to participation, a detailed verbal and written explanation of the study procedure was provided, and written informed consent obtained.

4.2.1 Study Design and Protocol

Thirteen females (age 25 ± 5 years; BMI 23.2 ± 1.4 kg/m^2) and ten males (age 22 ± 3 years; BMI 23.1 ± 1.5 kg/m^2) were enrolled in this study. All participants were healthy and free of any known cardiovascular, neurological or metabolic diseases. Female participants had a regular menstrual cycle (<34 days in length) and were not taking any hormonal contraceptive medication. Participants were informed to abstain from alcohol consumption and vigorous exercise for 24 hours, in addition to caffeine consumption for 12 hours and food consumption for 2 hours prior to the study.

Participants were asked to attend the laboratory for either two or three separate sessions for males and females, respectively: a familiarisation session, and either one or two separate experimental testing sessions. During the familiarisation session, participants were asked to lie in a supine position for ~20 minutes while instrumented with equipment (detailed below). Once instrumented, they performed the cerebrovascular responsiveness tests as detailed previously (Section 3.4.1; Figure 4.1). Once the familiarisation session was satisfactorily completed (i.e., suitable Doppler signals detected and no adverse effects to the CO_2 stimulus), participants were invited back for the experimental testing sessions. Female participants were asked to attend twice, during the early follicular and ovulatory phases of the menstrual cycle and were provided with an oral thermometer and calendar to record basal core temperature throughout their cycle. They were instructed to measure basal core temperature upon waking each morning, prior to consumption of food or water to ensure accurate measurement. The early follicular phase was defined as days 1-4 of the menstrual cycle, where day 1 is the first day of menstruation. Ovulation was defined

as the 48-hour period after a sustained >0.5 °C increase in basal core temperature, approximately halfway through the cycle. To confirm that participants were ovulating, an ovulation test (Clearblue®) was performed following an increase in basal core temperature.

Upon arrival at an experimental testing session, baseline measures of body mass and hydration status (urine sample) were taken. Participants were fitted with thermistors to measure core and skin temperature (detailed below) before dressing in a water-perfused tube-lined suit (Med-Eng, Ottawa, Canada) covering the entire body, excluding the head, face, hands and feet. Water circulating within the perfusion suit was altered to control skin and core temperatures. Thermo-neutral water (34 °C) perfused the suit while participants lay supine for a minimum of 20 minutes during which they were instrumented for measures of cerebro- and cardiovascular function (detailed below). Normothermic cerebrovascular-CO_2 responsiveness tests (Figure 4.1) were then performed, after which passive heat stress was induced by circulating ~49 °C water through the suit until core body temperature (T_C) increased 1 °C. Once the T_C target was reached, the suit's water temperature was lowered by ~2 °C to prevent further increases in T_C during heat stress cerebrovascular-CO_2 responsiveness tests.

4.2.2 Equipment and Outcome Measures

Beat-to-beat middle and posterior cerebral artery blood flow velocity (MCA*v*, PCA*v*) were assessed using transcranial Doppler (TCD; Doppler-Box X, DWL, Compumedics Ltd, Germany) with a 2-MHz probe placed over each temporal window. Ultrasound gel

was placed on the probes and held in place with a headset. Where possible the right MCA and left PCA were insonated, however the PCA could not be positively identified in some participants in which case the MCA was insonated on both sides. Search and identification procedures were performed as detailed previously (Section 3.3.1).

Beat-to-beat blood pressure was measured using a finger cuff on the middle finger of the left hand (Portapres, Finapres, Medical System BV, The Netherlands). Respiratory rate and volume were measured using a heated pneumotachograph (3813 Series, Hans Rudolph Inc, Kansas, USA) attached to a facemask, while fractional changes in inspired and expired O_2 and CO_2 were measured via a sample line attached to the facemask and a gas analyser (ML206, ADInstruments Ltd, New Zealand). Measures were recorded at 1k Hz via an analogue-to-digital converter (Powerlab, ADInstruments) and displayed in real time and stored for offline analysis using commercially available software (LabChart v7.3.5, ADInstruments). Calibration of equipment was performed before each testing session.

T_C was measured using a rectal thermistor (General Purpose Temperature Probe 400TM, Mon-a-therm®, Covidien, Mansfield, MA, USA), and mean skin temperature measured by the weighted average of four thermistors attached to the skin of the calf, thigh, bicep and chest (Grant EUS-U, Grant Instruments Ltd., Cambridge, United Kingdom). Both skin and core temperatures were logged and displayed in real time during testing (Grant 2020 Series Squirrel Data Logger, Grant Instruments, Cambridge, United Kingdom).

4.2.3 Data Analysis

Data from 60 s of the baseline period, 30 s of the hypercapnic stages (from 2:30 min – 3:00 min; Burley et al., 2020), and >20 s of data at the target end-tidal CO_2 during the hypocapnic stages, were extracted and used in the statistical analyses.

The slope of the cerebral blood flow (CBF) response to changes in $P_{ET}CO_2$ was calculated to give an estimation of cerebrovascular-CO_2 responsiveness in the MCA and PCA (MCA*v*-CO_2 responsiveness; PCA*v*-CO_2 responsiveness). Cerebrovascular responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were determined by the slope of the CBF response from stages Hyper1 to Hyper2, Hypo1 to Hypo2, and Hypo1 to Hyper1, respectively (see Figure 4.1).

The pulsatility index in the MCA and PCA (MCA*v*-PI; PCA*v*-PI) was calculated as (systolic CBF*v* – diastolic CBF*v*) / mean CBF*v*. An index of cerebrovascular conductance (CVC) was also calculated from the ratio of CBF to mean arterial pressure (MAP), allowing examination of cerebrovascular-CO_2 responsiveness without changes in MAP influencing cerebral blood flow velocity. An estimation of CVC to CO_2 was expressed as the change in CVC per mmHg change in $P_{ET}CO_2$ (MCA*v*-CVC-CO_2 responsiveness, PCA*v*-CVC-CO_2 responsiveness).

4.2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 8.0.0, GraphPad Software, San Diego, CA, USA). Two-way repeated measures ANOVAs were used for all outcome variables. Main and interaction effects of sex (2 levels: males

vs EF or males vs O) and thermal condition (2 levels: normothermia vs heat stress) were tested. Simple linear regressions were performed on cerebrovascular-CO_2 responsiveness outcome measures to obtain a best-fit value of the CBF response across the $P_{ET}CO_2$ range (stages Hypo1, Hyper1 and Hyper2; see Figure 4.1). The line of best fit is reported as $y = bx + a$, where b is the slope of the line and a is the *y*-intercept when $x=0$. The slope and *y*-intercept values were compared between sex (males vs EF; males vs O) and thermal conditions (normothermia vs heat stress). Data are presented as means ± SD. Statistical significance were based on an α-level of 0.05.

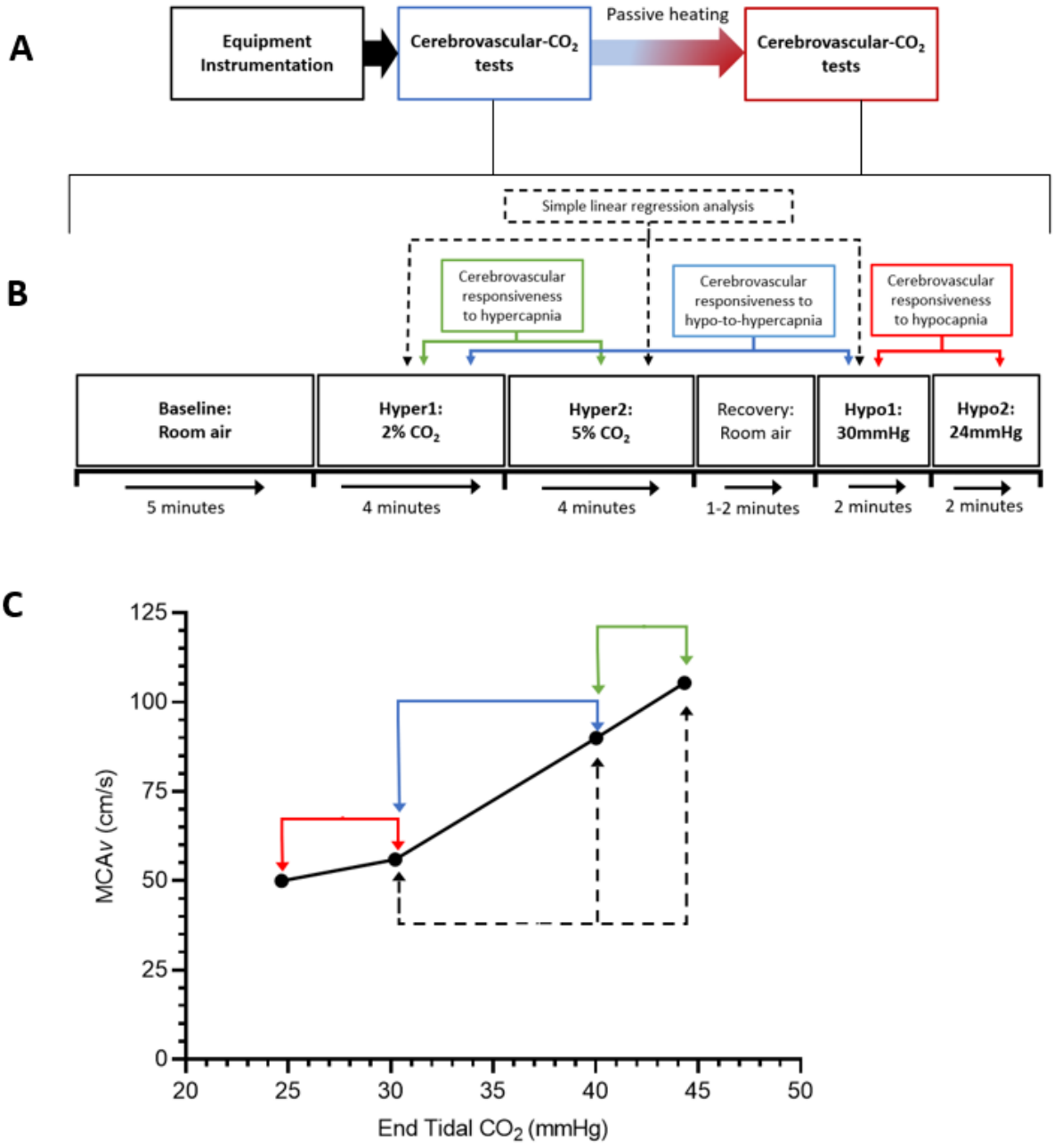

Figure 4.1 Schematic of the study protocol. Panel **A** provides an overview of the experimental session, with Panel **B** shows the cerebrovascular-CO_2 responsiveness tests protocol (performed twice, during normothermia and passive heat stress conditions). Panel **C** shows an example graph of the cerebrovascular responsiveness slope. Green, blue, and red arrows indicate points of data extraction to calculate the cerebrovascular responsiveness to hypercapnia (Hyper1 to Hyper2), hypo-to-hypercapnia (Hypo1 to Hyper1), and hypocapnia (Hypo1 to Hypo2), respectively. Dashed back arrows indicate points of data extraction used in simple linear regression analysis.

4.3 Results

Of the thirteen female participants enrolled in the study, eight completed both experimental sessions, and five completed one session. Subsequently, the number of participants completing the EF and O testing sessions were 12 and 9, respectively. Of the ten males enrolled, one was excluded due a baseline $P_{ET}CO_2$ below that of the Hyper1 target $P_{ET}CO_2$.

4.3.1 Males vs. Females during Early Follicular (EF) phase

4.3.1.1 Baseline Measures

Baseline responses can be found in Table 4.1. With heat stress, core body temperature increased (+1.1 ± 0.1 °C; *thermal condition*: $p<.01$) and skin temperature increased (+3.2 ± 0.8 °C; *thermal condition*: $p<.01$) to a similar extent in both males and females during EF (s*ex*: $p>.14$; *sex x thermal condition*: $p>.30$). With heat stress, heart rate increased (+26 ± 11 bpm; *thermal condition*: $p<.01$) and mean arterial pressure decreased (-12 ± 11 mmHg; *thermal condition*: $p<.01$) when compared to normothermia, with no difference between males and females during EF phase (*sex:* $p>.72$; *sex x thermal condition: p*>.29). End-tidal CO_2 was lower during heat stress (-1.9 ± 2.9 mmHg; *thermal condition*: $p=.01$) with no difference between males and females during EF (*sex*: $p=.17$; *sex x thermal condition: p*=.18). MCA*v* was on average higher in females, although this difference did not reach the threshold for significance ($p=.06$). Heat stress lowered MCA*v* similarly for both sexes (-9 ± 10 cm/s; *thermal condition*: $p<.01$; *sex x thermal condition*: $p=.10$). PCA*v* was significantly greater in females during EF compared to males (+8 ± 3 cm/s; *sex*: $p=.02$), decreasing with heat

stress to a similar extent in both groups (-6 ± 8 cm/s; *thermal condition*: *p*=.01; *sex x thermal condition*: *p*=.61). MCA*v*-PI and PCA*v*-PI increased during heat stress (+0.17 ± 0.14 and +0.24 ± 0.26, respectively; *thermal condition*: *p*<.01) with no difference between males and females during EF (*sex*: *p*>.17; *sex x thermal condition: p*>.14). There was a significant interaction for MCA*v*-CVC (*sex x thermal condition*: *p*=.05), with post hoc analysis showing that in normothermia, MCA*v*-CVC was greater in females during EF compared to males (*p*=.03), however, during heat stress there was no difference between sexes (*p*=.46). PCA*v*-CVC was greater in females during EF compared to males (+0.13 ± 0.05 cm/s/mmHg; *sex*: *p*=.02) in both thermal conditions (*thermal condition: p*=.30; *sex x thermal condition: p*=.33). There was no difference in ventilation between males and females during EF (*sex*: *p*=.55) or thermal conditions (*thermal condition: p*=.17; *sex x thermal condition*: *p*=.47).

Table 4.1 Baseline thermoregulatory, cardiovascular, respiratory, and cerebrovascular responses during normothermia and passive heat stress in males and females during two phases of the menstrual cycle.

	Females, Early Follicular (n = 12)		Females, Ovulatory (n = 9)		Males (n = 9)	
	Normo-thermia	Heat Stress	Normo-thermia	Heat Stress	Normo-thermia	Heat Stress
T_C (°C)	36.8 ± 0.1	37.9 ± 0.2#	37.1 ± 0.2	38.1 ± 0.3#	36.9 ± 0.2	38.1 ± 0.2#
T_S (°C)	34.1 ± 0.6	37.1 ± 0.9#	33.8 ± 0.8	37.6 ± 0.7#	33.9 ± 0.4	37.4 ± 0.5#
Heart Rate (bpm)	66 ± 13	90 ± 13#	66 ± 9	94 ± 8#	62 ± 4	91 ± 9#
Mean Arterial Pressure (mmHg)	76 ± 16	66 ± 11#	79 ± 14	66 ± 9#	77 ± 9	63 ± 8#
Ventilation (L/min)	5.8 ± 1.1	6.9 ± 2.7	6.2 ± 1.3	7.7 ± 2.8	6.6 ± 1.4	7.0 ± 2.3
End-Tidal CO_2 (mmHg)	37.8 ± 2.0	35.1 ± 4.3#	36.8 ± 3.7	34.7 ± 6.1#	38.6 ± 2.9	37.8 ± 2.4#
MCA*v* (cm/s)	83 ± 18	71 ± 20#	79 ± 14*	73 ± 16*	66 ± 8	62 ± 9#
PCA*v* (cm/s)	56 ± 8*	50 ± 10#*	53 ± 11	48 ± 11	47 ± 6	43 ± 5#
MCA*v*-PI	0.72 ± 0.12	0.89 ± 0.20#	0.79 ± 0.23	0.95 ± 0.30#	0.83 ± 0.12	1.00 ± 0.11#
PCA*v*-PI	0.76 ± 0.17	0.94 ± 0.29#	0.74 ± 0.07	0.92 ± 0.19#	0.85 ± 0.23	1.21 ± 0.50#
MCA*v*-CVC (cm/s/ mmHg)	1.12 ± 0.26*	1.09 ± 0.31	1.01 ± 0.18	1.12 ± 0.26#	0.86 ± 0.09	0.98 ± 0.14#
PCA*v*-CVC (cm/s/ mmHg)	0.77 ± 0.16*	0.77 ±0.16*	0.67 ± 0.17	0.74 ± 0.18#	0.60 ± 0.06	0.67 ± 0.09#

T_C, core body temperature; T_S, mean skin temperature; MCA*v*, middle cerebral artery velocity; PCA*v*, posterior cerebral artery velocity; MCA-PI, middle cerebral artery pulsatility index; PCA-PI, posterior cerebral artery pulsatility index; MCA*v*-CVC, middle cerebral artery conductance; PCA*v*-CVC, posterior cerebral artery conductance. Values are means ± SD, n-1 for PCA outcome variables. *significantly different from males; #significantly different from normothermia.

4.3.1.2 Middle Cerebral Artery Responsiveness

MCA*v*-CO_2 responsiveness to hypercapnia, hypocapnia, and hypo-to-hypercapnia were not significantly different between males and females during EF (*sex*: $p>.06$) or between thermal conditions (Figure 4.2; *thermal condition*: $p>.22$; *sex x thermal condition*: $p>.28$). Simple linear regression analysis of MCA*v*-CO_2 responsiveness found similar slopes ($p=.30$) but differing *y*-intercepts ($p<.01$), with females during EF having a higher *y*-intercept compared to males and heat stress having a lower *y*-intercept compared to normothermia in both sexes (EF normothermia, $y=3.50x-49.76$; males normothermia, $y=2.57x-32.26$; EF heat stress, $y=3.30x-47.29$; males heat stress, $y=2.44x-29.75$).

With heat stress, MCA*v*-CVC-CO_2 responsiveness to hypo-to-hypercapnia increased compared to normothermia (0.05 ± 0.03 vs 0.03 ± 0.01 cm/s/mmHg, *thermal condition*: $p=.02$) in both males and females during EF (*sex*: $p=.09$; *sex x thermal condition*: $p=.62$). There was no difference in MCA*v*-CVC-CO_2 responsiveness to hypercapnia and hypocapnia between males and females during EF (*sex*: $p>.12$) or between thermal conditions (thermal condition: $p>.57$; *sex x thermal condition*: $p>.30$).

4.3.1.3 Posterior Cerebral Artery Responsiveness

There was a significant interaction for PCA*v*-CO_2 responsiveness to hypercapnia (Figure 4.3; *sex x thermal condition*: $p=.03$). Post hoc analysis showed that in males, PCA*v*-CO_2 responsiveness to hypercapnia decreased from normothermia to heat stress (-0.88 ± 0.89 cm/s/mmHg; $p<.01$), and during heat stress was lower in males compared to females during EF (1.62 ± 0.84 vs 2.77 ± 0.72 cm/s/mmHg, $p<.01$). In

females during EF, PCA*v*-CO_2 responsiveness to hypercapnia remained similar between thermal conditions (*p*=.98) and compared to males in normothermia (*p*=.63). PCA*v*-CO_2 responsiveness to hypocapnia and hypo-to-hypercapnia was lower in males when compared to females during EF (-0.58 ± 0.35 vs. -0.92 ± 0.33 cm/s/mmHg and 1.65 ± 0.37 vs 2.39 ± 1.00 cm/s/mmHg, respectively; *sex*: *p*<.05) in both thermal conditions (*thermal condition*: *p*>.58; sex *x thermal condition*: *p*>.20). Simple linear regression analysis of PCA*v*-CO_2 responsiveness found similar slopes (*p*=.08) but differing *y*-intercepts (*p*<.01), with females during EF having a higher *y*-intercept compared to males and heat stress having a lower *y*-intercept compared to normothermia in both sexes (EF normothermia, *y*=2.46*x*-36.35; males normothermia, *y*=1.83*x*-22.42; EF heat stress, *y*=2.28*x*-32.37; males heat stress, *y*=1.61*x*-17.39).

PCA*v*-CVC-CO_2 responsiveness to hypercapnia had a significant interaction effect (*sex x thermal condition*: p=.05). Post hoc analysis showed that in males, PCA*v*-CVC-CO_2 responsiveness to hypercapnia decreased from normothermia to heat stress (by -0.01 ± 0.01 cm/s/mmHg *p*=.05). In females during EF, PCA*v*-CVC-CO_2 responsiveness to hypercapnia remained similar between thermal conditions (*p*=.98) and was not different to males in normothermia (*p*=.47) or heat stress (*p*=.29). PCA*v*-CVC-CO_2 responsiveness to hypocapnia was greater in females during EF when compared to males (-0.014 ± 0.005 vs. -0.009 ± 0.005 cm/s/mmHg, *sex*: *p*=.01) in both thermal conditions (*thermal condition*: *p*=.65; *sex x thermal condition*: *p*=.23). There was no significant difference in PCA*v*-CVC-CO_2 responsiveness to hypo-to-hypercapnia between males and females during EF (*sex*: *p*=.10) or between thermal conditions (thermal condition: *p*=.06; *sex x thermal condition*: *p*=.49).

Table 4.2 Cerebrovascular-CO_2 responsiveness values for the middle cerebral artery (MCA*v*-CO_2 responsiveness) and posterior cerebral artery (PCA*v*-CO_2 responsiveness) during normothermia and heat stress in males and females during two phases of their menstrual cycle. 'Full range' refers to the linear regression line of best-fit which includes the Hypo1, Hyper1 and Hyper2 stages of the cerebrovascular responsiveness protocol.

		Early Follicular		*Ovulatory*		*Males*	
		Normothermia	Heat Stress	Normothermia	Heat Stress	Normothermia	Heat Stress
MCAv-CO_2 responsiveness (cm/s/mmHg)	Hypercapnia	3.61 ± 0.73	3.58 ± 1.16	3.97 ± 1.14	3.37 ± 1.27	3.15 ± 0.94	2.62 ± 1.47
	Hypocapnia	-1.06 ± 0.52	-1.15 ± 0.47	-1.60 ± 0.59*	-1.30 ± 0.53*	-0.78 ± 0.41	-0.81 ± 0.25
	Hypo-to-hypercapnia	3.13 ± 1.29	3.10 ± 1.61	3.43 ± 1.04*	3.00 ± 1.45*	2.40 ± 0.57	2.37 ± 0.36
	Full range	3.50 ± 0.92	3.30 ± 0.91	3.59 ± 0.99	3.45 ± 0.70	2.57 ± 0.56	2.44 ± 0.57
PCAv-CO_2 responsiveness (cm/s/mmHg)	Hypercapnia	2.83 ± 0.81	2.77 ± 0.72	3.21 ± 0.77*	2.98 ± 0.94*#	2.49 ± 0.33	1.62 ± 0.84*#
	Hypocapnia	-0.95 ± 0.38*	-0.88 ± 0.29*	-1.18 ± 0.36*	-0.92 ± 0.33*	-0.50 ± 0.27	-0.66 ± 0.42
	Hypo-to-hypercapnia	2.38 ± 0.47*	2.41 ± 1.37*	2.35 ± 1.16	1.77 ± 1.50	1.67 ± 0.45	1.64 ± 0.29
	Full range	2.46 ± 0.49	2.28 ± 0.79	2.54± 0.89	2.38 ± 0.77	1.83 ± 0.34	1.61 ± 0.30

Values are means ± SD. *significantly different from males; #significantly different to normothermia

4.3.2 Males vs. Females during the Ovulatory (O) phase

4.3.2.1 Baseline Measures

Baseline responses can be found in Table 4.1. With heat stress, core body temperature increased (+1.1 ± 0.1 °C; *thermal condition*: $p<.01$) and skin temperature increased (+3.6 ± 1.0 °C; *thermal condition*: $p<.01$) to a similar extent in both males and females during O (*sex*: $p>.50$; *sex x thermal condition*: $p>.08$). With heat stress, heart rate increased (+29 ± 11 bpm; *thermal condition*: $p<.01$) and mean arterial pressure decreased (-14 ± 8 mmHg; *thermal condition*: $p<.01$) when compared to normothermia, with no difference between males and females during O for either of these responses (*sex:* $p>.27$; *sex x thermal condition:* $p>.82$). With heat stress, end-tidal CO_2 decreased (-1.5 ± 2.4 mmHg; *thermal condition*: $p=.02$) in both males and females during O (*sex*: $p=.20$; *sex x thermal condition*: $p=.30$). MCA*v* was greater in females during O compared to males (76 ± 15 vs 64 ± 9 cm/s; *sex*: $p=.03$), decreasing with heat stress (-5 ± 9 cm/s; *thermal condition*: $p=.03$) to a similar extent in both groups (*sex x thermal condition*: $p=.73$). MCA*v*-PI and PCA*v*-PI increased during heat stress (+0.17 ± 0.14 and +0.26 ± 0.27, respectively; *thermal condition*: $p<.01$) with no difference between males and females during O (*sex*: $p>.15$; *sex x thermal condition:* $p>.20$). With heat stress, MCA*v*-CVC increased (+0.11 ± 0.12 cm/s/mmHg; *thermal condition*: $p<.01$) compared to normothermia in both males and females during O (*sex*: $p=.07$; *sex x thermal condition*: $p=.85$). With heat stress, both PCA*v* (-5 ± 9 cm/s; *thermal condition*: $p<.01$) and PCA*v*-CVC (-0.07 ± 0.09 cm/s/mmHg; *thermal condition*: $p<.01$) decreased, with this response being similar in males and females during O (*sex*: $p>.19$; *sex x thermal condition*: $p>.80$). There was no difference in ventilation between males and

females in O (*sex*: *p*=.98) or between thermal conditions (*thermal condition: p*=.10; *sex x thermal condition*: *p*=.34).

