

UNIVERSITY OF BIRMINGHAM

EXERCISE-INDUCED CHANGES IN CEREBRAL BLOOD FLOW: AN *IN VITRO* AND *IN VIVO* INVESTIGATION OF VASCULAR RESPONSE

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ABSTRACT

Greater physical fitness is linked to improved cerebrovascular health and brain function. However, acute responses to exercise can differ depending on exercise intensity, as well as by the specific exercise protocol used. While the endothelium plays an essential role in driving responses to stimuli and adaptation in the vasculature, the cellular responses to transient changes in circulating environment are poorly understood. This thesis investigated the effect of different exercise protocols on the responses seen in the cerebrovasculature and circulating environment *in vivo*, alongside the use of endothelial cell culture models to examine cellular responses to *ex vivo* application of plasma and serum samples taken from exercising participants.

Cerebral haemodynamic responses were largely comparable between moderate intensity continuous exercise and clinical guideline-based high intensity interval exercise protocols. In contrast, sprint-interval exercise induced a unique pattern in middle cerebral artery velocity, whereby velocity was suppressed during the interval effort but then “rebounded” during recovery. A novel, semi-automated analysis methodology was developed to explore this temporal pattern further, showing that while intensity does modulate rebound characteristics the factors influencing temporal patterns in cerebral blood flow are likely multi-faceted. Finally, significant, intensity-dependent changes in VEGF, BDNF, IGF-1 and lactate were seen in response to exercise. However, *ex vivo* treatment of endothelial cells with samples taken pre- and post-exercise did not reveal any significant impact on cellular function, although further research is needed to fully illuminate the role of the circulating microenvironment in altering acute endothelial function.

Collectively, these findings highlight that the choice of exercise protocol can have a profound impact on cerebrovascular haemodynamics and responses in the circulating microenvironment. While the contribution of these two domains to long-term adaptive benefits to cerebrovascular health remains to be determined, it may be that the optimal exercise bout to elicit both haemodynamic- and neurotropic-induced adaptive changes may be a hybrid of the protocols tested here. Finally, future research should embrace the exciting opportunities and challenges that the application of translational research presents, to continue exploring the impact that exercise has on the brain from a cellular and systemic perspective.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AIC	Akaike Information Criterion
ANOVA	Analysis Of Variance
AUC	Area Under Curve
B2B	Beat-To-Beat
BAEC	Bovine Aortic Endothelial Cells
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
BP	Blood Pressure
BSA	Bovine Serum Albumin
CDS	Continuous Data Smoothing
CLEC14a	C-Type Lectin Domain Containing 14a
CO_2	Carbon Dioxide
CT	Cycle Threshold
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular Signal-Regulated Kinase
FBS	Foetal Bovine Serum
FMD	Flow Mediated Dilation
fNIRS	Functional Near-Infrared Spectroscopy
H_2O	Water
HIF1α	Hypoxia-Inducible Factor 1 alpha
HIIT	High Intensity Interval Training
HR	Heart Rate
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intracellular Adhesion Molecule
IGF	Insulin-Like Growth Factor
IGFBP3	Insulin-Like Growth Factor Binding Protein 3
IL	Interleukin
IPC	Ischemic Preconditioning
K2EDTA	Potassium Ethylenediaminetetraacetic Acid
KDR	Kinase Insert Domain Receptor
KLF2	Krüppel-Like Factor 2
LH	Lithium Heparin
LMEM	Linear Mixed Effects Model

Abbreviation	Definition
LOESS	Locally Weighted Scatter-Plot Smoother
M199	Medium 199
MAP	Mean Arterial Pressure
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
MICT	Moderate Intensity Continuous Training
MRI	Magnetic-Resonance Imaging
NF-κB	Nuclear Factor κ-Light-Chain-Enhancer Of Activated B Cells
NO	Nitric Oxide
O_2	Oxygen
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PECAM-1	Platelet Endothelial Adhesion Molecule-1
$P_{ET}CO_2$	Partial Pressure Of End-Tidal Carbon Dioxide
PGI2	Prostacyclin
PI3K	Phosphoinositide 3-Kinase
RIPA	Radioimmunoprecipitation Assay Buffer
RNA	Ribonecleic Acid
ROUT	Robust Regression and Outlier Removal
RPE	Rate Of Perceived Exertion
RT	Reverse Transcription
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SIT	Sprint Interval Training
TBST	Tris-Buffered Saline with Tween 20
TCD	Transcranial Doppler Ultrasound
TNF	Tumor Necrosis Factor Alpha
TRPV4	Transient Receptor Potential Vanilloid-Type 4
VCAM	Vascular Cell Adhesion Molecule
VE-cadherin	Vascular Endothelial Cadherin
VEGF	Vascular Endothelial Growth
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
$\dot{V}O_2$	Rate of Oxygen Consumption
$\dot{V}CO_2$	Rate of Carbon Dioxide Production
$\dot{V}_E$	Minute Ventilation Rate
YAP	Yes1 Associated Transcriptional Regulator

1. GENERAL INTRODUCTION

1.1. Background and Existing Literature

1.1.1. General benefits of exercise

Regular exercise has been a key component of a healthy lifestyle for millennia. Indeed, a 'prescription' of regular exercise has been recommended both for general health and as a remedy to disease as far back as the ancient Greek physician and philosopher Hippocrates (460-370 BCE) and was first prescribed as part of a healthy lifestyle by the ancient Indian physician Sushrata in 600 BCE (Tipton, 2014). In modern history, exercise has played a less central role in maintaining health and preventing disease, with advances and development of pharmacological treatments becoming prevalent. During the mid-20th century, however, growing evidence not only for the general health benefits of exercise, but also its capacity to treat a broad range of lifestyle-related diseases led to its reintroduction as a form of medicine (Berryman, 2010) - ultimately leading to the introduction of key guidelines for medical practitioners. These guidelines were first proposed in the joint statement by the American College of Sport Medicine and the American Medical Association in 2007, which outlined that; in the words of Robert E. Sallis, *"if we had a pill that conferred all the proven health benefits of exercise, physicians would widely prescribe it to their patients and our healthcare system would see to it that every patient had access to this wonder drug".*

Over the past 20 years, research into the health benefits of exercise has grown significantly and there is now a large body of evidence supporting its role as both part of a healthy lifestyle and as a treatment across a broad range of diseases. Specifically, physical activity levels have been shown to significantly impact all-cause mortality (Lee *et al.*, 2012), alongside reducing the risk for a broad number of diseases including type

II diabetes (Colberg *et al.*, 2010), cardiovascular disease (Lavie *et al.*, 2019), hypertension (Huai *et al.*, 2013), and dementia (Heyn *et al.*, 2004). Notably, while a sedentary lifestyle; typified by regular, prolonged periods of sitting, has been associated with an increased hazard ratio for cardiovascular disease and all-cause mortality, there is growing evidence that regular exercise negates some of this risk, in a dose-dependent manner (Ekelund *et al.*, 2019). In relation to the brain, increased physical activity and improved fitness in older age has been shown to improve cognitive function/decline and memory in both healthy ageing populations (Voss *et al.*, 2020), and patients with Alzheimer's Disease and related disorders (Heyn *et al.*, 2004; Buchner, 2007). The clear benefits of maintaining physical fitness together with an increasingly sedentary and ageing population, highlight the need for the promotion and implementation of exercise-based interventions across the population. Such interventions have the potential to play a pivotal role in improving the health of the general population and reducing the burden that lifestyle-related disease places on health services.

1.1.2. Physical fitness, exercise training and the cerebrovasculature

Alongside the cognitive benefits of exercise, higher physical fitness and exercise training has the potential to positively impact cerebrovascular health. While no singular definition exists for this term, in general cerebrovascular health is considered to be the capacity for the vessels of the brain to respond to perturbations in flow and to the metabolic demands of the brain. Good cerebrovascular health, therefore, describes a vascular system that can effectively control blood flow to the brain, such that neurological function is not limited by its blood supply. Similarly, to its definition, no one

method has been developed to assess cerebrovascular health, although common approaches include the measurement of resting blood flow/velocity and responses to changes in factors regulating flow in the cerebral arteries branching from the Circle of Willis (Figure 1.1).

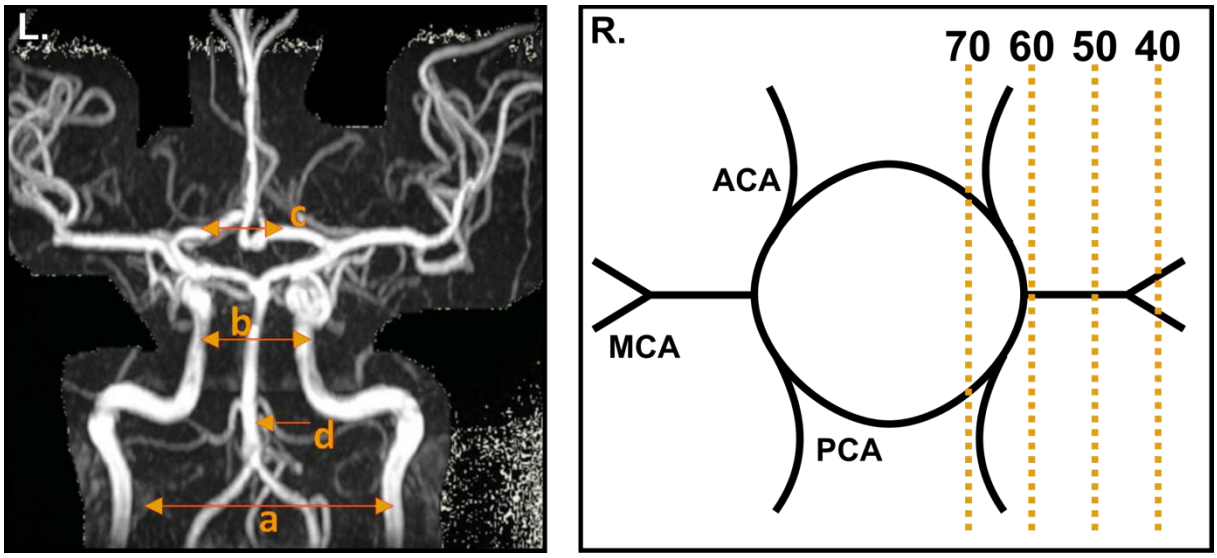


Figure 1.1 Schematic of the Circle of Willis cerebral blood vessels and example imaged using phase-contrast angiography
Phase contrast angiography image (**L**eft) shows the carotid artery (**a**), internal carotid arteries (**b**), middle cerebral arteries (**c**), and basilar artery (**d**), from an anterior position. Circle of Willis schematic (**R**ight) shows a graphical representation of the middle (MCA), anterior (ACA) and posterior cerebral arteries (PCA) in the transverse place form a superior position. Additionally, illustration is given of the approximate depths (in mm) at which these structures are insonated using transcranial Doppler ultrasound via the temporal window

The connection between exercise and cerebrovascular health is exemplified in the relationship between higher physical fitness and declines in cerebral blood flow velocity, in which higher fitness is associated with the attenuation of age-related decline in middle cerebral artery velocity (MCA_v) (Ainslie *et al.*, 2008). In a recent meta-analysis looking to identify the fitness and training-related benefits within the cerebrovasculature, Smith et al. (2021) found that higher fitness levels resulted in

significant declines in cerebrovascular resistance, alongside significant increases in cerebrovascular reactivity; a commonly used measure of cerebrovascular function. This difference was paired with a significant reduction in resting brain blood flow (as indexed by measures of MCA_v) in the highest fitness cohorts, compared to those with low fitness levels (Smith *et al.*, 2021).

The potential for greater physical fitness to improve cerebrovascular health and function has led to a number of studies looking to identify the benefits of exercise-related interventions across the population. Nevertheless, compared to cross-sectional research into the impact of fitness on cerebrovascular function, relatively few studies have been carried out on the longitudinal effects of exercise training (Smith et al. 2021). In fact, in their recent review of the literature, Smith *et al.* (2021) found no *significant* overall improvements in cerebrovascular reactivity or resting MCA_v across the nine studies utilising transcranial Doppler ultrasound (TCD) measures, nor the six studies using magnetic resonance imaging (MRI) based approaches.

A major shortcoming in the current literature is the high levels of heterogeneity seen across studies, firstly in the cohorts studied, ranging from postmenopausal women (Akazawa *et al.*, 2012; Bailey *et al.*, 2016*a*) to stroke (Ivey *et al.*, 2011) and breast cancer survivors (Northey *et al.*, 2019), with no use of healthy control arms in any study. Secondly, significant heterogeneity can be seen in the intervention strategies used, both in the length of the intervention; from 2-months (Akazawa *et al.*, 2012) to one year (Kaufman *et al.*, 2021), and in the format of the exercise studied; including high intensity interval training (HIIT) (Northey *et al.*, 2019), moderate intensity

continuous training (MICT) (Bailey *et al.*, 2016*a*; Northey *et al.*, 2019) and combined aerobic and strength training (Vicente-Campos *et al.*, 2012; Valkeinen *et al.*, 2013).

Current intervention studies suggest that short-term exercise training is not effective in improving resting measures of MCA_v, although improvements in cerebrovascular reactivity are seen over longer intervention periods (Smith *et al.*, 2021). Within this current literature, the largest improvements in cerebrovascular reactivity (measured through breath hold-induced hypercapnia) were seen in healthy older adults, following 7-months of moderate aerobic exercise 2–4 times per week (Vicente-Campos *et al.*, 2012). Similarly, Ivey *et al.* (2011) found that significant improvements in reactivity (measured through hypercapnic gas mixture inhalation) were comparable to the impact of statin prescription, following 6-months of moderate aerobic training 3-times per week in a cohort of stroke survivors. Contrastingly, shorter interventions in female breast cancer survivors, consisting of three months of either MICT or HIIT 3-times per week (Northey *et al.*, 2019), and in chronic kidney disease patients, consisting of four months of MICT 3-times per week (Stringuetta Belik *et al.*, 2018), found no consistent improvements in reactivity (measured by rebreathing and breath-hold methods, respectively). This disparity in reactivity responses highlights the impact that variety in intervention durations can have on potential benefits and, alongside heterogeneity in methodology and differences in primary outcome measures, highlights the need for further studies within the field. In particular, studies need to include more consistent use of healthy control populations and implement standardised methods for assessing cerebrovascular function.

1.1.3. Functional and structural adaptive responses to exercise

Alongside broad discussion of heterogeneity within the current literature, Smith *et al.* (2021) highlighted the limitations in our current understanding of the adaptive process within the vessels of the cerebrovasculature. They highlighted that while responses to exercise interventions have not been shown in literature to date, the time course for physiological and structural adaptations in the cerebrovasculature is not currently well understood. Developing our understanding of this time course is essential to both explaining the effects of training interventions and to determining the efficacy of particular interventions.

In the peripheral vasculature, functional and structural changes follow distinct patterns of response and contribute varying amounts to overall adaptations over the time course of an exercise intervention (Green *et al.*, 2017). Initially adaptations are primarily functional in nature, typically measured through changes in flow mediated dilation (FMD) of the brachial artery, peaking after approximately four weeks before gradually declining towards pre-intervention levels of function over further periods of intervention. Contrastingly, structural adaptations develop more linearly over the course of intervention, beginning to increase after ~2-weeks and continuing to increase over the course of an intervention. As a result, longer exercise interventions (8–10 weeks) may show no clear improvement in the FMD response despite beneficial adaptation, as these are largely structural in nature (Tinken *et al.*, 2010). If similar response patterns are seen in the cerebrovasculature, then the timing of measures taken during exercise interventions could play a crucial role in the differences seen across the literature to date.

The complexity of the cerebrovasculature, both in anatomy and regulation, makes determining the pattern and driving factors in adaptive response to exercise significantly more difficult to determine than in the case of the periphery. Whilst it is possible that adaptations are taking place within the cerebral arteries themselves, in a similar manner to those seen in peripheral conduit arteries, elevations in cerebral artery blood flow velocity has been shown to induce flow-dependent benefits across the brain's vasculature in animal models of exercise training (Bolduc *et al.*, 2013). Furthermore, it has been suggested that elevations in flow during exercise could stimulate both angiogenesis at the capillary level and neurogenesis within the brain and blood brain barrier, alongside increases in diameter (arteriogenesis) in existing arteries (Schmidt *et al.*, 2013). Adaptation of this nature could induce significant improvements in cerebrovascular function and health independently of remodelling of the cerebral arteries themselves, as the neurovascular unit is central to responses to both changes in $P_{ET}CO_2$ and blood pressure (Willie *et al.*, 2014). As such, adaptation may follow a distinct pattern and consist of a more complex combination of contributing factors compared to those seen in the periphery, potentially requiring significantly longer periods of exercise training more typical of habitual/life-long physical activity rather than relatively short exercise interventions. If this is the case, it would go some way as to explaining the equivocal findings seen between cross-sectional and longitudinal research to-date (Smith *et al.*, 2021).

Understanding the adaptive response of the cerebrovasculature to exercise is made more difficult still by the use of TCD, as this method determines the velocity of cerebral

blood flow and as such can be influenced to an unknown extent by changes in artery diameter (Ainslie & Hoiland, 2014). As a result, if adaptive responses to exercise interventions results in a change in overall vessel diameter or in greater vasodilatory responses to hypercapnia, then adaptations to exercise may be masked when assessed by TCD. While this does not eliminate the utility of TCD in the assessment of cerebrovascular responses and functional adaptations to training interventions, future research should look to include alternative measures that are either less dependent on uniform vasodilatory responses or that can directly measure vessel diameter and vasodilation. Clear guidelines have now been developed for the application of a number of different alternative measures of cerebrovascular function, including measures of cerebrovascular responsiveness using magnetic resonance imagery (Burley *et al.*, 2021) and duplex Doppler of the internal carotid artery (Carr *et al.*, 2020). Adoption of these methods will also be essential to understanding the relative contribution of structural remodelling within the cerebral arteries, as regardless of the accuracy of TCD-based measures, their lack of spatial resolution makes them effective only in determining functional adaptive responses.

1.1.4. Acute cerebrovascular responses to exercise

Regardless of the functional or structural nature of the adaptations that take place over a given training period, for adaptations to occur a sufficient acute stimulus must be provided within each exercise bolus. While the cellular mechanisms for adaptation within the cerebrovasculature are not fully understood, a key factor in driving these adaptations is the transient alteration of blood flow and the mechanical forces of shear stress and cyclic strain that are applied to the endothelium (Green *et al.*, 2017).

The capacity for exercise to drive these haemeodynamic responses has been studied in the most part during steady-state cycling, in which a clearly defined, biphasic pattern of responses has been established (Smith & Ainslie, 2017). Specifically, as exercise intensity increases cerebral perfusion is seen to increase continuously from “low” (20 – 40% maximal aerobic power (W_{max})) to “moderate” intensity (50 – 80% W_{max}), before gradually declining with further increases in intensity, dropping below resting levels as intensity approaches maximal aerobic capacity (Smith & Ainslie, 2017). The factors driving these responses are multi-faceted, however, most prominent among them is the impact of changes in the arterial partial pressure of CO_2 (P_aCO_2). This relationship is most commonly shown in the reciprocal temporal patterns in end-tidal CO_2 ($P_{ET}CO_2$); as a proxy measure of P_aCO_2 (Jones *et al.*, 1979), which increase steadily with intensity up to ventilatory threshold (~60-70% W_{max}), before declining at higher intensities as a result of hyperventilation-induced hypocapnia (Smith & Ainslie, 2017). It is worth noting, however, that most studies on incremental exercise have focused on cycling and the relative contribution of P_aCO_2 over other factors regulating CBF may differ across other modalities. For example, Furlong *et al.* (2020) demonstrated that while incremental exercise responses in $P_{ET}CO_2$ were closely matched between running and cycling modalities, running showed a continuous, linear increase in MCA_v contrasted with a typical biphasic response in cycling. This may be in part due to running-specific induction of rhythmic fluctuations in blood pressure, known as the “beat” phenomenon (Palatini *et al.*, 1989), which has been shown to translate to similar rhythmic patterns in MCA_v (Lyngeraa *et al.*, 2013). However, comparable MCA_v between running and cycling at moderate intensities, paired with elevations at higher

intensity being largely restricted to participants with higher aerobic fitness levels (Furlong *et al.*, 2020), suggests that this difference in responses between modalities cannot be entirely driven by differences in blood pressure. This highlights the potential for exercise modality to alter cerebrovascular responses to exercise and the potential for other regulatory factors to outweigh the regulatory capacity of reductions in $P_{ET}CO_2$ at maximal intensities.

Alongside the intensity and modality, the design of a given exercise protocol (e.g. continuous vs. interval-based exercise) can also play a key role in the haemodynamic responses seen. These differences were first identified in relation to peripheral vascular function and the varying impact that acute bouts of exercise have on post-exercise changes in flow mediated dilation (FMD). While 30-minutes of continuous exercise at higher intensities (>70% maximum heart rate) has been shown to lead to reductions in FMD capacity (Birk *et al.*, 2013), high intensity interval-based exercise (10 intervals of 1-minute at 80% W_{max}) appears to elicit similar acute increases in FMD responses to those seen following moderate intensity continuous exercise (Currie *et al.*, 2012). Importantly, these differences have also been shown to translate to clear differences in peripheral vascular function over the course of training protocols, as high intensity interval training has been shown to be as effective an intervention as moderate intensity continuous exercise (Ramos *et al.*, 2015), whereas continuous high intensity training elicits no clear improvements in function (Goto *et al.*, 2003). While these studies show the potential for protocol design to influence vascular responses to exercise, the mechanisms driving these responses differ markedly from those in the cerebrovasculature, with peripheral vascular responses being largely dependent on

local shear rate and changes on oxidative stress (Dawson *et al.*, 2013), as opposed to the more complex regulatory factors discussed above that relate to the cerebrovasculature. Most notably, oxidative stress is thought to play a pivotal role in protocol-dependent responses in the periphery (Green *et al.*, 2017), which, unlike the highly transient impact of changes in CO_2, are cumulative in their nature and impact. For this reason, it is essential that the potential impact of differing exercise protocols is directly investigated in the cerebrovasculature.

To date, despite the growing popularity of interval-based exercise strategies, relatively few studies have considered the impact and efficacy of interval-based protocols when targeting cerebrovascular function. In a recent meta-analysis, Whitaker *et al*. (2020) explored both acute and chronic impact of high-intensity interval-based exercise on markers of cerebrovascular blood flow and function. The authors highlighted that at the point of writing only three acute intervention studies had been conducted into cerebrovascular responses to high-intensity interval exercise. Within these three studies there was clear heterogeneity in the study cohort; with one study looking at prepubertal children (Tallon *et al.*, 2019) and two in adults (Monroe *et al.*, 2016; Burma *et al.*, 2020*b*, 2020*a*). Additionally, no consistent outcome measures were reported across studies; ranging from changes in oxygenated haemoglobin measured via near-infrared spectroscopy (Monroe *et al.*, 2016) to measures of MCA_V (Tallon *et al.*, 2019; Burma *et al.*, 2020*a*) and post-exercise changes in cerebrovascular reactivity (Burma *et al.*, 2020*b*) and dynamic autoregulation measures via TCD (Burma *et al.*, 2020*a*). Despite this variability within the limited studies conducted to date, consistent reductions are seen in both cerebrovascular blood flow and post-exercise functional

outcomes (Whitaker *et al.*, 2020), most likely driven by the concomitant reduction in $P_{ET}CO_2$ as a result of hyperventilation-induced hypocapnia (Tallon *et al.*, 2019; Burma *et al.*, 2020*a*).

The impact of a single sprint bout has also been investigated in two further studies (Curtelin *et al.*, 2018; Labrecque *et al.*, 2020). While these studies did not look to emulate potential intervention strategies, they provide key insights into the impact of high intensity intervals on the cerebrovasculature. In both studies, a single 30-second sprint interval was carried out on a cycle ergometer from a stationary start, at either W_{max} (Labrecque *et al.*, 2020) or at an "all-out" intensity (Curtelin *et al.*, 2018). These 30-second intervals were seen to induce a novel pattern in cerebral blood flow velocity, characterised by an elevation in MCA_v at the onset of the sprint, suppression during the interval itself, and followed by a significant rebound at the start of the recovery period. The driving factors behind this response appear to be multi-faceted, with initial elevations driven largely by elevations in blood pressure rather than CO_2, in a pressure-passive manner (Labrecque *et al.*, 2020). Conversely, subsequent suppression and rebound responses are consistent with hyperventilation-induced hypocapnia and post-exercise increases in $P_{ET}CO_2$, despite consistent elevations in blood pressure across the sprint bout and a subsequent, sustained drop during recovery (Curtelin *et al.*, 2018; Labrecque *et al.*, 2020). The rapid haemodynamic changes seen over the course of these high-intensity sprint efforts reinforces early concerns and discussion surrounding the risks that such protocols pose to the cerebrovasculature and the potential for hyperperfusion injuries to occur (Lucas *et al.*, 2015). This is particularly important if the use of interval-based approaches is going to

be promoted across the population (Gillen & Gibala, 2018), including within clinical populations that may have compromised cerebrovascular function and health (Calverley *et al.*, 2020). Additionally, the clear temporal pattern seen in sprint-interval exercise has not been seen in research into longer interval-based or lower intensity exercise, suggesting that either the high intensity or short duration of sprint-based exercise is key to driving these novel responses. As such, further research is needed to fully understand not only the efficacy of interval-based approaches in comparison to continuous exercise, but also how protocol design may modulate the impact of exercise on cerebral haemodynamics.

Finally, while most studies have explored high-intensity intervals one study has looked to determine whether exercise being conducted in intervals, rather than as a continuous effort, may influence cerebrovascular response at the same exercise intensity. Klein et al. (2019) investigated the impact of 10 minutes of moderate intensity exercise (60% W_{max}) on MCA_v, conducted either continuously or in 1-minute intervals separated by 1 minute of rest, in young and older men. They found that while comparable responses were seen in the older cohort, in young men continuous exercise resulted in significantly higher MCA_v from the second minute of exercise onwards, while consistent elevations across repeated intervals were comparable with those seen within the first minute of the continuous condition (Klein *et al.*, 2019). These responses were associated with similar patterns of response in both $P_{ET}CO_2$ and mean arterial pressure, which alongside continuous exercise driving significantly higher heart rate and RPE responses (Klein *et al.*, 2019), suggests that the short nature of 1-minute intervals results in a less pronounced elevation of MCA_V and therefore potentially

reduces the impact of key factors regulating the cerebrovasculature. Nevertheless, the authors also found that despite smaller absolute responses *during* the exercise intervals, when comparable recovery periods were taken into account, the interval-based exercise condition drove larger cumulative changes in MCA_v, as a result of elevations in blood velocity being maintained during resting recovery periods (Klein *et al.*, 2019). This study demonstrates that both intensity and protocol choice play a clear role in the impact that exercise can have on the cerebrovasculature, and the potential complexity introduced by interval-based approaches. However, it remains to be seen what impact this has in higher intensity exercise, during which hyperventilation-induced hypocapnia plays a more central role in regulating responses.

1.2. Summary

Exercise has clear health benefits, including improvements in cardiovascular health and all-cause mortality. These benefits extend to the cerebrovasculature, with greater physical fitness linked with attenuated age-related functional decline and improved cerebrovascular disease outcomes. Central to the long-term benefits of physical activity is the capacity for an acute bout of exercise to drive sufficient changes in physiology and subsequently stimulate adaptive signalling pathways. Within the vasculature, the key physiological change comes in the form of transient increases in flow, which have the potential to induce adaptation within the endothelium through upregulation of mechanosensory pathways in response to elevations in shear stress and cyclic strain (Green *et al.*, 2017). In the cerebrovasculature these responses are governed by a unique network of regulatory mechanisms, central to which is the

sensitivity of the neurovascular unit to changes in CO_2. As exercise intensity increases towards aerobic capacity, hyperventilation-induced hypocapnia leads to significant reductions in cerebral blood flow (Smith & Ainslie, 2017) and as a result a reduction in vertebral blood flow is seen.

However, a significant number of questions remain unanswered in relation to the cerebrovascular impact of exercise. How do differences in intensity and protocol impact acute changes in blood flow during exercise? How do haemodynamic responses compare to changes in circulating signalling factors; that may also be capable of influencing the brain and cerebrovasculature (Voss *et al.*, 2013*b*)? Similarly, given that the mechanisms that link transient responses to exercise with the adaptive benefits of regular exercise training have largely been adapted from research into peripheral vascular adaptation, or from non-exercising models of vascular adaptation to various stimuli. Will further research into the specific cerebrovascular responses to exercise validate these interpretations? Can translational research help link *in vivo* observations with *in vitro* mechanisms?

The overarching objective of this thesis is to consider these questions. The next chapter (Chapter 2) will take the form of a review of the current literature, outlining the mechanisms at play in a range of interventions with potential to benefit the vasculature and laying out a clear road map for better integration of translational research into the field of vascular physiology. Chapter 3 is a general methods chapter that details the central methodological approaches used in the experimental chapters that follow.

Chapter 4 directly compares three commonly utilised exercise approaches, both in their capacity to drive changes in cerebrovascular haemodynamics and changes in the circulating profile of key neurologically and vascularly active signalling factors.

Chapter 5 shows the impact that exercise intensity can play on the rebound profile first identified by Curtelin et al. (2018), as well as establishing a clear and reproducible method for analysing temporal patterns in vascular data.

Chapter 6 looks to investigate the capacity for transient changes in circulating microenvironment to impact endothelial activity, through the *ex vivo* application of serum and plasma samples taken from exercising participants within Chapter 1, to cultured endothelial cells.

Finally, Chapter 7 will provide general discussion and conclusion for all thesis chapters, examining the key findings of each data chapter and providing an overview of the implications and future directions for both *in vivo* and *in vitro* research into exercise in the context of cerebrovascular physiology.

2. NON-PHARMACOLOGICAL INTERVENTIONS FOR VASCULAR HEALTH AND THE ROLE OF THE ENDOTHELIUM

2.1. General introduction and scope of review

As the highway connecting every organ within the human body, the vascular system plays a unique and central role in maintaining health, while simultaneously holding the potential to disrupt multiple regions when not optimally functioning. As a result, vascular dysfunction plays a central role in disease progressions throughout the body, including in cardiovascular disease, stroke, and dementia (Toth *et al.*, 2017; Kraus *et al.*, 2019). The dangers of poor vascular health are becoming increasingly apparent in the modern world, as lifestyle-related factors play an ever more central role in all-cause mortality, and both communicable and cancer-related deaths are replaced by cardiovascular disease as the number one cause of mortality across the lifespan (Roth *et al.*, 2020). The deleterious impact that poor vascular health has on an individual's systemic health and quality of life, in addition to the escalating global burden of disease caused by poor vascular health, makes it imperative that effective and accessible interventions are prioritised to minimise the impact of vascular decline.

This review aims to examine the potential for non-pharmacological interventions to improve vascular function through acute changes in blood flow and its impact on the endothelium. The mechanisms driving these beneficial responses will be defined, alongside the potential for changes in the circulating molecular environment to modulate flow-dependent responses. In addition, we will look to explore the limitations in our current knowledge and understanding of how changes in flow affect the endothelium, particularly concerning the impact of non-pharmacological interventions. This will allow the development of more intervention-specific in vitro research methods and reduce our reliance on translating research from disease and dysfunctional flow

models. Dietary interventions will not be discussed within the scope of this review, as while these interventions have the potential to improve vascular function they act primarily through alternative mechanisms to those seen in exercise and other flow-dependent strategies and have been extensively reviewed elsewhere (Rees *et al.*, 2018; Brandhorst & Longo, 2019; Weaver *et al.*, 2020). Additionally, due to a lack of *in vivo* studies in humans, it is not possible to explore responses within smooth muscle cells and further work is required in this area.

2.2. Vascular responses to non-pharmacological interventions

2.2.1. Non-pharmacological interventions

The cumulative nature of vascular decline, coupled with the cost and potential health implications involved in drug-based treatment, have led to growing interest in non-pharmacological interventions strategies. These strategies have the added benefit of being applicable in a community setting, without the need for clinical diagnosis and can be utilised by the general public in a preventative manner, as has been shown in other lifestyle-related diseases such as type II diabetes (Schwarz *et al.*, 2012). Moreover, when utilised as “lifestyle” interventions, non-pharmacological approaches can have a holistic effect in improving both vascular function and general health (Dagogo-Jack *et al.*, 2010). These approaches include, but are not limited to, exercise-based interventions, heat therapy (e.g. hot water or sauna bathing), hypoxia-based interventions, ischemic preconditioning, and pneumatic compression.

As physical activity plays such a central role in vascular health, it is of little surprise that exercise-based interventions are one of the most commonly utilised non-pharmacological approaches for improving vascular function (Green & Smith, 2018). The clear potential in targeting physical activity levels to improve vascular function is coupled with the relative ease with which a cohort can be categorised, and intervention success can be quantified through measures of vascular function change alongside fitness testing, for example. While the impact of exercise is widespread and can result in changes to a large number of factors with vascular effects (Fiuza-Luces *et al.*, 2018), it is the impact it has on blood flow and the resulting shear stress (frictional force exerted on the vessel wall) and cyclic strain (repetitive/beat-to-beat stretching force) within the vasculature that is central to the benefits seen in vascular health (Green *et al.*, 2017). Indeed, increases in flow provide an essential stress stimulus for vascular responses to exercise as highlighted when restricting exercise-induced flow elevation limits subsequent adaptive improvements in flow-mediated dilation (FMD) (Tinken *et al.*, 2010; Birk *et al.*, 2012). Additionally, the changes in FMD following acute exercise have also been shown to correlate with basal changes seen following repeated bouts of exercise (i.e. training), demonstrating the role that transient responses to flow play in adaptive responses to interventions (Dawson *et al.*, 2018).

Alongside exercise-based strategies, there is now increasing interest in the potential for alternative strategies to exhibit significant benefits in the vasculature, to provide more options both in the general population and in those with significant barriers to physical activity (Allen & Morey, 2010). While the nature of these interventions differ significantly, a common central tenant is their capacity to drive acute elevations in

blood flow during an acute bout and the role that the cumulative effects of these transient changes play in adaptive improvements in vascular function (Cheng & MacDonald, 2018). As such it is important to consider the wealth of research around exercise and blood flow (Green *et al.*, 2017), to understand how these transient changes may lead to adaptive benefits and how alternative approaches may compare to exercise in their capacity to alter vascular health [e.g. passive heating vs. exercise (Francois & Thomas, 2016; Akerman *et al.*, 2019)]. Through a greater understanding of the role that non-pharmacological interventions play in altering flow we can build a better picture of how these strategies may complement one another or be used in combination with exercise – especially in populations with reduced physical capacity due to injury or disability. It is particularly important to understand the mechanisms driving the endothelium-dependent responses to these stimuli, as the endothelium plays an essential role in flow-response and sits as the interface between the circulating environment and the vasculature. *In vitro* research specific to the impact of non-pharmacological interventions is somewhat lacking, and it is therefore imperative to look at the full spectrum of work examining the influence of flow on the endothelium, to build a better understanding of how these interventions may drive endothelium-dependent adaptive responses.

2.2.2. Evidence for flow mediated adaptations in vivo

The central role of flow in driving beneficial adaptations has been well demonstrated in the context of exercise, with evidence demonstrating an elevation in blood flow throughout the body during exercise (Padilla *et al.*, 2011). While the specific flow response is highly dependent on the choice of protocol, during moderate-intensity

continuous cycling, two-fold increase in brachial artery blood flow (Thijssen *et al.*, 2009) and 25% increases in middle cerebral artery velocity are seen (Weaver *et al.*, 2021). Improvements in vascular function are also observed following exercise training, with significant improvements seen in basal brachial artery function measured by FMD (Early *et al.*, 2017). Remodelling of the vasculature is also observed with regular exercise, with both prolonged exercise training (>3-months) and comparison between active and sedentary individuals showing marked increases in femoral artery diameter and a reduction in media-intima thickness (Dinenno *et al.*, 2001). Similarly, in the cerebrovasculature, a recent meta-analysis highlighted that improved cerebrovascular function, as indexed by measures of cerebrovascular reactivity, is seen in individuals with higher fitness (Smith *et al.*, 2021). The evidence supporting the flow-dependent nature of exercise responses has been discussed at length in previous reviews, including the demonstration of the pivotal role that shear stress and endothelium-dependent mechanisms play in adaptive improvements in vascular function due to exercise interventions (Green *et al.*, 2017).

Elevations in blood flow are seen across a wide range of other non-pharmacological interventions, either through the specific design of interventions to alter blood flow, or through the manipulation of one or more factors that result in changes in flow. Of the intervention strategies that directly alter blood flow, the two supported by the greatest wealth of evidence are intermittent pneumatic compression and ischemic preconditioning. Pneumatic compression-based interventions involve the use of automated cuff-inflation systems, in which blood flow is increased through a cyclical pattern of inflation and deflation. This approach has been shown to drive positive

responses in increasing shear rate in the compressed limb, with 2-fold elevations in mean shear rate during early compression and maintenance of elevated shear when compressions are carried out at a low frequency (3-second inflation, 17-second deflation) (Sheldon *et al.*, 2012). Repeated exposure to pneumatic compression has shown significant improvements in markers of vascular function (Sheldon *et al.*, 2013), as well as the potential to drive elevations in basal flow rate (Kakkos *et al.*, 2005). However, to date the majority of studies have been conducted across a broad range of clinical and aged populations, with a focus on functional outcomes specific to the population's clinical outcomes (Sheldon *et al.*, 2013). This, combined with a lack of standardised compression protocols, means that further research is needed to fully understand the chronic benefits of pneumatic compression on vascular function, as well as allow comparison with other non-pharmacological interventions.

Similarly, ischemic preconditioning (IPC); characterised by short periods (4-5 minutes) of peripheral ischemia through inflation of a cuff to supra-systolic pressure, has also been linked to benefits in vascular function (Jones *et al.*, 2014). Initially designed as a protective treatment against ischemic reperfusion injuries, the impact of brief periods of blood flow restriction and subsequent reperfusion have subsequently been shown to have a broader effect on endothelial function. Acutely, alongside the direct manipulation of blood flow within the preconditioned limb (depicted in Figure 2.1), IPC leads to vasodilation and increased blood flow in the contralateral limb (Enko *et al.*, 2011). Over repeated treatments, IPC has been shown to increase endothelium-dependent forearm blood flow by ~30% in the preconditioned limb (Kimura *et al.*, 2007), alongside significant improvements in both preconditioned and contralateral

arm FMD (Jones *et al.*, 2014). The majority of current research has focused on the impact of IPC protocols on arterial function in the reconditioned and contralateral limbs, while recent findings have shown conflicting results in changes in dynamic cerebral autoregulation following a single IPC stimulus (Guo *et al.*, 2019; Carter *et al.*, 2020). Future research should look to clarify these conflicting findings, as well as expanding our understanding of how the IPC stimulus may affect a broad range of vascular beds.

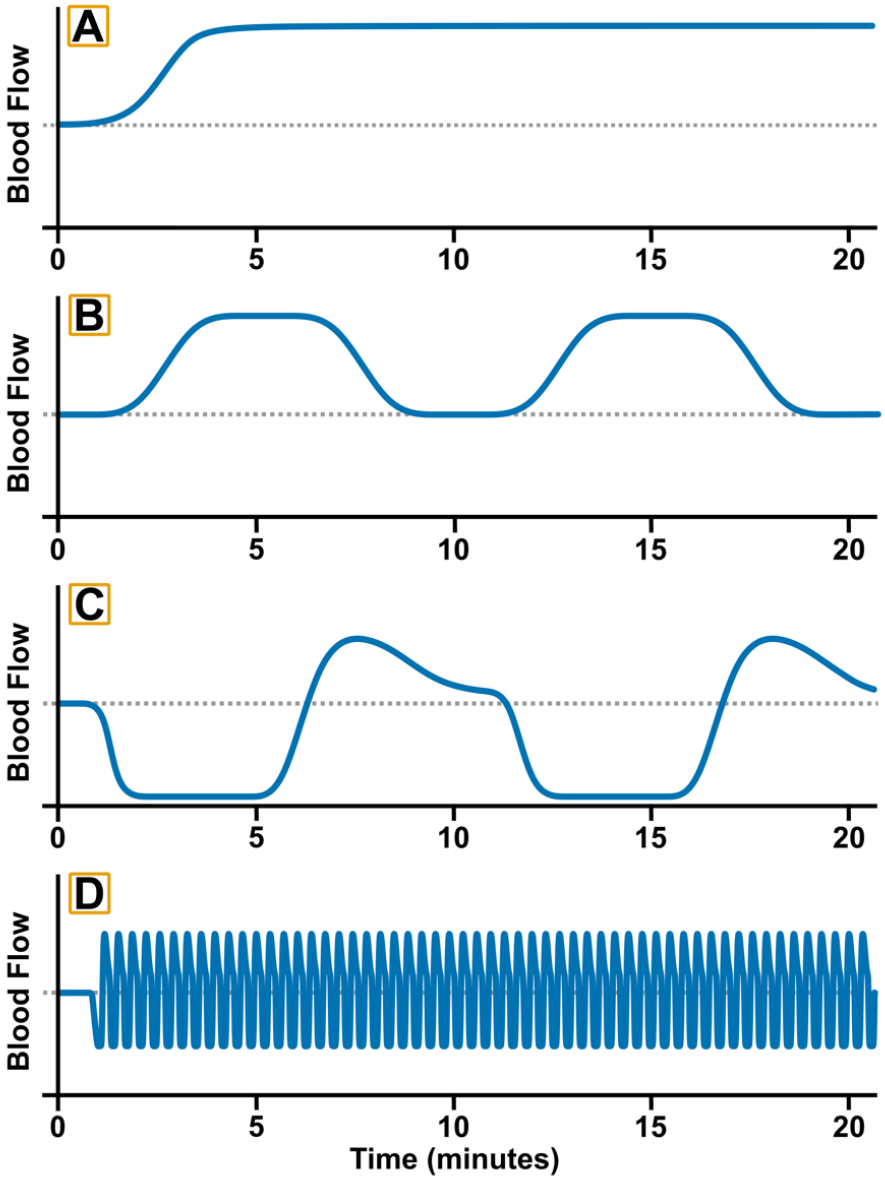


Figure 2.1 Representative flow patterns.
Illustration of typical flow patterns seen across different intervention strategies, resting baseline is shown as a dashed (---) line in all plots. **A**) Prolonged elevated flow patterns as are commonly seen during interventions such as steady-state exercise or continuous exposure to heat stimuli (e.g. hot water bathing). **B**) Interval or intermittent increases in flow, separated by a return to baseline, as is typical in interval-based exercise responses or in intermittent exposure to environmental stimuli. **C**) Flow response seen during ischemic preconditioning, typified by a prolonged period of very low flow during the ischemic period, followed by a rapid elevation and steady return towards baseline during the cuff-release period. **D**) Pneumatic compression induced flow patterns modelled on a low-frequency treatment pattern of repeated 4-second inflations and 16-second deflations.

Acute elevations in flow can also be induced by the manipulation of the external environment, either through changes in temperature, humidity and air composition (hypoxia); via immersion in water; or by a combination of these manipulations. Heat-based interventions have been shown to drive significant changes in blood flow, comparable to those seen in exercise, across a range of stimulus types, including hot water bathing (Thomas *et al.*, 2017), sauna bathing (Li *et al.*, 2020) and the utilisation of water perfused body suits (Chiesa *et al.*, 2016). The primary aim of such heat-based interventions is to induce a significant elevation in core temperature and many of these strategies have also shown the potential to drive adaptive improvements in endothelial function (Cheng & MacDonald, 2018). These adaptive responses include elevation in basal cerebral blood flow alongside improvements in cerebrovascular conductance and FMD in the brachial artery (Bailey *et al.*, 2016*b*). While the majority of research into water immersion for vascular health has utilised hot water conditions, immersion in thermoneutral water also has the potential to increase acute peripheral blood flow (Ayme *et al.*, 2014) and cerebral blood flow (Carter *et al.*, 2014), thus providing a stimulus for flow-mediated adaptations regardless of water temperature. Studies have also investigated the potential benefits of short-term hypoxia, in part due to its potential benefits in preconditioning against ischemic injury, and also for the potential benefits of low dose hypoxia in the wider vasculature (Navarrete-Opazo & Mitchell, 2014). While the impact of exposure to hypoxia is systemic and thus affect a wide range of physiological systems, evidence does show that this stimulus can produce elevations in blood flow, through endothelium-dependent release of nitric oxide and prostaglandins (Dinenno, 2016). Within the cerebrovasculature, acute hypoxia also induces changes in vascular function in order to maintain tissue oxygenation, including

increases in blood flow in the vertebral artery (Ogoh *et al.*, 2013) and vasodilation of the cerebral arteries (Wilson *et al.*, 2011). Although these acute hypoxic responses show the potential for elevations in flow, to date little is known about the efficacy of hypoxia as an intervention strategy. However, research in rodent models has shown positive responses to intermittent hypoxic training in both cognitive and cerebrovascular components of Alzheimer's disease (Manukhina *et al.*, 2016). Further research is needed to determine whether these responses will translate into hypoxic stimuli as an effective intervention for improving vascular function in humans.

Repeated exposure to many of these acute stimuli appear to stimulate similar adaptive responses, although the depth and breadth of our understanding of the processes driving these changes does vary significantly between interventions. Much like the weight of evidence for functional responses, the largest body of evidence surrounding the process and mechanisms of adaption can be found for exercise-based approaches. In a review of these processes, Green et al. (2017) explored the biphasic nature of adaptations to exercise. They highlighted that shorter intervention periods display a largely functional response phenotype, typified by increases in endothelium-dependent vasodilatory response and improvements in circulating inflammatory cytokines, oxidative stress markers and vasodilatory factors. This initial response appears to then plateau at ~4 weeks, with further improvements being produced by way of structural changes in lumen diameter, wall:lumen ratio and vessel elasticity (Green *et al.*, 2017). Across non-exercise strategies, the balance of functional and structural responses to interventions are best understood within the field of passive heating, as studies have demonstrated improvements in both endothelial functional

responses and flow mediated dilation, as well as structural improvements in elasticity and wall:lumen ratio over relatively short (8 week) intervention periods (Brunt *et al.*, 2016*b*). Nonetheless, the amount of data on these responses is somewhat smaller compared to the exercise context and there is still debate as to whether this pattern of response is likely to be seen across all heating modalities (Cheng & MacDonald, 2018).

While further research is needed across the majority of flow inducing non-pharmacological interventions, our ability to quantify these responses *in vivo* is relatively well developed, particularly in relation the peripheral arteries of the legs and arms. This is due to well established guidelines for utilisation of Doppler ultrasound to assess flow mediated dilation (i.e. FMD) of peripheral conduit arteries (Thijssen *et al.*, 2011), alongside pulse wave velocity measures to assess stiffness (Milan *et al.*, 2019). Similarly, functional responses to interventions within the brain can be assessed through the effective utilisation of transcranial Doppler ultrasound (Willie *et al.*, 2011), alongside more advanced techniques such as magnetic resonance imagery. In combination these measures can begin to build a full picture of the acute and chronic responses to non-pharmacological interventions across a wide range of spatial and temporal resolutions within the brain (Tymko *et al.*, 2018; Burley *et al.*, 2022). The non-invasive nature of many of these techniques, alongside improvements in affordability have allowed the development of a better understanding of the gross adaptative responses within the vasculature, across a range of populations and intervention strategies.

To date the majority of research has focused on arterial responses to interventions, either in the periphery or cerebrovasculature, and changes in functional outcomes relating to arterial adaptation (e.g. FMD and cerebrovascular reactivity). However, there is growing interest in examining vascular responses outside of these regions, including within the microvascular of the coronary and cutaneous circulation. While acute flow responses are difficult to determine in humans, increases in the size and vasodilatory capacity of the coronary arteries are seen following exercise training (Laughlin *et al.*, 2012). Within the cutaneous circulation in humans, exercise and passive heating stimuli significantly increased perfusion rate/vascular conductance, as assessed by Laser Doppler (Crandall & Wilson, 2014; Green *et al.*, 2017), alongside improvements in microvascular nitric oxide-mediated vasodilation following chronic interventions (Brunt *et al.*, 2016*a*; Green *et al.*, 2017). In addition to improving current understanding within specific vascular beds, greater breadth is needed in the range of vascular beds studied within intervention-based research. This is particularly important given the capacity for interventions to alter the distribution of blood flow across the vasculature. The focus of the current review is on the flow changes typically seen in the peripheral and cerebral circulation, as this is where the greatest level of understanding of acute and chronic flow responses is seen *in vivo*. However, future research must look to better characterise flow responses outside of these regions and to establish the link between these responses and the potential *in vitro* mechanisms driving them.

While we can characterise real-time flow responses *in vivo* within a particular vessel, how interventions may alter vascular function at the cellular level is not well

understood. This is particularly important in relation to the endothelium, which is pivotal in driving vascular responses to interventions, and plays a central role in responding to changes in both flow and the circulating environment (Cahill & Redmond, 2016). This is made more complex by the non-uniform patterns seen in acute responses between interventions and in the impact that changes in protocol can have on flow profiles (Figure 2.1). Understanding the cellular responses in both an acute and adaptive context is central to understanding the relationship between acute changes in flow and subsequent improvements in vascular health. This understanding can then be integrated into the discussion surrounding the capacity to predict adaptive responses to an intervention by assessing the acute vascular response during a single visit (Dawson *et al.*, 2018). The nature of the vascular system makes studying cellular responses *in vivo* incredibly challenging and we must look to *in vitro* models of the endothelium and flow response to build a complete picture of how the vasculature is responding to flow altering interventions.

2.3. Mechanisms driving adaptation to flow

2.3.1. Flow detection and response in endothelial cells

The vascular endothelium plays a central role in vessel function as the interface between the vessel and circulating blood, exemplified by the capacity for endothelial cells to respond to changes in blood flow. Early research during the 1980's identified the essential function of the endothelium in driving vasodilation, initially through endothelial ablation studies within canines (Pohl *et al.*, 1986). Concurrently, *in vitro* research began to identify the functional components of the endothelium that drive

relaxation of the smooth muscle cell layer and subsequent vasodilation. This included the identification and functional relevance of key vasoactive compounds, including nitric oxide (NO) (Palmer *et al.*, 1987), prostacyclin (PGI_2) (Moncada *et al.*, 1976) and endothelin-1 (Yanagisawa *et al.*, 1988). Alongside these vasoactive compounds, the endothelium was also shown to respond to shear stress through the opening of ion channels and subsequent hyperpolarization (Olesen *et al.*, 1988), a key component in endothelium-dependent hyperpolarization, leading to subsequent hyperpolarization and relaxation of smooth muscle (Garland & Dora, 2017). Subsequent research has identified a highly complex system of mechanosensitive proteins within the endothelium, that can detect the haemodynamic forces of shear stress and cyclic strain, and modulate responses to flow through mechanotransduction pathways in a force-dependent manner.

Among the first proteins identified to be playing a role as mechanosensors were members of the glycocalyx glycoprotein layer that lines the internal surface of vessels at the interface between the blood and endothelial cell layer. These proteins were shown to respond to flow through the transduction of force to the cell membrane via glypican coreceptors or to the cytoskeleton via syndecan coreceptors (Baratchi *et al.*, 2017). Numerous further mechanosensory proteins have since been shown to demonstrate an essential role in mechanotransduction, both in the detection of forces exerted on the cell and in the transduction of force from force sensors such as those within the glycocalyx. These include the VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial growth factor receptor 2 and 3 (VEGFR2/3) (VE-cadherin–PECAM-1–VEGFR2/3) mechanosensory complex (Tzima

et al., 2005), caveolar proteins (Ariotti & Parton, 2013), mechanosensitive ion channels (Baratchi *et al.*, 2017), integrins (Janoštiak *et al.*, 2014) and extracellular matrix component proteins (Figure 2.2) (Baratchi *et al.*, 2017).

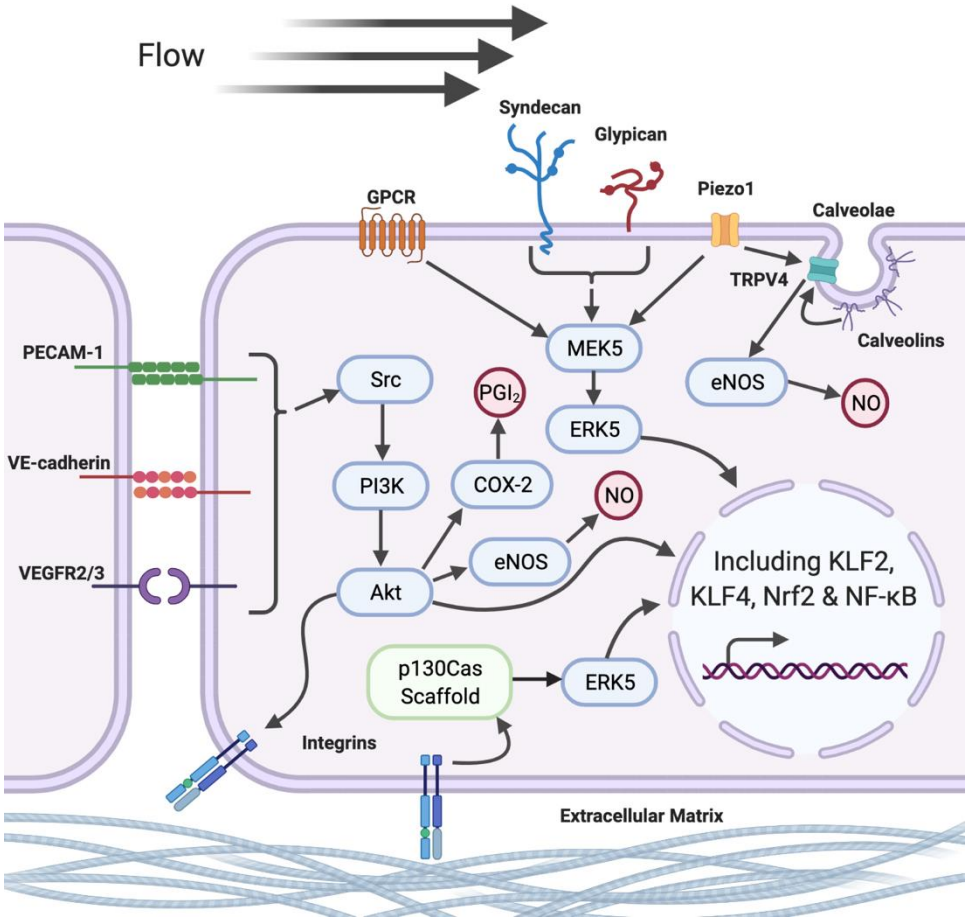


Figure 2.2 Flow-responsive mechanosensory pathways.
Changes in flow are detected within the endothelial cell through several mechanosensory pathways. There are broadly four key signalling cascades regulating gene expression. Membrane bound mechanosensory receptors, including G-protein coupled receptors (GPCR), Piezo1 and transient receptor potential vanilloid-type 4 (TRPV4) ion channels and glycocalyx members (including syndecan and glypican), induce changes in gene expression via the mitogen-activated protein kinase pathway (MEK5/ERK5). In addition, the VE-cadherin–PECAM-1–VEGFR2/3 mechanosensory complex [comprising of VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial growth factor receptor 2 and 3 (VEGFR2/3)] can induce a cascade of responses, through the activation of Src-dependent phosphatidylinositol-4,5-bisphosphate 3- kinase (PI3K) and subsequent activation of the protein kinase Akt. Integrin-associated pathways can also induce signalling cascade responses via the p130Cas Scaffold and ERK5, either in response to activation via Akt signalling or through detection in changes in extracellular matrix structures in response to flow. Finally, activation of calveolins within membrane calveolae are also capable of inducing vasodilatory responses, via the activation of membrane-bound calcium (Ca^{2+}) channels. Cumulatively, these signalling cascades induce changes in gene expression and acute vasodilatory responses via elevation in nitric oxide (NO) and prostacyclin (PGI_2) production. Created with BioRender.com.

While blood flow *in vivo* produces simultaneous shear stress and cyclic strain forces within blood vessels, these two forces are often addressed separately within *in vitro* research. Much of the research investigating the endothelial responses to mechanical force has been conducted using shear stress alone, due to the greater ease with which shear stress can be manipulated by the exposure of endothelial cells grown on a non-flexible surface to media under flow. As depicted in Figure 2.2, at the onset of shear stress the mechanical force exerted on the glycocalyx layer and other luminal mechanosensors activates the mitogen activated protein kinase (MAPK) extracellular signal-related kinase 5 (ERK5), via MAP/ERK kinase 5 (MEK5) (Le *et al.*, 2017). Simultaneously, elevated flow appears to drive shear-related responses within calveolae located within the endothelial membrane, through the activation of calveolin proteins, in particular calveolin-1 (Cav-1) (Sowa, 2012). Both of these pathways have also been shown to play a key role in regulating responses in mechanosensitive ion channels and as such the potential for shear stress to drive calcium-dependent endothelial hyperpolarisation and subsequent activation of vasodilatory signalling mechanisms (Baratchi *et al.*, 2017; Behringer, 2017). Among these mechanosensitive ion channels, the transient receptor potential cation channel V4 (TRPV4) and Piezo1 have been identified as key mechanosensors and form the simplest mechanism for force-detection within the endothelium. Both Piezo1 and TRPV4 have been shown to increase endothelial permeability upon initiation of shear stress and appear to both play an essential role in subsequent calcium influx (Gerhold & Schwartz, 2016). Recent research has also shown that TRPV4 is activated following the activation of Piezo1 (Swain & Liddle, 2021) and is enhanced by activation and co-localisation with Cav-1

(Lu *et al.*, 2017), suggesting that TRPV4 may play a central role in calcium-dependent flow responses.

Alongside the activation of luminal membrane proteins, flow-induced shear stress has also been shown to drive endothelial responses through the activation of adhesion molecules involved in cell-cell and cell-extracellular matrix (ECM) interactions. The most well studied of these pathways is the VE-cadherin–PECAM-1–VEGFR2/3 mechanosensory complex comprising of VE-cadherin, platelet endothelial cell adhesion molecule 1 (PECAM-1) and vascular endothelial growth factor receptor 2 and 3 (VEGFR2/3), which was first identified by Tzima et al. (2005). In response to increased shear, VE-cadherin transfers tension to PECAM-1 and facilitates the association between PECAM-1 and VEGFR2/3 (Conway *et al.*, 2013). This leads to the activation of signalling cascades through phosphoinositide 3-kinase (PI3K) (Jin *et al.*, 2003), integrin-associated pathways (Hahn & Schwartz, 2009) and subsequently altering gene expression (Figure 2.2; see Section 2.3.2). The essential role that this complex plays in mechanotransduction was demonstrated initially in bovine aortic endothelial cells (BAEC); in which loss of VE-cadherin or PECAM-1 resulted in loss of PI3K responses (Tzima *et al.*, 2005). Subsequently, similar PI3K responses under flow have also been seen in human umbilical vein endothelial cells (HUVEC) under the inhibition of all three components within the VE-cadherin–PECAM-1–VEGFR2/3 complex (Jin *et al.*, 2003; Coon *et al.*, 2015; Russell-Puleri *et al.*, 2017).

Evidence also suggests that shear stress can be detected through conformational changes in the extracellular matrix and ECM-cell interactions. However, this is less

well understood, in large part due to the high level of complexity and regional specificity (Wheeler *et al.*, 2014) that likely enables highly specific responses in different vascular beds (Baratchi *et al.*, 2017). Although much research is still needed to understand the scope and role of the ECM in flow responses, integrins appear to play a pivotal role in this mechanosensory mechanism. Specifically, integrins mediate interactions between the ECM and mechanosensory properties of PECAM-1 (Collins *et al.*, 2014), and enable ECM-dependent mechanotransduction and subsequent signalling pathways via the p130Cas scaffold complex (Janoštiak *et al.*, 2014). First identified by Yamada et al. (2006), the p130Cas scaffold complex has been shown to associate with cell membrane integrins and other focal adhesions (Defilippi *et al.*, 2006; Baratchi *et al.*, 2017), through which changes in ECM conformation can drive phosphorylation of the CAS substrate domain and the induction of ERK and other signalling pathways (*reviewed in* Janoštiak et al., 2014), and subsequent changes in gene expression (see Section 2.3.2). This unique mechanosensory pathway has the potential to alter flow-dependent responses across different regions, due to the complexity and variability in ECM composition across the vasculature (Collins *et al.*, 2014) and warrants further investigation, particularly when looking to understand flow responses within a specific vascular bed.

As mentioned, the majority of our understanding surrounding these mechanosensory pathways comes from the application of shear stress. However, growing evidence indicates that cyclic strain has a unique impact on endothelial responses to mechanical force (Meza *et al.*, 2019). Cyclic strain in isolation has been shown to activate a number of mechanosensory pathways, through stretch-activated ion channels, ECM-cell

adhesions (via the activation of integrins), and cell-cell interactions (via the VE-cadherin–PECAM-1–VEGFR2/3 mechanosensory complex; (Jufri *et al.*, 2015)). Collectively, these mechanisms enable cyclic strain to induce significant signalling responses and have been shown to induce changes in cell morphology and orientation in the absence of shear stress (Bernardi *et al.*, 2018). When investigated in combination with shear stress the two haemodynamic forces have an additive effect on mechanosensory signalling pathways, although this response is not fully synergistic in nature, most likely due to the overlap in the pathways responsible for detecting shear and strain (Meza *et al.*, 2019). Furthermore, the interaction between these forces becomes more complex when considering the relative strength and dynamics of each force and the structural variability seen across the vasculature (Gray & Stroka, 2017).

2.3.2. Signal cascade responses to flow exposure

The endothelial response to changes in blood flow, and the associated mechanosensory response to changes in shear stress and cyclic strain, is highly dependent on the nature of the flow change itself (i.e. the pattern of flow) both in terms of acute signalling responses and subsequent adaptive changes. While the mechanosensory pathways are activated under different flow profiles and the nature of the relationship between different sensors is not well understood, clear differences are seen in subsequent signal pathway responses between flow patterns (Figure 2.3) (Nakajima & Mochizuki, 2017). *In vitro* modelling has been used to identify the nature of these differences, including mimicking *in vivo* disease states; such as ischemic reperfusion injury, or structural variations that can alter regional flow *in vivo*; such as stenoses, bifurcations or arched structures (e.g. aortic arch) (Gray & Stroka, 2017).

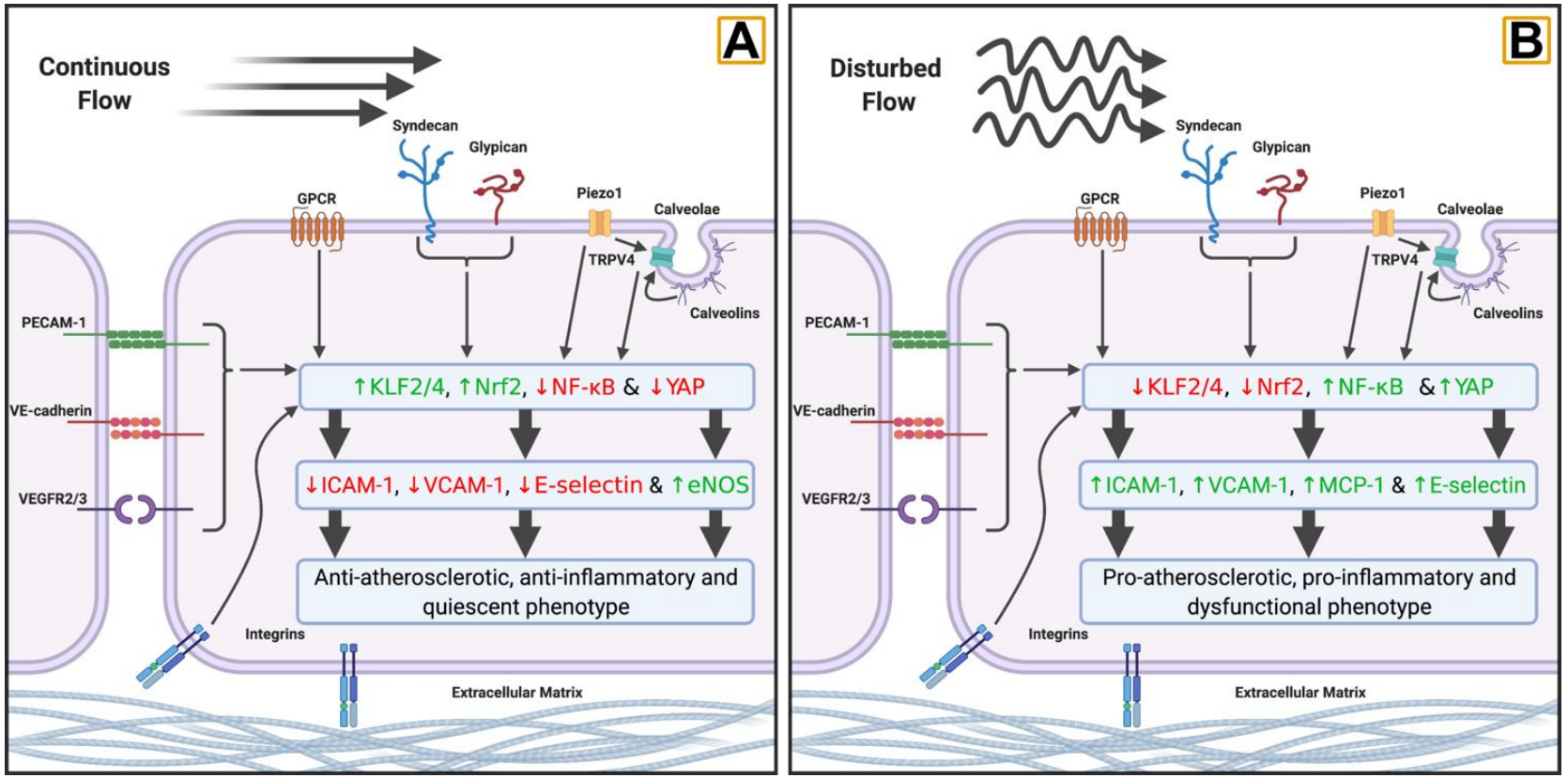


Figure 2.3 Differences in typical response between continuous and disturbed flow models.
A) Prolonged continuous and elevated flow results in the promotion of a quiescent endothelial cell phenotype through the upregulation of Krüppel-like factor 2 and 4 (KLF2/4) and Nuclear factor-E2-related factor 2 (Nrf2), while suppressing the activity of Nuclear-factor kappa B (NF-κB) and Yes-associated protein 1 (YAP), via the array of mechanosensory pathways detailed in Figure 1. **B**) Disturbed flow, characterised by oscillatory patterns of low or reversing shear stress, activates mechanosensory pathways through many of the same receptors as seen in continuous flow, however, these patterns drive opposing responses to those seen in continuous flow leading to the promotion of a dysfunctional endothelial phenotype. Created in BioRender.com.

Distinct endothelial phenotypes are seen across the vasculature, as a result of regional and tissue-specific variability in both local microenvironment and the function of the endothelium within different tissues (*reviewed in* Aird, 2007a, 2007b). Broadly speaking this heterogeneity has been observed in endothelial cells from different vascular beds (e.g. arteries vs. veins; Aird, 2007b); across different specialised tissue beds (heart, lungs, kidneys, liver and brain) (Cleuren *et al.*, 2019; Jambusaria *et al.*, 2020); and even in different regions within the same vessels (Simmons *et al.*, 2012). Crucially the plasticity of the endothelium to adapt to changes in stimuli can result in the rapid loss of the *in vivo* phenotype when the cells are cultured *in vitro* (Aird, 2007*b*; Kuosmanen *et al.*, 2017). This plasticity can be beneficial in developing cell culture models, particularly where cells from relatively accessible sources, such as HUVEC isolated from umbilical cord, can be manipulated to express phenotypes akin to those found in less accessible vascular beds. This is seen in HUVEC-based models of arterial flow, where arterial levels of shear stress (approx. 2.0 Pa or 20 dyn/cm^3), of either a constant or pulsatile nature (Figure 2.4), induces a well-characterised pattern of response (discussed below) comparable to those seen in BAEC (Conway *et al.*, 2013) and other arterial cell types (Luu *et al.*, 2010). Conversely, while plasticity enhances our ability to induce a particular phenotype through the manipulation of culture conditions, it also makes the maintenance of *in vivo* phenotypes in isolated cells or the modelling of specific vascular beds more complicated (Aird, 2007*b*). For example, in modelling of the blood-brain barrier researchers must consider the selection of appropriate endothelial cell sources; the essential role of astrocytes, pericytes and other cells of the neurovascular unit; alongside appropriate simulation of the microenvironment on both sides of the barrier (Sivandzade & Cucullo, 2018). To

date, the majority of studies investigating flow and intervention responses have focused on a relatively small pool of endothelial cell types and generally under linear, arterial flow. While this is in keeping with the majority of research in human *in vivo* research, future considerations (see Section 2.4) must look to expand the field in keeping with the advancement in technology facilitating the measurement of flow outside of conduit arteries (Baratchi *et al.*, 2017).

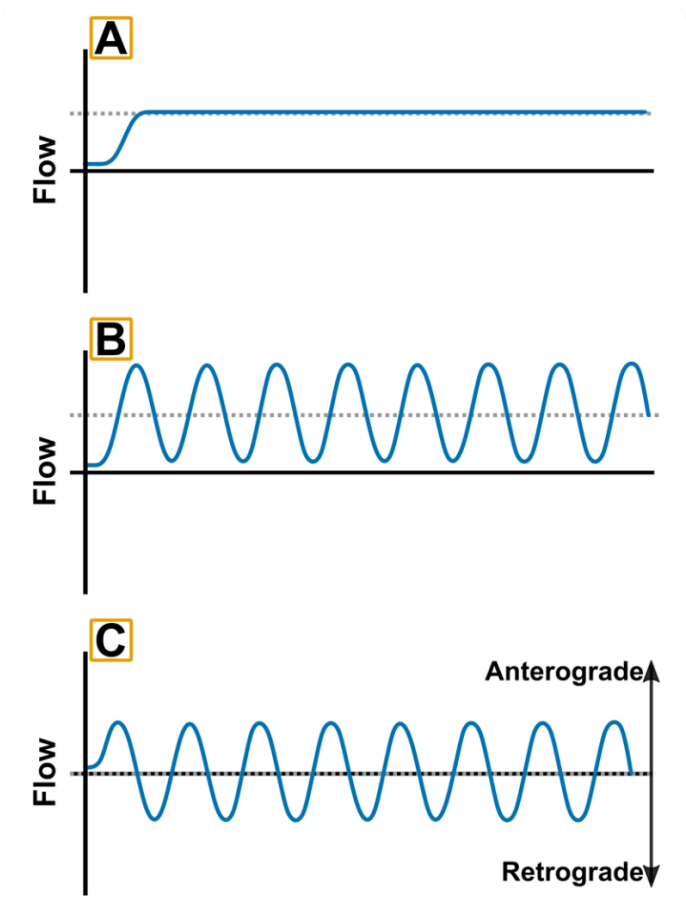


Figure 2.4 Flow patterns within continuous and disturbed cell culture conditions. Illustration of simple flow patterns utilised to induce continuous and disturbed responses within endothelial cells. Mean flow rate induced by each pattern is shown as a dashed (---) line in all plots. Continuous, elevated flow responses can be induced by the application of laminar (**A**) or pulsatile (**B**) flow patterns in cell culture. Pulsatile flow can also be utilised to simulate disturbed conditions, by the application of pulsatile profiles at low average flow rate (**C**), resulting in anterograde and retrograde flow components.

Continuous elevation of flow induces rapid responses in the expression of key pro-quiescent transcription factors (Figure 2.3a), most prominently krüppel-like factor 2 and 4 (KLF2 & KLF4) and nuclear factor E2-related factor 2 (Nrf2) via the ERK5 signalling pathway (Fledderus *et al.*, 2008). While elevation in the activity of nuclear-factor kappa-B (NF-κB) and the flow-responsive transcriptional co-regulator YAP, key factors in pro-inflammatory pathways, are also seen under continuous flow, this is highly transient and is followed by rapid reduction in activity over time (Nakajima & Mochizuki, 2017). Combined with the rapid activation of p130Cas signalling pathways through the VE-cadherin–PECAM-1–VEGFR2/3 complex and ECM-cell associated integrins, KLF2 mediates changes in vascular tone through the upregulation of endothelial nitric oxide (NO) synthase (eNOS) (Baratchi *et al.*, 2017). Simultaneously, these signalling responses have been linked to upregulation of anti-inflammatory factors; including eNOS and thrombomodulin (Lin *et al.*, 2005); suppression of inflammatory molecules; including intercellular cell adhesion molecule 1 (ICAM-1), VCAM-1 and E-selectin (Baratchi *et al.*, 2017; Ajami *et al.*, 2017); and improvements in redox balance via the Nrf2-antioxidant response (Fledderus *et al.*, 2008).

