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In-exercise vascular shear rate during acute continuous and interval exercise: impact upon endothelial function and MiR-21

Gemma K. Lyall[1]; Matthew J. Davies[1]; Carrie Ferguson[1]; Karen E. Porter[2]; Karen M. Birch[1]

[1] School of Biomedical Sciences, Faculty of Biological Sciences & Multidisciplinary Cardiovascular Research Centre, University of Leeds, UK

[2] Leeds Institute of Cardiovascular and Metabolic Medicine & Multidisciplinary Cardiovascular Research Centre, University of Leeds, UK

Running Head: In-exercise vascular shear rate

Corresponding Author:

Karen Birch

School of Biomedical Sciences,

Faculty of Biological Sciences &

Multidisciplinary Cardiovascular Research Centre,

University of Leeds, Leeds, LS2 9JT, UK

0113 343 6713 k.m.birch@leeds.ac.uk

Abstract

Background: Endothelial cell phenotype and endothelial function are regulated by hemodynamic forces, particularly wall shear stress (WSS). During a single bout of exercise, the specific exercise protocol can affect in-exercise WSS patterns, and consequently endothelial function. MicroRNAs might provide a biomarker of in-exercise WSS pattern, to indicate whether a specific exercise bout will have a positive effect on endothelial function. We evaluated the effect of acute interval (IT) and continuous (CON) in-exercise WSS patterns upon post-exercise endothelial function and circulating miR-21 expression. **Methods and Results:** 13 participants performed CON and 3 different IT exercise protocols matched for duration and intensity, on separate days. Oxygen uptake, heart rate and brachial artery blood flow were recorded throughout exercise. Brachial artery flow mediated dilation (FMD) was performed pre and 15 min post exercise. Plasma samples were acquired pre and 6 hours post exercise to determine miR-21 expression. In-exercise shear-rate (SR) patterns (a surrogate of WSS) differed according to the CON or IT work rate profile. In-exercise anterograde SR was greater in CON than IT ($P<0.05$), retrograde SR was equivalent between exercise protocols ($P>0.05$). Oscillatory shear index was higher during IT versus CON ($P<0.05$). Post-exercise FMD increased (pre 7.08±2.95, post 10.54±4.24%, $P<0.05$), whilst miR-21 expression was unchanged (pre 12.0±20.7 %cel-miR-39, post 11.1±19.3 %cel-miR-39, $P>0.05$); with no effect of exercise protocol ($P>0.05$). **Conclusions:** CON and IT exercise induced different SR patterns, but equivalent improvements in acute endothelial function. The absence of change in miR-21 expression suggests miR-21 is not a suitable biomarker of exercise-induced SR.

New & Noteworthy

Interval exercise has the potential to negatively impact vascular adaptations due to repeated oscillations in vascular shear. We are the first to continuously assess exercise-induced shear throughout different acute exercise protocols and examine its relationship with acute endothelial

function and a circulating biomarker of shear (miR-21). These experiments provide clear data indicating enhancement of the acute vascular response from differing interval exercise protocols, with the study also providing detailed vascular and shear responses for future reference.

Keywords: interval exercise; endothelial function; FMD; microRNA; shear rate

Introduction

Atherosclerosis is the underpinning pathology of cardiovascular disease (CVD). Endothelial dysfunction is the earliest stage in the silent development of atherosclerosis (24, 38) and has been established as an independent risk factor for CVD (5, 19). Assessment of endothelial function thus provides independent prognostic information beyond traditional CVD risk factors in both healthy and patient populations (29, 40). Additionally, as the asymptomatic nature of atherosclerosis can last many years, blood biomarkers as complementary indices of endothelial function are highly sought after; however currently there is no such circulating biomarker.