4.3.2.2 Middle Cerebral Artery Responsiveness

MCA*v*-CO_2 responsiveness to hypocapnia and hypo-to-hypercapnia was greater in females during the O phase compared to males (Figure 4.2; -1.45 ± 0.56 vs. -0.79 ± 0.33 cm/s/mmHg and 3.21 ± 1.24 vs. 2.38 ± 0.46 cm/s/mmHg, respectively, *sex*: *p*<.05) in both thermal conditions (*thermal condition*: *p*>.20; *sex x thermal condition: p*=.13). There was no difference in MCA*v*-CO_2 responsiveness to hypercapnia between males and females during O (*sex*: *p*=.14) or between thermal conditions (*thermal condition: p*=.06; *sex x thermal condition*: *p*=.90). Simple linear regression analysis of MCA*v*-CO_2 responsiveness found similar slopes (*p*=.11) but differing *y*-intercepts (*p*<.01), with females during O having a higher *y*-intercept compared to males and heat stress having a lower *y*-intercept compared to normothermia in both sexes (O normothermia, *y*=3.59*x*-50.48; males normothermia, *y*=2.57*x*-32.26; O heat stress, *y*=3.45*x*-49.33; males heat stress, *y*=2.44*x*-29.75).

MCA*v*-CVC-CO_2 responsiveness to hypocapnia was greater in females during the O phase compared to males (-0.02 ± 0.02 vs -0.01 ± 0.01 cm/s/mmHg; *sex*: *p*<.01) in both thermal conditions (*thermal condition*: *p*=.35; *sex x thermal condition*: *p*=.32). There was no difference in MCA*v*-CVC-CO_2 responsiveness to hypercapnia and hypo-to-hypercapnia between males and females during O (*sex*: *p*>.58) or between thermal conditions (thermal condition: *p*>.33; *sex x thermal condition*: *p*>.41).

4.3.2.3 Posterior Cerebral Artery Responsiveness

PCA*v*-CO_2 responsiveness to hypercapnia was greater in females during O compared to males (Figure 4.3; 3.10 ± 0.84 vs. 2.06 ± 0.76 cm/s/mmHg; *sex*: $p<.01$), decreasing with heat stress (-0.56 ± 0.93 cm/s/mmHg; *thermal condition*: $p=.03$) to a similar extent in both groups (*sex x thermal condition*: $p=.17$). PCA*v*-CO_2 responsiveness to hypocapnia was greater in females during O compared to males (-1.05 ± 0.36 vs. -0.58 ± 0.35 cm/s/mmHg; *sex*: $p<.01$) in both thermal conditions (*thermal condition*: $p=.69$; *sex x thermal condition*: $p=.09$). There was no difference in PCA*v*-CO_2 responsiveness to hypo-to-hypercapnia between males and females during O (*sex*: $p=.19$) or between thermal conditions (thermal condition: $p=.46$; *sex x thermal condition*: $p=.50$). Simple linear regression analysis of PCA*v*-CO_2 responsiveness found similar slopes ($p=.11$) but differing *y*-intercepts ($p<.01$), with females during O having a higher *y*-intercept compared to males and heat stress having a lower *y*-intercept compared to normothermia in both sexes (O normothermia, $y=2.54x-37.53$; males normothermia, $y=1.83x-22.42$; O heat stress, $y=2.38x-33.92$; males heat stress, $y=1.61x-17.39$).

There was a significant interaction effect for PCA*v*-CVC-CO_2 to hypercapnia (*sex x thermal condition*: $p=.01$). Post hoc analysis showed that in males, PCA*v*-CVC-CO_2 responsiveness to hypercapnia decreased with heat stress (by -0.01 ± 0.01 cm/s/mmHg: $p=.03$) and was lower in males compared to females during O during heat stress (0.02 ± 0.01 vs 0.03 ± 0.01 cm/s/mmHg, $p=.01$). In females during O, PCA*v*-CVC-CO_2 responsiveness to hypercapnia remained similar between thermal conditions ($p=.55$) and there was no difference between groups in normothermia ($p=.97$). PCA*v*-CVC-CO_2 responsiveness to hypocapnia was greater in females during

O compared to males (-0.02 ± 0.01 vs -0.01 ± 0.01 cm/s/mmHg; *sex*: *p*=.01) in both thermal conditions (*thermal condition*: *p*=.30; *sex x thermal condition*: *p*=.13). There was no difference in PCA*v*-CVC-CO_2 responsiveness to hypo-to-hypercapnia between females during O and males (sex: *p*=.96) or between thermal conditions (thermal condition: *p*=.89; *sex x thermal condition*: *p*=.39).

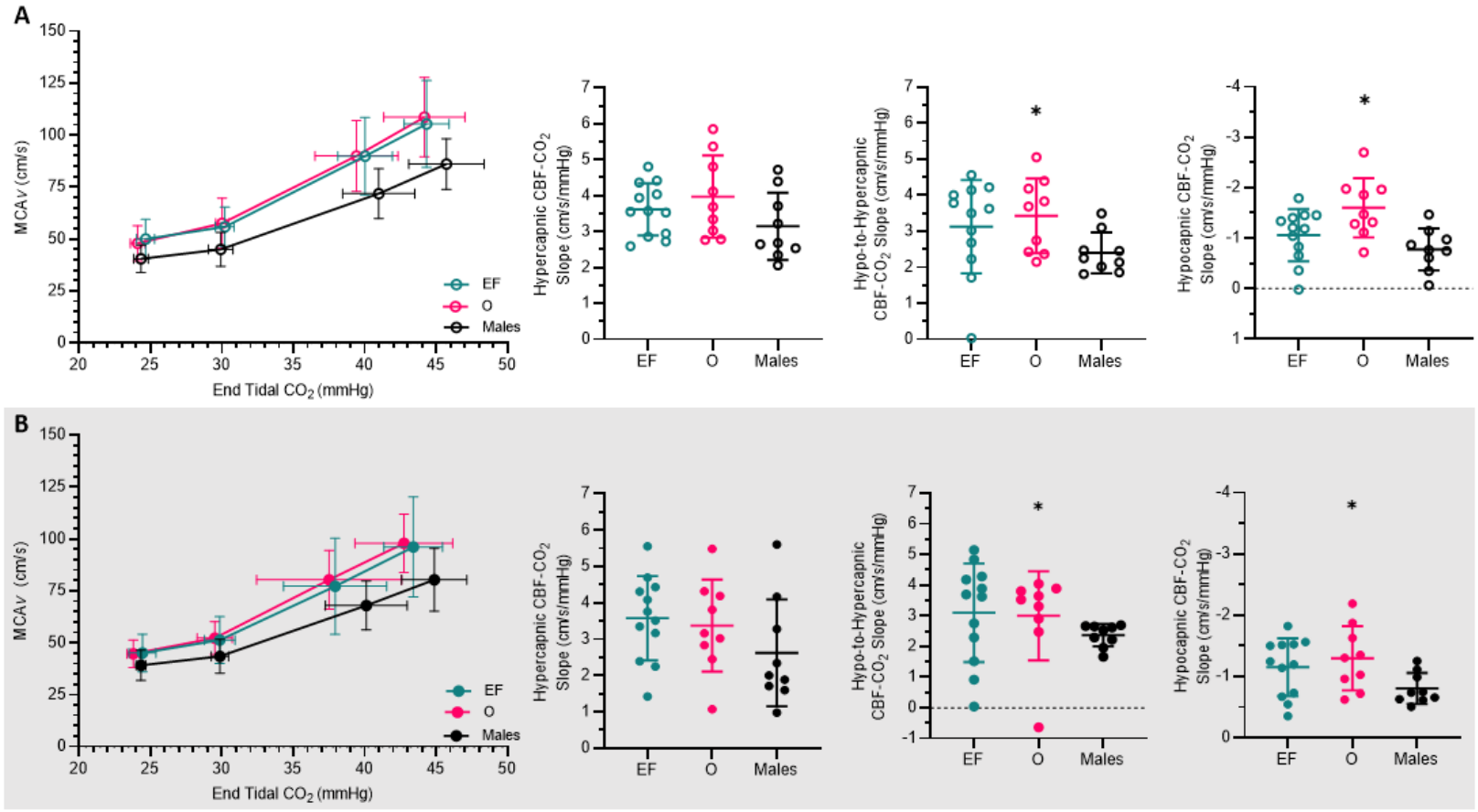

Figure 4.2 The middle cerebral artery blood flow velocity (MCA*v*) slope at hyper-, hypo-to-hyper- and hypocapnia (MCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in males (n=9), and females during the early follicular (EF; n = 12) and ovulatory (O; n = 9) phases of the menstrual cycle. *significantly different to males.

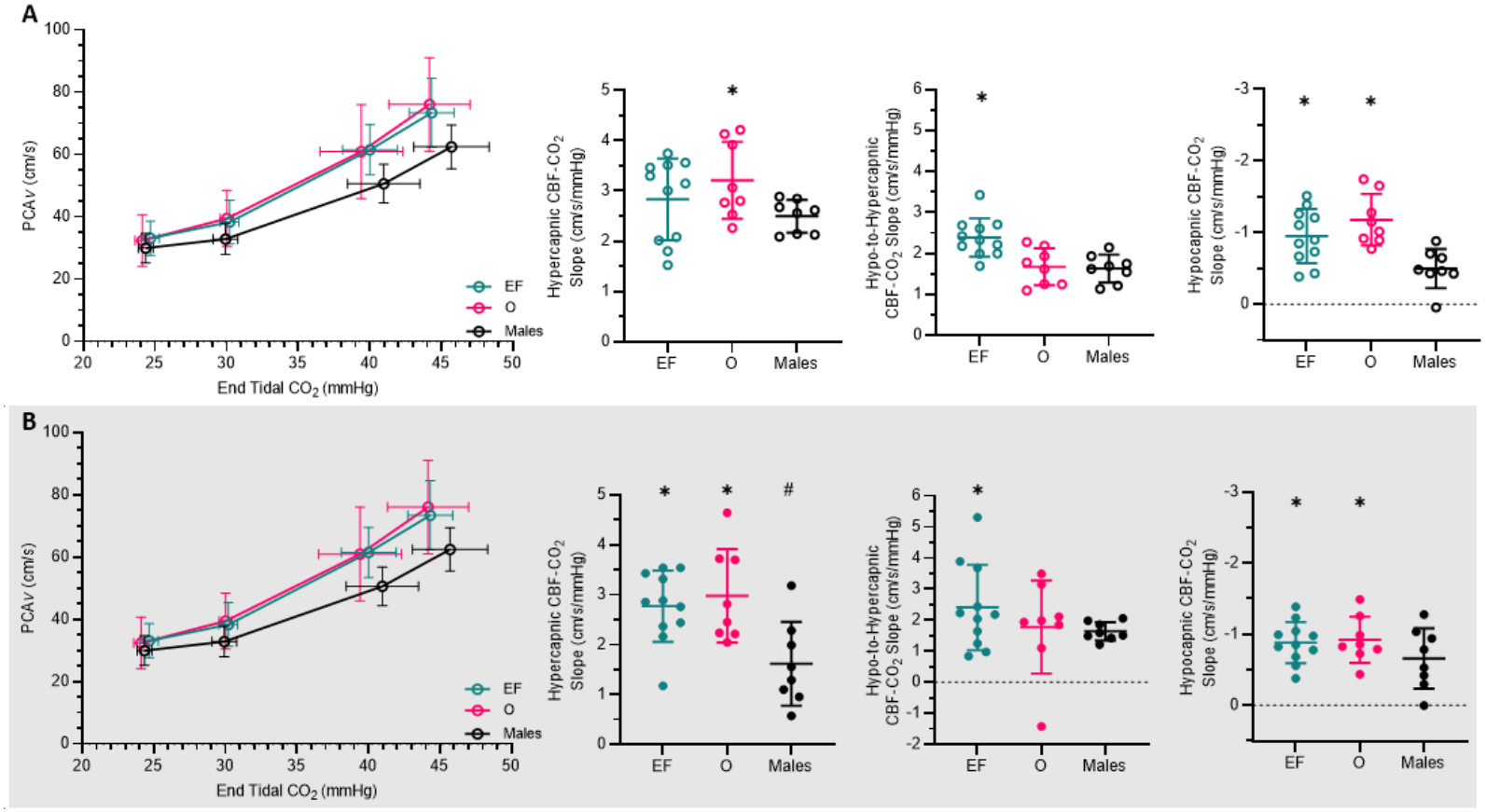

Figure 4.3 The posterior cerebral artery blood flow velocity (PCA*v*) slope at hyper-, hypo-to-hyper- and hypocapnia (PCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in males (n = 8), and females during the early follicular (EF; n = 11) and ovulatory (O; n = 8) phases of the menstrual cycle. *significantly different to males; #significantly different to normothermia.

4.4 Discussion

The main findings of this study were that: 1) regardless of thermal condition, MCA*v* responsiveness to hypercapnia and hypocapnia was similar between males and females during the early follicular (EF) phase. Conversely, PCA*v* responsiveness to hypo-to-hypercapnia and to hypocapnia was lower in males compared to females during the EF phase; 2) regardless of thermal condition, MCA*v* responsiveness to hypo- and to hypo-to-hypercapnia were lower in males compared to females during the ovulatory (O) phase. However, PCA*v* responsiveness to hypercapnia was lower in males compared to females during the O phase. Finally, 3) PCA*v* responsiveness in the hypercapnic range was differentially affected by heat stress in males as compared to females in the EF phase. Collectively, these findings indicate that sex can influence cerebrovascular-CO_2 responsiveness, dependent on the phase of the menstrual cycle. Furthermore, there appears to be regional sex differences in cerebrovascular-CO_2 responsiveness. Finally, heat stress appears to differentially affect the vasodilatory capacity of the PCA between males and females during the EF phase.

4.4.1 Effect of sex on cerebrovascular-CO_2 responsiveness

It was hypothesised that cerebrovascular-CO_2 responsiveness would be similar between males and females during the EF phase, and lower in males compared to females in the O phase of the menstrual cycle since greater circulating oestrogen has been shown to promote vasoactive factors. Previous research investigating sex differences have shown cerebrovascular-CO_2 responsiveness to be either greater in females compared to males (Kastrup et al., 1997; Oláh et al., 2000), similar between

the sexes (Favre et al., 2020; Peltonen et al., 2015), or greater in males compared to females (Miller et al., 2019). Differences between studies in outcome measure (i.e., cerebral blood flow vs. cerebral blood flow velocity), control of the menstrual cycle (i.e., no control vs. early follicular phase or non-active pill phase of oral contraception), and the method of examining cerebrovascular responsiveness (i.e., drug-induced vasodilation vs. CO_2 manipulation) may account for the discrepancy in reported results. As hypothesised, the present study found no differences in MCA*v* responsiveness between males and females during the EF phase. PCA*v* responsiveness to hypercapnia was also similar between males and females during EF, however PCA*v* responsiveness to hypo-to-hyper and hypocapnia was lower in males compared to females in the EF phase under both normothermic and heat stress conditions. Previous studies have primarily only insonated the MCA and investigated the cerebrovascular vasodilatory response (i.e., hypercapnia), while the present study investigates both the MCA and PCA at four points across the CO_2 range. While the present study did not formally compare the MCA*v*-CO_2 and PCA*v*-CO_2 responsiveness outcomes, the present observations indicate possible regional differences in cerebrovascular-CO_2 responsiveness to hypocapnia between males and females (discussed further below), and that this difference is present even during the EF phase of the menstrual cycle. Subsequently, studies investigating cerebrovascular responsiveness to hypocapnia in the posterior circulation should consider the different responses exhibited by males and females.

In line with our hypothesis, the present study found MCA*v* responsiveness to hypocapnia was lower in males compared to females in the ovulatory phase of the

menstrual cycle. Circulating oestrogen is associated with lower cerebrovascular tone (Krause et al., 2006) and greater basal cerebral blood flow velocity in the internal carotid artery (Krejza et al., 2001) and the MCA (Peltonen et al., 2016), which may have allowed females in the ovulatory phase to have a greater vasoconstrictive capacity in response to the hypocapnic stimulus. Conversely, circulating testosterone increases cerebrovascular tone (Gonzales et al., 2004), acting in an opposing manner to oestrogen and potentially limiting the cerebrovascular responsiveness in males. In support of this, the present study found resting cerebral blood flow velocity in the MCA was significantly greater in females during O, and approaching significance during EF, compared to males. Consistent with the MCA, PCA*v* responsiveness to hypo- and hypercapnia were also lower in males compared to females during the O phase. Although there was no significant difference in resting PCA*v* between males and females during O, females still had a higher resting PCA*v* than males on average (53 ± 11 cm/s vs. 47 ± 6 cm/s; p=.18). As such, cerebrovascular-CO_2 responsiveness appears to be similar in the anterior and posterior between males and females during O. However, in females during EF, only PCA*v* responsiveness to hypocapnia was shown to be greater than males, indicating lower oestrogen in the EF phase attenuates sex differences in MCA*v* responsiveness. This may indicate that regional differences in cerebrovascular-CO_2 responsiveness between males and females are driven by not only differences in circulating oestrogen levels, but also differences in circulating levels of other sex hormones. Indeed, the effects of both oestrogen and testosterone contribute to cerebrovascular tone in both males and females (Krause et al., 2011). As such, it is likely the ratio of circulating oestrogen *and* testosterone in males and females influence observed differences in basal cerebral blood flow and cerebrovascular-CO_2

responsiveness in the current study. Additionally, absolute levels of circulating oestrogen and progesterone throughout the menstrual cycle is particularly varied between females (Windham et al., 2002). Subsequently, the effects of sex hormones on the cerebrovasculature are likely to be highly individualistic, occurring along a continuum rather than as definitive male-female differences specific to certain phases of the menstrual cycle.

In the current study, sex differences in cerebrovascular-CO_2 responsiveness appeared to differ between the anterior and posterior circulation. MCA*v*-CO_2 responsiveness remained similar between males and females, apart from a lower MCA*v* responsiveness to hypocapnia in males compared to the females in the O phase. However, PCA*v*-CO_2 responsiveness was predominantly lower in males compared to females during both the EF and O phase. Previous studies have only insonated the MCA due to its accessible location close to the temporal window. Early cerebrovascular studies assumed that vascular responses measured in the MCA were an adequate representation of the global cerebrovascular response (Ainslie & Duffin, 2009). However, regional differences in cerebrovascular responsiveness to CO_2 have since been reported, with the posterior circulation having a blunted hypercapnic response compared to the anterior circulation (Sato et al., 2012; Skow et al., 2013). Conversely, Perko et al. (2011) found cerebrovascular responsiveness to L-arginine administration (i.e., a drug-induced vasodilatory response) was greater in the PCA as compared to the MCA, as well as greater in females compared to males for both vessels. These conflicting results may arise from different insonated vessels (e.g., PCA, basilar artery) or the differing vasoactive stimuli employed (e.g., hyperoxic rebreathing, L-arginine

administration). Regional differences in the vasoconstrictive capacity of the cerebrovasculature have been examined less extensively, with one previous study showing similar levels of responsiveness to hypocapnia between the MCA and PCA (Willie et al., 2012). Differences in vessel size between the MCA and PCA may be a contributing factor to possible region-specific responses, with the MCA having a greater diameter and subsequently, greater flow compared to the PCA. Further, known sex differences in vessel size may exacerbate this regional difference (Müller et al., 1991). While the present study did not directly compare the MCA*v*- and PCA*v*-CO_2 responsiveness outcomes, the observed sex differences in PCA*v*-CO_2 responsiveness appeared more robust than that observed within the MCA, potentially indicating regional differences in CBF regulation between males and females, although further investigation across the CO_2 range is warranted to confirm this.

4.4.2 Effect of thermal condition on cerebrovascular-CO_2 responsiveness

It was hypothesised that in heat stress, females would have greater cerebrovascular responsiveness to CO_2 than males. At present, the majority of studies investigating passive heat stress and cerebrovascular control consider only males, and existing data makes it unclear how thermoregulation and cerebrovascular regulation interact (Barnes & Charkoudian, 2021). For example, previous studies have reported cerebrovascular-CO_2 responsiveness to increase (Lucas et al., 2008), decrease (Ogoh et al., 2014), or remain similar (Low et al., 2008) during heat stress when compared to normothermia. These contradictory results may arise from the order in which cerebrovascular-CO_2 responsiveness tests were performed. For example, Ogoh et al. (2014) performed the hypo- and hypercapnic challenges in a randomised order, while

Low et al. (2008) performed hypocapnic followed by hypercapnic breathing challenges. Indeed, there is now evidence to indicate prior hypocapnia can attenuate the subsequent hypercapnic cerebrovascular response (Brothers et al., 2014). However, despite performing hypercapnia prior to hypocapnia, the present study found MCA*v* responsiveness during heat stress to be unchanged compared to normothermia and, in contrast to our hypothesis, similar between males and females under heat stress conditions. In contrast, males had a reduced PCA*v* responsiveness to hypercapnia during heat stress when compared to normothermia, and PCA*v* responsiveness to hypercapnia during heat stress was lower in males compared to females in the EF phase in the current study. Heat stress-induced regional differences in cerebrovascular-CO_2 responsiveness have been reported, with enhanced responsiveness in the external carotid artery (ECA) and reduced responsiveness in the internal carotid artery (ICA; Ogoh et al., 2014). Since the ECA mainly supplies the cutaneous circulation, increased ECA responsiveness during heat stress likely acts as a thermoregulatory mechanism to aid heat dissipation, and ICA responsiveness decreases to aid in the CBF regulatory response to heat stress (Ogoh et al., 2013). The present study indicates heat stress also causes regional differences in cerebrovascular responsiveness in the intracranial arteries. Since the MCA and PCA originate from different extracranial arteries (the ICA and basilar artery, respectively), region-specific heat stress responses in the intracranial vessels might be expected.

Previous studies examining cerebrovascular responsiveness to CO_2 during heat stress have primarily only examined males, and those that do include females do not formally compare the sexes (Barnes & Charkoudian, 2021). We found heat stress caused no

change in PCA*v* responsiveness for females in either the EF or O phase. However, in line with our hypothesis, males had a lower PCA*v*-CO_2 and PCA*v*-CVC-CO_2 responsiveness to hypercapnia during heat stress compared to the EF phase, and a lower PCA*v*-CVC-CO_2 responsiveness to hypercapnia compared to the O phase. An index of CVC-CO_2 responsiveness allows for examination of cerebrovascular responsiveness without heat-induced reductions in mean arterial pressure influencing the observed cerebral blood flow velocity response. The reduced PCA-CVC-CO_2 responsiveness could indicate that males have greater vasodilation in the PCA during heat stress, reducing their capacity for further dilation in response to hypercapnia. Cerebrovascular tone could not be directly measured in the present study (i.e., blood velocity measures only), and while heat stress caused a decrease in resting PCA*v* and increase in PI, there was no significant difference in this decrease between males and females. The observed sex-specific change in PCA*v*-CO_2 responsiveness during heat stress indicates a difference between males and females in cerebrovascular regulation to a thermal stressor.