Contrastingly, oscillatory or disturbed flow patterns (exemplified by repetitive changes from high-to-low flow or anterograde-retrograde flow, Figure 2.4) drive relatively small responses in KLF2/4 and Nrf2. Disturbed flow patterns trigger significant elevations in pro-atherosclerotic and pro-inflammatory signalling pathways (Figure 2.3b); most prominently via Nf-κB and YAP (Nakajima & Mochizuki, 2017). Sustained elevation in both factors under disturbed flow leads to a significantly more pronounced inflammatory response (Nakajima *et al.*, 2017); including increases in ICAM-1, VCAM-

1, E-selectin and Monocyte Chemoattractant Protein-1 (MCP-1) (Chiu & Chien, 2011). This difference in signalling response is driven by differences in mechanosensory activity between laminar and disturbed flow, demonstrated in the contrasting role of PECAM-1 between the two conditions. Under elevated laminar flow, acute increases in PECAM-1 signalling are essential for both acute shear-dependent vasodilatory responses (Bagi *et al.*, 2005; Russell-Puleri *et al.*, 2017) and subsequent vascular remodelling under exposure to chronic stimuli (Tzima *et al.*, 2005). While under pathological and disturbed flow, PECAM-1 plays an essential role in NF- κB activation and downstream over-expression of adhesion molecules (Tzima *et al.*, 2005). Further, in PECAM-1 knock-out mice disturbed flow has been linked to reductions in vessel diameter (Chen *et al.*, 2010) and atherosclerosis severity (Harry *et al.*, 2008). In combination the changes induced by prolonged disturbed flow leads to the establishment of a dysfunctional, pro-atherosclerotic endothelium exhibiting elevated levels of inflammation, oxidative stress and vasoconstriction (Chiu & Chien, 2011). While this phenotype is likely typical under chronic, pathological conditions, such as atherosclerosis, a lack of current research makes understanding the impact of shorter periods of oscillatory flow difficult. Given the positive impact that pneumatic compression can have on vasodilatory function, it is likely that shorter, transient periods of flow of this nature have a different impact on endothelial response. This makes comparison between *in vitro* research and the potential mechanisms driving responses to intervention difficult (Table 2.1) and warrants further research into the impact of transient exposure to oscillatory flow.

Table 2.1. Comparison of *in vitro* endothelial cell and *in* vivo vascular adaptive responses to common stimuli found during non-pharmacological interventions

Stimulus	Interventions	*In Vitro*	*In Vivo*
Continuous elevation in flow	• Exercise • Water immersion • Heat therapy	↑ eNOS ↓ inflammatory cytokines ↑ antioxidants ↑ anti-inflammatory molecules	↑ basal blood flow ↑ maximal flow-mediated dilation ↑ cerebrovascular responsiveness
Intermittent elevation in flow	• Exercise • Heat therapy	???	???
Oscillatory flow	• Pneumatic compression	*Prolonged exposure:* ↑ inflammatory cytokines ↑ adhesion molecules ↑ vasoconstriction factors	↑ basal blood flow Improved functional outcomes
Loss of flow	• Ischemic preconditioning	*Prolonged exposure:* ↑ inflammatory cytokines ↑ cellular dysfunction ↑ cell death	↑ endothelium-dependent blood flow ↑ maximal flow-mediated dilation
Hypoxia	• Ischemic preconditioning • Hypoxia	↑ inflammatory cytokines ↑ oxidative stress ↑ angiogenic factors ↑ eNOS	???
Increased temperature (Febrile/~39ºC)	• Heat therapy	↓ IL-6 response to TNFa ↓ future hyperthermic damage HSP protection against inflammation	↑ basal blood flow ↑ maximal flow-mediated dilation ↑ cerebrovascular conductance

Abbreviations: eNOS = endothelial nitric oxide synthase; IL = interleukin; VEGF = vascular endothelial growth factor; heat shock protein 72 = HSP72; tumor necrosis factor alpha = TNFα. ↑ = increase; ↓ = decrease; ??? = response currently unknown. References for the stated responses can be found in Sections 2.2.2, 2.3.2 & 2.3.3.

Less well studied *in vitro* is the endothelial response to changes in flow pattern in cultures grown under flow conditions and how short, transient changes may lead to subsequent adaptive changes in endothelial function. These transient changes in flow are a key focal point within *in vivo* human research and a central tenant of the mechanisms driving adaptations to a range of interventions including exercise (Green *et al.*, 2017), passive heating (Cheng *et al.*, 2019) and pneumatic compression (Credeur *et al.*, 2019) (see Section 2.2.2). While limited research has been conducted to date, *in vitro* research has shown the potential for acute changes in flow to stimulate beneficial signalling pathways within the endothelium. In endothelial cells preconditioned to arterial levels of shear stress, further elevations in shear have been shown to stimulate increases in the expression of a range of genes including KLF2 and KLF4, alongside the upregulation of eNOS activity and the promotion of an anti-inflammatory and atheroprotective phenotype (Zhang & Friedman, 2012). Furthermore, elevation in the frequency of pulsatile flow; as would be seen during interventions with the capacity to elevate heart rate, lead to increases in the expression of angiogenic and cell proliferation genes, as well as upregulation of vascular endothelial growth factor (VEGF) (Zhang & Friedman, 2013). These studies demonstrate that elevations or changes in flow conditions can drive significant mechanosensitive response, whether in cells previously cultured under static or continuous flow conditions. Additionally, they provide a potential mechanism for the vascular changes seen under interventions that drive continuous flow elevations (Table 2.1), and align with the improvements in NO-mediated vasodilatory function that are seen following interventions such as passive heating and exercise (Green *et al.*, 2017; Cheng *et al.*, 2019). The capacity for increases in both shear rate and pulsatile

frequency to induce changes in endothelial responses also demonstrates the complex nature of flow responses within the endothelium and the need for specific models of intervention responses. Future research must also consider the impact of the duration of flow changes on subsequent responses, as most interventions induce changes in magnitude and frequency of pulsatile flow, over a relatively short time-course and often in a non-continuous manner (see Figure 2.1).

While most prominently studied from the perspective of disease, research has been conducted into the impact that reduction or loss of flow during ischemic reperfusion can have on previously perfused cells *in vitro*. Under ischemic reperfusion the endothelium sees a rapid elevation in ROS and suppression of vasodilation via eNOS uncoupling, coupled with a transition into a more pro-thrombotic and pro-inflammatory state, via the upregulation of hypoxia-inducible factor 1 (HIF-1α) and NF-κB (Hernández-Reséndiz *et al.*, 2018). While ischemic events *in vivo* consist of both a loss or significant reduction of flow and exposure to a hypoxic environment, cessation of flow alone has been shown to drive significant signalling responses including the activation of NADPH oxidase 2 (NOX2) and subsequent production of ROS, the induction of pro-inflammatory transcription factors (including HIF-1α and NF-κB) (Chatterjee, 2018) and the downregulation of KLF2 (Matharu *et al.*, 2008). Although prolonged, uncontrolled periods of ischemia can have severe pathological complications, including cellular dysfunction, cardiovascular complications and death, shorter controlled periods of ischemia in the form of ischemic preconditioning are thought to elicit beneficial adaptations. These adaptations include improving vascular function within both the periphery and cardiac circulation and improving outcomes in

future ischemic reperfusion injury (Tapuria *et al.*, 2008). It has been suggested that these benefits result from the lower level of cellular distress caused by short bouts of ischemia, which are insufficient to impact on cell viability and death, but capable of driving adaptive responses to condition the endothelium against the potential damage of further ischemic events (Kalogeris *et al.*, 2012; Hernández-Reséndiz *et al.*, 2018). This is seen in the time-course of the inflammatory responses seen following cessation of flow; as assessed by neutrophil adherence under TNFα exposure, in which relatively little change is seen over the first 30-minutes of flow cessation (Matharu *et al.*, 2008). However, further research is needed to formally address endothelial responses to brief periods of ischemia, as used in IPC interventions, as well as in relation to hypoxia and loss of flow both separately and in combination.

2.3.3. Signal cascade responses to changes in culture conditions

Alongside the impact that environmental manipulation (e.g. hypoxic conditioning or passive heating) can have on blood flow (see Section 2.2.2), environment-based interventions also induce significant changes in the internal environment within blood vessels and the endothelium (e.g. temperature, oxygen tension/availability). As in the case of ischemic preconditioning, much of our understanding regarding the impact that changes in temperature or oxygen availability have on the endothelium comes from research focused on pathological conditions. Interestingly, *in vitro* endothelial cells respond to hypoxia by elevating signalling via NF-κB and HIF-1α (D'Ignazio & Rocha, 2016) in a similar manner to the responses seen under ischemic conditions (Hernández-Reséndiz *et al.*, 2018), suggesting similar mechanisms of response whether oxygen is reduced via ischemia or environmental manipulation. *In vivo*

research has demonstrated clear differences between moderate (9–16% inspired O_2) and extreme (2–8% inspired O_2) hypoxic “doses”; including beneficial vs. detrimental impact on blood pressure and systemic inflammation (Navarrete-Opazo & Mitchell, 2014), akin to the differences seen between transient and sustained ischemic reperfusion (Kalogeris *et al.*, 2012). *In vitro*, similar differences were seen when HUVEC were incubated at 0, 5, & 10% O_2, across 6, 12 & 24-hour periods, with elevations in HIF-1α and NK-κB, subsequent increases in ICAM-1, VEGF and reductions in cell viability occurring in a time- and O_2 concentration-dependent manner (Baldea *et al.*, 2018). While this study shows the potential impact of dose and exposure duration, the majority of *in vitro* studies have focused on extreme hypoxia reflective of pathological conditions, and more refined, dose-dependent response experiments are needed to understand the potential beneficial and detrimental effects of these differences at the cellular level (Pavlacky & Polak, 2020).

Similarly, heat stress can alter endothelial signalling pathways and drive adaptive responses beyond those induced by changes in flow alone. The impact of hyperthermia on endothelial cells appears to be highly dependent on the temperature and conditions of the exposure. In addition to increases in eNOS expression and NO release (Harris *et al.*, 2003), severe hyperthermia (≥41ºC) also triggers up-regulation in adhesion molecules such as ICAM-1 (Lefor *et al.*, 1994), and induction of endothelial apoptosis via a p53-dependent mechanism (Gu *et al.*, 2014). This response appears to depend on the severity of the hyperthermic stimulus, as febrile temperatures (38-40ºC) drive little basal response in inflammatory cytokines (including IL-1B, IL-6 & IL-8) or adhesion molecules (including ICAM-1, VCAM-1 & PECAM-1) (Shah *et al.*, 2002).

This difference in basal response may also relate to the anti-inflammatory effects febrile temperatures have on the endothelium, such as a reduction in TNFα-induced IL-6 secretion (Hasday *et al.*, 2001). The duration of exposure to heat stress also appears to alter the severity and nature of endothelial responses, with little apoptotic cell death seen following 90-minutes at 42ºC (Liu *et al.*, 2015) and a reduction in TNFα-induced cell adhesion molecule expression after 60-minutes at 42ºC compared to normothermic controls (Nakabe *et al.*, 2007). NF-κB is thought to play a key role in regulating these responses to hyperthermia, both during exposure to heat stress and in the subsequent impact on endothelial function post-hyperthermia. Hyperthermia stimulates increases in NF-κB to protect against heat-induced apoptosis Liu et al. (2015), with NF-κB levels subsequently decreased following hypoxic-reoxygenation stimuli (Brunt *et al.*, 2018) and were linked to reductions in adhesion molecule responses to TNFα treatment (Nakabe *et al.*, 2007). It is possible that the divergent NF-kB responses to heat stress are analogous to those seen in response to changes in shear stress (see Section 2.3.2). Under this hypothesis, shorter or febrile temperature exposure may induce transient elevations in NF-κB that effectively "primes" the endothelium and promotes an anti-inflammatory response, while prolonged exposure to high temperatures leads to continuous elevation in NF-κB and promotion of a pro-inflammatory, apoptotic phenotype. Future research should examine this hypothesis and determine the role that magnitude and duration play in hyperthermic responses. This is particularly important if we wish to interpret the impact of heat-based interventions, as typically small changes in core temperature (~1-2ºC) are induced for short periods, while most *in vitro* research to date has focused on longer exposures above 40ºC.

To summarise, across the wide range of unique intervention strategies utilised for vascular health, clear similarities can be seen in the capacity for dose-dependent responses within the endothelium, whether the dose response is related to magnitude of flow change, duration of stimulus or the level of deviation from “normal”/“healthy” conditions. These response patterns highlight the importance of specifically designing experiments to study the impact of interventions on the endothelium, rather than relying on the re-interpretation of findings from dysfunction or disease-based models.

2.3.4. Interaction with signalling factors, inflammatory cytokines and the circulating environment

Adding further complexity to our understanding of interventions and their effects on the endothelium is the manner in which different strategies may alter the circulating environment; specifically in the form of changes in circulating signalling molecules and vasoactive substances occurring *in vivo*. These changes can be: (i) induced locally by the endothelium itself in response to changes in flow; particularly in relation to the release of vasoactive substances and inflammatory cytokines; (ii) produced by other tissues and released into the circulation, or (iii) induced in an endocrine manner within the endothelium as a positive feedback loop response to circulating signalling factors released from other tissues.

In vivo the endothelium is rarely impacted in isolation and more commonly changes in flow are driven alongside changes in both proximal and distal tissues, making the role of circulating signalling factors central to understanding the holistic impact of non-

pharmacological interventions. The importance of changes in circulating factors is seen across a range of intervention and is best evidenced in exercise and passive heating, which despite their differences in aetiology (e.g. active recruitment of muscle contraction vs passive exposure to a heat stimulus), both produce marked elevations in core temperature and blood flow (Naylor *et al.*, 2011; Francois & Thomas, 2016). Despite the methodological contrast between these two approaches, similar responses are seen not only in haemodynamic responses (see Section 2.2.2), but also in vascular biomarkers including interleukin-6 (IL-6), VEGF and heat shock protein 72 (HSP72) (Henstridge *et al.*, 2016; Hoekstra *et al.*, 2018; Akerman *et al.*, 2019). The similarities in the known acute response to these two strategies has led to suggestions that they may induce similar vascular benefits to one another, as a result of comparable elevations in heart rate and increases in both core and skeletal muscle temperature during acute bouts (*reviewed* in Hoekstra et al., 2020). Interestingly, acute circulating factor responses are also seen during hypoxic stimuli (Table 2.2), including elevations in both HSP72 and VEGF (Taylor *et al.*, 2010; Burki & Tetenta, 2014). These complementary acute responses across the three intervention strategies is matched in their responses to repeated exposure, most notably in the reduction in circulating markers of inflammation across exercise training (Palmefors *et al.*, 2014), hot water bathing (Hoekstra *et al.*, 2020) and dose-dependently in intermittent hypoxia (Navarrete-Opazo & Mitchell, 2014).

Table 2.2. Acute responses in circulating factors seen in exercise-, heat- and hypoxia-based interventions in humans.

Factor	Exercise	Heat	Hypoxia
IL-1Rα	Y[1]	Y[5]	Y (chronic)[8]
IL-1β	Y[1]	?	?
IL-6	Y[2]	Y[5,6]	Y(chronic)[8]
IL-8	Y[2]	Y[5]/N[7]	?
IL-10	Y[2]	?	?
VEGF	Y[3]	N[7]	Y[9]
HSP72	Y[4]	Y[6]	Y[10]
TNFα	Y[1]	N[5,7]	N[9]

Factors are marked dependent on whether an elevation (**Y**) or no change (**N**) is seen with a given intervention stimulus. If responses are only seen over chronic exposure (>24-hours) to a stimulus then this is also denoted (**chronic**). Factors are also marked if the response is not well understood (**?**).
Abbreviations: IL = interleukin; VEGF = vascular endothelial growth factor; heat shock protein 72 = HSP72; tumor necrosis factor alpha = TNFα.
References: [1](Ostrowski *et al.*, 1999); [2](Kasapis & Thompson, 2005); [3](Landers-Ramos *et al.*, 2014); [4](Henstridge *et al.*, 2016); [5](Leicht *et al.*, 2015); [6](Faulkner *et al.*, 2017); [7](Kuhlenhoelter *et al.*, 2016); [8](Hartmann *et al.*, 2000); [9](Burki & Tetenta, 2014); [10](Taylor *et al.*, 2010)

One possible explanation for the similarities in both acute and chronic responses to a wide variety of intervention strategies is the central role that hypoxia-inducible factor 1α (HIF-1α) plays in angiogenesis and vascular function. *In vivo* human research has demonstrated that both acute and chronic exposure to heat or hypoxia results in similar elevations in extracellular HIF-1α (Lee *et al.*, 2016), while elevations in skeletal muscle due to localised hypoxia during exercise have been suggested to play an essential role in adaptation to training (Lindholm & Rundqvist, 2016). Although it is not possible to elucidate the impact that changes in HIF-1α have on the endothelium directly *in vivo*, HIF-1α plays a clear role in endothelial function *in vitro*. Indeed, the HIF-family of proteins have been highlighted as potential master regulators for angiogenesis during vascular development (Hashimoto & Shibasaki, 2015), driving elevations in NO

production via eNOS; the release of pro-angiogenic factors such as VEGF, and activation of inflammatory pathways via increase interleukin release (Krock *et al.*, 2011). Research in rodent models has also shown that hypoxia-inducible factors are essential to the acute inflammatory response (including IL-4, IL-6, and IL-10) following ischemic preconditioning (Cai *et al.*, 2013; Yang *et al.*, 2018) and the subsequent beneficial signalling responses via Akt (Du *et al.*, 2020), further demonstrates the central role that HIF signalling plays. The similarities and potential role of hypoxia-inducible factors across different intervention stimuli may be pivotal in the cross-over seen between many of the interventions discussed within this review, such as the cross-adaptation following heat acclimation interventions driving improvement in exercise tolerance in hot and hypoxic conditions (Lee *et al.*, 2016; Gibson *et al.*, 2017).

This is not to say that changes in the circulating environment are not highly dependent on the specific parameters of a given intervention, in much the same manner as is seen in the responses to flow between interventions. This is demonstrated in the variable responses to changing parameters within a single intervention type, such as the protocol-dependent responses in circulating IL-6, TNFα, VEGF and other factors seen following exercise-based interventions (Palmefors *et al.*, 2014; Ramos *et al.*, 2015) and the temperature-dependent response in HSP70 seen following active heat acclimation (Lovell *et al.*, 2007). The complexity of the circulating environment makes determining the full scope of a given intervention's impact very difficult, let alone the potential variations in response within and between different intervention paradigms. This is particularly true in relation to the current approach taken to understanding the circulating environment. This is often done by targeting specific biomarkers of

response (e.g. VEGF, HSP's, HIF-1α, etc.), which by their very nature are likely to display similar responses to interventions that have been selected specifically for their ability to stimulate vascular responses. To address this dilemma, future research must look to expand the breadth of targets investigated within the circulation, as can be achieved through the utilisation of omics approaches that allow for the full scope of responses within the circulation to be determined (Hoffman, 2017).

Our understanding of the impact that many circulating factors have on the endothelium at the cellular level suffers from a similar issue, in that the molecular response to many factors is well understood in isolation and in relation to pathological conditions. However, our knowledge of the cellular response to the collective changes seen during non-pharmacological interventions is much less developed. This is exemplified in our understanding of the impact that inflammatory cytokines have on the endothelium, which have been studied and reviewed at length in relation to vascular dysfunction and disease (Sprague & Khalil, 2009). Elevated inflammatory cytokine levels have been shown to play a central role in endothelial dysfunction through disruption of vasodilatory mechanisms, sustained upregulation of angiogenic factors and activation of NF-κB signalling pathways (Sprague & Khalil, 2009). Additionally, prolonged periods of cytokine elevation are thought to promote the development of hypertension (Pioli & de Faria, 2019), atherosclerosis (Tedgui & Mallat, 2006) and chronic vascular dysfunction (Steyers & Miller, 2014). When we look to interpret the potential role that these mechanisms play within interventions, similarities can be drawn in the transient response to interventions such as exercise and passive heat exposure (Kasapis & Thompson, 2005; Hoekstra *et al.*, 2018); in which significant elevations in inflammatory

cytokines likely elicit similar signalling responses to those seen in inflammatory disease or dysfunction. However, over repeated exposure to these strategies a reduction is seen in basal inflammatory cytokine profiles (Gleeson *et al.*, 2011; Hoekstra *et al.*, 2020), in stark contrast to the response seen in dysfunction. This is likely due to the transient nature of exposure during interventions compared to chronic elevations seen in pathological states. It has been suggested that this difference is key to the anti-inflammatory benefits of a number of interventions (Hoekstra *et al.*, 2020), in a similar manner to the transient periods of reduced flow seen in ischemic preconditioning that drives beneficial adaptive responses in vascular function (Kalogeris *et al.*, 2012; Hernández-Reséndiz *et al.*, 2018). This also highlights the importance of considering differences in the nature, magnitude and duration of changes in the circulating environment during interventions, especially when looking to compare the potential mechanisms driving endothelial responses from pathophysiological models and studies.

The importance of intervention-specific study design is made clear when changes in circulating environment are examined in concert with flow. For example, the endothelial cell response to even a small number of inflammatory cytokines (TNFα and IL-1β) is drastically altered when cells are preconditioned under arterial levels of shear stress (Luu *et al.*, 2010). Furthermore, when the pattern of flow is modulated during exposure to TNFα, a significant difference is seen in the response. For example, significant increases in neutrophil-adherence are seen when preconditioned cells are exposed to ischemic flow conditions (Sheikh *et al.*, 2003), demonstrating the potential for markedly different responses dependent on blood flow conditions. Alongside this reciprocal

relationship between circulating factors and flow, VEGF has the potential to interact with flow-responses through its role in the assembly of the p130Cas complex (Evans *et al.*, 2017) and interaction with other mechanosensory signal pathways via binding to VEGF receptors including VEGFR2/3 (Olsson *et al.*, 2006). Adding further complexity still, alongside the interaction between flow and circulating factors, there is also significant potential for interplay between the factors themselves, this is seen in the case of IGF-1 and VEGF as, like many growth factors, they share common signalling pathways through PI3K – Akt and MEK1/2 – ERK1/2, resulting in significant overlap in their signalling responses (Figure 2.5) (Olsson *et al.*, 2006; Rivera *et al.*, 2011; Bach, 2015). Similarly, heat shock proteins, particularly HSP90 (Figure 2.5), have been shown to directly interact with HIF-1α and VEGF signalling; through stabilization of HIF-1α (Minet *et al.*, 1999) and increased VEGFR2 receptor presentation (Bruns et al., 2012). Finally, as has been discussed in relation to differences in response to flow (see section 2.3.2), endothelial heterogeneity can also impact endothelial responses to circulating signalling factors, including variability in the magnitude and scope of VEGF-induced MAPK signalling response across BAEC, HUVEC and HMVEC cell types (Yashima *et al.*, 2001). Taken together, these interactions further emphasise the importance of understanding the complete spectrum of changes seen within the circulation *in vivo* as well as the collective effects of these changes when modelling endothelial response *in vitro*.

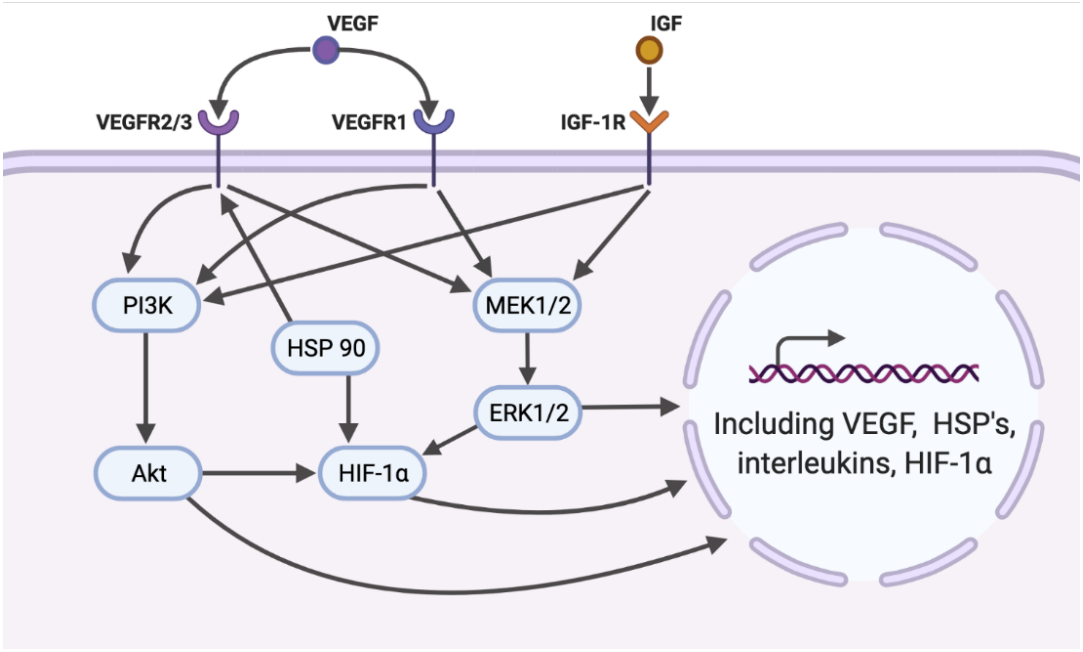


Figure 2.5 Shared signalling pathways between circulating factors and the proteins they upregulate.
Vascular endothelial growth factor (VEGF) and insulin-growth factor (IGF-1) both bind to unique membrane receptors (VEGFR1, VGEFR2/3 & IGF-1R) while sharing common signalling pathways within the endothelium, via the phosphatidylinositol-4,5-bisphosphate 3- kinase (PI3K) and activation of Akt, as well as through the mitogen-activated protein kinase pathway MEK1/2–ERK1/2. These signalling pathways can induce significant upregulation of key proteins including VEGF, heat shock proteins (HSP's), interleukins and hypoxia-inducible factor 1 alpha. HSP's and HIF-1α are also seen to be elevated in response to environmental changes such hypoxia and hyperthermia, and both proteins have to capacity to increase responses to circulating factors via interaction with receptor presentation, signal pathways or both. Created in BioRender.

The development of specific, well-designed cell culture experiments that incorporate core acute intervention factors/responses and better model the physiological environment of these scenarios is key to identifying how changes in circulating environment and flow responses drive beneficial vascular adaptations.

2.4. Current Advances and Future Directions

While we can draw inference as to the vascular effects of non-pharmacological interventions from our understanding of basic and pathological flow responses, more targeted studies are needed to fully understand the mechanisms driving beneficial responses to these interventions. This is particularly pertinent for interventions that share physiological responses with pathological conditions such as passive hyperthermia and ischemic preconditioning. While these interventions mimic the changes seen in disease states, responses are induced over a significantly more transient time course and in a more controlled fashion, and as a result likely induce different adaptive changes in the longer term (i.e. adaptation vs. maladaptation, as discussed in Section 2.3.2).

To improve on our current understanding, interventions should look to mimic the flow profiles seen within a given intervention, utilising the wealth of *in vivo* data already available on the nature of these responses. As already highlighted (see Section 2.3.2), both the magnitude and frequency of pulsatile flow can have an impact on endothelial responses (Zhang & Friedman, 2012, 2013) and future research should also consider and look to control the nature of both factors, to better mimic changes in blood flow and heart rate. Similarly, changes in flow should be studied in endothelial cells pre-conditioned to flow conditions representative of those seen at rest within the vessel of interest *in vivo* (e.g. 2.0 Pa shear in conduit arteries). These improvements are increasingly possible as a broad array of highly controllable flow culture apparatus are now widely available, both in the form of commercially available equipment and open-source, “do-it-yourself” solutions (Estrada *et al.*, 2011; Byun *et al.*, 2014). An example

for the potential of this approach is seen in Wang et al. (2016), in which Matlab-based flow simulations were utilised to demonstrate differences in actin microfilament formation and NO production under resting and exercising flow patterns, based on data taken from *in vivo* measurements of flow through the common carotid artery.

Physiologically relevant models are also essential in the wider context of environment-based interventions, as both baseline and treatment conditions are often not representative of the conditions seen *in vivo*, particularly within a healthy population. This is demonstrated in models of hypoxia, with normal/"normoxic" culture conditions generally set at 21% O_2 and 5% CO_2, which provides oxygenation levels well in excess of most physiological settings, and "hypoxic" conditions often at the other extreme, reducing oxygen levels closer to anoxic conditions, rather than those which are physiologically relevant to non-pathological conditions (Al-Ani *et al.*, 2018). Recently, the importance of maintaining and reporting consistent culture conditions in hypoxic experiments has become more prominent within the literature and researchers have begun to focus on developing more physiologically relevant conditions (Pavlacky & Polak, 2020) and cost-effective solutions for monitoring changes during experiments (Mathupala *et al.*, 2016). Similarly, the clear differences in endothelium responses dependent on hyperthermic conditions (see Section 2.3.3) highlights the need for more specific investigation of the responses to changes in environment, particularly in the context of intervention responses compared to pathological conditions, including considering how differences in duration and frequency of exposures may alter responses. Future studies should also aim to combine the different components of a given stimuli within a single experiment; for example, investigating the endothelial

responses to transient elevations in temperature and flow in combination, to understand whether these stimuli offer further benefits over each stimulus in isolation.

The incorporation of samples taken from *in vivo* experiments when simulating intervention conditions could also improve the physiological relevance of *in vitro* experiments. Alongside omics-based approaches to better understand the composition of these samples, this approach will also improve our understanding of how changes in the circulating microenvironment may interact with the endothelium, as well as how chronic interventions alter the nature of this microenvironment. This approach was recently taken by Brunt et al. (2018), who demonstrated that *ex vivo* serum application from participants following 8 weeks of passive heat therapy induced similar protective benefits in hypoxia-reoxygenation response to those seen in endothelial cells pre-conditioned at 39ºC. In future this approach could be utilised not only to model how repeated intervention visits may alter the baseline circulating microenvironment and its impact on endothelial cells in isolation, but also how changes seen within a single visit may alter the endothelial response to concurrent changes in flow, temperature or oxygen level.

Recent advances in cell culture techniques alongside increased affordability of cutting-edge approaches such as lab-on-chip systems, are likely to play an essential role in addressing the shortcomings within the current literature, particularly as *in vivo* research expands beyond large arterial vessels and incorporation of smooth muscle cells. Future research should look to incorporate these advanced culture methods and integrated approaches between *in vivo* and *in vitro* research, particularly focussing on

the selection of optimal culture conditions and accurate simulation of the magnitude, frequency and duration of intervention responses. This will help to build a more complete picture of the manner in which non-pharmacological interventions interact with the vascular endothelium and ultimately pave the way for the rapid analysis of *in vitro* endothelial responses in parallel with *in vivo* research.

2.5. Conclusion

Non-pharmacological interventions have the capacity to drive significant changes in blood flow and as a result induce beneficial adaptations within the endothelium. Our understanding of how the cellular mechanosensory mechanisms of the endothelium respond to flow are relatively well developed and, coupled with research into the impact that the circulating microenvironment has on the endothelium, we can begin to interpret how these mechanisms may be altered during interventions. However, research to date has largely focused on resting and pathological scenarios. Targeting future research to address the lack of studies into intervention responses will help to shed light on the potential benefits that non-pharmacological approaches can have on vascular health.

3. MATERIALS AND METHODS

3.1. Ethics

All human data and samples were obtained with written, informed consent in compliance with the Declaration of Helsinki (see Appendix 2). Approval was granted by the North East – Tyne & Wear South Research Ethics Committee (15/NE/0285); for isolation of umbilical cord derived endothelial cells (HUVEC), University of Birmingham Science, Technology, Engineering and Mathematics Ethical Review Committee (ERN_17-1570; ERN_19-1574PA1); for data collected in exercise-based studies, or University of Birmingham Local Ethical Review Committee (ERN_12-0079); for blood sampling.

3.2. Respiratory Gas Analysis

Breath-by-breath measures of respiratory gas exchange and ventilation were collected across all exercise-based participant visits using a metabolic cart (Vyntus CPX™, Vyaire Medical, Basingstoke, UK). Participants were fitted with face mask and head strap (both Hans Rudolph, Cranlea, Birmingham, UK), which was worn throughout the duration of each visit and connected to the metabolic cart by a twin tube sample line and DVT volume sensor via a silicone mask adapter (all Vyaire Medical). Continuous measures of ventilatory volume, oxygen and carbon dioxide were taken and analysed in real-time using SentrySuite® Software (Vyaire Medical), to obtain measures of minute ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (RER) and partial pressure of end-tidal carbon dioxide ($P_{ET}CO_2$). Data were exported for analysis using either 5-second averages or breath-by-breath measurements for analysis alongside TCD data.

3.3. Measurement of Cerebrovascular Blood Flow Velocity by Transcranial Doppler Ultrasound

3.3.1. Detection and measurement of cerebral blood flow velocity

Beat-by-beat middle cerebral artery blood flow velocity (MCA_v) was assessed by transcranial Doppler ultrasound (TCD) (Multi Dop X, DWL, Compumedics Ltd, Singen, Germany), using a 2 MHz ultrasound probe placed over the temporal ultrasound window to measure MCA_v bilaterally. Probes were prepared with ultrasound gel and held in place using a fully adjustable headset (DiaMon®, DWL). The middle cerebral artery was insonated following defined search and identification procedures in accordance with established guidelines (Willie *et al.*, 2011). Measures were recorded at 1 kHz through an analog-to-digital converter (Powerlab, ADInstruments, Sydney, Australia) and presented in real-time and stored for future analysis using dedicated signal analysis software (LabChart Pro 7, ADInstruments).

3.3.2. TCD Data Processing and Analysis

While the specifics of signal analysis varied between studies, the general process used for the processing and analysis of TCD data was kept uniform across all chapters. Three different processing methods were taken dependent on the aims and design of each study. Firstly, averages of MCA_v were taken at key time points (ranging from 30 seconds to 2 minutes) for assessment of the gross changes in cerebral blood flow velocity. Area under the curve (AUC) was calculated using automated peak analysis within LabChart (ADInstruments) to calculate a measure of total displacement, by the summation of the area under each individual systolic peak, from a baseline of 0 $cm \cdot s^{-1}$. Finally, MCA_v data were exported at 10 Hz or as beat-to-beat data for the analysis

of haemodynamic responses and time course analysis. The specific processing methods and subsequent analysis of data exported by these methods is detailed within each chapter.

3.4. Exercise Testing and Interventions

Exercise testing was carried using a stationary cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Physical fitness was assessed by ramp-incremental VO_{2peak} test to determine peak aerobic capacity, prior to participants taking part in any of the exercise interventions. This was carried out due to a level of physical fitness being set as an inclusion criteria and exercise intensity for the interventions being derived from the VO_{2peak} test outcomes. Briefly, VO_{2peak} tests consisted of a warm-up, in which exercise intensity (watts; W) was increased every minute; for a minimum of 5-minutes, until participants rated their effort as 11 on a Rating of Perceived Exertion (RPE) Scale (Borg, 1982). Participants then cycled continuously at a self-selected cadence, whilst intensity was increased by 30 W every 2-minutes, until volitional fatigue. Continuous measures of respiratory gas exchange were collected during all VO_{2peak} tests, as described in methods 3.2.

Breath-by-breath measures of $\dot{V}O_2$ were analysed to determine VO_{2peak}; taken as the highest 30-second average during the test. Maximum power output (W_{max}) was determined by averaging the intensity across the final 2-minutes, prior to volitional fatigue. A heart rate monitor chest strap (Polar, Warwick, UK) was worn across the duration of the VO_{2peak} test and data collected using the Polar Beat app (Polar) to

collect continuous measures of heart rate (HR), with maximum heart rate (HR_{max}) defined as the highest 30-second average during the test.

Exercise interventions were carried out across a range of intensities and durations dependent on the specific purpose of each study and its research aims. All interventions were categorised using the same nomenclature in order to maintain consistency and to ease discussion of the results between studies, as previously described by Weston et al. (2014). Steady-state exercise interventions were defined as moderate intensity, continuous training (MICT), with a fixed workload maintained over a prolonged duration (>20-minutes). Interval-based exercise was divided into two categories, dependent on both the intensity and duration of the exercise carried out. Longer, submaximal intervals based on clinical exercise guidelines were defined as high-intensity interval training (HIIT), whilst short intervals of <1-minute and bouts at supramaximal intensity were defined as sprint-interval training (SIT). Regardless of the type of exercise conducted within a given intervention, all exercise was preceded by a 5-minute warm-up period, identical to that used during VO_{2max} tests, and ended with a 3-minute active recovery period, cycling under minimal resistance (50 W). Measures of respiratory gas exchange and middle cerebral blood flow velocity were collected across all exercise sessions, as described in methods 3.2 and 3.3.

3.5. Blood Sampling and Processing

Venous blood samples were drawn via venepuncture or cannulation of a vein in the forearm.

Whole blood for serum was collected into uncoated plastic serum collection tubes (BD 367837; BD Vacutainer, Plymouth, UK) and kept at room temperature (18–23ºC) for 40-minutes to allow for coagulation to occur, before centrifugation at 5,000 g for 10-minutes at 4ºC, aliquoted into microcentrifuge tubes and stored at -80ºC until analysis.

Whole blood for plasma isolation was collected into vacutainer tubes containing an anti-coagulant coating. Either 1.6 $mg \cdot mL^{-1}$ potassium ethylenediaminetetraacetic acid (K_2EDTA) or 15 $IU \cdot mL^{-1}$ lithium heparin (LH) (BD Vacutainer) was used as an anti-coagulant dependent on the molecular outcome markers within a given study and any potential interactions between these molecules and the anti-coagulant components. Plasma was isolated by centrifugation and stored as described above for serum isolation.

3.6. Isolation and Culture of Human Umbilical Vein Endothelial Cells

3.6.1. Isolation of primary human umbilical endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from women undergoing elective caesarean section, as previously described (Munir *et al.*, 2015). Briefly, the umbilical cord vein was cannulated and phosphate buffered saline (without calcium or magnesium; PBS; Sigma-Aldrich) pumped through the vessel to remove any remaining blood. PBS was then aspirated, and the vein filled with 1 $mg \cdot mL^{-1}$ collagenase type Ia (Cat no. C2674, Sigma-Aldrich) diluted in PBS and incubated at 37ºC and 5% CO_2 for 15-minutes. Cords were then massaged for 2-minutes and endothelial cell suspension removed by flushing with PBS, followed by air

and then centrifuged at 250 g for 6-minutes. The resulting cell pellet was then resuspended in M199 culture media consisting of Medium 199 supplemented with 20% foetal bovine serum (FBS), 1 $\mu g \cdot mL^{-1}$ hydrocortisone, 10 $ng \cdot mL^{-1}$ epidermal growth factor (all Sigma-Aldrich), 100 $U \cdot mL^{-1}$ Penicillin-Streptomycin (Gibco, ThermoFisher, Loughborough, UK), and 2.5 $\mu g \cdot mL^{-1}$ amphotericin B (ThermoFisher). Endothelial cells were then culture to confluence (approximately 5 days) in 25 cm^2 culture flasks (T25 flasks; BD Falcon). All experiments using primary HUVEC isolated from umbilical cords were conducted using HUVEC at first passage.

3.6.2. Culture of cryopreserved human umbilical vein endothelial cells

Cryopreserved HUVEC primary cells were purchased from PromoCell (PromoCell, Heidelberg, Germany) and cultured according to supplier guidelines. Briefly, cells were supplied on dry ice in cryovials and were stored in liquid nitrogen until required. Cryovials were thawed for 2-minutes at 37ºC in a water bath, before being transferred to 25 cm^2 culture flasks containing Endothelial Cell Growth Medium (ECGM, PromoCell), consisting of Basal Medium with 0.02 $mL \cdot mL^{-1}$ fetal calf serum, 0.004 $mL \cdot mL^{-1}$ Endothelial Cell Growth Supplement, 0.1 $ng \cdot mL^{-1}$ epidermal growth factor, 1 $ng \cdot mL^{-1}$ basic fibroblast growth factor, 90 $\mu g \cdot mL^{-1}$ heparin, and 1 $\mu g \cdot mL^{-1}$ hydrocortisone (all PromoCell). All PromoCell supplied HUVEC were at passage 1 (P1) after thawing.

3.6.3. Subculture and cryopreservation of endothelial cells

PromoCell-sourced HUVEC were subcultured through 3 passages from the initial 25 cm^2 culture flask to a total of nine 75 cm^2 flasks at P4. HUVEC were then dispersed

(see Methods 3.6.4) and the resultant cell pellet resuspended in 1 mL of CryoSFM freezing medium (PromoCell), before being counted using an automated cell counter (Cellometer Auto T4, Nexcelom Biosciences) and the concentration adjusted to $1x10^6$ cells·mL^{-1}. The cell suspension was then aliquoted at 1 mL into 1.5 mL cryovials (Nalgene®, Sigma Aldrich) and slowly cooled to -80ºC before being stored in liquid nitrogen. For use in experiments, cryovials were thawed as described in Methods 3.6.2 and cultured in 25 cm^2 culture flasks, before being seeded to their experimental surface and used at P6.

3.6.4. Dispersal of endothelial cells for seeding onto new surfaces

Endothelial cells were first rinsed with EDTA for 30-seconds, which was then aspirated and replaced with 2.5 mg·mL^{-1} trypsin in EDTA (2:1 ratio) and incubated at room temperature until cells dissociated from the flask surface. Phase-contrast microscopy was used to monitor cell morphology and detachment from the plastic surface, at which point the reaction was quenched by the addition of either M199 culture media (for self-isolated HUVEC) or Medium 199 containing 20% FBS (for PromoCell HUVEC). The cell suspension was then centrifuged at 400 g for 6-minutes, supernatant discarded, and the resultant cell pellet resuspended in HUVEC culture media.

3.6.5. Seeding of endothelial cells onto multi-well plates

A single 25 cm^2 flask of HUVEC were trypsinised (as in Methods 3.6.4) and made up to 10 mL in M199 culture media or ECGM, with 0.5mL added to each of 20 wells of a 24-well plate or proportional volume for alternate well formats (BD Falcon). Plates were then incubated at 37ºC and 5% CO_2 for 24-hours before experiments were conducted.

3.7. Treatment of HUVEC with serum *ex vivo*

3.7.1. Culture of HUVEC for treatment with ex vivo serum

Endothelial cells were seeded onto the desired culture surface and cultured for a minimum of 24-hours prior to each experiment (see Methods 3.6.5). Serum samples (see Methods 3.5) were thawed at room temperature and supplemented into HUVEC media at the desired concentration. Cells were washed with PBS, to remove any residual FBS, before application of serum-supplemented media and incubation at 37ºC and 5% CO_2.

3.7.2. Assessment of the acute impact of human serum concentration on HUVEC

To assess the impact on morphology, mRNA expression and protein response, endothelial cells were treated with serum taking from participants at rest at final concentrations 0, 5, 10, 15, 20, and 25% in HUVEC media and incubated at 37ºC and 5% CO_2 for 2, 5, 10, 20, and 60-minute durations. Images were taken at 0- and 60-minutes using phase contrast microscopy (Olympus IX70, Olympus, Southend-on-Sea, UK) and at all time points mRNA and protein samples were isolated (see Methods 3.8.1 and 3.8.2.1).

3.8. Biochemical Sample Analysis

3.8.1. Messenger Ribonucleic Acid Isolation and Quantitative Polymerase Chain Reaction

Messenger ribonucleic acid (mRNA) was isolated from endothelial cells using RNeasy Mini Kit (Qiagen, Manchester, UK), according to the manufacturer's instructions. Adherent cells were isolated by washing with PBS followed by application of 350 µL of RLT lysis buffer (Qiagen) and stored at -80ºC. Samples were then thawed and homogenised by transferring lysate into a QIAshredder spin column (Qiagen) in a 2 mL collection tube, and centrifuged for 2-minutes at ≥8,000 g. The supernatant was then combined with 350 µL of 70% ethanol (1:1 ratio) and mixed by pipetting, before being transferred to a RNeasy spin column in a 2 mL collection tube and spun for 1-minute at ≥8,000 g. The column was then washed once with 500 µL RW1 buffer and twice with 500 µL RPE buffer, being spun for 1-minute at ≥8,000 g after the addition of each wash volume of RW1 and RPE buffers. Any flow through in these wash steps was discarded. The column was then dry spun for 2-minutes at ≥8,000 g to remove any residual ethanol, if ethanol was still olfactorily detectable in the sample the dry spin was repeated. RNA elution was then carried out by the addition of 30 µL of RNase-free water (Qiagen) and centrifuged for 1-minute at ≥8,000 g, to maximise mRNA yield the eluted sample was pipetted back into the column and the final spin step repeated. RNA concentration and purity were then assessed using a NanoDrop 1000 spectrophotometer (ThermoFisher). RNA was considered "pure" when the ratio of absorbance between 260 nm and 280 nm was greater than 2.0 and the ratio between 260 nm and 230 nm was in the range of 2.0–2.2.

Isolated mRNA was then reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, ThermoFisher, UK), following the manufacturer's instructions. Briefly, samples were diluted in RNase-free water (Qiagen) to a final concentration of 200 $ng \cdot mL^{-1}$. A reverse transcription (RT) Master Mix was made containing 3.2 µL of RNase-free water (Qiagen), 2 µL RT buffer, 0.8µL dNTP, 2 µL of random primers, 1 µL of RNaseOUT ribonuclease inhibitor and 1 µL of Multiscribe™ Reverse Transcriptase (all Applied Biosystems), to make a total volume of 10 µL per mRNA sample. 10 µL of sample and 10 µL of Master Mix were then combined in sterile PCR tubes (StarLab, Milton Keynes, United Kingdom). RT-PCR was carried out by cycling through 25ºC for 10-minutes, 37ºC for 120-minutes, 85ºC for 5-minutes, and then held at 4ºC, using a Veriti™ 96-well thermal cycler (Applied Biosystems).

Quantitative PCR (qPCR) was carried out using TaqMan Gene Expressions Assays according to the manufacturer's instructions using 384-well PCR reaction plates (all Applied Biosystems). Each sample was run in duplicate, with each reaction composed of 0.5µL cDNA, 5.5 µL TaqMan Master Mix, 3.9 µL RNase-free water, and 0.6µL FAM-labelled TaqMan gene expression assay on demand primer-probe conjugates (all Applied Biosystems), for each gene primer analysed (Table 3.1). Samples were run in triplicate as a singleplex on a QuantStudio 5 qPCR System (Applied Biosystems) for 40-cycles and analysed using ThermoFisher Connect™ (ThermoFisher). Threshold cycle (C_T) for expression across triplicates were averaged for each sample, with replicates outside of 1 C_T being discarded. Mean C_T was subtracted from the C_T value for *18S* as a housekeeping gene (ΔC_T). The ΔC_T for each experimental condition was

then subtracted from the ΔC_T for the control condition, to produce $\Delta\Delta C_T$ values for each condition. Relative changes in gene expression (i.e. fold change) was calculated using the following formula (Livak & Schmittgen, 2001).

$$Fold\ Change = 2^{-\Delta\Delta C_T}$$

Table 3.1 Primers used for human gene expression analysis.

Gene ID	TaqMan Assay ID	Amplicon Length
18S	Hs99999901_s1	187
KLF2	Hs00360439_g1	116
CLEC14A	Hs00545534_s1	81
HIF1A	Hs00153153_m1	76
VEGFA	Hs00900055_m1	59
KDR (VEGFR2)	Hs00911700_m1	83
IGFBP3	Hs00181211_m1	78

3.8.2. Protein Analysis of Biological Samples

3.8.2.1. Isolation of Intracellular Proteins from Endothelial Cells and Western Blotting

Intracellular protein fraction was isolated from endothelial cells by direct lysis of adherent cells from their culture surface. Radioimmunoprecipitation assay (RIPA) buffer was prepared containing 150 mM sodium chloride, 1% NP-40 detergent, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 5 mM Tris (pH 8.0) (all ThermoFisher). Prior to use in protein isolation, RIPA buffer was supplemented with cOmplete™ EDTA-free protease inhibitor cocktail and PhosSTOP™ phosphatase inhibitor cocktail (both Roche, Basel, Switzerland), with one tablet of each dissolved in

10 mL of RIPA buffer (Subedi *et al.*, 2019). Protein lysate was isolated by washing cells with ice-cold PBS for 1-minute, followed by application of ice-cold, inhibitor-supplemented RIPA buffer at a volume of approximately 150 µL per 1.0 x 10^6 cells, and incubation for 30-minutes on ice, before collection and storage at -80ºC.

Total protein concentration in samples was assessed by detergent compatible protein assay (*DC* Protein Assay, Bio-Rad, Watford, UK), following the manufacturers protocol. Briefly, BSA standards were prepared across a working range of 0–2 $mg \cdot mL^{-1}$ (0.125, 0.25, 0.5, 1 & 2 $mg \cdot mL^{-1}$) by dilution of a 2 $mg \cdot mL^{-1}$ BSA stock (ThermoFisher) in RIPA Buffer. Prior to being assayed, all samples were diluted 1:10 in de-ionised H_2O to a total volume of 40 µL and *DC* Assay Reagent A-S (Bio-Rad) were prepared fresh at a 50:1 ratio. Five microlitres of each standard or sample was then pipetted in triplicate into wells of a 96-well microplate, followed by 25 µL of A-S reagent mix and 200µL of *DC* Assay Reagent B (Bio-Rad). The microplate was then covered incubated for 5-minutes with agitation and 15-minutes rested at room temperature, after which absorbance was read at 630 nm using a microplate reader. Blank-corrected protein standard values were then used to produce a standard curve, from which the protein concentration of each sample was then calculated. Samples were then aliquoted and diluted to a standardised concentration of 1 $\mu g \cdot \mu L^{-1}$ and diluted at a ratio of 4:1 in Laemli Sample Buffer (Bio-Rad) containing 10% B-mercaptoethanol and denatured by heating at heated at 99ºC for 5-minutes, before being stored at -20ºC,

Changes in protein of interest were then assessed by Western Blot. To account for variations in loading, running, transfer and detection between gels, a single quality control sample (pooled lysate from untreated HUVEC) was run on all gels (Bass *et al.*, 2017). Samples were thawed, vortexed and 20 µL of the sample was loaded into Critereon™ TGX™ (Tris-Glycine eXtended) 4–20% precast gels (Bio-Rad) and run in Running Buffer containing 25 mM Tris Base, 190 mM glycine and 0.1% SDS in deionised H_2O (all Sigma Aldrich). Gels were run at a constant voltage of 140 V and amperage of 23 mA per gel until the dye front reached the bottom of the gel. Gels were then transferred to 0.45 µm pore PVDF membranes (Amersham™ Hybond®, Sigma Aldrich) using a transfer buffer containing 25 mM Tris Base and 190 mM glycine in deionised H_2O containing 20% methanol (all Sigma Aldrich). Membranes were layered into a blotting chamber, with the membrane layered in top of the protein gel, coupled either side with a layer of filter paper and a blotting pad. Proteins were transferred at 100 V and 300 mA for 60-minutes.

Membranes were then stained with Ponceau S (Sigma Aldrich) to check for successful transfer and imaged using an automated membrane imaging system (G:Box-Chemi-XRQ, Syngene, Cambridge, UK) then washed with deionised water before antibody staining. Each membrane was blocked in 0.1% Tween 20 tris-buffered saline (TBST, Pierce™, ThermoFisher) containing 3% fat free milk for 60-minutes at room temperature on a shaker and subsequently washed with TBST, following by 5-minutes in TBST on a shaker. If needed membranes were cut at this point to allow antibody staining of multiple proteins of different molecular weights. Primary antibodies for proteins of interest were diluted to the desired concentration (Table 3.2) in TBST

containing 3% BSA and incubated with the membrane overnight at 4ºC with constant agitation. Following overnight incubation, membranes were washed three times in TBST, with each wash cycle including a 5-minute soak period in TBST with continuous shaking. HRP-conjugated secondary antibodies, specific to the primary antibody species (Table 3.2) , were diluted to the required concentration in TBST containing 3% fat free milk and incubated with the membrane for 60-minutes at room temperature, before being washed in TBST as per primary antibody washing. The membrane was then developed using ECL Plus Substrate (Pierce™, ThermoFisher), according to the manufacturers guidelines and imaged using a digital imager, using automated exposure settings (Syngene).

Table 3.2 Antibodies used for relative protein quantification by Western Blot.

Antigen	Host & Isotype	Clonality	Clone No.	Concentration	Supplier
Primary Antibodies					
eNOS	Goat IgG	Polyclonal	N/A	0.5 $\mu g \cdot mL^{-1}$	R&D Systems
Phospho-eNOS (Ser1177)	Rabbit IgG	Monoclonal	C9C3	1:1000	Cell Signaling Technology®
VEGFR2	Goat IgG	Polyclonal	N/A	1.0 $\mu g \cdot mL^{-1}$	R&D Systems
Phospho-VEGFR2 (Y1214)	Rabbit IgG	Monoclonal	19A10	1:1000	Cell Signaling Technology®
Phospho-VEGFR2 (Y1214)	Rabbit IgG	Polyclonal	N/A	0.5 $\mu g \cdot mL^{-1}$	R&D Systems
Secondary Antibodies (HRP-conjugated)					
Anti-Rabbit IgG	Goat IgG	Polyclonal	N/A	1:10000	Cell Signaling Technology®
Anti-Goat IgG	Rabbit IgG	Polyclonal	N/A	1:10000	Invitrogen

Images were exported as .TIFF files and signal intensity was determined in 8-bit format in imageJ (Schneider *et al.*, 2012) by the quantification of the relative density of each band. Prior to analysis, signal values were normalised to the quality control sample, before being corrected to the signal intensity of the Ponceau S band at 40 kDa as a loading control (see equation below). Relative quantification of protein was then calculated by the subtraction of the corrected signal for each experimental condition from the corrected signal of the control condition.

$$Gel\ normalisation\ factor = \frac{N^{th}\ membrane\ control\ signal}{1^{st}\ membrane\ control\ signal}$$

$$Normalised\ signal = Gel\ normalisation\ factor\ \times\ Lane\ signal$$

$$Corrected\ signal = \frac{Normalised\ signal\ of\ interest}{Normalised\ Ponceau\ S\ signal}$$

3.8.3. Extracellular Secretome Analysis by Enzyme-Linked Immunosorbent Assay

Extracellular proteins were detected in plasma samples using DuoSet enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions (R&D Systems Inc, Abingdon, UK). ELISA microplates were prepared within 24-hours of utilisation and incubated with capture antibody overnight at room temperature, as per the manufacturers guidelines (R&D Systems). All wash steps were carried out using an automated plate washer (Wellwash™ Microplate Washer, Thermo Scientific, ThermoFisher), to dispense 3 volumes of 400 µL of wash buffer (0.05% Tween 20 in PBS; R&D Systems). The reagent diluent consisted of PBS containing either 5% BSA (Sigma Aldrich) or 5% Tween 20 (R&D Systems), dependent on the potential for cross-

reactivity between the protein of interest and bovine proteins within the BSA (see Table 3.3).

Optical density of the plate was determined at 450nm, with wavelength correction at 540 nm to correct for imperfections in the plate (FLUOstar Omega, BMG Labtech Ltd, Aylesbury, UK). Data was then processed by the averaging of duplicates for all samples and standards, followed by subtraction of the average of the zero standard. Four parameter logistic curve fitting was then applied using AssayFitPro (Assay Cloud, Nijmegen, Netherlands), to produce a standard curve from which the protein concentration within each sample could be calculated. Samples concentrations which fell outside the assay range (see Table 3.3) were re-analysed on a second ELISA plate, with samples which read above the higher limit of the range being diluted in accordance with manufacturer recommendations (R&D Systems).

Table 3.3 Assay range and reagent diluent used in DuoSet ELISA's.

Target Protein	Assay Range ($pg \cdot mL^{-1}$)		Reagent Diluent
	Lower Limit	Upper Limit	
VEGF	31.2	2000	BSA
BDNF	23.4	1500	BSA
IGF-1	93.8	6000	Tween 20

3.8.4. Photometric Assessment of Lactate

Aliquoted plasma was analysed for lactate content using an automated photometric-based clinical chemistry analyser (RX Daytona+, Randox, London, UK), according to the manufacturer's guidelines, using purpose made assay kits, standards and calibrations (all Randox). Briefly, a calibration curve was established automatically using Calibration Serum Level 3 (Randox) and quantification of lactate was determined enzymatically, by the conversion of lactate to pyruvate and subsequent photometric quantification of pyruvate production (Cat. LC 3980, Randox). Calibration settings were automatically validated against two known concentrations of lactate in serum (Assayed Multisera Level 2 and Level 3, Randox), prior to the quantification of lactate in duplicate within collected samples. The assay range for the assessment of lactate using this method is 0.1–19.7 $mmol \cdot L^{-1}$, if lactate concentration within a sample exceeded the upper limit of this range, then the sample was diluted and rerun, using the automated detection and dilution function within the analyser (RX Daytona+, Randox).

3.9. Statistical Analysis

Data are presented as mean and either standard deviation (SD) or confidence intervals (CI: [lower limit, upper limit]) throughout and an alpha value of 0.05 was used to determine significance of all statistical analyses, unless explicitly stated as otherwise. All data were analysed using R (R Core Team, 2019) and graphed in GraphPad Prism (Prism Version 8, GraphPad Software, San Diego, USA), Matlab (Matlab Version R2019b, Mathworks, Natick, USA), or R (R Core Team, 2019). For a full list of the packages utilised in analysis and plotting of data in R see Appendix 3.

Assumptions of normality were assessed by examination of skewness and kurtosis statistics; graphical interpretation of density-distribution and quantile-quantile plots; and analysis of Shapiro-Wilk's Test of normality. If skewness values were ≥0.5 in either the positive or negative direction, non-normally distributed data were transformed by logarithmic, square-root or inverted transformation based on the level and severity of skewness statistics (Tabachnick & Fidell, 2001). When appropriate, equal variance between groups was assessed by Levene's test for equal variance. If normality and/or equal variance assumptions were not met, then non-parametric statistics were applied for data analysis. When appropriate, outlying data points were identified and removed automatically in GraphPad Prism, using the ROUT method (Motulsky & Brown, 2006).

Normally distributed data were assessed using a range of parametric statistics. Mean differences at baseline and single factor repeated measures were analysed by either t-test; for comparisons with two levels, or one-way analysis of variance (ANOVA); for comparisons with more than 2 levels. Sphericity was assessed by Mauchly's Test and if significant Greenhouse-Geisser corrections were utilised for ANOVA results. Comparison of outcome measures with multiple factors or within datasets containing missing values was carried out by linear mixed effects models (LMEM), the specific design of each model was dependent on the study design, factors of interest and outcome measures used. In general, treatment or intervention and time variables were included as fixed factors, with participant as a random factor. Additional factors were assessed using Akaike's Information Critera (AIC) to determine inclusion and weighting of model errors (Symonds & Moussalli, 2011). Post hoc testing for

interactions within ANOVA and LMEM were carried out with pairwise comparison and Tukey Test adjustments for multiple comparisons.

Data found to be not normally distributed were assessed by non-parametric statistical analysis. One-way comparisons with 2 groups were carried out using the Mann-Whitney U test, using either Kruskal-Wallis one-way ANOVA (between groups) or Friedman Test (within groups) for one-way comparison when more than 2 groups were included. Multiple factor analysis of non-normally distributed data was carried out using rank-based non-parametric analysis as outlined by Noguchi et al. (2012), which allows for the comparison of up to three factors. Post hoc tests for interaction and were carried out with Bonferroni corrections for multiple comparisons.

The relationship between outcome measures was assessed by correlation using either Pearson's correlation coefficients (parametric analysis) or Spearman's correlations (non-parametric). The relationship between outcome measures and independent variables was investigated by either linear or multiple regression, with statistical analysis completed using the same methods as used for correlations.

4. CEREBRAL HAEMODYNAMIC AND NEUROTROPHIC FACTOR RESPONSES ARE DEPENDENT ON THE TYPE OF EXERCISE

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Weaver SR, Skinner BD, Furlong R, Lucas RAI, Cable NT, Rendeiro C, McGettrick HM & Lucas SJE (2021). Cerebral Hemodynamic and Neurotrophic Factor Responses Are Dependent on the Type of Exercise. *Front Physiol* **11,** 1–14.

The published form of this chapter is attached as an appendix for reference (Appendix 4).

4.1. Introduction

Physical activity is unique in its ability to positively impact human health and promote wellbeing across the lifespan (Pedersen & Saltin, 2015; O'Donovan *et al.*, 2017; Kraus *et al.*, 2019). These benefits are clearly seen within the brain, with higher physical fitness positively correlated with brain health and a reduction in risk of and progression rate across a number of neurological diseases (Kramer *et al.*, 2006; Bailey *et al.*, 2013; Burley *et al.*, 2016).

Traditional guidelines, typified by moderate intensity, continuous efforts of 30-minutes, elicit beneficial changes in resting cerebral blood flow, cognitive function, cardiovascular health and all-cause mortality (Garber *et al.*, 2011; Cai *et al.*, 2017; Alfini *et al.*, 2019). However, poor adherence to traditional exercise strategies, often characterized by a lack of time (Costello *et al.*, 2011), has resulted in increased interest in high intensity, interval-based strategies, which have shown similar health and fitness benefits compared to moderate intensity steady-state exercise (Stork *et al.*, 2017; Gibala & Little, 2020). Despite this, studies focusing on the cerebrovascular responses to these strategies are limited in number. While current evidence indicates comparable middle cerebral artery velocity (MCA_v) responses between continuous and interval-based moderate intensity exercise (Klein *et al.*, 2019), MCA_v has been shown to be suppressed over the course of a single 30-second all-out sprint bout, followed by a marked elevation in MCA_v during recovery (Curtelin *et al.*, 2018; Labrecque *et al.*, 2020). This reduction in response to high intensity interval-based strategies is in contrast to responses within the peripheral vasculature (Ramos *et al.*, 2015); likely driven by the unique response of the cerebrovasculature to exercise-induced hyperventilatory hypocapnia (Hellström *et al.*, 1996), as well as differences in

the regulatory responses to elevated systemic flow and blood pressure (Brassard *et al.*, 2017; Ogoh *et al.*, 2018; Calverley *et al.*, 2020). Regardless of the mechanisms driving these differences, it is essential that we understand how choice of exercise strategy can alter haemodynamic responses, as acute elevations in blood flow are likely pivotal in determining the adaptive benefits of longer training periods (Green *et al.*, 2017). Understanding this relationship is particularly important as research increasingly supports short (30 – 60-second) sprint interval training (SIT) protocols at supramaximal intensities, rather longer (~ 4-minute) high intensity efforts performed in guideline-based high intensity interval training (HIIT) (Weston *et al.*, 2014). While the current literature demonstrates the complex relationship between SIT and measures of cerebral haemodynamics (Curtelin *et al.*, 2018; Labrecque *et al.*, 2020), it is not clear whether these responses change over multiple bouts as is typical in training interventions, nor how SIT- and HIIT-based strategies may differ in their response.

Exercise also has the capacity to alter the profile of circulating signaling factors, including neurotrophic factors with the potential to act within the brain (Voss *et al.*, 2013*b*). Amongst these, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) are particularly relevant due to their ability to elicit a comprehensive profile of responses within the cerebrovasculature, blood brain barrier and in brain plasticity (Voss *et al.*, 2013*b*; Basso & Suzuki, 2017). Increases in all three of these factors have been demonstrated under a single exercise bout, with responses appearing to be intensity-dependent (Wahl *et al.*, 2010, 2014; Jeon & Ha, 2017; Reycraft *et al.*, 2020).

While the origin and brain-specific changes in many of these factors is not fully understood, particularly in relation to BDNF (Rasmussen *et al.*, 2009; Klein *et al.*, 2011), the impact that exercise can have on these factors is increasingly clear. In both animal models and humans, increases in BDNF have been associated with improvements in brain function, acute post-exercise cognitive function and subsequent adaptive benefits within the brain (Voss *et al.*, 2013*a*; Basso & Suzuki, 2017; Kujach *et al.*, 2020). Similarly, although acute changes in IGF-1 do not appear to correlate with changes in cognition (Tsai *et al.*, 2014), more chronically it has been suggested that IGF-1 is central to healthy cognitive ageing, due to its role in regulating metabolism and insulin resistance in the brain (Lewitt & Boyd, 2019). Further, IGF-1 signaling is essential to the maturation of BDNF and so may play a secondary role in stimulating it's release (Carro *et al.*, 2000). VEGF is most widely studied in terms of vascular endothelial regulation, stimulating angiogenic signal pathways and vascular adaptation to alterations in flow (Olsson *et al.*, 2006). A number of animal studies have also demonstrated its role within the brain, promoting angiogenesis within the cerebrovasculature (Morland *et al.*, 2017; Geiseler & Morland, 2018) and exhibiting neuroprotective effects when upregulated (Jin *et al.*, 2002; Góra-Kupilas & Jośko, 2005).

The evolving role of lactate as a signaling molecule raises interesting prospects within the brain, particularly considering the exponential increases seen at higher intensities (Hughson *et al.*, 1987). Increases in lactate alone have the capacity to significantly increase BDNF (Schiffer *et al.*, 2011) and have been associated with exercise-induced changes in circulating BDNF, IGF-1 and VEGF (Kujach *et al.*, 2020). Furthermore, lactate has been shown to modulate brain signaling pathways, crossing the blood brain

barrier and binding to hydroxycarboxylic acid receptor 1 (HCAR1) receptors that are highly expressed within the brain (Lauritzen *et al.*, 2014), driving responses relating to cerebral angiogenesis and stimulating cerebral VEGF expression (Morland *et al.*, 2017).

Given the potential impact of exercise intensity and protocol on cerebrovascular function, this study aimed to examine the MCA_v response to moderate intensity continuous exercise (MICT), guideline-based high intensity interval exercise (HIIT) and sprint interval exercise (SIT). Secondly, we aimed to explore how these exercise protocols may differentially alter the acute profile of key neurotrophic markers, which have previously been linked to exercise-induced changes in brain health. It was hypothesized that the pattern of middle cerebral artery velocity (MCA_v) response would differ between interval and continuous exercise, with SIT inducing the smallest increase from rest. In addition, we hypothesized that increases in circulating neurotrophic factors would be intensity dependent, with SIT inducing the greatest response.

4.2. Methods

4.2.1. Participants

Twenty-four regularly active participants were recruited for this study (see Table 4.1 for participant characteristics). All participants were healthy, with no history of cardiovascular, cerebrovascular or respiratory disease, and were not taking any medication (with the exception of oral contraception in female participants). Visits were carried out within an hour of the same time of day for each participant, with a minimum of 48 hours between sessions. The study was approved by the University of

Birmingham Ethics Committee (ERN-17_1570) and all participants gave written, informed consent prior to enrolling in the study, in adherence with the Declaration of Helsinki.

4.2.2. Experimental Protocol

Following enrolment participants attended the laboratory on four separate occasions to carry out: 1) a ramp-increment aerobic capacity test ($\dot{V}O_{2peak}$ test); 2) thirty minutes of moderate-intensity steady-state exercise (MICT); 3) four, 4-minute high intensity intervals (HIIT) based on a clinical intervention model (Weston *et al.*, 2014); and 4) four, 30-second all-out sprints (SIT) (Figure 4.1). Participants were asked to refrain from vigorous exercise or consuming alcohol for 24-hours and from consuming caffeine within 12-hours of each visit. In addition, participants were asked to record any food consumed on each visit day and to refrain from consuming food within 2-hours of testing beginning, with the same foods being consumed prior to subsequent sessions.

Table 4.1 Baseline characteristics and resting values for participants taking part in three different exercise protocols.

Baseline Characteristics (n = 24; 9	**Mean**	**SD**
Age (years)	23	5
Body Mass (kg)	73.0	11.1
$\dot{V}O_{2peak}$ (mL·min^{-1})	3185	544
$\dot{V}O_{2peak}$ (mL·kg^{-1}·min^{-1})	43.9	6.4
Resting Values		
MICT		
MCA_v (cm·s^{-1})	72.0	13.6
$P_{ET}CO_2$ (mmHg)	33.8	3.6
HR (beats·min^{-1})	68	10
BDNF (pg·mL^{-1})	112.5	75.8
IGF-1 (ng·mL^{-1})	1960	1350
VEGF (pg·mL^{-1})	456.5	980.6
Lactate (mM)	1.1	0.2
HIIT		
MCA_v (cm·s^{-1})	70.4	11.4
$P_{ET}CO_2$ (mmHg)	33.6	3.6
HR (beats·min^{-1})	67	12
BDNF (pg·mL^{-1})	134.0	91.6
IGF-1 (ng·mL^{-1})	1905	1160
VEGF (pg·mL^{-1})	404.9	770.8
Lactate (mM)	1.1	0.3
SIT		
MCA_v (cm·s^{-1})	70.5	11.8
$P_{ET}CO_2$ (mmHg)	33.4	3.2
HR (beats·min^{-1})	71	10
BDNF (pg·mL^{-1})	134.8	104.5
IGF-1 (ng·mL^{-1})	2101	1444
VEGF (pg·mL^{-1})	369.2	740.1
Lactate (mM)	1.3	0.9

Abbreviations: SD = standard deviation; MICT = moderate intensity continuous training; HIIT = high intensity interval training; SIT = sprint interval training; MCA_v = middle cerebral artery velocity; $P_{ET}CO_2$ = partial pressure of end-tidal carbon dioxide; HR = heart rate; BDNF = brain-derived neurotrophic factor; IGF-1 = insulin-like growth factor 1; VEGF = vascular endothelial growth factor.

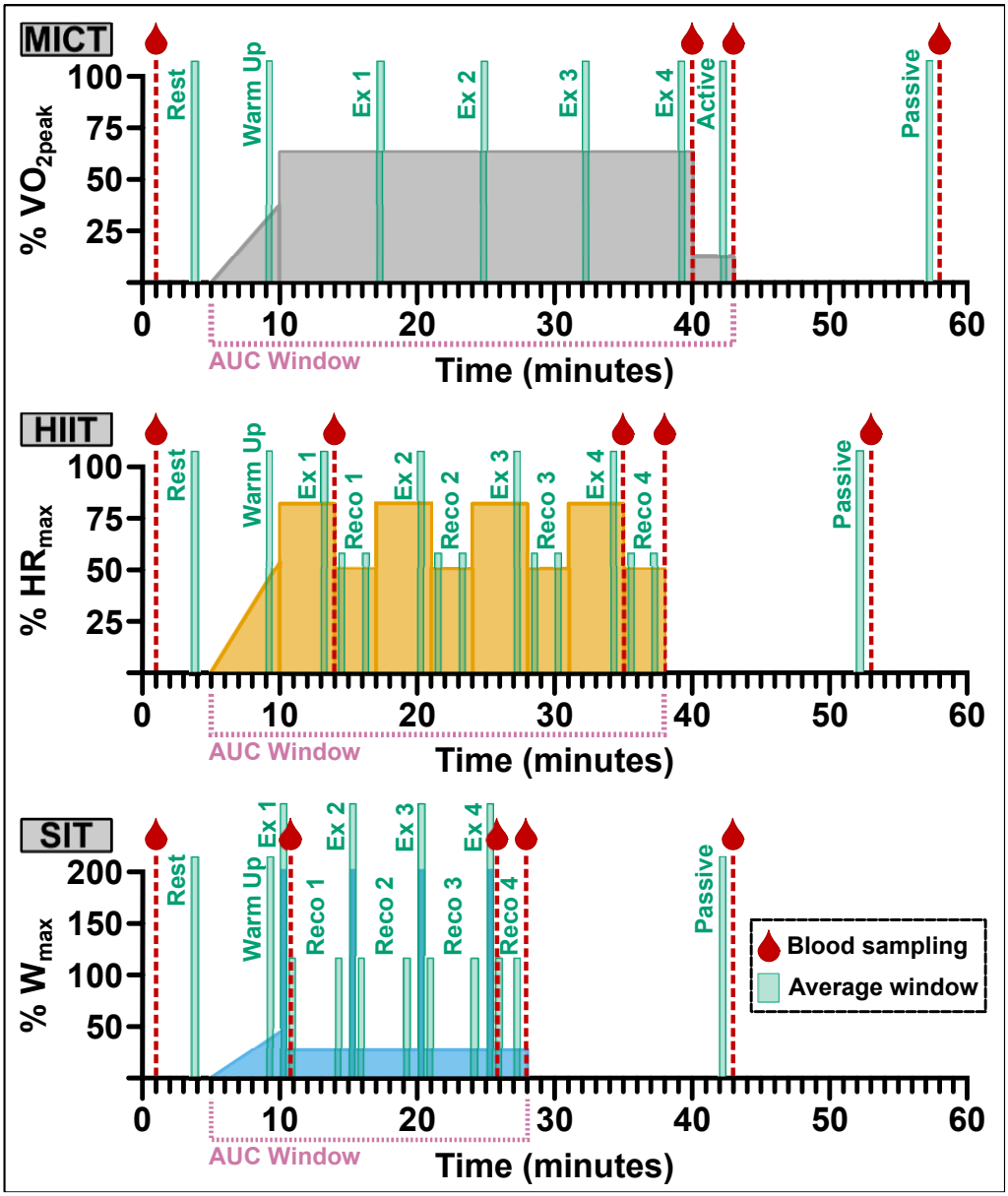


Figure 4.1 Study schematic showing exercise protocol and measurement time points for three exercise visits.

Participants took part in three separate exercise visits in a randomized order, consisting of 30 min of moderate, steady-state exercise at 65% $\dot{V}O_{2peak}$ (MICT); four 4 min high intensity intervals at 85% HR_{max} (HIIT); and four 30 s supramaximal sprint intervals at 200% W_{max} (SIT). All protocols were preceded by 5 min of seated rest and a 5 min warm-up, and followed by 3 min of active recovery at 50 W and 15 min of seated passive recovery. The timing of blood sampling (red droplet), data averaging windows, and labels for analysis (green boxes) and the period used in area under the curve (AUC) analysis (pink bracket) are included for all protocols. Averaging was carried out over 30-second windows across the course of each exercise protocol (Ex) and during the initial and final minute of recovery (Reco) following each HIIT and SIT bout. The final 30-second recovery window in both HIIT and SIT was taken as the active recovery timepoint, in conjunction with the active recovery window (Active) in MICT. VO2peak, maximum oxygen consumption; HRmax, maximum heart rate; Wmax, maximum aerobic power.

4.2.3. Maximal oxygen consumption test (VO_{2peak})

VO_{2peak} was assessed using a ramp-incremental exercise protocol on a cycle ergometer (Excalibur Sport, Lode, Netherlands). This test consisted of a 5-minute warm up until the participant indicated a rate of perceived exertion (RPE) of 11 on a Borg Visual Scale (Borg, 1982), followed by a 3-minute ramp protocol in which resistance was increased by 30 W every 3 minutes until volitional fatigue was reached, or the participant was not able to maintain a consistent cadence above 50 RPM. Oxygen consumption was continuously measured (Vyntus CPX, Vyaire Medical, UK) and $\dot{V}O_{2peak}$ was defined as the highest 30-second average achieved during the test. Continuous measures of heart rate (HR) (Polar H9 HR Sensor, Polar, Finland) were collected for determination of HR_{max}. Maximal power output (W_{max}) was determined as the power output achieved during the final stage of the VO_{2peak} test.