Endothelial cell (EC) phenotype and thus endothelial function is regulated by hemodynamic forces generated by blood flow in the lumen (6); in particular, endothelial wall shear stress (WSS) (10). The relative impact of endothelial WSS upon endothelial function is highly dependent upon blood flow velocity and flow profiles. High velocity, laminar WSS promotes an anti-atherogenic EC phenotype, partially mediated through increased production of nitric oxide (NO) (51). Conversely, low velocity and/or oscillatory WSS is associated with altered cell signaling pathways and a pro-atherogenic EC phenotype through upregulation of inflammatory factors, oxidative enzymes and vasoconstrictors (6).

Recently, mechano-sensitive microRNAs (miRs), small, non-coding RNAs which negatively regulate gene expression (7), expressed by ECs have been found to be responsive to both laminar and oscillatory WSS and contribute to the regulation of EC phenotype (27). MiR-21 in particular has been identified as an important epigenetic regulator of EC apoptosis and NO production, with its expression regulated by WSS (48). Overexpression of miR-21 in in response to differing shear profiles has been shown in previous studies, for example 24 hours of sustained oscillatory shear resulted in miR-21 overexpression and a subsequent proinflammatory endothelial response (50). On the other hand, 24 hours of unidirectional/laminar shear increased miR-21 expression creating an anti-inflammatory endothelial environment (48). It thus appears that overexpression of miR-21 can regulate endothelial phenotype, positively or negatively, via multiple downstream targets, of which nuclear receptor PPARα and PTEN are examples (48, 50), and that these downstream targets are influenced by differing shear signaling pathways. Importantly, unlike most miRs which are only detectable within cells, miR-21 is highly expressed in the circulation (2). Determining circulating miR-21 expression in conjunction with assessment of endothelial function may provide an insight into the endothelial environment in response to differing shear stimuli, thus offering miR-21 as a potential systemic blood borne biomarker.

Acute exercise offers the capacity to investigate the impact of different WSS patterns on both endothelial function and possible biomarkers of WSS, such as miR-21. Both acute and chronic continuous (CON) exercise improves endothelial function, assessed via flow mediated dilation (FMD) in the brachial artery, in healthy and patient populations (9, 14, 22, 25, 36, 43). It is purported that this improvement is driven by exercise-induced WSS mediated effects upon ECs, both in the active limb and in other systemic vessels (20). WSS patterns during exercise have only previously been examined for brief periods during acute continuous (CON) exercise (4, 15, 16, 21, 34, 44, 47). During rhythmic lower limb exercise WSS in the brachial artery has been seen to vary according to the blood flow response to exercise and the upstream thermoregulatory modification to systemic blood flow distribution and haemodynamics (42). For example, at the onset of exercise there is an immediate increase in retrograde WSS in the brachial artery which declines as exercise continues;, in contrast anterograde WSS increases throughout the exercise bout (42).

The acute impact of alternative modes of exercise upon WSS patterns and subsequent endothelial function is yet to be explored. For example, comparatively little is known regarding the effect of high intensity interval (IT) exercise regimes upon WSS patterns and subsequent endothelial function. As IT exercise consists of multiple transients between “work” and “recovery” throughout a single session, and the length of the recovery periods govern the magnitude of physiological recovery between each work bout, the pattern of WSS in the brachial artery may oscillate such that repeated transitions result in greater volumes of retrograde shear stress than seen in CON. Increased volumes of retrograde shear stress have been seen to be acutely detrimental to endothelial function (45) and EC phenotype (51). As circulating miR-21 concentration has been seen to be responsive to acute bouts of short duration exercise (2, 31, 32), potentially as a result of increased WSS throughout the arterial tree (48), its relationship with endothelial function may provide a biomarker to differentiate between these differing WSS patterns.

The aims of this study were thus twofold: (i) to characterize the acute in-exercise brachial artery WSS pattern induced by intensity and duration matched IT and CON exercise, and (ii) to investigate the effect of acute IT and CON in-exercise WSS patterns upon post-exercise endothelial function and circulating miR-21 levels. It was hypothesized that acute CON exercise would induce a more laminar WSS pattern increasing mir-21 expression, and because of the more anti-inflammatory (laminar WSS) exercise environment, increase endothelial function. On the other hand, acute IT exercise would induce a more oscillatory WSS pattern, increasing mir-21 expression, but because of the more pro-inflammatory environment (oscillatory WSS) exercise environment, decrease endothelial function.