There are established sex differences in blood pressure control between males and females. For example, young females appear to have less autonomic control of blood pressure (Christou et al., 2005) and lower muscle sympathetic nerve activity (Wehrwein et al., 2010) when compared to young men. These sex-specific differences in the control of blood pressure could have implications for cerebral blood flow regulation since blood flow is dependent on perfusion pressure. The present study found no differences between the sexes in baseline mean arterial pressure during passive heating, and differences in PCA*v*-CVC-CO_2 to hypercapnia between males

and females remained despite accounting for changes in blood pressure. This indicates that sex differences in blood pressure control does not translate to differences in cerebrovascular control, although the specific interaction between sex, blood pressure control, and cerebrovascular responsiveness warrants further investigation.

4.4.3 Considerations / Study limitations

Cerebral blood flow velocity is generally reported to be higher in females compared to males, although this is largely attributed to a smaller vessel diameter rather than hormonal differences between the sexes (Müller & Schimrigk, 1994). We found resting MCA*v* to be significantly greater in females during the O phase and approaching significance during the EF phase when compared to males, and PCA*v* only greater in females during the EF phase when compared to males. In order to account for sex differences in cerebral blood flow velocity resulting from vessel diameter, relative changes in cerebrovascular responsiveness can be reported. We have reported the absolute change in cerebrovascular-CO_2 responsiveness here as it is more pertinent to distinguishing clinically relevant changes in cerebrovascular function, as well as allowing for direct comparison to other populations. However, for completeness the relative changes in cerebrovascular-CO_2 responsiveness are reported in Appendix D.

We used Doppler ultrasound to measure blood flow velocity in the MCA and PCA. A primary assumption of TCD is that the insonated vessel maintains a constant diameter. While this assumption has been validated (Valdueza et al., 1997; Serrador et al., 2000), more recent MRI studies have reported changing vessel diameters in response to

changing CO_2 (Coverdale et al., 2014; Verbree et al., 2014), which may result in possible over- and under-estimations of CBF when CBF*v* is used as an index of absolute flow. Despite this, assessment of cerebrovascular responsiveness by TCD has been shown to offer valuable information on cerebrovascular health and function, provided data are interpreted with these limitations in mind (Willie et al., 2011).

Additionally, changes in $P_{ET}CO_2$ were used to calculate cerebrovascular responsiveness, based on the assumption that $P_{ET}CO_2$ accurately represents arterial CO_2. While these two variables can differ with metabolic CO_2 production and tidal volume, they do not differ by changes in breathing frequency (Jones et al., 1979) and has been validated over a range of core temperatures (Brothers et al., 2011).

In states of augmented hypo- or hypercapnia, the cerebrovasculature may reach a point of maximal constriction or dilation. The P_aCO_2 threshold for reaching the limit of the vessel calibre has been shown to be ~65 mmHg in the hypercapnic range and ~25 mmHg in the hypocapnic range (Harper & Glass, 1965). In the present study, $P_{ET}CO_2$ increased to ~44 mmHg during Hyper2, and decreased to ~24 mmHg during Hypo2. If the limit of the vessel calibre is reached, further diameter changes are not possible and a change in cerebrovascular-CO_2 responsiveness may not be represented by further changes in CBF*v*. To account for this, the Hypo2 stage was omitted from the simple linear regression, with analysis using the Hypo1, Hyper1 and Hyper 2 stages.

The present study employed a steady-state CO_2 technique to measure cerebrovascular-CO_2 responsiveness. This technique was chosen as it incorporates both the ventilatory and cerebrovascular response to a steady-state CO_2 stimulus.

However, ventilatory-CO_2 responsiveness has not been reported here as it was outside the remit of the thesis. To the best of our knowledge, sex differences in ventilatory-CO_2 responsiveness in conjunction with cerebrovascular-CO_2 responsiveness have not been examined. Females have previously been reported to have a lower ventilatory threshold in response to CO_2 when compared to males during a rebreathing protocol (MacNutt et al., 2012), suggesting differences in ventilatory control between the sexes. The use of different techniques to measure cerebrovascular- or ventilatory-CO_2 responsiveness (i.e., steady-state, rebreathing) may elicit different outcomes as they stimulate physiological systems in a different manner. For example, a rebreathing technique abolishes the PCO_2 gradient throughout the body (e.g., between end-tidal and arterial concentrations) and therefore, measures only the ventilatory response unaffected by the cerebrovascular response (Ainslie & Duffin, 2009). As such, the cerebrovascular outcomes reported herein may encapsulate possible sex differences in ventilatory responsiveness given the interaction between the vascular and the ventilatory response when using a steady-state CO_2 technique.

The present study did not control for several population demographics that may affect the reported outcome measures, including physical activity levels, sedentary behaviour, and training status. Greater aerobic fitness has previously been shown to be associated with lower baseline cerebral blood flow and greater cerebrovascular-CO_2 responsiveness (Burley et al., 2017; Foster et al., 2020). Therefore, differences in fitness levels between males and females may contribute to some of the observed variation in cerebrovascular outcomes between cohorts.

4.4.4 Summary

The findings of this study show that sex differences in cerebrovascular-CO_2 responsiveness are most evident during the ovulatory phase of the menstrual cycle. This supports previous evidence that increased oestrogen has a significant impact on the cerebrovascular function and regulation. Regional differences between the anterior and posterior circulation appear to be sex-specific, however further investigation formally comparing cerebral vessels is warranted. Diminished PCAv-CO_2 responsiveness to hypercapnia during heat stress is only evident in males, indicating that the cerebrovascular response to thermal stress is sex dependent.

5 THE INFLUENCE OF THE MENSTRUAL CYCLE ON CEREBROVASCULAR FUNCTION

5.1 Introduction

Sex differences in the rate and occurrence of cerebrovascular diseases (e.g., stroke and vascular dementia) indicate a possible role for sex hormones in brain vascular function and regulation. Increasing levels of oestrogen have been shown to lower cerebrovascular impedance (Krejza et al., 2004) and increase basal cerebral blood flow velocity (Krejza et al., 2001). Additionally, oestrogen has been reported to have neuroprotective effects, including suppression of the inflammatory response and influence on perfusion after ischaemic injury (Hurn et al., 1995; Santizo et al., 2002). These oestrogen-related effects are thought to result from the enhanced production and activity of vasodilatory factors associated with prolonged oestrogen exposure (e.g. endothelial NO synthase, prostacyclin pathways; Krause et al., 2006). However, the effects of progesterone on the cerebrovasculature are less clear, with the literature suggesting it both promotes and reduces the inflammatory response (Gibson et al., 2005; Sunday et al., 2006). Of note, progesterone is believed to counteract the vasodilatory properties of oestrogen (Krause et al., 2006). Thus, the opposing actions of oestrogen and progesterone may affect cerebrovascular function across the menstrual cycle.

The capacity for the cerebrovasculature to respond to vasoactive stimuli provides important information on brain vascular function. Cerebrovascular responsiveness to CO_2 is an indication of the vasodilatory reserve capacity (Hoiland et al., 2011), with a higher cerebrovascular-CO_2 responsiveness (when measured via TCD) associated with improved cerebrovascular function (Ainslie & Duffin, 2009; Willie et al., 2011).

Meanwhile, impairment has been shown to predict the risk of stroke in patients with carotid artery occlusion (Markus & Cullinane, 2001; Webster et al., 1995). Krejza and colleagues (2013) have shown cyclic increases in circulating oestrogen to be associated with a greater vasodilatory capacity in the extracranial arteries (measured using duplex Doppler-derived CBF). Conversely, Peltonen et al. (2016) found cerebrovascular-CO_2 responsiveness to hypercapnia to be similar between early and late follicular phases (using TCD-derived CBF*v*). Differences in the examined vessels and methods of assessment make it unclear how cerebrovascular responsiveness is changed across the menstrual cycle. Further, at present, no study has examined differences in the vasoconstrictive capacity of the cerebrovasculature between menstrual phases. Determining how acute fluctuations in oestrogen and progesterone influence cerebrovascular responsiveness across the entire CO_2 range will improve the understanding of their role in cerebrovascular health and disease.

Passive heat stress is an experimental model that acutely stresses the cerebrovasculature. Indeed, passive heat stress reduces cerebral blood flow and challenges cerebrovascular regulatory mechanisms, although existing data presents an unclear picture for how thermoregulation and cerebrovascular regulation interact (Barnes & Charkoudian, 2021). For example, cerebrovascular-CO_2 responsiveness has been shown to increase (Lucas et al., 2008), decrease (Ogoh et al., 2014), or remain similar (Low et al., 2008) during passive heat stress when compared to normothermic conditions. Furthermore, the majority of studies investigating passive heat stress and cerebrovascular control consider only males. Elevated oestrogen and progesterone (i.e., the mid luteal phase) is associated with an elevation in core body

temperature compared to the early follicular phase when hormone levels are comparatively low. This occurs alongside an integrative shift in thermoregulation; whereby onset of cutaneous vasodilation, sweating, shivering and cutaneous vasoconstriction shifts to a higher body temperature (Kolka & Stephenson, 1997). Furthermore, since elevated oestrogen has been shown to be associated with increases in cerebral blood flow (Krejza et al., 2004), the cerebrovascular response to heat stress may be differentially affected during high hormone (i.e. ovulatory) and low hormone (i.e. early follicular) phases of the menstrual cycle. Whether cerebrovascular-CO_2 responsiveness during passive heat stress is differentially affected with cyclic fluctuations in sex hormones across the menstrual cycle is not known. The addition of this environmental stressor may advance our understanding of the mechanisms underlying any observed differences in cerebrovascular regulation across the menstrual cycle.

Subsequently, this study aimed to compare cerebrovascular-CO_2 responsiveness during normothermia and passive heat stress conditions during: 1) the early follicular compared to the ovulatory phase, and 2) the early follicular compared to the mid luteal phase of the menstrual cycle. Previous studies have shown that oestrogen promotes vasodilatory pathways, whereas progesterone counteracts this and promotes vasoconstriction. As such, it was hypothesised that cerebrovascular responsiveness would be: 1) greater during ovulation compared to the early follicular phase; 2) similar between early follicular and mid luteal phases, and 3) greater during ovulation compared to the early follicular phase during passive heat stress.

5.2 Methods

Ethical approval was obtained for all experimental protocols and procedures by the University of Birmingham Ethics Committee (project code: ERN_15-1179). All testing took place in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Prior to participation, a detailed verbal and written explanation of the study procedure was provided, and written informed consent obtained.

5.2.1 Study Design and Protocol

Thirteen females (age 25 ± 5 years; BMI 23.2 ± 1.4 kg/m^2) participated in this study and are included in the analysis. All participants were healthy and free of any known cardiovascular, neurological or metabolic diseases. Participants had a regular menstrual cycle (<34 days in length) and were not taking any hormonal contraceptive medication. Participants were informed to abstain from alcohol consumption and vigorous exercise for 24 hours, in addition to caffeine consumption for 12 hours and food consumption for 2 hours prior to the study.

Participants were asked to attend the laboratory for four separate sessions: a familiarisation session, and three separate experimental testing sessions during the early follicular (EF), ovulatory (O), and mid-luteal (ML) phases of their menstrual cycle. During the familiarisation session, participants were asked to lie in a supine position for ~20 minutes while instrumented with equipment (detailed below). Once instrumented, they performed the cerebrovascular-CO_2 responsiveness tests as detailed previously (Section 3.4.1; Figure 5.2). Once the familiarisation session was

satisfactorily completed (i.e., suitable Doppler signals detected and no adverse effects to the CO_2 stimulus), participants were invited back for the experimental testing sessions. Participants were provided with an oral thermometer and calendar to record basal core temperature throughout their cycle. They were instructed to measure basal core temperature upon waking each morning, prior to consumption of food or water to ensure accurate measurement. The early follicular phase was defined as days 1-4 of the menstrual cycle, where day 1 is the first day of menstruation. Ovulation was defined as the 48-hour period after a sustained >0.5 °C increase in basal core temperature, approximately halfway through the cycle. To confirm that participants were ovulating an ovulation test (Clearblue®) was performed following an increase in basal core temperature. The mid-luteal phase was 8-10 days post-ovulation, depending on total cycle length.

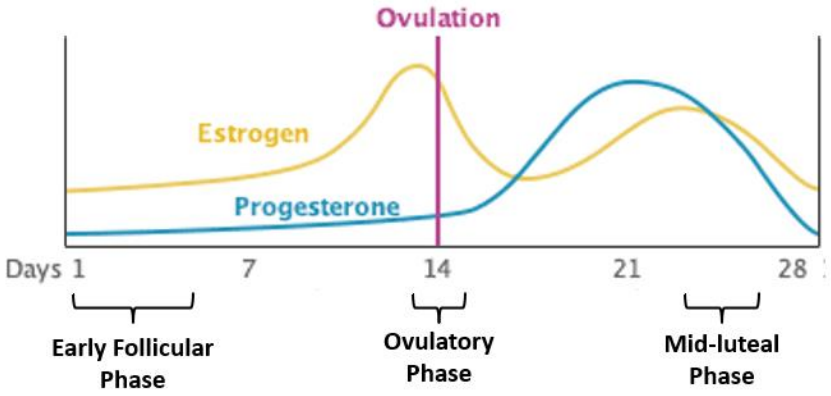

Figure 5.1 The hormone profile for a normally menstruating female. Adapted from womeninbalance.org.

Upon arrival at an experimental testing session, baseline measures of body mass and hydration status (urine sample) were taken. Participants were fitted with thermistors to measure core and skin temperature (detailed below) before dressing in a water-

perfused tube-lined suit (Med-Eng, Ottawa, Canada) covering the entire body, excluding the head, face, hands and feet. Water circulating within the perfusion suit was altered to control skin and core temperatures. Thermo-neutral water (34 °C) perfused the suit while participants lay supine for a minimum of 20 minutes during which they were instrumented for measures of cerebro- and cardiovascular function (detailed below). Normothermic cerebrovascular-CO_2 responsiveness tests (Figure 5.2) were then performed, after which passive heat stress was induced by circulating ~49 °C water through the suit until core body temperature (T_C) increased by 1 °C. Once the T_C target was reached, the suit's water temperature was lowered by ~2 °C to prevent further increases in T_C during heat stress cerebrovascular-CO_2 responsiveness tests.

5.2.2 Equipment and Outcome Measures

Beat-to-beat middle and posterior cerebral artery blood flow velocity (MCA*v*, PCA*v*) were assessed using transcranial Doppler (TCD; Doppler-Box X, DWL, Compumedics Ltd, Germany) with a 2-MHz probe placed over each temporal window. Ultrasound gel was placed on the probes and held in place with a headset. Where possible the right MCA and left PCA were insonated, however the PCA could not be positively identified in one participant, in which case the MCA was insonated on both sides. Search and identification procedures were performed as detailed previously (Section 3.3.1).

Beat-to-beat blood pressure was measured using a finger cuff on the middle finger of the left hand (Portapres, Finapres, Medical System BV, The Netherlands). Respiratory rate and volume were measured using a heated pneumotachograph (3813 Series,

Hans Rudolph Inc, Kansas, USA) attached to a facemask, while fractional changes in inspired and expired O_2 and CO_2 were measured via a sample line attached to the facemask and a gas analyser (ML206, ADInstruments Ltd, New Zealand). Measures were recorded at 1k Hz via an analogue-to-digital converter (Powerlab, ADInstruments) and displayed in real time and stored for offline analysis using commercially available software (LabChart v7.3.5, ADInstruments). Calibration of equipment was performed before each testing session.

T_C was measured using a rectal thermistor (General Purpose Temperature Probe 400TM, Mon-a-therm®, Covidien, Mansfield, MA, USA), and mean skin temperature measured by the weighted average of four thermistors attached to the skin of the calf, thigh, bicep and chest (Grant EUS-U, Grant Instruments Ltd., Cambridge, United Kingdom). Both skin and core temperatures were logged and displayed in real time during testing (Grant 2020 Series Squirrel Data Logger, Grant Instruments, Cambridge, United Kingdom).

5.2.3 Data Analysis

Data from 60 s of the baseline period, 30 s of the hypercapnic stages (from 2:30 min – 3:00 min; Burley et al., 2020), and >20 s of data at the target end-tidal CO_2 during the hypocapnic stages, were extracted and used in the statistical analyses.

The slope of the cerebral blood flow (CBF) response to changes in $P_{ET}CO_2$ was calculated to give an estimation of cerebrovascular-CO_2 responsiveness in the MCA and PCA (MCA*v*-CO_2 responsiveness; PCA*v*-CO_2 responsiveness). Cerebrovascular responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were

determined by the slope of the CBF response from stages Hyper1 to Hyper2, Hypo1 to Hypo2, and Hypo1 to Hyper1, respectively (see Figure 5.2).

The pulsatility index in the MCA and PCA (MCA*v*-PI; PCA*v*-PI) was calculated as (systolic CBF*v* – diastolic CBF*v*) / mean CBF*v*. An index of cerebrovascular conductance (CVC) was also calculated from the ratio of CBF to mean arterial pressure (MAP), allowing examination of cerebrovascular-CO_2 responsiveness without changes in MAP influencing cerebral blood flow velocity. An estimation of CVC to CO_2 was expressed as the change in CVC per mmHg change in $P_{ET}CO_2$ (MCA*v*-CVC-CO_2 responsiveness, PCA*v*-CVC-CO_2 responsiveness).

5.2.3.1 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 8.0.0, GraphPad Software, San Diego, CA, USA). To account for missing data, a mixed effects model was used for all outcome measures. Main and interaction effects of menstrual phase (2 levels: EF vs O or EF vs ML) and thermal condition (2 levels: normothermia vs heat stress) were tested. Simple linear regressions were performed on cerebrovascular-CO_2 responsiveness outcome measures to obtain a best-fit value of the CBF response across the $P_{ET}CO_2$ range (stages Hypo1, Hyper1 and Hyper2; see Figure 5.2). The line of best fit is reported as $y = bx + a$, where b is the slope of the line and a is the *y*-intercept when $x=0$. The slope and *y*-intercept values were compared between menstrual phases (EF vs O; EF vs ML) and thermal conditions (normothermia vs heat stress). Data are presented as means ± SD. Statistical significance were based on an α-level of 0.05.

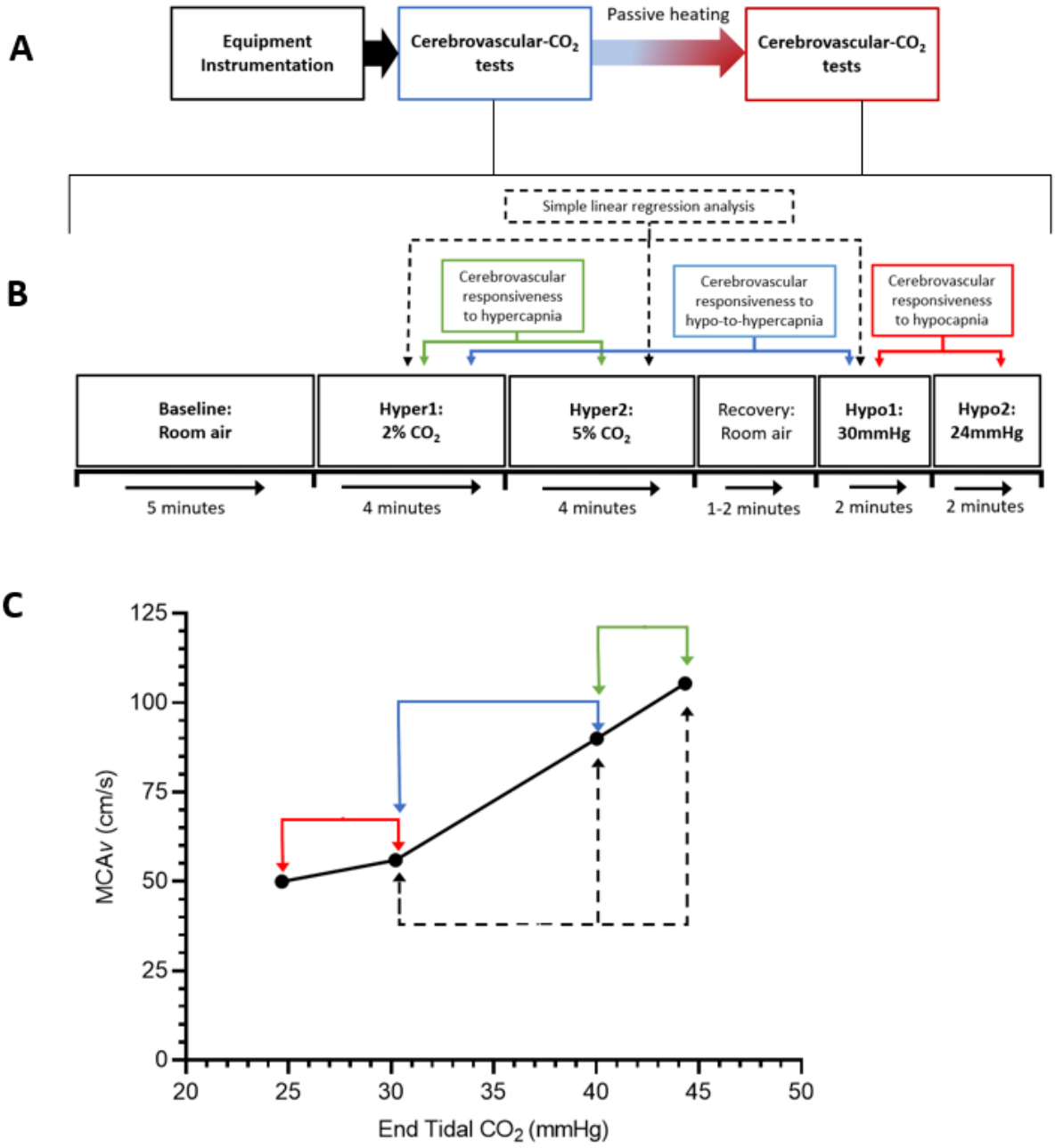

Figure 5.2 Schematic of the study protocol. Panel **A** provides an overview of the experimental session, with Panel **B** shows the cerebrovascular-CO_2 responsiveness tests protocol (performed twice, during normothermia and passive heat stress conditions). Panel **C** shows an example graph of the cerebrovascular responsiveness slope. Green, blue, and red arrows indicate points of data extraction to calculate the cerebrovascular responsiveness to hypercapnia (Hyper1 to Hyper2), hypo-to-hypercapnia (Hypo1 to Hyper1), and hypocapnia (Hypo1 to Hypo2), respectively. Dashed back arrows indicate points of data extraction used in simple linear regression analysis.

5.3 Results

Of the thirteen participants enrolled, seven completed all three experimental sessions, three participants completed two sessions, and three participants completed one session. Subsequently, the number of participants completing the EF, O and ML testing sessions were 12, 9 and 9, respectively.

5.3.1 Early Follicular vs. Ovulatory Phase

5.3.1.1 Baseline measures

Baseline responses can be found in Table 5.1. Core temperature was higher in O compared to the EF phase during normothermia and heat stress (+0.3 ± 0.2°C; *menstrual phase*: p=.03; *menstrual phase x thermal condition*: p=.34), with heat stress increasing core temperature similarly in both menstrual phases (EF: +1.1 ± 0.1°C, O: +1.0 ± 0.1°C; *thermal condition*: p<.01) Skin temperature increased with heat stress (+3.3 ± 0.9°C; *thermal condition*: p<.01) when compared to normothermia, with no difference between EF and O phases (*menstrual phase*: p=.78; *menstrual phase x thermal condition*: p=.19). With heat stress, heart rate increased (+26 ± 13 bpm; *thermal condition: p*<.01) and mean arterial pressure decreased (-11 ± 12 mmHg; *thermal condition: p*<.01) when compared to normothermia, with no difference between EF and O phases for both these responses (*menstrual phase: p*>.66; *menstrual phase x thermal condition: p*>.15*)*. End-tidal CO_2 was lower during heat stress (-2.4 ± 3.3 mmHg; *thermal condition: p*=.03), with no difference between menstrual phase *(menstrual phase*: p=.66; *menstrual phase x thermal condition: p*=.31). MCA*v*-PI and PCA*v*-PI increased during heat stress (+0.17 ± 0.14 and +0.18 ± 0.16, respectively;

thermal condition: $p<.04$) with no difference between EF and O phases (*menstrual phase*: $p>.12$; *menstrual phase x thermal condition:* $p>.57$). Ventilation, MCA*v*, PCA*v*, MCA*v*-CVC and PCA*v*-CVC were similar between menstrual phases (*menstrual phase:* $p>.15$*)* and thermal conditions (*thermal condition*: $p>.07$; *menstrual phase x thermal condition:* $p>.08$).