4.2.4. Experimental visits

All three experimental visits were carried out in a randomized, cross-over fashion, with the order of experimental visits for all participants determined *a priori* using customized R script (R Core Team, 2019). On arrival an intravenous catheter (20G, BD Venflon, UK) was placed in the antecubital vein of the forearm to enable blood sample collection during exercise, participants then rested in a supine position for a minimum of 10 minutes before resting blood samples were collected. Participants were instrumented for cerebrovascular and cardiorespiratory measures (detailed below) and sat quietly for 5 minutes while resting measures of MCA_v, HR and end-tidal carbon dioxide ($P_{ET}CO_2$) were collected. All exercise protocols were carried out at a self-selected cadence, included a 5-minute warm up identical to that used during $\dot{V}O_{2peak}$ testing, and ended with 3 minutes of active recovery at 50 W and a seated 15-minute passive

recovery period (Figure 4.1). Verbal encouragement was given throughout all exercise protocols and participants were given information about elapsed time at regular intervals.

4.2.4.1. MICT protocol

During MICT, participants carried out 30 minutes of continuous exercise at 65% of $\dot{V}O_{2peak}$. Resistance on the cycle ergometer was initially set between 55–60% of W_{max} and adjusted once cycling had commenced in order to attain 65% of $\dot{V}O_{2peak}$. Oxygen consumption was continuously monitored across the duration of the 30-minute period to ensure this intensity was maintained and cycling resistance adjusted as required.

4.2.4.2. HIIT protocol

HIIT consisted of four, 4-minute intervals at 85% HR_{max}, separated by 3 minutes of active recovery at 50 W, based on guideline recommendations and definitions of HIIT in clinical settings (Weston *et al.*, 2014). Initial workload during interval bout 1 was set at ~70% W_{max}, monitored and adjusted to achieve 85% HR_{max} in a similar manner to MICT workload. For remaining bouts, the initial intensity was set at the power output during the final 30 seconds of the preceding 4-minute bout and adjusted as necessary to maintain intensity at 85% HR_{max}.

4.2.4.3. SIT protocol

SIT intervals consisted of four, 30-second intervals at 200% of W_{max}, separated by 4.5 minutes of active recovery at 50 W; with the exception of the final recovery which was matched to MICT and HIIT at 3 minutes at 50 W. Following warm-up, the workload was reduced to 50 W for 30 seconds in order to ensure that the change in workload

from pre-interval to sprint was matched across all four intervals. Workload was ramped up 5 seconds prior to each sprint interval to account for a ~5–7-second delay in resistance on the cycle ergometer and intensity was dropped to 50 W as soon as the 30-second bout ended.

4.2.5. Measurements

4.2.5.1. Cerebral blood velocity

MCA_v was assessed bilaterally using a 2-MHz transcranial Doppler (TCD) ultrasound system (Multi Dop X, DWL, Compumedics Ltd., Germany). Ultrasound probes were attached to a fully adjustable headset (DiaMon, DWL) to allow for continuous measurement during exercise. The MCA was located via the temporal window using standardized search techniques, with depth, gain and filter settings matched within participants across visits (Willie *et al.*, 2011). Resting mean velocity values for right and left MCA_v were compared during rest to maximize comparability between sessions and data were visually inspected for signal drop-out or interference during analysis windows. Alongside TCD setting uniformity, these factors were used to determine consistency, with the most consistent MCA signal (left/right) across all three sessions used for data analysis.

4.2.5.2. Respiratory gases and ventilation rate

Breath-by-breath measures of respiratory gases were measured by collection of expired air through a facemask (Hans Rudolph, USA) and analysed continuously via a metabolic cart (Vyntus CPX, Vyaire Medical). Measures of minute ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}O_2$) and $P_{ET}CO_2$ were exported for later analysis.

4.2.5.3. Data Acquisition

MCA_v and HR data were recorded through an analogue-to-digital converter (Powerlab, ADInstruments, New Zealand) and stored for further analysis using dedicated signal analysis software (LabChart Pro v7, ADInstruments). $P_{ET}CO_2$ data were aligned with other signal data and processed alongside MCA_v to match time course and data averaging periods between the two.

4.2.5.4. Blood sampling and analysis

Blood samples were drawn from the antecubital vein and collected into two 6-mL vacutainers for the isolation of plasma, one containing K_2EDTA and one lithium heparin (LH) as an anticoagulant (both BD Vacutainer, UK). Samples were collected at rest, at the end of each exercise protocol, end of active recovery, end of passive recovery and in the case of HIIT and SIT an additional aliquot was taken following the initial interval bout (Figure 4.1). Samples were immediately centrifuged at 5,000 g, for 10 minutes at 4ºC; carefully aliquoted to avoid disruption of buffy coat and frozen at -80ºC for future analysis. All analyses were conducted using plasma isolated with lithium heparin as an anticoagulant, with the exception of VEGF, which was analysed using plasma isolated in K_2EDTA.

Plasma levels of VEGF, IGF-1 and BDNF were analysed by DuoSet Enzyme-Linked Immunosorbent Assay (ELISA) according to manufacturer's instructions (#DY293B, #DY291, #DY248 respectively, all R&D Systems, UK), with each sample run in duplicate on a single plate. Sample concentration was determined using standard curves produced by 4-parameter logistic curve-fitting and automated concentration analysis (AssayFit Pro, Assay Cloud, Netherlands). In the eventuality that all samples

from a single visit showed no detectable protein-of-interest, samples were re-run, using a new sample aliquot on a second plate alongside samples that reported reproducible levels of the given protein-of-interest. All sample aliquots used within these analyses were subject to a single freeze-thaw cycle.

Circulating levels of blood lactate were analysed using an automated photometric-based chemical analyzer (RX+ Daytone, Randox, UK), using purpose made assay kits, standards and reagents, according to the manufacturer's guidelines (Randox).

4.2.6. Data Processing and Statistical Analysis

4.2.6.1. Data Processing

Power output data was collected across all three experimental visits and averaged over 5-minute periods across the duration of MICT and over the duration of each bout of HIIT and SIT. Additionally, average power output was calculated over the complete duration of each protocol.

Haemodynamic and respiratory data were averaged over the penultimate 30 seconds of rest, warm-up, active and passive recovery. During the exercise efforts, data were averaged over 30 seconds at four time points (7.5-minute intervals) during MICT, over the penultimate 30 seconds of HIIT bouts, and across the full 30-second duration of SIT bouts. In addition, 30-second averages were obtained in the initial and final minute of recovery following each HIIT and SIT bout (RECO 1–4; see Figure 4.1).

MCA_v data were also exported at 1 Hz continuously from start of warm-up to end of active recovery to plot time-course data for the duration of each study visit, alongside

quantification of area-under-curve (AUC) for the same time-course, using Pulse Analysis software within Labchart (ADInstruments). AUC was calculated as total blood velocity ($cm \cdot s^{-1}$) over the total time period (s) and so is presented as a measure of displacement ($cm \cdot s^{-1} \cdot s = cm$).

4.2.6.2. Statistical Analysis

Assumptions of normality were assessed by graphical interpretation of density distribution and Q-Q plots, as well as by analysis of skewness, kurtosis and Shapiro-Wilk's Test statistics. Resting baseline data and power output data were compared by one-way analysis of variance (ANOVA), if sphericity assumptions were not met under Mauchly's Test then Greenhouse-Geisser corrections were utilized for ANOVA results.

Comparison of repeated-measures outcome data was carried out using linear mixed effects models (LMEM), with time and protocol included as fixed factors and participant ID as a random factor. Inclusion of sex and fitness ($\dot{V}O_{2peak}$) as factors was assessed using Akaike's Information Criteria (AIC) to determine inclusion and weighting of model errors for each outcome variable (Symonds & Moussalli, 2011). Post hoc testing for interactions within ANOVA and LMEM were carried out with pairwise comparison and Tukey Test adjustments for multiple comparison. All results are presented as mean ± standard deviation (mean±SD) unless otherwise stated.

4.3. Results

4.3.1. Exercise intensity during acute exercise

Mean power output across the duration of MICT was 148 ± 28 W and did not differ significantly across the duration of the protocol ($p > 0.05$). During HIIT, average power output was 198±45 W and remained consistent across bouts 1–3 (201 ± 44 W; 201 ± 46 W; 198 ± 46 W, respectively), although a significant decline was seen in bout 4 compared to the other 3 bouts (194 ± 47 W, $p \leq 0.02$). Due to the design of the SIT condition, average power output was matched across all four bouts (503 ± 103 W). Average power output was significantly lower in MICT than in either other condition ($p < 0.001$), and significantly greater in SIT than in HIIT ($p < 0.001$).

Oxygen consumption was significantly elevated from resting baseline across all three protocols ($p < 0.001$, Table 4.2). During MICT, $\dot{V}O_2$ remained relatively stable across the course of 30-minutes, although a small significant increase in the third time point (2063 ± 367 $mL \cdot min^{-1}$, $p \leq 0.03$) compared with time points one and two (1975 ± 354 $mL \cdot min^{-1}$ and 2006 ± 384 $mL \cdot min^{-1}$, respectively). Oxygen consumption during both HIIT and SIT was significantly greater than MICT at all exercising time points ($p \leq 0.046$, Table 4.2). During HIIT, $\dot{V}O_2$ was significantly lower in bout 1 (2327 ± 574 $mL \cdot min^{-1}$) than either bout 2 (2447 ± 511 $mL \cdot min^{-1}$, $p = 0.04$) or bout 3 (2483 ± 511 $mL \cdot min^{-1}$, $p = 0.007$). Similarly, SIT bout 1 (2291 ± 392 $mL \cdot min^{-1}$) was significantly lower than bout 2 (2512 ± 470 $mL \cdot min^{-1}$, $p \leq 0.001$) and bout 4 (2473 ± 502 $mL \cdot min^{-1}$, $p < 0.03$).

Table 4.2 Measures of oxygen consumption, minute ventilation and heart rate at rest and across the duration of three different exercise protocols.

	Rest	Ex 1	Ex 2	Ex 3	Ex 4
MICT					
$\dot{V}O_2$ ($mL \cdot min^{-1}$)	293 ± 112	1975 ± 354*$†	2006 ± 384*$†	2063 ± 367*$†	2023 ± 383*$†
$\dot{V}_E$ ($L \cdot min^{-1}$)	9.4 ± 3.1	55.1 ± 10.9*$†	56.1 ± 12.2*$†	57.7 ± 11.9*$†	57.4 ± 13.6*$†
HR ($beat \cdot min^{-1}$)	68 ± 10	144 ± 14*$†	147 ± 13*$†	150 ± 13*$†	151 ± 14*$†
HIIT					
$\dot{V}O_2$ ($mL \cdot min^{-1}$)	287 ± 69	2327 ± 574*	2447 ± 511*	2484 ± 511*	2396 ± 631*
$\dot{V}_E$ ($L \cdot min^{-1}$)	9.5 ± 2.1	70.0 ± 21.2*	76.9 ± 20.1*Ω	78.8 ± 21.3*Ω	77.9 ± 22.9*Ω
HR ($beat \cdot min^{-1}$)	67 ± 12	159 ± 10*	167 ± 10*	170 ± 12*	172 ± 11*
SIT					
$\dot{V}O_2$ ($mL \cdot min^{-1}$)	316 ± 69	2291 ± 392*	2512 ± 470*	2448 ± 529*	2473 ± 502*
$\dot{V}_E$ ($L \cdot min^{-1}$)	10.7 ± 2.4	83.3 ± 20.3*	101.3 ± 27.6*	106.0 ± 26.4*	109.8 ± 28.8*
HR ($beat \cdot min^{-1}$)	71 ± 10	164 ± 14*	169 ± 15*	171 ± 13*	171 ± 11*

Abbreviations: SD = standard deviation; MICT = moderate intensity continuous training; HIIT = high intensity interval training; SIT = sprint interval training; $\dot{V}O_2$ = oxygen consumption; $\dot{V}_E$ = minute ventilation; HR = heart rate. Significance (analysed by linear mixed model, $p<0.05$) between resting values and subsequent time points is denoted by ✱. Significant differences between protocols are denoted by **$** between MICT and HIIT; **†** between MICT and SIT; and **Ω** between HIIT and SIT.

4.3.2. Haemodynamics responses to acute exercise interventions

Baseline characteristics and resting values for MCA_v, $P_{ET}CO_2$ and HR during seated rest are displayed in Table 1. No significant differences were seen between any of these measures at rest ($p > 0.05$). Within-participant variability (coefficient of variability) among intervention visits was 5.5% for MCA_v, 4.8% for $P_{ET}CO_2$ and 7.2% for HR.

Assessment of model fit validated the inclusion of sex in linear modelling of MCA_v, $P_{ET}CO_2$ and HR. While significant interactions were seen between time and protocol across all outcome measures and data sets, inclusion of sex as a fixed factor did not reveal any significant main effect or interaction between sex and the time-by-protocol interaction effect. For this reason, all further analysis and interpretation of these data were carried out including male and female participants as a complete cohort.

Significant increases in MCA_v from rest during warm up ($p < 0.001$) were comparable across all exercise protocols (MICT: $\Delta 16.7 \pm 10.1$ cm·s^{-1}, HIIT: $\Delta 15.5 \pm 12.3$ cm·s^{-1}, SIT: $\Delta 15.3 \pm 9.3$ cm·s^{-1}, $p > 0.05$ between protocols). During MICT a sustained and uniform elevation in MCA_v was seen across all time points (mean across time points: 85.1 ± 16.2 cm·s^{-1}), with no significant changes across the 30-minute time course (Figure 4.2). While all HIIT bouts showed a sustained, significant elevation in MCA_v from rest (19–21%, $p < 0.001$), bout 1 showed significantly greater elevation in comparison to both HIIT bout 2 ($\Delta 6.3 \pm 6.4$ cm·s^{-1}, $p = 0.043$) and time point one during MICT ($\Delta 4.8 \pm 9.0$ cm·s^{-1}, $p = 0.047$). End-tidal CO_2 showed similar patterns in response across both MICT and HIIT, with significant elevations from baseline that were sustained across warm up and all exercising time points in both protocols (Figure

4.2). HIIT bout 1 also showed significantly greater increases in $P_{ET}CO_2$ than all other HIIT bouts (≥2.6 mmHg, $p < 0.001$), whilst a small significant difference was seen between bouts 3 and 4 and the comparable timepoints during MICT (Δ1.4 ± 2.8 mmHg and Δ1.8 ± 2.9 mmHg respectively, $p \leq 0.04$).

Compared to resting values, there was a significant elevation in MCA_v following the first 30-second SIT bout (Δ17.3 ± 11.6 cm·s^{-1}, $p < 0.001$), accompanied by a significant increase in $P_{ET}CO_2$ at the same time point (Δ4.5 ± 4.8 mmHg, $p < 0.001$). These changes were comparable to those seen during MICT and HIIT in terms of MCA_v (both $p > 0.05$), however, $P_{ET}CO_2$ was significantly lower in SIT than in both MICT (Δ2.3 ± 3.9 mmHg, $p = 0.004$) and HIIT (Δ3.3 ± 3.0 mmHg, $p < 0.001$). During subsequent SIT bouts, significant reductions in MCA_v were seen in comparison to bout 1 (≥11.7 cm·s^{-1}, $p < 0.001$; Figure 4.2), with no further elevations above baseline across bouts 2 – 4 ($p > 0.05$). Significant reductions in $P_{ET}CO_2$ were also seen with repeated bouts, resulting in a significant suppression in $P_{ET}CO_2$ below resting baseline from bout 2 onwards ($p \leq 0.004$; Figure 4.2). During recovery from sprint intervals, a pattern of elevated MCA_v and $P_{ET}CO_2$ was seen within the first minute of recovery following each SIT bout, which was not seen in the same window of time during HIIT (Figure 4.3). This “rebound” was only found to be significant in comparison to the related sprint bout following bout 1 (Bout vs. Recovery = Δ10.0 ± 7.9 cm·s^{-1}, $p < 0.001$), although smaller significant elevations above baseline were seen across all subsequent rebounds (9–14% above rest, $p \leq 0.04$). All four bouts showed significant elevations in $P_{ET}CO_2$ during the rebound phase both in comparison to the preceding sprint bout and to baseline measures (≥5.6 mmHg, $p<0.001$).

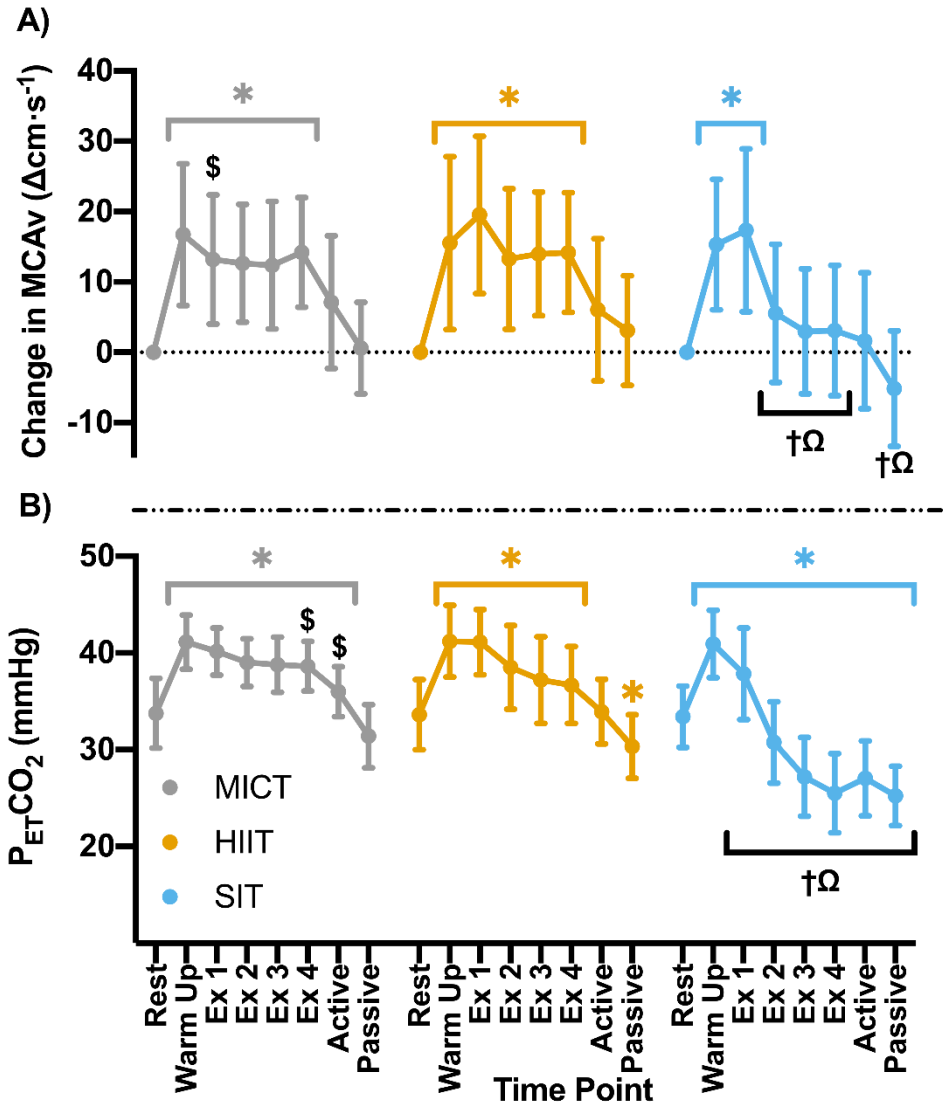


Figure 4.2 MCA_v and $P_{ET}CO_2$ responses across three exercise protocols.
(A) MCA_v was determined continuously by transcranial Doppler ultrasound (TCD) and presented as change in MCA_v from rest in each protocol for each participant. **(B)** $P_{ET}CO_2$ was determined through breath-by-breath respiratory gas exchange and ventilation. Data were averaged over 30 s of rest (rest); 5 min warm up (warm up); 3 min active recovery (active); and 15 min seated passive recovery (passive) in all protocols. Exercising averages (Ex 1–4) were taken every 7 min during 30 min of moderate intensity (65% VO2peak) steady state exercise (MICT); in the final minute of the four 4 min high intensity (85% HRmax) interval bouts (HIIT) and across the duration of four 30 s supramaximal (200% Wmax) sprint intervals (SIT). Data are presented as mean ± SD (n = 24; except SIT–Passive: n = 23). Significant differences (analysed by linear mixed models, $p < 0.05$) between resting values and subsequent time points are denoted by ✱. Significant differences between protocols are denoted by **$** between MICT and HIIT; **†** between MICT and SIT; and **Ω** between HIIT and SIT.

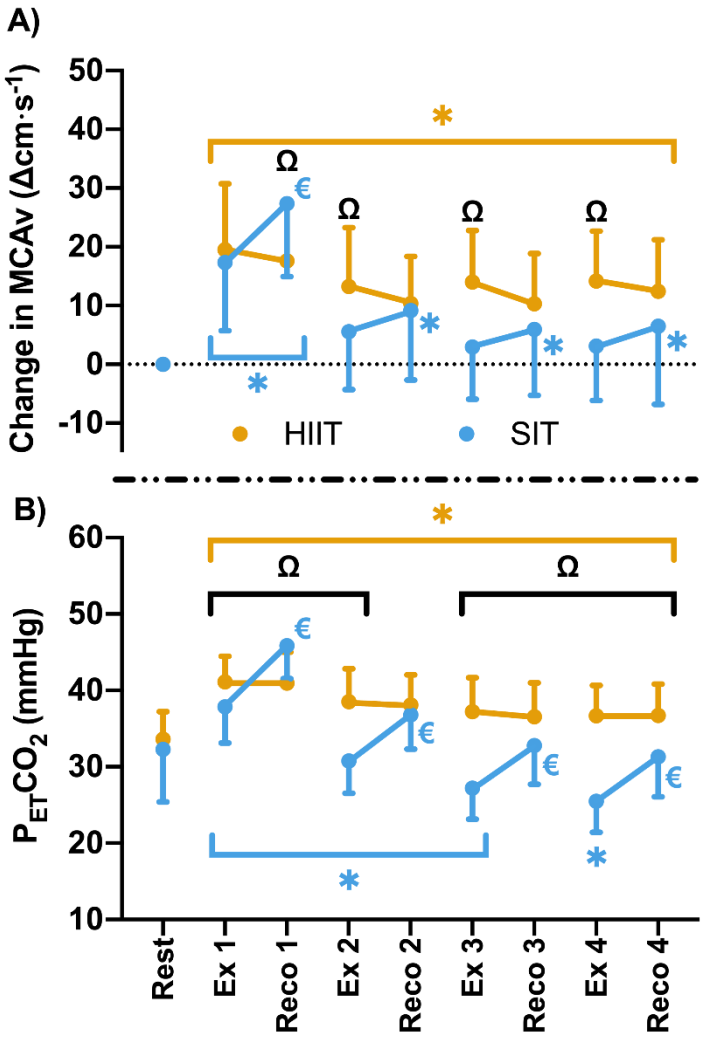


Figure 4.3 MCA_v and $P_{ET}CO_2$ responses during and immediately following bouts of HIIT and SIT.
(A) MCAv was determined continuously across all exercise sessions by transcranial Doppler ultrasound (TCD) and presented as change in MCA_v from rest in each session for each participant. **(B)** $P_{ET}CO_2$ was determined by measurement of breath-by-breath respiratory gas exchange and ventilation. Data were averaged for 1 min during seated rest (Rest); over 30 s during the final minute of four 4 min high intensity (85% HR_{max}) interval bouts (HIIT; Ex 1–4); across the duration of four 30 s supramaximal (200% W_{max}) sprint intervals (SIT; Ex 1–4); and over a 30 s period, 15 s into the recovery following each bout in both interval protocols (Reco 1–4). Data are presented as mean ± SD (n = 24). Significance (analysed by linear mixed model, $p < 0.05$) between time points is denoted by ✱ for differences from resting values; **€** for differences between interval (Ex) and recovery (Reco). Significant differences between protocols are denoted by **Ω**.

Analysis of $\dot{V}_E$ measures showed an intensity-dependent pattern across all three protocols, mirroring changes seen in $P_{ET}CO_2$. Specifically, significant elevations were seen in all three protocols ($p \leq 0.001$, Table 4.2), but with lower elevations in MICT than either other protocol at all time points ($p \leq 0.01$), and significantly greater $\dot{V}_E$ in SIT than in HIIT from bout 2 onwards ($p \leq 0.002$). While V_E remained relatively stable across MICT, significant differences were seen between bout 1 of HIIT (70.0 ± 21.2 $L \cdot min^{-1}$) and all subsequent HIIT bouts (minimum 76.9 ± 20.1 $L \cdot min^{-1}$, $p \leq 0.001$). Similarly, bout 1 of SIT (83.3 ± 20.2 $L \cdot min^{-1}$) was lower than all subsequent bouts (minimum 101.3 ± 27.6 $L \cdot min^{-1}$, $p \leq 0.001$), and bout 2 was lower than 4 (101.3 ± 27.6 $L \cdot min^{-1}$ and 109.8 ± 28.7 $L \cdot min^{-1}$; respectively, $p = 0.03$).

Area under the curve analysis of blood displacement across all sessions was carried out in eighteen participants, four participants were not included in this analysis due to temporary signal drop out during one or more sessions making the analysis of area under the curve across the full session unreliable. Displacement over the course of MICT was significantly higher than both HIIT ($1.66x10^5 \pm 3.12x10^4$ cm vs. $1.36x10^5 \pm 2.23x10^4$ cm, $p < 0.001$) and SIT ($8.14x10^4 \pm 1.46x10^4$ cm, $p < 0.001$) exercise protocols. Figure 4.4 shows the mean MCAv across the period used in AUC analysis, in comparison to resting values, demonstrating the difference in average response between the three exercise protocols and the unique pattern of response between sprint bouts and immediate recovery period.

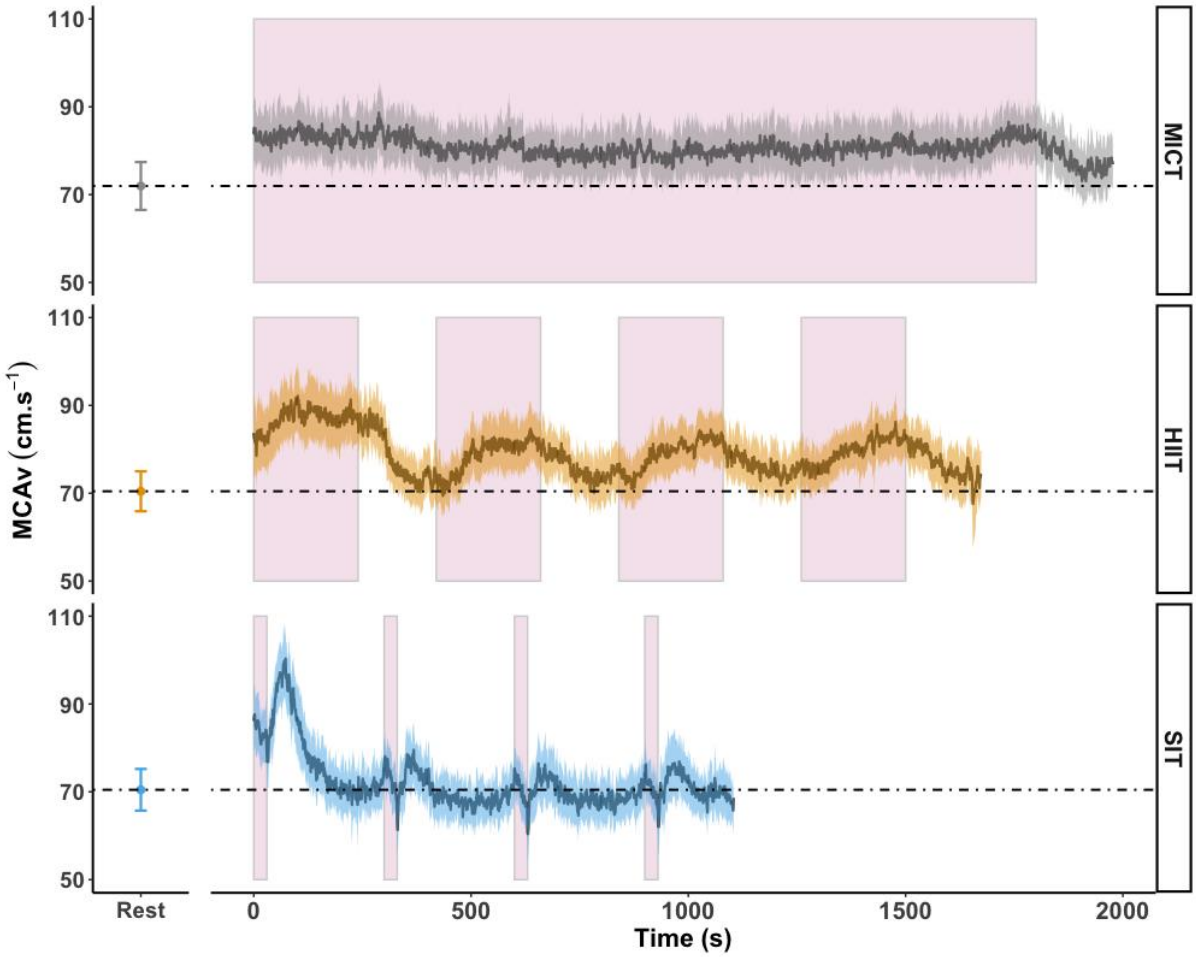


Figure 4.4 Mean MCA_v responses over the duration of three exercise protocols. MCA_v was determined continuously by transcranial Doppler ultrasound (TCD) and exported at 1 Hz frequency from the onset of each protocol after warm-up had been completed. Exercise protocols were: 30 min of moderate intensity (65% $\dot{V}O_{2peak}$) steady state exercise (MICT); four 4 min high intensity (85% HR_{max}) interval bouts (HIIT) separated by 3 min active recovery at 50 W and four 30 s supramaximal (200% W_{max}) sprint intervals (SIT) separated by 4.5 min active recovery at 50 W, with all protocols followed by 3 min of active recovery at 50 W. Data are presented as average ± 95% confidence intervals (n = 18), across the duration of each protocol including active recovery periods, with resting values averaged over 30 s of seated rest presented for comparison. These time periods were identical to those used in calculation of AUC. Continuous effort during MICT and the timing of the four interval periods in HIIT and SIT are highlighted in pink.

Heart rate data showed a clear pattern that was dependent on the intensity of the exercise stimulus within each of the protocols. Significant increases were seen from rest to the end of warm up across all exercise protocols (all $p < 0.001$, Table 4.2), whilst the elevation in heart rate was significantly higher during the warm up in SIT than in MICT (126 ± 15 vs. 118 ± 12 beat·min^{-1}, $p = 0.003$), but not in comparison to HIIT (121 ± 12 beat·min^{-1}, $p = 0.057$). All three protocols saw a significant increase in HR from warm up to exercise (all $p < 0.001$), with significantly lower HR in MICT across all time points than both HIIT (minimum difference of Δ16 ± 12 beats·min^{-1}, $p < 0.001$) and SIT (minimum Δ21 ± 14 beat·min^{-1}, $p < 0.001$). Within each protocol, no change was seen across time points in MICT or SIT, however, HIIT bout 1 exhibited significantly lower HR (159 ± 10 beats·min^{-1}) than in the subsequent bouts (bout 2: 167 ± 10 beats·min^{-1}; bout 3: 170 ± 12 beats·min^{-1}; bout 4: 172 ± 11 beats·min^{-1}, all $p < 0.04$ from bout 1).

4.3.3. Protocol-dependent changes in circulating factors

Blood samples were drawn from all 24 participants across all time points, with the exception of the final time point in MICT for 1 participant (cannula blockage). Of these, samples fell outside the limit of detection for the given test in 3 participants for BDNF (n = 21); 7 participants for IGF-1 (n = 17); 9 participants for VEGF (n = 15); and none for lactate (n = 24). Sample loss post collection for 1 participant for the SIT protocol resulted in a reduction in sample number for the analysis of VEGF (SIT: n = 14) and blood lactate (SIT: n = 23). No significant differences were seen in baseline measures for any of the blood markers ($p > 0.05$, Table 4.1). Model validation resulted in the inclusion of sex as a fixed factor in linear mixed modelling for BDNF alone, whilst

analyses of data for IGF-1, VEGF and blood lactate were carried out using base models alone.

Within the full cohort significant changes in BDNF were seen in SIT alone, with a significant elevation from rest at all later time points, peaking at the end of exercise (Δ149 ± 162% from rest, Figure 4.5) and remaining significantly elevated through to the end of passive recovery (all $p \leq 0.040$). While baseline measures of BDNF did not differ significantly between male and female participants, significant differences were seen between sexes in both MICT and SIT exercise protocols (Figure 4.6). Females exhibited significantly greater increases in BDNF compared to males during active ($p = 0.021$) and passive recovery ($p < 0.001$) in MICT and from the end of exercise onwards in SIT ($p < 0.001$). Marked differences in the response to each protocol were also seen between sexes. Within the female subgroup, increases in BDNF from rest were seen following active recovery ($p = 0.052$) reaching significance at the end of passive recovery ($p = 0.028$) during MICT; and significant elevations were also seen from the end of exercise onwards peaking at active recovery ($p \leq 0.002$) during SIT. Whereas within the male cohort changes in BDNF levels were only significant between rest and end of exercise in the SIT protocol ($p = 0.005$).

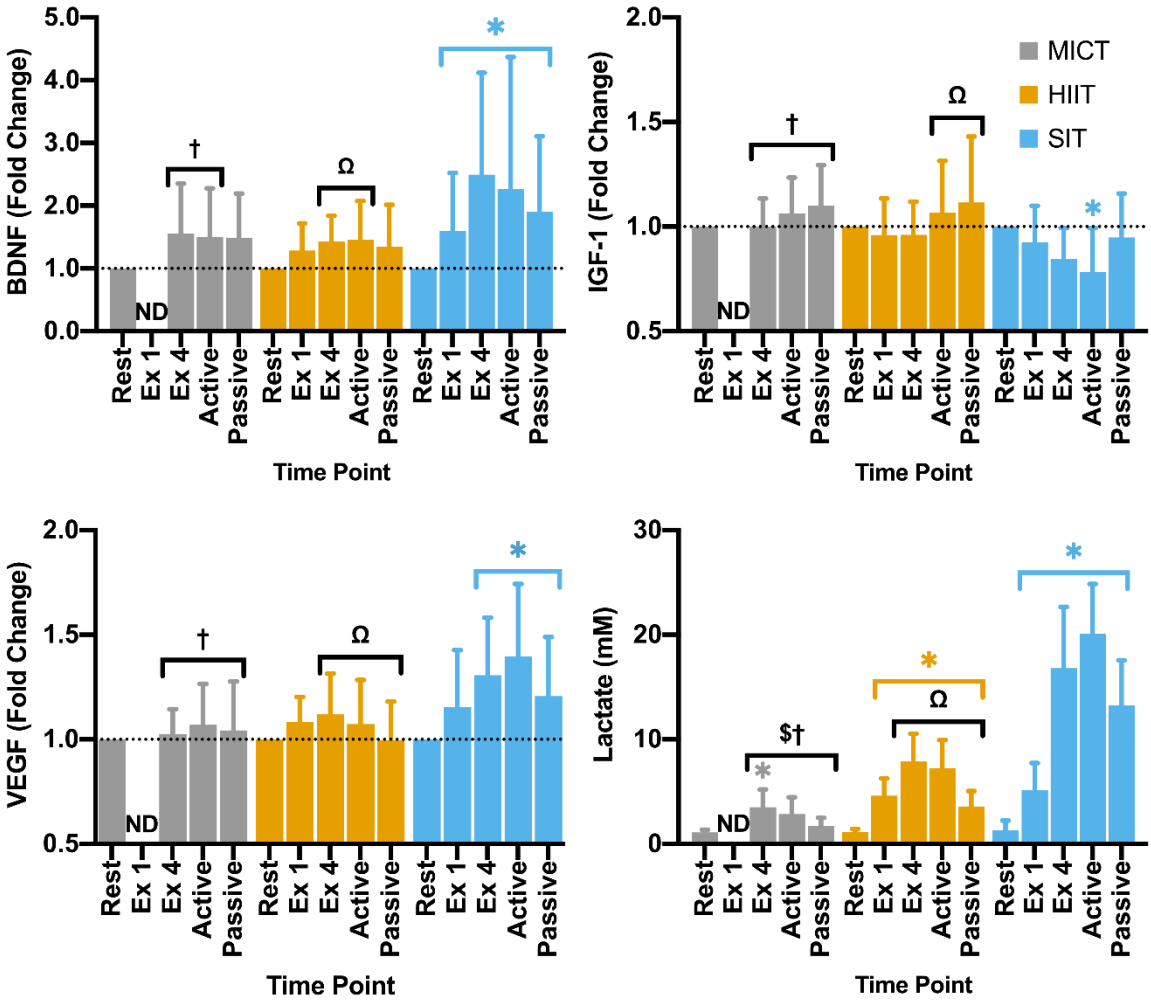


Figure 4.5 BDNF, IGF-1, VEGF, and lactate responses to three exercise protocols.
Blood plasma samples were taken during seated rest (Rest); immediately after the first interval exercise bout (Ex 1; HIIT and SIT only); at the end of exercise (Ex 4), end of active recovery (Active); and the end of passive recovery (Passive). Brain-derived neurotrophic factor (BDNF; n = 21), insulin-like growth factor 1 (IGF-1; n = 17), and vascular endothelial growth factor [VEGF; n = 15 (14 in SIT)] were analysed by ELISA. Blood lactate [n = 24 (23 in SIT)] was analysed using automated photometric analysis. Data are presented as mean ± SD. Significance (analysed by linear mixed model, $p < 0.05$) between resting values and subsequent time points is denoted by ✱. Significant differences between protocols are denoted by **$** between MICT and HIIT; **†** between MICT and SIT; and **Ω** between HIIT and SIT. **ND**, not determined.

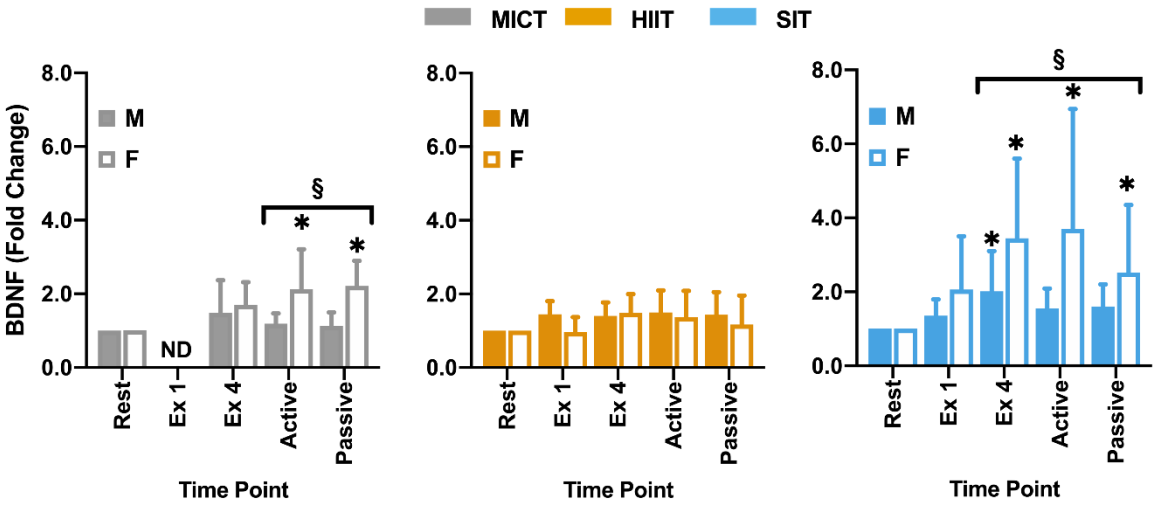


Figure 4.6 Comparison of BDNF responses between sexes across three exercise protocols.
Blood plasma samples were taken were taken during seated rest (Rest); immediately after the first interval exercise bout (Ex 1; HIIT and SIT only); at the end of exercise (Ex 4); end of active recovery (Active) and end of passive recovery (Passive). Brain-derived neurotrophic factor (BDNF; n = 21, 7 female) was analysed by ELISA. Data are presented as mean ± SD. Significance (analysed by linear mixed model, $p < 0.05$) between resting values and subsequent time points is denoted by ✱. Significant differences between sexes at a given time point are denoted by **§**. **M**, Male; **F**, Female; **ND**, not determined.

Changes in IGF-1 across the duration of visits were only found to be significant in the SIT protocol, with a pattern of reduction across the exercise period reaching significance at the end of active recovery ($p = 0.002$, Figure 4.5). While not significantly elevated from baseline values, changes in IGF-1 for both MICT and HIIT were relatively higher than SIT at the end of active ($p < 0.001$) and passive recovery (MICT vs SIT; $p = 0.023$ and HIIT vs SIT; $p = 0.011$), and at the end of exercise for MICT compared to SIT ($p = 0.018$).

Significant changes in VEGF from baseline were only observed for the SIT protocol, with significant elevations in VEGF seen from the end of exercise onwards (>23%; $p \leq 0.015$, Figure 4.5). Specifically, a significant difference in VEGF was seen between the immediate impact of a single SIT bout and further elevations in VEGF at the end of exercise ($p = 0.017$) and active recovery ($p < 0.001$). At all three of these time points, fold-change in VEGF was significantly greater than that of either MICT ($p \geq 0.036$) or HIIT ($p \geq 0.026$).

All three protocols increased blood lactate levels, but the magnitude of these changes varied between protocols. Specifically, while a significant elevation was seen in MICT at the end of exercise alone ($p = 0.005$), changes in HIIT and SIT were significant at all time points during and following the exercise stimulus (Figure 4.5). Further, whilst similar increases in lactate from rest were seen following the first bout of both HIIT ($\Delta 3.47 \pm 1.47$ mM, $p < 0.001$) and SIT ($\Delta 3.84 \pm 2.97$ mM, $p < 0.001$), significant divergence between these exercise protocols was seen after this time point. Elevations in blood lactate after the final bout of SIT were more than 2-fold greater than those seen in HIIT ($\Delta 15.51 \pm 6.14$ mM vs. $\Delta 6.95 \pm 2.35$ mM, $p < 0.001$), with

lactate peaking from rest in SIT at the end of active recovery (Δ18.81 ± 4.91 mM, $p < 0.001$) before declining during passive recovery in both the HIIT and SIT protocols (Figure 4.5).

4.4. Discussion

The primary purpose of this study was to compare how differences in exercise strategy may alter acute cerebrovascular responses and impact key circulating signaling factors with neurotrophic potential. Our main findings show that: a) significant elevations in MCA_v during MICT were matched by similar elevations in cerebral blood velocity for each bout of HIIT; b) SIT elicited a unique response in MCA_v, with only the initial bout inducing significant elevations in blood velocity from rest and a rebound in MCA_v occurring immediately following each SIT bout; and c) SIT alone drove significant changes in circulating levels of all investigated markers (BDNF, IGF-1, VEGF and blood lactate). These data demonstrate that both the cerebrovascular and circulating signal factor responses to exercise are highly dependent on the protocol that is being undertaken. As a result, it is essential that we consider the relative contribution that haemodynamic and circulating factor responses have on the brain and its vasculature to fully understand the efficacy of different exercise strategies.

4.4.1. Haemodynamic responses to acute exercise protocols

Our findings demonstrate that both MICT and HIIT exercise protocols elicited a similar magnitude of increase in cerebral blood velocity, although the pattern, duration and total blood displacement over these two protocols varied (as demonstrated in Figure 4.4). The importance of the total shear stimulus in comparison to peak changes has been demonstrated over short time periods (up to 60-seconds) in brachial artery flow

mediated dilatory (FMD) responses (Pyke & Tschakovsky, 2007), but to the best of our knowledge has not been investigated over longer time periods such as that represented here. While Hoiland et al. (2017) demonstrated the capacity for transient periods of hypercapnia (30-seconds) to induce an FMD response within the cerebrovasculature, a recent comparison of brachial and internal carotid artery FMD showed that the magnitude and pattern of dilatory responses was distinctly different between the two vessels (Carr *et al.*, 2020) and as such the impact of peak and total shear stimulus within the cerebrovasculature may be distinct from that seen within the periphery. Therefore, whether exercise-induced observations, both acute and chronic, based on FMD responses in the periphery translate to FMD responses in the cerebrovasculature remain to be determined. This distinction is particularly pertinent in relation to the SIT protocol, as total blood displacement was the lowest overall (as reflected by AUC), yet changes over the first sprint interval and immediate post-bout were among the largest changes at any time point across all three protocols (see Figure 4.4).

The similarity in the acute responses between MICT and HIIT within this study have clear clinical ramifications, as these data indicate that an interval-based approach has the potential to elicit comparable beneficial changes in the cerebrovasculature. Additionally, the slight differences seen in the initial bout of HIIT are likely linked to the lower heart rate within this bout, resulting from an under estimation of the required exercise intensity to elicit 85% HR_{max}, suggesting that a greater response may be seen at a lower intensity. This adds to a growing body of evidence supporting the utilization of interval-based exercise to target both general and vascular health outcomes (Weston *et al.*, 2014; Pedersen & Saltin, 2015), and complements the recent findings

of Klein et al. (2019) who demonstrated comparable responses to 10 minutes of work-matched continuous compared to intermittent exercise. This is an important step in increasing our understanding of how differences in the exercise protocol may affect cerebrovascular function (Calverley *et al.*, 2020). Future work is essential to understand the impact that acute differences in exercise protocol response may have on changes in cerebrovascular function over a training period, particularly given the potential for acute responses to act as markers of possible adaptive benefits (Dawson *et al.*, 2018; Voss *et al.*, 2020).

As expected, suppression of exercise-induced increases in MCA_v across the SIT protocol matched the overall lowering of $P_{ET}CO_2$, consistent with the hyperventilatory hypocapnia associated with high intensity exercise (Ogoh & Ainslie, 2010) and the central role of changes in arterial CO_2 in regulating cerebral blood flow (*reviewed in* Smith & Ainslie, 2017). Interestingly, the rebound in MCA_v immediately following each SIT bout also matched changes in $P_{ET}CO_2$ (Figure 4.3), while the greatest increases in both MCA_v and $P_{ET}CO_2$ were observed within the first bout. Thus, MCA_v responses during each bout and across all bouts appear to be strongly influenced by the changes in $P_{ET}CO_2$. While not investigated within the current study, oxidative-nitrosative stress has previously been linked to reductions in brachial artery endothelium-dependent vasodilation (Goto *et al.*, 2003) and impaired dynamic cerebral autoregulation (Bailey *et al.*, 2011) following high intensity exercise, and as such may also play some role in the MCA_v response we observed with the SIT protocol. However, further research is needed to determine the oxidative-nitrosative stress response to interval-based high intensity exercise and the role that this plays within the cerebrovasculature.

Labrecque et al. (2020) previously demonstrated similar rebound response with a single 30-second sprint bout and suggested that delays in the cerebral autoregulation may also contribute to this MCA_v rebound, which given the large elevations in blood pressure with SIT exercise seems likely. Unfortunately, we were not able to accurately determine blood pressure during sprint intervals at the intensity utilized within the current study. Interestingly, rebound responses were not observed with the longer intervals used in the HIIT protocol. Therefore, future research is needed to fully understand the potential for differences in both intensity and duration of the interval to maximize the potential benefits of sprint-interval exercise, including with reliable measuring of blood pressure during such sprint efforts.

4.4.2. Sprint-based intervals elicit unique patterns in neurotrophic and signaling factor response

Contrasting the changes seen in MCA_v, SIT alone induced significant changes in all assessed neurotrophic factors. Given the potential interaction between changes in these factors and exercise-induced improvements in cognitive function (Lista & Sorrentino, 2010; Skriver *et al.*, 2014), these findings raise the possibility that exercise protocol choice may alter the profile of exercise-related benefits in brain health. Whilst a clear response was seen in blood lactate across all protocols, those seen in SIT were greater than in either the MICT or HIIT exercise protocols, and this may have widespread benefits as lactate serves as a signaling molecule (Nalbandian & Takeda, 2016; Morland *et al.*, 2017). Additionally, elevations in lactate have been shown to drive subsequent elevations in circulating VEGF (Sameer Kumar *et al.*, 2007) and BDNF (Schiffer *et al.*, 2011), and as such may be key to changes in VEGF and BDNF being seen primarily within the SIT protocol alone.

The potential for lactate to stimulate VEGF release, alongside intensity-dependent upregulation within the skeletal muscle (Hoier & Hellsten, 2014), may help to explain the significant elevations in SIT alone. While the lack of VEGF modulation by either MICT or HIIT adds to previous literature showing contrasting findings across a range of exercise protocols and cohorts, likely due to the influence of confounding factors (Kraus *et al.*, 2004; Skriver *et al.*, 2014; Basso & Suzuki, 2017). While it was beyond the scope of this study to determine the influence of all confounding factors, both sex and fitness were taken into consideration for model fitting and neither resulted in improved model fit. Based on previous research, low basal levels of VEGF in combination with high interindividual variability may explain why a number of samples were found to fall below the limit of detection during analysis (Jelkmann, 2001). Despite this reduction in sample size, significant increases were seen with SIT, indicating that SIT may induce a more consistent response in VEGF compared to lower intensity exercise. These findings add to a growing body of evidence that high intensity, interval-based conditions, can have a significantly greater impact on transient responses in circulating VEGF (Wahl *et al.*, 2011; Kujach *et al.*, 2020).

Significant decreases in IGF-1 during SIT, alongside a pattern of suppression in HIIT, add to inconsistent responses in IGF-1 seen in previous research in which studies have demonstrate both a lack of response (Wahl *et al.*, 2010) and significant elevation in IGF-1 with sprint-based exercise (Kujach *et al.*, 2020). Interestingly, within the current study IGF-1 does appear to be rising during the recovery period of both interval-based protocols. Previous research has demonstrated dichotomous results in measures of IGF-1 taken during recovery, with those showing increases during

recovery finding peak changes within an hour of exercise (Kraemer *et al.*, 2017). The relationship between IGF-1 and its binding proteins in regulating signaling (Kraemer *et al.*, 2017); alongside the capacity for peripheral IGF-1 to be regulated by and transported into the brain in animal models (Fernandez & Torres-Alemán, 2012; Vecchio *et al.*, 2018) adds further complexity to exercise associated responses. For this reason, it is essential that future studies explore the role that exercise protocol choices play in modulating IGF-1 and associated binding proteins, particularly over longer periods of recovery.

Responses in BDNF within the current study are consistent with previous research in relation to the response to SIT (Kujach *et al.*, 2020) and the relative lack of response at lower intensities (Nofuji *et al.*, 2012; Reycraft *et al.*, 2020). One possible explanation for this lack of response at lower intensities is the relatively short half-life of BDNF in the circulation of ~2.7-minutes reported by some authors (Pardridge *et al.*, 1994), which would necessitate very high levels of release in order for accumulation of BDNF to be detected in plasma. However, more recent studies have disputed this half-life time, demonstrating half-life's as long as 60-minutes (Pan *et al.*, 1998; Khalin *et al.*, 2016). In addition, the potential for lactate to upregulate BDNF in a similar manner to VEGF (Schiffer *et al.*, 2011) also raises the potential role that intensity-related elevations in lactate may play in BDNF elevations. Interestingly, a significant difference was seen between male and female participants within the current study, with female participants showing greater responses in the recovery period during SIT and significant elevations in MICT that were not seen in male participants or the cohort as a whole (Figure 4.6). Previous studies have highlighted the potential for sex differences both at rest (Lommatzsch *et al.*, 2005; Piccinni *et al.*, 2008) and during

exercise (Serra-Millàs, 2016; Dinoff *et al.*, 2017). While in a recent meta-analysis, Dinoff et al. (2017) found significant elevations in BDNF in males alone, the authors highlighted that this may be due in part to a relative lack of studies including female cohorts. In combination with the contrasting findings of the present study and a general lack of studies across different exercise protocols, there is a clear need for further research into the potential role of sex differences in exercise-related BDNF responses. Within the current study female participation was not restricted by oral contraceptive use or menstrual cycle phases, so the role that cycle phase plays in these differences cannot be fully understood. This is particularly pertinent given the significant variations across the menstrual cycle highlighted by Begliuomini et al. (2007), thus further research is needed to explore the influence that this basal variability may play in exercise-induced responses within females and in comparison to male cohorts. The lack of BDNF modulation by HIIT also raises interesting questions for future study as to the role of exercise duration and intensity in stimulating changes in BDNF, with consideration as to how plasma half-life may dictate the capacity for exercise to drive accumulation within the circulation.

4.4.3. Limitations and Future Directions

While growing evidence supports the utilization of interval-based exercise, it is important to consider the potential risks of these strategies against the physiological benefits they may provide (Lucas *et al.*, 2015; Labrecque *et al.*, 2020). Encouragingly, recent studies into the risk between interval-based and traditional continuous exercise protocols for cardiovascular disease cohorts have shown that the two are of similar risk (Rognmo *et al.*, 2012; Wewege *et al.*, 2018). Our findings alongside the limited risk that clinical guideline-based intervals appear to pose, do suggest that interval-

based exercise can be safely used to positively impact cerebrovascular health. However, it is still important that the safety of such interventions are considered before they are applied across a broader population, including at risk cohorts.

The utilization of TCD to measure blood velocity as a proxy of cerebral blood flow has been widely debated (Ainslie & Hoiland, 2014). The potential for the MCA to change in diameter during exercise has significant ramifications for the use of TCD, particularly as the nature of the diameter change may depend on the specific exercise stimulus and these concerns must be taken into consideration when utilizing TCD (Hoiland & Ainslie, 2016; Verbree *et al.*, 2017). While alternative methods such as MRI, fNIRS and duplex ultrasound may present a more accurate or in depth examination of the cerebrovasculature, at present these methods cannot be employed across the range of exercise protocols examined in the current study (Ainslie & Hoiland, 2014).

The potential influence of changes in haemoconcentration as a result of fluid shift during exercise was not considered within the current study. While previous research has shown greater levels of hemoconcetration at higher intensities (Wahl *et al.*, 2010; Bloomer & Farney, 2013), the lack of responses in either MICT or HIIT, alongside significant reductions in IGF-1, contradict the potential for circulating responses to be purely haemoconcentration-dependent. Additionally, measures of BDNF were taken in plasma samples alone and not in serum, which the latter previously been shown to generate significantly higher yields of BDNF due to the contribution of platelet-derived BDNF (Serra-Millàs, 2016). However, our aim within the current study was to identify responses within the circulating levels of neurotrophic factors and their release from tissue and as such plasma-based measures of BDNF were chosen. While care was

taken in the preparation of plasma samples, so as to avoid the inclusion of platelets within the final sample, we did not include a second centrifugation step to eliminate any residual platelets from samples and as such this may have affected BDNF and other measures sensitive to platelet activity. However, preparation of plasma was carried out at higher speeds in a similar manner to a recently validated protocol for the removal of platelets by single-step centrifugation (Rikkert *et al.*, 2020).

As already discussed, female participants were included regardless of oral contraceptive usage and menstrual phase. While this limits our ability to determine the influence of sex hormones or menstrual cycle, this decision was made to address the studies aims in free-living individuals and to maximize recruitment of female participants (Stanhewicz & Wong, 2020). Furthermore, while numerous strategies have been suggested to control for the menstrual cycle within studies, the variability between individuals makes many of these strategies unreliable (Häggström, 2014; Sims & Heather, 2018). Nevertheless, our findings demonstrate the potential for sex differences to influence acute exercise responses within the vasculature, warranting further exploration of this interaction.

While this study demonstrates the clear difference in acute responses, it is not yet clear whether the differences between these acute bouts result in significant differences in a chronic context. As such, although growing evidence supports the concept that acute responses are often indicative of the chronic impact of interventions (Dawson *et al.*, 2018; Voss *et al.*, 2020), the exploration of the response to a prolonged training period across all three exercise approaches is clearly warranted. Similarly, the population studied within the current work were healthy, young and active, and so

future work is required to identify how differences across the population may alter this response.

4.4.4. Conclusion

In conclusion, similar acute haemodynamic responses are seen between traditional continuous and more recent guideline-based interval exercise strategies (4x4 minute bouts at 85% HR_{max}), indicating that this type of interval-based exercise approach may also be capable of inducing similar changes in cerebrovascular function over chronic training periods. While sprint-based intervals drove relatively limited changes in blood velocity compared to guideline-based interval and traditional continuous exercise strategies, these intervals induced large changes in circulating factors with a potential to significantly impact cerebrovascular health and function. This dichotomy in response between exercise strategies may provide an alternate or adjunct means for improving brain health through combining approaches to physical activity within interventions.

5. REBOUND CEREBRAL HAEMODYNAMICS DURING SPRINT-BASED EXERCISE

5.1. Introduction

As recommendations surrounding exercise continue to develop, an increasing focus is being given to the incorporation of interval-based exercise approaches. From a clinical perspective, these have traditionally involved relatively long intervals of around 4-minutes at sub-maximal intensities (~85% HR_{max}); however, there is increasing interest in the potential benefits of sprint-based approaches, more often seen in athletic training contexts (Weston *et al.*, 2014). This interest stems not only from the potential for these approaches to be more time-efficient than both steady-state exercise and clinical interval approaches; potentially improving feasibility across the general population, but also through their potential to elicit similar or even greater benefits across a range of health domains including metabolic health and aerobic fitness (Burgomaster *et al.*, 2005; Gibala & Little, 2020). More recently, research has begun to investigate the potential benefits and efficacy of these approaches in targeting vascular health (Rakobowchuk *et al.*, 2018), and a growing discussion has developed around the potential effects that high intensity interval exercise may have on cerebrovascular health (Lucas *et al.*, 2015; Calverley *et al.*, 2020; Whitaker *et al.*, 2020).

While short, very high intensity intervals are less well defined in their protocol than other approaches, research so far within the cerebrovascular field has focused largely on 30-second efforts at intensities ranging from sub-maximal efforts (Labrecque *et al.*, 2020) to maximum, “all-out” sprint efforts (Curtelin *et al.*, 2018), as was utilised in the previous chapter (Chapter 4; Weaver *et al.*, 2021). The unique response to bouts of sprint interval training (SIT) were first shown by Curtelin et al. (2018), who showed a clear rise in MCAv at the start of a single “all-out” sprint bout, followed by a rapid

reduction over the latter stages of the effort and a subsequent second elevation, or rebound, during the start of the recovery period. This rebound response was matched by similar rebound responses in $P_{ET}CO_2$, despite no clear elevation in mean arterial pressure (MAP) over the same period. While this rebound response was unique and presented within the figures of the published paper, Curtelin et al. (2018) did not investigate this phenomenon further or discuss potential driving factors.

Following on from the first identification of this rebound response, we conducted our own investigation into the cerebrovascular responses to three commonly utilised protocols, as detailed in the previous chapter (Chapter 4; Weaver *et al.*, 2021). Within our findings, a clear rebound response was evident across four SIT bouts at 200% W_{max}; although this response was most pronounced in the first bout, while no clear rebound response was seen over longer 4-minute intervals at 85% HR_{max}.

This response was investigated further by Labrecque et al. (2020), who looked more precisely at the nature of rebound responses, over the course of a single 30-second SIT bout at 100% W_{max}. They identified that maximum suppression of MCA_v was seen at 22-seconds into the sprint bout and that the subsequent rebound resulted in an increase of 43% in MCA_v at 44-seconds following the end of the exercise effort. Additionally, while $P_{ET}CO_2$ followed a similar pattern of response, the timing of this response was approximately 20-seconds faster than that of MCAv. By contrast, MAP increased significantly at the onset of the sprint interval and remained elevated throughout exercise, displaying no clear rebound during the recovery phase. The authors suggested that these differences across the three measures, may be evidence

of a delay in autoregulatory responses within the cerebrovasculature, with the sudden rise in BP driving significant elevations in MCAv initially and independently of $P_{ET}CO_2$.

To date, research on the response to SIT-based protocols has focused on “all-out” efforts; as are typical in athletic training (Curtelin *et al.*, 2018; Weaver *et al.*, 2021), or the response to a single bout in a highly controlled setting (Labrecque *et al.*, 2020). To better understand the rebound response observed during SIT-based protocols, further research is needed into how different intensities may modulate these responses across repeated bouts. Characterisation of rebound responses has also largely relied on blocked average data analysis, with averaging across relatively long time periods (e.g. 30-second averaging). While this is common practice when analysing cerebrovascular data collected during exercise, it may be limited in its efficacy in SIT-based studies, as this approach results in averaging across the course of an entire sprint bout. Some progress has been made in this regard, with Labrecque et al. (2020) taking a more continuous analysis approach allowing for a better understanding of the cerebrovascular kinetics across the course of the sprint and rebound period. Moving forwards, it is essential that the methods behind these analyses are well documented, such that a standardised approach can be developed. Indeed, this has been clearly demonstrated in relation to the study of exercise onset kinetics for moderate intensity exercise, for which there is now a clear and established method for analysis (Billinger *et al.*, 2017), as well as in the extensive documentation and subsequent White Paper on the assessment of cerebral autoregulation using Transfer Function analysis (Claassen *et al.*, 2015). As exercise strategies become more complex, potentially involving a combination of different intensity and duration interval efforts, so too will the research looking to characterise responses to these stimuli. Consequently, it is

pivotal that a clear method be developed for the analysis of data from such studies, so that findings can be easily interpreted and replicated.

5.2. Aims and Hypothesis

General exercise recommendations are becoming broader and more flexible, such that SIT-based approaches are becoming mainstream. As such, understanding the responses to these exercise protocols within the cerebrovasculature is becoming increasingly critical. Therefore, the aim of the present study was to explore how differences in exercise intensity during 30-second sprint intervals alter cerebrovascular haemodynamic responses, and more specifically the presence and nature of a rebound-based response. Secondarily, we aim to develop a semi-automated method for the processing of kinetic responses to sprint-interval exercise and establish the efficacy of different processing methods for the accurate analysis of rebound responses.

It was hypothesised that the greatest rebound responses would be seen at the highest intensities, particularly following the initial sprint bout; while the lower intensities would elicit more consistent responses across multiple bouts, due to a reduction in hyperventilatory hypocapnia and associated reductions in MCA_v response.

5.3. Methods

5.3.1. Study Design

Following enrolment participants attended the laboratory for a total of five visits. Visit one consisted of an incremental $\dot{V}O_{2peak}$ test to determine aerobic fitness, as well as to establish a W_{max} on which the intensity of sprint-intervals was based. During this visit all participants also completed fitness and activity questionnaires (Appendix 5Appendix 6).

The following four experimental visits involved four 30-second sprint-intervals at either 80%, 120%, 160%, or 200% of W_{max} (Figure 5.1). The order of the four experimental visits was conducted in a randomised order, with the order for each participant determined *a priori* (as described in Materials & Methods Section 3.4). Throughout the course of each experimental visit, continuous measures of MCA_v and $P_{ET}CO_2$ were collected (see Materials & Methods 3.2 & 3.3.1) and stored for analysis.

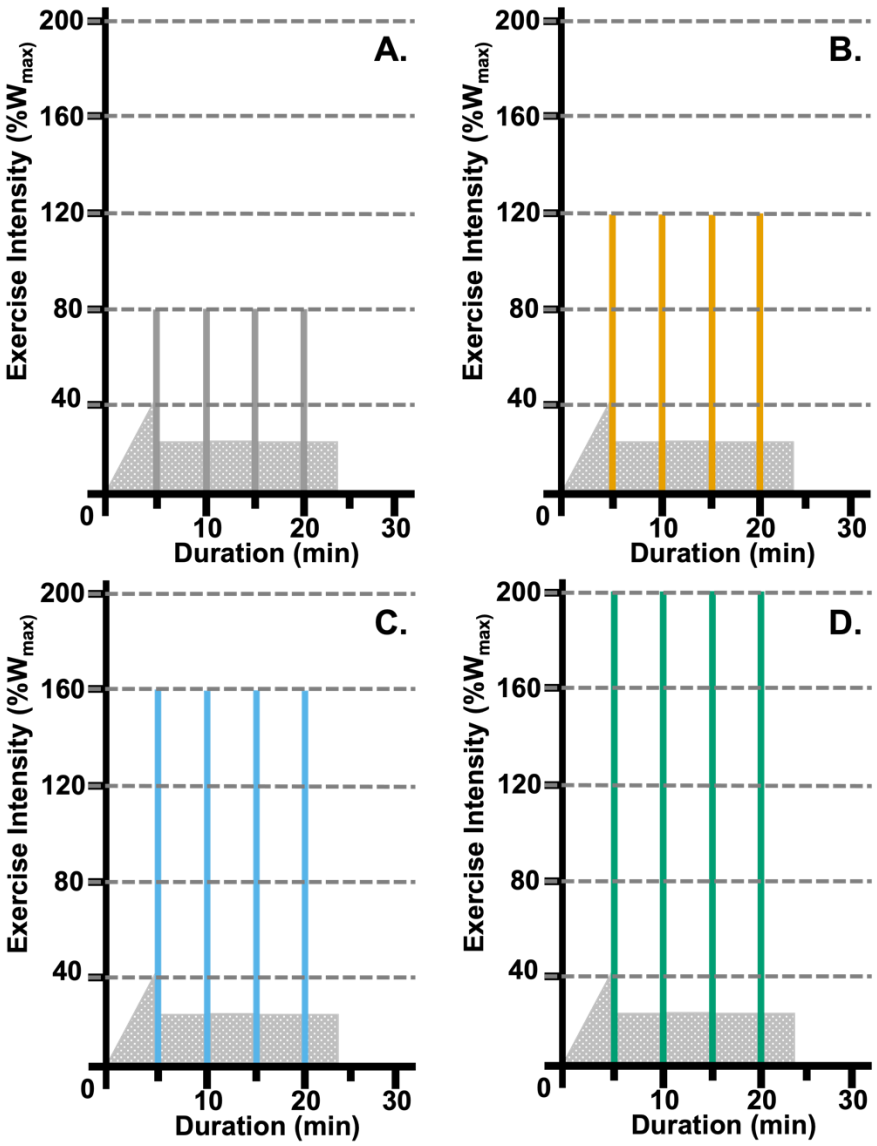


Figure 5.1 Study schematic showing exercise intensity variation in repeated sprint-intervals.
Participants took part in four separate experimental visits, consisting of four 30-second sprint intervals at either 80% (**A**), 120% (**B**), 160% (**C**) or 200% (**D**) of W_{max}. Each sprint-interval visit was preceded by 5-minutes warm up and followed by 3-minutes of active recovery, with sprint intervals separated by four and a half minutes of active recovery at 50W.

5.3.2. Method Development

To allow for the identification, characterisation and quantification of temporal patterns following sprint intervals, novel methods for pre-processing data were paired with an automated feature selection process. The development of these methods is outlined in the following section and full scripts are available at https://github.com/src-weaver/thesis_rebound.

5.3.2.1. Pre-processing of MCA_V measures

Continuous MCA_V data collected via TCD presents significant challenges to analysis at high temporal resolution, including signal drop-out; external interference; and most notably the variation in data over the course of each cardiac cycle (Figure 5.2). To minimise the impact that these variations have in the downstream analysis, continuous data were pre-processed to reduce noise and improve the identification of key components of the sprint rebound response. The approach taken to pre-process data followed one of two pipelines, either based on the smoothing of continuously exported data (CDS approach) or on the interpolation of beat-to-beat exported data (B2B approach).

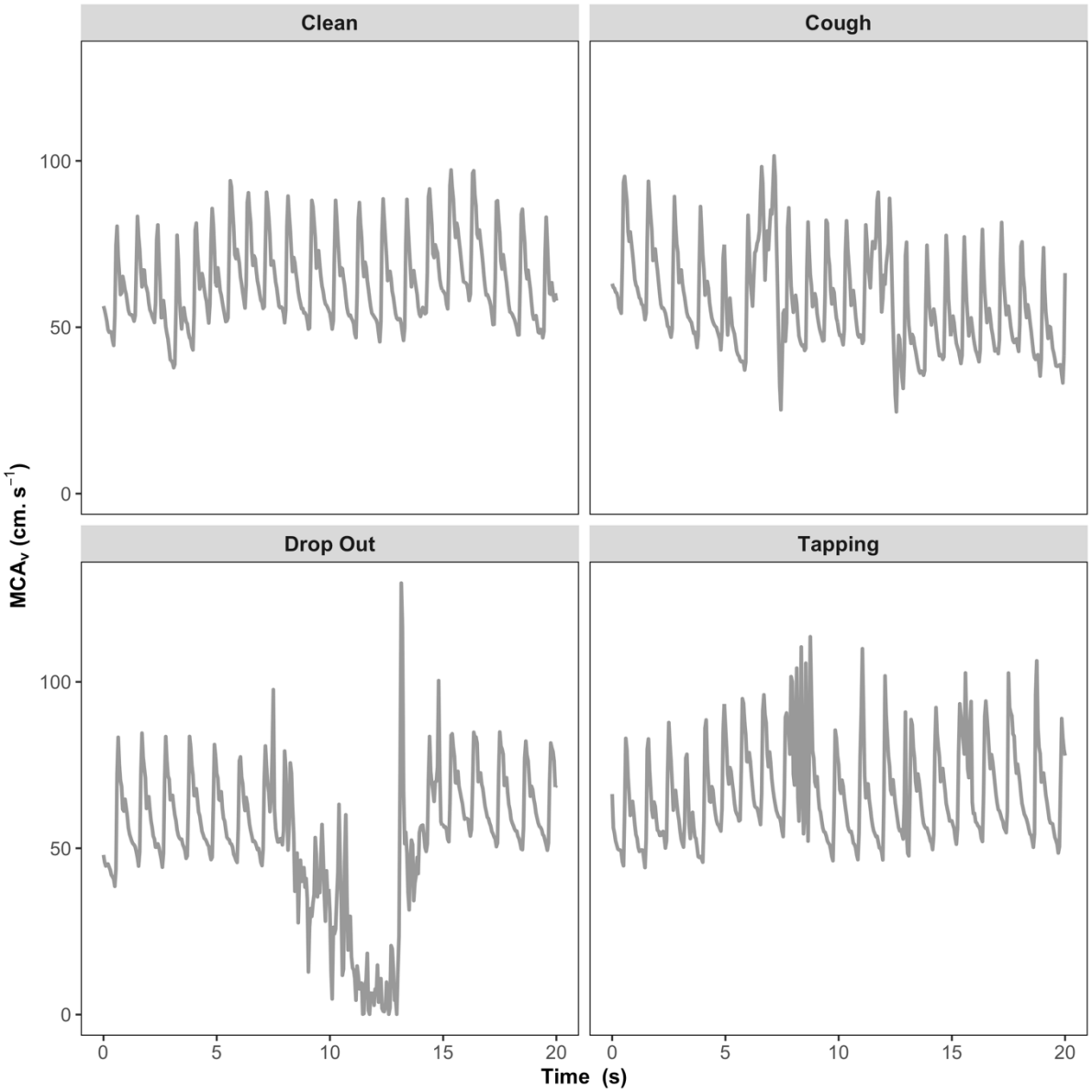


Figure 5.2 Characteristic MCA_v patterns induced by varying sources of interference

Representative resting measures of MCA_v collected by TCD and exported at 20 Hz, showing either clean TCD signal data (**Upper Left**), or with the induction of visible noise artefacts through asking the participant to cough (**Upper Right**); lifting and replacing the TCD probe on the temporal window (**Lower Left**); or by tapping on the outer surface of the TCD probe (**Lower Right**).

Through the CDS approach, data were exported at a sampling rate of 20 Hz and subsequently smoothed to reduce signal noise, using rolling averages. This method applies continuous averaging to each point within a given data set, with averaging occurring symmetrically over a given window, such that a 10-second window would average across the 5-seconds preceding and following each given data point. This approach is highly dependent on the size of the averaging window, with shorter windows providing higher temporal resolution but less effective smoothing, while longer windows are more effective in reducing the impact of noise but more susceptible to over-smoothing and elimination of physiologically relevant variation within the data set (see Figure 5.3). For this reason, multiple averaging window sizes were applied to the data to determine the impact that varying this factor had on resultant feature selection, with 10-second (CDS-10) and 30-second windows (CDS-30) carried forward to feature selection and for comparison to the B2B approach.