Methods

Participants

Thirteen healthy participants volunteered for this study (9 male: 4 female, mean ± standard deviation (SD): age 22 ± 3 years, BMI 23.6 ± 2.1 kg/m^2). All participants were free of current or previous risk factors associated with cardiovascular and respiratory diseases and metabolic disorders. Participants were non-smokers and were not taking prescription medications. Females were tested during the same phase of their menstrual cycle and those taking hormonal contraceptives were tested during the same phase of their oral contraceptive use. The University of Leeds ethics committee approved the study protocols which adhered to the declaration of Helsinki. Written informed consent was gained prior to data collection.

Experimental Protocol

Participants attended the laboratory on five separate occasions, each separated by >48 hours. Protocols were completed in a quiet, darkened, temperature controlled laboratory (22-24°C). The initial visit comprised a pre-exercise health screening followed by a standard ramp incremental exercise test (RIT) on a semi-recumbent cycle ergometer. The RIT was used to characterize participant's aerobic function and identify the appropriate work rates for the subsequent exercise protocols, as described in detail below. Following this visit, participants completed four different exercise protocols in a random order. These four visits were conducted following >8 hours of overnight fasting and abstinence from caffeine, alcohol and exercise training for 24 hours. At each of the four visits, participants provided a 10 ml venous blood sample from the antecubital fossa on the arm not used for FMD assessment. Participants then rested supine for >10 min prior to ultrasound recordings of brachial artery FMD. A >3 min warm up (unloaded cycling) and 24 min semi-recumbent cycling exercise protocol was then completed. During each exercise protocol the right arm was extended and supported at the level of the heart. Heart rate (HR), breath-by-breath pulmonary gas exchange and Duplex ultrasound of the brachial artery were recorded throughout the exercise period. Upon cessation of the exercise protocol, a cool down (>2 min unloaded cycling) was completed. Following 15 min supine rest, a post-exercise FMD assessment was completed. Finally, a second venous blood sample was obtained 6 hours post cessation of the exercise protocol. This sampling time point was chosen to be consistent with previous literature assessing circulating microRNA expression in-vivo following acute exercise (39).

Exercise Protocols

Breath by breath pulmonary gas exchange (MedGraphics D-Series, Medical Graphics Corporation, St Paul, MN, USA) was assessed during a standard RIT on a semi-recumbent ergometer (Angio, Lode BV, Groningen, Netherlands) to measure peak oxygen uptake ($\dot{V}O_{2peak}$) and estimate lactate threshold (LT). LT was calculated using the V-slope method and identified the target $\dot{V}O_2$, which was used to select the work rates for each of the four exercise protocols. The four exercise protocols were thus matched for exercise intensity ($\dot{V}O_2$ in the heavy intensity domain (49)) and duration (24 min) but differed in pattern and work rate. The protocols are displayed in **Figure 1** and were defined as (i) continuous exercise (CON), (ii) 4 x 180 s of work each interspersed with 180 s of 10 W active recovery (LONG IT), (iii) 12 x 60 s of work with 60 s 10 W active recovery (SHORT IT) and (iv) 4 x 180 s work with 180 s recovery at work rate equivalent to 70% LT (LONG IT 70). The inclusion of the Long IT 70 protocol was to maintain an increased $\dot{V}O_2$ during the recovery periods and therefore elevate the mean $\dot{V}O_2$ and energy expenditure of the session to more closely match the CON and Short IT exercise protocols.