Table 5.1 Baseline thermoregulatory, cardiovascular, respiratory, and cerebrovascular responses during normothermia and passive heat stress in females during three stages of the menstrual cycle.

	Early Follicular (n = 12)		Ovulatory (n = 9)		Mid Luteal (n = 9)	
	Normo-thermia	Heat Stress	Normo-thermia	Heat Stress	Normo-thermia	Heat Stress
T_C (°C)	36.8 ± 0.1	37.9 ± 0.2#	37.1 ± 0.2*	38.1 ± 0.3*#	36.9 ± 0.3	38.0 ± 0.3#
T_S (°C)	34.1 ± 0.6	37.1 ± 0.9#	33.8 ± 0.8	37.6 ± 0.7#	34.0 ± 0.6	37.4 ± 0.7#
Heart Rate (bpm)	66 ± 13	90 ± 13#	66 ± 9	94 ± 8#	68 ± 11	93 ± 9#
Mean Arterial Pressure (mmHg)	76 ± 16	66 ± 11#	79 ± 14	66 ± 9#	85 ± 14	61 ± 12#
Ventilation (L/min)	5.8 ± 1.1	6.9 ± 2.7	6.2 ± 1.3	7.7 ± 2.8	6.0 ± 1.2	7.3 ± 1.8
End-Tidal CO_2 (mmHg)	37.8 ± 2.0	35.1 ± 4.3#	36.8 ± 3.7	34.7 ± 6.1#	36.4 ± 2.7*	33.6 ± 5.0*
MCA*v* (cm/s)	83 ± 18	71 ± 20#	79 ± 14	73 ± 16	81 ± 18	68 ± 23#
PCA*v* (cm/s)	56 ± 8	50 ± 10#	53 ± 11	48 ± 11	55 ± 14	45 ± 11#
MCA*v*-PI	0.72 ± 0.12	0.89 ± 0.20	0.79 ± 0.23	0.95 ± 0.30	0.73 ± 0.14	0.88 ± 0.21
PCA*v*-PI	0.76 ± 0.17	0.94 ± 0.29#	0.74 ± 0.07	0.92 ± 0.19#	0.72 ± 0.06	0.84 ± 0.11#
MCA*v*-CVC (cm/s/mmHg)	1.12 ± 0.26	1.09 ± 0.31	1.01 ± 0.18	1.12 ± 0.26	0.98 ± 0.23	1.15 ± 0.44
PCA*v*-CVC (cm/s/mmHg)	0.77 ± 0.16	0.77 ± 0.16	0.67 ± 0.17	0.74 ± 0.18	0.66 ± 0.19	0.77 ± 0.22

T_C, core body temperature; T_S, mean skin temperature; MCA*v*, middle cerebral artery velocity; PCA*v*, posterior cerebral artery velocity; MCA*v*-PI, middle cerebral artery pulsatility index; PCA*v*-PI, posterior cerebral artery pulsatility index; MCA*v*-CVC, middle cerebral artery conductance; PCA*v*-CVC, posterior cerebral artery conductance. Values are means ± SD, n-1 for PCA outcome variables. *significantly different from early follicular; #significantly different from normothermia.

5.3.1.2 Middle Cerebral Artery Responsiveness

MCA*v*-CO_2 responsiveness to hypocapnia was greater during O when compared to the EF phase (Figure 5.3; +0.35 ± 0.46 cm/s/mmHg; *menstrual phase*: *p*<.01) in both thermal conditions (*menstrual phase x thermal condition: p*=0.12). MCA*v*-CO_2 responsiveness to hypercapnia and hypo-to-hypercapnia was similar between EF and O phases (*menstrual phase*: *p>.67)* and thermal conditions (Table 5.2; *thermal condition*: *p*>.27.; *menstrual phase x thermal condition*: *p*>.41). Simple linear regression analysis of MCA*v*-CO_2 responsiveness found similar slopes (*p*=.97) and *y*-intercepts (*p*=.36) between menstrual phase and thermal conditions (EF normothermia, *y*=3.50*x*-49.76; O normothermia, *y*=3.59*x*-50.48; EF heat stress, *y*=3.30*x*-47.29; O heat stress, *y*=3.45*x*-49.33).

MCA*v*-CVC-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia was not significantly changed between EF and O phases (*menstrual phase*: *p*>.06*)* and thermal conditions (*thermal* condition: *p*>.21; menstrual *phase x thermal condition*: *p*>.45).

5.3.1.3 Posterior Cerebral Artery Responsiveness

PCA*v*-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were similar between EF and O phases (*menstrual phase*: *p*>.29*)*, and between thermal conditions (Figure 5.4; *thermal condition: p*>.14; *menstrual phase x thermal condition*: *p*>.44). Simple linear regression of PCA*v*-CO_2 responsiveness found similar slopes (*p*=.92) and *y*-intercepts (*p*=.49) between menstrual phases and thermal conditions

(EF normothermia, y=2.46x-36.35; O normothermia, y=2.54x-37.53; EF heat stress, y=2.28x-32.37; O heat stress, y=2.38x-33.92).

With heat stress, PCA*v*-CVC-CO_2 responsiveness to hypocapnia decreased when compared to normothermia (-0.01 ± 0.01 vs -0.02 ± 0.00 cm/s/mmHg; *thermal condition*: p=.04) in both menstrual phases (*menstrual phase:* p=.45; *menstrual phase x thermal condition:* p=.52). PCA*v*-CVC-CO_2 responsiveness to hypercapnia and hypo-to-hypercapnia was similar between menstrual phases (*menstrual phase*: p>.17) and thermal conditions (*thermal condition:* p>.48; *menstrual phase x thermal condition*: p>.20).

Table 5.2 Cerebrovascular responsiveness values for the middle cerebral artery (MCA*v*-CO2 responsiveness) and posterior cerebral artery (PCAv-CO_2 responsiveness) during normothermia and heat stress in females during three phases of the menstrual cycle. 'Full range' refers to the linear regression line of best-fit which includes the Hypo1, Hyper1 and Hyper2 stages of the cerebrovascular responsiveness protocol.

Values are means ± SD. *significantly different from early follicular

		Early Follicular		*Ovulatory*		*Mid Luteal*	
		Normothermia	Heat Stress	Normothermia	Heat Stress	Normothermia	Heat Stress
MCAv-CO_2 responsiveness (cm/s/mmHg)	Hypercapnia	3.61 ± 0.73	3.58 ± 1.16	3.97 ± 1.14	3.37 ± 1.27	3.60 ± 1.68	3.29 ± 1.07
	Hypocapnia	-1.06 ± 0.52	-1.15 ± 0.47	-1.60 ± 0.59*	-1.30 ± 0.53*	-1.18 ± 0.76	-0.72 ± 0.57
	Hypo-to-hypercapnia	3.13 ± 1.29	3.10 ± 1.61	3.43 ± 1.04	3.00 ± 1.45	3.65 ± 1.10	3.34 ± 1.80
	Full range	3.50 ± 0.92	3.30 ± 0.91	3.59 ± 0.99	3.45 ± 0.70	3.55 ± 1.01	3.51 ± 0.94
PCAv-CO_2 responsiveness (cm/s/mmHg)	Hypercapnia	2.83 ± 0.81	2.77 ± 0.72	3.21 ± 0.77	2.98 ± 0.94	2.49 ± 0.91	2.58 ± 1.15
	Hypocapnia	-0.95 ± 0.38	-0.88 ± 0.29	-1.18 ± 0.36	-0.92 ± 0.33	-0.82 ± 0.45	-1.02 ± 0.50
	Hypo-to-hypercapnia	2.38 ± 0.47	2.41 ± 1.37	2.35 ± 1.16	1.77 ± 1.50	2.55 ± 0.92	3.87 ± 3.11
	Full range	2.46 ± 0.49	2.28 ± 0.79	2.54± 0.89	2.38 ± 0.77	2.43 ± 0.81	2.35 ± 0.82

5.3.2 Early Follicular vs. Mid-Luteal Phase

5.3.2.1 Baseline Measures

Baseline responses can be found in Table 5.1. With heat stress, core body temperature increased (+1.1 ± 0.1 °C; *thermal condition*: $p<.01$) and skin temperature increased (+3.2 ± 0.9 °C; *thermal condition*: $p<.01$) compared to normothermia in both EF and ML phases (*menstrual phase*: $p>.16$; *menstrual phase x thermal condition*: $p>.48$). With heat stress, heart rate increased (+24 ± 12 bpm; *thermal condition:* $p<.01$) and mean arterial pressure decreased (-12 ± 15 mmHg, *thermal condition*: $p<.01$) compared to normothermia with no significant difference between EF and ML phases for both these responses (*menstrual phase*: $p>.51$; *menstrual phase x thermal condition*: $p>.07$). End-tidal CO_2 was unchanged with heat stress (*thermal condition:* $p=.11$) but was lower in the ML phase compared to EF (-1.2 ± 2.3 mmHg; *menstrual phase:* $p=.04$; *menstrual phase x thermal condition*: $p>.67$). Heat stress decreased MCA*v* and PCA*v* compared to normothermia (-12.6 ± 11.0 cm/s and -8.0 ± 9.4 cm/s; *thermal condition*: $p<.01$ and $p=.01$, respectively), with no difference between menstrual phase (*menstrual phase*: $p>.51$*; menstrual phase x thermal condition*: $p>.44$). MCA*v*-PI and PCA*v*-PI increased during heat stress (+0.17 ± 0.14 and +0.14 ± 0.14, respectively; *thermal condition*: $p<.01$) with no difference between EF and ML phases (*menstrual phase*: $p>.39$; *menstrual phase x thermal condition:* $p>.07$). Ventilation, MCA*v*-CVC, and PCA*v*-CVC were similar between menstrual phases (*menstrual phase:* $p>.51$), and thermal conditions (*thermal condition:* $p>.06$; *menstrual phase x thermal condition:* $p>.11$).

5.3.2.2 Middle cerebral artery responsiveness

MCA*v*-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia was similar between EF and ML phases (*menstrual phase*: *p*>.10*)* and thermal conditions (Figure 5.5; *thermal condition: p*>.14*; menstrual phase x thermal condition*: *p*>.11). Simple linear regression of MCA*v*-CO_2 responsiveness found similar slopes (*p*=.98) and intercepts (*p*=.29) between menstrual phases and thermal conditions (EF normothermia, *y*=3.50*x*-49.76; ML normothermia, *y*=3.55*x*-49.51; EF heat stress, *y*=3.30*x*-47.29; ML heat stress, *y*=3.51*x*-55.41).

With heat stress, MCA*v*-CVC-CO_2 responsiveness to hypo-to-hypercapnia was greater when compared to normothermia (0.09 ± 0.11 vs 0.04 ± 0.01 cm/s/mmHg; *thermal condition: p*=.04) in both menstrual phases (*menstrual phase*: *p*=.17; *menstrual phase x thermal condition*: *p*=.17). MCA*v*-CVC-CO_2 responsiveness to hypercapnia and hypocapnia was similar between EF and ML phases (*menstrual phase*: *p*>.15*)*, and between thermal conditions (*thermal condition: p*>.16; *menstrual phase x thermal condition*: *p*>.36).

5.3.2.3 Posterior Cerebral Artery Responsiveness

PCA*v*-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were similar between EF and ML phases (*menstrual phase*: *p*>.20*)* and thermal conditions (Figure 5.6; *thermal condition: p*>.20*; menstrual phase x thermal condition*: *p*>.26). Simple linear regression of PCA*v*-CO_2 responsiveness found similar slopes (*p*=.97) and intercepts (*p*=.52) between menstrual phases and thermal conditions (EF

normothermia, y=2.46x-36.35; ML normothermia, y=2.43x-34.80; EF heat stress, y=2.28x-32.37; ML heat stress, y=2.35x-34.35).

PCAv-CVC-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were similar between EF and ML phases (*menstrual phase*: p>.37*)* and thermal conditions (*thermal* condition: p>.06; menstrual *phase x thermal condition*: p>.12).

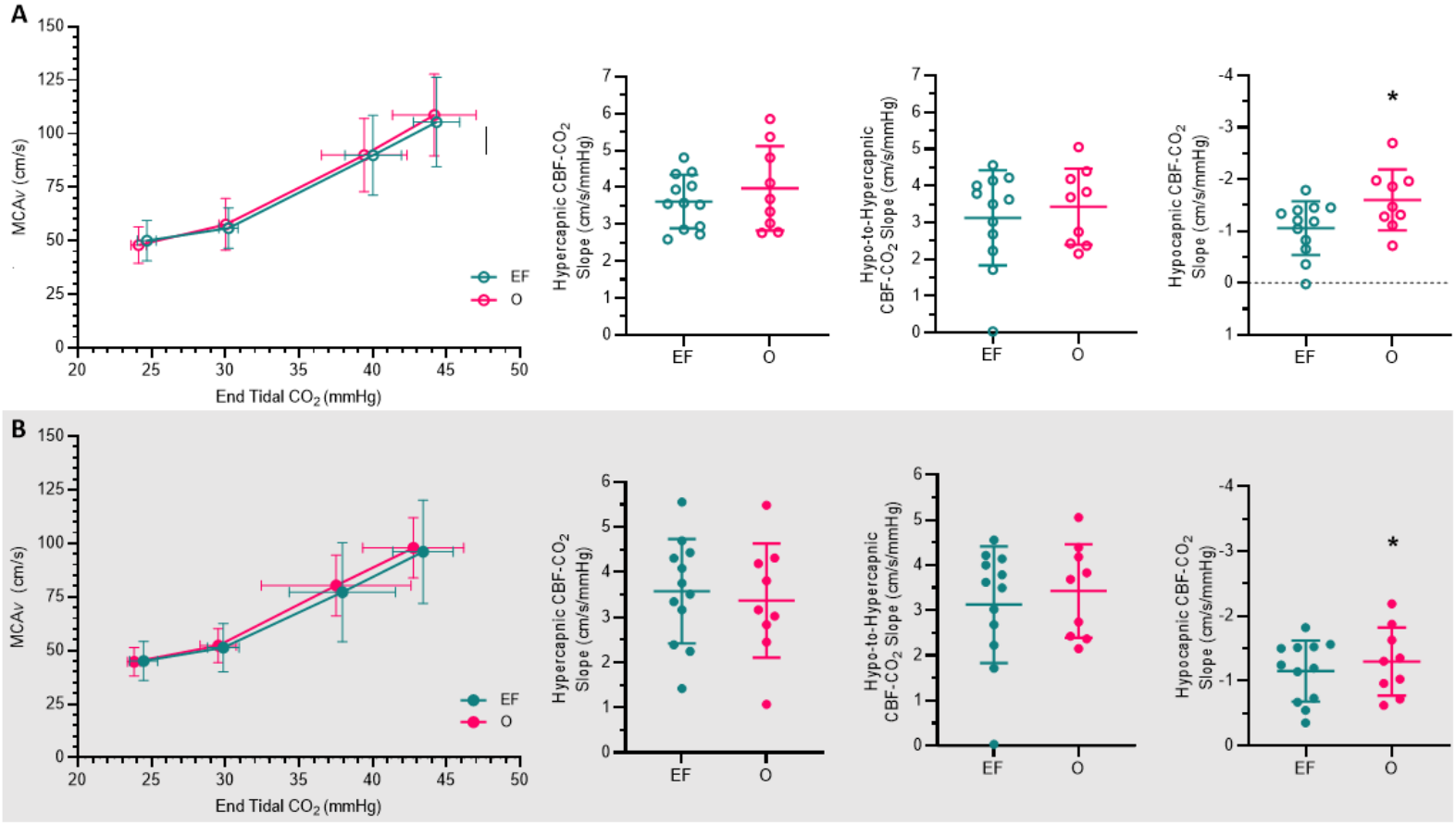

Figure 5.3 The middle cerebral artery blood flow velocity (MCA*v*) slope at hyper-, hypo-to-hyper, and hypocapnia (MCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in females during the early follicular (EF; n = 12) and ovulatory (O; n = 9) phases of the menstrual cycle. *significantly different to EF.

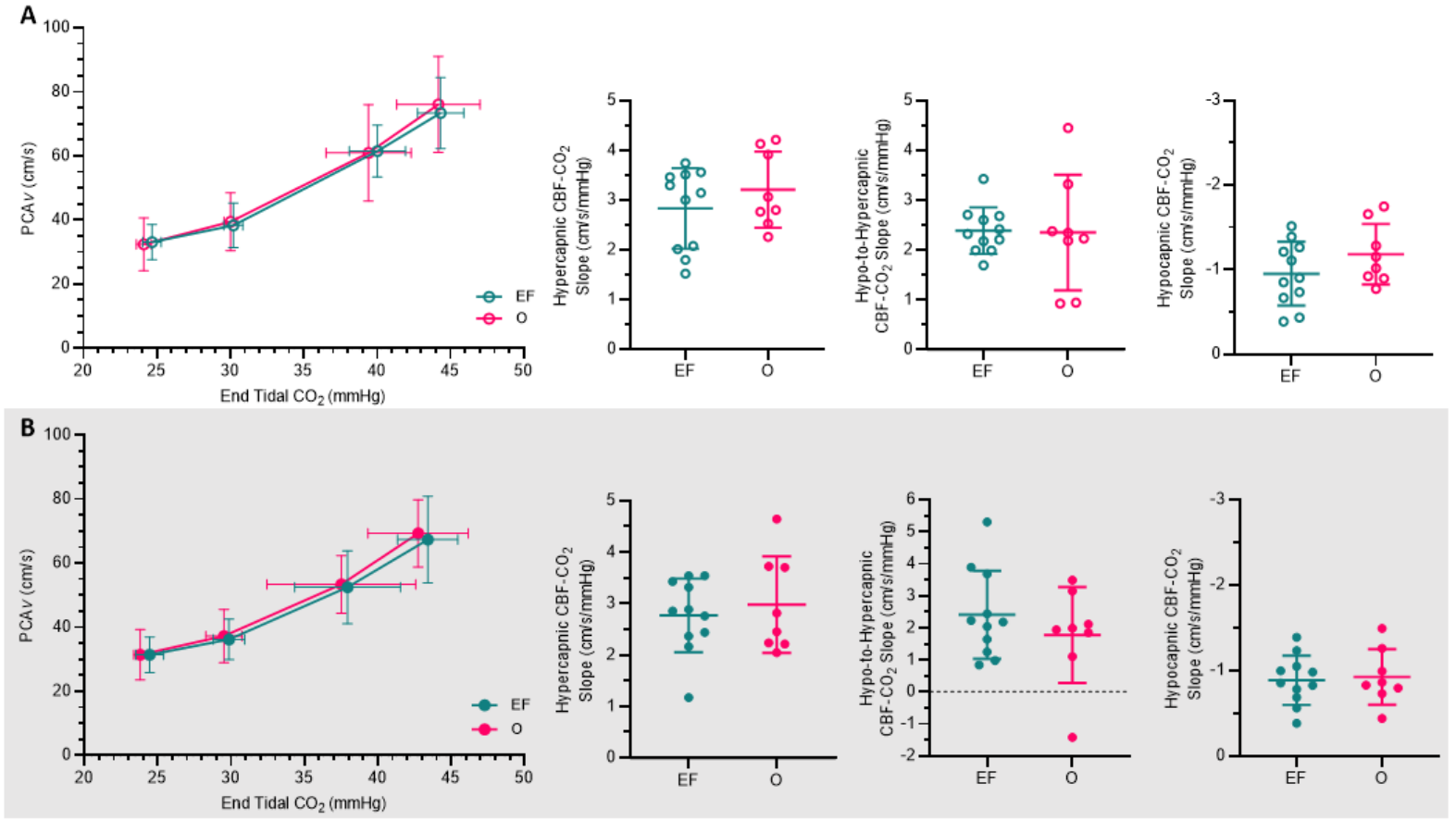

Figure 5.4 The posterior cerebral artery blood flow velocity (PCA*v*) slope at hyper-, hypo-to-hyper-, and hypocapnia (PCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in females during the early follicular (EF; n = 11) and ovulatory (O; n = 8) phases of the menstrual cycle.

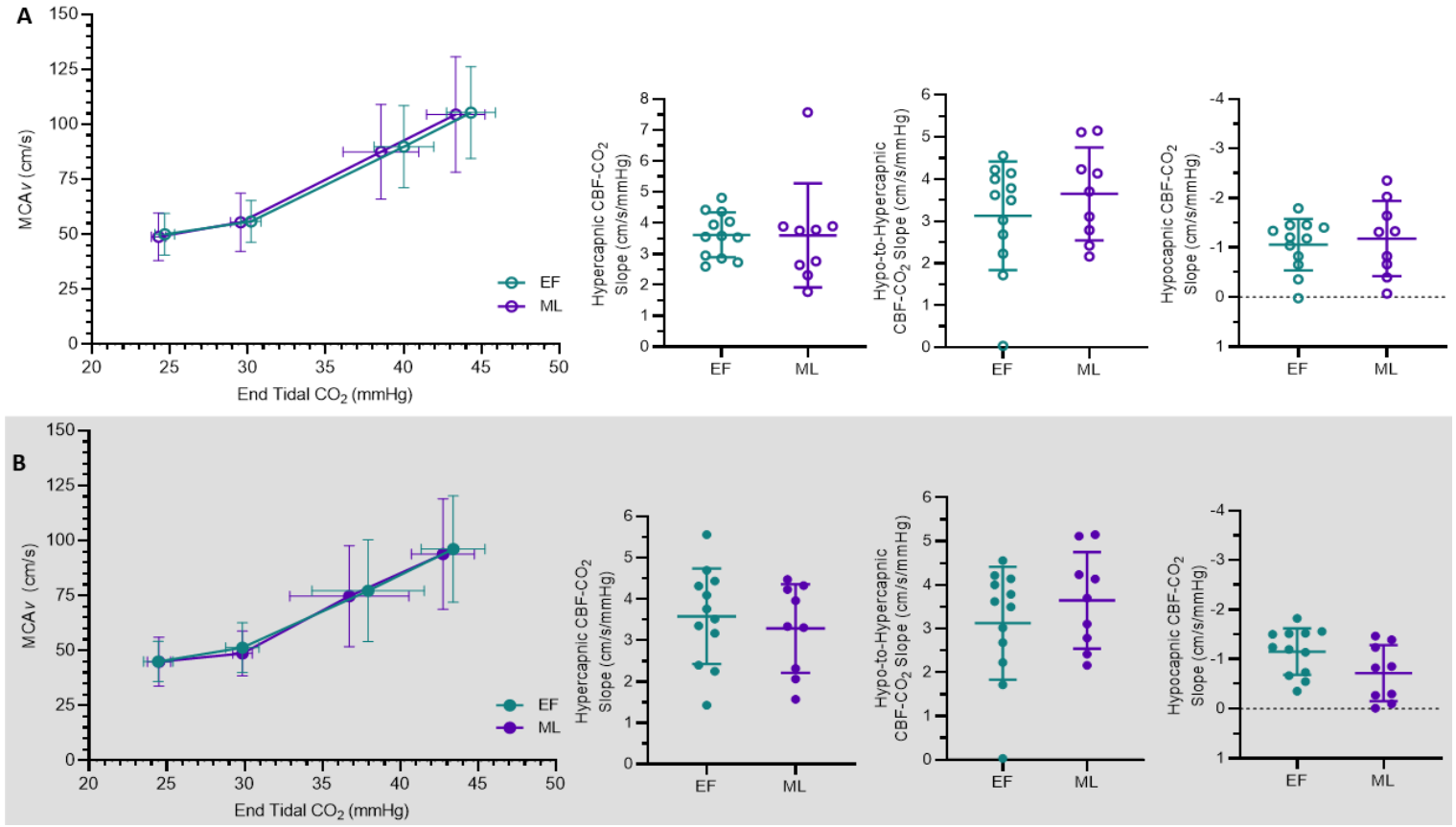

Figure 5.5 The middle cerebral artery blood flow velocity (MCA*v*) slope at hyper-, hypo-to-hyper-, and hypocapnia (MCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in females during the early follicular (EF; n = 12) and mid-luteal (ML; n = 9) phases of the menstrual cycle.