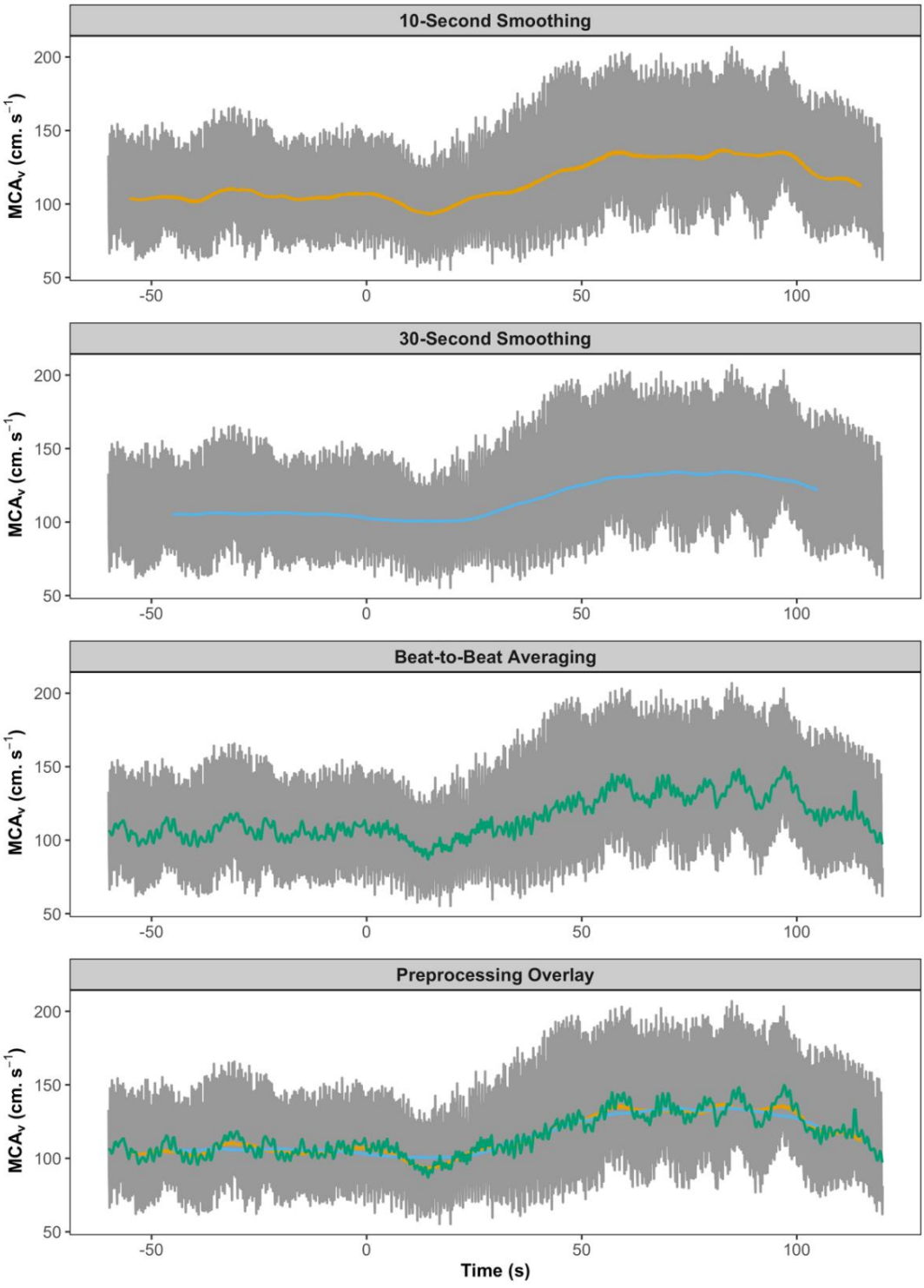


Figure 5.3 Representative plots of pre-processing methods (Participant SI002) MCA_v was determined continuously by transcranial Doppler ultrasound (TCD) and subsequently exported and processed following either the continuous data smoothing approach with a 10- (1st plot – **orange**) or 30-second window (2nd plot – **blue**) or the beat-to-beat approach (3rd plot – **green**). Data are plotted overlaying raw MCA_v data exported at 20 Hz (shown in **grey**) and are shown individually (top three plots) or in combination (bottom plot).

The B2B approach utilises native beat detection functions within the Blood Pressure Module in LabChart Pro 7. This function provides a means for the detection of the systolic peak within each cardiac cycle, with data then averaged across each beat and exported for further processing. All default settings were maintained within the peak selection parameters, with the exception of cycle duration which was limited to 1.5-seconds (minimum detectable cardiac cycle of 40 beats·min^{-1}) in order to minimise the influence of signal drop-out.

To reduce the impact of random noise and non-physiological variation in beat-to-beat data, all data sets were processed through time series outlier detection and removal using the tsoutliers function within the Forecast package in R (Hyndman *et al.*, 2021). Briefly, this process detrends time series data by fitting a locally weighted smoothing (LOESS) curve to each individual data set. The residual component of this detrending process is then used to detect outliers, by identifying data points that sit outside the 90th percentile of the residual mean ($\pm 2(q_{0.9} - q_{0.1})$). The effectiveness of this process depends on the overall level of noise within each dataset, as the greater the level of noise the larger the given quantile range will be. Within the current data set, the impact that the level of noise can have on outlier detection can be seen in Figure 5.4 and Figure 5.5 (see Appendix 7Appendix 20 for all individual data inspection plots).

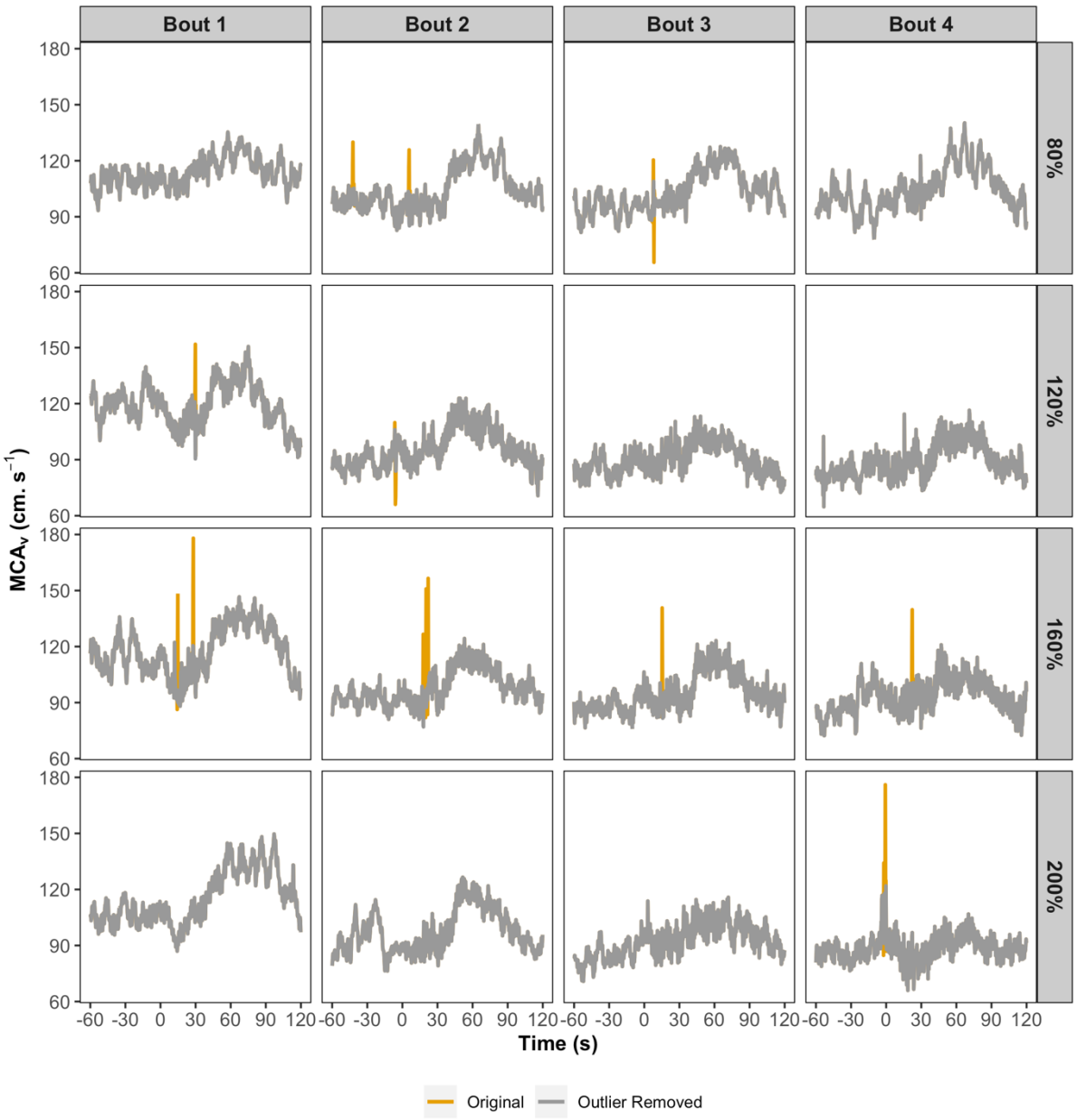


Figure 5.4 Representative plots of data following outlier removal (Participant SI002)
MCA_V was determined continuously by transcranial Doppler ultrasound (TCD) and subsequently exported and processed following the beat-to-beat approach (**orange**). Following initial pre-processing potential outliers were detected and removed from the data (**grey**). Data are presented from a single participant as individual data from each of four bouts (**left – right**) across 30-second sprint intervals at 80%, 120%, 160%, and 200% of W_{max} (**top – bottom**).

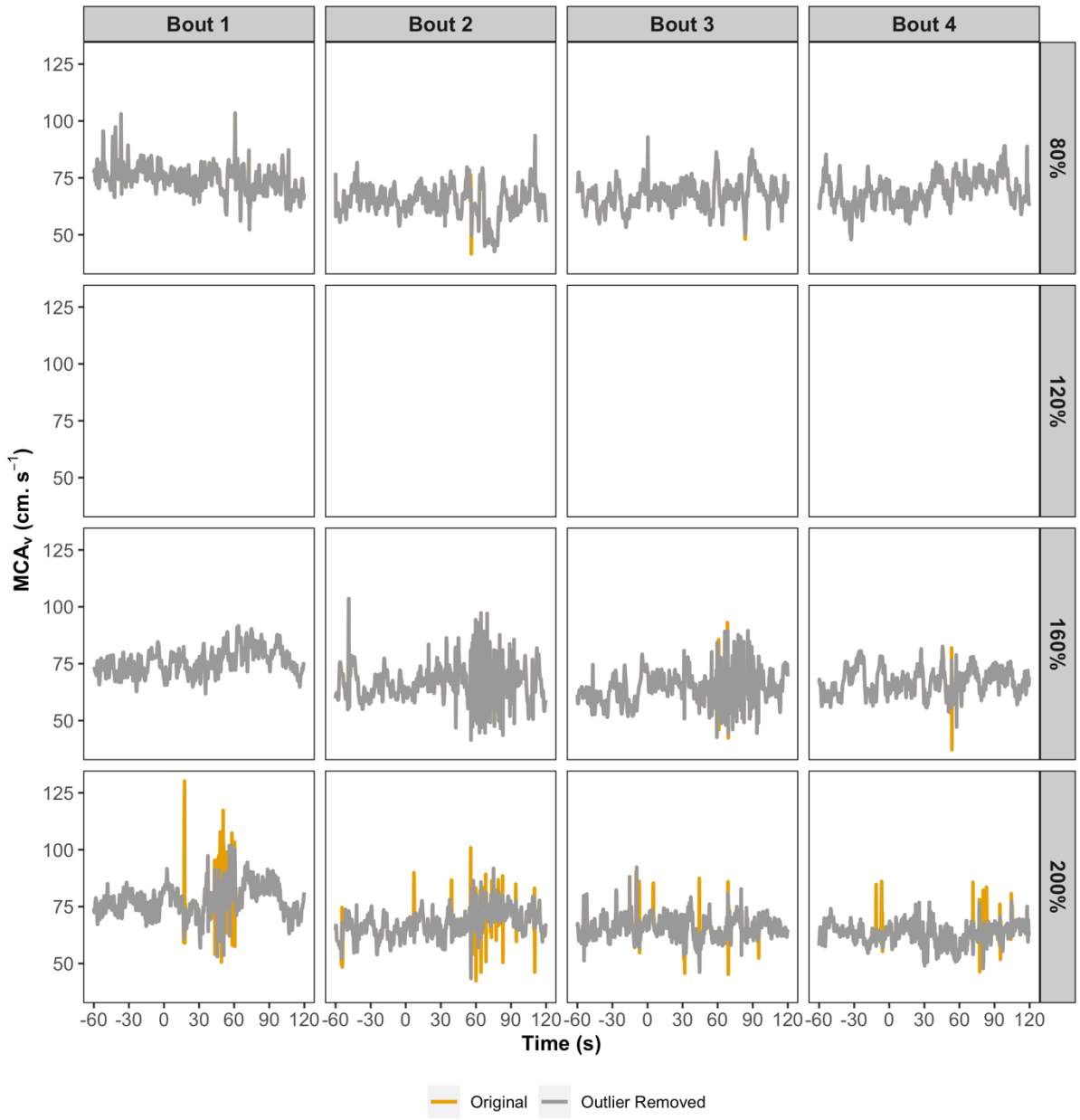


Figure 5.5 Representative plots of data following outlier removal (Participant SI014)

MCA_V was determined continuously by transcranial Doppler ultrasound (TCD) and subsequently exported and processed following the beat-to-beat approach (**orange**). Following initial pre-processing potential outliers were detected and removed from the data (**grey**). Data are presented from a single participant as individual data from each of four bouts (**left – right**) across 30-second sprint intervals at 80%, 120%, 160%, and 200% of W_{max} (**top – bottom**). Empty panels are shown in conditions where data were not collected from the participant.

Following outlier removal, beat-to-beat data were interpolated by fitting cubic spline (third-order polynomials) between each beat average and resampled at a sampling frequency of 4Hz. This was done to standardise sample frequency, so as to limit the influence of heart rate on subsequent feature selection processes, and to align the pre-processing procedures with those used in the standardised procedures used within transfer function analysis (Claassen *et al.*, 2015). Interpolated, resampled data were then passed to the feature selection pipeline alongside data produced via the CDS approach.

5.3.2.2. Pre-processing of $P_{ET}CO_2$

Measures of $P_{ET}CO_2$ taken using a Vyntus metabolic cart (see Materials & Methods 3.2) are collected on a breath-by-breath basis and as such this fixes the maximum sampling rate at this frequency. For this reason, $P_{ET}CO_2$ data were pre-processed in an identical manner to the B2B approach, with breath-by-breath data being screened for outliers and subsequently interpolated and resampled at 4Hz, prior to being passed through the feature selection pipeline.

5.3.2.3. Feature Selection

Previous analysis of the rebound response seen during sprint-intervals showed a defined pattern of suppression of MCA_v during the interval itself, followed by a rebound in blood velocity early within the recovery phase (Labrecque *et al.*, 2020; Weaver *et al.*, 2021). To identify this response within continuous datasets in an automated, unbiased manner all datasets were passed through a feature selection pipeline.

The start of each sprint interval was detected by identification of markers placed within each dataset during data collection. This time point was then used to extract a data window spanning 1-minute prior and 2-minutes after the onset of the interval and the time course for each window was standardised to zero at the onset of the given interval. To detect potential rebound suppression the minimum point within 40-seconds of interval onset was automatically identified and both the time and magnitude of this minimum point were extracted for analysis. Potential rebound peaks were then identified by detection of the maximum point over the remaining duration of the window, with time and magnitude extracted for analysis.

Following feature selection CDS and B2B pre-processing approaches were compared to determine accuracy, reliability, and comparability of each approach. Firstly, each participants data were visual inspected, with individual visit and bout data plotted over the course of each bout and labelled with minimum and maximum rebound markers (Figure 5.6; Appendix 21–Appendix 34). Secondly, approaches were quantitatively compared through correlation analysis of timing and magnitude of extracted features.

Additionally, fixed 30-second averages were taken across the duration of each sprint bout and between 15- and 45-seconds into active recovery from each bout. CDS-30 data were then compared with these fixed averages, to compare the continuous approaches taken within this chapter to those used in previous research (Chapter 4; Weaver *et al.*, 2021).

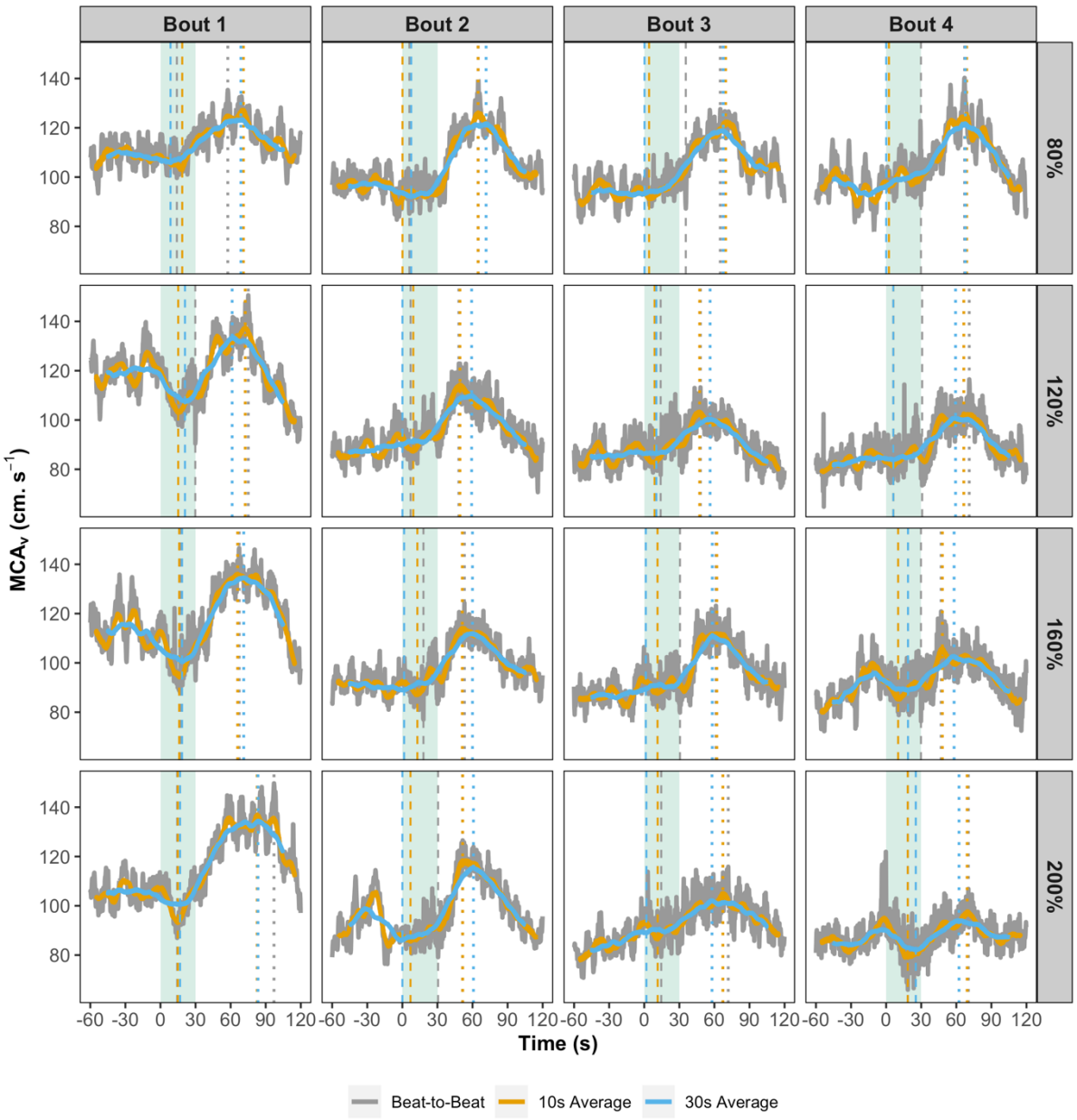


Figure 5.6 Comparison of feature selection following different pre-processing approaches (Participant SI002)

MCA_v was determined continuously by transcranial Doppler ultrasound (TCD) and subsequently exported and processed following either the continuous data smoothing approach with a 10- (**orange**) or 30-second window (**blue**) or the beat-to-beat approach (**grey**). Data are presented from a single participant as individual data from each of four bouts (**left – right**) across 30-second sprint intervals at 80%, 120%, 160%, and 200% of W_{max} (**top – bottom**). Time points are normalised to the start of each 30-second interval (**green box**). Feature selection results for minimum (**dashed line**) and maximum time points (**dotted line**) are shown for each pre-processing approach. Plots for each participant are automatically generated during signal processing, to allow for visual inspection of feature selection processes.

5.4. Results

To determine the comparability across pre-processing approaches, individual data inspection was paired with statistical comparison of feature selection following each method. This data will be presented first within the following section, to outline the justification for taking the B2B approach forward for subsequent analysis of rebound responses.

5.4.1. Comparison of Pre-Processing Approaches

Visual inspection of individual feature selection (Appendix 21–Appendix 34) showed clear disparity in the timing of both minimum and maximum points between CDS and B2B approaches, as well as variation in timing between 10- and 30-second rolling averages within the CDS approach. Amongst this variability, the most common differences were seen in the detection of the timing of the minimum point for each sprint interval, in which differences were seen across all three visualised comparisons (B2B, 10-second and 30-second CDS). These differences were most apparent in bouts featuring smaller observable rebound responses, in which the longer 30-second averaging window appeared to smooth out the minimum point, resulting in the minimum point being detected at the earliest possible time point (i.e. 0 seconds into the interval). Additionally, the higher temporal resolution of the B2B approach led in some instances to erroneous variability in the data being identified as either the minimum or maximum time point. An example of this is presented in Figure 5.6, where the minimum point detected for participant 2 within bout 1 at 120% W_{max} is likely an erroneous value.

Correlation analysis of the timing of both minimum and maximum points showed significant correlation between all pre-processing approaches. More specifically, significant correlations were seen between 10- and 30-second averaging data for the CDS approach, both overall and within each experimental visit (Figure 5.7 & Figure 5.8). When comparing feature timing between B2B and CDS approaches, maximum point timing was significantly correlated between both 10- and 30-second CDS data and B2B data across all visits (Figure 5.8). However, correlations were weaker between CDS and B2B data in minimum point timing, with no significant relationship seen between 10-second CDS and B2B data at 80% W_{max}, or between 30-second CDS and B2B data at 200% W_{max} (Figure 5.7 & Figure 5.8).

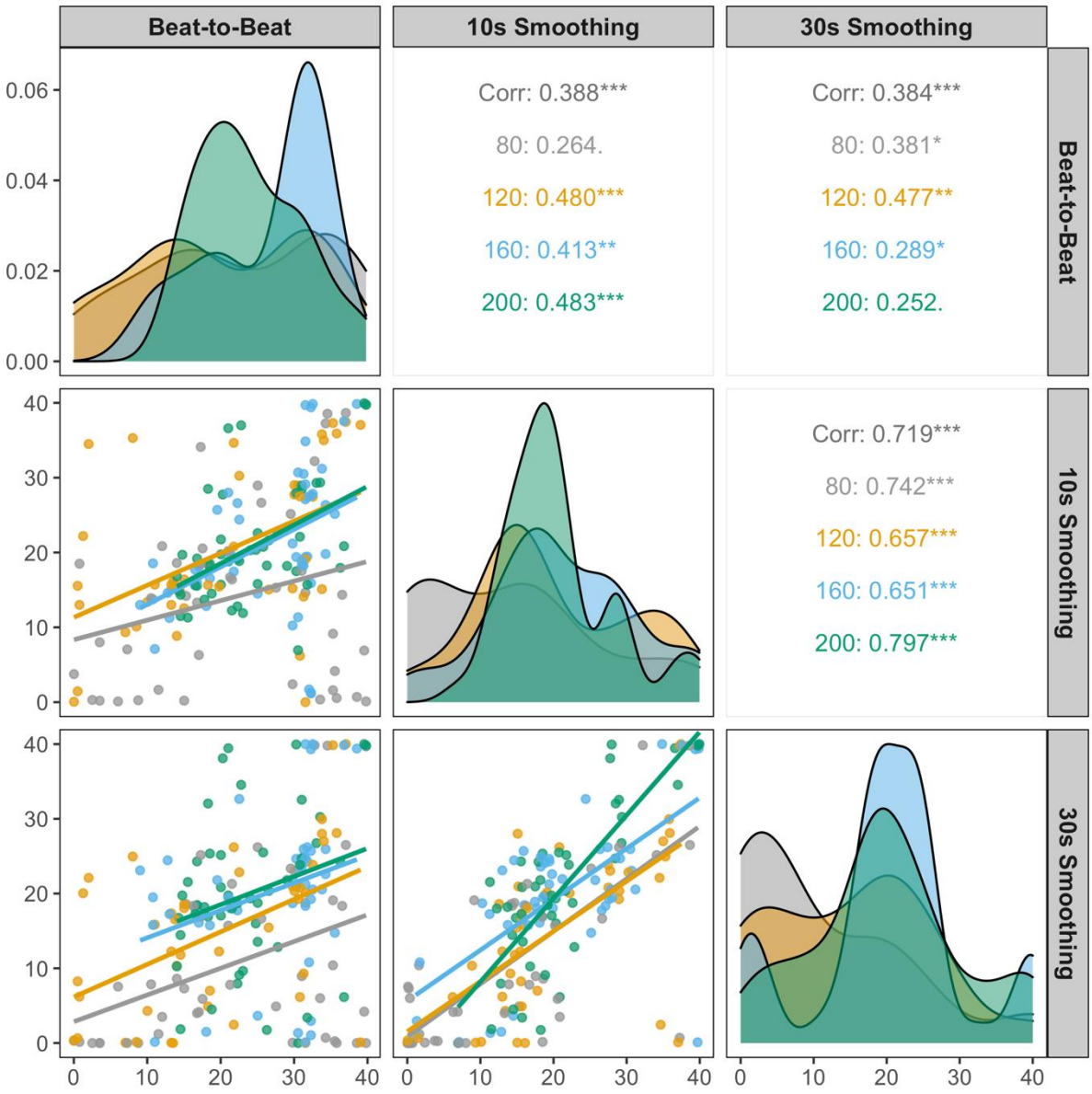


Figure 5.7 Correlation matrix for minimum point timing across pre-processing approaches

Minimum point timing results following feature selection were analysed by correlation between each pre-processing approaches, both in relation to 80% (80 – **grey**), 120% (120 – **orange**), 160% (160 – **blue**) and 200% W_{max} (200 – **green**) interval intensities, as well as collectively across intensities (corr). Correlation coefficients between approaches are shown in the upper right three panels, with significance denoted by * ($p<0.01$), ** ($p<0.001$) or *** ($p<0.0001$) where appropriate. Diagonal (top right – bottom left) panels show frequency distributions for minimum point timing, grouped by intensity, with time (seconds) on the x-axis and frequency (count) on the y-axis. Correlation plots between approaches and line of best fit (grouped by exercise intensity) are shown in the bottom left three panels, with time (seconds) on both axes.

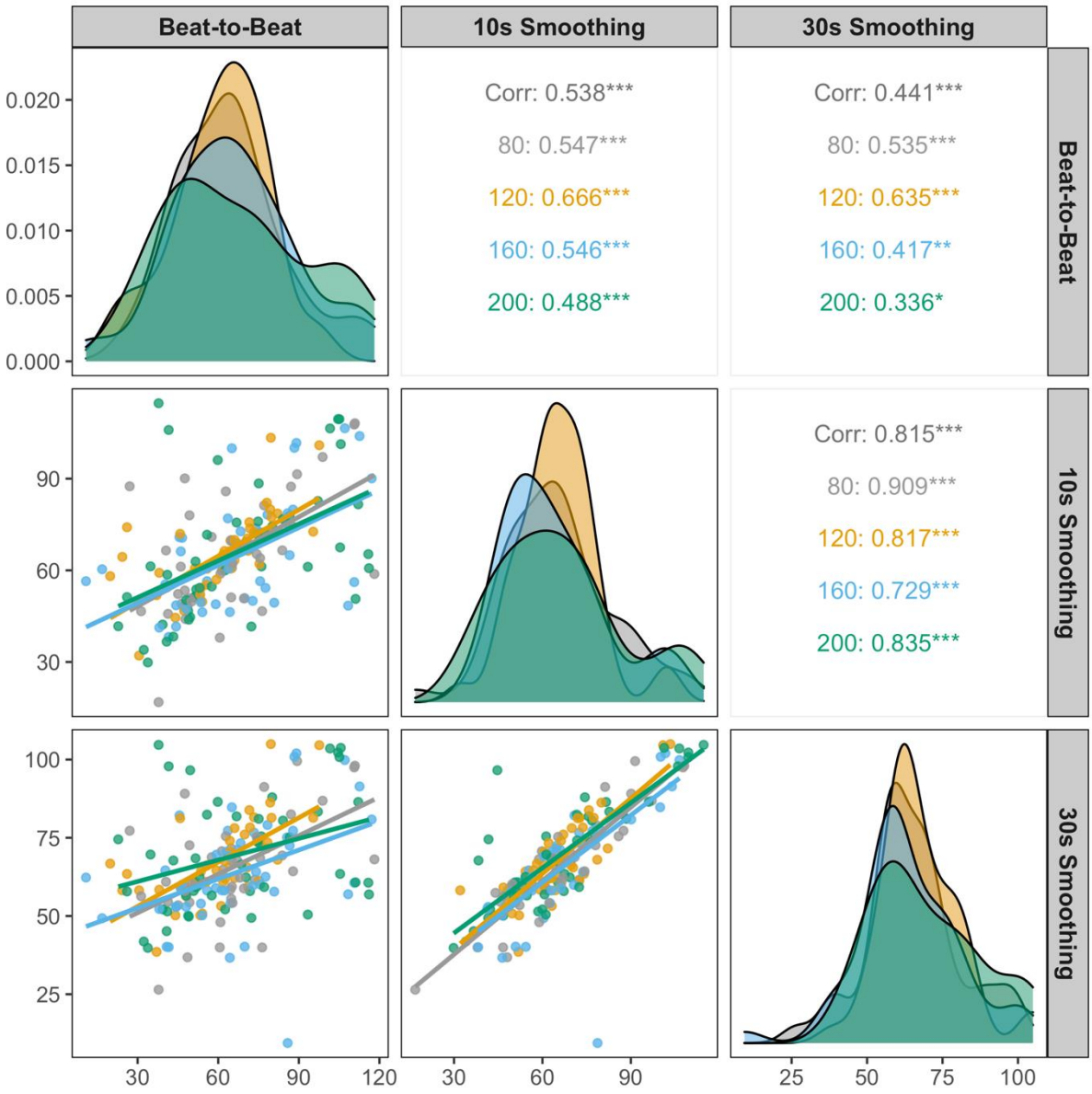


Figure 5.8 Correlation matrix for maximum point timing across pre-processing approaches

Maximum point timing results following feature selection were analysed by correlation between each pre-processing approaches, both in relation to 80% (80 – **grey**), 120% (120 – **orange**), 160% (160 – **blue**) and 200% W_{max} (200 – **green**) interval intensities, as well as collectively across intensities (corr). Correlation coefficients between approaches are shown in the upper right three panels, with significance denoted by * ($p<0.01$), ** ($p<0.001$) or *** ($p<0.0001$) where appropriate. Diagonal (top right – bottom left) panels show frequency distributions for maximum point timing, grouped by intensity, with time (seconds) on the x-axis and frequency (count) on the y-axis. Correlation plots between approaches and line of best fit (grouped by exercise intensity) are shown in the bottom left three panels, with time (seconds) on both axes.

Significant correlations were also seen in the magnitude of both minimum and maximum points between all pre-processing approaches. Specifically, both overall and visit specific correlations were found to be significant for all comparisons of minimum value selection (Figure 5.9), alongside R^2 greater than 0.90 in all comparisons, with the exception of correlation within the 160% W_{max} visit between the B2B approach and both the 10-second ($R^2 = 0.88$, $p < 0.01$) and 30-second CDS approach ($R^2 = 0.89$, $p < 0.01$). For the maximum value selection, significant correlations were found between all three approaches, both overall and in comparison within visit ($R^2 > 0.90$, - < 0.01; Figure 5.10).

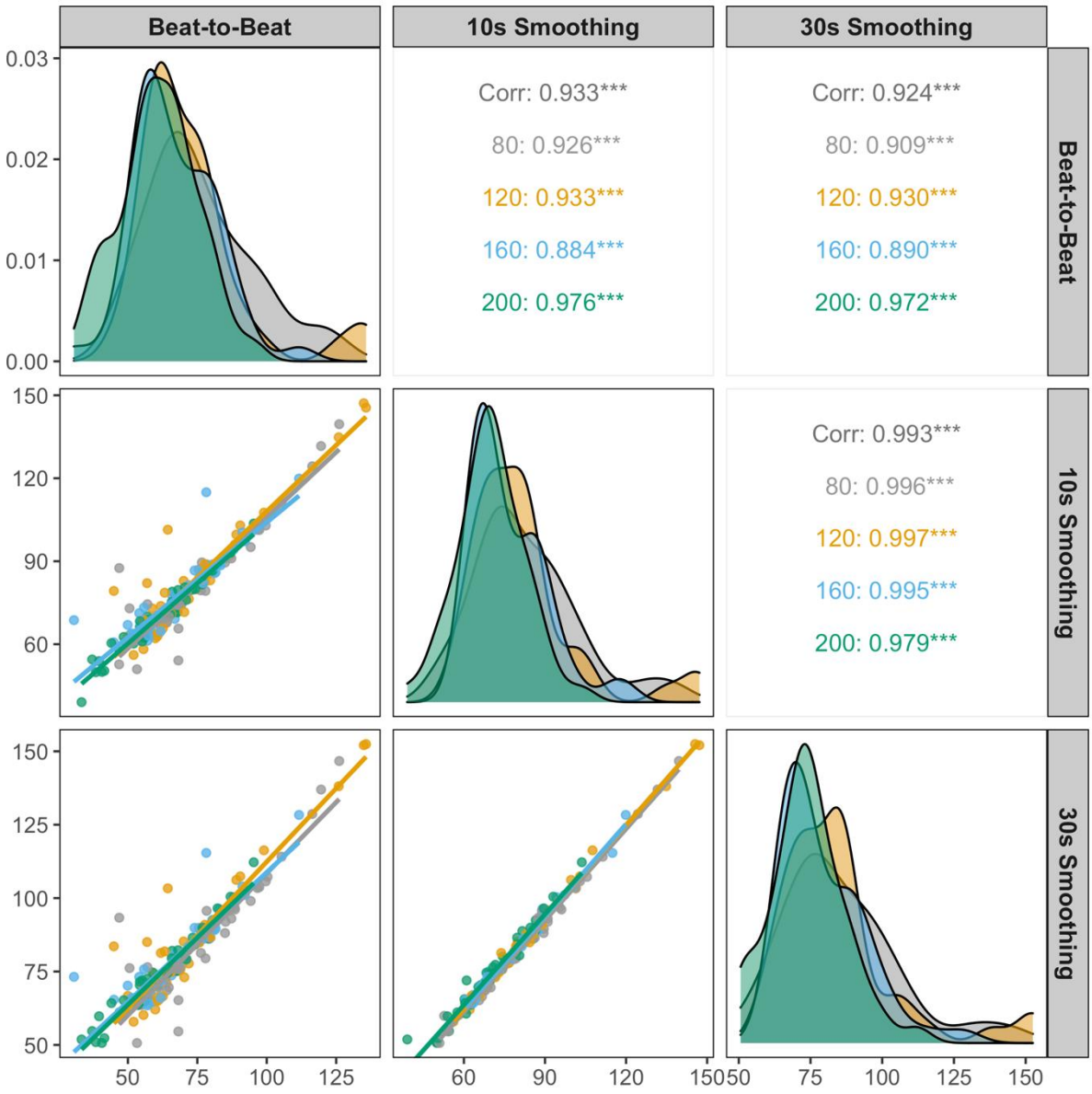


Figure 5.9 Correlation matrix for minimum point magnitude across pre-processing approaches

Minimum point magnitude results following feature selection were analysed by correlation between each pre-processing approaches, both in relation to 80% (80 – **grey**), 120% (120 – **orange**), 160% (160 – **blue**) and 200% W_{max} (200 – **green**) interval intensities, as well as collectively across intensities (corr). Correlation coefficients between approaches are shown in the upper right three panels, with significance denoted by * (p<0.01), ** (p<0.001) or *** (p<0.0001) where appropriate. Diagonal (top right – bottom left) panels show frequency distributions for minimum point magnitude, grouped by intensity, with MCA_v ($cm \cdot s^{-1}$) on the x-axis and frequency (count) on the y-axis. Correlation plots between approaches and line of best fit (grouped by exercise intensity) are shown in the bottom left three panels, with MCA_v ($cm \cdot s^{-1}$) on both axes.

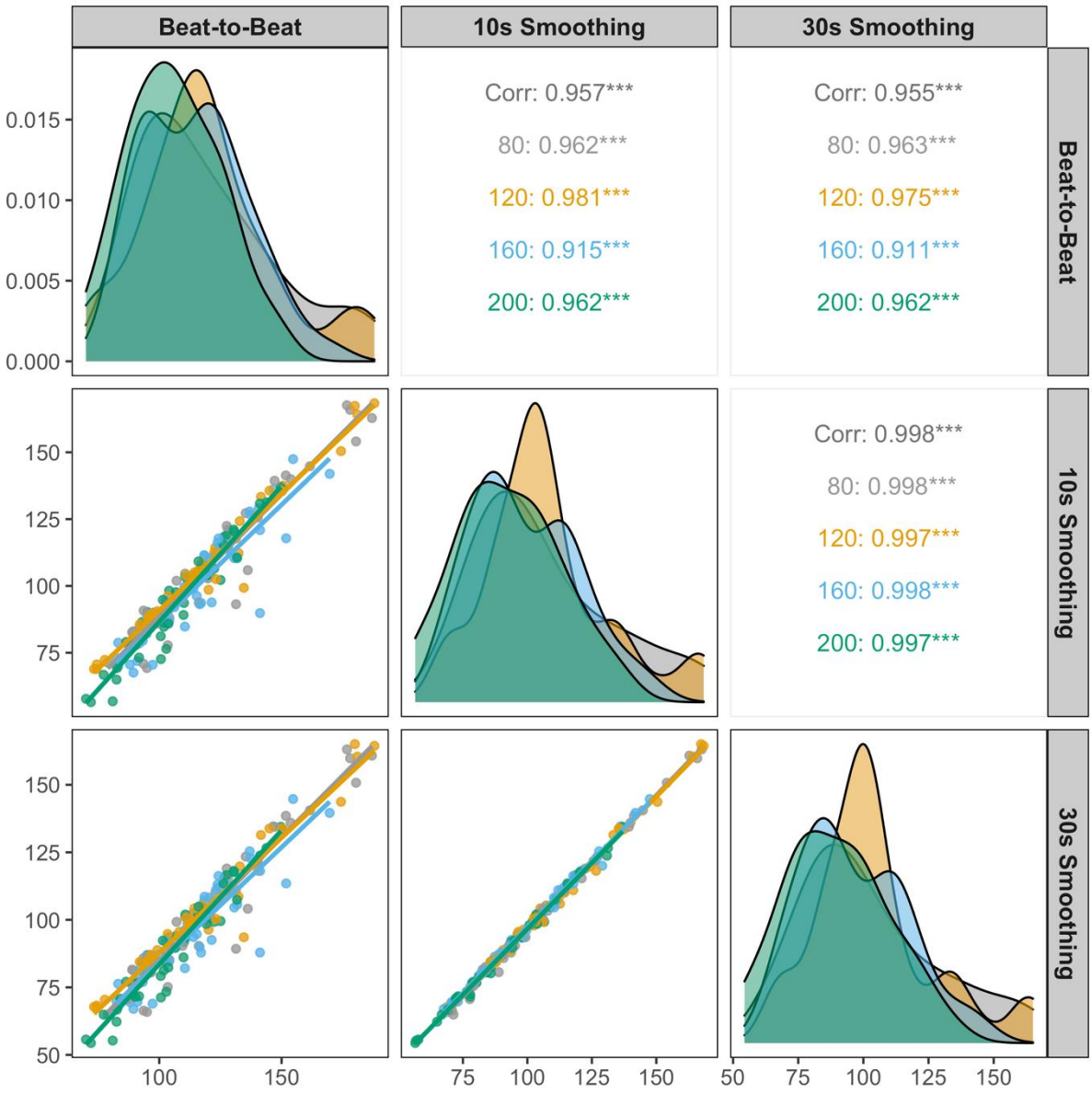


Figure 5.10 Correlation matrix for maximum point magnitude across pre-processing approaches

Maximum point magnitude results following feature selection were analysed by correlation between each pre-processing approaches, both in relation to 80% (80 – **grey**), 120% (120 – **orange**), 160% (160 – **blue**) and 200% W_{max} (200 – **green**) interval intensities, as well as collectively across intensities (corr). Correlation coefficients between approaches are shown in the upper right three panels, with significance denoted by * (p<0.01), ** (p<0.001) or *** (p<0.0001) where appropriate. Diagonal (top right – bottom left) panels show frequency distributions for maximum point magnitude, grouped by intensity, with MCA_v ($cm \cdot s^{-1}$) on the x-axis and frequency (count) on the y-axis. Correlation plots between approaches and line of best fit (grouped by exercise intensity) are shown in the bottom left three panels, with MCA_v ($cm \cdot s^{-1}$) on both axes.

5.4.2. Comparison with previous analysis approach

In comparison between feature selection using CDS-30 data and fixed averages, high levels of correlation were seen across all intensities, with significant correlations in both minimum ($r = 0.987$, $p < 0.01$) and maximum point magnitude ($r = 0.994$, $p < 0.01$). As a result, high levels of agreement were seen in both mean and median, with values within 2 $cm \cdot s^{-1}$ across both minimum and maximum points. Similarly, while a broad range of timings was seen in automated feature selection from CDS-30 data, overall timing points were within 4-seconds of fixed average timing for minimum points (16.7 ± 11.4 s; median = 18.3 s) and 7-seconds for maximum points (66.5 ± 16.2 s; median = 63.2 s).

5.4.3. Intensity-dependent variation in rebound responses

Data produced through the B2B approach was taken forward for full analysis of rebound responses. This choice was taken as higher temporal resolution and reduced smoothing effects allow for the detection of smaller, more rapidly changing responses within the data.

5.4.3.1. Magnitude of MCAv rebound response

Rebound responses in MCA_v were seen across all four intensities, although the pattern of response magnitude across bouts differed in an intensity-dependent manner (Figure 5.11). Specifically, the magnitude of rebound response for the 80% W_{max} session was seen to increase over the course of repeated bouts, with the largest change in MCA_v from minimum to maximum seen in bout 4 (Δ44.1 $cm \cdot s^{-1}$ compared to Δ39.2 $cm \cdot s^{-1}$ for bout 1). Conversely, the rebound responses declined over the course of multiple bouts at 200% W_{max}, with the largest rebound response seen in bout

1 (Δ52.3 $cm \cdot s^{-1}$ compared to Δ38.7 $cm \cdot s^{-1}$ for bout 4). Statistical analysis showed significant main effects for visit, bout, and between minimum and maximum points (all $p < 0.01$), however, no significant interactions were found between any of these three factors.

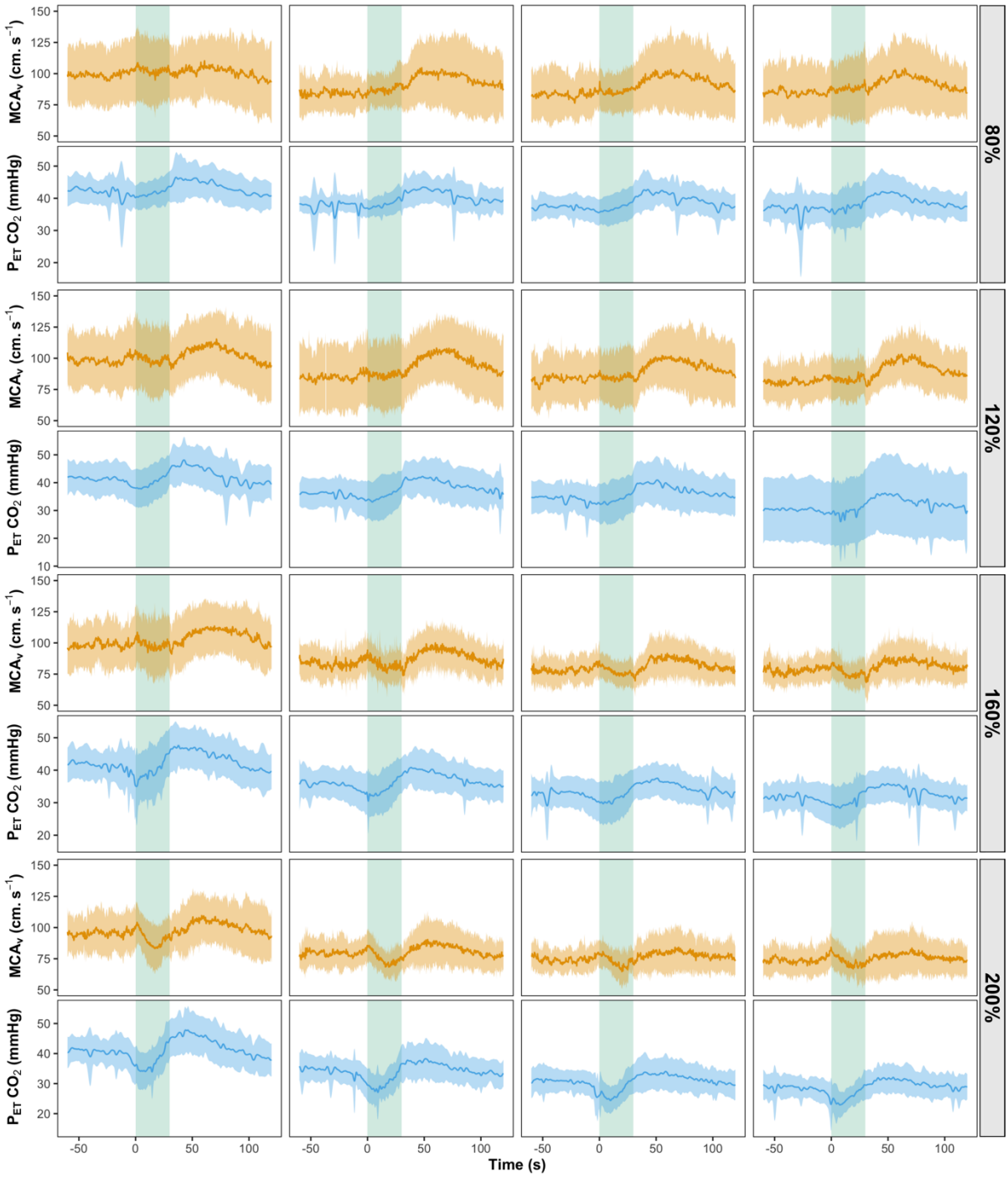


Figure 5.11 Mean $P_{ET}CO_2$ and MCA_V responses across four repeated sprint bouts at four intensities

MCA_V and $P_{ET}CO_2$ were determined continuously and processed using the B2B approach. Exercise protocols were four 30-second sprint intervals at 80%, 120%, 160%, and 200% W_{max} separated by 4.5 min active recovery at 50 W, with all protocols followed by 3 min of active recovery at 50 W. Data are presented as 60-seconds prior and 120-seconds following each sprint bout, averaged across participants. Shaded area (green) denotes the period for each sprint interval.

Analysis of main effects showed that MCA_v values within the 80% W_{max} visit (97.7 ± 32.3 cm·s^{-1}) were significantly higher than those in visits at any other intensity (120%: 96.0 ± 33.5 cm·s^{-1}; 160%: 90.7 ± 30.0 cm·s^{-1}; 200%: 83.8 ± 27.9 cm·s^{-1}; all $p < 0.01$), while MCA_v in 200% W_{max} visits was significantly lower than in all other visits ($p \leq 0.03$). Across the four bouts, regardless of intensity and minimum/maximum point, MCA_v was significantly greater for bout 1 (102.0 ± 31.8 cm·s^{-1}) than in any other bout (Bout 2: 91.4 ± 31.8 cm·s^{-1}; Bout 3: 88.0 ± 29.6 cm.s^{-1}; Bout 4: 85.7 ± 29.8 cm·s^{-1}; all $p < 0.01$), with MCA_v values also significantly greater in bout 2 compared to bout 4 alone ($p = 0.02$). Overall minimum point MCA_v values were significantly lower than those at the maximum point (69.0 ± 17.8 cm·s^{-1} vs. 115.0 ± 24.4 cm·s^{-1} respectively; $p < 0.01$).

5.4.3.2. Magnitude of PETCO2 rebound response

The changes in PETCO2 followed a similar pattern of response to that of MCA_v, with the most prominent rebound seen in early bouts at higher intensities and a notable reduction in the magnitude of rebounds in later bouts at these intensities. Linear mixed effect models showed significant interactions between visit and bout ($p = 0.01$) and between visit and minimum/maximum point ($p < 0.01$), alongside significant main effects for all three factors (all $p < 0.01$). However, no significant interactions were seen between sprint bout and the minimum/maximum point, or in the combination of all three factors (Figure 5.12).

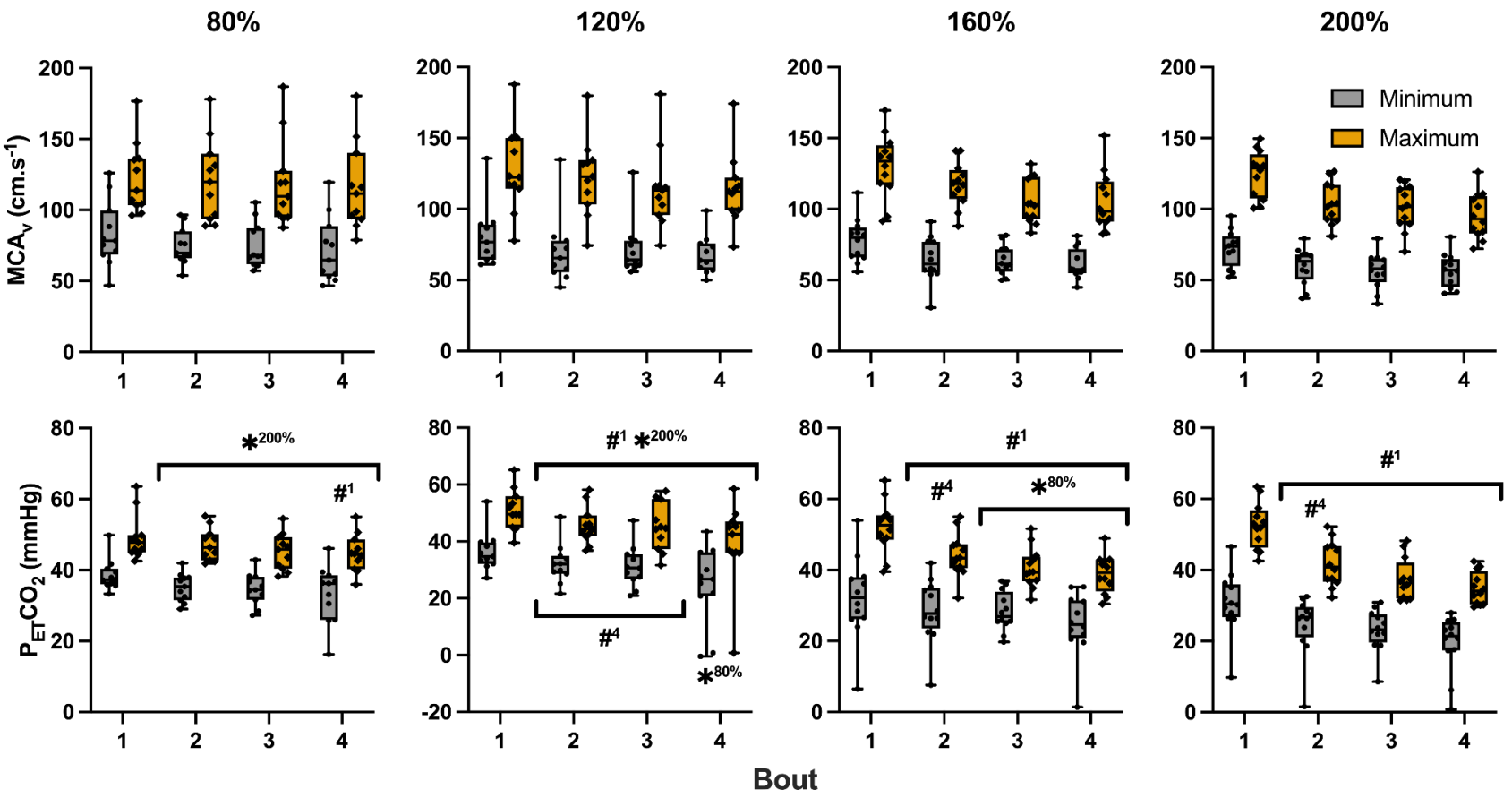


Figure 5.12 Magnitude of rebound responses during repeated sprint intervals at different intensities
MCA_V and $P_{ET}CO_2$ were determined continuously by transcranial Doppler ultrasound and breath-by-breath measurement, respectively. Following pre-processing by the B2B approach, automated feature selection was carried out to determine minimum and maximum points. Magnitude of minimum (**grey**) and maximum (**orange**) points are shown as box and whisker plots of median (horizontal line), interquartile range (box) and minimum-maximum range (whisker) alongside individual data points. Significant overall differences (analysed by linear mixed models, $p < 0.05$) between exercise intensities are denoted by ✱, with superscript signifying comparison intensity. Significant overall differences between bouts are denoted by #, with superscript signifying comparison bout.

While no differences were seen between visits in bout 1, $P_{ET}CO_2$ was seen to be significantly different between visits in bouts 2 – 4. In bout 2, $P_{ET}CO_2$ was significant lower at 200% W_{max} (33.1 ± 11.4 mmHg) than at 80% (41.2 ± 7.5 mmHg; $p < 0.01$) and 120% (38.9 ± 9.6 mmHg; $p < 0.01$). Similarly, lower values were seen at 200% (30.2 ± 9.3 mmHg) than at 80% (39.9 ± 7.2 mmHg; $p < 0.01$) and 120% W_{max} (38.1± 10.6 mmHg; $p < 0.01$) in bout 3, alongside significantly lower values at 160% compared to 80% (34.4 ± 8.3 mmHg, $p = 0.02$). In bout 4, $P_{ET}CO_2$ was significantly greater at 80% W_{max} (39.0 ± 9.0 mmHg) than at any other intensity (120%: 32.0 ± 15.8 mmHg; 160%: 31.8 ± 10.3 mmHg; 200%: 27.1 ± 10.2 mmHg; all $p < 0.01$), alongside significantly lower values at 200% than either at 120% ($p = 0.01$) or 160% W_{max} ($p = 0.02$).

Within the 80% W_{max} visit, significant differences were only seen between bouts 1 and 4 ($p = 0.02$), however, at higher intensities significant differences were seen across a larger number of bouts. Specifically, at 120% W_{max}, $P_{ET}CO_2$ declined significantly following bout 1 ($p \leq 0.02$) and was significantly lower in bout 4 than in any other bout ($p < 0.01$). Similarly, at both 160% and 200% W_{max} a significant decline was seen following bout 1 ($p < 0.01$), which continued to decline over the course of repeated bouts, with significant reductions seen between bout 2 and bout 4 at both intensities (160%: $p = 0.03$; 200%: both $p < 0.01$).

A significant interaction effect between minimum/maximum point and visit showed significant rebound responses across all intensities. The interaction effect was mediated by differences in the magnitude of changes across between visits. Specifically, the magnitude of this rebound was lowest at 80% W_{max} (min: 35.4 ± 5.7

mmHg; max: 46.6 ± 5.5 mmHg; $p < 0.01$) and increased through 120% (min: 31.0 ± 9.9 mmHg; max: 45.3 ± 10.3 mmHg; $p < 0.01$) and 160% (min: 28.4 ± 9.2 mmHg; max: 43.9 ± 7.9 mmHg; $p < 0.01$), with the greatest responses seen at 200% W_{max} (min: 24.4 ± 8.7 mmHg; max: 41.6 ± 8.9 mmHg; $p < 0.01$). Both minimum and maximum points also differed significantly between visits. Across all intensities, minimum point $P_{ET}CO_2$ reached a lower value in higher compared to lower intensity visits, with these differences being significant between all intensities, except for the comparison between 120% and 160% W_{max} ($p = 0.07$). Correspondingly, maximum point values were also lower in higher compared to lower intensity visits. However, these differences were only found to be significant when comparing values at 200% to those at 80% and 120% W_{max} (both $p < 0.01$).

5.4.3.3. Timing of MCAv and $P_{ET}CO_2$ rebound responses

The timing of both minimum and maximum points within all rebound responses remained relatively unchanged across visits and bouts, with no significant differences seen in either MCA_v or $P_{ET}CO_2$ (Figure 5.13). The exception to this uniformity was found within differences in timing between minimum and maximum point, in which a significant main effect showed minimum point timing being significantly earlier than timing of maximum points across all visits and bouts ($p < 0.01$).

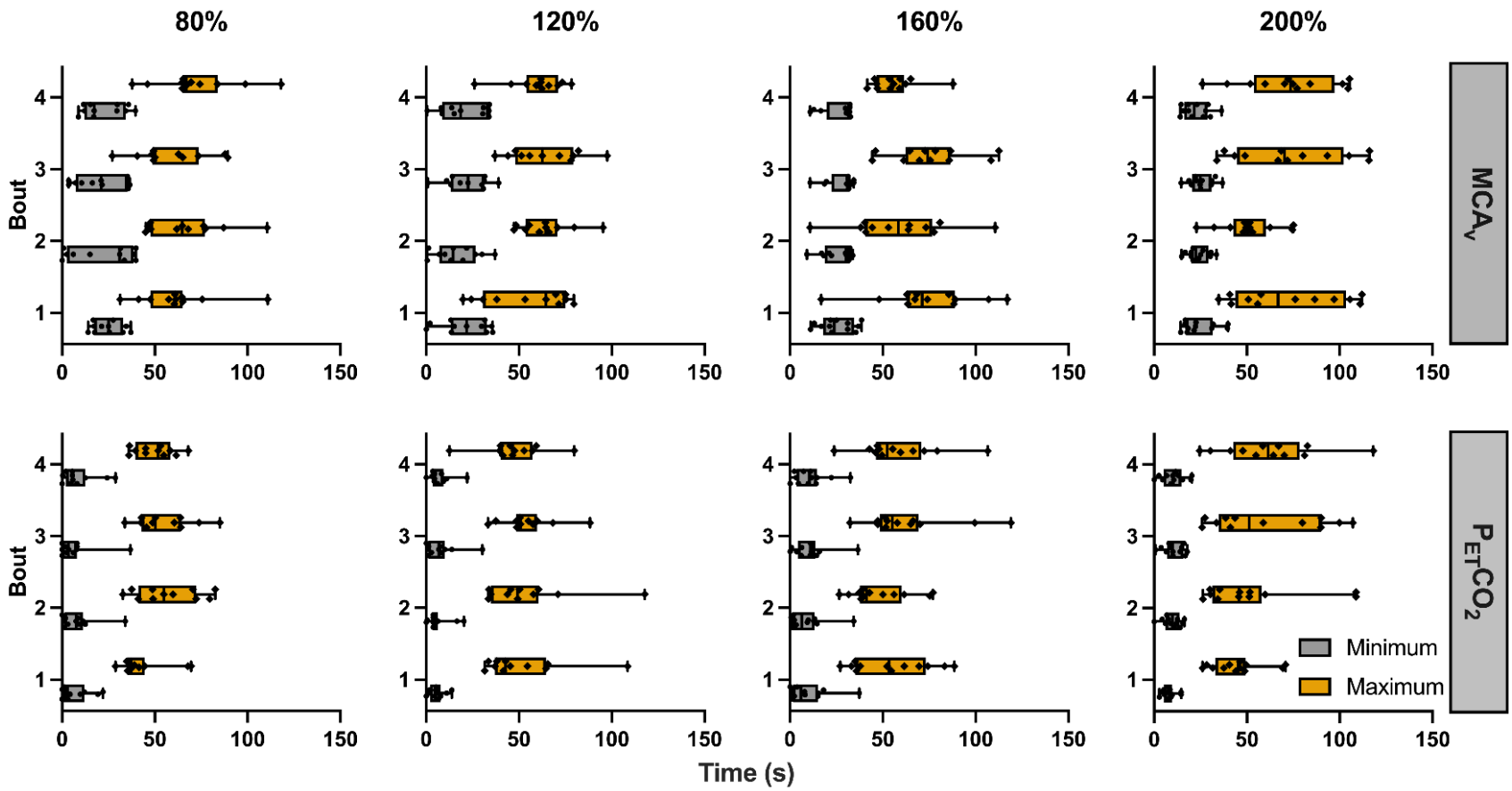


Figure 5.13 Timing of rebound responses during repeated sprint intervals at different intensities
MCA_V and $P_{ET}CO_2$ were determined continuously by transcranial Doppler ultrasound and breath-by-breath measurement, respectively. Following pre-processing by the B2B approach, automated feature selection was carried out to determine minimum and maximum points. Timing of minimum (**grey**) and maximum (**orange**) points are shown as box and whisker plots of median (horizontal line), interquartile range (box) and minimum-maximum range (whisker) alongside individual data points. Significant overall differences (analysed by linear mixed models, $p < 0.05$) between exercise intensities are denoted by ✱, with superscript signifying comparison intensity. Significant overall differences between bouts are denoted by #, with superscript signifying comparison bout.

5.5. Discussion

This study demonstrates the capacity for simple analysis processes to be used to effectively investigate temporal patterns in haemodynamics, as well as providing a clear description of more thorough methods that can be used to standardise future research analysis. The main findings of these analyses were that acute sprint bouts at a range of intensities are capable of inducing MCA_V rebound responses across multiple bouts and that the amplitude and pattern of these responses over repeated efforts appears to feature an intensity-dependent component.

5.5.1. Approaches to time series analysis

The analysis of temporal patterns in physiological data is relatively well established, although relatively specialised in the field of cerebrovascular physiology, with analysis largely limited to transfer function analysis as a means of assessing autoregulatory function (Claassen *et al.*, 2015) and more recently in the assessment of exercise onset kinetics (Billinger *et al.*, 2017, 2021). Traditionally, time-series analysis of physiological data has largely been restricted to clinical settings and evaluation of long-term trends and variability in time series, most notably the analysis of heart rate variability across a range of clinical settings (Kennedy & Turley, 2011; Faes *et al.*, 2019; Solís-Montufar *et al.*, 2020). Additionally, the majority of time series analyses both within academic and non-academic fields has focused on the analysis of predictive variables (e.g. transfer functions or heart rate variability) or on the potential to forecast future trends from time series data. Recently, attention has been given to the analysis of temporal patterns and features within time series data rather than consolidating data into single

outcome measures (Jebb *et al.*, 2015). Building on recent work by Labcreque *et al.* (2020), this study demonstrates the potential for these approaches to be taken in an exercise and vascular physiology context.

The approach taken within this chapter shows the potential for physiological time series, such as those collected during acute exercise, to be analysed at a significantly greater temporal resolution than has been common-place in previous research. While a similar approach was adopted in the work of Labrecque et al. (2020), the authors did not provide a clear and reproducible description of the method used for processing and selecting features within their data. Therefore, a primary purpose of this chapter was to develop a clear framework for analysing time series data collected via TCD and discuss the limitations and benefits of different pre-processing approaches, such that future research can utilise and build upon these processes in a similar manner to that taken in transfer function analysis (Claassen *et al.*, 2015). Two separate methods were used to pre-process time series data prior to feature selection, both of which were utilised to provide a uniform sampling rate and to smooth out both non-physiological noise and the high frequency oscillations of the cardiac cycle. The CDS approach provides a simple and clear method for time series preparation, while the B2B approach, standardises temporal pattern analysis to previously developed methods and provides greater control for the influence of the cardiac cycle within each data set.

By their nature physiological time series are often noisy and, regardless of the presence of predictable variation such as that introduced by the cardiac cycle, require smoothing to reduce the impact of noise within the data set. The simplest way to reduce the impact of noise is to smooth data using a moving average, as was done

within the CDS approach. This appears to be relatively effective in relation to the current dataset, as while the influence of the cardiac cycle of largely eliminated, the overall trend within each window is largely protected (Figure 5.11). As would be predicted, the larger the window that is applied to the data the more these features are smoothed out of the data, which means that the selection of an optimal window length is essential to strike a balance between limiting the influence of noise on feature selection and minimising the loss of physiological trends within each data set. While several different window lengths were investigated within the CDS approach, this was by no means exhaustive and future work should look to systematically identify the best smoothing approach if continuously exported data is to be processed in this manner. More complex smoothing approaches will also likely improve the capacity for noise elimination without altering trends within data, however, a clear and pre-determined set of criteria must first be established to avoid introducing confirmation bias into the processing of continuous data.

The CDS approach may also lead to over-smoothing regardless of method, as the application of smoothing processes to continuously exported raw data runs the risk of violating the assumptions of smoothing procedures. Most notably the assumption that data are locally stationary, in that the data do not have seasonal (i.e. regular rhythmic patterns) or trend (i.e. constant long-term patterns) components, or that these are accounted for when smoothing (Jebb *et al.*, 2015). However, the CDS approach was taken as a preliminary method for pre-processing for two reasons. Firstly, this approach is in keeping with the commonly taken approach to block averaging commonly used to analyse cerebrovascular haemodynamics, where a fixed averaging window is taken across raw data without removal of the cardiac cycle seasonal

component. Secondly, by providing validation of a relatively simple approach to time series analysis alongside the more complex processes within the B2B approach, we have demonstrated a simple method for a broader community of researchers to access time series analysis and to begin to explore data at higher temporal resolutions.

The B2B approach takes a more haemodynamic specific approach to time series analysis, utilising more complex pre-processing to remove the seasonal component of the time series by averaging data over each cardiac cycle. This approach has become the supported standard within transfer function analysis and has been discussed in detail in the white paper on transfer function analysis procedures (Claassen *et al.*, 2015). For the purposes of the haemodynamic analyses done in this chapter, the initial pre-processing recommendations from this white paper were followed (Recommendations 4–7), both to take an evidence-backed approach and to maximise repeatability in future research. Nevertheless, one amendment was made to recommended procedures, as across several data sets significant noise was found on visual inspection of waveforms. To counter this, LOESS curve detrending and outlier detection was applied prior to interpolation, to limit the influence of signal drop out and other forms of noise in later analysis. This approach was generally successful in eliminating significant non-physiological noise, although it failed to detect and remove outliers when repeated signal drop out was present within the data (Figure 5.5). This is indicative of the limitations of outlier removal, rather than selection of an inappropriate removal method, and highlights the importance of both visual data inspection and appropriate pre-processing. While it is not possible to eliminate all noise from physiological data, particularly when collecting data during high intensity

exercise, future research should look to minimise the influence it has on data analysis by following best practice for the collection of data via TCD (Willie *et al.*, 2011) and through robust inspection and outlier removal of data, as demonstrated within this chapter.

Following pre-processing, feature selection was carried out using automated selection of minimum points and maximum points that fitted within the confines of predetermined parameters. The choice of parameters was based on the rebound responses reported in previous literature (Curtelin *et al.*, 2018; Labrecque *et al.*, 2020; Weaver *et al.*, 2021) and ensured that the points selected for approach comparison and subsequent analysis were unbiased and aligned with previous research. However, this approach relies on the accuracy and availability of previous research; on which parameters can be set and cannot be broadly applied to feature selection when looking at novel temporal patterns. There is also an innate bias in selecting features in this way, as it requires the researcher to decide on the presence and importance of features and, particularly when looking at new and novel features, the likelihood of type I error is relatively high (Lanovaz & Hranchuk, 2021). To avoid this issue, future research should look to employ more complex methods for feature identification. For example, researchers could be blinded during feature identification and selection processing, in similar manner to the process recommended during flow mediated dilation analysis for peripheral vascular function (Corretti *et al.*, 2002). Alternatively, multiple researchers could be asked to carry out feature identification independently and subsequently compare and come to a consensus, as is commonly done in screening of papers for meta-analyses (Higgins *et al.*, 2021). The use of machine learning may allow for more rapid identification and selection optimisation, particularly when exploring large data

sets, and could allow for feature selection without the need for excessive smoothing or pre-determination of key features (i.e., unsupervised feature learning) (Längkvist *et al.*, 2014). However, taking this approach requires significantly greater technical knowledge and risks limiting usability within the wider academic community.

Regardless of the potential limitations or further development needed within this area of data analysis, the approach taken within the present study demonstrates the effectiveness of automated approaches to time series analysis. While future research should look to optimise and advance these processes to maximise their utility across different domains within the field of vascular physiology, the basic principles outlined within this study aim to promote the use of time series analysis within physiology and emphasises to accessibility and feasibility of such approaches to better explore temporal patterns in complex data.

5.5.2. Rebound haemodynamics on sprint interval exercise

The feature selection process was largely successful in identifying both the trough and peak (i.e. minimum and maximum values) within rebound responses, and clearly highlights the presence of such responses across both multiple intensities and repeated bouts. The 200% W_{max} intervals exhibited a similar response to those seen in previous data at supramaximal intensities (Weaver *et al.*, 2021), with a gradual reduction in the rebound response alongside a decrease in overall MCA_v with repeated bouts, characteristic of the hyperventilation-induced hypocapnia response associated with high intensity exercise (Ogoh & Ainslie, 2010). Interestingly, while similar responses were evident at 160% and to some extent in later bouts at 120% W_{max},

submaximal intensity sprint efforts showed a slightly increased rebound response during the second bout, with no subsequent reduction over repeated bouts.

These differences are echoed in the response patterns seen within $P_{ET}CO_2$, with relatively little suppression over repeated bouts at the two lower intensities, suggesting that relatively little change in hyperventilatory response is seen over four repeated bouts of 30-seconds at these intensities. One potential cause of these differences is in the matched recovery period over the four intensities. The impact of recovery period duration on recovery of heart rate, oxygen consumption and other cardiovascular responses is a complex topic and a still evolving field (Buchheit & Laursen, 2013). However, longer periods of recovery have been shown to be needed at higher intensities for recovery of heart rate (Seiler *et al.*, 2007), while longer recovery periods between all-out sprint efforts significantly reduces heart rate and VO_2 at the end of recovery, despite not impacting responses during subsequent bouts (Smilios *et al.*, 2018). Therefore, if less complete recovery is seen between bouts at higher intensities, this may explain the residual hyperventilatory hypocapnia and smaller rebound responses seen following four and a half minutes of recovery during 200% intervals compared to those carried out 80% W_{max}. Future research should also look to incorporate greater bout numbers alongside variations in recovery duration to determine whether modifying these two factors may contribute to hyperventilatory hypocapnia responses. Alternatively, researchers may look to pre-fatigue individuals using higher intensity intervals, followed by intervals at lower intensity, to determine whether fatigue must be combined with supramaximal intensities to see a loss of rebound response.

Within the initial hypotheses, it was predicted that the presence of rebound responses would be intensity dependent, with the presence and magnitude of responses being linked with higher sprint intensity. While some variation is seen across different intensities, rebound responses were not unique to higher intensity intervals and no significant difference was seen in the amplitude of responses. Further, the presence of rebound responses during repeated bouts was more consistent at lower intensities. Additionally, the work of Labrecque *et al.* (2020) showed that a single 30-second sprint bout at 100 W_{max} is also sufficient to induce significant rebound responses following the end of the sprint effort. These findings suggest that even sub-maximal sprint efforts may be capable of inducing transient elevations in blood flow and drive comparable adaptive responses to those seen in all-out sprint efforts, with the additional benefit of these responses being inducible over repeated sprint efforts. However, whether such rebound responses are adaptive (or maladaptive) for the vasculature remains speculative, as intervention studies in humans focused on this question have not been conducted. Nevertheless, while it is not well understood whether transient or sustained elevations are blood flow are optimal for driving adaptive changes in function, *in vitro* research does suggest that mechanosensory responses are greatest at the onset of changes in flow (see Chapter 2). Furthermore, ischemic preconditioning (IPC); which induces a temporal pattern similar to the suppression and subsequent rebound responses shown in the present study, has been shown to drive improvements in endothelial function after 7-days of IPC consisting of 5 repeated bouts of occlusion-reperfusion (Jones *et al.*, 2014). While further research is needed, sprint interval exercise may drive similar adaptive responses to those seen in IPC, particularly when interval intensity is optimised to drive significant rebound responses across repeated bouts.

The capacity for lower intensity intervals to induce rebound responses raises the question as to what the driving factor in sprint rebound responses is, as intensity alone cannot explain this phenomenon. Interestingly, previous research showed no rebound responses in 4-minute interval exercise at similar intensities (Weaver *et al.*, 2021). The disparity between responses under these two protocols indicates that not only the intensity but also the duration of interval bouts may play a significant role in the presence of rebound responses. The mechanism driving this difference between interval durations is not immediately apparent. It may be that performing high intensity efforts for longer periods of time results in stabilisation of respiratory and cerebrovascular responses during the bout, which subsequently limits the reactive response during the early stages of recovery. Alternatively, as the rebound responses seen during 30-second intervals appear to be driven by post-exercise increase in $P_{ET}CO_2$ (Labrecque *et al.*, 2020), if longer interval durations result in prolonged hyperventilatory hypocapnia this could prevent rapid changes of $P_{ET}CO_2$ at the onset of recovery and thereby eliminates the subsequent rebound in MCA_v. Future studies are needed to determine the relationship between interval duration and rebound responses following high intensity efforts, as well as how the effects of duration interact with the intensity-dependent variations shown within the current study.