In-exercise assessment of blood flow

The same site of the upper right arm as used in the FMD assessment was used for all in-exercise recordings. The ultrasound (Vivid E9, GE Healthcare, Milwaukee, WI, USA) was operated in duplex mode to obtain continuous second by second recordings of brachial diameter and blood flow velocity during the exercise protocols. Recording, using a 10 MHz linear array probe, started during the final 30 s of warm up and ended 1 min into cool down. Images were recorded directly onto the ultrasound in consecutive 4 min loops and Vascular Imager (MIA, Coralville, IA, USA) utilized to calculate anterograde and retrograde (decelerative) shear rate (SR). SR was used as a surrogate of WSS and, as viscosity was not assessed in the current study, was used as an indicator of the frictional force of blood flow (33). The term SR will thus be used hereafter. Intra-rater reliability CV for anterograde and retrograde SR were 23.8% and 24.5% respectively.

Assessment of endothelial function

FMD procedures were conducted in accordance with previous guidelines (46) with an intra-rater FMD reliability of 11.4%. Resting brachial artery diameter was recorded in duplex in the distal third of the upper right arm for 20 s at 15 frames per second using the Vivid E9. Immediately following this, a blood pressure cuff placed on the right forearm, distal to the ultrasound probe, was rapidly inflated to >220 mmHg for 5 min. Recording of diameter and blood flow velocity began 30 s prior to cuff deflation and continued for 150 s post cuff deflation. The sample volume was adjusted to account for vessel diameter and the entirety of blood flow through the vessel. An insonation of 60^{O} was achieved for all measurements of blood flow velocity and did not vary between participants or protocols.

Plasma sampling and quantification of microRNA-21 expression

Blood samples were collected in standard EDTA treated vacutainers, stored on ice and processed within 2 hours of collection. Samples were centrifuged for 20 min at 1900xg at 4°C to obtain plasma. The extracted plasma was centrifuged for 10 min at 16000xg at 4°C, producing platelet free plasma which was aliquoted and immediately frozen at -80°C.

Total RNA was extracted from thawed platelet free plasma and cel-miR-39 spike-in control was added using a miRNeasy serum/plasma kit (Qiagen, Maryland, USA). Extracted RNA was once again stored at -80^{O}C. To quantify circulating miR-21 levels within the extracted RNA, standard reverse transcription-quantitative real time polymerase chain reaction (RT-qPCR) was used with TaqMan probes and primer sets (TaqMan Small RNA assays transcription kit, Applied Biosciences, Foster City, USA) for miR-21 (000397), with cel-miR-39 (000200) acting as a control. A 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA) assessed relative quantification of miR-21 compared to cel-miR-39.

Analysis of data

All measures of vessel diameter and blood flow velocity were assessed offline using commercial automated edge detection and wall tracking software (Brachial Analyzer for Research, MIA, Coralville, IA, USA). Resting diameter was determined as a mean of the diameter across the 20 s recording period, peak diameter as the greatest diameter recorded following cuff deflation and relative FMD as:

Relative FMD (%) = (peak diameter – baseline diameter) / baseline diameter * 100.

Peak hyperemia as the stimulus for vasodilatation following occlusion was determined as the highest blood flow velocity in the first 10 s following cuff deflation, whilst area under the shear rate curve from cuff release to 60 s (AUC_{60}) and 90 s (AUC_{90}) post cuff release were also calculated. Peak SR was calculated as:

Peak shear rate (s^{-1}) = 8 * (peak hyperemia / baseline diameter).

During the exercise protocols anterograde and retrograde SR were calculated using:

Shear rate (s^{-1}) = (mean blood velocity / mean diameter) * 8.

Oscillatory Shear Index (OSI) was utilized to indicate laminar (0-0.5 a.u.) or oscillatory (> 0.5 a.u.) blood flow (32, 36):

OSI (a.u.) = retrograde SR / (retrograde SR + anterograde SR).

Statistical Analysis

Statistical analysis was completed using SPSS Statistics 21 (IBM, Chicago, IL, USA). Data were assessed for normality using the Shapiro-Wilk test and log transformed if not normally distributed. Variables measured pre-exercise and during exercise (e.g. total SR, mean SR, mean $\dot{V}O_2$, mean HR etc.) were compared between protocols via a repeated measures one-way ANOVA.