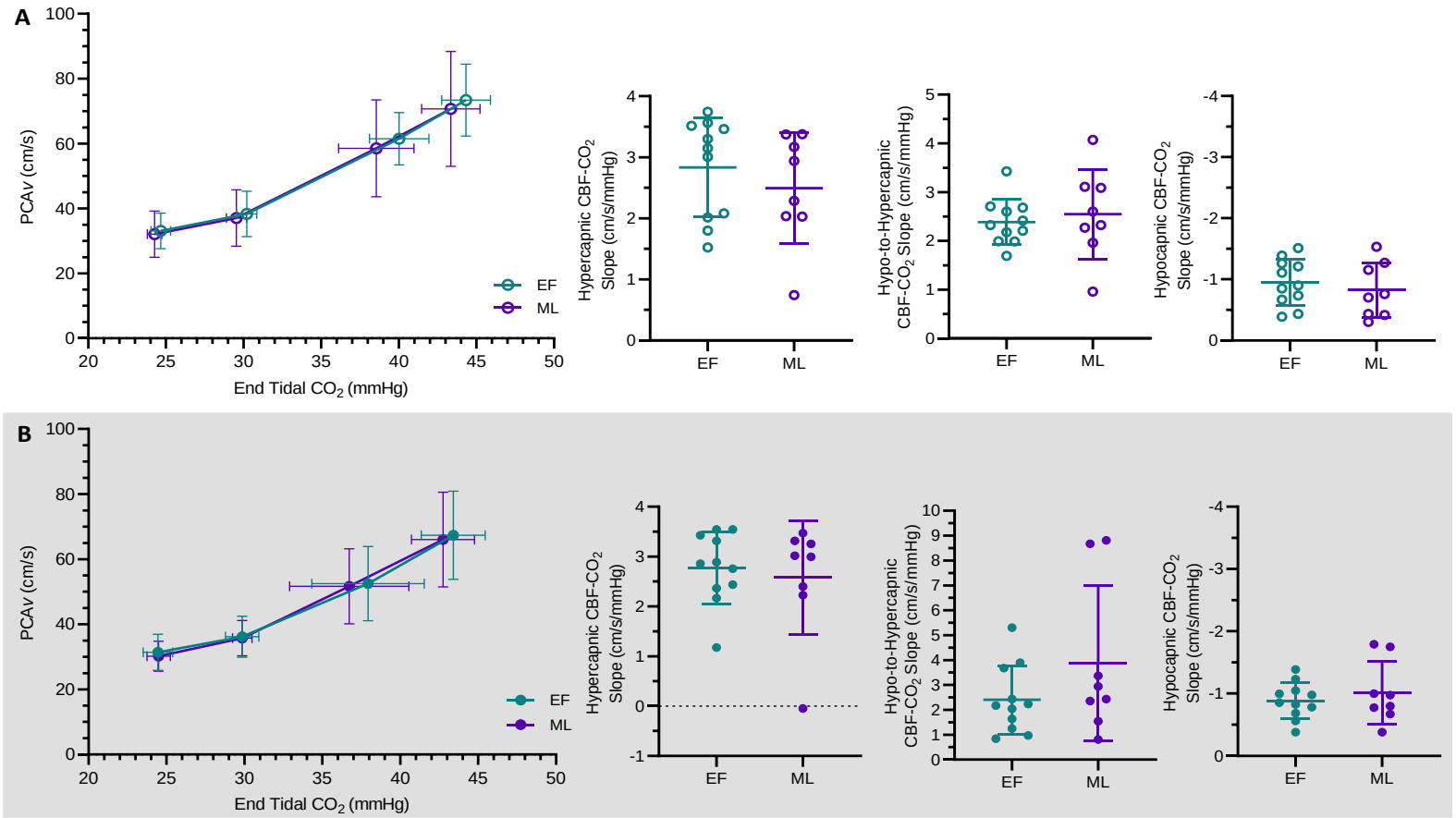

Figure 5.6 The posterior cerebral artery blood flow velocity (PCA*v*) slope at hyper-, hypo-to-hyper, and hypocapnia (PCA*v*-CO_2 responsiveness) under normothermic (Panel A) and passive heat stress (Panel B) conditions in females during the early follicular (EF; n = 11) and mid-luteal (ML; n = 8) phases of the menstrual cycle.

5.4 Discussion

The main findings of this study were that 1) MCA*v*-CO_2 responsiveness to hypocapnia was greater during the ovulatory phase compared to the early follicular phase, and 2) cerebrovascular-CO_2 responsiveness during heat stress was unaffected by menstrual phase. These findings indicate that greater levels of oestrogen during the O phase have a significant effect on the vasoconstrictive capacity of the MCA, while greater levels of progesterone may counteract the effect of oestrogen during the ML phase. Additionally, while the response to passive heat stress is similar across the menstrual cycle, there does appear to be regional differences in cerebrovascular-CO_2 responsiveness between normothermic and heat stress conditions.

5.4.1 Effect of menstrual phase on cerebrovascular-CO_2 responsiveness

It was hypothesised that cerebrovascular-CO_2 responsiveness would be greater during phases of increased circulating levels of oestrogen, since oestrogen is known to promote eNOS production and prostacyclin pathways, thereby reducing cerebrovascular tone and increasing vasodilation. Previous studies have reported that vasodilatory cerebrovascular responsiveness is both greater during ovulation (Krejza et al., 2013) and similar between ovulatory and early follicular phases (Peltonen et al., 2016), although these studies used different vasoactive stimuli (acetazolamide and indomethacin, respectively) than used in the current study (CO_2). Importantly, using a CO_2 stimulus allowed us to examine both the vasodilatory and vasoconstrictive responses. Our findings showed no difference in cerebrovascular responsiveness to hypercapnia or hypo-to-hypercapnia between phases (i.e., EF vs. O and EF vs. ML),

but a greater MCA*v*-CO_2 responsiveness to hypocapnia during ovulation compared to the early follicular phase. Circulating oestrogen is associated with lower cerebrovascular tone (Krause et al., 2006) and therefore, greater basal cerebral blood flow velocity in the internal carotid artery (Krejza et al., 2001) and the MCA (Peltonen et al., 2016) that may in turn allow for a greater vasoconstrictive capacity in response to hypocapnia. However, the present study found no difference in resting cerebral blood flow velocity or PI in the MCA or PCA between EF and O phases. Since TCD was used to insonate the vessels, changes in vessel diameter could not be measured, which would provide a more accurate indication of changes in flow velocity and cerebrovascular tone. At present, it appears unlikely that differences in baseline haemodynamics between menstrual phases cause the observed greater MCA*v*-CO_2 responsiveness to hypocapnia in O.

Cerebrovascular-CO_2 responsiveness in the PCA remained similar between menstrual phases, indicating that MCA*v* and PCA*v* responsiveness to hypocapnia respond independently to changing oestrogen levels. Regional differences in cerebrovascular responsiveness have previously been reported, with the posterior circulation having a blunted hypercapnic response compared to the anterior circulation (Sato et al., 2012; Skow et al., 2013), while other findings suggest no differences in regional cerebrovascular-CO_2 responsiveness (Willie et al., 2012). However, these studies either did not control for the menstrual cycle or included females in only the early follicular phase, making comparisons with the present study difficult. While the present study did not directly compare the MCA and PCA, the enhanced MCA*v*-CO_2

responsiveness to hypocapnia indicates the MCA may be more greatly affected by fluctuations in oestrogen compared to the PCA.

It was hypothesised that cerebrovascular-CO_2 responsiveness would remain similar during EF and ML phases as progesterone appears to blunt the vasodilatory effect of oestrogen (Krause et al., 2006). In support of previous literature (Hazlett & Edgell, 2018), we found no difference in cerebrovascular-CO_2 responsiveness outcome measures between EF and ML phases. The physiological pathways by which progesterone acts on the cerebrovasculature are less understood, with the majority of information arising from animal models. However, it is generally understood that progesterone acts as an antagonist to oestrogen, attenuating the anti-inflammatory response post-ischaemic brain injury (Sunday et al., 2006), and negating the vasodilatory effects of oestrogen in the peripheral vasculature (Miner et al., 2011). While caution needs to be taken comparing the findings of the present study to such models, one could speculate that the rise in circulating progesterone during the ML phase appears to attenuate any effect high oestrogen levels may have on the cerebrovascular-CO_2 responsiveness outcomes assessed here. Furthermore, when examining effects of the menstrual cycle in humans it may be more pertinent to consider the ratio of progesterone to oestrogen due to their antagonistic relationship. Therefore, more research is needed to better understand the combined effects of changing oestrogen and progesterone levels throughout the menstrual cycle on cerebrovascular function.

5.4.2 Effect of thermal condition on cerebrovascular-CO_2 responsiveness

We found no differences in cerebrovascular-CO_2 responsiveness to heat stress between menstrual phases, despite a greater core body temperature during ovulation compared to the early follicular phase. This indicates that changes in sex hormones throughout the menstrual cycle do not appear to alter cerebrovascular-CO_2 responsiveness during heat stress. This does not, however, indicate that sex hormones do not have any effect on this response, only that the natural changes in hormone levels across the menstrual cycle are insufficient to produce an observable influence. Indeed, the thermoregulatory mechanisms regulating CBF during thermal stress (e.g., blood pressure, arterial CO_2) are likely too robust to observe any influence of oestrogen and progesterone on cerebrovascular-CO_2 responsiveness. It is possible that if sex hormone levels were manipulated to a supra-physiological level (i.e., ovarian hyperstimulation) a different outcome may be observed.

MCA*v*-CVC-CO_2 responsiveness to hypo-to-hypercapnia was greater during heat stress compared to normothermia. Conversely, PCA*v*-CVC-CO_2 responsiveness to hypocapnia was lower during thermal stress than normothermic conditions. Importantly, an index of CVC-CO_2 responsiveness allows for examination of cerebrovascular responsiveness without the reduction in mean arterial pressure induced by passive heat stress influencing the observed cerebral blood flow velocity response. As previously discussed, regional differences in cerebrovascular-CO_2 responsiveness have been reported in both males and females when the menstrual cycle is not controlled for (Sato et al., 2012; Skow et al., 2013). Heat-stress-induced differences in regional cerebrovascular-CO_2 responsiveness have also been

previously reported in males, with heat stress enhancing responsiveness in the external carotid artery and reducing responsiveness in the internal carotid artery, as compared to normothermia (Ogoh et al., 2014). While we did not directly compare MCA*v* and PCA*v* responsiveness, the present study indicates potential differences between the response of intracranial vessels to changing end-tidal CO_2 in menstruating females during passive heating. Further investigation of these potential regional effects in females is needed to increase our understanding of how the heat stress response interacts with changing oestrogen and progesterone levels, and how this differs across the cerebrovasculature.

5.4.3 Considerations / Study limitations

We used Doppler ultrasound to measure blood flow velocity in the MCA and PCA. A primary assumption of TCD is that the insonated vessel maintains a constant diameter. While this assumption has been validated (Valdueza et al., 1997; Serrador et al., 2000), more recent MRI studies have reported changing vessel diameters in response to changing CO_2 (Coverdale et al., 2014; Verbree et al., 2014) resulting in possible over- and under-estimations of CBF when CBF velocity is used as a direct index of absolute flow. Despite this, assessment of cerebrovascular-CO_2 responsiveness by TCD has been shown to offer valuable information on cerebrovascular health and function, provided the derived data are interpreted with these limitations in mind (Willie et al., 2011).

Additionally, changes in $P_{ET}CO_2$ were used to calculate cerebrovascular-CO_2 responsiveness, based on the assumption that $P_{ET}CO_2$ accurately represents arterial

CO_2 (P_aCO_2). While these two variables can differ with metabolic CO_2 production and tidal volume, they do not differ by changes in breathing frequency (Jones et al., 1979) and has been validated over a range of core temperatures (Brothers et al., 2011).

In states of augmented hypo- or hypercapnia, the cerebrovasculature may reach a point of maximal constriction or dilation. The P_aCO_2 threshold for reaching the limit of the vessel calibre has been shown to be ~65mmHg in the hypercapnic range and ~25mmHg in the hypocapnic range (Harper & Glass, 1965). In the present study, $P_{ET}CO_2$ increased to ~44mmHg during Hyper2, and decreased to ~24mmHg during Hypo2. If the limit of the vessel calibre is reached, further diameter changes are not possible and a change in cerebrovascular-CO_2 responsiveness may not be represented by further changes in CBF velocity. To account for this, the Hypo2 stage was omitted from the simple linear regression, with analysis using the Hypo1, Hyper1 and Hyper 2 stages.

The present study employed a steady-state CO_2 technique to measure cerebrovascular-CO_2 responsiveness. This technique was chosen as it incorporates both the ventilatory and cerebrovascular response to a steady-state CO_2 stimulus. However, ventilatory-CO_2 responsiveness has not been reported here as it was outside the remit of the thesis. To the best of our knowledge, sex differences in ventilatory-CO_2 responsiveness in conjunction with cerebrovascular-CO_2 responsiveness have not been examined. Previously, no differences have been found in the ventilatory threshold between menstrual phases when a rebreathing protocol is employed (MacNutt et al., 2012), suggesting that if a sex hormone-mediated effect on ventilatory control is present, acute hormone fluctuations across the cycle are insufficient to alter the

response. The use of different techniques to measure cerebrovascular- or ventilatory-CO_2 responsiveness (i.e., steady-state, rebreathing) may elicit different outcomes as they stimulate physiological systems in a different manner. For example, a rebreathing technique abolishes the PCO_2 gradient throughout the body (e.g., between end-tidal and arterial concentrations) and therefore, measures only the ventilatory response unaffected by the cerebrovascular response (Ainslie & Duffin, 2009). As such, the present findings should be considered with the methodology used in mind, and that outcomes may differ when reported alongside ventilatory responsiveness or if a different technique is used.

The present study did not control for several population demographics that may affect the reported outcome measures, including physical activity levels, sedentary behaviour, and training status. Greater aerobic fitness has previously been shown to be associated with lower baseline cerebral blood flow and greater cerebrovascular-CO_2 responsiveness (Burley et al., 2017; Foster et al., 2020). Therefore, differences in fitness may contribute to some of the observed variation between cohorts.

5.4.4 Summary

The findings of this study show that natural fluctuations in sex hormones across the menstrual cycle have minimal influence on cerebrovascular-CO_2 responsiveness. Enhanced cerebrovascular responsiveness to hypocapnia during ovulation supports previous evidence that oestrogen has the greatest impact on the cerebrovasculature, and that progesterone counteracts this during the mid-luteal phase. The response of the cerebrovasculature to passive heat stress does not differ between menstrual

phases. However, regional differences identified between the anterior and posterior circulation during heat stress warrant further investigation.

6 THE INFLUENCE OF MENOPAUSE ON CEREBROVASCULAR FUNCTION

6.1 Introduction

The risk of stroke increases with age, with this risk rising disproportionately in older females compared to older males. More specifically, despite being lower in females compared to males during middle age, the risk of stroke doubles for females in the first 10 years post-menopause (Lisabeth & Bushnell, 2012), a time when ovarian hormone levels abruptly and chronically decrease. Ovarian hormones (i.e., oestrogen, progesterone) have been reported to have a number of neuroprotective effects, including suppression of the inflammatory response and influence perfusion after ischaemic injury (Hurn et al., 1995; Santizo et al., 2002). Oestrogen acts both directly and indirectly on the vasculature by enhancing production and activity of vasodilatory factors (e.g., endothelial NO synthase, prostacyclin pathways; Krause et al., 2006). The effect of progesterone on the vasculature is far less clear, with evidence suggesting it both promotes and reduces the inflammatory response (Gibson et al., 2005; Sunday et al., 2006). Nevertheless, overall ovarian hormones are generally understood to positively influence cerebrovascular function. Indeed, the decline in oestrogen throughout menopause has been associated with a decline in peripheral artery endothelial function (Moreau et al., 2012). However, recent evidence shows little association between the commonly applied cerebral and peripheral measures of vascular responsiveness (i.e. MCA*v*-CO_2 vs. flow mediated dilatation (FMD) in the brachial artery) (Carr et al., 2020). Therefore, whether the changing hormone profile associated with the menopausal transition influences cerebrovascular function and regulation in post-menopausal females is unclear.

The capacity for the cerebrovasculature to respond to vasoactive stimuli provides important information on brain vascular function. Cerebrovascular responsiveness to CO_2 is an indication of the vasodilatory reserve capacity (Hoiland et al., 2011), with a higher cerebrovascular-CO_2 responsiveness (when measured via TCD) associated with improved cerebrovascular function (Ainslie & Duffin, 2009; Willie et al., 2011). Few studies have compared cerebrovascular-CO_2 responsiveness between pre- and post-menopausal females. Those that do report either no difference between pre- and post-menopausal females (Brislane et al., 2020; Mitsis et al., 2007), or lower cerebrovascular-CO_2 responsiveness in post-menopausal females when compared to both younger and age-matched pre-menopausal females (Matteis et al., 1998). However, methodology inconsistencies between these studies (e.g., CO_2 inhalation, breath-holding index) make it challenging to determine possible menopause-related differences in cerebrovascular-CO_2 responsiveness. Indeed, two recent reviews of the literature concluded that the inconsistent reporting of cerebrovascular outcomes alongside insufficient high-quality evidence means the physiological implications of menopause on cerebrovascular-CO_2 responsiveness is yet to be elucidated (Ruediger et al., 2021; Skinner et al., 2021), and further investigation is necessary.

Cerebral autoregulation is the intrinsic ability of the cerebrovasculature to maintain adequate perfusion despite changes in blood pressure (Lassen, 1959). Reduced effectiveness of this regulatory mechanism renders the brain more susceptible to both hypo- and hyperperfusion injury. The chronic decline in oestrogen throughout menopause has been associated with a chronic elevation in blood pressure (Rosenthal & Oparil, 2000). Despite this, whether cerebral autoregulation differs in post-

menopausal compared to pre-menopausal females remains largely overlooked. To the best of our knowledge, only one study has directly compared dynamic cerebral autoregulation in these cohorts, reporting no difference between pre- and post-menopausal females (Brislane et al., 2020). However, pre-menopausal females were only tested in the early follicular phase of the menstrual cycle when circulating oestrogen and progesterone are lowest. As such it remains unknown how cerebral autoregulation differs between post-menopausal and pre-menopausal females across different phases of the menstrual cycle (e.g., mid-luteal).

Subsequently, this study aimed to compare cerebrovascular-CO_2 responsiveness and cerebral autoregulation in 1) post-menopausal females compared to pre-menopausal females in the early follicular phase, and 2) post-menopausal females compared to pre-menopausal females in the mid-luteal phase of the menstrual cycle. Previous studies have shown that oestrogen promotes vasodilatory pathways and has beneficial effects on the cerebrovasculature, while the effects of progesterone are less clear. As such, it was hypothesised that cerebrovascular-CO_2 responsiveness and cerebral autoregulation would be greater in pre-menopausal compared to post-menopausal females, regardless of menstrual phase.

6.2 Methods

Ethical approval was obtained for all experimental protocols and procedures by the University of Birmingham Ethics Committee (project code: ERN_15-1179). All testing took place in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Prior to participation, a detailed verbal and written

explanation of the study procedure was provided, and written informed consent obtained.

6.2.1 Study Design and Protocol

Nine **pre**-menopausal females (age 20 ± 3 years; BMI 22.7 ± 1.8 kg/m^2) and six **post**-menopausal females (age 57 ± 2 years; BMI 23.9 ± 0.9 kg/m^2) participated in this study and were included in the analysis. All participants were healthy and free of any known cardiovascular, neurological or metabolic diseases. **Pre**-menopausal participants had a regular menstrual cycle (<34 days in length) and were not taking any hormonal contraceptive medication. **Post**-menopausal participants were included irrespective of HRT status. Participants were informed to abstain from alcohol consumption and vigorous exercise for 24 hours, in addition to caffeine consumption for 12 hours and food consumption for 2 hours prior to the study.

Participants were asked to attend the laboratory for either one (**post**-menopausal) or two (**pre**-menopausal, during the EF and ML phases of the menstrual cycle) experimental sessions. Prior to experimental sessions all participants completed a familiarisation session, during which participants were asked to lie in a supine position for ~20 minutes while instrumented with equipment (detailed below). Once instrumented, they performed the cerebrovascular-CO_2 responsiveness tests as detailed previously (Section 3.4.1; Figure 6.1). Participants were also familiarised with the squat-to-stand procedure (Section 3.4.2). Once the familiarisation session was satisfactorily completed (i.e., suitable Doppler signals detected and no adverse effects to the CO_2 stimulus), participants were invited back for their experimental testing

session(s). Participants were provided with an oral thermometer and calendar to record basal core temperature throughout their cycle. They were instructed to measure basal core temperature upon waking each morning, prior to consumption of food or water to ensure accurate measurement. The early follicular phase was defined as days 1-4 of the menstrual cycle, where day 1 is the first day of menstruation. The mid-luteal phase was defined as 8-10 days post-ovulation (ovulation identified by a sustained >0.5°C increase in basal core temperature), depending on total cycle length.

Upon arrival at an experimental testing session, participants lay supine for a minimum of 20 minutes during which they were instrumented for measures of cerebro- and cardiovascular function (detailed below). Cerebrovascular-CO_2 responsiveness tests were then performed (Figure 6.1). This was followed by repeated squat-to-stand manoeuvres performed at two different frequencies (5s and 10s cycles) for 5 minutes each (described previously; Section 3.4.2).

6.2.2 Equipment and Outcome Measures

Beat-to-beat middle cerebral artery blood flow velocity (MCA*v*) was assessed using transcranial Doppler (TCD; Doppler-Box X, DWL, Compumedics Ltd, Germany) with a 2-MHz probe placed over the temporal window. Ultrasound gel was placed on the probes and held in place with a headset. Search and identification procedures were performed as detailed previously (Section 3.3.1).

Internal carotid artery (ICA) blood flow was assessed using Duplex Doppler ultrasound (Terason uSmart 3300; Teratech, USA) with a 10 MHz linear array ultrasound transducer probe and an insonation angle of 60°. Measurements were made 1-2cm

proximal to the carotid bulb. Screenshots of the ICA were taken during baseline measurements to ensure the same location was insonated during 5% CO_2 inhalation. Technical guidelines published by Thomas and colleagues (2015) were adhered to in order to standardise measurements and improve accuracy.

Beat-to-beat blood pressure was measured using a finger cuff on the middle finger of the left hand (Finapres NOVA, Finapres Medical Systems, The Netherlands). Respiratory rate and volume were measured using a heated pneumotachograph (3813 Series, Hans Rudolph Inc, Kansas, USA) attached to a facemask, while fractional changes in inspired and expired O_2 and CO_2 were measured via a sample line attached to the facemask and a gas analyser (ML206, ADInstruments Ltd, New Zealand). Measures were recorded at 1k Hz via an analogue-to-digital converter (Powerlab, ADInstruments) and displayed in real time and stored for offline analysis using commercially available software (LabChart v7.3.5, ADInstruments).