Previous research looking at temporal patterns in single sprint bouts showed an initial elevation in MCA_v that was comparable in amplitude to those seen in recovery rebound responses (Labrecque *et al.*, 2020). This biphasic pattern was shown to be driven by two differing factors. Specifically, the initial elevations in MCA_v at the onset of the sprint bout were driven by simultaneous elevations in MAP, despite a reduction in $P_{ET}CO_2$,

while rebound response post-sprint were accompanied by clear elevations in $P_{ET}CO_2$, without an elevation in MAP. Further, the magnitude of the two peaks were similar for the initial and recovery MCAv data. By contrast, the findings within the current study did not exhibit this same biphasic pattern, with only a small elevation at the onset of bouts seen in higher intensity sprints, if at all, and these increases did not match the amplitude of elevations in sprint rebound responses (Figure 5.11). Most notably, no clear onset response was seen at 80% and 120% W_{max}, the two intensities that most closely matched the intensity used by Labrecque *et al.* (2020). The likely explanation for the differences in onset response between these two studies lies in the specific design of the exercise protocols. Labrecque *et al*. (2020) had participants rest passively for 3-minutes prior to the start of the sprint bout, while the current study design had participants commence each bout from a standardised workload of 50W. While not measured within the current study, the temporal patterns shown by Labrecque *et al.* (2020) indicate that the key driving factor is likely the influence that protocol choice had on changes in blood pressure. The impact that differences in exercise protocol have on blood pressure is not well understood, however, the impact that changes in posture, movement and force production can have on blood pressure and MCA_v has been explored in other areas of research. For example, during squat-stand manoeveurs the engagement of quadriceps alone during the squat phase, without any additional loading, results in increases in MAP of ~20–30 mmHg and ~40–60 $cm.s^{-1}$ elevations in MCA_v (Barnes *et al.*, 2017). Significant research has also been conducted into the temporal patterns in both blood pressure and MCA_v during resistance exercise. During isometric leg extensions at 50% of maximal voluntary contraction, a significant elevation in MCA_v (~20 $cm.s^{-1}$) immediately after the onset of contraction, paired with elevations in MAP (~30 mmHg), are followed by subsequent

significant decline in both factors as the contraction is held (Perry *et al.*, 2020). These changes are highly dependent on the nature of the exercise, with MAP changes being both intensity- and duration-dependent (Perry & Lucas, 2021). While these responses to squat-stand manouvers and resistance exercise cannot be directly translated to the differences seen between sprint interval protocols, they do demonstrate that the onset of muscle contraction results in large elevations in MAP and MCA_v, and the intensity-dependent nature of these changes is likely responsible for the differences seen between sprint intervals carried out from a resting position or a “rolling start”. Future research should look to answer this question more specifically, by characterising the force production and changes in blood pressure that are seen with different pre-sprint protocols and how these differences may impact cerebrovascular haemodynamics.

Understanding these differences in protocol are not only essential to better determine how we can optimise exercise protocols to drive improvements in cerebrovascular function, but also to identify and mitigate the potential risks that interval-induced changes in haemodynamics may place on the cerebrovasculature. Rapid elevations in cerebrovascular blood flow (i.e. hyperperfusion) during interval-based exercise have been suggested to increase the risk of damage to the cerebrovasculature and blood brain barrier, particularly if these changes are not met with a comparable neuroprotective response (Lucas *et al.*, 2015; Labrecque *et al.*, 2020). While the capacity for the cerebrovasculature to regulate blood flow in response to fluctuations in blood pressure is still widely debated, a growing consensus within the field is that the capacity for dynamic autoregulation to maintain stable cerebral blood flow is challenged as the rate of change in blood pressure increases (Claassen *et al.*, 2021). For this reason, although research to date shows comparable adverse event risk

between high intensity interval training and moderate intensity continuous exercise (Rognmo *et al.*, 2012; Wewege *et al.*, 2018), caution should be taken in recommending short, sprint-based interval exercise to high-risk cohorts until the nature and mechanisms driving sprint rebound responses have been fully understood.

5.5.2.1. Limitations and Future Directions

As already mentioned, measures of blood pressure were not collected within the current study. While this measurement would have provided a more complete discussion of the mechanisms driving rebound response, reliable measurement of continuous, beat-to-beat blood pressure during supramaximal intensities is not possible without the utilisation of invasive measures, such as those used by Curtelin et al. (2018) in their examination of Wingate sprint responses. This is largely due to the reliance on finger cuff inflation for the non-invasive measurement of blood pressure. This measurement requires the participant to avoid tightly gripping the handlebar of the cycle ergometer, to minimise noise and maintain a clear signal during sprint bouts. Interestingly, research has shown that reliable, non-invasive measure of blood pressure can be obtained at higher, non-maximal intensities (Labrecque *et al.*, 2020) and future research should look to incorporate this measure whenever feasible.

The current study utilises data taken from a pilot cohort, including incomplete participant datasets, due to unforeseen circumstances that curtailed data collection (i.e. restricted laboratory access due to the COVID pandemic response). Nevertheless, these data were sufficient to demonstrate clear and consistent rebound responses, comparable at 200% W_{max} to those seen in previous research (Curtelin *et al.*, 2018; Weaver *et al.*, 2021). However, it is likely that the current data lacked

sufficient power to statistically detect more subtle differences in rebound responses between bouts and intensities. For this reason, while the data collected have clearly demonstrated the efficacy and utility of the novel analytical approach developed within this chapter, future research is needed to build upon these findings in a larger, well-powered cohort, alongside expansion of this method into other exercising contexts.

Finally, the application of TCD as a proxy for cerebral blood flow has been shown to have inherent limitations, due to the capacity for the middle cerebral artery to vasodilate in response to increases in blood pressure and circulating CO_2 (Hoiland & Ainslie, 2016; Verbree *et al.*, 2017). The utility of TCD in light of this limitation has been discussed at length previously (Ainslie & Hoiland, 2014), however, it is important to highlight that the probable change in diameter during exercise would be vasodilatory in nature and this would result in underestimation of blood flow values from measures of MCA_v. Further, while alternative approaches including MRI and duplex ultrasound may offer a more accurate or higher resolution examination of cerebral blood flow, their flexibility and feasibility in an exercise context is limited and as such TCD is still the most efficacious measure within exercise-based studies (Ainslie & Hoiland, 2014).

5.5.3. Conclusion

The current study demonstrates that sprint-based intervals of 30-seconds can induce rebound responses in measures of MCA_v and $P_{ET}CO_2$ across a wide range of intensities and over multiple bouts. Lower intensities appear to drive more reproducible rebound responses across multiple bouts, whereas higher intensity bouts result in a steady decline in rebound amplitude as a result of increased hyperventilatory hypocapnia. The methods used to examine these responses present

a new and highly adaptable approach to the analysis of cerebrovascular data. The detailed presentation of the methods used, data derived and discussion around this approach is intended to allow future research to utilise and build upon these methods, to develop novel insights into the temporal patterns induced by exercise and other acute interventions.

6. THE IMPACT OF CHANGES IN CIRCULATING MICROENVIRONMENT ON THE ENDOTHELIUM: AN *EX VIVO* STUDY

6.1. Introduction

As demonstrated in Chapter 4, exercise has the capacity to drive transient changes in a wide range of circulating molecules, with varying responses seen dependent on the exercise protocol employed. Many of these molecules have been suggested to drive beneficial responses within the brain and cerebrovasculature (Voss *et al.*, 2013*b*) and, as discussed in Chapter 2, the direct impact of many of these vasoactive and neurotrophic factors on the endothelium has also been investigated in an *in vitro* context. The potential influence of the circulating microenvironment is complex, not only having the potential to induce unique responses through binding to factor-specific receptors; including the VEGF and IGF signalling pathways (Delafontaine *et al.*, 2004; Simons *et al.*, 2016), but also through the modulation of mechanosensitive pathways. For example, KLF2, which plays a central role in responses to elevation in flow, has been seen to also be downregulated in response to IL-1B (SenBanerjee *et al.*, 2004), while CELC14a; which drives angiogenic responses in low and perturbed flow, has been shown to interact with both VEGF receptor pathways (Lee *et al.*, 2017) and HSP70-1A-induced angiogenesis (Jang *et al.*, 2017). To date, the majority of this research has focused on the isolated impact of a single or small number of factors in highly controlled concentrations, rather than the influence that the full complement of changes seen *in vivo* can have on the endothelium and how differences in the responses seen across different interventions or exercise protocols may alter this cellular response.

One means by which to evaluate the response of the endothelium to changes seen in the circulating environment is the *ex vivo* application of serum or plasma taken from participants. *Ex vivo* serum application is seen most prominently in clinical research

settings, where the method has been used in efforts to improve transplant and stem cell treatment, through the culturing of cells for transplant in human serum (Castells-Sala *et al.*, 2017; Saury *et al.*, 2018). However, this approach has also been adapted to better understand cellular responses to intervention-induced changes in the circulating microenvironment, particularly in a human context where the isolation of cells from many tissues is difficult or not possible. Pioneering work by Bruckbauer & Zemel (2011) in skeletal muscle established the utility of *ex vivo* approaches within physiology, where the impact of dairy consumption in humans was analysed in relation to its effects on skeletal muscle function *in vitro*. Subsequently, similar approaches have been used to determine the effects of age-related differences in circulating microenvironment (Kalampouka *et al.*, 2018) and dietary changes on muscle function (Carson *et al.*, 2018; Lees *et al.*, 2021). While this approach has seen limited application in vascular physiology, Brunt et al. (2018) recently highlighted the potential utility of *ex vivo* serum application to study the endothelial impacts of heat therapy in limiting the inflammatory and oxidative stressor effects of hypoxia in HUVEC.

6.1.1. Aims & Hypothesis

The aim of this study was to investigate changes in endothelial gene and protein expression in response to the *ex vivo* application of samples taken from participants over the duration of three different exercise protocols (detailed in Chapter 4.2.4). We hypothesised that the application of samples taken following a sprint-interval training (SIT) protocol would have the greatest impact on endothelial gene and protein expression, due to the presence of the greatest changes in circulating factors within this condition (Chapter 4.3.2).

6.2. Methods

Within the following experiments, *ex vivo* application of serum and plasma (see Materials & Methods 3.7) was used to model endothelial responses when exposed to the circulating environment seen during acute bouts of exercise. HUVEC were treated with samples taken from 8 participants (4 female) at rest (denoted as Pre within this chapter) and immediately following active recovery (denoted as Post), across three different acute exercise bouts (MICT, HIIT and SIT; see Chapter 4.2.4). Participant samples were selected based on age, relative fitness and BMI, with selection being matched across male and female participants (see for Appendix 35 details). Average measures of BDNF, VEGF, IGF-1 and lactate for this sub-cohort are shown in Table 6.1.

Table 6.1 Pre- and post-exercise values for circulating factors measured in participant samples used in comparison of HUVEC responses.

	MICT		HIIT		SIT	
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**
Plasma-Derived Measures						
BDNF (n=7) ($pg \cdot mL^{-1}$)	103.6 ± 57.6	209.7 ± 156.8	142.5 ± 103.0	181.1 ± 133.2	139.6 ± 141.2	273.6 ± 196.7
VEGF (n=6) ($pg \cdot mL^{-1}$)	863.9 ± 1498.1	785.9 ± 1298.0	704.1 ± 1153.1	928.1 ± 1565.6	703.5 ± 1190.2	1126.48 ± 2000.6
IGF-1 (n=5) ($ng \cdot mL^{-1}$)	3312.1 ± 1663.6	3312.1 ± 1422.7	2806.1 ± 1495.3	2949.3 ± 1086.1	3283.8 ± 2059.9	2296.8 ± 725.1
Serum-Derived Measures						
Lactate (n=8) (mM)	1.1 ± 0.2	2.7 ± 1.3	1.1 ± 0.3	7.1 ± 3.1	1.8 ± 1.6	20.6 ± 4.2

The impact of *ex vivo* treatments were assessed across exercise conditions in relation to changes in HUVEC gene and protein expression (see Materials & Methods 3.8). With all data analysed by linear mixed effect model (Materials & Methods 3.9), with

treatment condition (Control; MICT, HIIT & SIT; Pre & Post) and treatment duration as fixed factors and participant ID as a random factor. Post hoc testing for interactions were carried out with pairwise comparison and Tukey Test adjustments for multiple comparison. All results are presented as mean ± standard deviation (mean±SD) unless otherwise stated.

6.3. Results

6.3.1. Gene expression responses to ex vivo serum treatment

To study changes in gene expression, commercially available HUVEC were treated with 20% serum collected from participants for 10-120 minutes (Figure 6.1Figure 6.6). Linear mixed effects modelling showed a significant effect of treatment over control, but no differences between treatment conditions, for response in *CLEC14a* ($p < 0.01$; Figure 6.1), *IGFBP3* ($p = 0.02$; Figure 6.3), and *KLF2* ($p = 0.05$; Figure 6.4). These responses were seen alongside significant effects across treatment duration for *CLEC14a* ($p < 0.01$), and *KLF2* ($p = 0.04$), without significant differences within treatment conditions across exposure durations. Serum treatment had no significant effects on expression of *HIF1α* (Figure 6.2), *VEGFA* (Figure 6.5), or *KDR*/*VEGFR2* (Figure 6.6), irrespective of either treatment condition or exposure time.

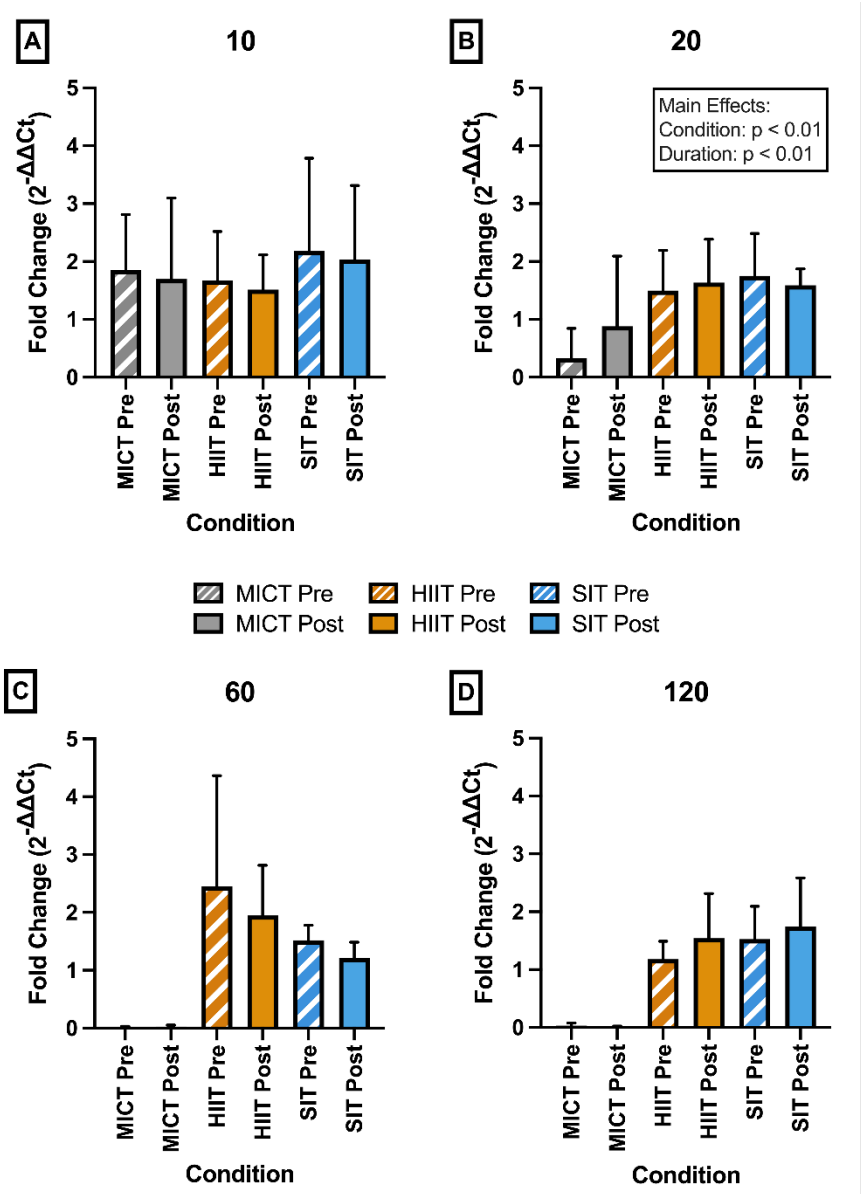


Figure 6.1 Effect of *ex vivo* serum treatment on *CLEC14a* expression.
HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *CLEC14a* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of A:MICT Pre (n = 6); B:MICT Post, D:HIIT Pre, D:SIT Pre and D:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

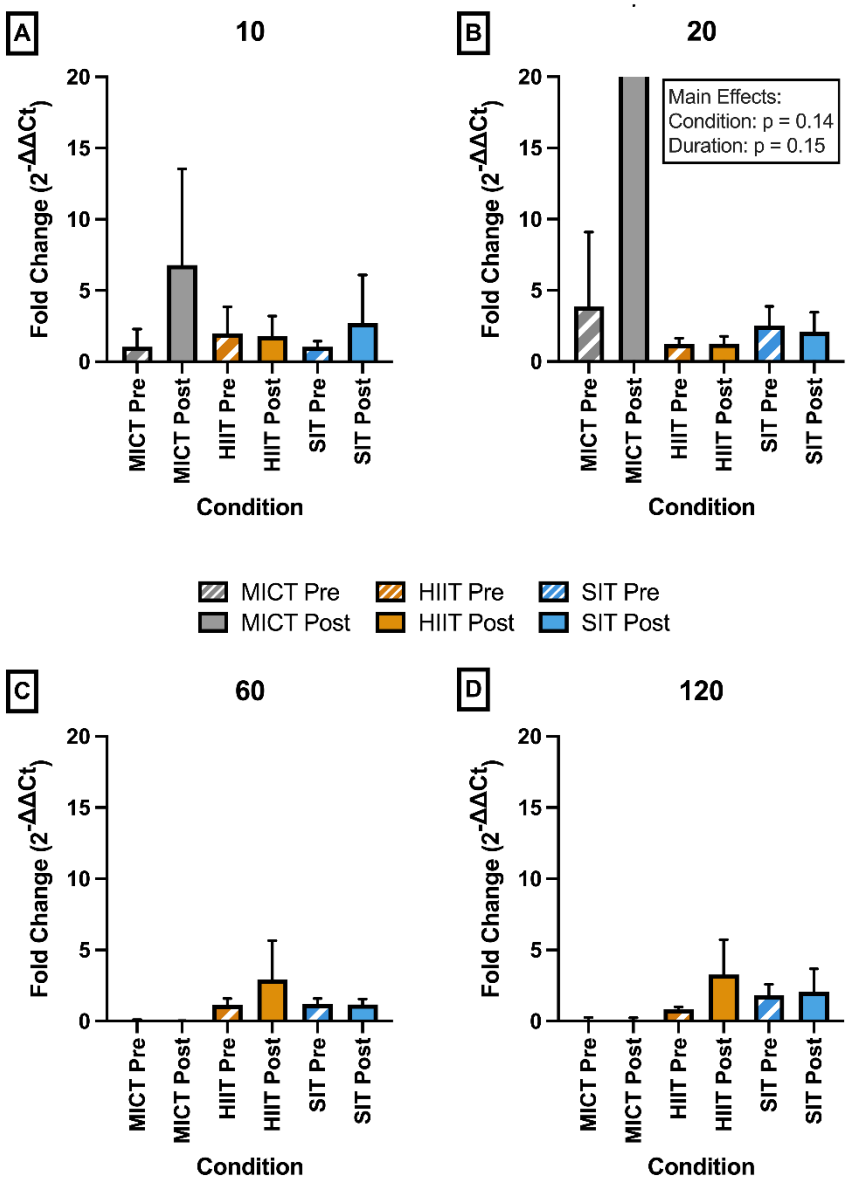


Figure 6.2 Effect of *ex vivo* serum treatment on *HIF1α* expression.
HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *HIF1α* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of D:MICT Pre (n = 4); A:MICT Pre (n = 5); C:MICT Pre, C:MICT Post, D:MICT Post, B:HIIT Pre, C:HIIT Pre, D:HIIT Pre, B:HIIT Post, A:SIT Pre, C:SIT Pre, (n = 6); and A:HIIT Pre, A:HIIT Post, C:HIIT Post, A:SIT Post, B:SIT Post, C:SIT Post and D:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

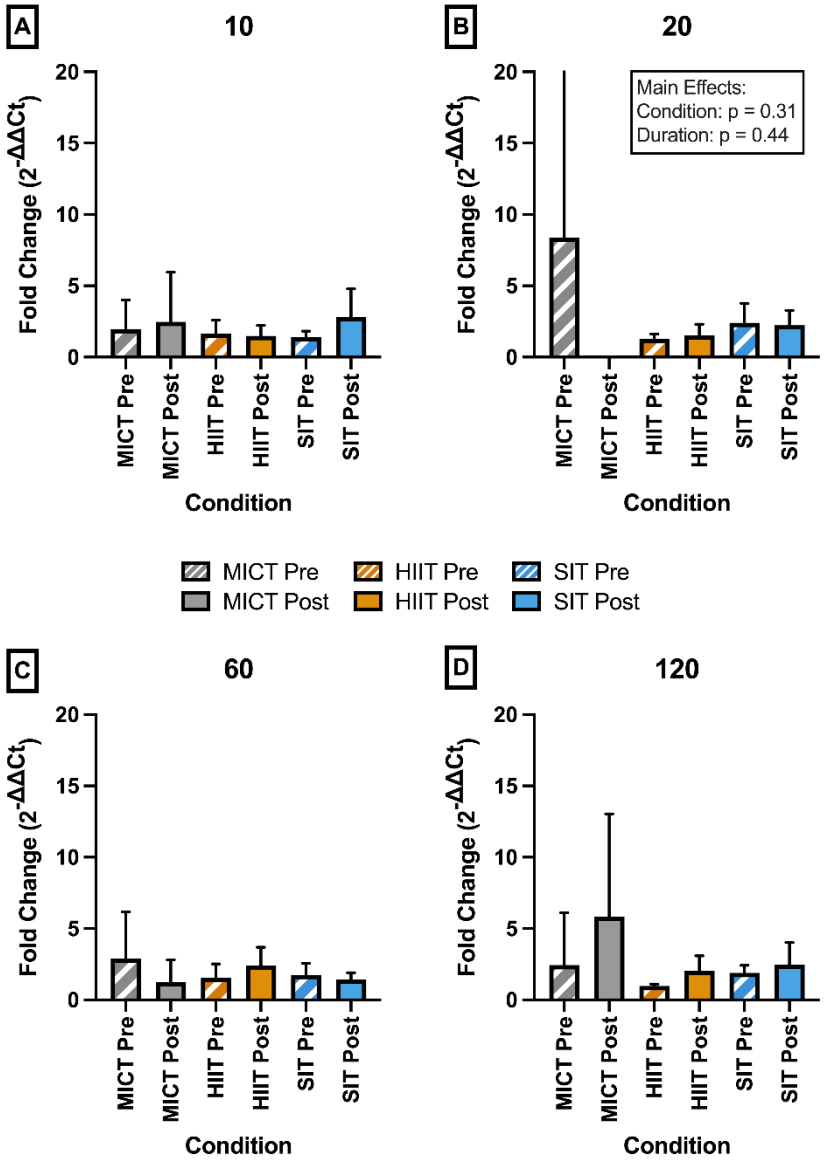


Figure 6.3 Effect of *ex vivo* serum treatment on *IGFBP3* expression.
HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *IGFBP3* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of A:MICT Pre (n = 2); B:MICT Pre (n = 3); C:MICT Pre, A:MICT Post, B:MICT Post, C:MICT Post (n = 4); A:MICT Pre (n = 5); D:HIIT Pre, A:SIT Pre, D:SIT Pre (n = 6); C:HIIT Pre, C:SIT Post; D:MICT Pre, D:SIT Post (n = 7); and D:MICT Post (n = 8). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

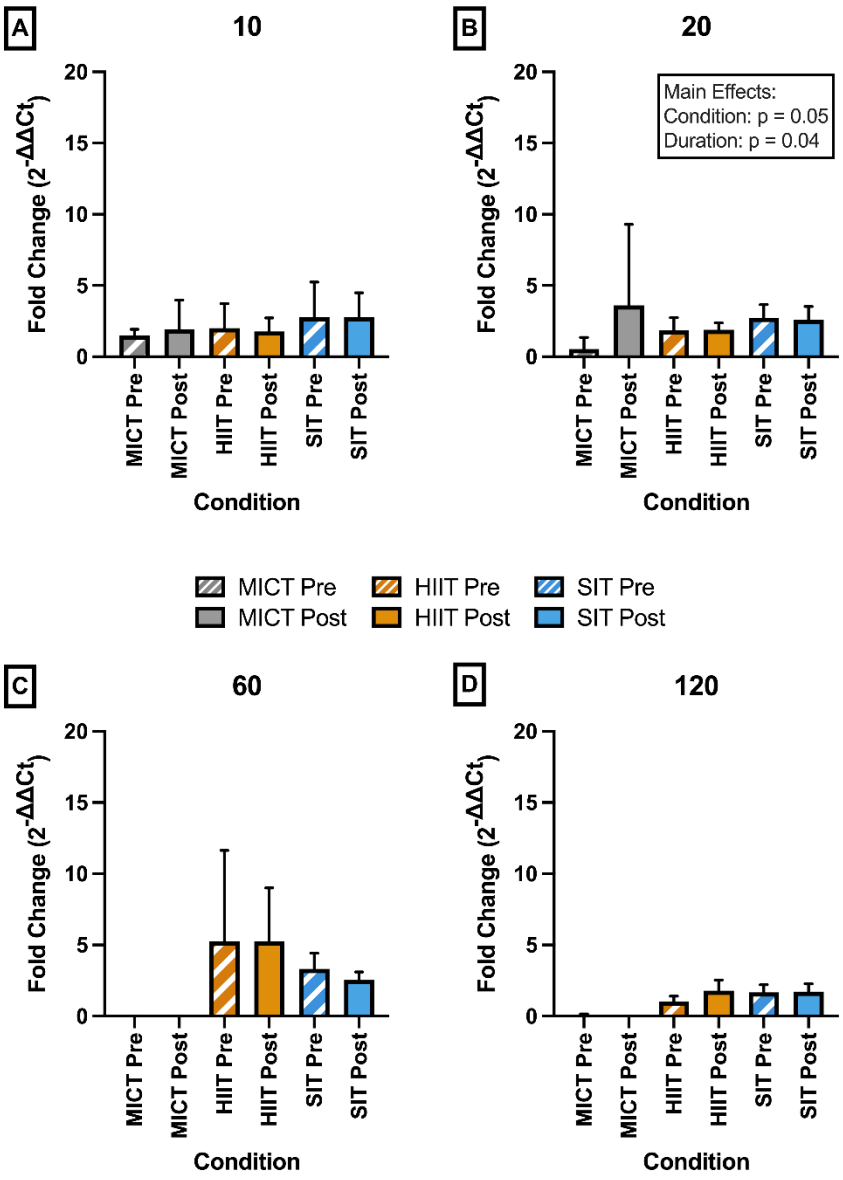


Figure 6.4 Effect of *ex vivo* serum treatment on *KLF2* expression.
HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *KLF2* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of D:MICT Pre, D:SIT Post (n = 6); A:MICT Pre, B:MICT Pre, D:MICT Post, D:HIIT Pre, D:HIIT Post and C:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

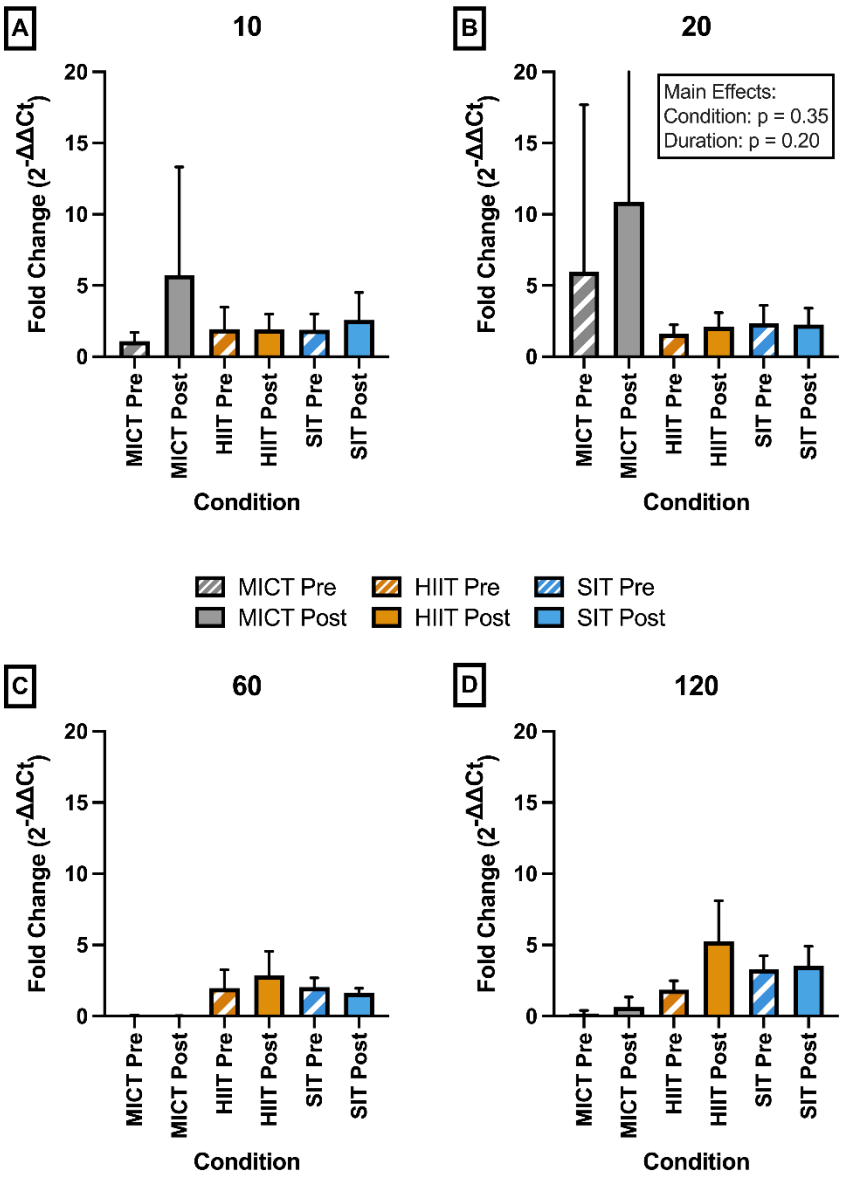


Figure 6.5 Effect of *ex vivo* serum treatment on *VEGFA* expression.
HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *VEGFA* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of D:MICT Pre (n = 5); A:MICT Pre, B:MICT Pre, C:MICT Pre, D:SIT Post (n = 6); A:MICT Post, B:MICT Post, C:MICT Post, C:HIIT Pre, D:HIIT Pre, A: SIT Pre, D:SIT Pre, C:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

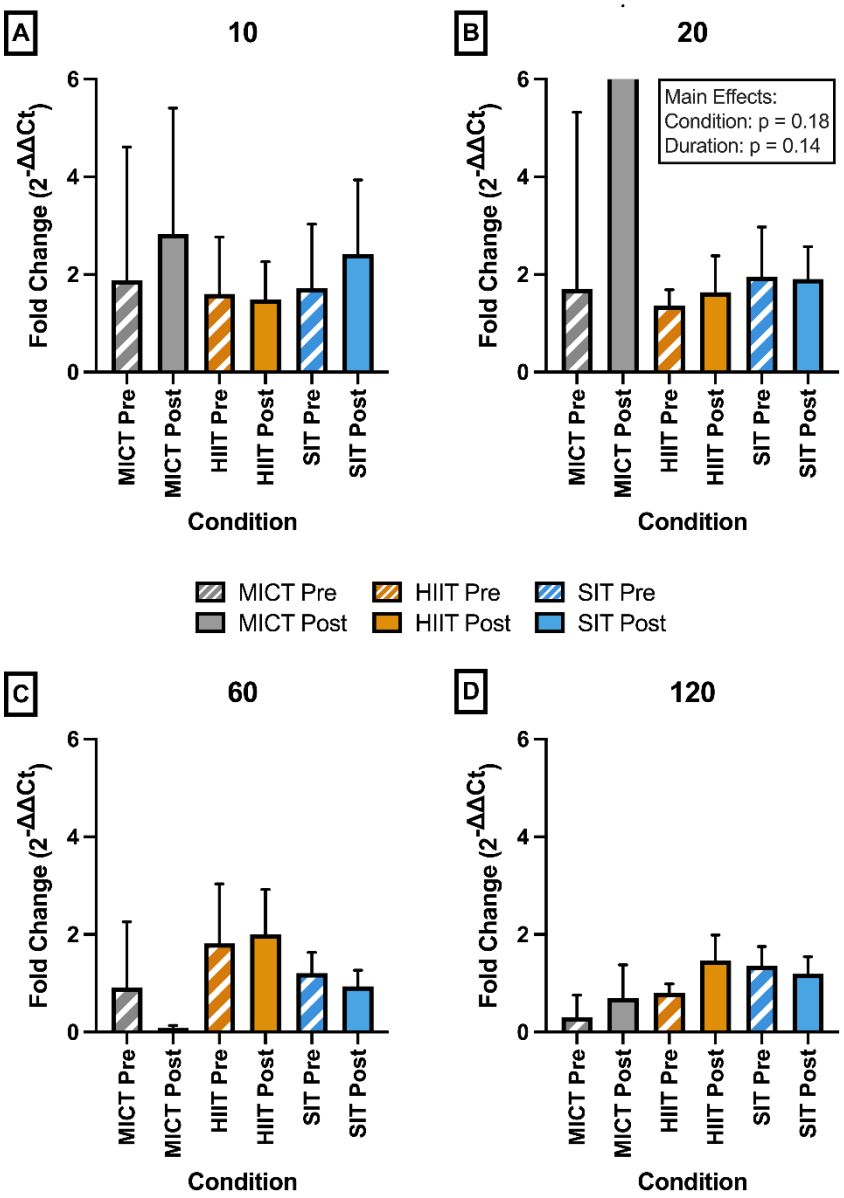


Figure 6.6 Effect of *ex vivo* serum treatment on *KDR/VEGFR2* expression. HUVEC were treated with 20% serum samples and cultured for 10 (**A**), 20 (**B**), 60 (**C**), and 120-minutes (**D**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *KDR/VEGFR2* was examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of D:MICT Pre (n = 5); A:MICT Pre, B:MICT Pre, C:MICT Post, D:MICT Post, D:SIT Post (n = 6); C:MICT Pre, A:MICT Post, B:MICT Post, B:HIIT Pre, D:HIIT Pre, A: SIT Pre, D:SIT Pre, C:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

To explore the potential impact of longer serum treatment durations on gene expression, HUVEC were treated with the same *ex vivo* serum samples for 240 and 360-minutes. Linear mixed effect modelling showed significant effects for treatment over control for *CLEC14a* ($p = 0.03$; Figure 6.7) and *KLF2* ($p = 0.04$; Figure 6.8), alongside a significant effect across treatment duration for *IGFBP3* ($p = 0.03$; Figure 6.7), however, statistical analysis showed no significant interactions between treatment conditions or durations. Further, prolonged serum treatment did not significantly alter gene expression of *HIF1α* (Figure 6.7), *VEGFA* (Figure 6.8), or *KDR*/*VEGFR2* (Figure 6.8). While non-significant elevations in gene expression were seen in MICT Pre and HIIT Post after 360-minutes of treatment across all genes, these were accompanied by large increases in standard deviation at these data points (Figure 6.7Figure 6.8). Similarly, greater increases were visible after 360-minutes compared to 240-minutes across all genes and treatment conditions, however, these increases were matched by increases in variability.

Overall, these findings suggest that although treatment with human serum did result in changes in gene expression, these changes were not highly variable across participant samples and did not display clear differences in effect between pre- and post-exercise conditions.

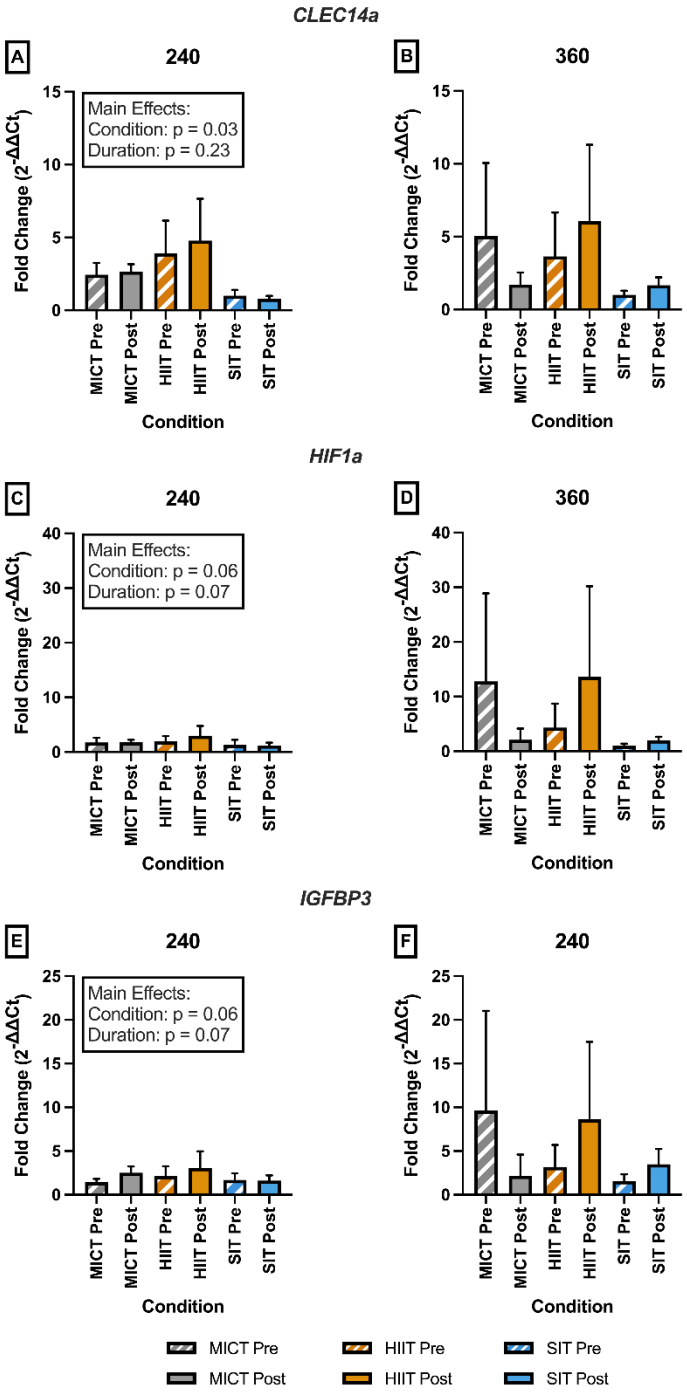


Figure 6.7 Effect of prolonged *ex vivo* serum treatment on *CLEC14a, HIF1a,* and *IGFBP3* gene expression.

HUVEC were treated with 20% serum samples and cultured for 240 (**A, C, E**) or 360-minutes (**B, D, F**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *CLEC14a, HIF1a,* and *IGFBP3* were examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of A:SIT Post, B:MICT Post, D:MICT Post, F:MICT Post (n = 6); B:SIT Post, C:SIT Post, D:SIT Post, E:MICT Pre, E:SIT Post and F:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

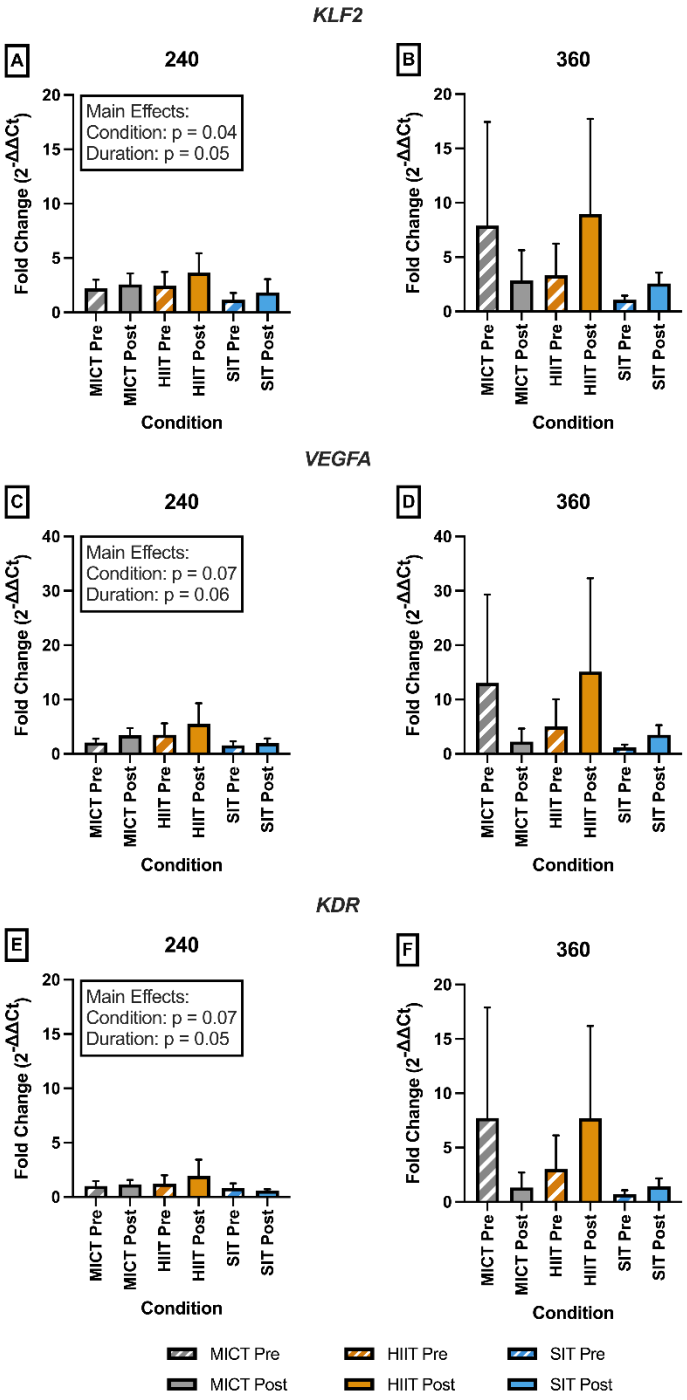


Figure 6.8 Effect of prolonged *ex vivo* serum treatment on *KLF2, VEGFA* and *KDR* gene expression.

HUVEC were treated with 20% serum samples and cultured for 240 (**A, C, E**) or 360-minutes (**B, D, F**). Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of MICT, HIIT, and SIT exercise sessions. Gene expression for *KLF2, VEGFA* and *KDR* were examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, with the exception of B:MICT Post, D:MICT Post, E:SIT Post, F:MICT Post (n = 6); A:SIT Pre, A:SIT Post, B:MICT Pre, B:SIT Post, C:MICT Pre, C:SIT Post, D:SIT Post and F:SIT Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with main effects shown inset and significant interaction effects denoted by ✱.

6.3.2. Ex vivo treatment with plasma in comparison to serum treatment

Previous research has shown that significant differences are seen in the molecular and metabolite composition of plasma and serum (Yu *et al.*, 2011; Lima-Oliveira *et al.*, 2018). Given the lack of clear responses to serum treatment, the potential for plasma to induce different changes in gene expression was explored. To determine this, the impact of Pre and Post samples within the SIT condition were re-assessed at 60 and 120-minutes using plasma samples taken from the same cohort of participants as previously used in serum treatments.

Treatment with plasma resulted in a more consistent profile of gene expression across conditions and treatment durations, with relatively small fold changes from control shown across all genes (Figure 6.9). Linear mixed effects modelling showed significant effects for treatment over control for *HIF1a* ($p = 0.03$; Figure 6.9B) and significant effects for treatment duration in *VEGFA* ($p = 0.02$; Figure 6.9B). While significant interaction effects were seen in *CLEC14a* only (Figure 6.9A), in which a significant interaction effect between treatment condition and exposure time was found. Post-hoc analysis showed a significant increase in expression of *CLEC14a* from SIT Pre to Post after 60-minutes of treatment ($p = 0.03$), coupled with a significant reduction in expression in SIT Post after a further 60-minutes of treatment ($p = 0.02$; Figure 6.9A).

Correlation analysis of the fold change in gene expression for all targets in response to treatment with plasma and serum revealed no significant correlation between the two sample types (all $p \geq 0.20$; Figure 6.10).

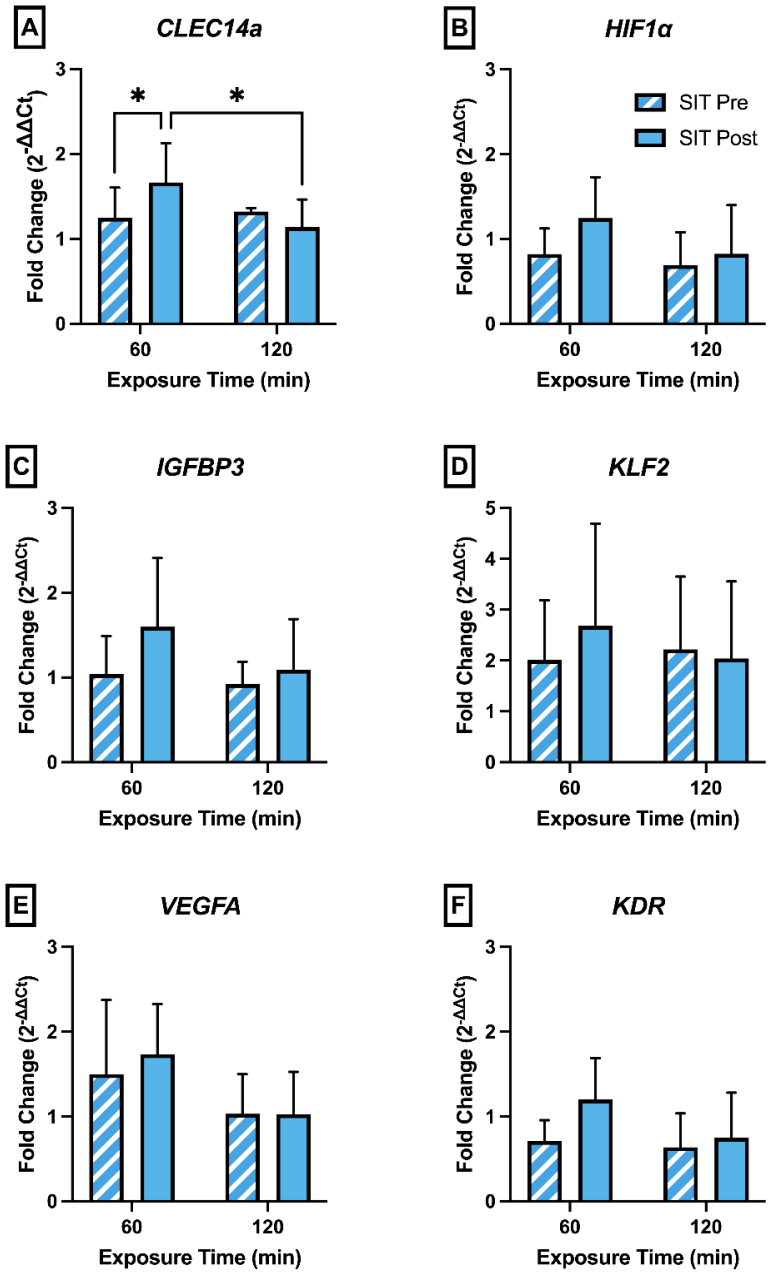


Figure 6.9 Effects of *ex vivo* plasma treatment of HUVEC on their gene expression.
HUVEC were treated with 20% plasma samples and cultured for 60 or 120-minutes. Serum samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of SIT exercise sessions. Gene expression for *CLEC14a* (**A**), *HIF1a* (**B**), *IGFBP3* (**C**), *KLF2* (**D**), *VEGFA* (**E**) and *KDR* (**F**) were examined by qPCR. Outliers were determined by ROUT analysis and resulted in n = 8 for all datasets, except for A:120 Pre, C:120 Pre (n = 6); C:120 Post, E: 120 Pre, E:60 Post and E:120 Post (n = 7). Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with significant interaction effects denoted by ✱.

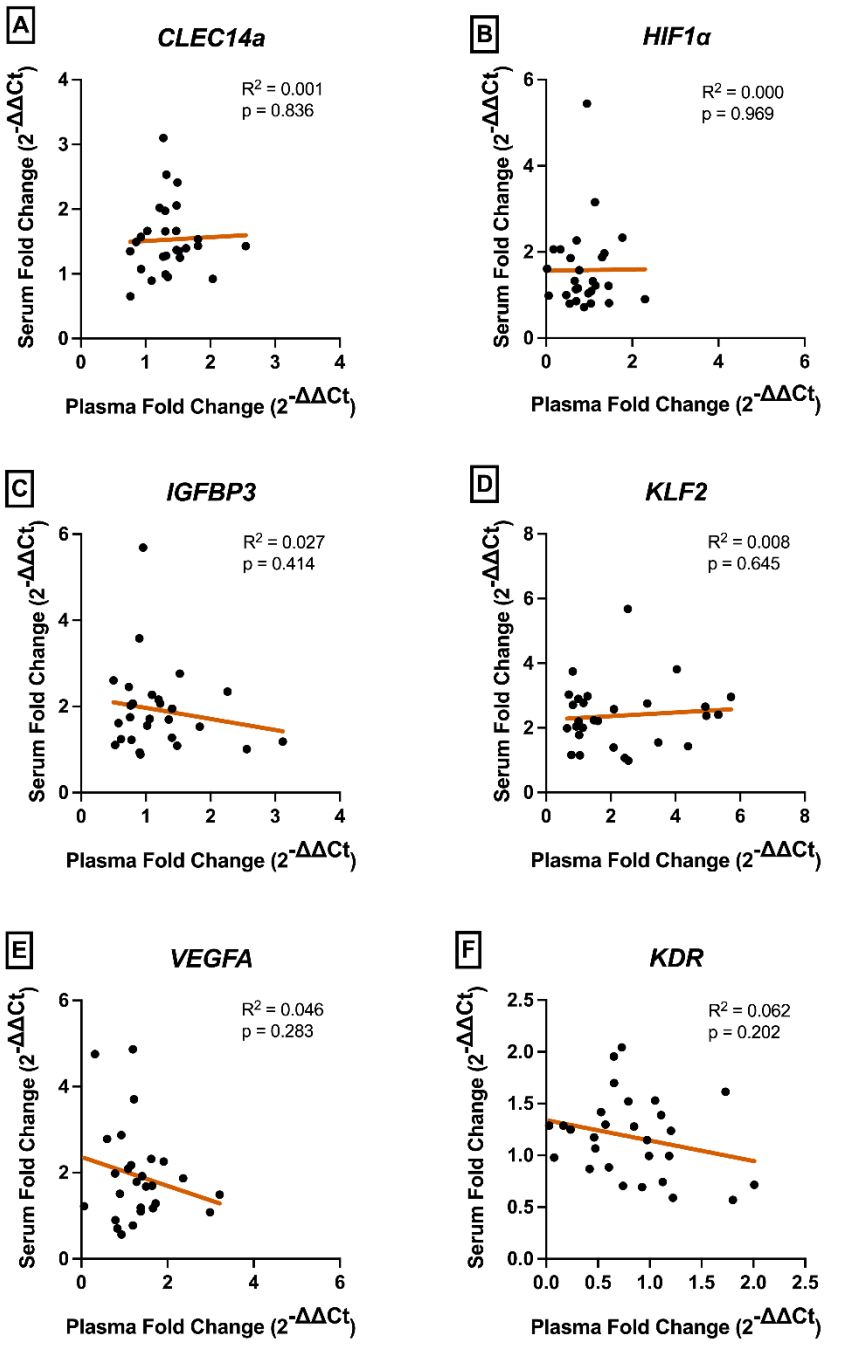


Figure 6.10 Correlation between *ex vivo* plasma and serum treatment of HUVEC on their gene expression.
HUVEC were treated with either 20% plasma (**x-axis**) or 20% serum (**y-axis**) and cultured for 60 or 120-minutes. Samples were taken from 8 participants (4 female) during rest (**Pre**) and after (**Post**) completion of SIT exercise sessions. Gene expression for *CLEC14a* (**A**), *HIF1α* (**B**), *IGFBP3* (**C**), *KLF2* (**D**), *VEGFA* (**E**) and *KDR* (**F**) were examined by qPCR. Outliers were removed prior to correlation using ROUT analysis and only data with both plasma and serum values were plotted. Red lines denote linear regression line of fit, with related R^2 and p-value for the line inset.

6.3.3. Protein activation in response to ex vivo plasma treatment

To explore the effects of exercise-induced changes in circulating secretome, non-commercially isolated HUVEC were treated with previously collected human plasma samples. Preliminary experiments showed that application of plasma supplemented media (M199 basal media containing 20% human plasma and 100 U·mL^{-1} Penicillin-Streptomycin) resulted in reversal of the anti-coagulant effects of EDTA resulting in media coagulation (Figure 6.11). To mitigate this reversal, sodium heparin (Sigma-Aldrich) was added to plasma samples prior media preparation at a final concentration of 90 µg·mL^{-1}, to match the hepain content within Endothelial Cell Growth Medium (PromoCell).

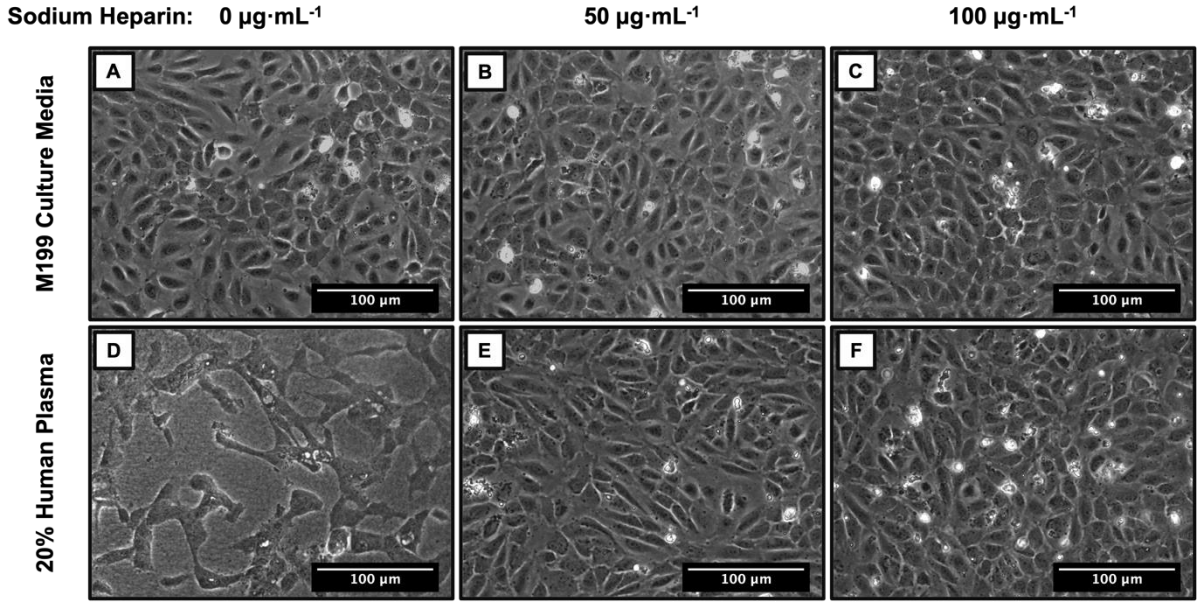


Figure 6.11 Effects of treating HUVEC with sodium heparin supplemented human plasma
HUVEC were cultured for 24-hours in either M199 culture media (A – C) or M199 basal media supplemented with 20% human plasma and 100 U·mL^{-1} Penicillin-Streptomycin (D – F). Sodium heparin was added in a subset of experiments at a concentration of either 50 µg·mL^{-1} (B & E) or 100 µg·mL^{-1} (C & F), to determine the capacity for coagulating effects of plasma supplementation to be reversed. Scale bar = 100 µm.

To assess protein activation and signal cascade responses in HUVEC following *ex vivo* plasma treatment, cell lysates were analysed by western blot for phosphorylated and total content of Akt, ERK1/2, eNOS and VEGFR2. Prior to analysis of protein content in experimental samples, antibodies were validated for function and specificity to their respective target proteins (Figure 6.12–Figure 6.15). Both phosphorylated and total protein antibodies showed specific binding for Akt at 60 kDa (Figure 6.12), eNOS at 140 kDa (Figure 6.13) and ERK1/2 at 42 kDa (Figure 6.14). However, on membranes incubated with antibodies targeting p-Akt^{Ser473} (Figure 1.12A), total-eNOS (Figure 1.13B) and p-ERK1/2$^{Thr202/Tyr204}$ (Figure 1.14A) an additional band was seen at ~50-60 kDa in plasma supplemented media lanes. This is most likely the result of non-specific bindings to residual albumin within these samples; given its abundance, comparable molecular weight (~66 kDa) and known presence as a residual protein in plasma sample preparation (Geyer *et al.*, 2019). Additionally, while membranes incubated with p-eNOS^{Ser1177} antibody showed targeted binding at 140 kDa (see red box in Figure 1.13A), high levels of non-specific binding were also seen (see purple box in Figure 1.13A). For this reason, all antibodies in future analysis were diluted in TBST containing 3% BSA to block non-specific binding.

The total-VEGFR2 antibody showed specificity for VEGFR2 at 230 kDa alongside non-specific binding at 50 kDa (Figure 6.15C), which exhibited a stronger signal than that of the total-VEGFR2 band, leading to membranes being cut at 100 kDa to improve VEGFR2-specific signal intensity (Figure 6.15D). Two different antibodies targeting p-VEGFR2^{Tyr1175} were tested to determine the capacity for changes in VEGFR2 phosphorylation to be detected in HUVEC cell lysates. However, neither antibody was able to measure detectable levels of p-VEGFR2^{Tyr1175} in untreated HUVEC or in

HUVEC treated with either plasma supplemented media from resting participants for 1-hour or 100 U·mL^{-1} TNFα for 4-hours (Figure 6.15A & B).

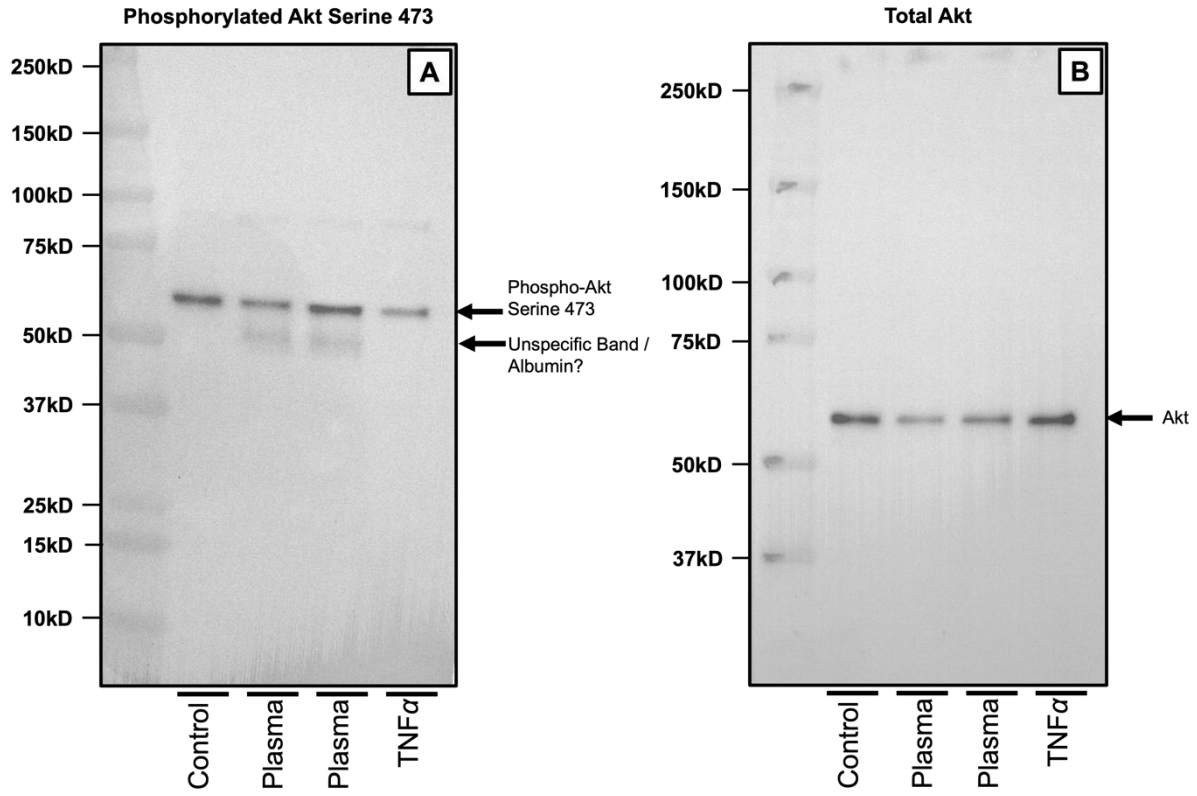


Figure 6.12 Phosphorylated and total Akt antibody validation
HUVEC cell lysates were prepared for Western Blotting following culture in M199 culture media without treatment (**Control**), after treatment with plasma supplemented media for 1-hour (**Plasma**), or following treatment with 100 U·mL^{-1} TNFα for 4-hours (**TNFα**). Lysates were run on 4–20% precast Tris-Glycine gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham^{TM}) and incubated with primary antibodies diluted in TBST specific to phosphorylated Akt^{Ser473} (**A**) or total Akt (**B**) (both Cell Signalling Technologies), followed by incubation with primary host-specific HRP-conjugated secondary antibodies.

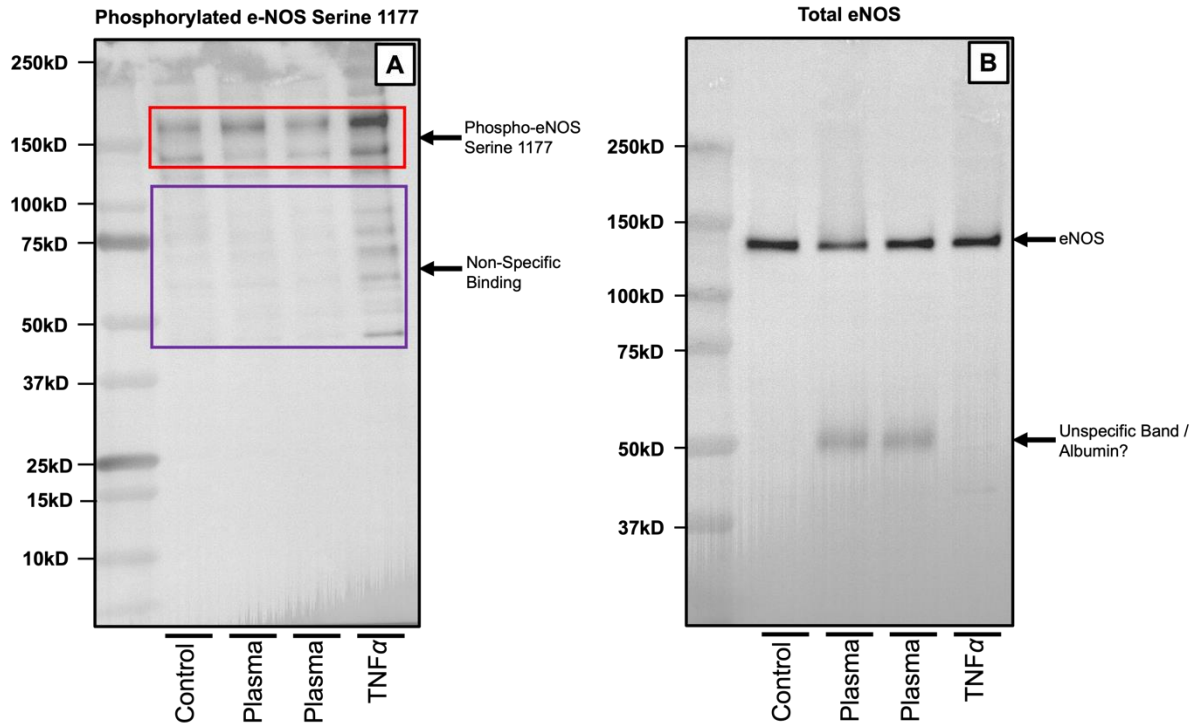


Figure 6.13 Phosphorylated and total eNOS antibody validation
HUVEC cell lysates were prepared for Western Blotting following culture in M199 culture media without treatment (**Control**), after treatment with plasma supplemented media for 1-hour (**Plasma**), or following treatment with 100 $U \cdot mL^{-1}$ TNFα for 4-hours (**TNFα**). Lysates were run on 4–20% precast Tris-Glycine gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham^{TM}) and incubated with primary antibodies diluted in TBST specific to phosphorylated eNOS^{Ser1177} (**A – red box**; Cell Signalling Technologies) or total eNOS (**B**; R&D Systems), followed by incubation with primary host-specific HRP-conjugated secondary antibodies. A region of non-specific binding under p- eNOS^{Ser1177} incubation is also highlighted (**A – purple box**).

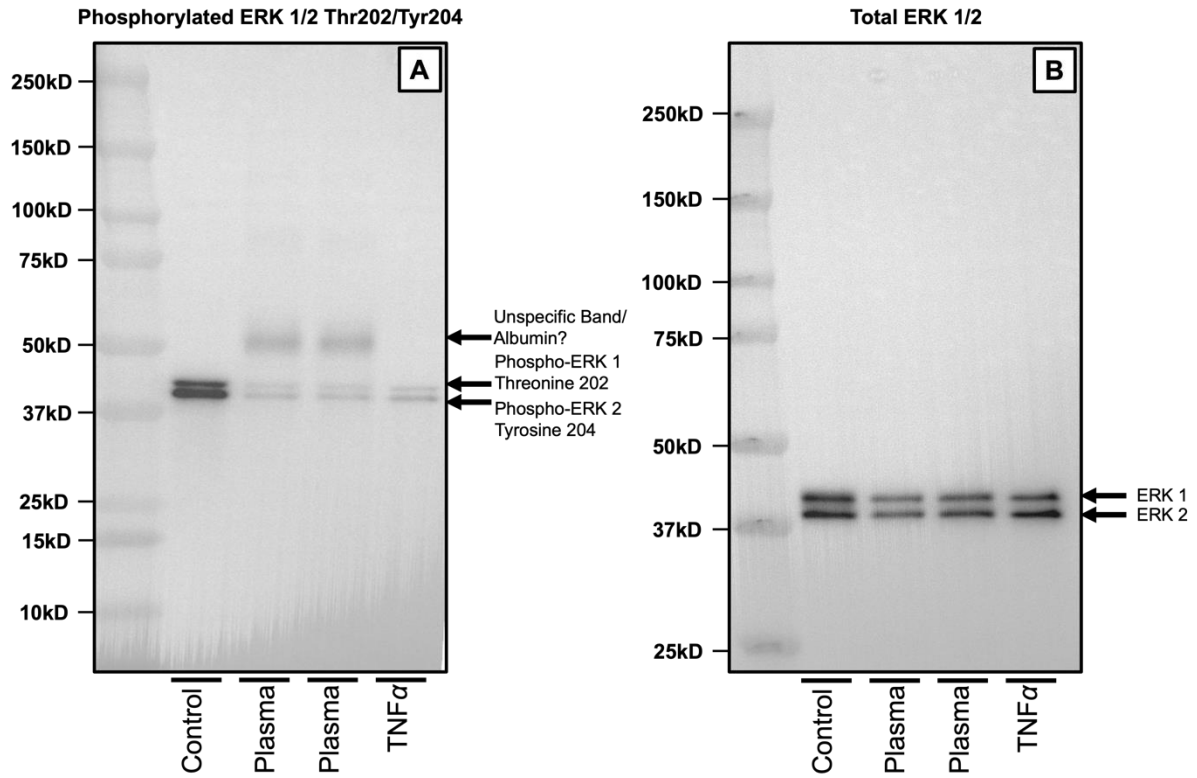


Figure 6.14 Phosphorylated and total ERK1/2 antibody validation
HUVEC cell lysates were prepared for Western Blotting following culture in M199 culture media without treatment (**Control**), after treatment with plasma supplemented media for 1-hour (**Plasma**), or following treatment with 100 $U \cdot mL^{-1}$ TNFα for 4-hours (**TNFα**). Lysates were run on 4–20% precast Tris-Glycine gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham^{TM}) and incubated with primary antibodies diluted in TBST specific to phosphorylated ERK1/2$^{Thr202/Tyr204}$ (**A**) or total ERK1/2 (**B**) (both Cell Signalling Technologies), followed by incubation with primary host-specific HRP-conjugated secondary antibodies.

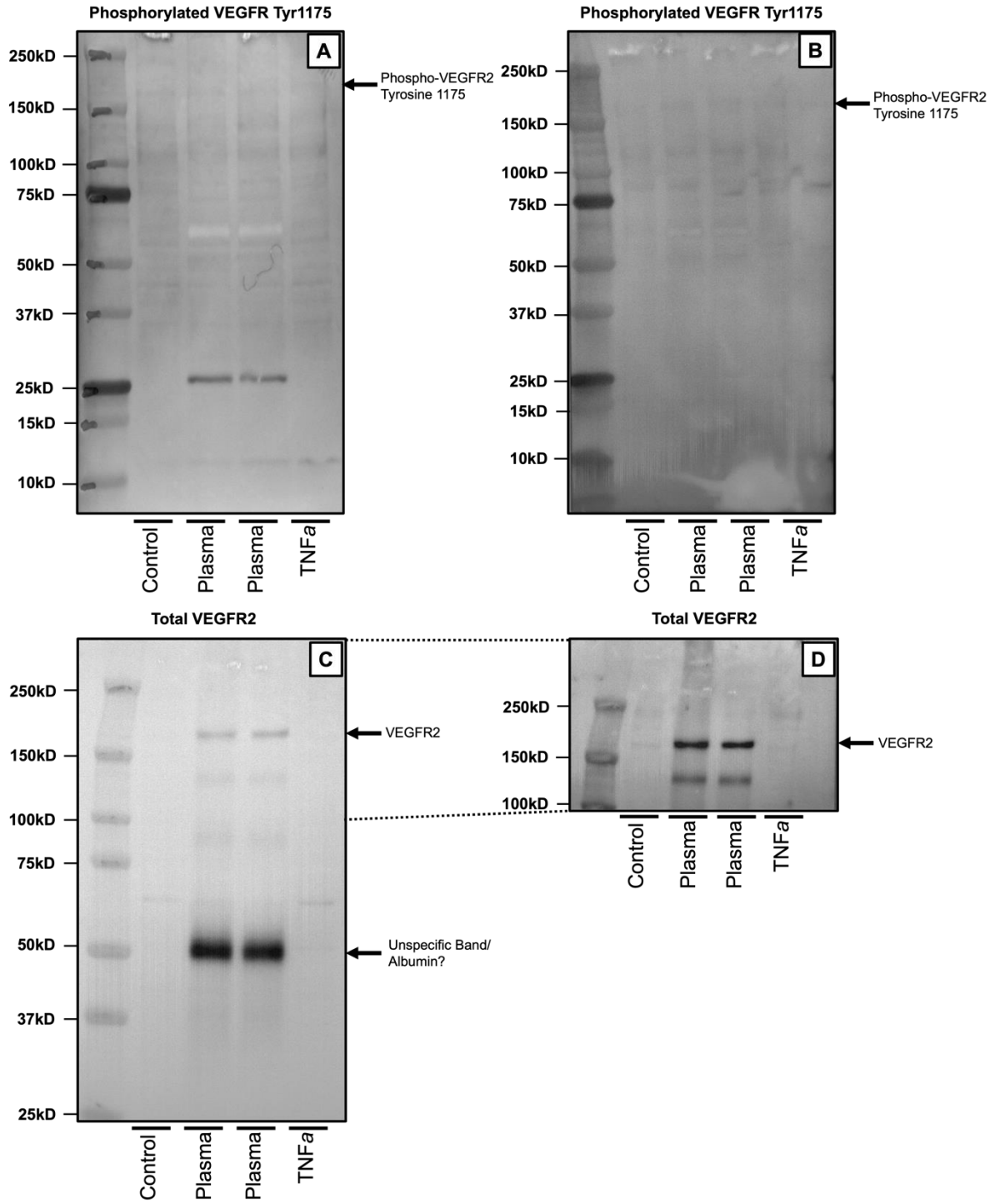


Figure 6.15 Phosphorylated and total VEGFR2 antibody validation
HUVEC cell lysates were prepared for Western Blotting following culture in M199 culture media without treatment (**Control**), after treatment with plasma supplemented media for 1-hour (**Plasma**), or following treatment with 100 $U \cdot mL^{-1}$ TNFα for 4-hours (**TNFα**). Lysates were run on 4–20% precast Tris-Glycine gels (Bio-Rad) and transferred to nitrocellulose membranes (Amersham™) and incubated with primary antibodies diluted in TBST specific to phosphorylated $VEGFR2^{Tyr1175}$ (**A**; Cell Signalling Technologies, **B**; R&D Systems) or total VEGFR2 (**C&D**; R&D Systems), followed by incubation with primary host-specific HRP-conjugated secondary antibodies.

Having validated antibodies, HUVEC responses to plasma samples taken pre- and post-exercise were assessed over 15- and 60-minutes of treatment. Shorter 15-minute treatments were chosen to assess the highly transient responses seen following *in vitro* treatment of endothelial cells with growth factors, such as the impact of VEGF on VEFGR2 and ERK activation (Song & Finley, 2018). While 60-minute treatments were selected to determine the more gradual responses to factors such as lactate, which drives elevations in Akt signaling after >30-minutes of treatments (Ruan & Kazlauskas, 2013).

Changes in Akt activation were consistent between 15- and 60-minute treatment durations, in which linear mixed effects modelling showed significant elevations in p-Akt$^{Ser\ 473}$/total-Akt in SIT compared to both control and the MICT and HIIT treatment conditions (all $p<0.01$; Figure 6.16C). However, the relative contribution of changes in p-Akt^{Ser473} or total-Akt to these differences in activation did appear to vary between treatment durations, as well as by condition. In the SIT condition, 15-minute plasma treatment led to increased Akt activation primarily through increases in the level of p-Akt^{Ser473}, with particularly large responses in a single participant's SIT Post sample leading to significant differences between Pre and Post (Figure 6.16A). In contrast, 60-minute treatments drove elevations in p-Akt^{Ser473} across all sample conditions (Figure 6.16D), counteracted by comparable elevations in total-Akt in MICT and HIIT conditions (Figure 6.16E) and thus resulting in no changes in overall activation in either of these conditions. Despite these differences across treatment conditions, across all three conditions no significant differences were seen between pre- and post-exercise plasma treatment.