To assess the impact of exercise protocol upon miR-21 expression and FMD and its associated measures, a linear mixed model was conducted with time (pre vs. post) and protocol (CON vs. Long IT vs. Short IT vs. Long IT 70) treated as fixed factors. Resting brachial artery caliber influences the vasodilation of the brachial artery following FMD (1), therefore resting brachial artery diameter was used as a covariate during analysis of FMD. Brachial artery diameter assessed second-by-second throughout each exercise protocol did not change during exercise. Additionally, resting brachial artery diameter did not differ pre- to post-exercise (*P=0.86*) or between exercise protocols (*P=0.99; Table 1*). For assessment of miR-21 expression, pre-exercise miR-21 expression was used as the covariate. Bonferroni post-hoc analysis was performed when significant effects were found. Pearson correlations were used to identify relationships between normally distributed variables. All data are presented as mean ± standard deviation (SD). An *a priori* analysis using GLIMMPSE (https://glimmpse.samplesizeshop.org/#/), revealed a sample size of 13 would be required to find a difference in FMD following exercise of 3% between two of the four protocols, assuming a power of 0.8, alpha of 0.5 and a SD of 3.0.

Results

Shear rate patterns during exercise

Patterns of anterograde and retrograde SR mirrored the work rate profile of the exercise (**Figure 2A**) undertaken and were consistent with patterns of forward and decelerative blood flow, HR (**Figure 3A**) and $\dot{V}O_2$ (**Figure 3B**). However, in contrast to $\dot{V}O_2$ and HR, both of which reached a steady-state (CON) or pseudo-steady-state (IT) as expected during exercise in the heavy intensity exercise domain (47) (**Figure 3**), anterograde SR continued to increase throughout each exercise protocol (**Figure 2A I-IV**). Retrograde SR stabilized over time in each protocol following an initial increase (**Figure 2A I-IV**).

Total, mean, maximum and minimum volume of retrograde SR did not differ between protocols (*P>0.05;* **Figure 2B**). Whilst minimum anterograde SR did not differ between protocols (*P>0.05;* **Figure 2B**), total, mean and maximum anterograde SR were greater in CON (total: $14x10^5 \pm 4x10^5$ s^{-1}, mean: 1044 ± 297 s^{-1}, max: 1892 ± 408 s^{-1}) than in Long IT (total: $10x10^5 \pm 3x10^5$ s^{-1}, mean: 803 ± 251 s^{-1}, max: 1403 ± 441 s^{-1}; *P<0.05;* **Figure 2B**), with mean and maximum anterograde SR in CON also greater than Short IT (mean: 859 ± 265 s^{-1}, max: 1584 ± 430 s^{-1}; *P<0.05;* **Figure 2B**). Additionally, mean and maximum anterograde SR was greater in Long IT 70 (mean: 963 ± 224 s^{-1}, max: 1738 ± 435 s^{-1}) than in Long IT (*P<0.05;* **Figure 2B**). The pooled protocol mean HR was correlated with the pooled protocol mean and total anterograde (mean: *r=0.61, P<0.001*; total: *r=0.57, P<0.001*) and retrograde SR (mean: *r=-0.64, P<0.001*; total: *r=-0.68, P<0.001*).

Oscillatory Shear Index

Maximum and minimum OSI did not differ between the four exercise protocols (*P>0.05*; **Figure 4**). However, mean OSI was lower in CON (0.22 ± 0.06 a.u.) than in Long IT (0.27 ± 0.07 a.u.) and Short IT (0.27 ± 0.07 a.u.; *P<0.05;* **Figure 4**). Importantly, a period of time with an OSI >0.5 (indicating periods of purely oscillatory shear) occurred in each protocol (**Figure 4**). This time was not different between protocols with a pooled protocol mean of 26.8 ± 32.2 s (*P>0.05*). An OSI >0.5 typically occurred during the first 360 s of each protocol equating, to CON 69%, Long IT 61%, Short IT 49% and Long IT 70 66% of the 360 s (P>0.05).