6.2.3 Data Analysis

Data from 60 s of the baseline period, 30 s of the hypercapnic stages (from 2:30 min – 3:00 min; (Burley et al., 2020), and >20 s of data at the target end-tidal CO_2 during the hypocapnic stages, were extracted and used in the statistical analyses. The slope of the cerebral blood flow (CBF) response to changes in $P_{ET}CO_2$ was calculated to give an estimation of cerebrovascular-CO_2 responsiveness in the MCA (MCA*v*-CO_2 responsiveness). MCA*v*-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were determined by the slope of the CBF response from stages Hyper1 to Hyper2, Hypo1 to Hypo2, and Hypo1 to Hyper1, respectively (see Figure 6.1). An

index of cerebrovascular conductance (CVC) was also calculated from the ratio of CBF to mean arterial pressure (MAP), allowing examination of cerebrovascular responsiveness without changes in MAP influencing cerebral blood flow velocity. The pulsatility index in the MCA (MCA*v*-PI) was calculated as (systolic MCA*v* – diastolic MCA*v*) / mean MCA*v*. An estimation of CVC to CO_2 was expressed as the change in CVC per mmHg change in $P_{ET}CO_2$ (MCA*v*-CVC-CO_2 responsiveness).

Ultrasound images of the ICA were captured using video-recording software and analysed using an edge-detection and wall-tracking software (Cardiovascular Suite, Quipu, Italy). Doppler flow velocity waveforms were automatically traced and mean blood flow velocity (cm/s) calculated. From the 2-minute recording, blood vessel diameter (cm) and blood flow velocity (cm/sec) from >20s of the baseline period and Hyper2 were used to calculate blood flow (see Figure 6.1). The relative change in ICA diameter from baseline to Hyper2 was also calculated.

Cerebral autoregulation data were analysed using Ensemble-R software (Elucimed Ltd, New Zealand). Beat-to-beat blood pressure and flow velocity values were spline interpolated and re-sampled for spectral and transfer function analyses (TFA) based on the Welch algorithm. This approach subdivides each selected recording period into successive overlapping windows. Data within each window were then linearly detrended and passed through a Hanning window before fast Fourier transform analysis. TFA coherence, gain and phase of the forced MAP oscillations were sampled at the point estimate of the driven frequency (0.05 Hz and 0.10 Hz). These point estimates were selected as they are in the very low (0.02-0.07 Hz) and low (0.07-0.20

Hz) frequency ranges where dynamic cerebral autoregulation is thought to be most operant (Smirl et al., 2015).

6.2.4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (Version 8.0.0, GraphPad Software, San Diego, CA, USA). Unpaired t-tests were used to compare baseline data and cerebrovascular-CO_2 responsiveness outcome measures between groups (PM vs EF; PM vs ML). Simple linear regressions were performed on MCA*v*-CO_2 responsiveness to obtain a best-fit value of the MCA*v* response across the $P_{ET}CO_2$ range (stages Hypo1, Hyper1 and Hyper2; see Figure 6.1). The line of best fit is reported as $y = bx + a$, where b is the slope of the line and a is the *y*-intercept when $x=0$. The slope and *y*-intercept values were compared between groups (PM vs EF; PM vs ML).

It was not possible to insonate the ICA in all participants and subsequently statistical analyses were not performed. Similarly, data from some participants were excluded from TFA analysis (detailed below) and statistical analyses were not performed.

Data are presented as means ± SD. Statistical significance were based on an α-level of 0.05.

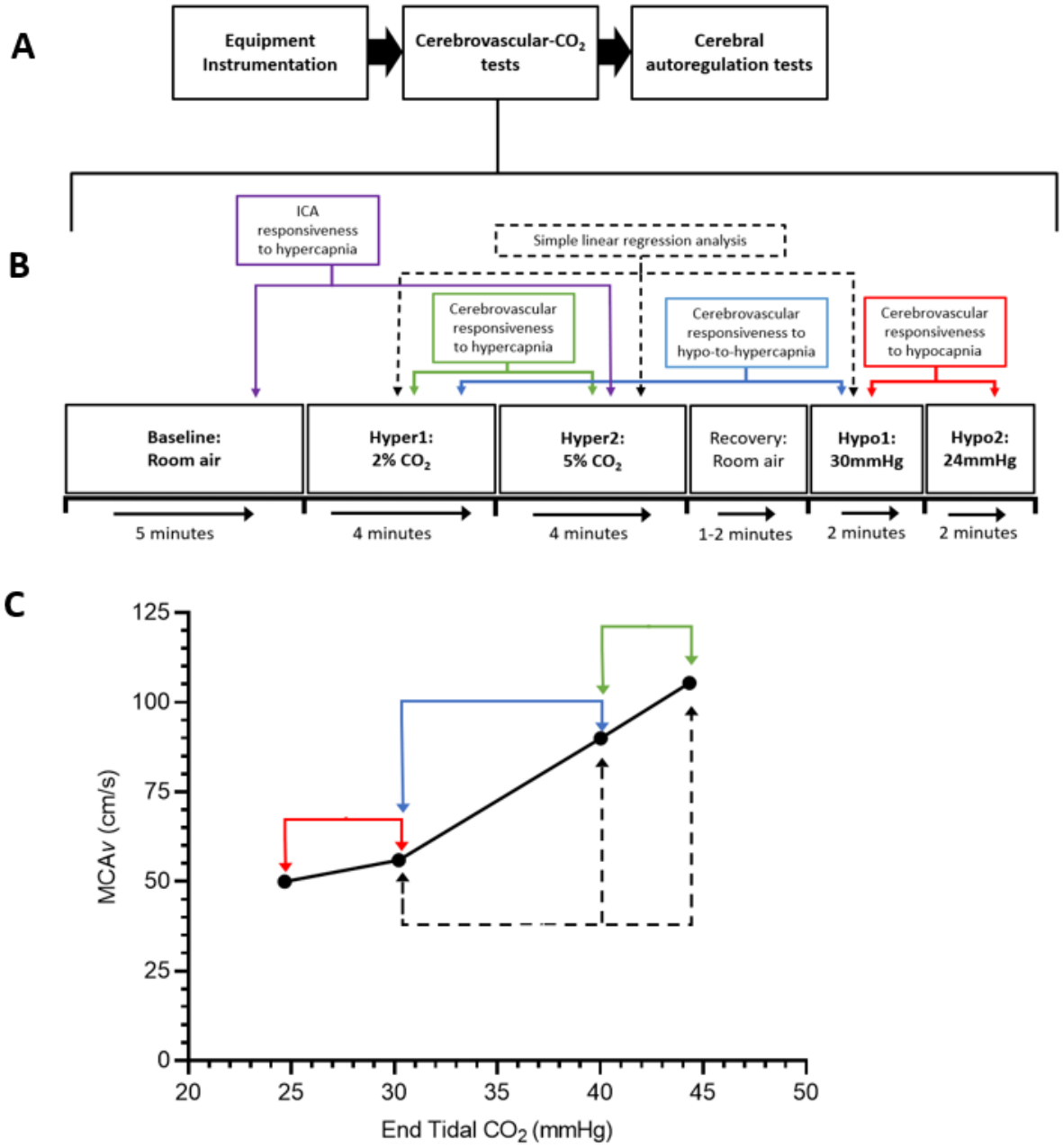

Figure 6.1 Schematic of the study protocol. Panel **A** provides an overview of the experimental session, with Panel **B** shows the cerebrovascular-CO_2 responsiveness tests protocol. Panel **C** shows an example graph of the cerebrovascular responsiveness slope. Green, blue, and red arrows indicate points of data extraction to calculate the cerebrovascular responsiveness to hypercapnia (Hyper1 to Hyper2), hypo-to-hypercapnia (Hypo1 to Hyper1), and hypocapnia (Hypo1 to Hypo2), respectively. Dashed back arrows indicate points of data extraction used in simple linear regression analysis. Purple arrows indicate points of data extraction to calculate the internal carotid artery (ICA) responsiveness to hypercapnia.

6.3 Results

Of the nine **pre**-menopausal participants enrolled, nine completed experimental sessions in the early follicular (EF) phase and six completed experimental sessions in the mid-luteal (ML) phase of the menstrual cycle. Of the six **post**-menopausal females who were enrolled and completed the experimental session, three were taking HRT medication.

Data for one **post**-menopausal participant was excluded from the cerebrovascular-CO_2 responsiveness data (MCA*v*-CO_2 and ICA-CO_2 responsiveness outcome measures) due to equipment error. Additionally, the ICA could only be insonated and acceptable images recorded in four **post**-menopausal females, and four and three **pre**-menopausal females during the EF and ML phases, respectively.

Data for five **post**-menopausal females, alongside six and three **pre**-menopausal females during the EF and ML phases, respectively, were deemed suitable for transfer function analysis in the 0.05 Hz frequency. Data for six **post**-menopausal females, and seven and five **pre**-menopausal females during the EF and ML phases, respectively, were acceptable for transfer function analysis in the 0.10 Hz frequency.

Table 6.1 Baseline cardiovascular, respiratory, and cerebrovascular responses in **post**-menopausal (n=6) and **pre**-menopausal females during the early follicular (n=9) and mid-luteal (n=6) phases of the menstrual cycle.

	Pre-Menopausal females		
	Early Follicular (n = 9)	Mid-Luteal (n = 6)	**Post**-Menopausal females (n = 6)
Heart Rate (bpm)	64 ± 7	66 ± 10	58 ± 6
Mean Arterial Pressure (mmHg)	83 ± 5*	81 ± 10*	92 ± 7
Ventilation (L/min)	4.9 ± 1.0*	3.8 ± 0.9	3.0 ± 1.2
End-Tidal CO_2 (mmHg)	38.1 ± 3.0	40.2 ± 1.3	38.5 ± 1.9
MCA*v* (cm/s)	70 ± 16	67 ± 16	65 ± 12
MCA*v*-PI	0.71 ± 0.13	0.75 ± 0.04	0.73 ± 0.11
MCA*v*-CVC (cm/s/mmHg)	0.86 ± 0.17	0.80 ± 0.13	0.70 ± 0.12
ICA blood flow (ml/min) [#]	412 ± 241	468 ± 192	480 ± 106

MCA*v*, middle cerebral artery velocity; MCA*v*-PI, middle cerebral artery pulsatility index; MCA*v*-CVC, middle cerebral artery conductance. ICA, internal carotid artery. Values are means ± SD, **significantly different from post-menopause.* [#]ICA blood flow data reported in four post-menopausal females, and four and three pre-menopausal females during the early follicular and mid-luteal phases, respectively.

*6.3.1 **Post**-Menopausal Females vs. **Pre**-Menopausal Females during the Early Follicular Phase*

6.3.1.1 Baseline Data

Baseline data can be found in Table 6.1. Mean arterial pressure was +9 ± 3 mmHg higher (p<.01) and ventilation -1.9 ± 0.6 L/min lower (p=.01) in **post**-menopausal females when compared to **pre**-menopausal females during EF. Heart rate, end-tidal CO_2, MCA*v*, MCA*v*-PI, and MCA*v*-CVC were not significantly different between **post**-

menopausal and **pre**-menopausal females during EF (p>.08). ICA blood flow appeared to be greater in **post**-menopausal females compared to **pre**-menopausal females during EF, as reflected by 3 out of 4 **post**-menopausal females having an ICA blood flow above the mean of the **pre**-menopausal females during EF at baseline.

6.3.1.2 MCAv Responsiveness

MCAv-CO_2 responsiveness to hypercapnia was greater in **pre**-menopausal females during EF when compared to **post**-menopausal females (Figure 6.2; 4.10 ± 1.30 vs 2.57 ± 0.72 cm/s/mmHg; p=.03). MCAv-CO_2 responsiveness to hypocapnia and hypo-to-hypercapnia were similar between groups (p>.27). Simple linear regression analysis of MCAv-CO_2 responsiveness found similar slopes (p=.41) and y-intercepts (p=.08) between **pre**-menopausal females during EF and **post**-menopausal females (EF, y=3.15x-47.89; PM, y=2.42x-28.82).

MCAv-CVC-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were not significantly different between **pre**-menopausal females during EF and **post**-menopausal females (p>.06).

6.3.1.3 ICA Responsiveness

The relative change in ICA diameter in response to hypercapnia (baseline-Hyper2; Table 6.2) appeared to be greater in **post**-menopausal females compared to **pre**-menopausal females during EF, as reflected by 3 out of 4 **post**-menopausal females having a change in ICA diameter above the mean of the **pre**-menopausal females during EF. Similarly, ICA-CO_2 responsiveness to hypercapnia (Table 6.2) appeared to be greater in **post**-menopausal females compared to **pre**-menopausal females during

EF, as reflected by 3 out of 4 **post**-menopausal females having an ICA-CO_2 responsiveness above the mean of the **pre**-menopausal females during EF. Individual ICA blood flow responses at baseline and during Hyper2 are displayed in Figure 6.3.

6.3.1.4 Cerebral Autoregulation

The gain, phase and coherence for repeated squat-to-stand manoeuvres are displayed in Figure 6.4. During 0.05 Hz squat-to-stand manoeuvres absolute gain (**Pre**: 0.50 ± 0.23 cm/s/mmHg; **Post**: 0.48 ± 0.13 cm/s/mmHg), normalised gain (**Pre**: 0.87 ± 0.19 %/mmHg; **Post**: 0.86 ± 0.17 %/mmHg) and coherence (**Pre**: 0.73 ± 0.16; **Post**: 0.79 ± 0.12) appeared similar between **pre**-menopausal females during EF and **post**-menopausal females. Phase appeared to be greater in **pre**-menopausal females during EF compared to **post**-menopausal females (**Pre**: 2.15 ± 0.65 radians; **Post**: 1.79 ± 0.48 radians), as reflected by 4 out of 5 **pre**-menopausal females having a phase above the mean of the **post**-menopausal females. During 0.10 Hz squat-to-stand manoeuvres phase (**Pre**: 1.87 ± 1.66 radians; **Post**: 1.40 ± 1.31 radians) and coherence (**Pre**: 0.62 ± 0.09; **Post**: 0.58 ± 0.12) appeared similar between **pre**-menopausal females during EF and **post**-menopausal females. Absolute gain (**Pre**: 0.80 ± 0.25 cm/s/mmHg; **Post**: 0.53 ± 0.11 cm/s/mmHg) and normalised gain (**Pre**: 1.35 ± 0.19 %/mmHg; **Post**: 1.00 ± 0.22 %/mmHg) appeared to be greater in **pre**-menopausal females during EF compared to **post**-menopausal females, as reflected by 5 out of 7 and 7 out of 7 **pre**-menopausal females having an absolute gain and normalised gain, respectively, above the mean of the **post**-menopausal females.

Table 6.2 Cerebrovascular responsiveness in the middle cerebral artery (MCA*v*-CO_2 responsiveness) and internal carotid artery (ICA-CO_2 responsiveness, ICA diameter), in post-menopausal females and pre-menopausal females during the early follicular and mid-luteal. 'Full range' refers to the linear regression line of best-fit which includes the Hypo1, Hyper1 and Hyper2 stages of the cerebrovascular responsiveness protocol. ICA-CO_2 responsiveness and ICA diameter changes refer to responses between the baseline and Hyper2 stages.

		Pre-Menopausal Females		**Post**-Menopausal Females
		Early Follicular	Mid Luteal	
MCA*v*-CO_2 responsiveness (cm/s/mmHg)	Hypercapnia	4.10 ± 1.30*	4.16 ± 1.28*	2.57 ± 0.72
	Hypocapnia	-0.88 ± 0.37	-1.23 ± 0.48	-0.85 ± 0.66
	Hypo-to-hypercapnia	2.87 ± 0.88	2.26 ± 1.13	2.36 ± 0.54
	Full range	3.15 ± 0.86	2.89 ± 1.10	2.42 ± 0.57
ICA-CO_2 responsiveness (ml/min/mmHg) [#]	Hypercapnia	19.00 ± 17.96	12.25 ± 11.90	29.30 ± 13.69
ICA diameter (% change) [#]	Hypercapnia	-0.5 ± 2.3	1.2 ± 4.2	2.3 ± 9.8

Values are means ± SD. *significantly different from post-menopausal females. [#]ICA blood flow data reported in four post-menopausal females, and four and three pre-menopausal females during the early follicular and mid-luteal phases, respectively.

6.3.2 ***Post***-Menopausal Females vs. ***Pre***-Menopausal Females during the Mid-Luteal Phase

6.3.2.1 Baseline data

Baseline data can be found in Table 6.1. Mean arterial pressure was 11 ± 5 mmHg higher (p<.05) in **post**-menopausal females when compared to **pre**-menopausal females during ML. Heart rate, ventilation, end-tidal CO_2, MCA*v*, MCA*v*-PI, and MCA*v*-CVC were not significantly different between **post**-menopausal females and **pre**-menopausal females during ML (p>.08). ICA blood flow appeared to be similar between **post**-menopausal females and **pre**-menopausal females during ML.

6.3.2.2 MCAv Responsiveness

MCA*v*-CO_2 responsiveness to hypercapnia was greater in **pre**-menopausal females during ML when compared to **post**-menopausal females (Figure 6.2; 4.16 ± 1.28 vs 2.57 ± 0.72 cm/s/mmHg; p=.04). MCA*v*-CO_2 responsiveness to hypocapnia and hypo-to-hypercapnia were similar between groups (p>.30). Simple linear regression analysis of MCA*v*-CO_2 responsiveness found similar slopes (p=.62) and y-intercepts (p=0.64) between **post**-menopausal and **pre**-menopausal females during ML (ML, y=2.89x-44.60; PM, y=2.42x-28.82).

MCA*v*-CVC-CO_2 responsiveness to hypercapnia, hypocapnia and hypo-to-hypercapnia were not significantly different between **post**-menopausal and **pre**-menopausal females during ML (p>.06).

6.3.2.3 ICA Responsiveness

The relative change in ICA diameter in response to hypercapnia (baseline-Hyper2) appeared to be similar between **post**-menopausal females and to **pre**-menopausal females during ML (Table 6.2). ICA-CO_2 responsiveness to hypercapnia (Table 6.2) appeared greater in **post**-menopausal females compared to **pre**-menopausal females during ML, as reflected by 4 out of 4 **post**-menopausal females having an ICA-CO_2 responsiveness above the mean of the **pre**-menopausal females during ML. Individual ICA blood flow responses at baseline and during Hyper2 are displayed in Figure 6.3.

6.3.2.4 Cerebral Autoregulation

The gain, phase and coherence for repeated squat-to-stand manoeuvres are displayed in Figure 6.4. During 0.05 Hz squat-to-stand manoeuvres absolute gain (**Pre**: 0.51 ± 0.20 cm/s/mmHg; **Post**: 0.48 ± 0.13 cm/s/mmHg), normalised gain (**Pre**: 0.95 ± 0.19 %/mmHg; **Post**: 0.86 ± 0.17 %/mmHg), coherence (**Pre**: 0.81 ±0.10; **Post**: 0.79 ± 0.12), and phase (**Pre**: 1.78 ± 0.85 radians; **Post**: 1.79 ± 0.48 radians) appeared similar between **post**-menopausal females and **pre**-menopausal females during ML. During 0.10 Hz squat-to-stand manoeuvres phase (**Pre**: 1.05 ± 1.30 radians; **Post**: 1.40 ± 1.31 radians) appeared similar between **post**-menopausal and **pre**-menopausal females during ML. Coherence (Pre: 0.49 ± 0.05; Post: 0.58 ± 0.12) appeared greater in **post**-menopausal females compared to **pre**-menopausal females during ML, as reflected by 5 out of 6 **post**-menopausal females having a coherence above the mean of the **pre**-menopausal females. Absolute gain (**Pre**: 0.63 ± 0.13 cm/s/mmHg; **Post**: 0.53 ± 0.11 cm/s/mmHg) and normalised gain (**Pre**: 1.20 ± 0.44 %/mmHg; **Post**: 1.00 ± 0.22 %/mmHg) appeared to be greater in **pre**-menopausal females during ML compared to

post-menopausal females, as reflected by 4 out of 5 **pre**-menopausal females having an absolute gain and normalised gain above the mean of the **post**-menopausal females.

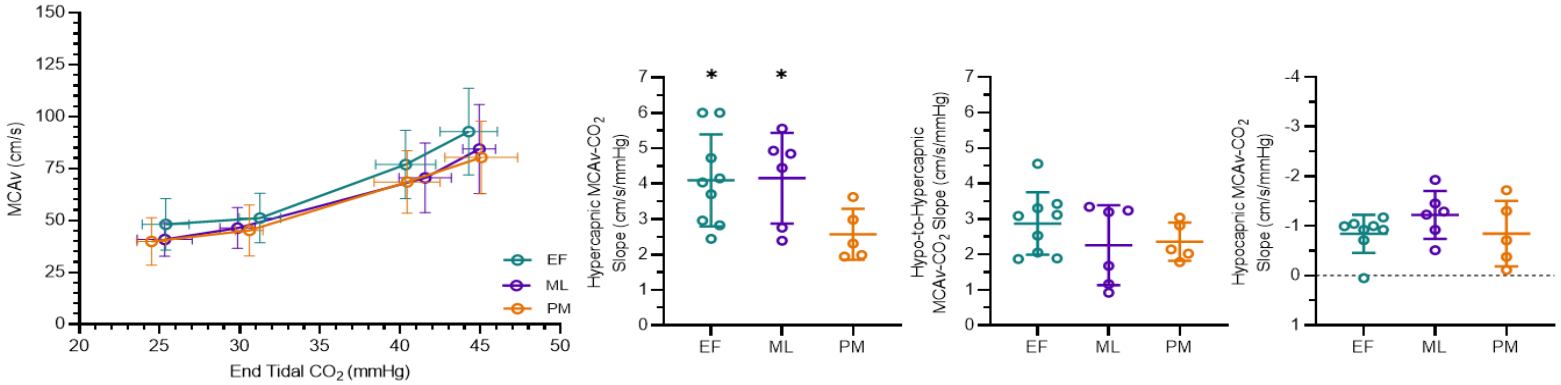

Figure 6.2 The middle cerebral artery blood flow velocity (MCA*v*) slope at hyper-, hypo- and hypo-to-hypercapnia (MCA*v*-CO_2 responsiveness) in pre-menopausal females during the early follicular (EF; n = 9) and mid-luteal (ML; n = 6) phase of the menstrual cycle, and in post-menopausal females (n = 5).

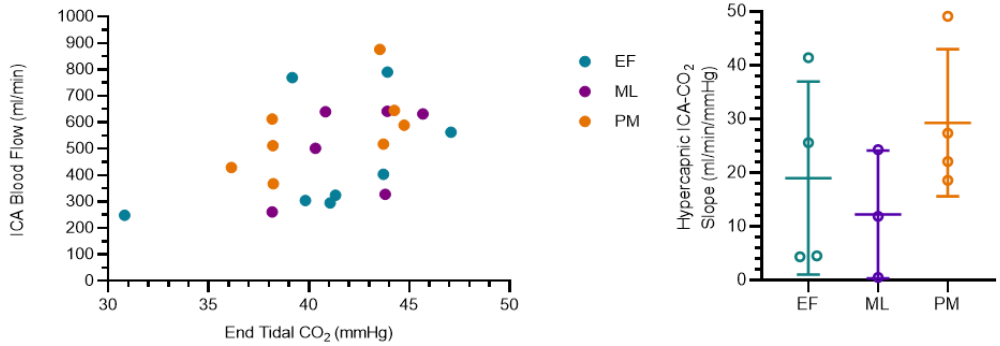

Figure 6.3 Individual internal carotid artery blood flow (ICA) at baseline and in response to 5% CO_2 inhalation in post-menopausal females (PM; n = 4), and pre-menopausal females during the early follicular (EF; n = 4) and mid-luteal (ML; n = 3) phase of the menstrual cycle.