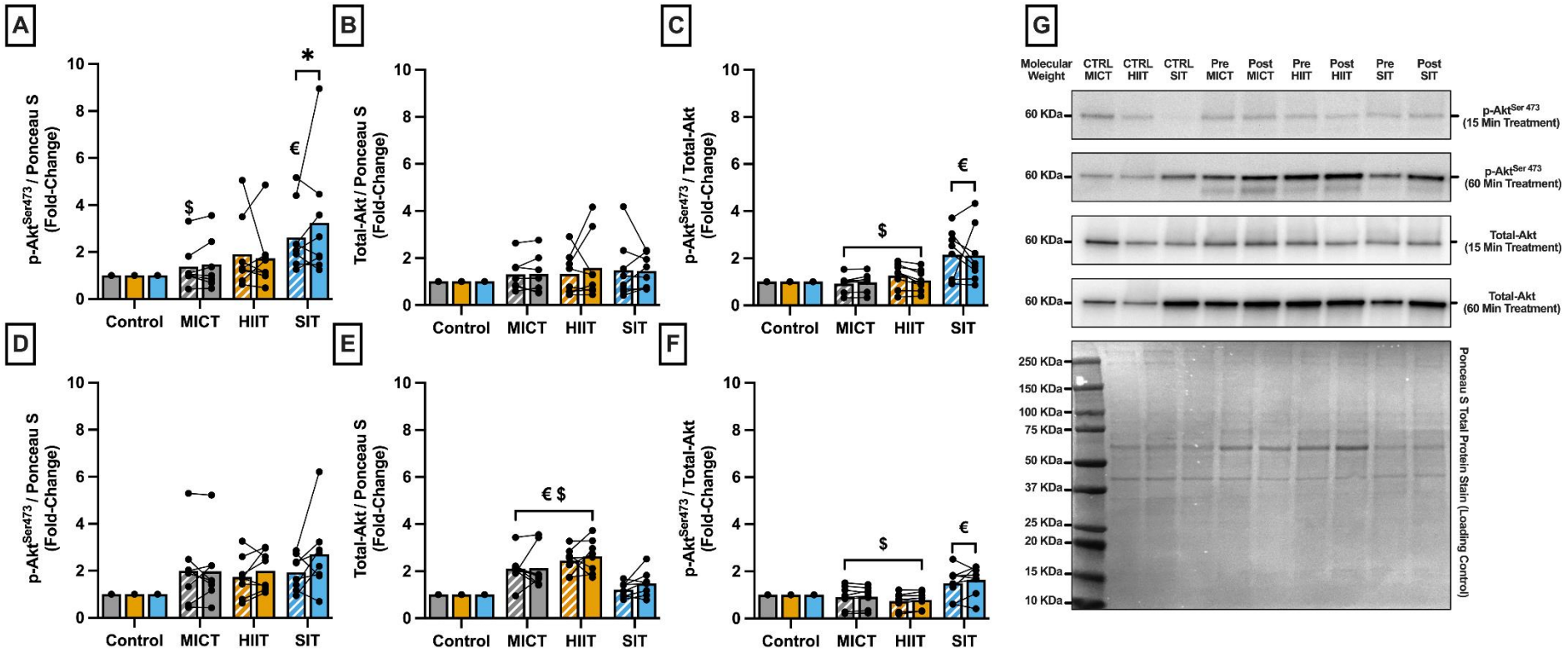


Figure 6.16 Effects of *ex vivo* plasma treatment on Akt phosphorylation and total protein content
HUVEC were treated with 20% plasma samples and cultured for 15- (**A–C**) and 60-minutes (**D-F**). Plasma samples were taken from 8 participants (4 female) during rest (**dashed**) and after (**solid**) completion of MICT, HIIT, and SIT exercise sessions. Control lysates were taken from donor matched HUVEC cultured in M199 culture media without supplementation. Protein phosphorylation and total expression for Akt was examined by Western Blotting (**G**). Data are presented as fold change from the average of matched controls. Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with significance denoted by ✱ for differences between pre and post, **€** for differences from control, **$** for differences from SIT, # for differences from HIIT and **†** for differences from MICT **.** N = 8 for all samples except for SIT samples over 15-minute treatments (n = 7, 3 female).

Responses in eNOS to 15- or 60-minutes of *ex vivo* plasma treatment were markedly less uniform than those seen in Akt, with high levels of inter-participant variability in p-eNOS^{Ser1177} (Figure 6.17A&D) and total-eNOS (Figure 6.17B&E) under both 15- and 60-minute treatments. Despite this variability, significant increases in eNOS activation were seen in the SIT condition following 15-minute treatment over both control and SIT conditions (Figure 6.17C). Additionally, linear mixed effects modelling revealed a significant interaction between condition and treatment in eNOS activation, more specifically, while still significant over control the SIT treatment condition resulted in a significant reduction in eNOS activity from pre- to post-exercise plasma treatments ($p < 0.01$). Within 60-minute treatments, no significant interaction was seen between treatment conditions, likely driven by high levels of variability across p-eNOS^{Ser1177} and total-eNOS (Figure 6.17D&E). However, linear mixed effects modelling revealed significant effects of treatment over control across all measures, indicating that the application of plasma treatments alone induced significant changes in eNOS. In particular and regardless of treatment condition, 60-minutes of treatment led to increases in total-eNOS not seen under 15-minute treatments, which in combination with smaller increases in p-eNOS^{Ser1177} led to notable reductions in eNOS activity.

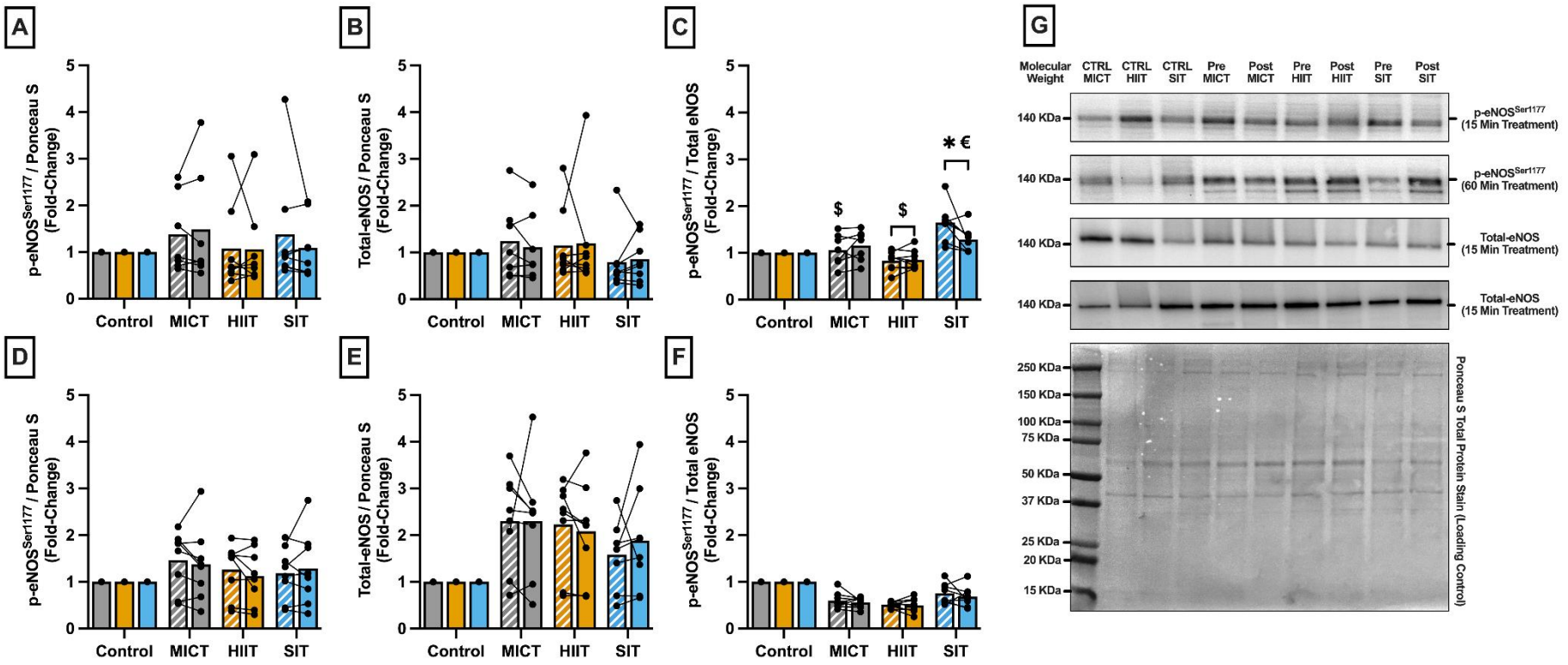


Figure 6.17 Effects of *ex vivo* plasma treatment on eNOS phosphorylation and total protein content
HUVEC were treated with 20% plasma samples and cultured for 15- (**A–C**) and 60-minutes (**D-F**). Plasma samples were taken from 8 participants (4 female) during rest (**dashed**) and after (**solid**) completion of MICT, HIIT, and SIT exercise sessions. Control lysates were taken from donor matched HUVEC cultured in M199 culture media without supplementation. Protein phosphorylation and total expression for eNOS was examined by Western Blotting (**G**). Data are presented as fold change from the average of matched controls. Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with significance denoted by ∗ for differences between pre and post, **€** for differences from control, **$** for differences from SIT, # for differences from HIIT and **†** for differences from MICT. N = 8 for all samples except for SIT samples over 15-minute treatments (n = 7, 3 female).

Plasma treatment resulted in clear variations in ERK1/2 response between conditions over 15-minutes, potentially driven by donor-variations in response to human plasma (Figure 6.18A-C). In MICT and HIIT reductions were seen in comparison to control in p-ERK1/2$^{Thr202/Tyr204}$ (Figure 6.18A) alongside elevations in total-ERK1/2; which were found to be significant in the case of MICT Pre and both HIIT conditions (Figure 6.18B). These responses led to reduced ERK-1/2 activation in both conditions, which reached significance in the HIIT Post condition ($p = 0.04$; Figure 6.18C). Contrastingly, significant elevations in p-ERK1/2$^{Thr202/Tyr204}$ ($p < 0.01$; Figure 6.18A) coupled with a slight reduction in total-ERK1/2 led to significant increases in ERK1/2 activation in the SIT condition (all $p < 0.01$; Figure 6.18). Over 60-minute treatments more consistent responses were seen, with reductions in p-ERK1/2$^{Thr202/Tyr204}$ and small increases in total-ERK1/2 which led to an overall reduction in ERK1/2 activation across all conditions compared to control (Figure 6.18).

Interestingly, while p-VEGFR2^{Tyr1175} was not detectable across any control or treatment conditions, total-VEGFR2 was detected in both 15- and 60-minute treatments, in contrast with a lack of detectable levels in control samples (Figure 6.19). This indicates that while plasma treatments were not sufficient to induce elevations in VEGFR2 activation, they may have been capable of altering total content over relatively short periods.

Overall, responses to plasma treatment appeared to be largely driven by changes induced by treatment with plasma following initial culture with FBS supplementation, rather than in specific differences between samples taken pre- or post-exercise.

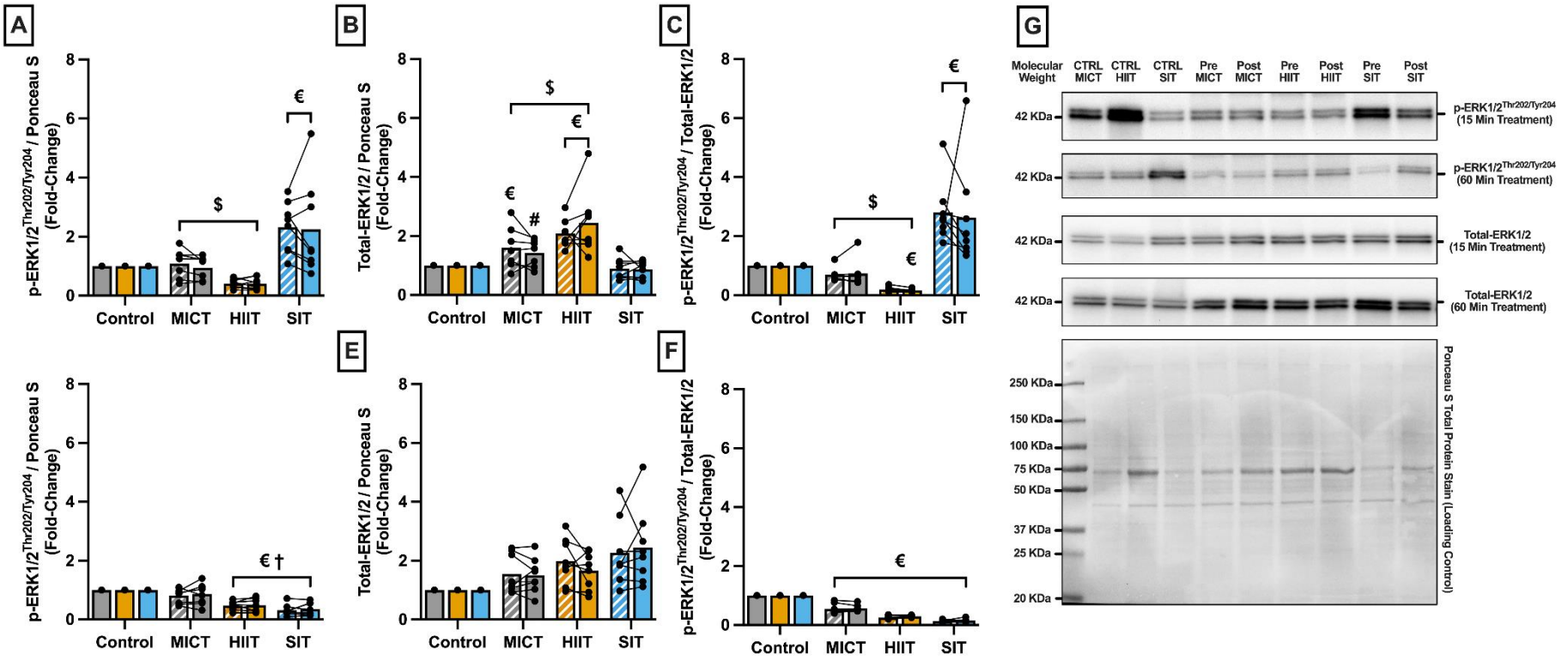


Figure 6.18 Effects of *ex vivo* plasma treatment on ERK1/2 phosphorylation and total protein content
HUVEC were treated with 20% plasma samples and cultured for 15- (**A–C**) and 60-minutes (**D–F**). Plasma samples were taken from 8 participants (4 female) during rest (**dashed**) and after (**solid**) completion of MICT, HIIT, and SIT exercise sessions. Control lysates were taken from donor matched HUVEC cultured in M199 culture media without supplementation. Protein phosphorylation and total expression for ERK1/2 was examined by Western Blotting(**G**). Data are presented as fold change from the average of matched controls. Statistical significance (α = 0.05) was assessed by linear mixed effect modelling, with significance denoted by ✱ for differences between pre and post, **€** for differences from control, **$** for differences from SIT, # for differences from HIIT and **†** for differences from MICT. N = 8 for all samples except for SIT samples over 15-minute treatments (n = 7, 3 female).

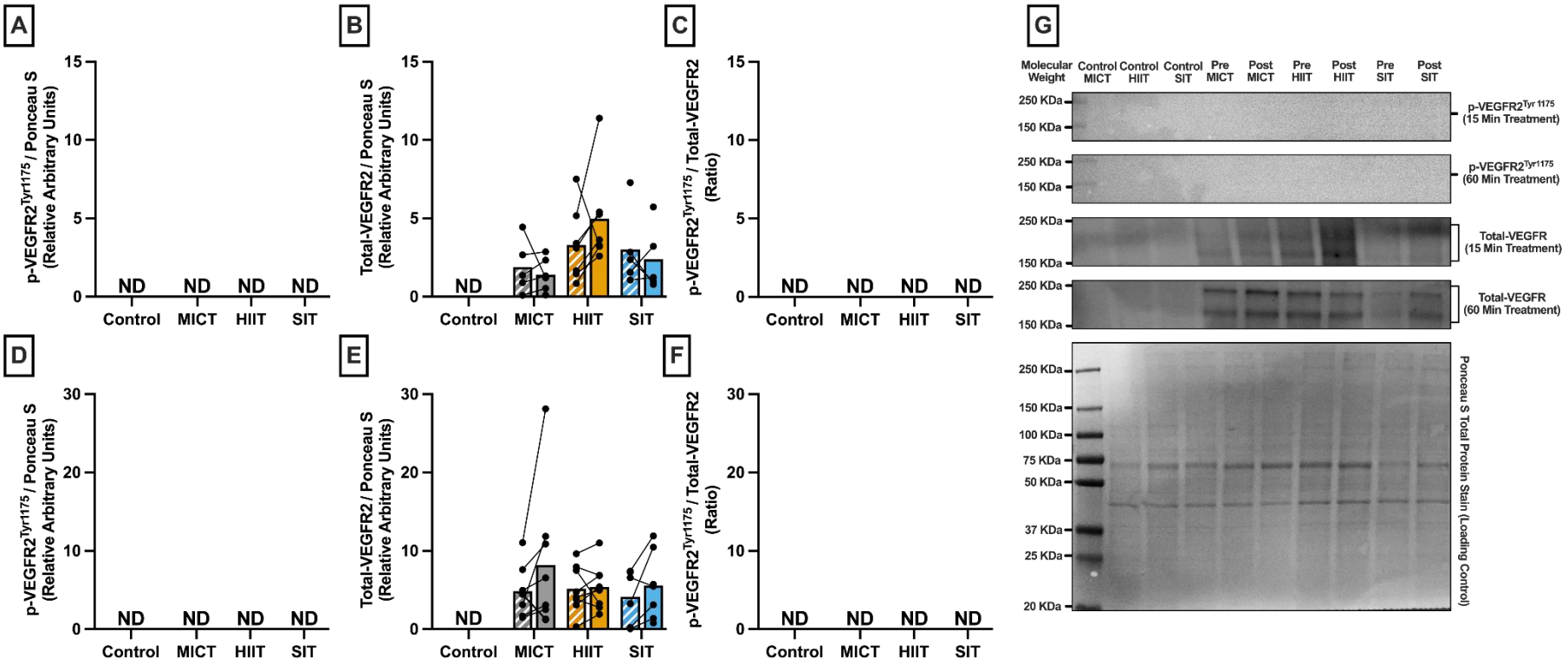


Figure 6.19 Effects of *ex vivo* plasma treatment on VEGFR2 phosphorylation and total protein content
HUVEC were treated with 20% plasma samples and cultured for 15- (**A–C**) and 60-minutes (**D–F**). Plasma samples were taken from 8 participants (4 female) during rest (**dashed**) and after (**solid**) completion of MICT, HIIT, and SIT exercise sessions. Control lysates were taken from donor matched HUVEC cultured in M199 culture media without supplementation. Protein phosphorylation and total expression for VEGFR2 was examined by Western Blotting (**G**). Data are presented as relative arbitrary units (signal band intensity/Ponceau S). Total VEGFR2 was not detectable in control samples but was detected after 15-minute treatment in MICT Pre (n = 5) and Post (n = 6); HIIT Pre and Post (n = 7); and SIT Pre and Post (n = 5), as well as after 60-minutes of treatment in MICT Pre and Post (n = 8), HIIT Pre and Post (n = 8); and SIT Pre (n = 6) and Post (n = 7). VEGFR2 phosphorylation was not detectable (**ND**) in any samples.

6.4. Discussion

This study aimed to investigate changes in endothelial gene and protein expression, in response to *ex vivo* application of serum and plasma samples taken from participants following acute exercise bouts. In response to *ex vivo* serum treatment, no consistent changes in HUVEC gene expression were seen, although high levels of variability were seen between participants. The potential for prolonged exposure to induce more cohesive changes in expression was also investigated, however, a lack of clear responses were maintained from 10 to 360 minutes of treatment. Interestingly, while responses were still largely lacking, a transition from the application of serum to plasma yielded more consistent changes in gene expression, including small but significant increases in the expression of *CLEC14a*. The impact of plasma treatment on the VEGFR2 signalling pathway was also explored, however, similarly to the responses seen in gene expression, no significant differences were seen between treatment with samples taken pre- and post-exercise. Interestingly, the use of multiple HUVEC donors within the protein-related experiments did highlight that significant donor-variation was seen in the gross responses to treatment with human plasma.

6.4.1. The impact of the circulating microenvironment on gene expression

The vascular benefits of exercise are thought to be primarily linked to the impact of acute changes in blood flow and the effects that this haemodynamic stimulus has on mechanosensory pathways within the endothelium (Green *et al.*, 2017). The precise mechanisms driving these changes are likely multifaceted and dependent on the nature and temporal patterns of the induced changes in flow (see Chapter 2). However, the clear role of haemodynamic stimuli overall, suggests that flow-sensitive

pathways are likely upregulated during exercise and that these are central to the adaptive process. Furthermore, as discussed in Chapter 2, acute exercise also has the potential to induce endothelial stress responses, particularly when patterns in flow are non-uniform or turbulent (see Chapter 2). To understand how the circulating microenvironment may interact with endothelial responses to flow, gene expression targets were chosen that have previously been shown to be flow-modulated either under continuous high rates of shear (*KLF2*) (Dekker *et al.*, 2002), or under perturbed/low shear rate (*CLEC14a*) (Mura *et al.*, 2012). In addition, genes of interest were chosen that are involved in key processes also central to endothelial responses to acute changes in shear, such as angiogenesis and inflammation (*VEGF* & *VEGFR2* (Olsson *et al.*, 2006), *IGFBP3* (Granata *et al.*, 2007), *HIF1α* (Pugh & Ratcliffe, 2003)) .

Although no study to date has investigated endothelial gene expression responses to *ex vivo* serum or plasma treatment, several studies have explored the expression profile responses that single molecules can have on the endothelium. These molecules include many of the circulating factors seen to be altered in response to exercise, such as lactate and VEGF, which were seen to increase in the current study (Table 6.1). Prolonged treatment (>6 hours) of HUVEC with 10 mM lactate has been shown to significantly upregulate the expression of *HIF-1α* (Sonveaux *et al.*, 2012), as well as *VEGF* and *VEGFR2* (Sameer Kumar *et al.*, 2007), promoting a pro-angiogenic phenotype within the endothelium. Similarly, VEGF has been shown to play a central role in angiogenesis within the endothelium, with treatment at 100 $ng \cdot mL^{-1}$ driving sustained upregulation of the expression of angiogenic and stress-responses genes including *IGFBP3* (Schweighofer *et al.*, 2009) and *HIF-1α* (Rivera *et al.*, 2011). Within

the current study, however, no clear changes were seen in the expression of any of the target genes, with highly variable responses seen across all targets, particularly when treatments were conducted with *ex vivo* serum. There are several possible factors involved in this variability, not least the complex composition of samples collected from free-living participants, which is evident in the variance of levels of VEGF, IGF-1 and BDNF (Table 6.1). Beyond the small number of quantified factors, it is highly likely that similar levels of heterogeneity are to be found in the broad array of circulating molecules not measured within these samples. The impact that this has on the results of *ex vivo* treatment is also dependent on the distribution of the mean of each composite factor, since if the distribution is skewed in favour of more pronounced changes between time points, then significant responses are likely to be seen and vice versa. Future research should look to comprehensively profile samples used in *ex vivo* treatments; to help clarify potential causes of variability, alongside designing specific *in vivo* studies for the collection of samples for *ex vivo* application (see Section 6.4.3); to minimise the variability in samples from the outset.

While the variability in the samples used in the current study has clear implications in the subsequent findings, the overall lack of differential response between pre- and post-exercise conditions indicates that the differences in circulating microenvironment over short treatment durations did not significantly alter endothelial gene expression. However, the focus within the current study was on the transient changes that occur during and immediately post-exercise and their potential to induce changes in gene expression, in a similar manner to the suggested role that acute alterations in flow are thought to be indicative of the potential long-term benefits of a given protocol (Dawson *et al.*, 2018). Beyond these transient responses, a single exercise bout is also capable

of driving more sustained changes in the circulating microenvironment, particularly in response to high intensity or exhaustive exercise. The most consistently studied of these effects is the impact that exercise can have on elevations in inflammatory cytokines, which exhibit a unique pattern of prolonged elevation post-exercise, dependent on both the specific cytokine as well as the intensity and duration of the exercise bout (Pedersen & Toft, 2000). In a recent review, Cerqueira et al. (2020) showed that sustained elevations in both pro-inflammatory (IL-6 and IL-8) and anti-inflammatory cytokines (IL-10) are seen for up to 2-hours post-exercise, however it should be noted that these responses were highly variable across studies. Nonetheless, this does introduce an alternative mechanism by which exercise-induced changes in circulating microenvironment may alter endothelial gene expression, as the endothelium is highly sensitive to changes in inflammation (Pober & Sessa, 2007). Furthermore, while inflammatory responses are one clear avenue for future study, post-exercise and recovery related changes in a range of factors, including elevations in growth hormone and IGF-1 (Kraemer *et al.*, 2017) - both of which are thought to play a role in angiogenesis and endothelial function (Bach, 2015). Therefore, future application of *ex vivo* research may help in developing our understanding of how post-exercise changes in the circulating environment alter gene expression, beyond the transient influence of perturbations in flow.

6.4.2. Transient changes in circulating microenvironment do not alter the VEGFR2 signalling pathway

Given the highly transient nature of many of the changes seen in the circulating microenvironment during exercise, the most physiologically relevant question may lie in how these changes alter signalling pathways over a relatively short time period.

Within the current study, the signalling pathway of focus was that of VEGFR2 and the downstream signalling proteins Akt and ERK1/2 (Simons *et al.*, 2016), which play an essential role in regulating endothelial function and the upregulation of angiogenesis. VEGFR2 is activated by two distinct mechanisms within the endothelium, with ligand-dependent activation occurring in response to binding of VEGF to VEGFR2 on the endothelial cell membrane (Ferrara *et al.*, 2003) and ligand-independent activation in response to changes in shear stress (Tzima *et al.*, 2005). This makes the VEGFR2 pathway an ideal target when looking to determine the potential for the circulating microenvironment to modulate flow-dependent responses within the endothelium, as it can act to translate both forms of stimuli into subsequent changes in endothelial function. Alongside VEGFR2 and its signalling pathway, potential changes in eNOS were investigated. eNOS plays an essential role within the endothelium, both in driving NO-mediated vasodilation (Tousoulis *et al.*, 2011) and as a modulator of angiogenesis and cell permeability (Fukumura *et al.*, 2001). Moreover, eNOS has also been shown to be activated by the VEGFR2 signalling pathway in both a ligand-dependent (Cai *et al.*, 2006) and -independent manner (Jin *et al.*, 2003), demonstrating the potential for both changes in circulating microenvironment and blood flow to alter similar endothelial functional pathways.

Within the current study, however, no significant changes in either the activation or total levels of VEGFR2 were seen in response to exercise-induced changes in plasma composition, across either 15- or 60-minute treatments. As such, it appears that changes in circulating microenvironment induced by a single bout of exercise are not sufficient to stimulate the VEGFR2 pathway. One potential explanation for this lack of response lies in the relatively low concentrations of VEGF within the treatment plasma

(Table 6.1), which at an average concentration after addition to media of ~200 pg·mL^{-1} is 50- to 500-fold lower than the 10 or 100 ng·mL^{-1} concentration commonly used in studies involving VEGF treatment (Cai *et al.*, 2006; Dellinger & Brekken, 2011). Although VEGFR2 activation has been seen at lower concentrations of VEGF, including similar responses at 5 ng·mL^{-1} as those seen at 100 ng·mL^{-1} (Cai *et al.*, 2006), the concentrations used in the current study may still be insufficient to induce detectable upregulation of VEGFR2. Additionally, no detectable upregulation was seen in Akt or ERK1/2 signalling between pre- and post-exercise conditions, which are key components of VEGFR2-dependent changes in gene expression and increased eNOS activity (Simons *et al.*, 2016). Therefore, in combination with the capacity for plasma treatment to induce detectable changes in both Akt and ERK1/2 in comparison to untreated controls without any clear effect of exercise condition, it is unlikely that endothelial responses to the changes in circulating microenvironment are driven by changes in the activity of VEGFR2 or its downstream signalling network. Future research should, however, look to confirm this hypothesis through treatment of endothelial cells with smaller concentrations of VEGF in isolation, similar to those seen in exercising conditions, to determine whether the activation of the VEGF signalling pathway is isolated to pathologically relevant concentrations.

Plasma samples used in the current study also contained significant and relatively uniform differences in the concentration of lactate between treatment conditions, which has previously been shown to promote angiogenesis via VEGFR2. While the concentrations seen within these samples was lower than that typically seen in traditional *in vitro* studies (~4 mM compared to 10 mM (Sameer Kumar *et al.*, 2007; Ruan & Kazlauskas, 2013)), it is possible that these levels are sufficient to activate

VEGFR2 related pathways. In support of this idea, Beckert et al. (2006) demonstrated that, while dose-dependent in nature, treatment of HUVEC with lactate at concentrations as low as 5 mM was sufficient to induce significant increases in VEGF, alongside increase in endothelial cell migration. One potential issue with this concept is that the upregulation of VEGF and subsequent VEGFR2 activation has been shown to be dependent on activation of the Akt signalling pathway (Ruan & Kazlauskas, 2013), which was not seen in the present study. However, even at concentrations of 10 mM, Akt responses to lactate in HUVEC are relatively slow, with clear elevations not seen until 60 minutes of treatment and not peaking until 120 minutes (Ruan & Kazlauskas, 2013). Given the relatively low concentrations seen within the current sample pool, it is possible that activation of this pathway was occurring, but was not yet detectable, particularly given the high level of variability in *ex vivo* treatment compared to direct treatment with lactate. For this reason, longer treatment periods are needed to determine whether lactate at lower concentrations, such as those seen in the samples used here, can have a notable impact on Akt-dependent VEGFR2 and eNOS upregulation. Additionally, it may be that the lack of detectable changes in VEGFR2 activation is partly the result of a limit in methodological sensitivity, given the lack of detectable control samples in either p-VEGFR2^{Tyr1175} or total-VEGFR2, rather than purely a lack of response to treatment and in combination. These questions warrant further study.

The role of eNOS is essential to the vasodilatory capacity of the vasculature and has the potentially to be differentially affected by exercise (Higashi & Yoshizumi, 2004). *In vitro*, not only is eNOS upregulated in response to changes in flow, as a result of the activation of a complex network of mechanosensory pathways (Fleming, 2010), but

has also been seen to be activated in response to changes in the circulating microenvironment, including in response to increases in VEGF (Cai *et al.*, 2006) and lactate (Sonveaux *et al.*, 2012). In an exercising context, less focus has been given to the role of the circulating microenvironment in the modulation of eNOS-dependent vasodilation. This is primarily due to evidence showing that transient elevations in flow are essential to the adaptive benefits of exercise in the peripheral vasculature, demonstrated by the lack of exercise-induced adaptations in the brachial artery when blood flow is restricted during the exercise bout (Green *et al.*, 2017). Nonetheless, changes in the circulating microenvironment are thought to play a detrimental role in the relative lack of peripheral vascular responses to high intensity continuous exercise, through the accumulation of systemic oxidative stress (Goto *et al.*, 2007). Interestingly, the only significant impact of *ex vivo* plasma treatment was found in the form of a small, but significant reduction in eNOS activity between pre- and post-exercise SIT conditions after 15 minutes of treatment (Figure 6.17). While by no means definitive, it may be that while lower intensity continuous (MICT) or interval-based exercise (HIIT) does not significantly alter eNOS activity, changes in the circulating microenvironment during sprint-based protocols acould inhibit the function of eNOS. If this is the case, these findings would suggest that the theory that interval-based exercise ameliorates the negative impact of high intensity exercise on endothelium-dependent vasodilation is limited in its scope and that at supra-maximal intensities, sufficient stress is induced as to overcome the recovery benefits of the interval format. There is a clear need for further research to test this hypothesis, firstly, to confirm that the previous concept of accumulated oxidative stress proposed by Goto et al. (Goto *et al.*, 2007) stands true when tested in an *ex vivo* context, through the collection and application of plasma samples following both continuous and interval-based exercise. And secondly, to

determine whether increases in markers of oxidative stress are seen following SIT-based protocols and how the specifics of this protocol lead to responses comparable to continuous high intensity efforts.

6.4.3. Current limitations and future considerations in the application of ex vivo approaches

The most notable and consistent change in the current study was seen within protein activation and the impact that treatment of HUVEC with human plasma had on proteins of interest, regardless of the specific source of the samples. Furthermore, these responses were seen to be donor-dependent in nature, indicating that differences are detectable in the response of HUVEC donors to human plasma. This highlights a major issue, not only as it increases the variability within the outcome measure when multiple donors are used, as can been seen between MICT, HIIT and SIT conditions across protein-related outcome measures (Figure 6.16–Figure 6.19), but these responses have the potential to cloak differences in response between conditions. Donor-variations have previously been seen in HUVEC and other primary cell types, including small but detectable differences in inflammatory responses to treatment with TNFα or interferon-γ (Suárez *et al.*, 2007). Most notably, however, are the differences seen between HUVEC collected from male and female donors, in which female donor HUVEC have been shown to exhibit significantly greater enrichment of 77 immune-related genes (Lorenz *et al.*, 2015). Of these, 20 were regulated by shear, resulting in a significantly greater shear-related response in female HUVEC alongside greater tolerance of serum starvation conditions (Lorenz *et al.*, 2015). These sex differences have also been seen in the function of HUVEC, with female HUVEC producing higher levels of basal eNOS expression, alongside greater migratory and proliferative

properties (Addis *et al.*, 2014). While the sex of HUVEC donors used within the current study were not known, it may be that these differences are at least partly responsible for the donor-dependent differences seen in protein expression.

Regardless of the role of donor-variability, future studies should take steps to limit the influence that this factor can have on experimental outcomes. One solution to this issue is to use a single HUVEC donor, however, this increases the potential for erroneous responses to play a significant role in research findings, as may be present within the gene expression findings within the current study. Alternatively participant samples could be randomly allocated to each donor rather than allocation being on a condition-dependent basis, which would allow for donor variation to be included while minimising potential influence over results. However, this is also potentially flawed as it still increases the risk of a cloaking effect and increased variability being introduced across all experimental outcomes. A more viable solution may be the treatment of HUVEC using pooled samples from multiple participants, as this would allow for multiple donors to be used and help to facilitate the inclusion of multiple conditions being present within each HUVEC donor. This does eliminate the capacity for individual responses to be detected, however, which was the reason this approach was not taken in the current study. Nevertheless, future research may look to utilise this approach in combination with more comprehensive analysis of each donors circulating microenvironment prior to combining samples, or alternatively, use this approach to screen a broader number of conditions before carrying out additional experiments with individual samples. Additionally, regardless of the approach taken, researchers may also look to culture HUVEC in pooled serum or plasma from human

donors prior to experimental treatment, to further minimise the impact of donor-variation in the general response to culture under serum/plasma.

While the presence of biological variability between donors likely plays a considerable role in the lack of clear findings within the current study, experimental design considerations need to be acknowledged. Most notably, the samples used within the current research were collected as part of a previous study (Chapter 4; Weaver *et al.*, 2021) and as such the collection of both serum and plasma were not planned for *ex vivo* application *a priori*. As a result, relatively low volumes of both sample types (1–2 mL per participant and time point) were available for utilisation within the current study, which gave considerable limitations to the breadth and depth of possible experiments. This is particularly the case when considering application of samples across multiple time points, as well as limiting the total RNA and protein yields and subsequent capacity for re-analysis. The potential impact of this is particularly apparent in gene expression analysis, in which “not detected” samples are seen (Figure 6.1Figure 6.6) as well as a lack of detectable bands in Western Blot analysis of p-VEGFR2^{Tyr1177} and total VEGFR2 (Figure 6.19). A key consideration for both qPCR and Western Blotting are the concentration and total amount of sample used, with higher concentrations or totals providing greater potential for detection, particularly when targets of interest are found in lower abundance (Bass *et al.*, 2017; Taylor *et al.*, 2019). To eliminate this issue in future research, specific *in vivo* studies should be designed, or the use of *ex vivo* approaches should be decided *a priori* to allow for larger sample volume collection. This would also allow for a greater number of treatment durations and specific selection of sample time points, to maximise the potential changes in circulating microenvironment.

To date, *ex vivo* research in physiology has largely focused on responses to dietary manipulations, in which participant diet is controlled and postprandial blood samples are collected to determine the potential influence of gross changes in circulating macromolecules (Bruckbauer & Zemel, 2011; Wang *et al.*, 2011; Carson *et al.*, 2018). The exception to this is the work of Brunt and colleagues (2018) who demonstrated the potential of *ex vivo* serum application within the context of the endothelial response to a hypoxic stimuli and the potential for heat therapy to exhibit vasoprotective properties under these conditions. These authors used serum taken from participants prior to and following 8-weeks of heat therapy, with the serum used to treat HUVEC for 24-hours prior to a hypoxic stimuli. Their results showed a protective benefit against hypoxia-induced elevations in NF-κB activation and oxidative stress (Brunt *et al.*, 2018). Nonetheless, across all previous studies the samples utilised have been taken at rest and from participants taking part in experiments involving either a single, well controlled change in circulating microenvironment; in the case of dietary interventions, or a prolonged intervention stimulus; which had previously been shown to induce distinct adaptive vascular responses (Brunt *et al.*, 2016*a*, 2016*b*). This approach provides relatively good levels of control and, alongside selection of a relatively homogenous cohort, helps to minimise the variability introduced in the application of samples taken from human participants. In contrast, the current study involved a relatively heterogenous cohort, containing both female and male participants, in a free-living setting and, as such, this was likely a key driver in the lack of clear endothelial responses. To better emulate the success of these approaches in the study of acute exercise responses, future research should look to take steps to reduce sample variability, including conducting initial studies in relatively homogenous

cohorts, under well characterised exercise stimuli and with clear *a priori* sampling approach.

6.4.4. Conclusion

To the best of our knowledge, this is the first study to apply *ex vivo* research to understand how exercise-induced transient changes in circulating microenvironment affects the endothelium. We have shown that in response to three distinct acute exercise bouts, no significant changes are seen in gene expression related to angiogenesis, vasculogenesis or inflammation. Furthermore, while treatment with plasma resulted in an overall increase in VEGFR2 signalling pathway activity, in a donor-dependent manner, the only response to exercise-induced changes in microenvironment were found in the inhibition of eNOS following treatment with samples from sprint-based interval exercise. Finally, we have highlighted the clear need for further development of *ex vivo* research approaches and made recommendations as to the first steps in improving the broader application of these methods.

7. GENERAL DISCUSSION

7.1. Summary of Main Findings

The overall aim of this thesis was to identify the impact that acute exercise can have on cerebrovascular physiology, both in terms of haemodynamic stimulus and the potential influence of the circulating microenvironment. To achieve this, a translational approach was taken combining both *in vivo* exercise-based approaches and *in vitro* studies of the endothelium. Collectively, these studies highlighted the complexity and transient nature of physiological responses to exercise and the capacity for both minor and major modifications in exercise protocol to significantly alter these responses. The main findings of this thesis are:

- Continuous moderate intensity and clinical guideline-based high intensity interval exercise induce comparable elevations in MCA_v during exercise;
- Sprint interval exercise drives notably reduced haemodynamic responses during the exercise bout itself, but was countered by a greater impact on the circulating microenvironment;
- Sprint intervals induced a novel rebound pattern of MCA_v that appeared to be both intensity- and duration-dependent, and
- *Ex vivo* application of samples taken immediately post-exercise did not appear to induce angiogenic or vasodilatory responses within the endothelium

7.2. Context of Findings Within the Literature

7.2.1. Implications of exercise protocol on the cerebrovascular benefits of exercise

The capacity for exercise to drive elevations in MCA_v has been well documented and explored in the context of exercise intensity for nearly 30 years (Moraine *et al.*, 1993), with a clearly established inverted-U pattern of response seen as intensity moves from

light, through moderate, to high intensities (Smith & Ainslie, 2017). However, growing interest in the potential benefits of interval-based exercise have highlighted the paucity of data beyond continuous exercise responses within the cerebrovasculature (Lucas *et al.*, 2015; Calverley *et al.*, 2020).

The data presented within Chapter 4 demonstrate that protocol choice can have a considerable impact on cerebral haemodynamics and illustrate the importance of directly assessing how differences in protocol may affect cerebrovascular responses to exercise. While this was not the first study to investigate the influence of acute interval-based exercise (Klein *et al.*, 2019), it was the first and currently only study to directly compare MCA_v responses during commonly applied continuous and interval-based approaches (Weaver *et al.*, 2021). Importantly, the findings of this study highlighted that clinical guideline-based interval exercise provided a similar stimulus to traditional moderate-intensity continuous protocols and, as such, is likely to drive a similar adaptive stimulus. In contrast, supramaximal sprint intervals drove a unique pattern of suppression and rebound in MCA_v over the course of repeated bouts. This highlights the importance of considering the temporal pattern of haemodynamic responses, rather than just the magnitude of change seen within a particular averaging window, as continuous responses cannot be assumed when the exercise bout itself is not continuous in nature.

The presence of a rebound response following 30-second cycling efforts appears to be a consistent phenomenon within the literature, with single bouts at both supramaximal/"all-out" (Curtelin *et al.*, 2018) and maximal aerobic intensity (Labrecque *et al.*, 2020) exhibiting similar patterns in MCA_v to those seen in Chapter

4. To explore the factors driving this phenomenon, Chapter 5 investigated how altering the intensity of sprint intervals may modulate the rebound response across multiple sprint bouts. Interestingly, and contrary to initial hypotheses, consistent rebound responses were seen across the full range of intensities explored. Furthermore, while the greatest amplitude of change was seen at the maximum intensity (200% W_{max}), more consistent rebound responses were seen at lower intensities. In contrast with the findings of Labrecque et al. (2020), consistent "pressure passive" peaks in MCA_v were not seen at the onset of sprint bouts, which are likely the result of differences in blood pressure perturbations when bouts are initiated from a stationary or "rolling" start. In combination, these findings show that rebound responses cannot be fully explained by the higher intensity at which sprint intervals are often conducted, and that even minor changes in sprint protocol may drive marked changes in the haemodynamic response to this exercise format.

7.2.2. Advances in analytical approach for haemodynamic data

Adoption of more complex analytical approaches when handling cerebrovascular data provides greater clarity and temporal resolution, which is not achievable under traditional approaches to data handling. Such approaches have led to significant advances in other areas of cerebrovascular physiology, most notably in relation to the assessment of autoregulation through transfer function analysis, for which widely adopted guidelines have now been established (Claassen *et al.*, 2015). In an exercising context, attempts have also been made to move beyond simple analysis including the use of area under the curve analysis as a measure of total MCA_v response within both Chapter 4 of this thesis and in previous research (Klein *et al.*, 2019). Additionally, Billinger et al. (2017) have recently developed automated methods

for the characterisation and quantification of exercise onset responses, as a potential marker of cerebrovascular health in older adults (Billinger *et al.*, 2017) and over the course of stroke recovery (Billinger *et al.*, 2021). However, to date little has been done to better understand and quantify the spectrum of unique temporal patterns seen in haemodynamic data during exercise.

The method development process outlined in Chapter 5 looked to address this shortcoming and provide clear guidance to other researchers who may want to adopt similar approaches in future research. Within this chapter, consideration was given to the potential benefits and limitations of different approaches, including the quality of data produced, impact on physiological relevance and ease of use. The latter is of particular importance if temporal pattern analysis is to become more common place, as unlike previously developed approaches (e.g. transfer function analysis) these methods do not produce specific outcome metrics, limiting the scope for dedicated software to remove the need for research involvement in the analytical process. To this effect, alongside step-by-step description of the analytical process, where possible the approaches were adopted from transfer function guidelines (Claassen *et al.*, 2015) and the R scripts used has been made publicly available (https://github.com/src-weaver/thesis_rebound).

7.2.3. Changes in the circulating microenvironment and their impact on the endothelium

Alongside assessment of haemodynamic responses, the impact that exercise has on circulating neurotrophic factors were also determined in Chapter 4. The role of these factors reaches beyond the vasculature, having been proposed as a possible

mechanism by which exercise can directly alter brain structure and function (Voss *et al.*, 2013*b*). The results of Chapter 4 showed that the impact of exercise on circulating lactate, VEGF, IGF-1 and BDNF were largely intensity-dependent, with the greatest responses observed in the SIT condition. This finding illustrates the complexity of the interaction between exercise and physiology *in vivo*, as a dichotomy was seen between the responses in the circulating microenvironment and MCA_v. One possible implication from this is that while lower intensity exercise provides the greater benefits within the cerebrovasculature, higher intensity exercise such as SIT is capable of driving improvements within the brain. Alternatively, given the potential role of circulating factors such as VEGF in driving angiogenesis within the cerebrovasculature (Morland *et al.*, 2017; Geiseler & Morland, 2018), it may be that the changes in circulating microenvironment during exercise provides an alternative mechanism for cerebrovascular adaptation. To capitalise on this, interventions should combine protocols such as MICT and HIIT that have the potential to drive elevations in flow, with higher intensity, sprint-based intervals, to gain the benefits of both haemodynamic and neurotrophic factor stimuli.

To explore the potential for these changes in circulating microenvironment to stimulate responses within the endothelium, the studies in Chapter 6 treated HUVEC with *ex vivo* serum and plasma samples taken pre- and immediately post-exercise in Chapter 4. In contrast to the hypothesis that *ex vivo* treatment would induce pre-post exercise changes in flow-responsive pathways, the findings of this chapter showed no significant effects of sample collection condition on HUVEC gene or protein expression. Taken at face value, these findings indicate that changes in the circulating environment post-exercise do not have the capacity to induce changes in endothelial

phenotype, which in turn limits the potential for this to represent a novel path for vascular adaptive benefits in exercise when flow responses are not seen. However, the experiments within Chapter 6 are novel in their application of *ex vivo* cell culture methods in the study of transient changes in the circulating microenvironment, and significant optimisation of these methods are required before definitive conclusions can be drawn. Most notably, the need for greater consideration of donor-variability in the use of primary endothelial cells (Addis *et al.*, 2014) and the importance of *a priori* design of experiments so that due consideration can be given to cohort selection and the timing and volume of sample collection.

7.3. Future Directions

While the findings of this thesis build upon the current literature and have sought to develop innovative translational approaches to further our understanding of exercise and its role in the cerebrovasculature, the adoption of these approaches provides a myriad of further research opportunities.

1. Given the apparent complexity in the relationship between exercise protocol and changes in MCA_V, highlighted in Chapter 4, future research should look to characterise the individual contribution that intensity, duration, and interval pattern play in driving these responses.
2. Based on the findings in Chapter 5, the influence of interval intensity does not fully explain the rebound phenomenon and further research is needed to determine how other components of sprint-interval exercise (e.g. interval duration) alter this unique haemodynamic pattern.

3. The establishment of standard methodology for the processing, analysis and presentation of haemodynamic data will help to accelerate the adoption of more complex analytical approaches and improve repeatability within the field.
4. The findings and discussion in Chapter 6 may act as a primer for future use of *ex vivo* approaches in exercise-related translational research, the first steps in which must involve the optimisation of collection and treatment protocols.

7.4. Summary

Overall, this thesis found that the exercise protocol plays a central role in the cerebral haemodynamic response to acute exercise, both in the magnitude of the change in MCA_v and in the temporal pattern that these changes exhibit. The finding that both moderate intensity continuous exercise and clinical guideline-based interval protocols exhibit relatively similar total increases in MCA_v from baseline, suggests that the growing preference for interval-based exercise will not alter the cerebrovascular benefits of the traditional exercise guidelines. The observation of a unique rebound pattern within sprint-based exercise led to further investigation of the factors that may be governing this response, which highlighted that this rebound was both intensity and duration dependant. Along with these novel observations, a new methodology for the characterisation and analysis of temporal patterns in haemodynamic data was also developed. Sprint-intervals were also seen to induce more pronounced changes in the circulating microenvironment. However, the results of *ex vivo* studies do not currently indicate that these changes would provide an alternative mechanism for vascular adaptation.

In conclusion, exercise drives complex changes in both cerebral haemodynamics and the circulating microenvironment, which should be taken into consideration when looking to target brain vascular health. The findings and methods presented within this thesis provides clear examples of how protocol changes can alter the nature of exercise responses, as well as providing clear directions for further exploration. Future research should look to incorporate translational research methods in the context of exercise and the cerebrovasculature, to better explore the multi-dimensional nature of the field.

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8. APPENDIX

Appendix 1 Consent form for Chapter 4

UNIVERSITY OF BIRMINGHAM

School of Sport, Exercise and Rehabilitation Sciences

Participant Consent Form

Study Title: *Investigating the interaction between exercise mode and exercise intensity on brain vascular health.*

Investigators: Dr Sam Lucas, Dr Rebekah Lucas, Ms Claire Burley, Mr Sam Weaver, Mr Rhodri Furlong, Ms Gabriella Imi

Participant Name & ID: _____

Initial each box

1. I have read the study information sheet (ERN 17-1570 Version 2) and have discussed the experiment with one of the above named investigators, who have explained the procedures to my satisfaction.

2. I understand that I am volunteering to participate in the experiment by my choice and that I may stop and withdraw from the experiment at any time.

    a. I am volunteering for study 1: (Initial this box to choose study 1)

    b. I am volunteering for study 2: (Initial this box to choose study 2)

    c. I am volunteering for study 3: (Initial this box to choose study 3)

3. I confirm that I have not been treated for any cardiovascular, metabolic, neurological or respiratory conditions in the past.

4. I understand that the data collected during the study may be looked at by responsible individuals from the University of Birmingham where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data and understand that any information will be kept strictly confidential.

5. I understand that my digital data will be stored for a minimum of 10 years in accordance with University of Birmingham policies on password protected systems accessible only to research personnel associated with this study. I agree to this.

6. I understand that my questionnaire data will be stored for a minimum of 10 years in accordance with University of Birmingham policies in a locked cabinet only accessible to the research personnel associated with this study. I agree to this.

7. I would like to receive a summary of the study findings Yes / No (circle your response)
    a. If you would like to receive a summary of findings please provide contact details below:
    Postal Address: _____
    _____
    Email Address: _____

8. I agree to participate in this study.

Name of Participant (PRINT) Date Signature

Name of Researcher (PRINT) Date Signature

Appendix 2 Consent form for Chapter 5

UNIVERSITY OF BIRMINGHAM

School of Sport, Exercise and Rehabilitation Sciences

Participant Consent Form

Study title: Effect of sprint-interval exercise on brain blood flow and release of neurovascular adaptive signalling factors (ERN_ 19-1574PA1)

Investigators: Dr Sam Lucas, Dr Martin Whitham, Mr Sam Weaver, Miss Hannah-Jade Parker

Participant Name & ID: _____

Initial each box

1. I have read the study information sheet (ERN_19-1574PA1) and have discussed the experiment with one of the above named investigators, who have explained the procedures to my satisfaction.

2. I understand that I am volunteering to participate in the experiment by my choice and that I may stop and withdraw from the experiment at any time.

    a. I am volunteering for Part A: (Initial this box to choose Part A)

    b. I am volunteering for Part B: (Initial this box to choose Part B)

3. I confirm that I have not been treated for any cardiovascular, metabolic, neurological or respiratory conditions in the past.

4. I understand that the data collected during the study may be looked at by responsible individuals from the University of Birmingham where it is relevant to my taking part in this research. I give permission for these individuals to have access to my data and understand that any information will be kept strictly confidential.

5. I understand that my digital data will be stored for a minimum of 10 years in accordance with University of Birmingham policies on password protected systems accessible only to research personnel associated with this study. I agree to this.

6. I understand that my questionnaire data will be stored for a minimum of 10 years in accordance with University of Birmingham policies in a locked cabinet only accessible to the research personnel associated with this study. I agree to this.

7. I would like to receive a summary of the study findings Yes / No (circle your response)
    a. If you would like to receive a summary of findings please provide contact details below:
    Postal Address: _____
    _____
    Email Address: _____

8. I agree to participate in this study.

Name of Participant (PRINT)	Date	Signature
Name of Researcher (PRINT)	Date	Signature

Appendix 3 List of R packages used in this thesis

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Appendix 4 Published peer-reviewed manuscript relating to Chapter 4

frontiers in Physiology

ORIGINAL RESEARCH
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Cerebral Hemodynamic and Neurotrophic Factor Responses Are Dependent on the Type of Exercise

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This study examined acute cerebral hemodynamic and circulating neurotrophic factor responses to moderate intensity continuous exercise (MICT), guideline-based high intensity interval exercise (HIIT), and sprint interval exercise (SIT). We hypothesized that the pattern of middle cerebral artery velocity (MCAv) response would differ between interval and continuous exercise, with SIT inducing the smallest increase from rest, while increases in neurotrophic factors would be intensity-dependent. In a randomized crossover design, 24 healthy adults (nine females) performed three exercise protocols: (i) MICT (30 min), (ii) HIIT (4 × 4 min at 85% HR_{max}), and (iii) SIT (4 × 30 s supramaximal). MCAv significantly increased from rest across MICT ($\Delta 13.1 \pm 8.5$ cm·s^{-1}, $p < 0.001$) and all bouts of HIIT ($\Delta 15.2 \pm 9.8$ cm·s^{-1}, $p < 0.001$), but only for the initial bout of SIT ($\Delta 17.3 \pm 11.6$ cm·s^{-1}, $p < 0.001$). Immediately following each interval bout, MCAv increased (i.e., rebounded) for the SIT (9–14% above rest, $p \leq 0.04$), but not HIIT protocol. SIT alone induced significant elevations from rest to end-exercise in vascular endothelial growth factor (VEGF; $\Delta 28 \pm 36\%$, $p = 0.017$) and brain-derived neurotrophic factor (BDNF, $\Delta 149\% \pm 162\%$, $p < 0.001$) and there were greater increases in lactate than in either other protocol (>5-fold greater in SIT, $p < 0.001$), alongside a small significant reduction at the end of active recovery in insulin-like growth factor 1 (IGF-1, $\Delta 22 \pm 21\%$, $p = 0.002$). In conclusion, while the nature of the response may differ, both guideline-based and sprint-based interval exercise have the potential to induce significant changes in factors linked to improved cerebrovascular and brain health.

Keywords: cerebrovascular, exercise, hemodynamic, neurotrophic factor, physiology

INTRODUCTION

Physical activity is unique in its ability to positively impact human health and promote wellbeing across a lifespan (Pedersen and Saltin, 2015; O'Donovan et al., 2017; Kraus et al., 2019). These benefits are clearly seen within the brain, with higher physical fitness positively correlated with brain health and a reduction in risk and progression rate of a number of neurological diseases (Kramer et al., 2006; Bailey et al., 2013; Burley et al., 2016).

Traditional guidelines, typified by moderate intensity, and continuous efforts of 30 min, elicit beneficial changes in resting cerebral blood flow, cognitive function, cardiovascular health, and all-cause mortality (Garber et al., 2011; Cai et al., 2017; Alfini et al., 2019). However, poor adherence to traditional exercise strategies, often characterized by a lack of time (Costello et al., 2011), has resulted in increased interest in high intensity, interval-based strategies, which have shown similar health and fitness benefits compared to moderate intensity steady-state exercise (Stork et al., 2017; Gibala and Little, 2020). Despite this, studies focusing on the cerebrovascular responses to these strategies are limited in number. While current evidence indicates comparable middle cerebral artery velocity (MCAv) responses between continuous and interval-based moderate intensity exercise (Klein et al., 2019), MCAv has been shown to be suppressed over the course of a single 30 s all-out sprint bout, followed by a marked elevation in MCAv during recovery (Curtelin et al., 2018; Labrecque et al., 2020). This reduction in response to high intensity interval-based strategies is in contrast to responses within the peripheral vasculature (Ramos et al., 2015), likely driven by the unique response of the cerebrovasculature to exercise-induced hyperventilatory hypocapnia (Ogoh and Ainslie, 2010), as well as differences in the regulatory responses to elevated systemic flow and blood pressure (Brassard et al., 2017; Ogoh et al., 2018; Calverley et al., 2020). Regardless of the mechanisms driving these differences, it is essential that we understand how choice of exercise strategy can alter hemodynamic responses, as acute elevations in blood flow are likely pivotal in determining the adaptive benefits of longer training periods (Green et al., 2017). Understanding this relationship is particularly important as research increasingly supports short (30–60 s) sprint interval training (SIT) protocols at supramaximal intensities, rather than longer (∼4 min) high intensity efforts performed in guideline-based high intensity interval training (HIIT) (Weston et al., 2014). While the current literature demonstrates the complex relationship between SIT and measures of cerebral hemodynamics (Curtelin et al., 2018; Labrecque et al., 2020), it is not clear whether these responses change over multiple bouts as is typical in training interventions, nor how SIT- and HIIT-based strategies may differ in their response.

Exercise also has the capacity to alter the profile of circulating signaling factors, including neurotrophic factors with the potential to act within the brain (Voss et al., 2013b). Amongst these, brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF) are particularly relevant due to their ability to elicit a comprehensive profile of responses within the cerebrovasculature, blood-brain barrier, and in brain plasticity (Voss et al., 2013b; Basso and Suzuki, 2017). Increases in all three of these factors have been demonstrated under a single exercise bout, with responses appearing to be intensity-dependent (Wahl et al., 2010, 2014; Jeon and Ha, 2017; Reycraft et al., 2020).

While the origin and brain-specific changes in many of these factors is not fully understood, particularly in relation to BDNF (Rasmussen et al., 2009; Klein et al., 2011), the impact that exercise can have on these factors is increasingly clear. In both animal models and humans, increases in BDNF have been associated with improvements in brain function, acute post-exercise cognitive function, and subsequent adaptive benefits within the brain (Voss et al., 2013a; Basso and Suzuki, 2017; Kujach et al., 2020). Similarly, although acute changes in IGF-1 do not appear to correlate with changes in cognition (Tsai et al., 2014), more chronically it has been suggested that IGF-1 is central to healthy cognitive aging, due to its role in regulating metabolism and insulin resistance in the brain (Lewitt and Boyd, 2019). Further, IGF-1 signaling is essential to the maturation of BDNF and so may play a secondary role in stimulating its release (Carro et al., 2000). VEGF is most widely studied in terms of vascular endothelial regulation, stimulating angiogenic signal pathways and vascular adaptation to alterations in flow (Olsson et al., 2006). A number of animal studies have also demonstrated its role within the brain, promoting angiogenesis within the cerebrovasculature (Morland et al., 2017; Geiseler and Morland, 2018) and exhibiting neuroprotective effects when upregulated (Jin et al., 2002; Góra-Kupilas and Jośko, 2005).

The evolving role of lactate as a signaling molecule raises interesting prospects within the brain, particularly considering its exponential increases seen at higher intensities (Hughson et al., 1987). Increases in lactate alone have the capacity to significantly increase BDNF (Schiffer et al., 2011) and have been associated with exercise-induced changes in circulating BDNF, IGF-1, and VEGF (Kujach et al., 2020). Furthermore, lactate has been shown to modulate brain signaling pathways, crossing the blood-brain barrier and binding to HCAR1 receptors that are highly expressed within the brain (Lauritzen et al., 2014), driving responses relating to cerebral angiogenesis and stimulating cerebral VEGF expression (Morland et al., 2017).

Given the potential impact of exercise intensity and protocol on cerebrovascular function, this study aimed to examine the MCAv response to moderate intensity continuous exercise (MICT), guideline-based high intensity interval exercise (HIIT), and sprint interval exercise (SIT). Secondly, we aimed to explore how these exercise protocols may differentially alter the acute profile of key neurotrophic markers, which have previously been linked to exercise-induced changes in brain health. It was hypothesized that the pattern of middle cerebral artery velocity (MCAv) response would differ between interval and continuous exercise, with SIT inducing the smallest increase from rest. In addition, we hypothesized that increases in circulating neurotrophic factors would be intensity-dependent, with SIT inducing the greatest response.

MATERIALS AND METHODS

Participants

Twenty-four regularly active participants were recruited for this study (see **Table 1** for participant characteristics). All participants were healthy, with no history of cardiovascular, cerebrovascular, or respiratory disease, and were not taking any medication (with the exception of oral contraception in female participants). Visits were carried out within an hour of the same time of day for each participant, with a

TABLE 1 | Baseline characteristics and resting values for participants taking part in three different exercise protocols.

Baseline characteristics (*n* = 24; 9 female)	**Mean**	***SD***
Age (years)	23	5
Body Mass (kg)	73.0	11.1
VO_{2peak} (mL·min^{-1})	3,185	544
VO_{2peak} (mL·kg^{-1}·min^{-1})	43.9	6.4
Resting values		
MICT		
MCAv (cm·s^{-1})	72.0	13.6
$P_{ET}CO_2$ (mmHg)	33.8	3.6
HR (beats·min^{-1})	68	10
BDNF (pg·mL^{-1})	112.5	75.8
IGF-1 (ng·mL^{-1})	1,960	1,350
VEGF (pg·mL^{-1})	456.5	980.6
Lactate (mM)	1.1	0.2
HIIT		
MCAv (cm·s^{-1})	70.4	11.4
$P_{ET}CO_2$ (mmHg)	33.6	3.6
HR (beats·min^{-1})	67	12
BDNF (pg·mL^{-1})	134.0	91.6
IGF-1 (ng·mL^{-1})	1,905	1,160
VEGF (pg·mL^{-1})	404.9	770.8
Lactate (mM)	1.1	0.3
SIT		
MCAv (cm·s^{-1})	70.5	11.8
$P_{ET}CO_2$ (mmHg)	33.4	3.2
HR (beats·min^{-1})	71	10
BDNF (pg·mL^{-1})	134.8	104.5
IGF-1 (ng·imL^{-1})	2,101	1,444
VEGF (pg·mL^{-1})	369.2	740.1
Lactate (mM)	1.3	0.9

SD, standard deviation; MICT, moderate intensity continuous training; HIIT, high intensity interval training; SIT, sprint interval training; MCAv, middle cerebral artery velocity; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; HR, heart rate; BDNF, brain-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; VEGF, vascular endothelial growth factor.

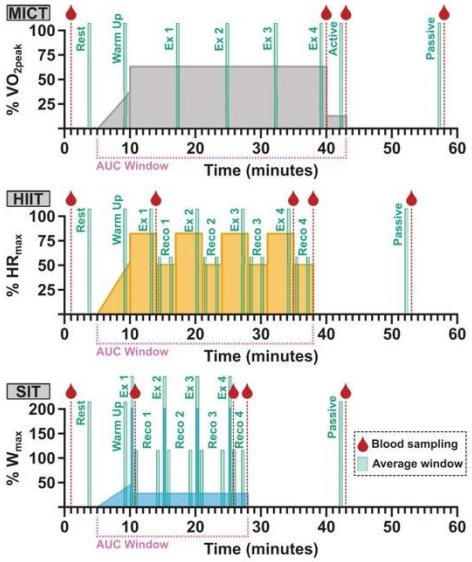


FIGURE 1 | Participants took part in three separate exercise visits in a randomized order, consisting of 30 min of moderate, steady-state exercise at 65% VO_{2peak} (MICT); four 4 min high intensity intervals at 85% HR_{max} (HIIT); and four 30 s supramaximal sprint intervals at 200% W_{max} (SIT). All protocols were preceded by 5 min of seated rest and a 5 min warm-up, and followed by 3 min of active recovery at 50 W and 15 min of seated passive recovery. The timing of blood sampling (red droplet), data averaging windows, and labels for analysis (green boxes) and the period used in area under the curve (AUC) analysis (pink bracket) are included for all protocols. Averaging was carried out over 30-second windows across the course of each exercise protocol (Ex) and during the initial and final minute of recovery (Reco) following each HIIT and SIT bout. The final 30-second recovery window in both HIIT and SIT was taken as the active recovery timepoint, in conjunction with the active recovery window (Active) in MICT. VO_{2peak}, maximum oxygen consumption; HR_{max}, maximum heart rate; W_{max}, maximum aerobic power.

minimum of 48 h between sessions. The study was approved by the University of Birmingham Ethics Committee (ERN-17_1570) and all participants gave written, informed consent prior to enrolling in the study, in adherence with the Declaration of Helsinki.

Experimental Protocol

Following enrolment, participants attended the laboratory on four separate occasions to carry out: (1) a ramp-increment aerobic capacity test (VO_{2peak} test); (2) 30 min of moderate-intensity steady-state exercise (MICT); (3) four 4 min high intensity interval (HIIT) workouts based on a clinical intervention model (Weston et al., 2014); and (4) four 30 s all-out sprints (SIT) (**Figure 1**). Participants were asked to refrain from vigorous exercise or consuming alcohol for 24 h and from consuming caffeine within 12 h of each visit. In addition, participants were asked to record any food consumed on each visit day and to refrain from consuming food within 2 h of testing beginning, with the same foods being consumed prior to subsequent sessions.

Maximal Oxygen Consumption Test (VO_{2peak})

VO_{2peak} was assessed using a ramp-incremental exercise protocol on a cycle ergometer (Excalibur Sport, Lode, Netherlands). This test consisted of a 5 min warm up until the participant indicated a rate of perceived exertion (RPE) of 11 on a Borg Visual Scale (Borg, 1982), followed by a 3 min ramp protocol in which resistance was increased by 30 W every 3 min until volitional fatigue was reached, or the participant was not able to maintain a consistent cadence above 50 RPM. Oxygen consumption was continuously measured (Vyntus CPX, Vyaire Medical, United Kingdom) and VO_{2peak} was defined as the highest 30

s average achieved during the test. Continuous measures of HR (Polar H9 HR Sensor, Polar, Finland) were collected for determination of HR_{max}. Maximal power output W_{max} was determined as the power output achieved during the final stage of the VO_{2peak} test.

Experimental Visits

All three experimental visits were carried out in a randomized, cross-over fashion, with the order of experimental visits for all participants determined *a priori* using customized R script (R Core Team, 2019). On arrival, an intravenous catheter (20G, BD Venflon, United Kingdom) was placed in the antecubital vein of the forearm to enable blood sample collection during exercise; participants then rested in a supine position for a minimum of 10 min before resting blood samples were collected. Participants were instrumented for cerebrovascular and cardiorespiratory measures (detailed below) and sat quietly for 5 min while resting measures of middle cerebral artery velocity (MCAv), heart rate (HR), and end-tidal carbon dioxide ($P_{ET}CO_2$) were collected. All exercise protocols were carried out at a self-selected cadence, included a 5 min warm up identical to that used during VO_{2peak} testing, and ended with 3 min of active recovery at 50 W and a seated 15 min passive recovery period (**Figure 1**). Verbal encouragement was given throughout all exercise protocols and participants were given information about elapsed time at regular intervals.

MICT Protocol

During MICT, participants carried out 30 min of continuous exercise at 65% of VO_{2peak}. Resistance on the cycle ergometer was initially set between 55 and 60% of W_{max} and adjusted once cycling had commenced in order to attain 65% of VO_{2peak}. Oxygen consumption was continuously monitored across the duration of the 30 min period to ensure this intensity was maintained and cycling resistance was adjusted as required.

HIIT Protocol

HIIT consisted of four 4 min intervals at 85% HR_{max}, separated by 3 min of active recovery at 50 W, based on guideline recommendations and definitions of HIIT in clinical settings (Weston et al., 2014). Initial workload during interval bout 1 was set at ∼70% W_{max}, and monitored and adjusted to achieve 85% HR_{max} in a similar manner to the MICT workload. For remaining bouts, the initial intensity was set at the power output during the final 30 s of the preceding 4 min bout and adjusted as necessary to maintain intensity at 85% HR_{max}.

SIT Protocol

SIT intervals consisted of four 30 s intervals at 200% of W_{max}, separated by 4.5 min of active recovery at 50 W, with the exception of the final recovery which was matched to MICT and HIIT at 3 min at 50 W. Following warm-up, the workload was reduced to 50 W for 30 s in order to ensure that the change in workload from pre-interval to sprint was matched across all four intervals. Workload was ramped up 5 s prior to each sprint interval in order to account for a ∼5–7 s delay in resistance on the cycle ergometer and intensity was dropped to 50 W as soon as the 30 s bout ended.

Measurements

Cerebral Blood Velocity

MCAv was assessed bilaterally using a 2 MHz transcranial doppler (TCD) ultrasound system (Multi Dop X, DWL, Compumedics Ltd., Germany). Ultrasound probes were attached to a fully adjustable headset (DiaMon, DWL) to allow for continuous measurement during exercise. The MCA was located via the temporal window using standardized search techniques, with depth, gain, and filter settings matched within participants across visits (Willie et al., 2011). Resting values for right and left MCAv were compared during rest to maximize comparability between sessions, with the most consistent MCA (right/left) signal across all three sessions used for data analysis.

Respiratory Gases and Ventilation Rate

Breath-by-breath measures of respiratory gases were measured by collection of expired air through a facemask (Hans Rudolph, United States) and analyzed continuously via a metabolic cart (Vyntus CPX, Vyaire Medical). Measures of minute ventilation (V_E), oxygen consumption (VO_2), and $P_{ET}CO_2$ were exported for later analysis.

Data Acquisition

MCAv and HR data were recorded through an analog-to-digital converter (Powerlab, ADInstruments, New Zealand) and stored for further analysis using dedicated signal analysis software (LabChart Pro v7, ADInstruments). $P_{ET}CO_2$ data were aligned with other signal data and processed alongside MCAv to match time course and data averaging periods between the two.

Blood Sampling and Analysis

Blood samples were drawn from the antecubital vein and collected into two 6 mL vacutainers for the isolation of plasma, one containing K_2EDTA and one lithium heparin (LH) as an anticoagulant (both BD Vacutainer, United Kingdom). Samples were collected at rest, at the end of each exercise protocol, end of active recovery, end of passive recovery, and in the case of HIIT and SIT an additional aliquot was taken following the initial interval bout (**Figure 1**). Samples were immediately centrifuged at 5,000 g, for 10 min at 4°C, carefully aliquoted to avoid disruption of buffy coat, and frozen at −80°C for future analysis. All analyses were conducted using plasma isolated with LH as an anticoagulant, with the exception of VEGF, which was analyzed using plasma isolated in K_2EDTA.

Plasma levels of VEGF, IGF-1, and BDNF were analyzed by DuoSet ELISA according to manufacturer's instructions (#DY293B, #DY291, #DY248, respectively, all R&D Systems, United Kingdom), with each sample run in duplicate on a single plate. Sample concentration was determined using standard curves produced by 4-parameter logistic curve-fitting and automated concentration analysis (AssayFit Pro, Assay Cloud, Netherlands). In the eventuality that all samples from a single visit showed no detectable protein-of-interest, samples were re-run, using a new sample aliquot on a second plate alongside samples that reported reproducible levels of the given protein-of-interest. All sample aliquots used within these analyses were subject to a single freeze-thaw cycle.

Circulating levels of blood lactate were analyzed using an automated photometric-based chemical analyzer (RX+ Daytone, Randox, United Kingdom), using purpose made assay kits, standards, and reagents, according to the manufacturer's guidelines (Randox).

Data Processing and Statistical Analysis

Data Processing

Power output data was collected across all three experimental visits and averaged over 5 min periods across the duration of MICT and over the duration of each bout of HIIT and SIT. Additionally, average power output was calculated over the complete duration of each protocol.

Hemodynamic and respiratory data were averaged over the penultimate 30 s of rest, warm-up, and active and passive recovery. During the exercise efforts, data were averaged over 30 s at four time points (7.5 min intervals) during MICT, over the penultimate 30 s of HIIT bouts, and across the full 30 s duration of SIT bouts. In addition, 30 s averages were obtained in the initial and final minute of recovery following each HIIT and SIT bout (RECO 1–4; see **Figure 1**).

MCAv data were also exported at 1 Hz continuously from the start of warm-up to the end of active recovery to plot time-course data for the duration of each study visit, alongside quantification of area-under-curve (AUC) for the same time-course, using Pulse Analysis software within Labchart (ADInstruments). AUC was calculated as total blood velocity ($cm{\cdot}s^{-1}$) over the total time period (s) and so is presented as a measure of displacement ($cm{\cdot}s^{-1} \cdot s = cm$).

Statistical Analysis

Assumptions of normality were assessed by graphical interpretation of density distribution and Q-Q plots, as well as by analysis of skewness, kurtosis, and Shapiro-Wilk's Test statistics. Resting baseline data and power output data were compared by one-way analysis of variance (ANOVA); if sphericity assumptions were not met under Mauchly's Test then Greenhouse-Geisser corrections were utilized for ANOVA results.

Comparison of repeated-measures outcome data was carried out using linear mixed effects models (LMEM), with time and protocol included as fixed factors and participant ID as a random factor. Inclusion of sex and fitness (VO_{2peak}) as factors was assessed using Akaike's Information Criteria (AIC) to determine inclusion and weighting of model errors for each outcome variable (Symonds and Moussalli, 2011). *Post hoc* testing for interactions within ANOVA and LMEM were carried out with pairwise comparison and Tukey Test adjustments for multiple comparison. All results are presented as mean ± standard deviation (mean ± *SD*) unless otherwise stated.

RESULTS

Exercise Intensity During Acute Exercise

Mean power output across the duration of MICT was 148 ± 28 W and did not differ significantly across the duration of the protocol ($p > 0.05$). During HIIT, average power output was 198 ± 45 W and remained consistent across bouts 1–3 (201 ± 44 W; 201 ± 46 W; 198 ± 46 W, respectively), although a significant decline was seen in bout 4 compared to the other 3 bouts (194 ± 47 W, $p \leq 0.02$). Due to the design of the SIT condition, average power output was matched across all four bouts (503 ± 103 W). Average power output was significantly lower in MICT than in either other condition ($p < 0.001$), and significantly greater in SIT than in HIIT ($p < 0.001$).

Oxygen consumption was significantly elevated from resting baseline across all three protocols ($p < 0.001$, **Table 2**). During MICT, $\dot{V}O_2$ remained relatively stable across the course of 30 min, although there was a small significant increase in the third time point (2,063 ± 367 $mL{\cdot}min^{-1}$, $p \leq 0.03$) compared with time points one and two (1,975 ± 354 $mL{\cdot}min^{-1}$ and 2,006 ± 384 $mL{\cdot}min^{-1}$, respectively). Oxygen consumption during both HIIT and SIT was significantly greater than MICT at all exercising time points ($p \leq 0.046$, **Table 2**). During HIIT, $\dot{V}O_2$ was significantly lower in bout 1 (2,327 ± 574 $mL{\cdot}min^{-1}$) than either bout 2 (2,447 ± 511 $mL{\cdot}min^{-1}$, $p = 0.04$) or bout 3 (2,483 ± 511 $mL{\cdot}min^{-1}$, $p = 0.007$). Similarly, SIT bout 1 (2,291 ± 392 $mL{\cdot}min^{-1}$) was significantly lower than bout 2 (2,512 ± 470 $mL{\cdot}min^{-1}$, $p \leq 0.001$) and bout 4 (2,473 ± 502 $mL{\cdot}min^{-1}$, $p < 0.03$).

Hemodynamics Responses to Acute Exercise Interventions

Baseline characteristics and resting values for MCAv, $P_{ET}CO_2$, and HR during seated rest are displayed in **Table 1**. No significant differences were seen between any of these measures at rest ($p > 0.05$). Within-participant variability (coefficient of variability) among intervention visits was 5.5% for MCAv, 4.8% for $P_{ET}CO_2$, and 7.2% for HR.

Assessment of model fit validated the inclusion of sex in linear modeling of MCAv, $P_{ET}CO_2$, and HR. While significant interactions were seen between time and protocol across all outcome measures and data sets, inclusion of sex as a fixed factor did not reveal any significant main effect or interaction between sex and the time-by-protocol interaction effect. For this reason, all further analysis and interpretation of these data were carried out including male and female participants as a complete cohort.

Significant increases in MCAv from rest during warm up ($p < 0.001$) were comparable across all exercise protocols (MICT: Δ16.7 ± 10.1 $cm{\cdot}s^{-1}$, HIIT: Δ15.5 ± 12.3 $cm{\cdot}s^{-1}$, SIT: Δ15.3 ± 9.3 $cm{\cdot}s^{-1}$, $p > 0.05$ between protocols). During MICT a sustained and uniform elevation in MCAv was seen across all time points (mean across time points: 85.1 ± 16.2 $cm{\cdot}s^{-1}$), with no significant changes across the 30 min time course (**Figure 2**). While all HIIT bouts showed a sustained, significant elevation in MCAv from rest (19–21%, $p < 0.001$), bout 1 showed significantly greater elevation in comparison to both HIIT bout 2 (Δ6.3 ± 6.4 $cm{\cdot}s^{-1}$, $p = 0.043$) and time point one during MICT (Δ4.8 ± 9.0 $cm{\cdot}s^{-1}$, $p = 0.047$). End-tidal CO_2 showed similar patterns in response across both MICT and HIIT, with significant elevations from baseline that were sustained across warm up and all exercising time points in both protocols (**Figure 2**). HIIT bout 1 also showed significantly greater increases in $P_{ET}CO_2$ than

TABLE 2 | Measures of oxygen consumption, minute ventilation, and heart rate at rest and across the duration of three different exercise protocols.

	Rest	Ex 1	Ex 2	Ex 3	Ex 4
MICT					
VO_2 (mL·min^{-1})	293 ± 112	1,975 ± 354*$†	2,006 ± 384*$†	2,063 ± 367*$†	2,023 ± 383*$†
V_E (L·min^{-1})	9.4 ± 3.1	55.1 ± 10.9*$†	56.1 ± 12.2*$†	57.7 ± 11.9*$†	57.4 ± 13.6*$†
HR (beat·min^{-1})	68 ± 10	144 ± 14*$†	147 ± 13*$†	150 ± 13*$†	151 ± 14*$†
HIIT					
VO_2 (mL·min^{-1})	287 ± 69	2,327 ± 574*	2,447 ± 511*	2,484 ± 511*	2,396 ± 631*
V_E (L·min^{-1})	9.5 ± 2.1	70.0 ± 21.2*	76.9 ± 20.1* Ω	78.8 ± 21.3* Ω	77.9 ± 22.9* Ω
HR (beat·min^{-1})	67 ± 12	159 ± 10*	167 ± 10*	170 ± 12*	172 ± 11*
SIT					
VO_2 (mL·min^{-1})	316 ± 69	2,291 ± 392*	2,512 ± 470*	2,448 ± 529*	2,473 ± 502*
V_E (L·min^{-1})	10.7 ± 2.4	83.3 ± 20.3*	101.3 ± 27.6*	106.0 ± 26.4*	109.8 ± 28.8*
HR (beat·min^{-1})	71 ± 10	164 ± 14*	169 ± 15*	171 ± 13*	171 ± 11*

*SD, standard deviation; MICT, moderate intensity continuous training; HIIT, high intensity interval training; SIT, sprint interval training; VO_2, oxygen consumption; V_E, minute ventilation; HR, heart rate. Significance (analyzed by linear mixed model, $p < 0.05$) between resting values and subsequent time points is denoted by *. Significant differences between protocols are denoted by $ between MICT and HIIT; † between MICT and SIT; and Ω between HIIT and SIT.*

all other HIIT bouts (≥2.6 mmHg, $p < 0.001$), whilst a small significant difference was seen between bouts 3 and 4 and the comparable timepoints during MICT (Δ1.4 ± 2.8 mmHg and Δ1.8 ± 2.9 mmHg respectively, $p \leq 0.04$).

Compared to resting values, there was a significant elevation in MCAv following the first 30 s SIT bout (Δ17.3 ± 11.6 cm·s^{-1}, $p < 0.001$), accompanied by a significant increase in $P_{ET}CO_2$ at the same time point (Δ4.5 ± 4.8 mmHg, $p < 0.001$). These changes were comparable to those seen during MICT and HIIT in terms of MCAv (both $p > 0.05$), however, $P_{ET}CO_2$ was significantly lower in SIT than in both MICT (Δ2.3 ± 3.9 mmHg, $p = 0.004$) and HIIT (Δ3.3 ± 3.0 mmHg, $p < 0.001$). During subsequent SIT bouts, significant reductions in MCAv were seen in comparison to bout 1 (≥11.7 cm·s^{-1}, $p < 0.001$; **Figure 2**), with no further elevations above baseline across bouts 2–4 ($p > 0.05$). Significant reductions in $P_{ET}CO_2$ were also seen with repeated bouts, resulting in a significant suppression in $P_{ET}CO_2$ below resting baseline from bout 2 onwards ($p \leq 0.004$; **Figure 2**). During recovery from sprint intervals, a pattern of elevated MCAv and $P_{ET}CO_2$ was seen within the first minute of recovery following each SIT bout, which was not seen in the same window of time during HIIT (**Figure 3**). This "rebound" was only found to be significant in comparison to the related sprint bout following bout 1 (Bout vs. Recovery = Δ10.0 ± 7.9 cm·s^{-1}, $p < 0.001$), although smaller significant elevations above baseline were seen across all subsequent rebounds (9–14% above rest, $p \leq 0.04$). All four bouts showed significant elevations in $P_{ET}CO_2$ during the rebound phase both in comparison to the preceding sprint bout and to baseline measures (≥5.6 mmHg, $p < 0.001$).

Analysis of V_E measures showed an intensity-dependent pattern across all three protocols, mirroring changes seen in $P_{ET}CO_2$. Specifically, significant elevations were seen in all three protocols ($p \leq 0.001$, **Table 2**), but with lower elevations in MICT than either other protocol at all time points ($p \leq 0.01$), and significantly greater V_E in SIT than in HIIT from bout 2 onwards ($p \leq 0.002$). While V_E remained relatively stable across MICT, significant differences were seen between bout 1 of HIIT (70.0 ± 21.2 L·min^{-1}) and all subsequent HIIT bouts (minimum 76.9 ± 20.1 L·min^{-1}, $p \leq 0.001$). Similarly, bout 1 of SIT (83.3 ± 20.2 L·min^{-1}) was lower than all subsequent bouts (minimum 101.3 ± 27.6 L·min^{-1}, $p \leq 0.001$), and bout 2 was lower than 4 (101.3 ± 27.6 and 109.8 ± 28.7 L·min^{-1}; respectively, $p = 0.03$).

Area under the curve analysis of blood displacement across all sessions was carried out in eighteen participants; four participants were not included in this analysis due to temporary signal drop out during one or more sessions, making the analysis of area under the curve across the full session unreliable. Displacement over the course of MICT was significantly higher than both HIIT ($1.66 \times 10^5 \pm 3.12 \times 10^4$ cm vs. $1.36 \times 10^5 \pm 2.23 \times 10^4$ cm, $p < 0.001$) and SIT ($8.14 \times 10^4 \pm 1.46 \times 10^4$ cm, $p < 0.001$) exercise protocols. **Figure 4** shows the mean MCAv across the period used in AUC analysis, in comparison to resting values, demonstrating the difference in average response between the three exercise protocols and the unique pattern of response between sprint bouts and immediate recovery period.

Heart rate data showed a clear pattern that was dependent on the intensity of the exercise stimulus within each of the protocols. Significant increases were seen from rest to the end of the warm up across all exercise protocols (all $p < 0.001$, **Table 2**), whilst the elevation in heart rate was significantly higher during the warm up in SIT than in MICT (126 ± 15 vs. 118 ± 12 beat·min^{-1}, $p = 0.003$), but not in comparison to HIIT (121 ± 12 beat·min^{-1}, $p = 0.057$). All three protocols saw a significant increase in HR from warm up to exercise (all $p < 0.001$), with significantly lower HR in MICT across all time points than both HIIT (minimum difference of Δ16 ± 12 beats·min^{-1}, $p < 0.001$) and SIT (minimum Δ21 ± 14 beat·min^{-1}, $p < 0.001$). Within each protocol, no change was seen across time points in MICT or SIT, however, HIIT bout 1 exhibited significantly lower HR (159 ± 10 beats·min^{-1}) than in the subsequent bouts (bout 2: 167 ± 10 beats·min^{-1}; bout 3: 170 ± 12 beats·min^{-1}; bout 4: 172 ± 11 beats·min^{-1}, all $p < 0.04$ from bout 1).

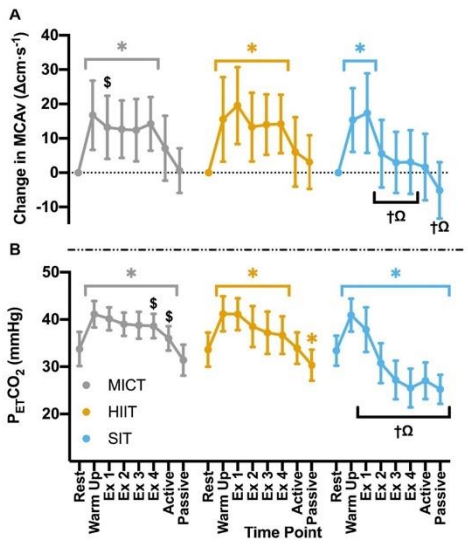


FIGURE 2 | (A) MCAv was determined continuously by transcranial doppler ultrasound (TCD) and presented as change in MCAv from rest in each protocol for each participant. **(B)** $P_{ET}CO_2$ was determined through breath-by-breath respiratory gas exchange and ventilation. Data were averaged over 30 s of rest (rest); 5 min warm up (warm up); 3 min active recovery (active); and 15 min seated passive recovery (passive) in all protocols. Exercising averages (Ex 1–4) were taken every 7 min during 30 min of moderate intensity (65% VO_{2peak}) steady state exercise (MICT); in the final minute of the four 4 min high intensity (85% HR_{max}) interval bouts (HIIT) and across the duration of four 30 s supramaximal (200% W_{max}) sprint intervals (SIT). Data are presented as mean ± *SD* (n = 24; except SIT–Passive: n = 23) and were analyzed by linear mixed models. Significant differences (analyzed by linear mixed model, $p < 0.05$) between resting values and subsequent time points are denoted by *. Significant differences between protocols are denoted by $ between MICT and HIIT; † between MICT and SIT; and Ω between HIIT and SIT.

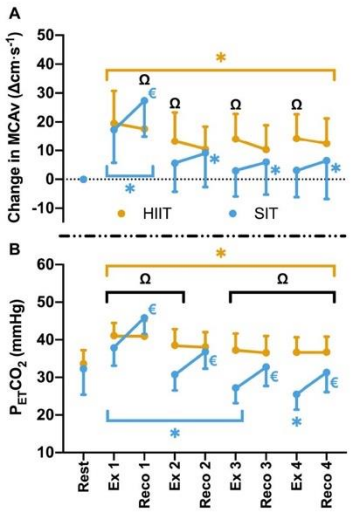


FIGURE 3 | (A) $P_{ET}CO_2$ was determined by measurement of breath-by-breath respiratory gas exchange and ventilation. **(B)** MCAv was determined continuously across all exercise sessions by transcranial doppler ultrasound (TCD) and presented as change in MCAv from rest in each session for each participant. Data were averaged for 1 min during seated rest (Rest); over 30 s during the final minute of four 4 min high intensity (85% HR_{max}) interval bouts (HIIT; Ex 1–4); across the duration of four 30 s supramaximal (200% W_{max}) sprint intervals (SIT; Ex 1–4); and over a 30 s period, 15 s into the recovery following each bout in both interval protocols (Reco 1–4). Data are presented as mean ± *SD* (n = 24). Significance (analyzed by linear mixed model, $p < 0.05$) between time points is denoted by * for differences from resting values; € for differences between interval (ex) and recovery (reco). Significant differences between protocols are denoted by Ω.

Protocol-Dependent Changes in Circulating Factors

Blood samples were drawn from all 24 participants across all time points, with the exception of the final time point in MICT for one participant (cannula blockage). Of these, samples fell outside the limit of detection for the given test in three participants for BDNF (n = 21), seven participants for IGF-1 (n = 17), nine participants for VEGF (n = 15), and none for lactate (n = 24). Sample loss post collection for one participant for the SIT protocol resulted in a reduction in sample number for the analysis of VEGF (SIT: n = 14) and blood lactate (SIT: n = 23). No significant differences were seen in baseline measures for any of the blood markers ($p > 0.05$, **Table 1**). Model validation resulted in the inclusion of sex as a fixed factor in linear mixed modeling for BDNF alone, whilst analyses of data for IGF-1, VEGF, and blood lactate were carried out using base models alone.

Within the full cohort, significant changes in BDNF were seen in SIT alone, with a significant elevation from rest at all later time points, peaking at the end of exercise (Δ149 ± 162% from rest, **Figure 5**) and remaining significantly elevated through to the end of passive recovery (all $p \leq 0.040$). While baseline measures of BDNF did not differ significantly between male and female participants, significant differences were seen between sexes in both MICT and SIT exercise protocols (**Figure 6**). Females exhibited significantly greater increases in BDNF compared to males during active ($p = 0.021$) and passive recovery ($p < 0.001$) in MICT and from the end of exercise onwards in SIT ($p < 0.001$). Marked differences in the response to each protocol were also seen between sexes. Within the female subgroup, increases in BDNF from rest were seen following active recovery ($p = 0.052$), reaching significance at the end of passive recovery ($p = 0.028$) during MICT; significant elevations were also seen from the end of exercise onwards, peaking at active recovery ($p \leq 0.002$) during SIT. Whereas within the male cohort, changes in BDNF levels were only significant between rest and end of exercise in the SIT protocol ($p = 0.005$).

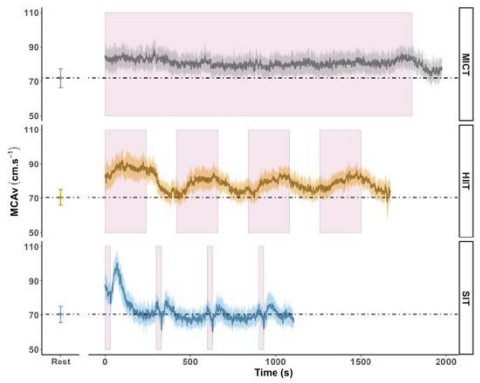


FIGURE 4 | MCAv was determined continuously by transcranial doppler ultrasound (TCD) and exported at 1 Hz frequency from the onset of each protocol after warm-up had been completed. Exercise protocols were: 30 min of moderate intensity (65% VO_{2peak}) steady state exercise (MICT); four 4 min high intensity (85% HR_{max}) interval bouts (HIIT) separated by 3 min active recovery at 50 W and four 30 s supramaximal (200% W_{max}) sprint intervals (SIT) separated by 4.5 min active recovery at 50 W, with all protocols followed by 3 min of active recovery at 50 W. Data are presented as average ± 95% confidence intervals (n = 18), across the duration of each protocol including active recovery periods, with resting values averaged over 30 s of seated rest presented for comparison. These time periods were identical to those used in calculation of area under the curve (AUC). Continuous effort during MICT and the timing of the four interval periods in HIIT and SIT are highlighted in pink.

Changes in IGF-1 across the duration of visits were only found to be significant in the SIT protocol, with a pattern of reduction across the exercise period reaching significance at the end of active recovery (p = 0.002, **Figure 5**). While not significantly elevated from baseline values, changes in IGF-1 for both MICT and HIIT were relatively higher than SIT at the end of active ($p < 0.001$) and passive recovery (MICT vs. SIT; p = 0.023 and HIIT vs. SIT; p = 0.011), and at the end of exercise for MICT compared to SIT (p = 0.018).

Significant changes in VEGF from baseline were only observed for the SIT protocol, with significant elevations in VEGF seen from the end of exercise onwards (>23%; $p \leq 0.015$, **Figure 5**). Specifically, a significant difference in VEGF was seen between the immediate impact of a single SIT bout and further elevations in VEGF at the end of exercise (p = 0.017) and active recovery ($p < 0.001$). At all three of these time points, fold-change in VEGF was significantly greater than that of either MICT ($p \geq 0.036$) or HIIT ($p \geq 0.026$).

All three protocols increased blood lactate levels, but the magnitude of these changes varied between protocols. Specifically, while a significant elevation was seen in MICT at the end of exercise alone (p = 0.005), changes in HIIT and SIT were significant at all time points during and following the exercise stimulus (**Figure 5**). Further, whilst similar increases in lactate from rest were seen following the first bout of both HIIT (Δ3.47 ± 1.47 mM, $p < 0.001$) and SIT (Δ3.84 ± 2.97 mM, $p < 0.001$), significant divergence between these exercise protocols was seen after this time point. Elevations in blood lactate after the final bout of SIT were more than 2-fold greater than those seen in HIIT (Δ15.51 ± 6.14 vs. Δ6.95 ± 2.35 mM, $p < 0.001$), with lactate peaking from rest in SIT at the end of active recovery (Δ18.81 ± 4.91 mM, $p < 0.001$) before declining during passive recovery in both the HIIT and SIT protocols (**Figure 5**).

DISCUSSION

The primary purpose of this study was to compare how differences in exercise strategy may alter acute cerebrovascular responses and impact key circulating signaling factors with neurotrophic potential. Our main findings show that: (a) significant elevations in MCAv during MICT were matched by similar elevations in cerebral blood velocity for each bout of HIIT; (b) SIT elicited a unique response in MCAv, with only the initial bout inducing significant elevations in blood velocity from rest and a rebound in MCAv occurring immediately following each SIT bout; and (c) SIT alone drove significant changes in circulating levels of all investigated markers (BDNF, IGF-1, VEGF, and blood lactate). These data demonstrate that both the cerebrovascular and circulating signal factor responses to exercise are highly dependent on the protocol that is being undertaken. As a result, it is essential that we consider the relative contribution that hemodynamic and circulating factor responses have on the brain and its vasculature to fully understand the efficacy of different exercise strategies.

Hemodynamic Responses to Acute Exercise Protocols

Our findings demonstrate that both MICT and HIIT exercise protocols elicited a similar magnitude of increase in cerebral blood velocity, although the pattern, duration, and total blood displacement over these two protocols varied (as demonstrated in **Figure 4**). The importance of the total shear stimulus in comparison to peak changes has been demonstrated over short time periods (up to 60 s) in brachial artery flow mediated dilatory (FMD) responses (Pyke and Tschakovsky, 2007), but to the best of our knowledge has not been investigated over longer time periods, such as that represented here. While Hoiland et al. (2017) demonstrated the capacity for transient periods of hypercapnia (30 s) to induce an FMD response within the cerebrovasculature, a recent comparison of brachial and internal carotid artery FMD showed that the magnitude and pattern of dilatory responses was distinctly different between the two vessels (Carr et al., 2020) and as such the impact of peak and total shear stimulus within the cerebrovasculature may be distinct from that seen within the periphery. Therefore, whether exercise-induced observations, both acute and chronic, based on FMD responses in the periphery are translated to FMD responses in the cerebrovasculature remain to be determined. This distinction is particularly pertinent in relation to the SIT protocol, as total blood displacement was the lowest overall (as reflected by AUC), yet changes over the first sprint interval and immediate post-bout

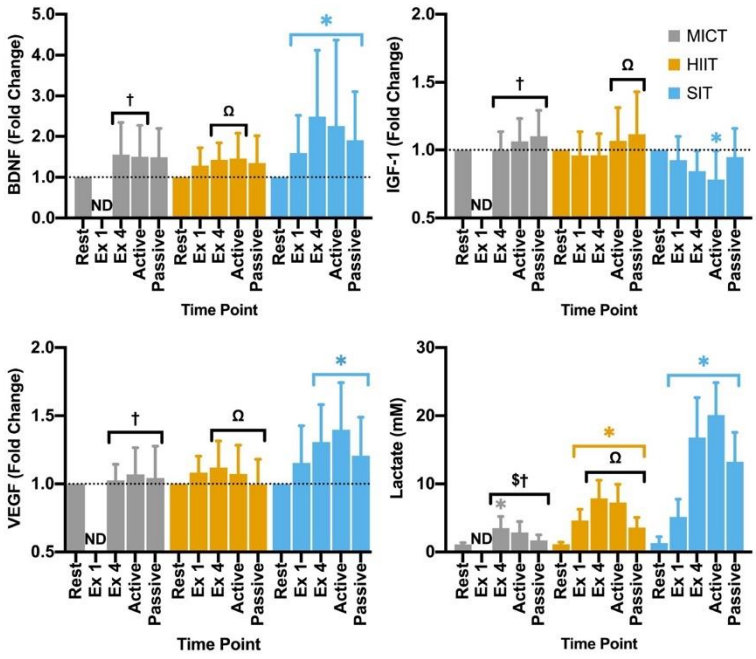


FIGURE 5 | Blood plasma samples were taken during seated rest (Rest); immediately after the first interval exercise bout (Ex 1; HIIT and SIT only); at the end of exercise (Ex 4, end of active recovery (Active); and the end of passive recovery (Passive). Brain-derived neurotrophic factor (BDNF; $n = 21$), insulin-like growth factor 1 (IGF-1; $n = 17$), and vascular endothelial growth factor [VEGF; $n = 15$ (14 in SIT)] were analyzed by ELISA. Blood lactate [$n = 24$ (23 in SIT)] was analyzed using automated photometric analysis. Data are presented as mean ± *SD*. Significance (analyzed by linear mixed model, $p < 0.05$) between resting values and subsequent time points is denoted by *. Significant differences between protocols are denoted by $ between MICT and HIIT; † between MICT and SIT; and Ω between HIIT and SIT. ND, not determined.

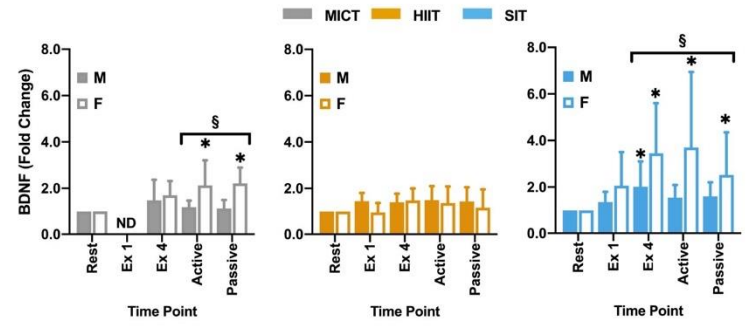


FIGURE 6 | Blood plasma samples were taken were taken during seated rest (Rest); immediately after the first interval exercise bout (Ex 1; HIIT and SIT only); at the end of exercise (Ex 4); end of active recovery (Active)' and end of passive recovery (Passive). Brain-derived neurotrophic factor (BDNF; $n = 21$, 7 female) was analyzed by ELISA. Data are presented as mean ± *SD*. Significance (analyzed by linear mixed model, $p < 0.05$) between resting values and subsequent time points is denoted by *. Significant differences between sexes at a given time point are denoted by §. M, Male; F, Female; ND, not determined.

were among the largest changes at any time point across all three protocols (see **Figure 4**).

The similarity in the acute responses between MICT and HIIT within this study have clear clinical ramifications, as these data indicate that an interval-based approach has the potential to elicit comparable beneficial changes in the cerebrovasculature. Additionally, the slight differences seen in the initial bout of HIIT are likely linked to the lower heart rate within this bout, resulting from an underestimation of the required exercise intensity to elicit 85% HR_{max}, suggesting that a greater response may be seen at a lower intensity. This adds to a growing body of evidence supporting the utilization of interval-based exercise to target both general and vascular health outcomes (Weston et al., 2014; Pedersen and Saltin, 2015), and complements the recent findings of Klein et al. (2019) who demonstrated comparable responses to 10 min of work-matched continuous compared to intermittent exercise. This is an important step in increasing our understanding of how differences in the exercise protocol may affect cerebrovascular function (Calverley et al., 2020). Future work is essential to understand the impact that acute differences in exercise protocol response may have on changes in cerebrovascular function over a training period, particularly given the potential for acute responses to act as markers of possible adaptive benefits (Dawson et al., 2018; Voss et al., 2020).

As expected, suppression of exercise-induced increases in MCAv across the SIT protocol matched the overall lowering of $P_{ET}CO_2$, consistent with the hyperventilatory hypocapnia associated with high intensity exercise (Ogoh and Ainslie, 2010) and the central role of changes in arterial CO_2 in regulating cerebral blood flow (reviewed in Smith and Ainslie, 2017). Interestingly, the rebound in MCAv immediately following each SIT bout also matched changes in $P_{ET}CO_2$ (**Figure 3**), while the greatest increases in both MCAv and $P_{ET}CO_2$ were observed within the first bout. Thus, MCAv responses during each bout and across all bouts appear to be strongly influenced by the changes in $P_{ET}CO_2$. While not investigated within the current study, oxidative-nitrosative stress has previously been linked to reductions in brachial artery endothelium-dependent vasodilation (Goto et al., 2003) and impaired dynamic cerebral autoregulation (Bailey et al., 2011) following high intensity exercise, and as such may also play some role in the MCAv response we observed with the SIT protocol. However, further research is needed to determine the oxidative-nitrosative stress response to interval-based high intensity exercise and the role that this plays within the cerebrovasculature.

Labrecque et al. (2020) previously demonstrated a similar rebound response with a single 30 s sprint bout and suggested that delays in the cerebral autoregulation may also contribute to this MCAv rebound, which, given the large elevations in blood pressure with SIT exercise, seems likely. Unfortunately, we were not able to accurately determine blood pressure during sprint intervals at the intensity utilized within the current study. Interestingly, rebound responses were not observed with the longer intervals used in the HIIT protocol. Therefore, future research is needed to fully understand the potential for differences in both intensity and duration of the interval to maximize the potential benefits of sprint-interval exercise, including with reliable measuring of blood pressure during such sprint efforts.

Sprint-Based Intervals Elicit Unique Patterns in Neurotrophic and Signaling Factor Response

Contrasting the changes seen in MCAv, SIT alone induced significant changes in all assessed neurotrophic factors. Given the potential interaction between changes in these factors and exercise-induced improvements in cognitive function (Lista and Sorrentino, 2010; Skriver et al., 2014), these findings raise the possibility that exercise protocol choice may alter the profile of exercise-related benefits in brain health. Whilst a clear response was seen in blood lactate across all protocols, those seen in SIT were greater than in either the MICT or HIIT exercise protocols, and this may have widespread benefits as lactate serves as a signaling molecule (Nalbandian and Takeda, 2016; Morland et al., 2017). Additionally, elevations in lactate have been shown to drive subsequent elevations in circulating VEGF (Sameer Kumar et al., 2007) and BDNF (Schiffer et al., 2011), and as such may be key to changes in VEGF and BDNF being seen primarily within the SIT protocol alone.

The potential for lactate to stimulate VEGF release, alongside intensity-dependent upregulation within the skeletal muscle (Hoier and Hellsten, 2014), may help to explain the significant elevations in SIT alone. The lack of VEGF modulation by either MICT or HIIT adds to previous literature showing contrasting findings across a range of exercise protocols and cohorts, likely due to the influence of confounding factors (Kraus et al., 2004; Skriver et al., 2014; Basso and Suzuki, 2017). While it was beyond the scope of this study to determine the influence of all confounding factors, both sex and fitness were taken into consideration for model fitting and neither resulted in improved model fit. Based on previous research, low basal levels of VEGF in combination with high interindividual variability may explain why a number of samples were found to fall below the limit of detection during analysis (Jelkmann, 2001). Despite this reduction in sample size, significant increases were seen with SIT, indicating that SIT may induce a more consistent response in VEGF compared to lower intensity exercise. These findings add to a growing body of evidence that high intensity, interval-based conditions can have a significantly greater impact on transient responses in circulating VEGF (Wahl et al., 2011; Kujach et al., 2020).

Significant decreases in IGF-1 during SIT, alongside a pattern of suppression in HIIT, add to inconsistent responses in IGF-1 seen in previous research in which studies have demonstrated both a lack of response (Wahl et al., 2010) and significant elevation in IGF-1 with sprint-based exercise (Kujach et al., 2020). Interestingly, within the current study, IGF-1 does appear to be rising during the recovery period of both interval-based protocols. Previous research has demonstrated dichotomous results in measures of IGF-1 taken during recovery, with those showing increases during recovery finding peak changes within an hour of exercise (Kraemer et al., 2017). The relationship between IGF-1 and its binding proteins in regulating signaling

(Kraemer et al., 2017); alongside the capacity for peripheral IGF-1 to be regulated by and transported into the brain in animal models (Fernandez and Torres-Alemán, 2012; Vecchio et al., 2018) adds further complexity to exercise associated responses. For this reason, it is essential that future studies explore the role that exercise protocol choices play in modulating IGF-1 and associated binding proteins, particularly over longer periods of recovery.

Responses in BDNF within the current study are consistent with previous research in relation to the response to SIT (Kujach et al., 2020) and the relative lack of response at lower intensities (Nofuji et al., 2012; Reycraft et al., 2020). One possible explanation for this lack of response at lower intensities is the relatively short half-life of BDNF in the circulation of ∼2.7 min reported by some authors (Pardridge et al., 1994), which would necessitate very high levels of release in order for accumulation of BDNF to be detected in plasma. However, more recent studies have disputed this half-life time, demonstrating half-lives as long as 60 min (Pan et al., 1998; Khalin et al., 2016). In addition, the potential for lactate to upregulate BDNF in a similar manner to VEGF (Schiffer et al., 2011) also raises the potential role that intensity-related elevations in lactate may play in BDNF elevations. Interestingly, a significant difference was seen between male and female participants within the current study, with female participants showing greater responses in the recovery period during SIT and significant elevations in MICT that were not seen in male participants or the cohort as a whole (**Figure 6**). Previous studies have highlighted the potential for sex differences both at rest (Lommatzsch et al., 2005; Piccinni et al., 2008) and during exercise (Serra-Millàs, 2016; Dinoff et al., 2017). While in a recent meta-analysis, Dinoff et al. (2017) found significant elevations in BDNF in males alone, the authors highlighted that this may be due in part to a relative lack of studies including female cohorts. In combination with the contrasting findings of the present study and a general lack of studies across different exercise protocols, there is a clear need for further research into the potential role of sex differences in exercise-related BDNF responses. Within the current study female participation was not restricted by oral contraceptive use or menstrual cycle phases, so the role that cycle phase plays in these differences cannot be fully understood. This is particularly pertinent given the significant variations across the menstrual cycle highlighted by Begliuomini et al. (2007), thus further research is needed to explore the influence that this basal variability may play in exercise-induced responses within females and in comparison to male cohorts. The lack of BDNF modulation by HIIT also raises interesting questions for future study as to the role of exercise duration and intensity in stimulating changes in BDNF, with consideration as to how plasma half-life may dictate the capacity for exercise to drive accumulation within circulation.

Limitations and Future Directions

While growing evidence supports the utilization of interval-based exercise, it is important to consider the potential risks of these strategies against the physiological benefits they may provide (Lucas et al., 2015; Labrecque et al., 2020). Encouragingly, recent studies into the risk between interval-based and traditional continuous exercise protocols for cardiovascular disease cohorts have shown that the two are of similar risk (Rognmo et al., 2012; Wewege et al., 2018). Our findings, alongside the limited risk that clinical guideline-based intervals appear to pose, do suggest that interval-based exercise can be safely used to positively impact cerebrovascular health. However, it is still important that the safety of such interventions are considered before they are applied across a broader population, including at-risk cohorts.

The utilization of TCD to measure blood velocity as a proxy of cerebral blood flow has been widely debated (Ainslie and Hoiland, 2014). The potential for the MCA to change in diameter during exercise has significant ramifications for the use of TCD, particularly as the nature of the diameter change may depend on the specific exercise stimulus and these concerns must be taken into consideration when utilizing TCD (Hoiland and Ainslie, 2016; Verbree et al., 2017). While alternative methods such as MRI, fNIRS, and duplex ultrasound may present a more accurate or in depth examination of the cerebrovasculature, at present these methods cannot be employed across the range of exercise protocols examined in the current study (Ainslie and Hoiland, 2014).

The potential influence of changes in hemoconcentration as a result of fluid shift during exercise was not considered within the current study. While previous research has shown greater levels of hemoconcentration at higher intensities (Wahl et al., 2010; Bloomer and Farney, 2013), the lack of responses in either MICT or HIIT, alongside significant reductions in IGF-1, contradict the potential for circulating responses to be purely hemoconcentration-dependent. Additionally, measures of BDNF were taken in plasma samples alone and not in serum, which the latter previously has been shown to generate significantly higher yields of BDNF due to the contribution of platelet-derived BDNF (Serra-Millàs, 2016). However, our aim within the current study was to identify responses within the circulating levels of neurotrophic factors and their release from tissue and as such plasma-based measures of BDNF were chosen. While care was taken in the preparation of plasma samples, so as to avoid the inclusion of platelets within the final sample, we did not include a second centrifugation step to eliminate any residual platelets from samples and as such this may have affected BDNF and other measures sensitive to platelet activity. However, preparation of plasma was carried out at higher speeds in a similar manner to a recently validated protocol for the removal of platelets by single-step centrifugation (Rikkert et al., 2020), although this protocol utilized a 20-minute centrifugation, compared to the 10-minute period used within the current study.

As already discussed, female participants were included regardless of oral contraceptive usage and menstrual phase. While this limits our ability to determine the influence of sex hormones or menstrual cycle, this decision was made to address the studies aims in including free-living individuals and to maximize recruitment of female participants (Stanhewicz and Wong, 2020). Furthermore, while numerous strategies have been suggested to control for the menstrual cycle within studies, the variability between individuals makes many of these strategies unreliable (Häggström, 2014; Sims and Heather, 2018). Nevertheless, our findings demonstrate the potential for sex differences to influence acute exercise responses within the vasculature, warranting further exploration of this interaction.

While this study demonstrates the clear difference in acute responses, it is not yet clear whether the differences between these acute bouts result in significant differences in a chronic context. As such, although growing evidence supports the concept that acute responses are often indicative of the chronic impact of interventions (Dawson et al., 2018; Voss et al., 2020), the exploration of the response to a prolonged training period across all three exercise approaches is clearly warranted. Similarly, the population studied within the current work were healthy, young, and active, and so future work is required to identify how differences across the population may alter this response.

CONCLUSION

In conclusion, similar acute hemodynamic responses are seen between traditional continuous and more recent guideline-based interval exercise strategies (4 × 4 min bouts at 85% HR_{max}), indicating that this type of interval-based exercise approach may also be capable of inducing similar changes in cerebrovascular function over chronic training periods. While sprint-based intervals drove relatively limited changes in blood velocity compared to guideline-based interval and traditional continuous exercise strategies, these intervals induced large changes in circulating factors with a potential to significantly impact cerebrovascular health and function. This dichotomy in response between exercise strategies may provide an alternate or adjunct means for improving brain health through combining approaches to physical activity within interventions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the University of Birmingham Ethics Committee. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Experiments were carried out in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham, UK. SW, RF, and SL: conception and design of the experiments. SW, BS, RF, RL, SL, NC, CR, and HM: pilot testing, design revision, and experiment preparation. SW, BS, RF, RL, and SL: data collection. SW and SL: data processing and analysis. SW, HM, and SL: manuscript preparation. All co-authors read, contributed with comments, and approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix 5 General Health Questionnaire

The University of Birmingham

School of Sport, Exercise and Rehabilitation Sciences

General Health Questionnaire

Participant ID: .....

Date: .....

Name of the responsible investigator for the study:.....

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed study is appropriate for you. Your answers will be kept strictly confidential.

You do not have to answer any questions that you are not comfortable answering, but omission of certain questions may mean that you are unable to take part in this study.

1.	You are.....	Male	Female
3.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
4.	Are you currently taking any medication?	YES	NO
5.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
6.	Has your doctor ever said you have “heart trouble”?	YES	NO
7.	Has your doctor ever said you have high blood pressure?	YES	NO
8.	Have you ever taken medication for blood pressure or your heart?	YES	NO
9.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
10.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
11.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO

13.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
14.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
15.	Do you suffer from asthma?	YES	NO
16.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
17.	Has your doctor ever said you have diabetes?	YES	NO
18.	Have you ever had heat stroke?	YES	NO
19.	Have you ever had viral hepatitis?	YES	NO
20.	If you are female, to your knowledge, are you pregnant?	YES	NO
21.	Do you have kidney or gut dysfunction?	YES	NO
22.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
23.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

YES	NO

Appendix 6 General Practice Physical Activity Questionnaire

General Practice Physical Activity Questionnaire

Date……

Participant ID:……..

1. Please tell us the type and amount of physical activity involved in your work.

		Please mark one box only
a	I am not in employment (e.g. retired, retired for health reasons, unemployed, full-time carer etc.)	
b	I spend most of my time at work sitting (such as in an office)	
c	I spend most of my time at work standing or walking. However, my work does not require much intense physical effort (e.g. shop assistant, hairdresser, security guard, childminder, etc.)	
d	My work involves definite physical effort including handling of heavy objects and use of tools (e.g. plumber, electrician, carpenter, cleaner, hospital nurse, gardener, postal delivery workers etc.)	
e	My work involves vigorous physical activity including handling of very heavy objects (e.g. scaffolder, construction worker, refuse collector, etc.)	

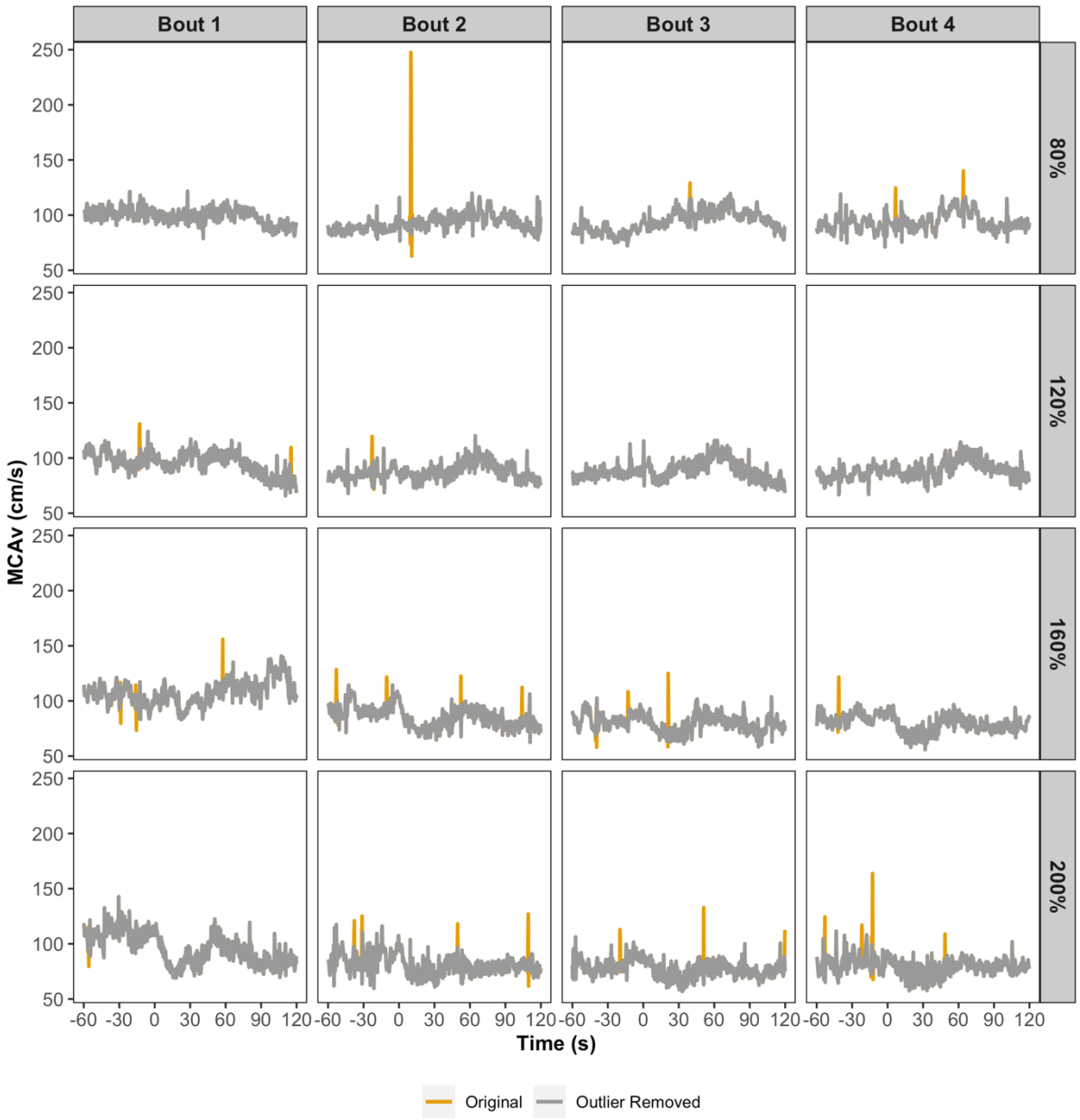
2. During the *last week*, how many hours did you spend on each of the following activities? *Please answer whether you are in employment or not*

Please mark one box only on each row

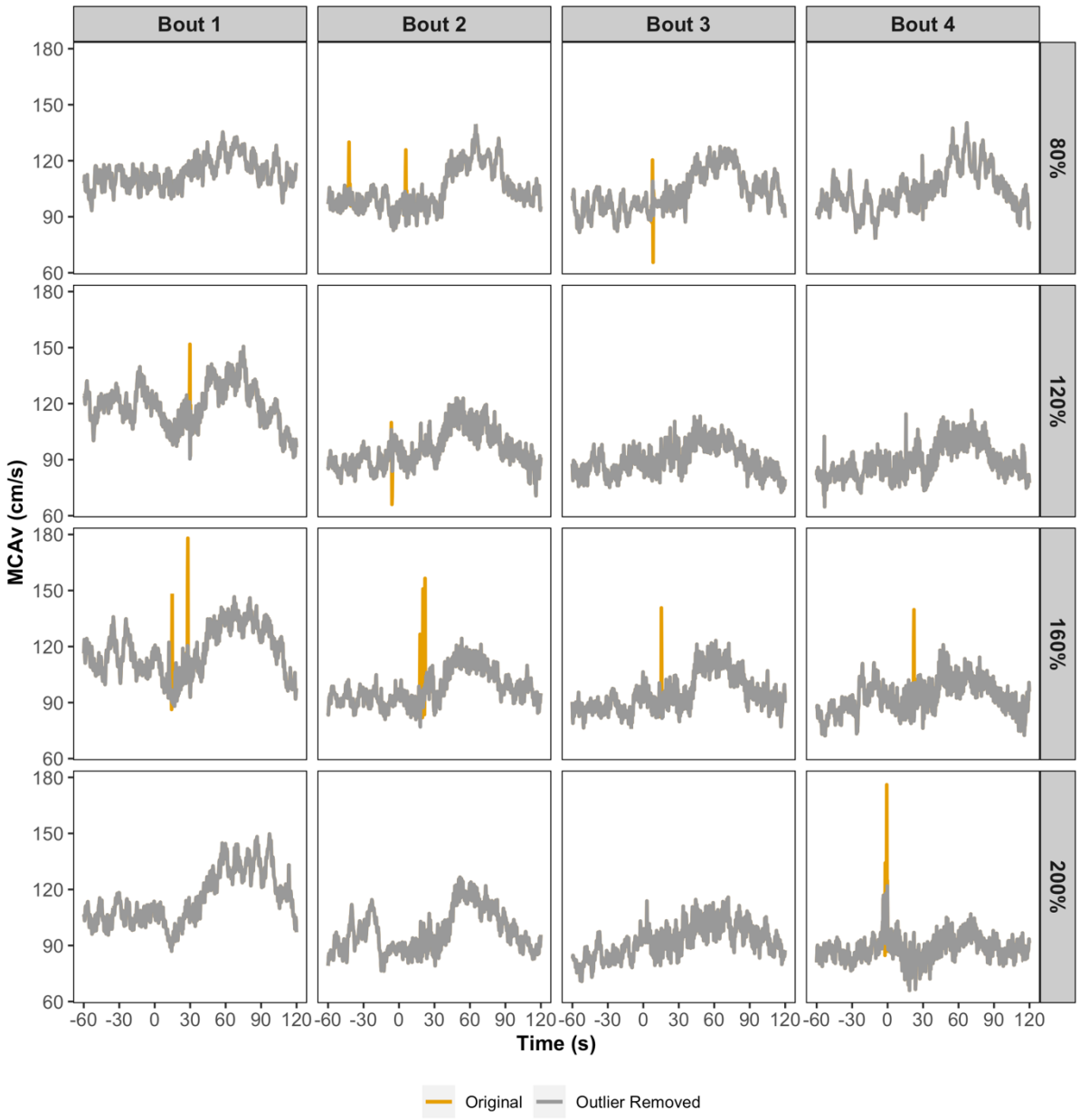
		None	**Some but less than 1 hour**	**1 hour but less than 3 hours**	**3 hours or more**
a	Physical exercise such as swimming, jogging, aerobics, football, tennis, gym workout etc.				
b	Cycling, including cycling to work and during leisure time				
c	Walking, including walking to work, shopping, for pleasure etc.				
d	Housework/Childcare				
e	Gardening/DIY				

3. How would you describe your usual walking pace? Please mark one box only.

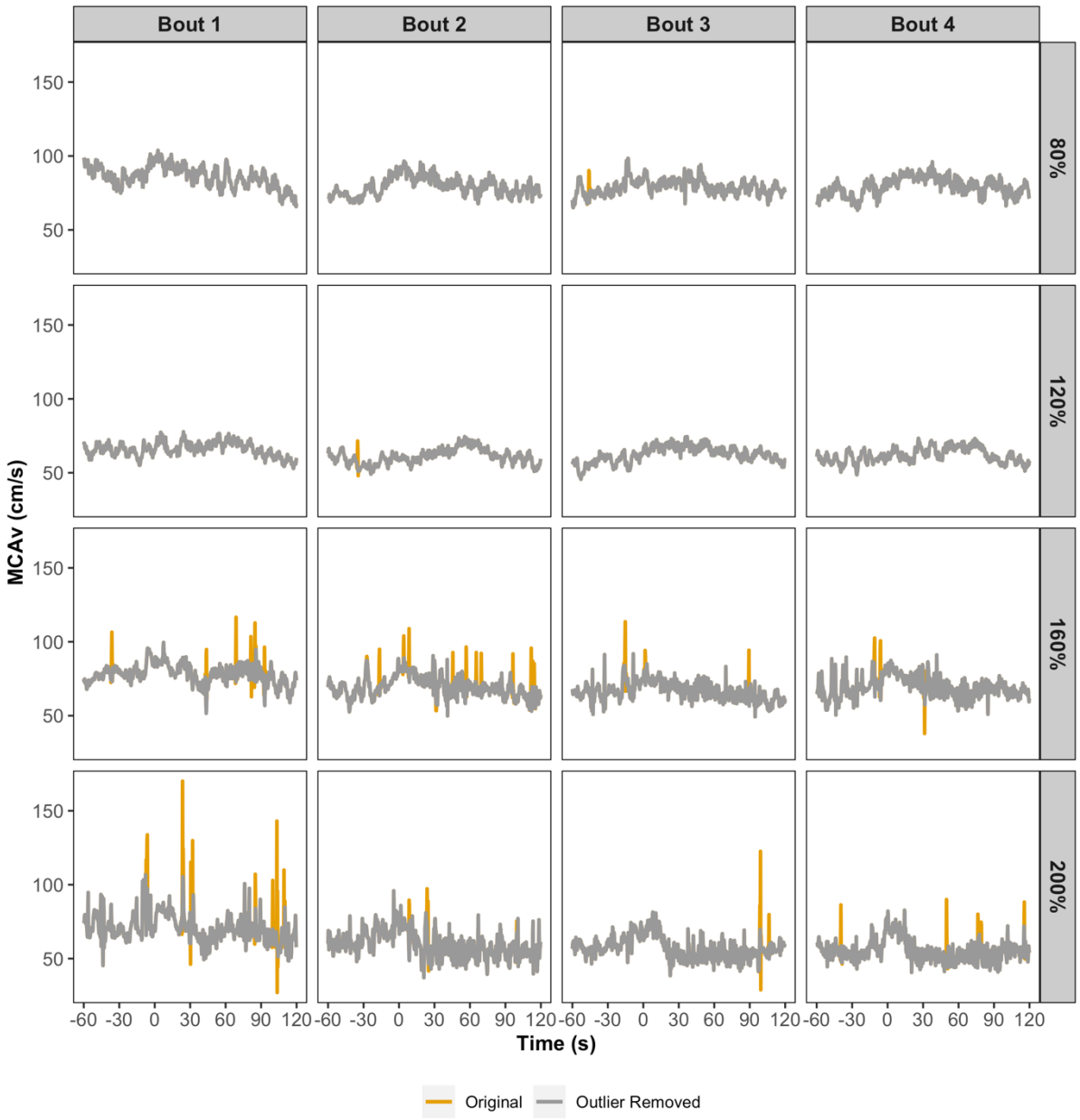
Slow pace (i.e. less than 3 mph)		Steady average pace	
Brisk pace		Fast pace (i.e. over 4mph)	



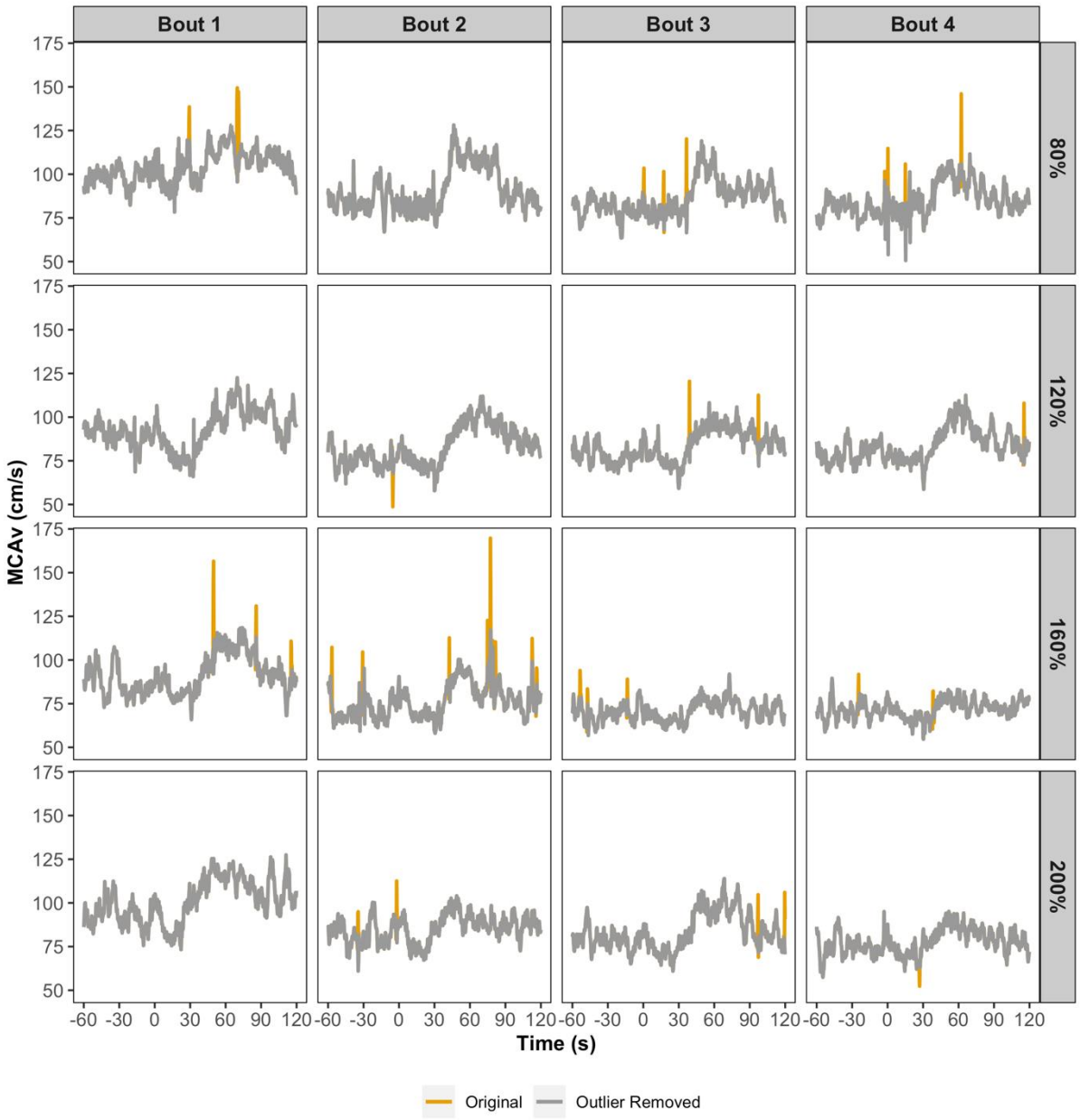
Appendix 7 Representative plots of data following outlier removal (Participant SI001)



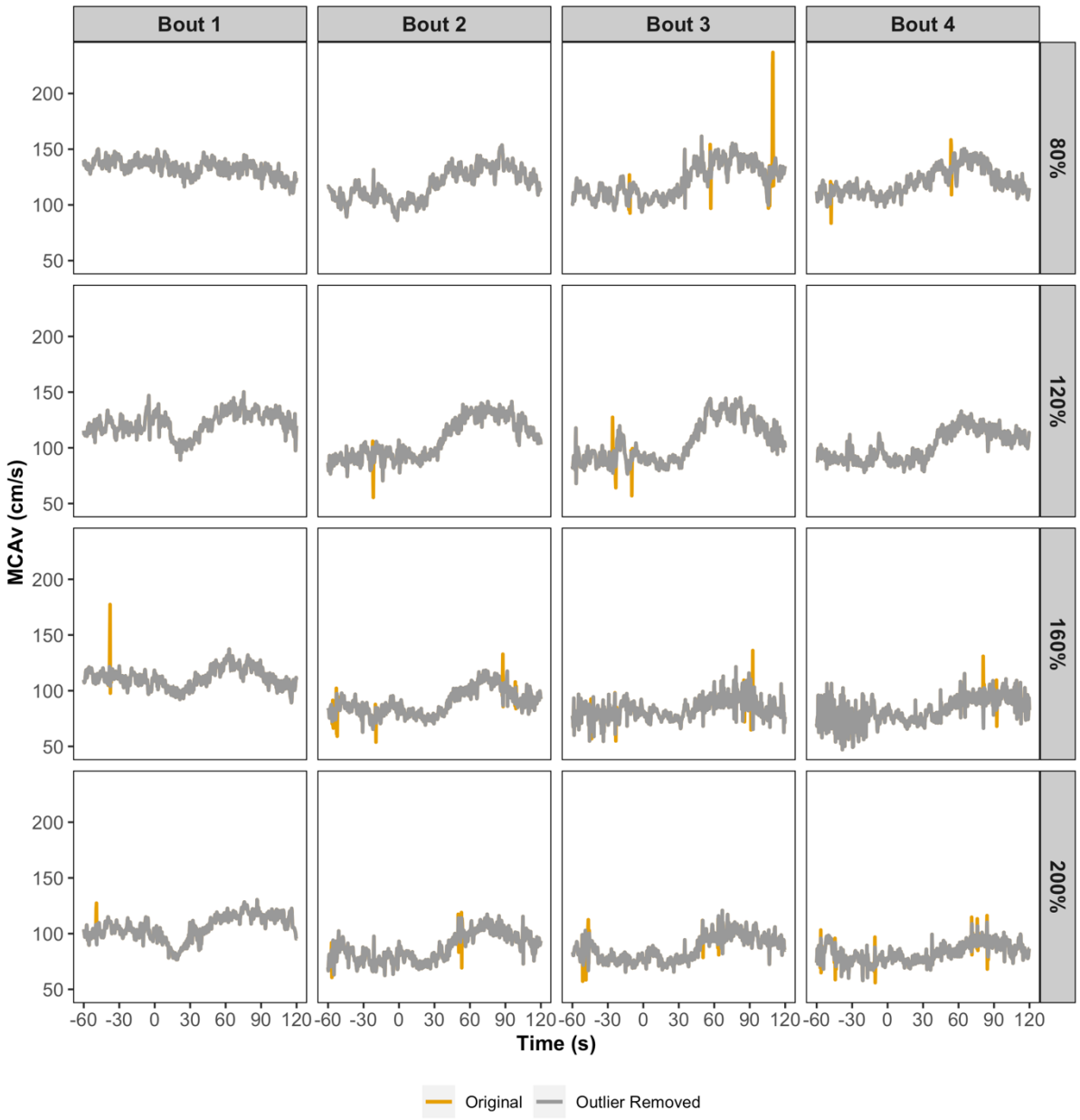
Appendix 8 Representative plots of data following outlier removal (Participant SI002)



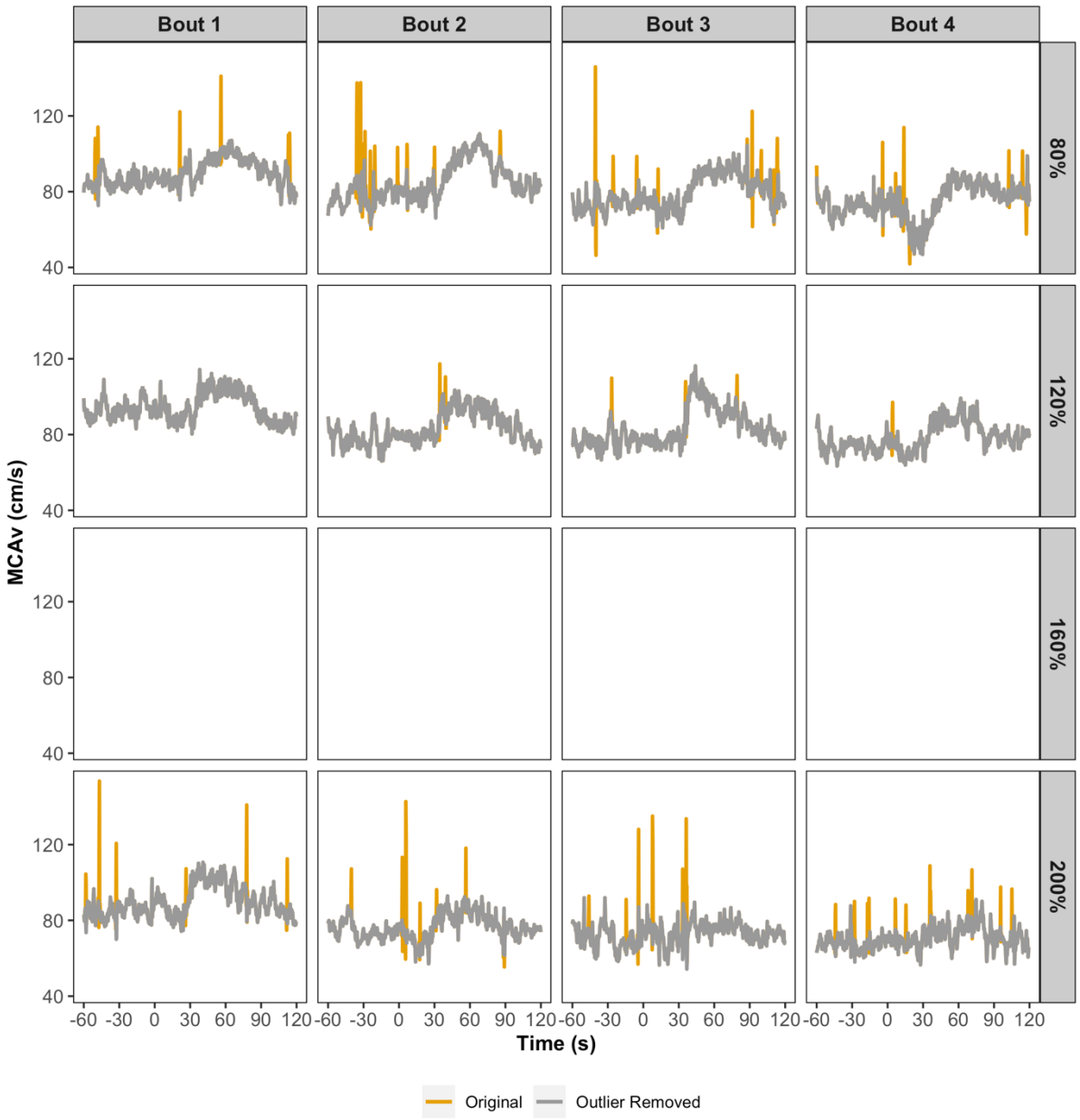
Appendix 9 Representative plots of data following outlier removal (Participant SI003)



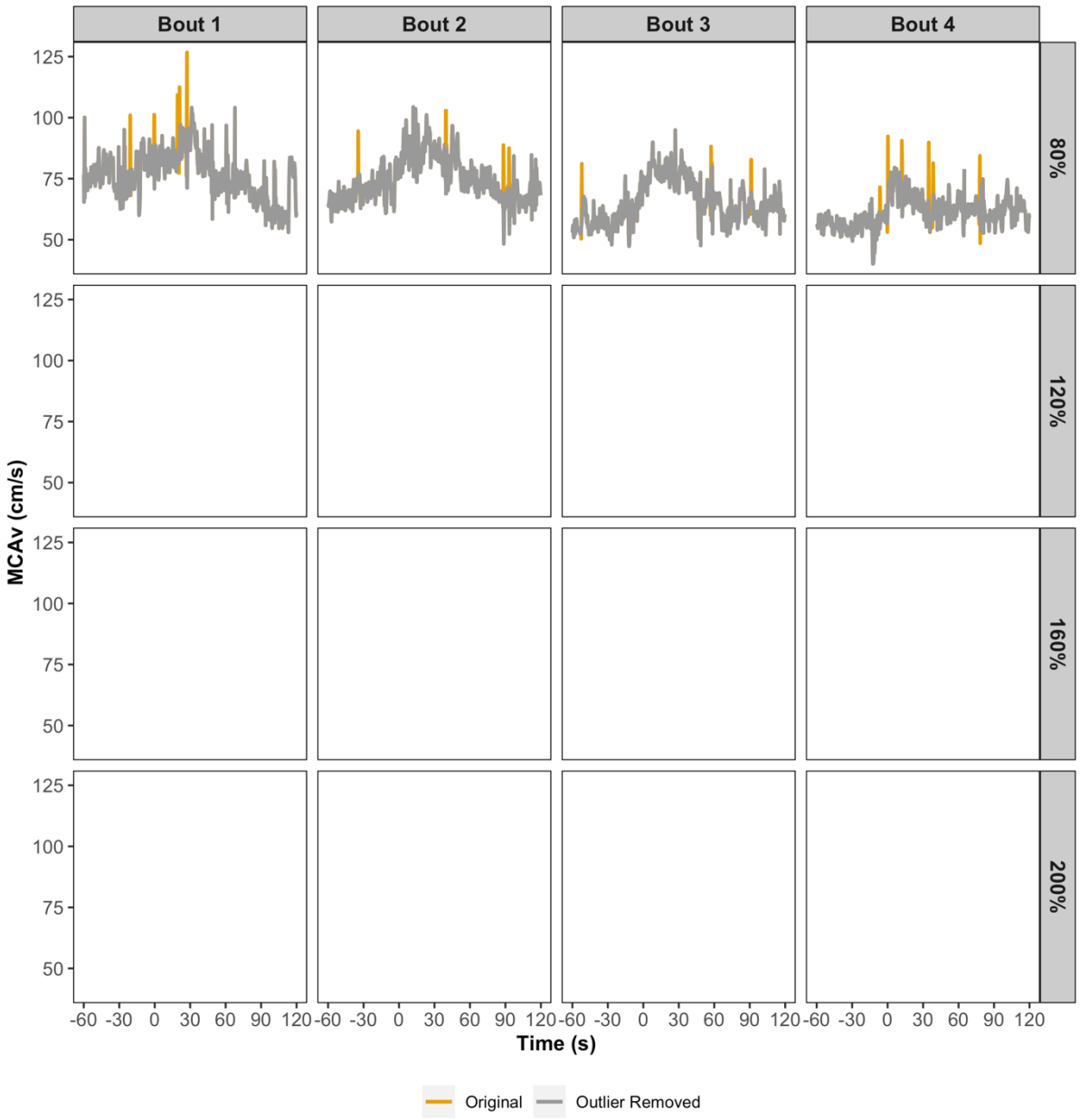
Appendix 10 Representative plots of data following outlier removal (Participant SI005)



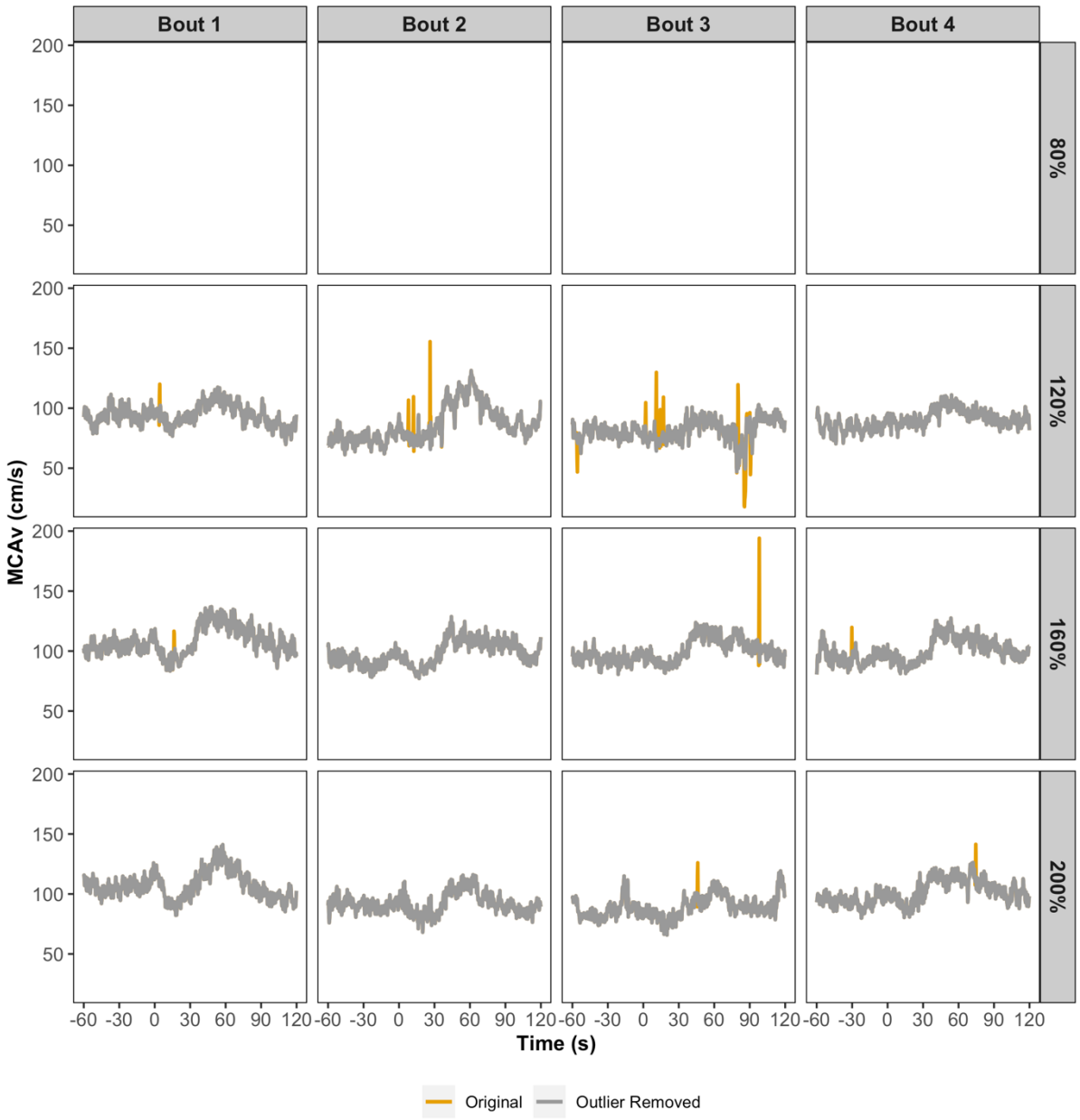
Appendix 11 Representative plots of data following outlier removal (Participant SI006)



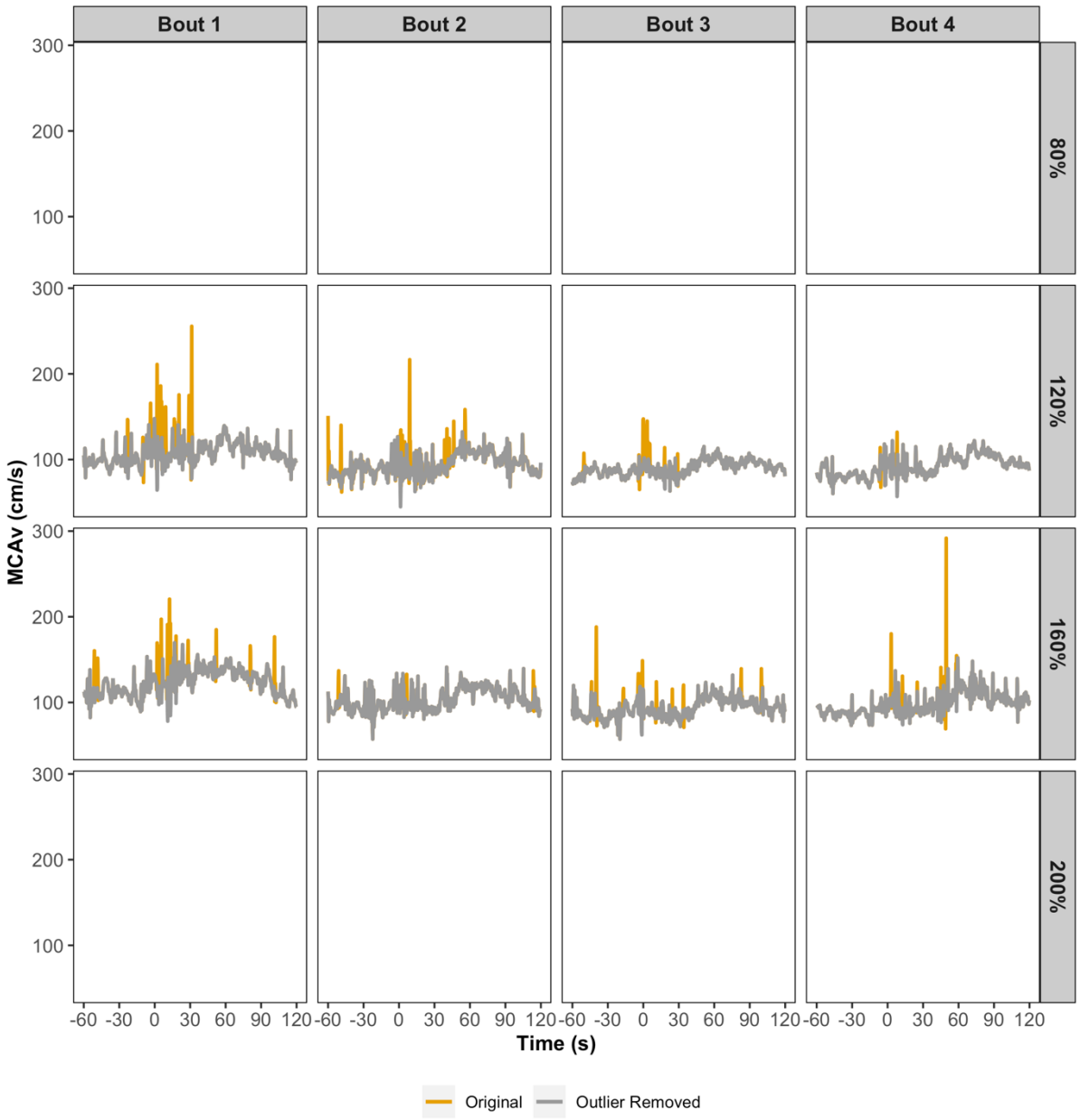
Appendix 12 Representative plots of data following outlier removal (Participant SI009)