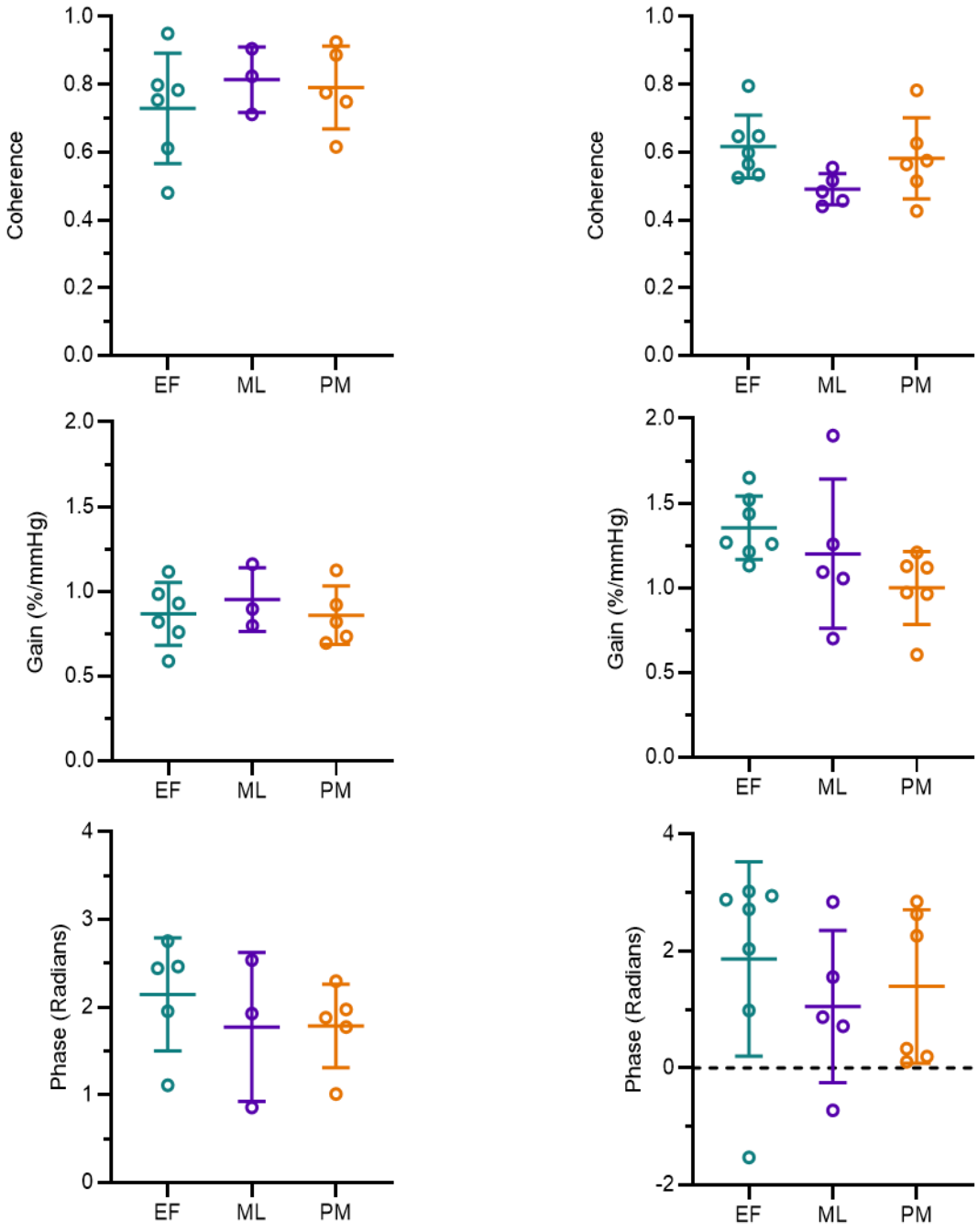

Figure 6.4 Transfer function analysis in pre-menopausal females during the early follicular (EF) and mid-luteal (ML) phase of the menstrual cycle, and in post-menopausal (PM) females performing repeated squat-to-stand manoeuvres. Coherence (top), normalised gain (middle) and phase (bottom) in the middle cerebral artery is shown for 0.05 Hz (left panel; EF n = 6, ML n = 3, PM n = 5) and 0.10 Hz (right panel; EF n = 7, ML n = 5, PM n = 6) frequency manoeuvres.

6.4 Discussion

The main finding of this study is that MCA*v*-CO_2 responsiveness to hypercapnia was greater in pre-menopausal females compared to post-menopausal females, irrespective of menstrual phase. Although statistical analysis could not be performed on all outcome measures, the preliminary data for ICA-CO_2 responsiveness to hypercapnia and cerebral autoregulation suggests differences between cohorts may be present. This can provide an important foundation to inform future research investigating cerebrovascular function in pre- and post-menopausal women.

6.4.1 Cerebrovascular-CO_2 responsiveness in pre- and post-menopausal females

It was hypothesised that cerebrovascular-CO_2 responsiveness would be greater in pre-menopausal females when compared to post-menopausal females regardless of menstrual phase, since circulating oestrogen levels are higher in pre-menopausal females (even in EF) and higher circulating oestrogen levels are associated with beneficial cerebrovasculature effects. Previous studies have reported cerebrovascular-CO_2 responsiveness in the MCA to be either similar between pre- and post-menopausal females (Brislane et al., 2020; Mitsis et al., 2007), or greater in pre-menopausal compared to post-menopausal females (Matteis et al., 1998). Of note, Brislane and colleagues (2020) performed voluntary hyperventilation to an end-tidal CO_2 <20 mmHg prior to the hypercapnic challenge. Not only has performing a hypocapnic challenge been shown to blunt the cerebrovascular response to a subsequent hypercapnic stimulus (Brothers et al., 2014), but the P_aCO_2 threshold for reaching the limit of the vessel calibre has been shown to be ~25 mmHg in the

hypocapnic range (Harper & Glass, 1965). Consequently, cerebrovascular-CO_2 responsiveness measures that include end-tidal CO_2 values below 25 mmHg may potentially mis-represent or skew the responsiveness outcome measure. Additionally, using a breath-holding method, Matteis and colleagues (1998) reported lower cerebrovascular responsiveness to hypercapnia in post-menopausal females when compared to both younger and age-matched pre-menopausal females. When calculating the breath-holding index, the duration of the breath-hold is used as an indication of the P_aCO_2 stimulus and thus, used to normalise changes in cerebral blood flow. The relationship between breath-holding time and P_aCO_2 has since been shown to be non-linear and highly variable between participants (Totaro et al., 1999), and therefore breath-holding cannot produce a standardised hypercapnic stimulus or be compared to other vasoactive stimuli (Fierstra et al., 2013). Consequently, the methodological issues in the few previous studies reporting cerebrovascular-CO_2 responsiveness determines that there is no clear consensus on the difference, if any, between pre- and post-menopausal females.

In the present study and in support of our hypothesis, MCA*v*-CO_2 responsiveness to hypercapnia was greater in pre-menopausal females during EF and ML when compared to post-menopausal females. Since oestrogen is known to promote eNOS production and prostacyclin pathways, thereby reducing cerebrovascular tone and increasing vasodilation (Krause et al., 2006), the abrupt decline in oestrogen over the menopausal transition may limit the vasodilatory capacity of the MCA. Conversely, MCA*v*-CVC-CO_2 responsiveness to hypercapnia was found to be similar between pre- and post-menopausal females. An index of CVC-CO_2 responsiveness allows for

examination of cerebrovascular responsiveness without the influence of differences in mean arterial pressure (MAP). As resting MAP was higher in post-menopausal females compared to pre- menopausal females during both the early follicular and mid-luteal phase, the blunted MCA*v*-CO_2 responsiveness to hypercapnia observed in post-menopausal females may be accounted for by increased blood pressure. Since the chronic decline in oestrogen throughout menopause has been associated with a chronic elevation in blood pressure (Rosenthal & Oparil, 2000), a decline in sex hormones may still be the driving factor behind the observed differences in MCA*v*-CO_2 responsiveness to hypercapnia.

To the best of our knowledge, ICA-CO_2 responsiveness has not been previously examined between pre- and postmenopausal females. In contrast to our hypothesis, the preliminary data presented here shows post-menopausal females to have an on-average greater ICA-CO_2 responsiveness to hypercapnia when compared to pre-menopausal females (as reflected by 3 out of 4, and 4 out of 4 post-menopausal females having an ICA-CO_2 responsiveness greater than the average for pre-menopausal females during EF and ML, respectively). Interestingly, this opposes the greater cerebrovascular responsiveness to hypercapnia we observed in pre-menopausal women in the MCA. ICA-CO_2 responsiveness to hypercapnia has previously been described as having a tendency (p=.094) towards being greater in older compared to younger males, while MCA*v* responsiveness to hypercapnia was similar between groups (Braz et al., 2017). Although speculative, since the MCA arises from the ICA a greater ICA-CO_2 responsiveness to hypercapnia may act as a compensatory mechanism to counteract a blunted MCA*v* responsiveness in post-

menopausal females. Since statistical analyses were not performed, the data reported in the present study is insufficient to determine differences in ICA-CO_2 responsiveness between pre- and post-menopausal females, and how this may differ from responsiveness in the MCA. Despite this, it can be used as a foundation to inform possible future research into intra- and extra-cranial cerebrovascular responsiveness in these cohorts.

The apparent differences in cerebrovascular-CO_2 responsiveness between the MCA and ICA in the present study support a growing body of evidence demonstrating regional differences in cerebrovascular responsiveness. Until recently, it had been assumed that vascular responses measured in the MCA were an adequate representation of the global cerebrovascular response (Ainslie & Duffin, 2009). However, regional differences in cerebrovascular responsiveness in young males and females have been reported, both between intracranial vessels, as well as between intra- and extracranial vessels (Sato et al., 2012). Cerebrovascular-CO_2 responsiveness in the extracranial vessels, such as the ICA, have been much less extensively studied in comparison to the MCA. While the present study did not formally compare the MCA and ICA, the possible opposing directional change observed in cerebrovascular responsiveness between vessels in post-menopausal females further indicates the MCA does not represent the global cerebrovascular response to a CO_2 stimulus. As such, further investigation into regional differences in cerebral blood flow regulation is warranted.

6.4.2 Cerebral Autoregulation in pre- and post-menopausal females

It was hypothesised that cerebral autoregulation would be improved in pre-menopausal females when compared to post-menopausal females, regardless of menstrual phase. Cerebral autoregulation has been shown to be similar across phases of the menstrual cycle (Favre & Serrador, 2019), but improved in females compared to males (Deegan et al., 2010; Favre & Serrador, 2019), indicating that acute fluctuations in sex hormones are insufficient to cause a change in a robust regulatory mechanism such as autoregulation. To the best of our knowledge only one study has reported cerebral autoregulation in post-menopausal females and pre-menopausal females during EF, indicating no difference between groups in the low-frequency range (Brislane et al., 2020), despite the chronic decline in sex hormones observed in the menopausal transition. The preliminary data presented in the present study appear to indicate differences in cerebral autoregulation may be present between pre- and post-menopausal females, and that these differences may be both menstrual phase and frequency dependent. Pre-menopausal females during EF appear to show improved buffering of blood pressure oscillations in the very-low frequency range (0.05 Hz) when compared to post-menopausal females. This was reflected in the on-average greater phase in pre-menopausal females, suggesting an improved vasoactive response to changes in pressure. In contrast, pre-menopausal females during ML appear to show similar cerebral autoregulation in the very-low-frequency range compared to post-menopausal females. In the low-frequency range (0.10 Hz), post-menopausal females appear to have a lower absolute and normalised gain compared to pre-menopausal females in both EF and ML phases, indicating an improved buffering efficiency of blood

pressure fluctuations into cerebral blood flow velocity. Of note, there are known differences in blood pressure control between pre- and post-menopausal females, with post-menopausal females shown to have a higher baroreflex “setpoint” (Peinado et al., 2017), as well as a higher baseline muscle sympathetic nerve activity compared to pre-menopausal females (Barnes et al., 2014). How this altered blood pressure control translates to differences in cerebral autoregulation is unknown. Further, due to the small sample size at present it would be speculative to conclude that cerebral autoregulation is definitively improved in one cohort over another, and further investigation is necessary.

6.4.3 Considerations / Study Limitations

The small sample size meant that statistical analyses could not be performed for all outcomes measures. As such, preliminary data only is reported for ICA responsiveness and cerebral autoregulation outcomes. Furthermore, post-menopausal females were not excluded if taking HRT. With the addition of more participants, we hope to provide a comparison between pre-menopausal females across the menstrual cycle and post-menopausal females both taking HRT and not taking HRT. Of the six post-menopausal females enrolled thus far three were on HRT medication, which may prove a confounding factor in the reported outcome measures. Indeed, HRT has been shown to be associated with greater resting cerebral blood flow (Słopień et al., 2003) and improved pulsatility index (an indication of downstream resistance and cerebral tissue perfusion (Skinner et al., 2021)). Consequently, the data presented in the present study should be interpreted with caution, and with the small sample size and HRT inclusion in mind.

We used Doppler ultrasound to measure blood flow velocity in the MCA. A primary assumption of TCD is that the insonated vessel maintains a constant diameter. While this assumption has been validated (Valdueza et al., 1997; Serrador et al., 2000), more recent MRI studies have reported changing vessel diameters in response to changing CO_2 (Coverdale et al., 2014; Verbree et al., 2014), resulting in possible over- and under-estimations of CBF when CBF velocity is used as an index of absolute flow. Despite this, assessment of cerebrovascular-CO_2 responsiveness by TCD has been shown to offer valuable information on cerebrovascular health and function, provided the derived data are interpreted with these limitations in mind (Willie et al., 2011).

Changes in $P_{ET}CO_2$ were used to calculate cerebrovascular responsiveness, based on the assumption that $P_{ET}CO_2$ accurately represents arterial CO_2 (P_aCO_2). While these two variables can differ with metabolic CO_2 production and tidal volume, they have been shown to not differ with changes in breathing frequency (Jones et al., 1979).

In states of augmented hypo- or hypercapnia, the cerebrovasculature may reach a point of maximal constriction or dilation. The P_aCO_2 threshold for reaching the limit of the vessel calibre has been shown to be ~65 mmHg in the hypercapnic range and ~25 mmHg in the hypocapnic range (Harper & Glass, 1965). In the present study, $P_{ET}CO_2$ increased to ~45 mmHg during Hyper2, and decreased to ~24mmHg during Hypo2. If the limit of the vessel calibre is reached, further diameter changes are not possible and a change in cerebrovascular responsiveness may not be represented by further changes in CBF velocity. To account for this, the Hypo2 stage was omitted from the simple linear regression, with analysis using the Hypo1, Hyper1 and Hyper 2 stages.

The present study employs a steady-state CO_2 technique to measure cerebrovascular responsiveness to CO_2. This technique was chosen as it incorporates both the ventilatory and cerebrovascular response to a CO_2 stimulus. The use of other techniques to measure cerebrovascular-CO_2 responsiveness may elicit different outcomes as they stimulate physiological systems in a different manner. For example, a rebreathing technique abolishes the PCO_2 gradient throughout the body (e.g., between end-tidal and arterial concentrations) and therefore measures only the ventilatory response unaffected by the cerebrovascular response (Ainslie & Duffin, 2009). As such, the present findings should be considered with the methodology used in mind, and that outcomes may differ when reported alongside ventilatory responsiveness or if a different technique is used.

The present study used squat-to-stand manoeuvres to drive oscillations in blood pressure, maximising the signal-to-noise ratio and thereby improving the coherence between the input and output signal. Using this approach, blood pressure oscillations are expected to elicit coherence values ~0.90 at the frequency of interest (Claassen et al., 2009; Smirl et al., 2015), which is greater than those observed in the present study (0.10 Hz manoeuvres - EF: 0.62 ± 0.09, ML: 0.49 ± 0.05, PM: 0.58 ± 0.12; 0.05 Hz manoeuvres - EF: 0.73 ± 0.16, ML: 0.81 ± 0.10, PM: 0.79 ± 0.12). However, similar coherence values to those presented here have been reported in pre- and post-menopausal cohorts performing 0.10 Hz squat-to-stand manoeuvres (pre-menopausal: 0.62 ± 0.12, post-menopausal: 0.67 ± 0.12; Brislane et al., 2020). Since the coherence metric provides an indication of the reliability of the transfer function

analysis, the phase and gain outcomes should be interpreted with caution in the present study.

The present study did not control for several population demographics that may affect the reported outcome measures. For example, physical activity levels, sedentary behaviour, and training status are likely to differ between pre- and post-menopausal females. Previously, ICA-CO_2 responsiveness to hypercapnia has been shown to be improved in aerobically trained compared to untrained males (Braz et al., 2017), and in older adults regular aerobic exercise is associated with greater vasodilatory capacity in the MCA (Barnes et al., 2013). Subsequently, possible differences in physical fitness between cohorts should be considered when interpreting the results, particularly the improved MCA*v*-CO_2 responsiveness to hypercapnia reported in pre-menopausal compared to post-menopausal females.

6.4.4 Summary

The findings of this study show that pre-menopausal females have an improved MCA*v*-CO_2 responsiveness to hypercapnia compared to post-menopausal females, irrespective of menstrual phase. This supports previous evidence that increased sex hormones have a positive influence on cerebrovascular function and regulation. The preliminary data for ICA-CO_2 responsiveness and cerebral autoregulation provide an important foundation to inform future research investigating cerebrovascular function in pre- and post-menopausal women.

7 GENERAL DISCUSSION

The incidence and prevalence of cerebrovascular diseases are increasing, with the overall risk of stroke and dementia higher in females compared to males (Seshadri & Wolf, 2007). The disproportionate increase in cerebrovascular disease risk factors in the first 10 years after menopause (Lisabeth & Bushnell, 2012) indicates a role for sex hormones in healthy cerebrovascular function and regulation. Despite a growing body of evidence in recent years, the exact role of oestrogen and progesterone on the cerebrovasculature is yet to be fully elucidated. Given this, the primary aim of this thesis was to assess the influence of sex hormones on cerebrovascular function and regulation. In order to do so a systematic review and meta-analysis (Chapter 2) was performed to synthesise and identify knowledge gaps in the current literature on cerebrovascular function and changing sex hormones. Subsequently, measures of cerebrovascular function were compared in females across the menstrual cycle (Chapter 5), and between males and females (Chapter 4) during normothermia and during a passive heat stress perturbation. Further, measures of cerebrovascular function were compared between post-menopausal females and pre-menopausal females during two phases of the menstrual cycle (Chapter 6).

7.1 Main Findings

The systematic review and meta-analysis presented in Chapter 2 established that hormone replacement therapy (HRT) in post-menopausal females has the potential to improve cerebrovascular function. This was reflected in a significant reduction in pulsatility index when compared to post-menopausal females not taking HRT. The effect of changes in sex hormones in hormone groups other than HRT (e.g., menstrual cycle, pregnancy) remains largely unclear, at least in part due to the substantial

heterogeneity in the current literature and the limited evidence for certain hormone groups in research (e.g., oral contraception, ovarian hyperstimulation).

Chapter 5 found MCA*v*-CO_2 responsiveness to hypocapnia was greater during the ovulatory phase compared to the early follicular phase, indicating that oestrogen has a significant effect on the vasoconstrictive capacity of the MCA. Cerebrovascular-CO_2 responsiveness remained similar between early follicular and mid-luteal phases, which may indicate that greater progesterone may counteract or dampen the effect of greater oestrogen. Finally, while the response to passive heat stress was similar across the menstrual cycle, there appeared to be regional differences in cerebrovascular-CO_2 responsiveness between normothermic and heat stress conditions.

Chapter 4 demonstrated that sex can influence cerebrovascular responsiveness to CO_2, and that this is menstrual cycle dependent and differs with the insonated vessel. When females are in the early follicular phase of the menstrual cycle, MCA*v*-CO_2 responsiveness to hypo- and hypercapnia were similar, and PCA*v*-CO_2 responsiveness to hypo-to-hypercapnia and to hypocapnia were greater, as compared to males. During the ovulatory phase, MCA*v*-CO_2 responsiveness to hypo- and to hypo-to-hypercapnia, and PCA*v*-CO_2 responsiveness to hypercapnia, were greater as compared to males. Furthermore, diminished PCA*v*-CO_2 responsiveness to hypercapnia during heat stress is only evident in males, indicating that the cerebrovascular-CO_2 response to thermal stress is sex dependent.

Finally, Chapter 6 demonstrated that pre-menopausal females have an improved MCA*v*-CO_2 responsiveness to hypercapnia compared to post-menopausal females,

irrespective of menstrual phase. Additionally, preliminary data for ICA-CO_2 responsiveness to hypercapnia appears to show a greater response in post-menopausal females compared to pre-menopausal females. Moreover, preliminary data appears to indicate that differences in cerebral autoregulation may be present between pre- and post-menopausal females, and these differences may be both menstrual phase and BP-oscillation frequency dependent. However, due to the small sample size, care should be taken when interpreting these results.

7.2 General Discussion

7.2.1 Cerebrovascular-CO_2 responsiveness

Cerebrovascular responsiveness to CO_2 measures the cerebral blood flow response to a changing CO_2 stimulus, providing a functional measure of cerebrovascular regulation that is commonly applied in healthy and clinical populations. Impairment of cerebrovascular responsiveness to a vasodilatory stimulus (i.e., increased CO_2, acetazolamide administration) has been shown to predict the risk of stroke in patients with carotid artery occlusion (Gupta et al., 2012; Markus & Cullinane, 2001; Webster et al., 1995), and therefore a greater cerebrovascular responsiveness to CO_2 is interpreted as improved cerebrovascular function. Few studies have investigated the influence of changing sex hormones on cerebrovascular-CO_2 responsiveness, and those that do employ different modes of assessment (e.g., CO_2 inhalation, breath-holding-index) making comparisons between studies difficult. Furthermore, both duration of the CO_2 stimulus and the timepoint used for analysis has recently been shown to significantly alter these cerebrovascular responsiveness values (Burley et

al., 2020). As such, a lack of high-quality evidence and the variance in cerebrovascular-CO_2 responsiveness methodology means the influence of changing sex hormones remains largely unknown.

		Chapter 4 (vs M)		Chapter 5 (vs EF)		Chapter 6 (vs PM)	
		EF	O	O	ML	EF	ML
Cerebrovascular-CO_2 responsiveness to **hypercapnia**	MCA*v*	↔	↔	↔	↔	↑	↑
	PCA*v*	↑	↑	↔	↔	-	-
	ICA	-	-	-	-	↓	↓
Cerebrovascular-CO_2 responsiveness to **hypo-to-hypercapnia**	MCA*v*	↔	↑	↔	↔	↔	↔
	PCA*v*	↑	↔	↔	↔	-	-
	ICA	-	-	-	-	-	-
Cerebrovascular-CO_2 responsiveness to **hypocapnia**	MCA*v*	↔	↑	↑	↔	↔	↔
	PCA*v*	↑	↑	↔	↔	-	-
	ICA	-	-	-	-	-	-

Figure 7.1 Observed differences in middle cerebral artery (MCA*v*), posterior cerebral artery (PCA*v*) and internal carotid artery (ICA) -CO_2 responsiveness between males and females (Chapter 4), across the menstrual cycle (Chapter 5) and between pre- and post-menopausal females (Chapter 6). EF, early follicular; O, ovulatory; ML, mid-luteal; M, males; PM, post-menopausal females. Black arrows indicate no difference between cohorts. Blue, red, and green arrows indicate a change during normothermic conditions, passive heat stress conditions, and both thermal conditions, respectively. Red box indicates preliminary data only.

Cerebrovascular-CO_2 responsiveness outcomes across all cohorts examined in this thesis are illustrated in Figure 7.1. As reported in Chapter 5, the acute, natural fluctuations in oestrogen and progesterone across the menstrual cycle appear to have minimal influence on cerebrovascular-CO_2 responsiveness. Previous studies have primarily looked at vasodilatory cerebrovascular-CO_2 responsiveness showing it to be both greater during ovulation (Krejza et al., 2013) and similar between ovulatory and

early follicular phases (Peltonen et al., 2016). We found no difference in the vasodilatory response between phases (i.e., EF vs. O and EF vs. ML). Importantly, we employed a CO_2 stimulus that also allowed assessment of the vasoconstrictive response of the cerebrovasculature. MCA*v*-CO_2 responsiveness to hypocapnia was greater during the ovulatory phase compared to the early follicular phase (Figure 7.1), indicating oestrogen has a significant effect on the vasoconstrictive capacity of the cerebrovasculature. However, progesterone appears to counteract the effects of oestrogen in the ML phase. To the best of our knowledge, this is the first study comparing cerebrovascular-CO_2 responsiveness in the hypocapnic range across the menstrual cycle. While it appears that acute changes in sex hormones are mostly insufficient to alter cerebrovascular-CO_2 responsiveness, the findings of Chapter 5 indicate that oestrogen can alter the vasoconstrictive capacity of the cerebrovasculature, which at present remains largely unstudied.

Sex differences in cerebrovascular-CO_2 responsiveness are reported to be present across the whole CO_2 range examined, although this is dependent on the phase of the menstrual cycle and the vessel insonated (Chapter 4). Previous research investigating sex differences have shown cerebrovascular-CO_2 responsiveness to be either greater in females compared to males (Kastrup et al., 1997; Oláh et al., 2000) or similar between the sexes (Favre et al., 2020; Peltonen et al., 2015). However, these studies either did not control for the menstrual cycle or studied females only during phases where sex hormones are likely to be lowest and there is likely to be the least difference between males and females. As shown in Figure 7.1 during the ovulatory phase, MCA*v*-CO_2 responsiveness to hypo- and to hypo-to-hypercapnia, and PCA*v*-CO_2

responsiveness to hypercapnia, were greater compared to males. Of interest, even during the early follicular phase when circulating sex hormones are lowest, females had a greater PCA*v*-CO_2 responsiveness to hypocapnia and hypo-to-hypercapnia when compared to males. This has important implications for the inclusion of female participants in experimental studies examining cerebrovascular function. At present, it is common practice to test female participants during the early follicular phase of the menstrual cycle to control for the 'confounding influence' of oestrogen and progesterone as much as possible, thus allowing female and male data to be combined. However, we report that even during menstrual cycle phases where sex hormones are expected to be lowest, females can have a greater PCA*v*-CO_2 responsiveness across the CO_2 range compared to males. As such, the insonated vessel should be considered and whether it is appropriate to combine male and female data when the cerebrovasculature may have a sex-specific response.

The findings in Chapter 6 indicate that the chronic decline in sex hormones associated with the menopausal transition has a detrimental effect on MCA*v*-CO_2 responsiveness to hypercapnia. Previous studies have reported cerebrovascular-CO_2 responsiveness in the MCA to be both similar between pre- and post-menopausal females (Brislane et al., 2020), and greater in pre-menopausal compared to post-menopausal females (Matteis et al., 1998), with methodological inconsistencies meaning the influence of menopause on cerebrovascular-CO_2 responsiveness is further confounded. We report a blunted MCA*v*-CO_2 responsiveness to hypercapnia in post-menopausal females compared to pre-menopausal females, which appears to be at least partially driven by a higher mean arterial pressure. Preliminary data appears to show ICA-CO_2

responsiveness to hypercapnia is greater in post-menopausal females compared to pre-menopausal females during both EF and ML phases (Figure 7.1), perhaps showing a compensatory improvement in function to account for a blunted MCA*v*-CO_2 responsiveness. Further investigation into cerebrovascular-CO_2 responsiveness in both intra- and extra-cranial vessels, and the differences from pre- to post-menopause is necessary to fully elucidate how cerebrovascular function changes across the female lifespan.

It is apparent across all cohorts examined in this thesis that regional differences in cerebrovascular-CO_2 responsiveness exist. Insonation of the MCA and PCA (Chapters 5 and 4) allowed assessment of cerebrovascular-CO_2 responsiveness in intracranial arteries in both the anterior and posterior circulation, in addition to the ICA (Chapter 6) as an index of cerebrovascular-CO_2 responsiveness in the extracranial arteries. Although this thesis did not formally compare between vessels, we report significant differences in MCA*v*-CO_2 responsiveness between EF and O phases of the menstrual cycle, and between males and females, that were not reflected in PCA*v*-CO_2 responsiveness. Furthermore, MCA*v*-CO_2 responsiveness was significantly greater in pre- compared to post-menopausal females while the ICA appeared to show the opposite response. Regional differences in cerebrovascular-CO_2 responsiveness have been reported previously with the posterior circulation having a blunted hypercapnic response compared to the anterior circulation (Sato et al., 2012; Skow et al., 2013), while other findings suggest no differences in regional cerebrovascular-CO_2 responsiveness (Willie et al., 2012). However, these studies were not investigating regional differences within the context of sex hormones, and therefore either did not

control for the menstrual cycle or included females in only the early follicular phase, thus making comparisons with data presented in this thesis difficult. It is common practice within the literature to insonate only the MCA, primarily due to its accessible location and the view that MCA responses can provide an adequate representation of the global cerebrovascular response. The data presented within this thesis indicates not only that cerebrovascular-CO_2 responsiveness differs depending on the insonated vessel, but that these differences may be sex and menstrual phase specific. Formal comparison between intra- and extracranial vessels will help to further elucidate regional differences in cerebrovascular-CO_2 responsiveness and how this varies with changing sex hormones.

As previously mentioned, there is currently no standardised approach to measuring cerebrovascular-CO_2 responsiveness in the literature. While a steady-state CO_2 stimulus is commonly employed, it has only recently been demonstrated that both the duration of the CO_2 stimulus and the timepoint used for analysis can significantly alter these cerebrovascular responsiveness values (Burley et al., 2020). Additionally, the MCA*v* response to a CO_2 stimulus has recently been shown to rarely reach steady-state (Koep et al., 2021), and therefore adoption of a set-time point to assess cerebrovascular-CO_2 responsiveness (as done so in this thesis) can produce substantial variability within a participant assessed multiple times. Subsequently, assessment of cerebrovascular responsiveness by analysing the dynamic “onset” kinetics to a stimulus is becoming more prominent in the literature (Billinger et al., 2017; Koep et al., 2021), although at present there is also no standardised protocol to this method of analysis. Since cerebrovascular-CO_2 responsiveness is a common

functional test to assess brain vascular health, determination and standardisation of the optimal method of analysis would be of significant value to the field.

At present, cerebrovascular responsiveness to a vasodilatory stimulus (e.g., increased CO_2, acetazolamide administration) is shown to predict the risk of cerebrovascular disease (Gupta et al., 2012; Markus & Cullinane, 2001; Webster et al., 1995). There remains little to no evidence that the same is true for the vasoconstrictive capacity of the cerebrovasculature, primarily due to the understudied nature of the hypocapnic range. Subsequently, while it is hypothesised that a greater vasoconstrictive capacity is also indicative of improved cerebrovascular function, it does not have the same body of evidence linking it to risk of cerebrovascular disease. Future studies may wish to examine cerebrovascular-CO_2 responsiveness to hypocapnia in clinical populations to strengthen this assumption.

7.2.2 Integrative cerebrovascular function

Control of cerebral blood flow involves a range of overlapping regulatory mechanisms that work together to ensure a sufficient supply of oxygen and nutrients are maintained, as well as to prevent hypo- or hyperperfusion injury. Since the cerebrovasculature is particularly sensitive to arterial CO_2, with both direct and indirect influences on cerebral blood flow regulation, examination of cerebrovascular-CO_2 responsiveness in isolation is a valuable measure of cerebral blood flow regulation. However, it is important to consider the integrative nature of the regulatory mechanisms governing the cerebrovasculature and that cerebrovascular-CO_2 responsiveness can only provide insight into one facet of cerebrovascular function. Cerebral autoregulation is another

key regulatory mechanism, working to maintain adequate cerebral perfusion despite changes in blood pressure. Hypercapnia has previously been shown to impair cerebral autoregulation (Maggio et al., 2013), illustrating how these mechanisms can interact. When examining cohorts where blood pressure is likely to differ (e.g., pre- and post-menopausal females), it is reasonable to conclude that cerebral autoregulatory mechanisms may be affected and that this may in turn have an influence on cerebrovascular-CO_2 responsiveness outcomes. Thus, examination of both cerebral autoregulation and cerebrovascular-CO_2 responsiveness can allow for a more complete assessment of cerebral blood flow regulation.

Chapter 6 reported cerebral autoregulation and cerebrovascular-CO_2 responsiveness in both pre- and post-menopausal females. While cerebral autoregulatory data is only preliminary at present, pre-menopausal females in the EF phase appeared to have a higher phase in the very-low frequency range compared to post-menopausal females, indicating better active vasodilation and constriction in response to changes in pressure. Conversely, in the low-frequency range, post-menopausal females appeared to show an improved gain compared to pre-menopausal females in both EF and ML phases, indicating an enhanced buffering efficiency of blood pressure fluctuations. Autoregulation in pre- and post-menopausal females is mostly unstudied in the literature, with only one study previously reporting no differences between post-menopausal females and pre-menopausal females during EF (Brislane et al., 2020). Due to the small sample size reported in Chapter 6, it would be speculative to definitively conclude that cerebral autoregulatory outcomes differ substantially between cohorts. Moreover, it is not possible to determine how the reported blunted

MCA*v*-CO_2 responsiveness and improved ICA-CO_2 responsiveness to hypercapnia are related to these autoregulatory outcomes. Subsequently, further investigation of cerebral autoregulation in pre- and post-menopausal females is warranted, with these data to be interpreted alongside cerebrovascular-CO_2 responsiveness.

Passive heat stress is an experimental model that can be used to acutely stress the cerebrovasculature. Since passive heating causes a reduction of arterial blood pressure and P_aCO_2 due to redistribution of cardiac output to the cutaneous circulation and hyperthermia-induced hyperventilation, it presents a unique challenge to cerebral blood flow regulation. The majority of studies investigating cerebrovascular function and passive heat stress have evaluated only males making it difficult to determine the role, if any, of sex hormones on cerebrovascular function during passive heating (Barnes & Charkoudian, 2021). Since elevated oestrogen has been associated with increased cerebral blood flow (Krejza et al., 2004), and females have a lower end-tidal CO_2 compared to males (Minhas et al., 2018), assessing cerebrovascular-CO_2 responsiveness during passive heat stress may uncover underlying sex or sex hormone-related differences in cerebrovascular function when examined during passive heating. Chapters 5 and 4 examined cerebrovascular-CO_2 responsiveness during normothermia and passive heat stress across the menstrual cycle and between the sexes, respectively. Overall, the response of the cerebrovasculature to passive heat stress was found to not differ across the menstrual cycle. However, we report a diminished PCA*v*-CO_2 responsiveness to hypercapnia during heat stress that is only evident in males, indicating that the cerebrovascular response to thermal stress can be sex dependent. As such, we conclude that acute, natural fluctuations in sex

hormones (i.e., across the menstrual cycle) are insufficient to influence the robust regulatory cerebrovascular response to heat stress, while there appears to be fundamental differences in this thermoregulatory response between males and females. Whether this is the result of different circulating levels of sex hormones between males and females, or due to another sex-related difference (e.g., structural differences) is yet to be determined.

7.3 Future directions

On the basis of the findings contained in this thesis and current gaps in the literature, several relevant questions have arisen and would provide logical next steps for future research.

1. As highlighted in Chapter 2, there is a distinct lack of research investigating cerebrovascular function in female cohorts of ovarian hyperstimulation and oral contraception. Future research in such cohorts will prove particularly pertinent for those undergoing IVF treatment and the considerable proportion of pre-menopausal females who take hormonal contraception. Additionally, studies examining the impact of sex-hormone related treatments or therapies (e.g., gender reassignment) would be particularly beneficial.
2. Further examination of regional differences in cerebrovascular-CO_2 responsiveness across intra- and extra-cranial vessels is necessary, alongside determination of sex-hormone related regional responses.
3. Based on the preliminary data reported in Chapter 6, further investigation into differences in cerebrovascular function and regulation in pre- and post-

menopausal females are warranted to further establish the link between menopause and cerebrovascular disease.

4. Establishment of a standardised protocol to measure cerebrovascular-CO_2 responsiveness thus allowing for comparison between studies and improved synthesis of knowledge regarding sex hormones and cerebrovascular function.
5. Finally, the data presented in this thesis could provide a valuable foundation for the calculation of statistical power in future randomised controlled trials. Larger scale studies examining the effects of sex hormone interventions on cerebrovascular function in both healthy and clinical populations will provide important information on how oestrogen and progesterone could be used in a therapeutic manner.

7.4 Summary

Overall, this thesis found that measures of cerebrovascular function can vary significantly between cohorts with low and high sex hormones (i.e., pre- and post-menopausal females), and indicates that regional differences in cerebrovascular-CO_2 responsiveness are present. The findings that pre-menopausal females exhibit improved intracranial cerebrovascular-CO_2 responsiveness compared to both young males and post-menopausal females supports the hypothesis that sex hormones play a protective role in cerebrovascular function. However, whether this extends to being a contributing factor to the disproportionate rise in cerebrovascular disease in females is yet to be determined.

7.5 Reflections of the researcher

Every PhD thesis, and every journey that came before it, is different. Briefly, I hope to provide some context for my own journey in the form of some lessons learned:

- Where possible, avoid doing a PhD during a global pandemic. I was fortunate that I had collected most of my data before March 2020, however, moving 200 miles away from the PGR community was not particularly conducive to writing.
- Turning a full-time PhD into a part-time PhD is not a sign of failure. The benefits of adequate down-time cannot be overstated, and the effort needed to self-fund a PhD should not be underestimated. Becoming part-time was a hard decision to make, but ultimately offered many new and exciting opportunities.
- Practice gets you closer to perfect. I am not a natural public speaker and, unfortunately, it seems the only way to improve is to speak in public. The list of conference presentations tell their own story of what an uphill battle this was.
- Don't be afraid to ask. I had, and sometimes still have, a fear of asking questions as I feel I should already know the answer. The caveat is to be prepared – always be ready for the answer to the question to be another question (the phrase "well, what do *you* think?" will stay with me for a while yet).

Before becoming a postgraduate student, I thought people who completed PhD's came pre-packaged with extraordinary skill and knowledge. Now that I've completed one, I'd love to say that is true, but it's not – at least not in my case. It took a lot of hard work, mistakes, and long days which, to be honest, makes this achievement feel all the more enjoyable.

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APPENDICES

Appendix A Systematic review and meta-analysis search strategy

	Medline	Web of Science	Embase
1	(Cerebrovascular Circulation or Cerebral Arter* OR Middle Cerebral Artery OR Posterior Cerebral Artery OR Carotid Artery, Internal OR Carotid Artery, External).ti,ab,kw	TS=("Cerebrovascular Circulation" OR "Cerebral Arter*" OR "Middle Cerebral Artery" OR "Posterior Cerebral Artery" OR "Carotid Artery, Internal" OR "Carotid Artery, External")	('brain circulation' or 'brain blood flow' or 'brain artery' or 'posterior cerebral artery' or 'middle cerebral artery' or 'external carotid artery' or 'internal carotid artery').ti,ab,kw.
2	(Gonadal Steroid Hormones or sex or menstrual cycle or follicular phase or luteal phase or ovulat* or estradiol or estradiol congeners or progesterone congeners or luteinizing hormone or follicle stimulating hormone or premenopause or perimenopause or postmenopause or menopaus* or hormone replacement therapy or Fertilization in Vitro or Ovulation Induction or ovulation inhibition or contraceptive agents, female or Contraceptives, Oral, Hormonal or pregnancy).ti,ab,kw	TS=("Gonadal Steroid Hormones" or sex or "menstrual cycle" or "follicular phase" or "luteal phase" or ovulat* or estradiol or "estradiol congeners" or "progesterone congeners" or "luteinizing hormone" or "follicle stimulating hormone" or premenopause or perimenopause or postmenopause or menopaus* or "hormone replacement therapy" or "Fertilization in Vitro" or "Ovulation Induction" or "ovulation inhibition" or "contraceptive agents, female" or "Contraceptives, Oral, Hormonal" or pregnancy)	('Sex hormone' or sex or 'menstrual cycle' or 'follicular phase' or 'luteal phase' or ovulation or estradiol or progesterone or 'luteinizing hormone' or follitropin or menopause or postmenopause or climacterium or premenopause or 'estrogen therapy' or 'hormone substitution' or 'in vitro fertilization' or pregnancy or 'ovulation induction' or 'ovulation inhibition' or 'hormonal contraception' or 'contraceptive agent').ti,ab,kw.
3	1 AND 2	#2 AND #1	1 AND 2
4	3 NOT (fetus or fetal or child* or neonatal).ti,ab,kw	#3 NOT TS=(fetal or fetus or neonatal or child*) AND #3 Not TI=(fetal or fetus or neonatal or child*)	3 NOT (fetus or fetal or child* or neonatal).ti,ab,kw
5	4 NOT (animal* or rat*).ti,kw	#4 NOT TS=(animal* or rat*) AND #4 NOT TI=(animal* or rat*)	4 NOT (animal* or rat*).ti,kw

Appendix B Level of evidence criteria for included studies.

Level 1 (High):	Level 2 (Moderate):	Level 3 (Low):
A control group was used	Pre-/post- and/or repeated measures design was used	Post-test only OR cross-sectional design was used
A pre-/post- or repeated-measures design was used	A control or comparison group may have been used, but was not required	Case Studies (individual or very small cohort)
Groups were randomised	Groups were not required to be randomised	Uncontrolled study
Example: Randomised Control Trial	A retrospective design may be used	A retrospective design may be used
	Examples: Cohort, Case-Control, Time Series studies	Example: Cross-sectional study

Appendix C Hormone replacement therapy (HRT) characteristics for included HRT studies.

Study	Study Intervention - HRT type	Time on HRT Prior to Study	Time Since Menopause
Acar et al. (2005)	Intranasal 17β-oestradiol (300μg)	Participants not receiving HRT prior to study	Mean ± SD: 3.6 ± 2.6 years
Cacciatore et al. (1998)	Assigned either oral oestradiol (2 mg/d; 12 d/month), combined with oral norethisterone acetate (1 mg/d; 10 d/month) and followed by oestradiol (1 mg/d; 6d/month), or transdermal oestradiol (50μg/d; 14d/month), combined with norethisterone acetate 0.25 mg/g; 14 d/month).	Participants either not receiving HRT prior to study or had a minimum washout period of 2 months.	Range: 6 months - 5 years
Cagnacci et al. (2000)	Continuous transdermal HRT (50 mg/day oestradiol, plus 10 mg/day medroxyprogesterone acetate for 12 days every 28 days)	Participants were on HRT for at least 6 months prior to the study.	Range: 1 - 4 years
Clapauch et al. (2007)	Intranasal 17β-oestradiol (300μg)	Participants were using either transdermal oestrogen, or transdermal oestrogen with cyclical dihydrogesterone prior to study. Time period not stated.	Mean ± SD: 6.4 ± 3.5 years
Crook et al. (1991)	Assigned transdermal oestradiol (50μg/d) for weeks 1-6, combined with medroxyprogesterone acetate (10mg/d; 12d/month) for 12 days a month for weeks 10-21.	Not stated.	Median (range): 24 months (8-96 months)
Darj et al. (1999)	Oral 17β-oestradiol (2mg/d; 12d/month), combined with norethisterone acetate (1mg/d; 10d/month), followed by 17β-oestradiol (1mg/d; 6 d/month)	Not stated.	Mean (range): 38.1 months (6 - 108 months)

Guvenal et al. (2009)	Assigned to either conjugated equine oestrogens (0.625mg/d) or combined with medroxyprogesterone acetate (2.5 mg/d)	Participants not receiving HRT prior to study.	Not stated.
Huang et al. 2009	17β-oestradiol (2mg/d) with norethisterone acetate (1mg/d)	Participants not receiving HRT prior to study.	Mean: 1.5 years
Jackson & Vyas (1998)	Oral oestradiol (2 mg daily)	Participants either not receiving HRT prior to study or had a minimum washout period of 12 months.	Median (range): 11 (1-28) years
Lazar et al. (2004)	Oral oestradiol (2mg/d) and norethisterone acetate (1mg/day)	Participants either not receiving HRT prior to study or had a minimum washout period of 6 months.	Mean ± SD: 6.6 ± 6.1 years
Naessen & Bakos (2001)	A 20mg oestrogen implant placed subdermally every 6 months	Participants had an average HRT duration of 18.8 years (range 5.8 –33.9 years) prior to the study.	Not stated.
Pan et al. (2002)	Conjugated equine oestrogens (0.625mg/d) combined with medroxyprogesterone acetate (5 mg/d)	Participants either not receiving HRT prior to study or had a minimum washout period of 3 months.	Mean ± SD: 1.8 ± 2.8 years
Penotti et al (1996)a	Transdermal 17β-oestradiol (50 µg/d), combined with medroxyprogesterone acetate (10mg/d) for 12 days every two months.	Participants had received HRT for at least 1 year, but less than 2 years prior to the study.	Mean ± SD (range): 4.71 ± 2.03 years (3 - 12 years)
Penotti et al. (1998)	Transdermal 17β-oestradiol (50 µg/d)	Not stated.	Not stated.
Persico et al. (2005)	Continuous oestradiol transdermal supplementation (50 µg/d) combined with	Participants not receiving HRT prior to study.	Mean ± SD (range): 3.3 ±

	medroxyprogesterone acetate (10 mg/d; 12d every 2 months)		1.5 years (1–8 years)
Vidovic et al. (2001)	Combined oral oestradiol (2mg/d) and norethisterone acetate (1mg/d)	Participants not receiving HRT prior to study.	Minimum of 12 months.
Wender et al. (2011)	Assigned to either conjugated equine oestrogens (0.625mg/d) or combined with medroxyprogesterone acetate (2.5 mg/d)	Participants either not receiving HRT prior to study or had a minimum washout period of 6 months.	Mean ± SD: CEE group 5.12 ± 4.95 years; combined CEE+MPA group 5.76 ± 4.29 years

Appendix D Cerebrovascular responsiveness values (relative change) for the middle cerebral artery (MCA*v*-CO_2, MCA*v*-CVC-CO_2) and posterior cerebral artery (PCA*v*-CO_2, PCA*v*-CVC-CO_2) during normothermia and heat stress in males and females during two phases of their menstrual cycle. Values are means ± SD. *significantly different from males; # significantly different to normothermia

		Early Follicular		*Ovulatory*		*Males*	
		Normothermia	Heat Stress	Normothermia	Heat Stress	Normothermia	Heat Stress
MCAv-CO_2 responsiveness (%)	Hypercapnia	3.9 ± 1.4	5.1 ± 1.6*#	4.8 ± 1.8	4.7 ± 1.5	4.5 ± 1.0	3.5 ± 1.8
	Hypocapnia	-1.9 ± 0.8	-2.2 ± 0.6	-2.7 ± 0.6*	-2.5 ± 0.9*	-1.7 ± 0.8	-1.8 ± 0.3
	Hypo-to-hypercapnia	6.2 ± 1.3	4.0 ± 1.3*#	5.9 ± 1.5	3.7 ± 1.2*#	5.5 ± 1.7	5.5 ± 1.2
PCAv-CO_2 responsiveness (%)	Hypercapnia	4.4 ± 0.9	5.6 ± 1.0*	4.9 ± 1.5*	6.7 ± 3.3*	4.9 ± 0.5	3.5 ± 1.7#
	Hypocapnia	-2.4 ± 0.7	-2.4 ± 0.7	-3.2 ± 1.6*	-2.5 ± 0.7*	-1.5 ± 0.9	-2.0 ± 1.1
	Hypo-to-hypercapnia	6.4 ± 1.7	4.2 ± 1.8#	6.0 ± 1.9	3.1 ± 2.5*#	5.1 ± 1.1	5.3 ± 1.3
	Hypercapnia	2.2 ± 1.0*	2.9 ± 1.4	3.4 ± 1.8	3.3 ± 1.6	3.9 ± 1.1	2.3 ± 2.0#

MCAv-CVC-CO_2 responsiveness (%)	Hypocapnia	-2.5 ± 0.8	-2.1 ± 0.9	-3.2 ± 1.3*	-2.5 ± 1.3*	-2.0 ± 0.9	-2.0 ± 0.8
	Hypo-to-hypercapnia	5.4 ± 1.3	6.4 ± 2.4#	4.7 ± 1.3	4.6 ± 4.9	4.6 ± 1.4	6.0 ± 1.1#
PCAv-CVC-CO_2 responsiveness (%)	Hypercapnia	2.8 ± 1.4*	3.3 ± 1.6	3.3 ± 1.5	5.4 ± 3.4*	4.3 ± 0.6	2.2 ± 1.2#
	Hypocapnia	-2.9 ± 0.7	-2.4 ± 0.9	-3.9 ± 1.8*	-2.3 ± 1.1#	-2.0 ± 1.0	-2.3 ± 0.8
	Hypo-to-hypercapnia	5.4 ± 1.3	6.5 ± 3.2#	5.3 ± 1.7	3.6 ± 6.6	4.3 ± 1.1	5.9 ± 1.1#