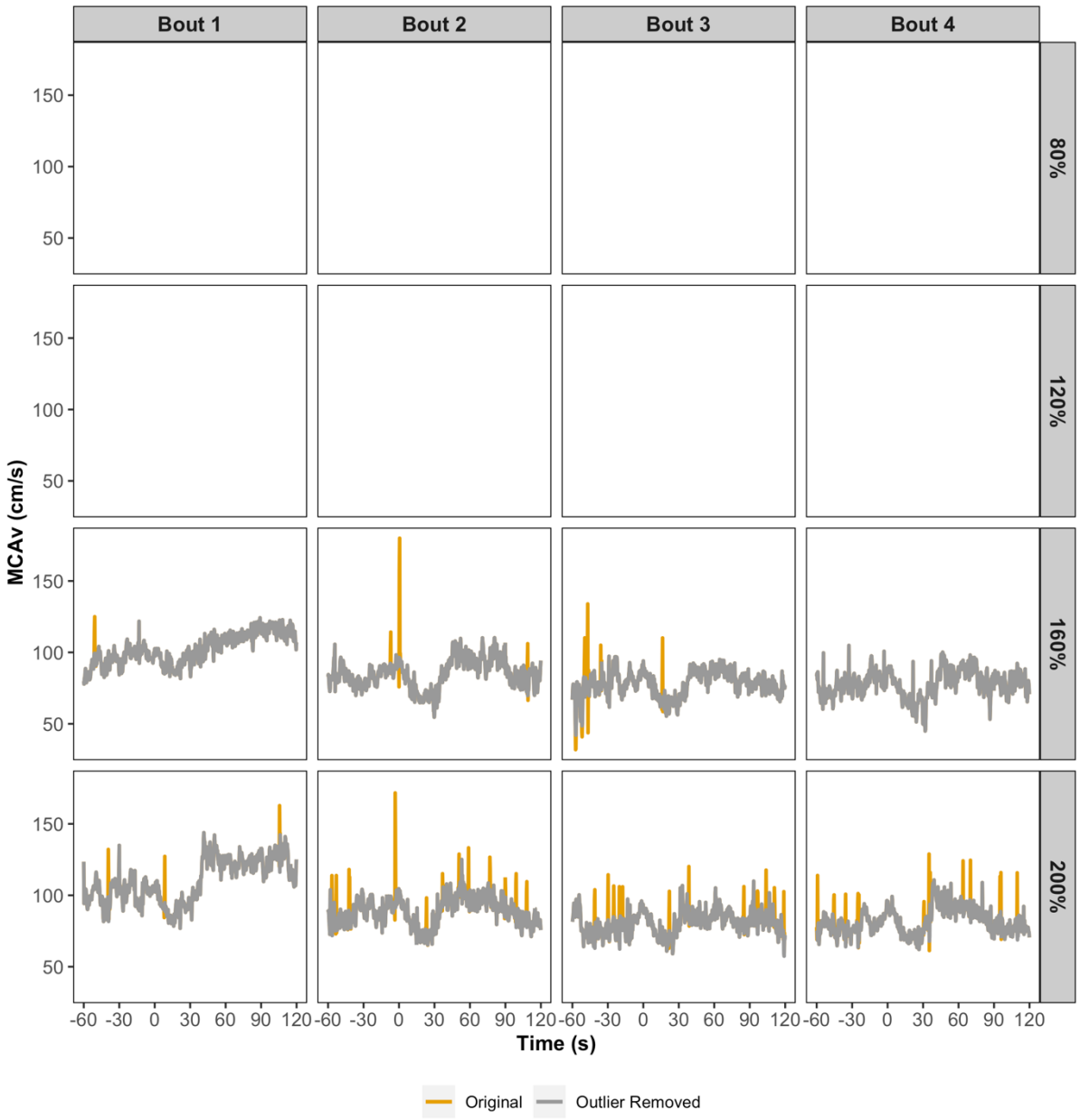
Appendix 13 Representative plots of data following outlier removal (Participant SI010)



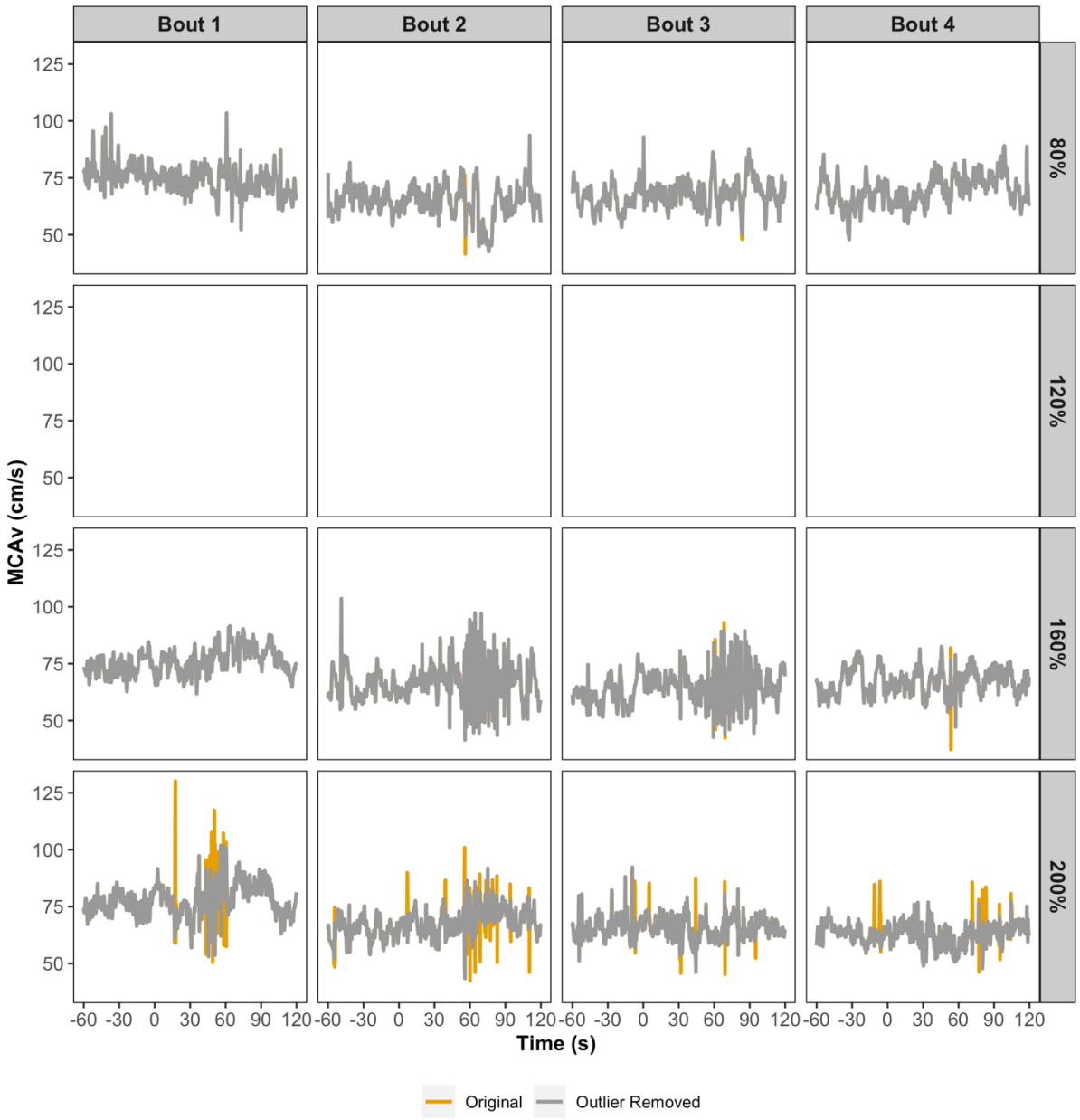
Appendix 14 Representative plots of data following outlier removal (Participant SI011)



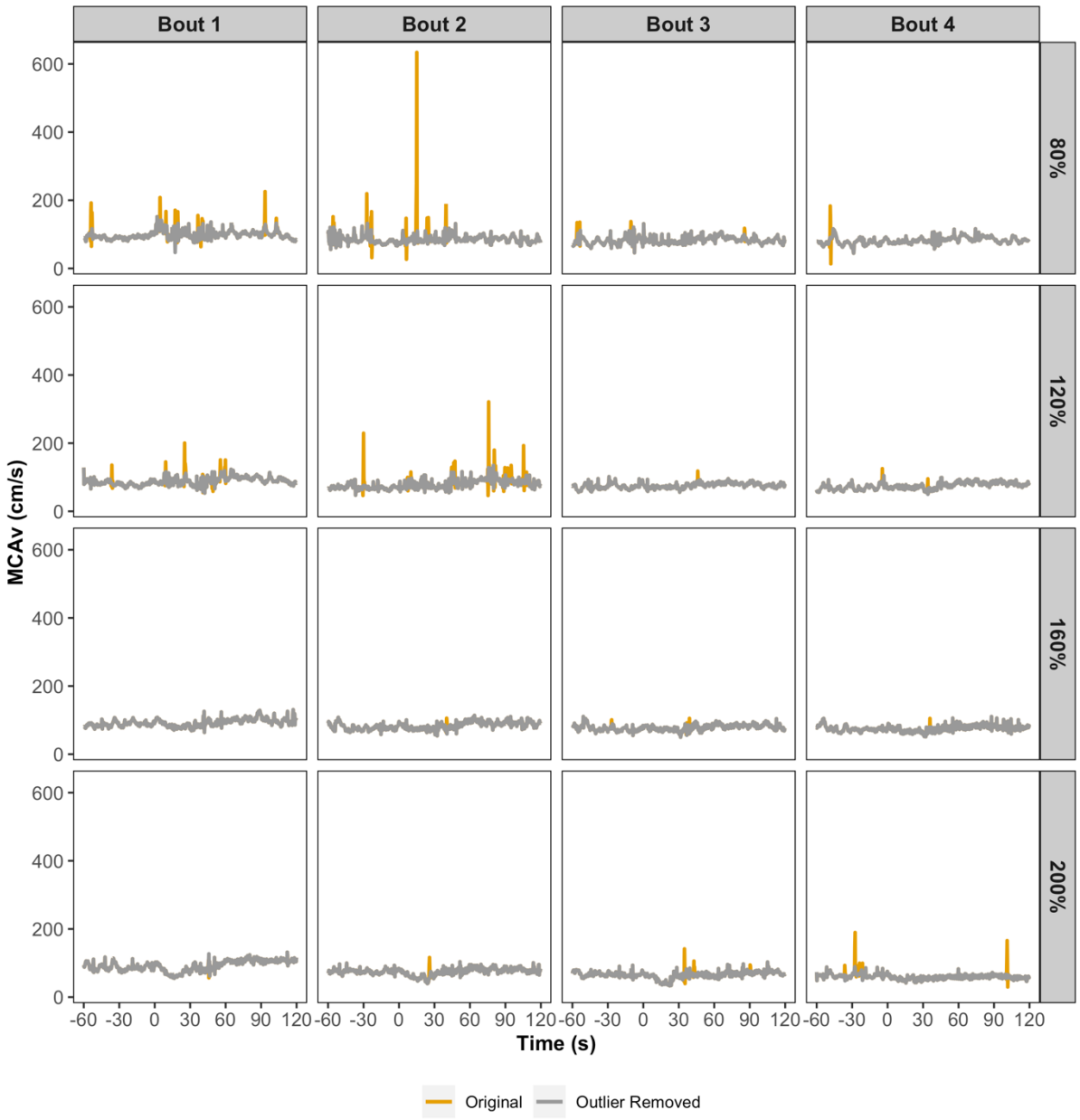
Appendix 15 Representative plots of data following outlier removal (Participant SI012)



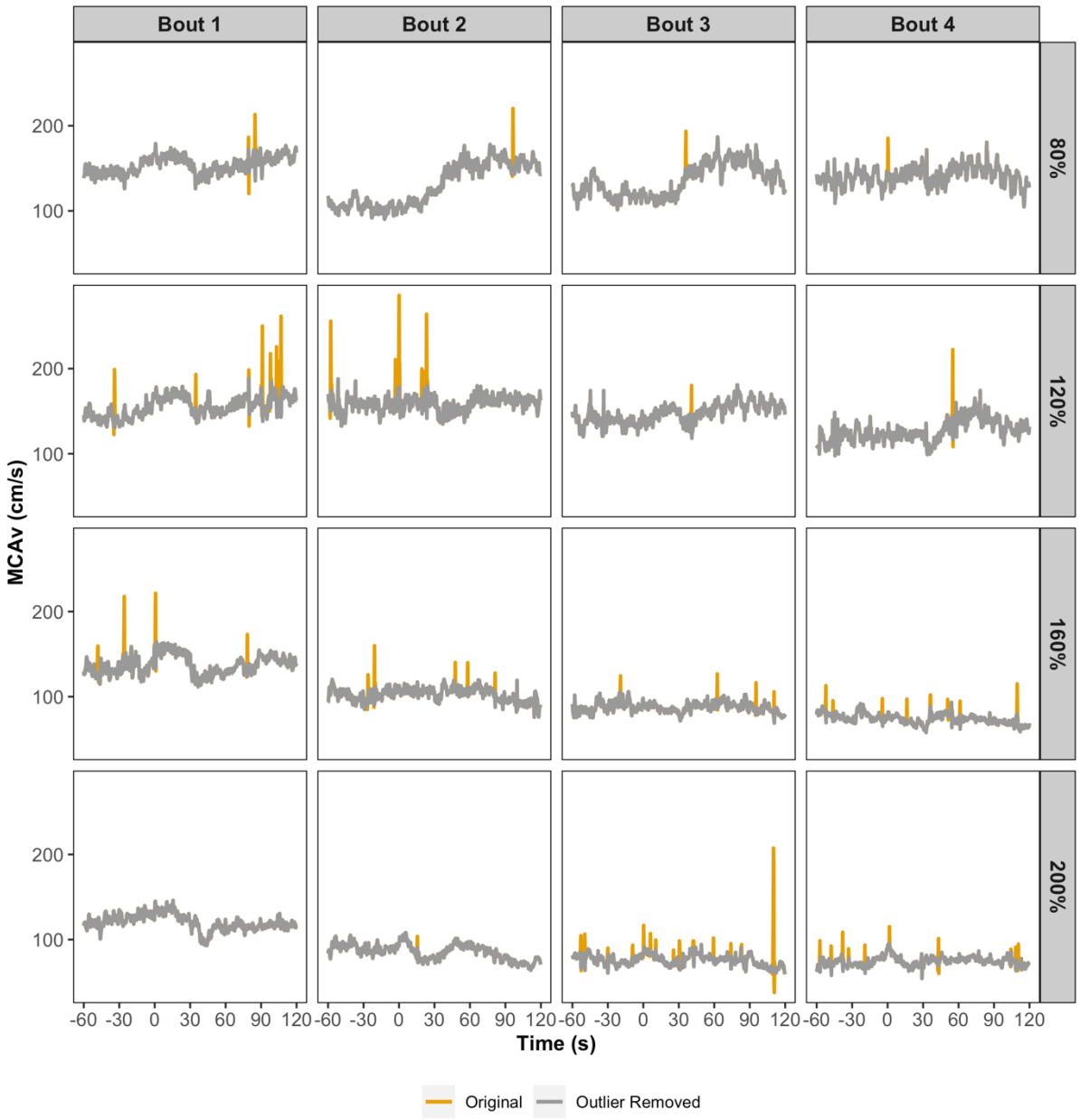
Appendix 16 Representative plots of data following outlier removal (Participant SI013)



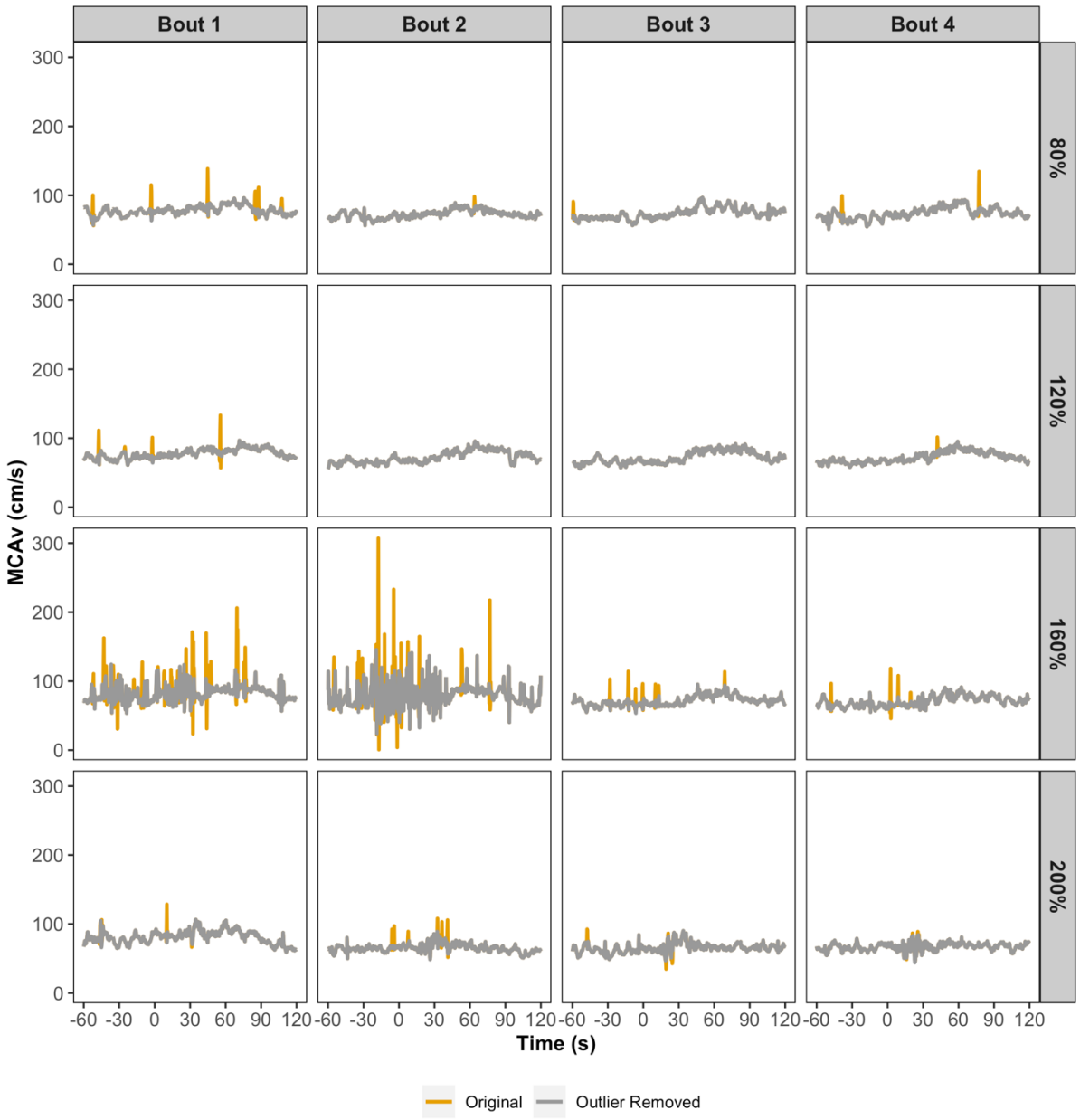
Appendix 17 Representative plots of data following outlier removal (Participant SI014)



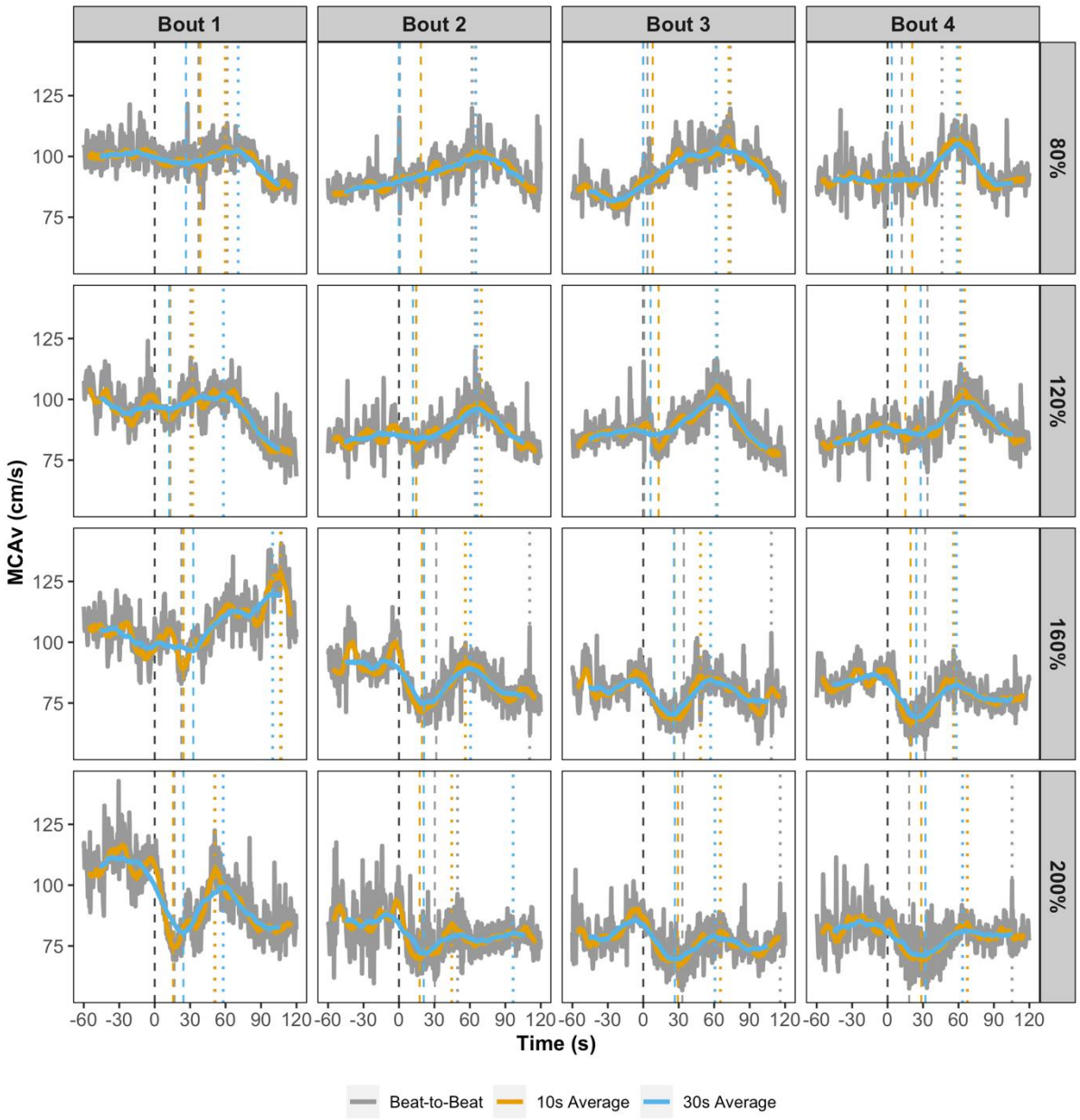
Appendix 18 Representative plots of data following outlier removal (Participant SI015)



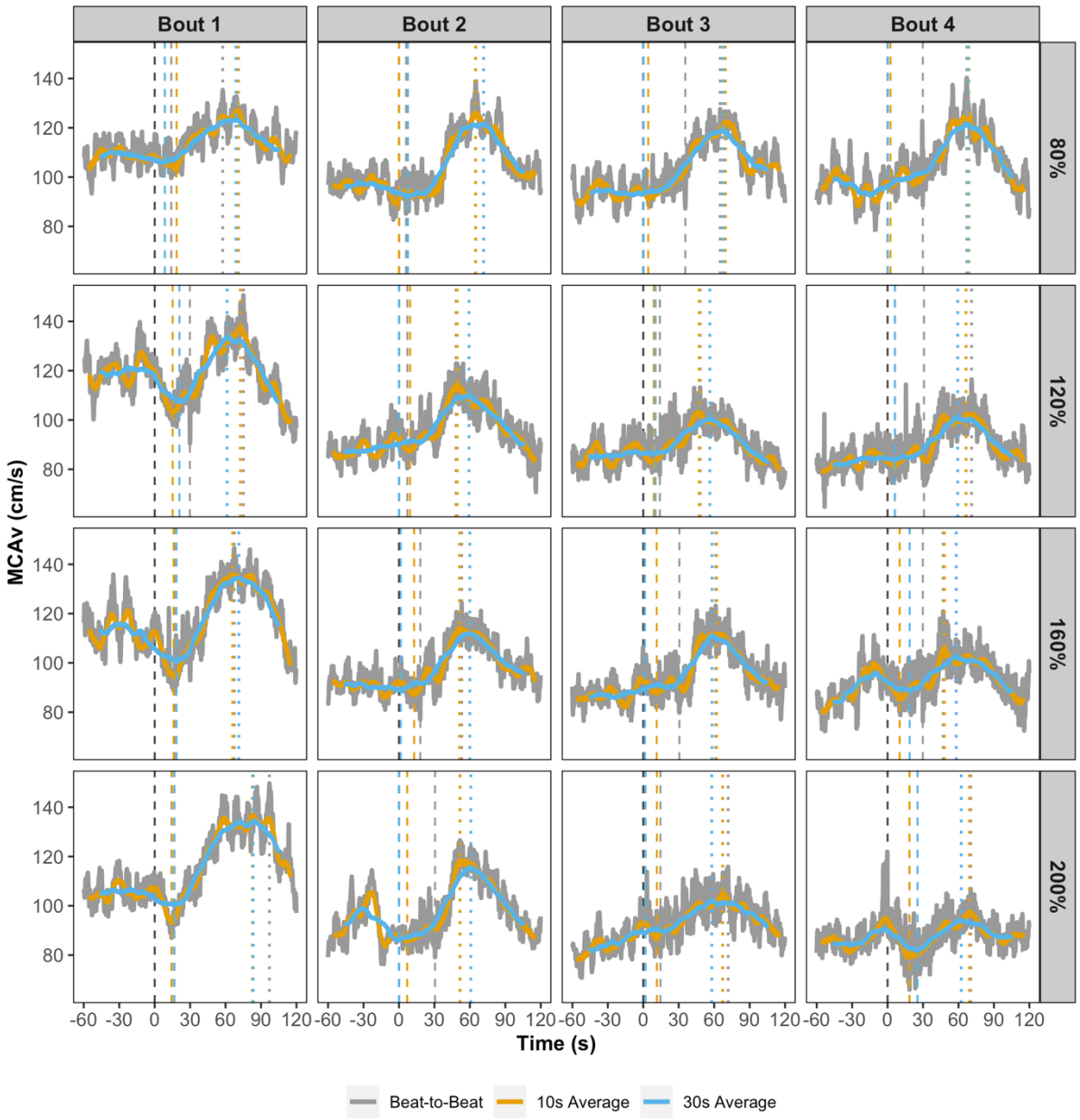
Appendix 19 Representative plots of data following outlier removal (Participant SI017)



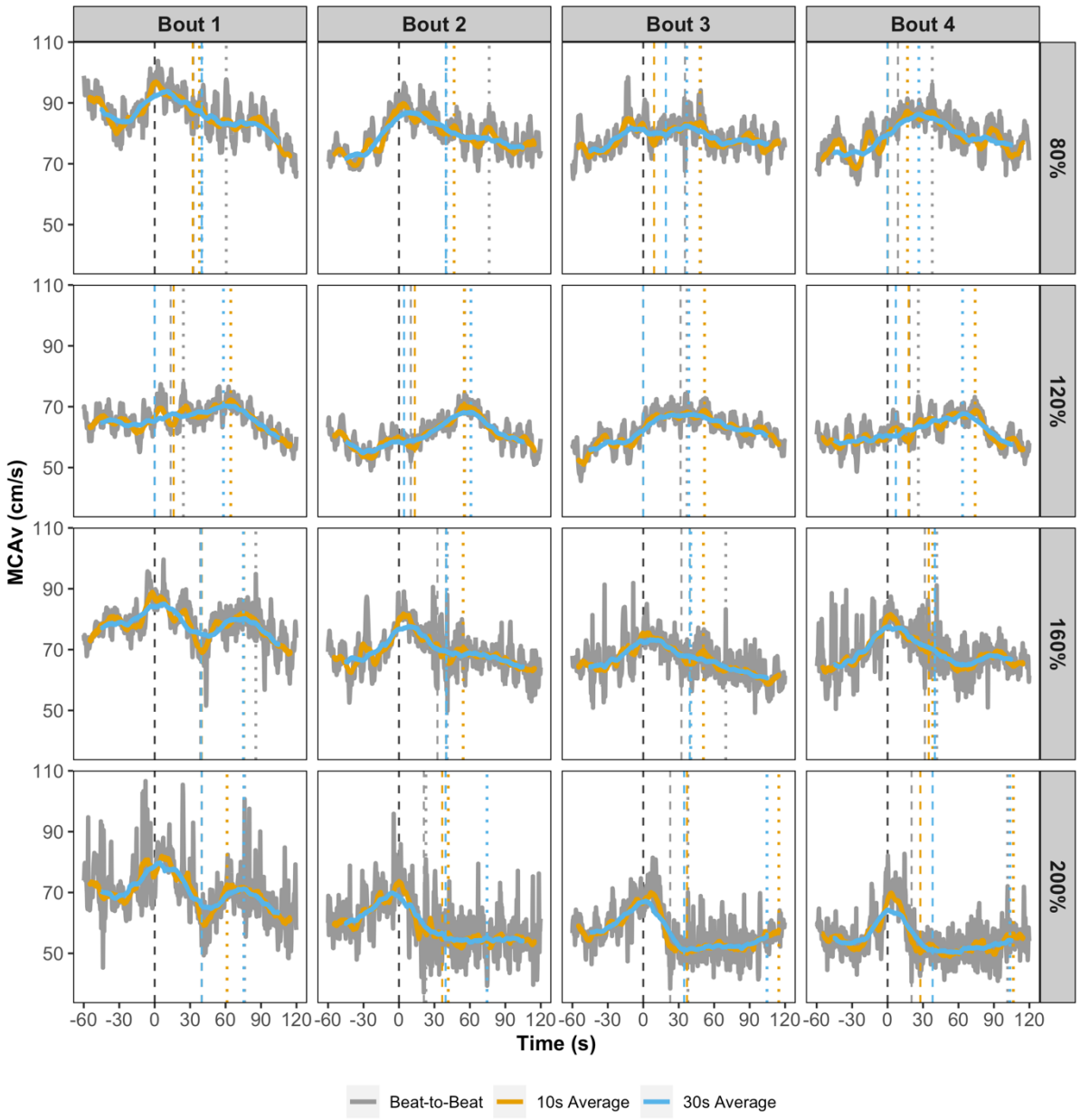
Appendix 20 Representative plots of data following outlier removal (Participant SI018)



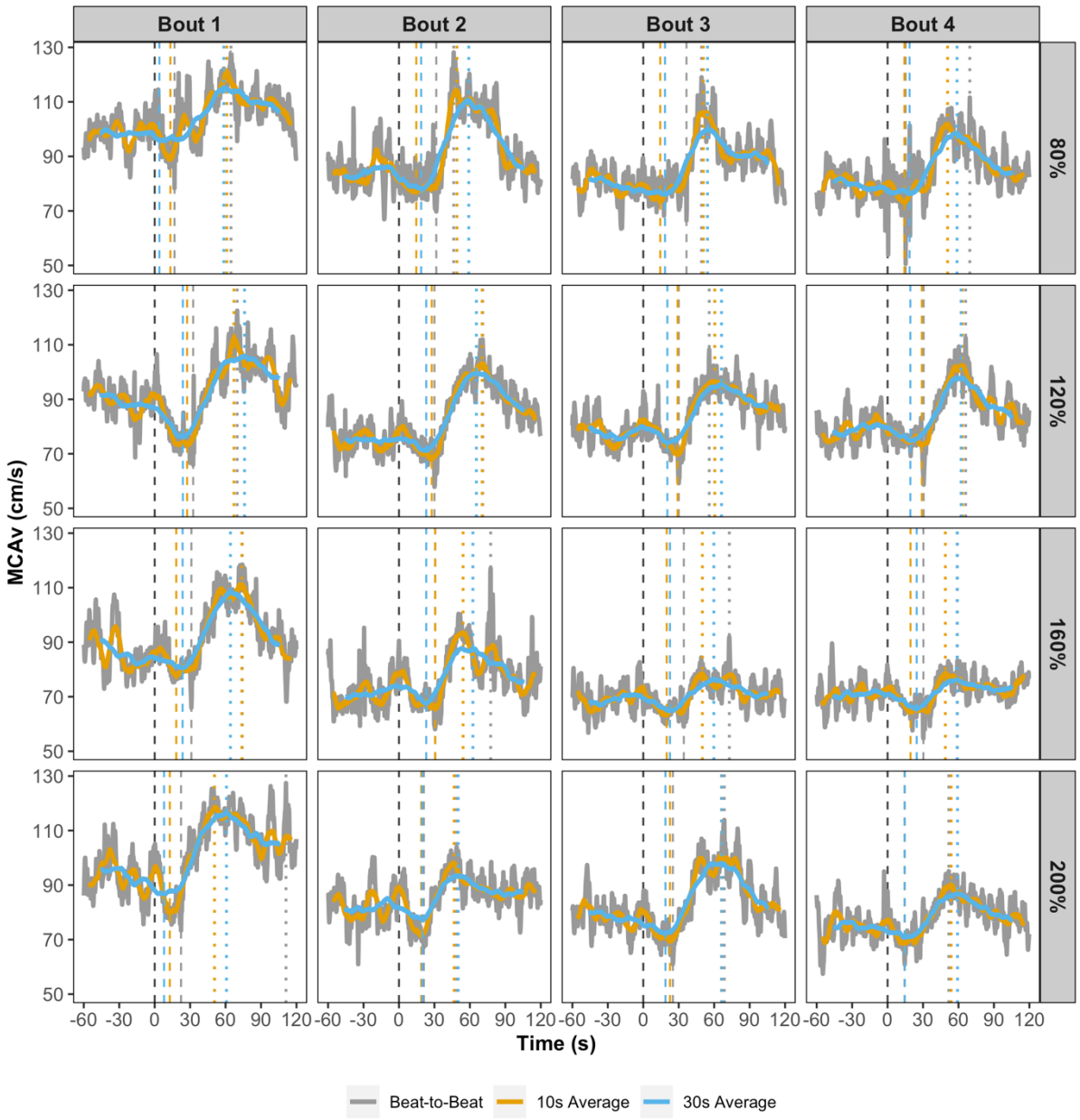
Appendix 21 Comparison of feature selection following different pre-processing approaches (Participant SI001)



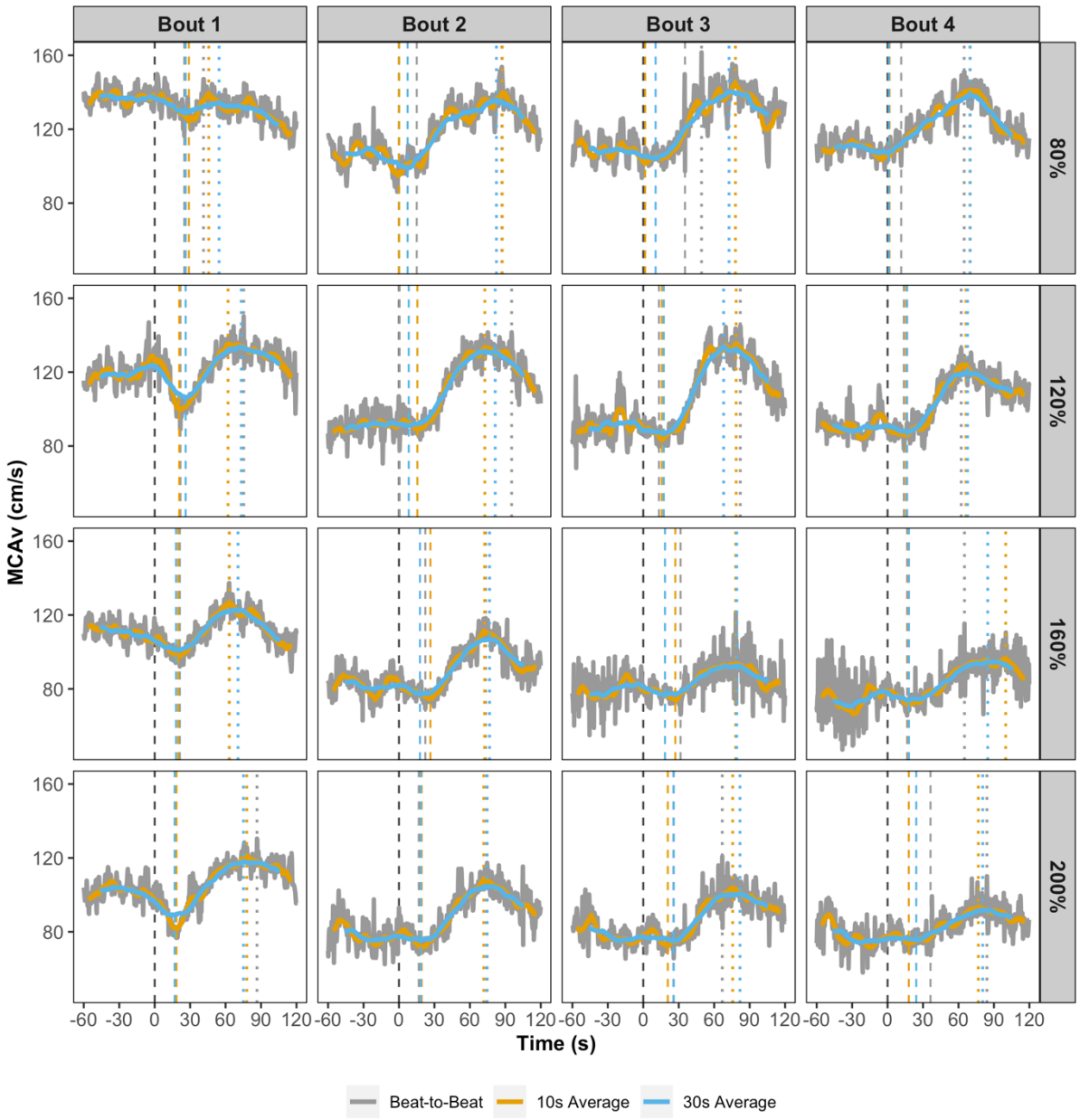
Appendix 22 Comparison of feature selection following different pre-processing approaches (Participant SI002)



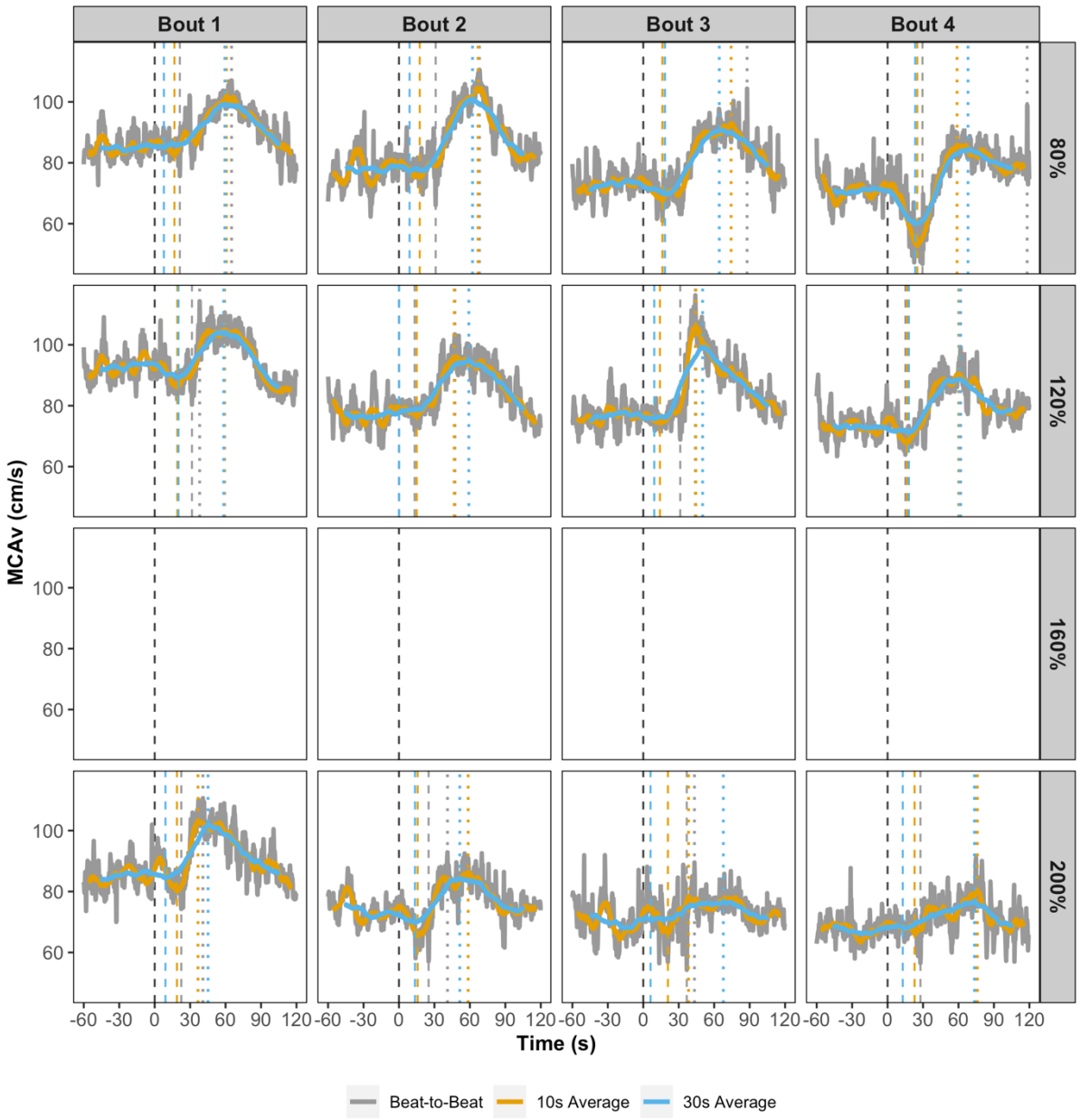
Appendix 23 Comparison of feature selection following different pre-processing approaches (Participant SI003)



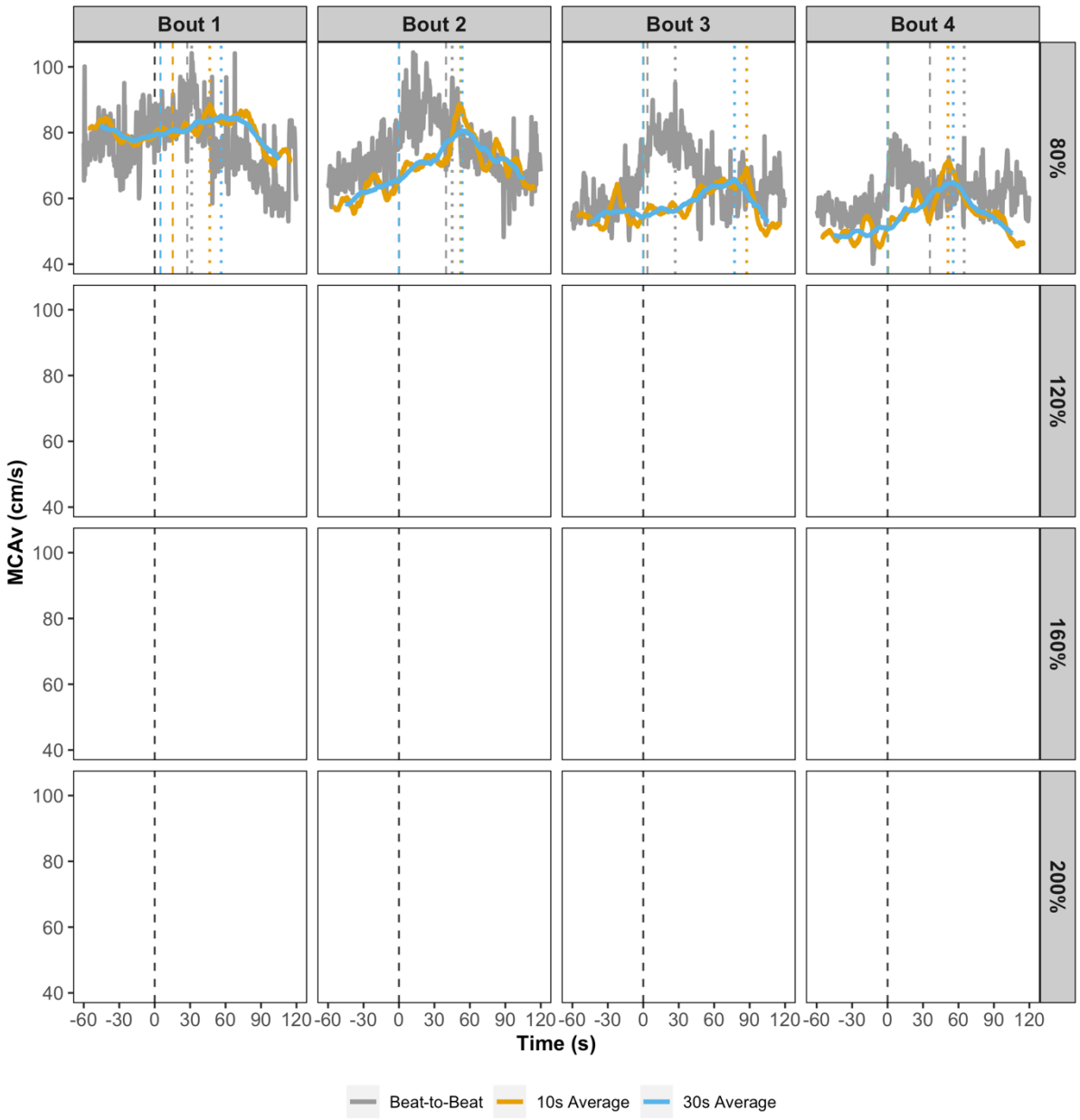
Appendix 24 Comparison of feature selection following different pre-processing approaches (Participant SI005)



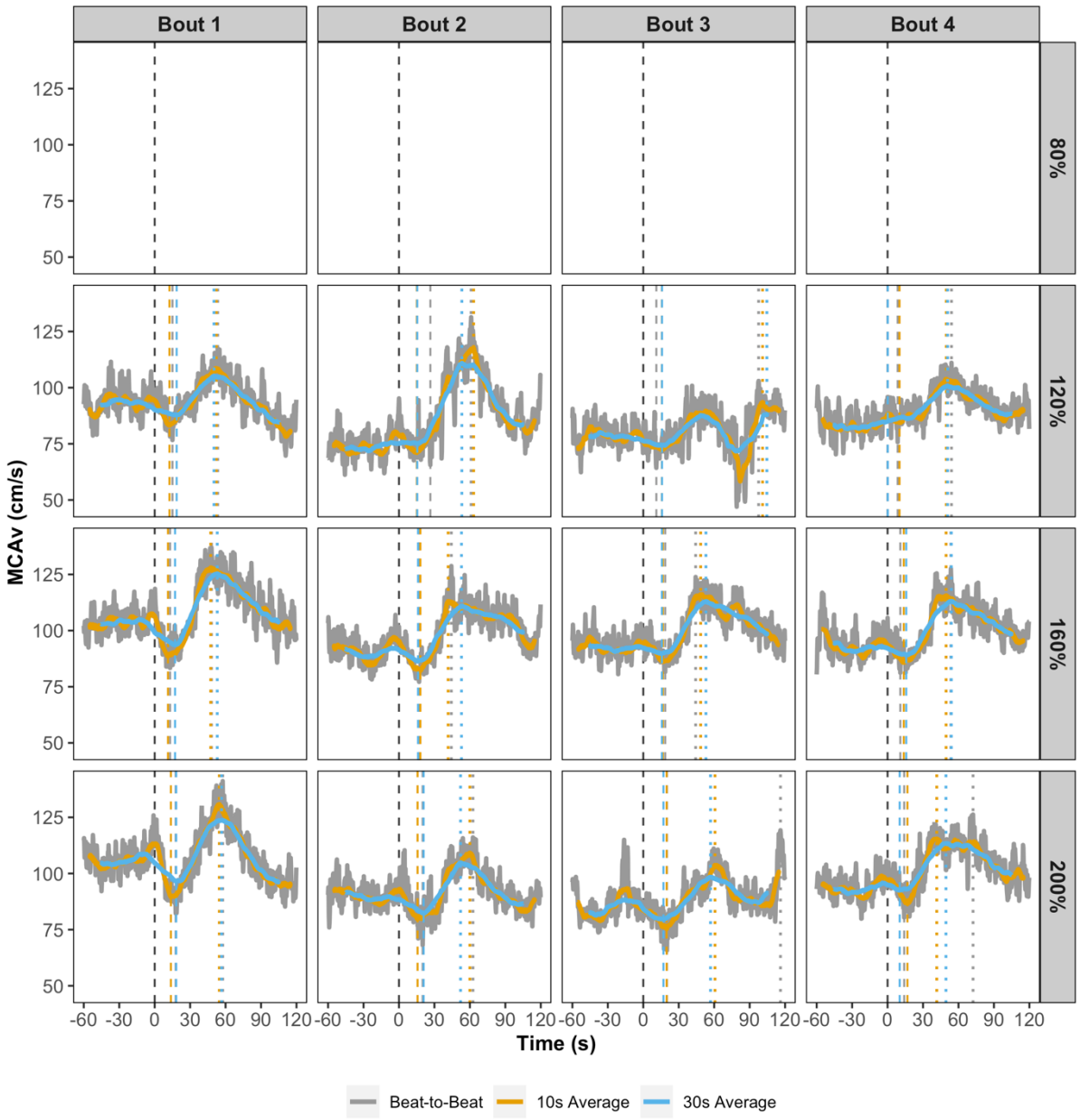
Appendix 25 Comparison of feature selection following different pre-processing approaches (Participant SI006)



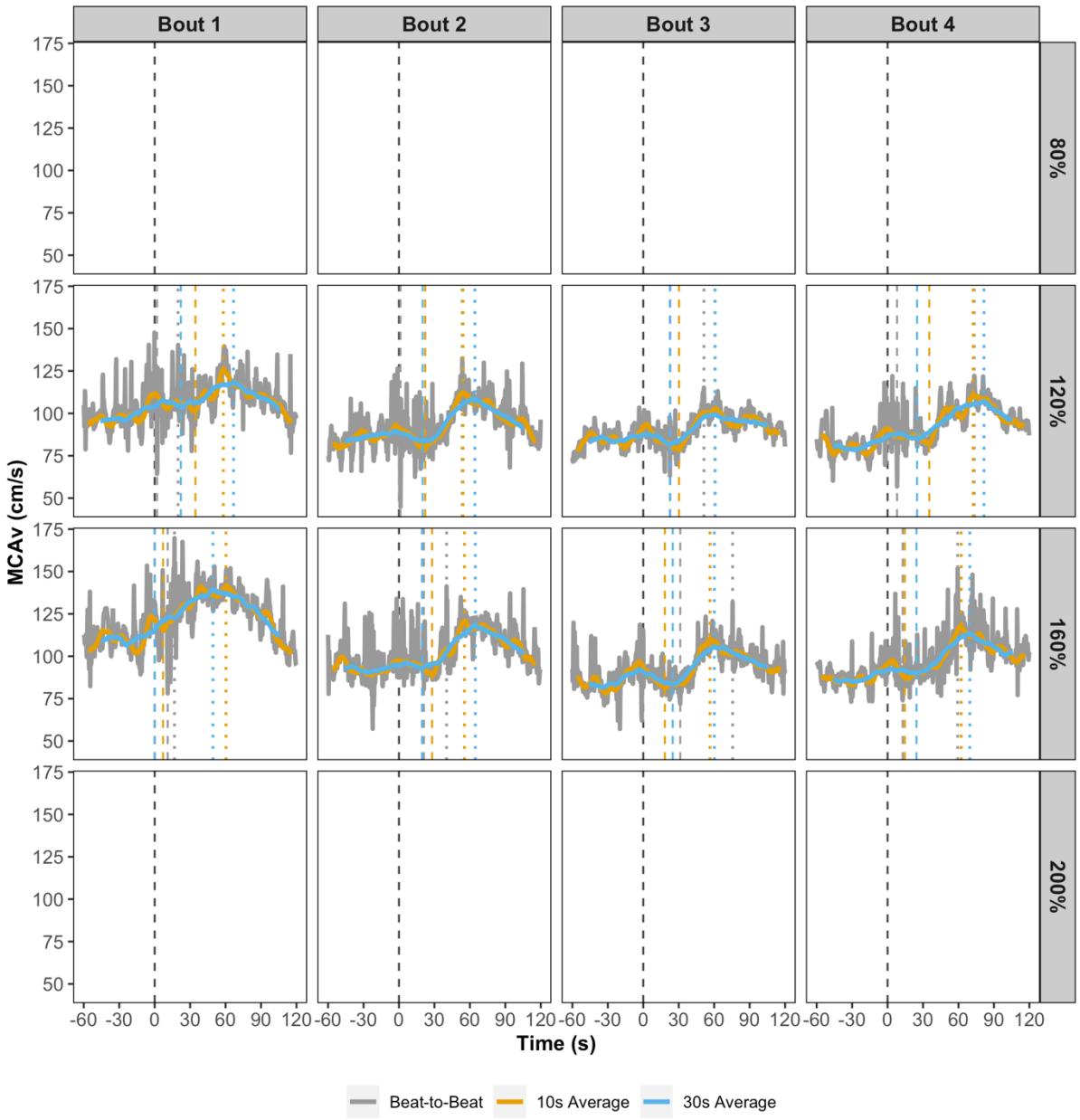
Appendix 26 Comparison of feature selection following different pre-processing approaches (Participant SI009)



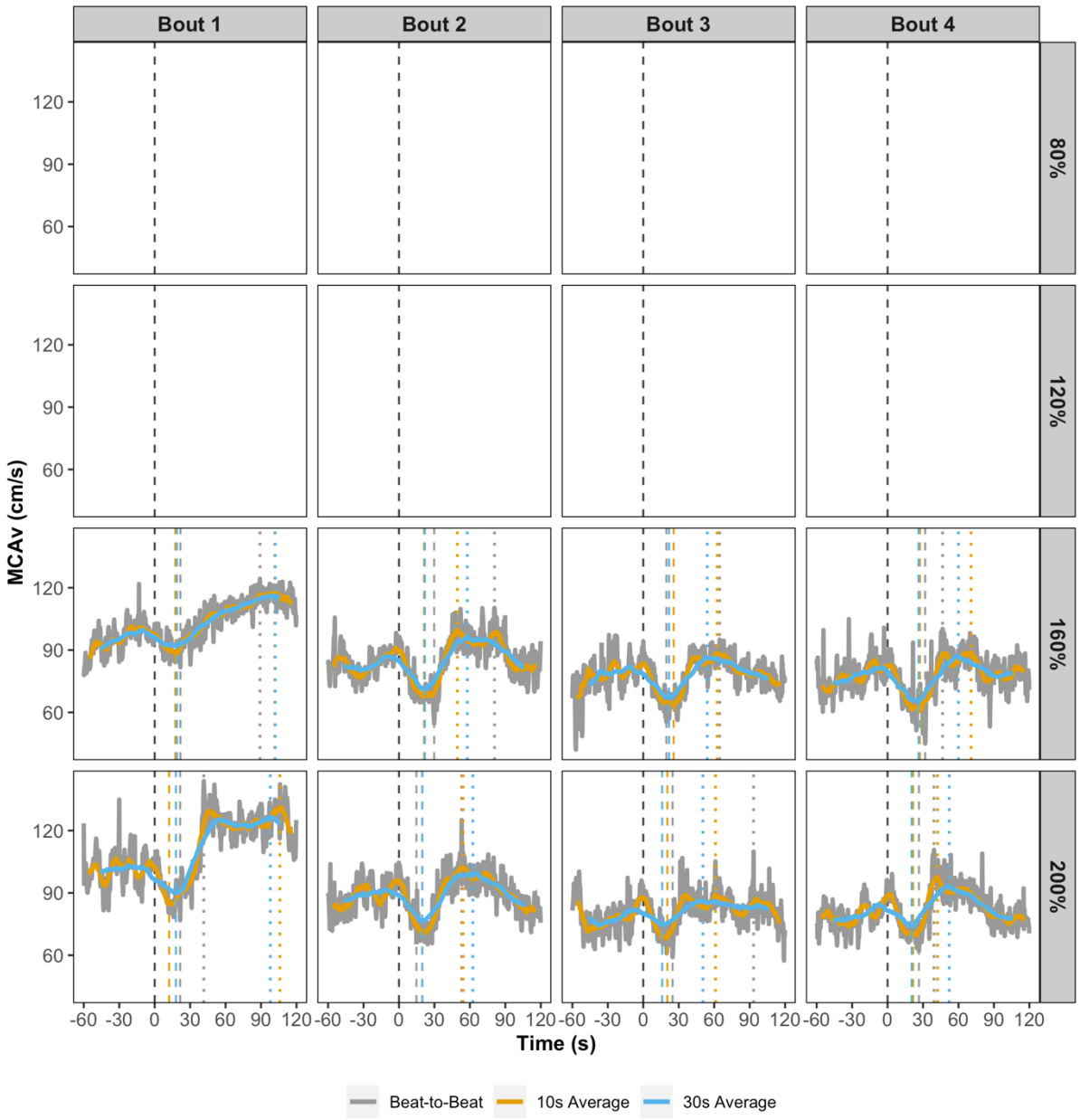
Appendix 27 Comparison of feature selection following different pre-processing approaches (Participant SI010)



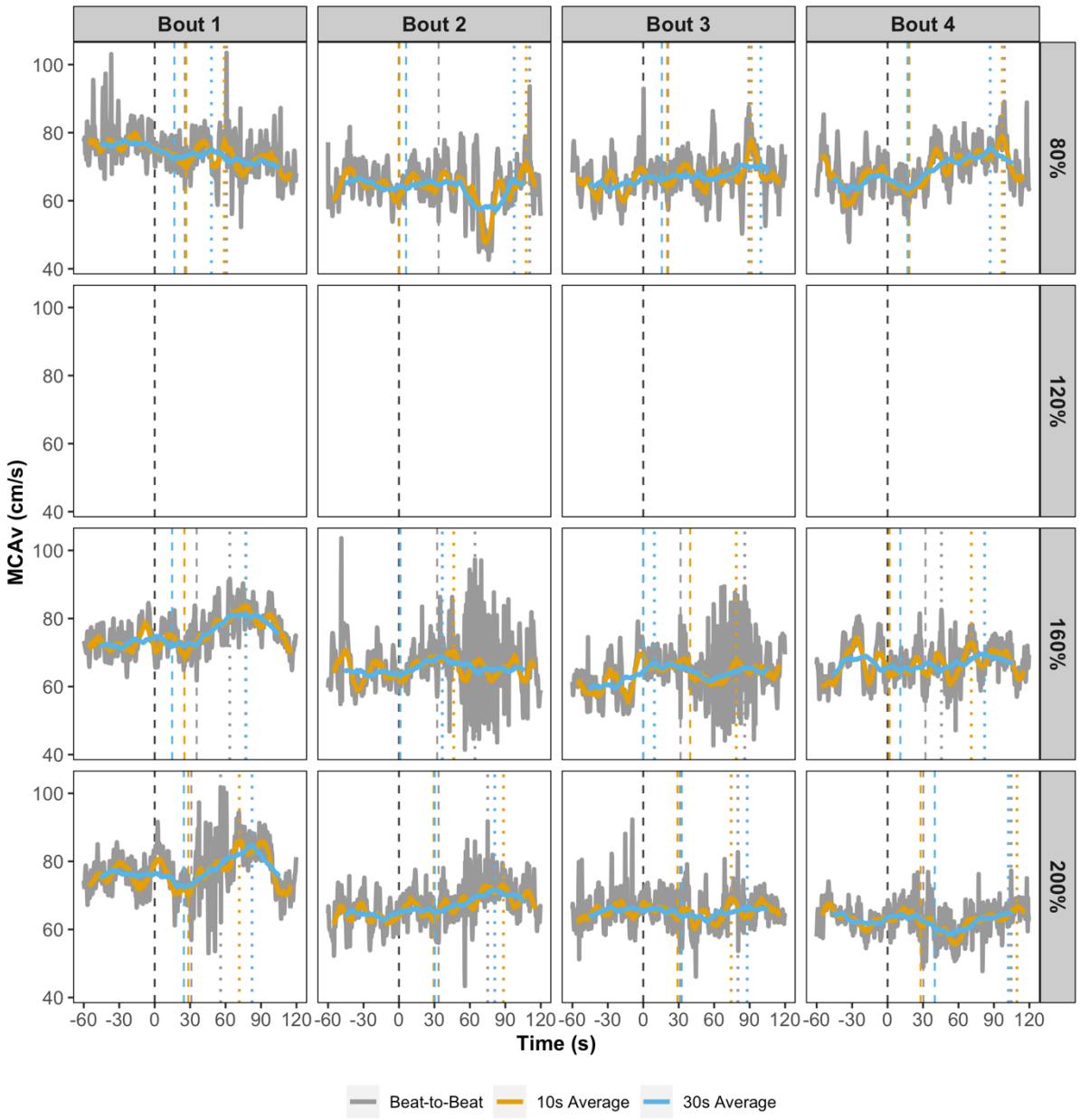
Appendix 28 Comparison of feature selection following different pre-processing approaches (Participant SI011)



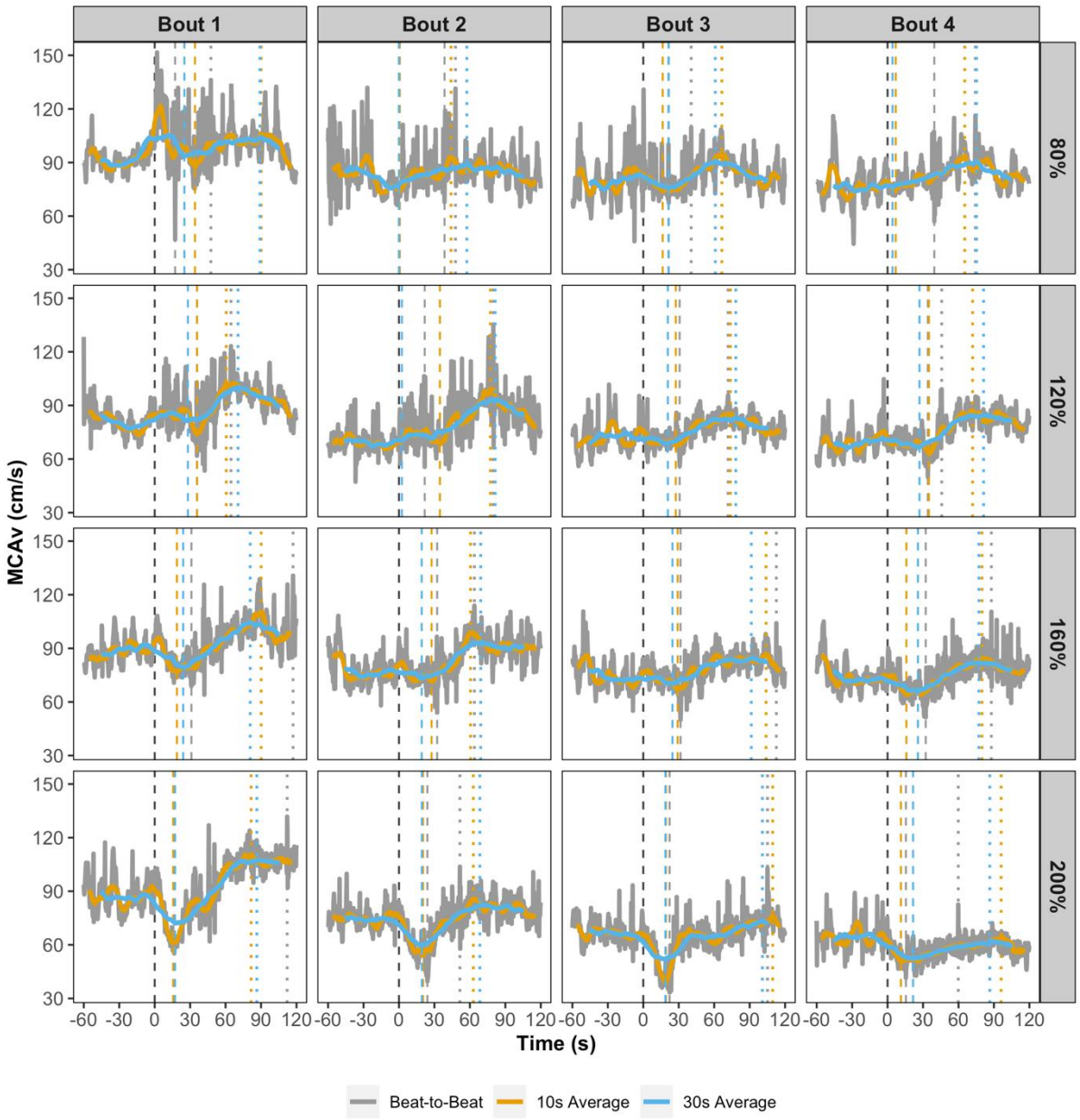
Appendix 29 Comparison of feature selection following different pre-processing approaches (Participant SI012)



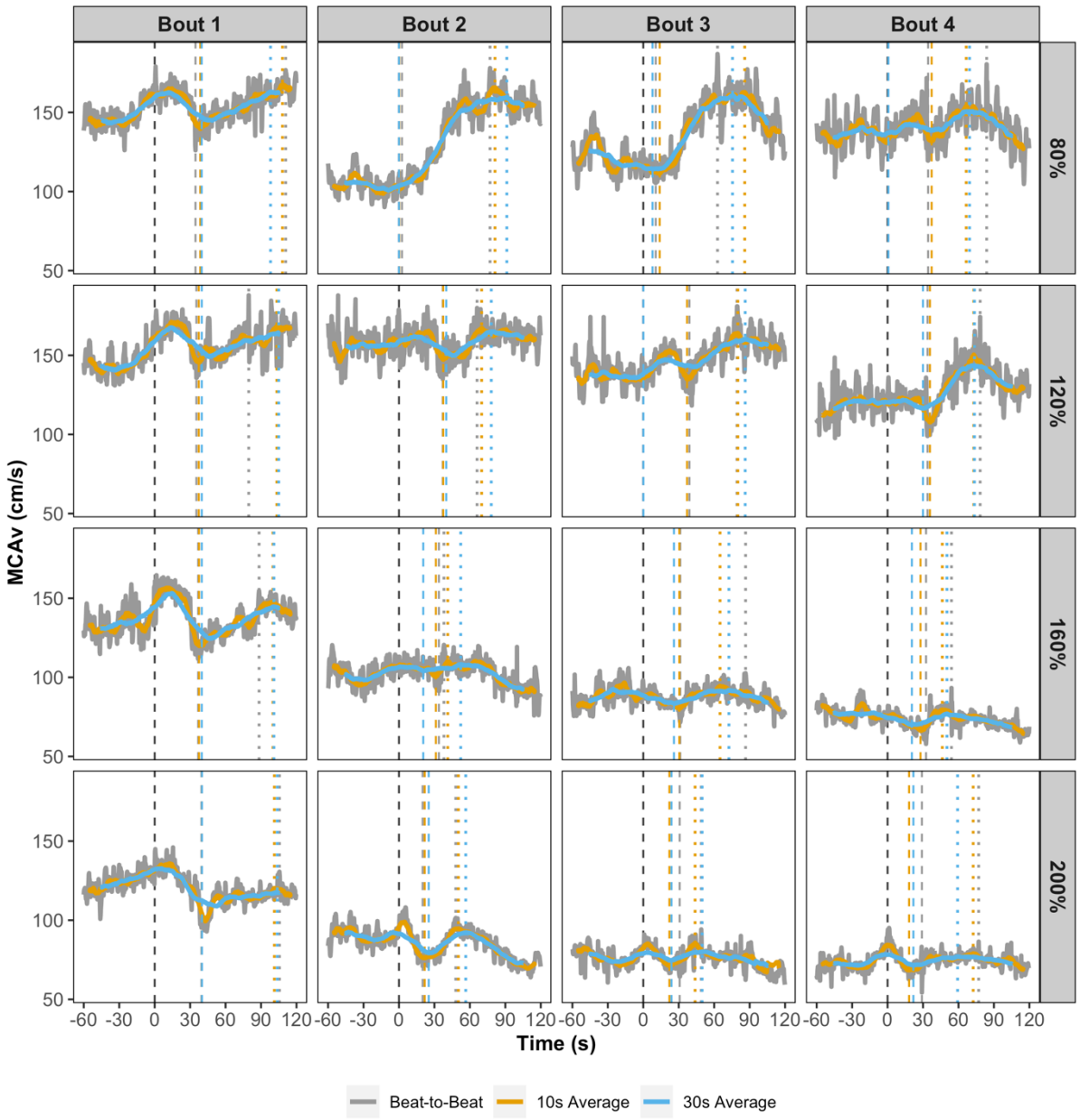
Appendix 30 Comparison of feature selection following different pre-processing approaches (Participant SI013)



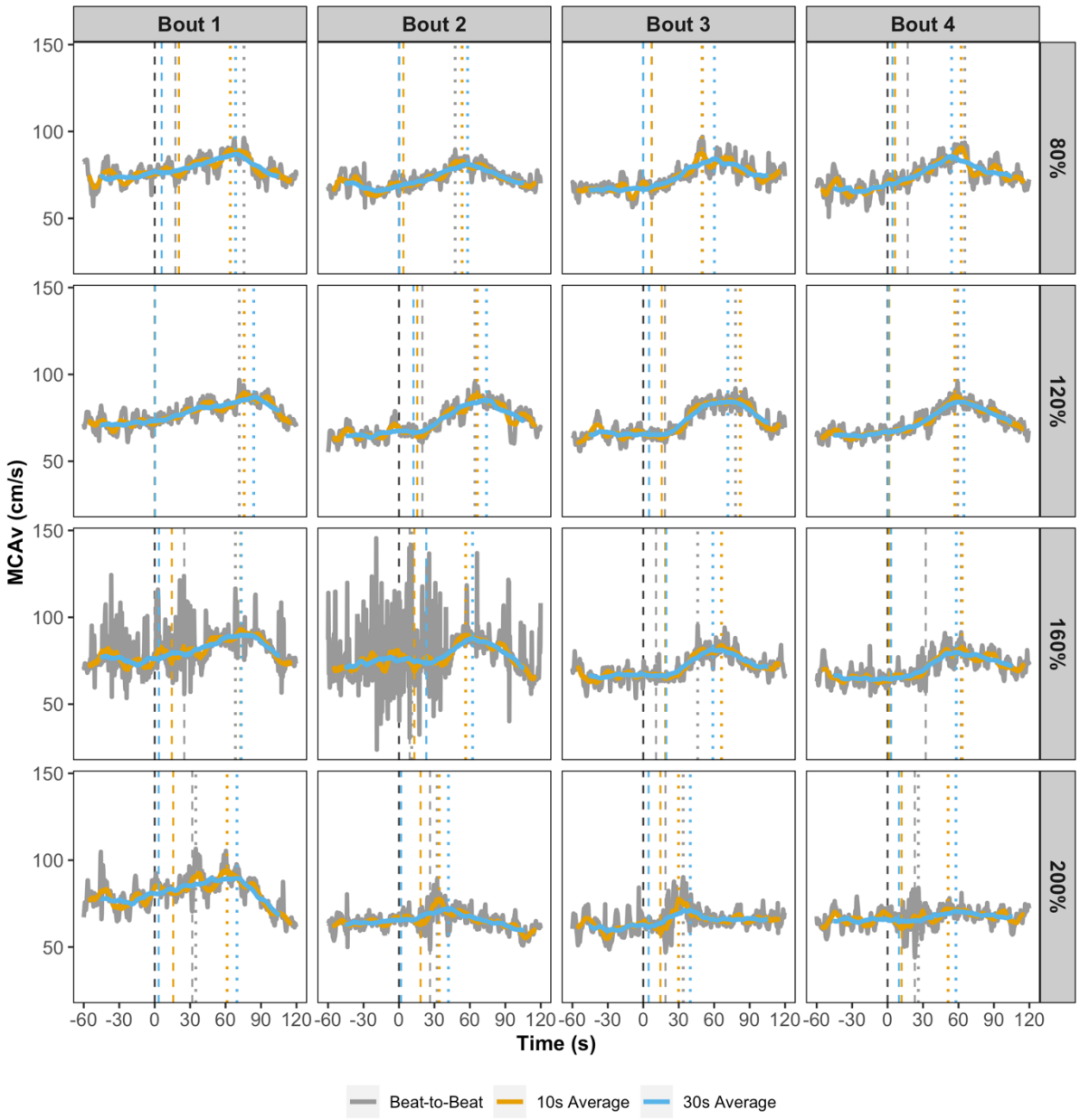
Appendix 31 Comparison of feature selection following different pre-processing approaches (Participant SI014)



Appendix 32 Comparison of feature selection following different pre-processing approaches (Participant SI015)



Appendix 33 Comparison of feature selection following different pre-processing approaches (Participant SI017)



Appendix 34
Comparison of feature selection following different pre-processing approaches (Participant SI018)

Appendix 35 Participant characteristics for samples used in Chapter 6

Participant ID	Sex	Age (years)	BMI (kg·m^2)	$\dot{V}O_{2peak}$
SI012	F	19	23.76	42.58
SI016	M	18	20.82	48.17
SI018	M	26	25.77	53.96
SI025	M	25	23.59	64.11
SI026	M	22	22.21	53.74
SI027	F	22	22.84	44.69
SI028	F	24	21.09	46.47
SI029	F	21	23.45	41.97