Acute brachial artery endothelial function

Following acute exercise, absolute and relative FMD were increased by 0.14 ± 0.01 mm (time effect *P<0.05*; **Table 1**) and 3.36 ± 0.48 % (time effect *P<0.001*; **Table 1**), respectively, with no difference between exercise protocols (time x protocol *P>0.05)*. The vasodilatory stimuli recorded during the FMD procedure differed pre to post exercise, with an increase in peak SR values (peak hyperemia, peak SR, time to peak dilation; time effect *P<0.05;* **Table 1**) and a reduction in total volume of SR (AUC_{60}; time effect *P<0.05*; **Table 1**) although not SR AUC_{90} (time effect *P>0.05*; **Table**

1). These stimuli were not different between protocols (time x protocol P>0.05; **Table 1**). Adjusting FMD for peak SR did not alter the result (data not shown).

MicroRNA – 21 expression

Circulating plasma levels of miR-21 reported as a percentage of cel-miR-39 showed no pre-exercise differences between exercise protocols (*P>0.05*). Circulating plasma levels of miR-21 (%cel-miR-39) were unaffected by acute exercise, irrespective of exercise protocol (time effect *P>0.05*; time x protocol *P>0.05*; **Table 1**).

Discussion

This study is, to our knowledge, the first to explore a potential relationship between exercise-induced SR, acute endothelial function and circulating miR-21 expression. The key findings are: 1) exercise protocols produced distinct anterograde and retrograde SR patterns; 2) acute brachial artery endothelial function improved following all exercise protocols; 3) expression of circulating miR-21 was unaffected by exercise, regardless of the exercise-induced SR pattern. In agreement with the original hypothesis, the IT exercise protocols, when matched for the same metabolic intensity as CON, produced differing SR patterns resulting in a greater OSI. Importantly, and in contrast to our original hypothesis, these differences in SR pattern did not differentially affect endothelial function or circulating miR-21 expression.

Acute endothelial function

Previous studies have shown acute bouts of exercise to illicit different post-exercise FMD responses; either increases, decreases or no change, as previously reviewed in detail (11). Acute exercise conducted at higher exercise intensity has been seen in a previous study to lead to an early decrease in post-exercise FMD, when compared to moderate intensity (3). Notably, Birk et al. (3) reported that the post-exercise decrease in FMD was greatest after 30 min of cycling at 85% HR_{max}

but still decreased, although to a lesser extent after cycling at 70% HR_{max}. The immediate impairment in endothelial function following acute high intensity exercise has been hypothesized to be a result of either increased oxidative stress, substrate depletion, retrograde SR, decreased SR stimulus during FMD or a reduction in EC sensitivity to SR (11). In the present study, FMD increased 15 min following acute high intensity exercise irrespective of protocol.

In this study, the SR stimulus during FMD assessment was maintained following acute exercise and whilst retrograde SR was induced during exercise, it did not have a detrimental impact upon FMD. The current study did not assess EC sensitivity, substrate depletion or oxidative stress and therefore determining the potential causes of differences in acute endothelial response to high intensity exercise between this and previous studies is difficult. It has been purported that high intensity exercise is associated with greater oxidative stress resulting in attenuation of FMD via reductions in the bioavailability and production of nitric oxide (37). Antioxidant supplementation prevents FMD attenuation following high intensity exercise (41), and habitual exercise training improves antioxidant defense (13). This was demonstrated by Hwang et al. (23) whereby trained individuals did not show an immediate decrease in FMD following acute exercise, in contrast to untrained individuals. Participants in the present study were moderately well trained (mean $\dot{V}O_{2peak}$ 46.4±5.5 ml/kg/min), suggesting potentially high antioxidant defense resulting in increased FMD response to high intensity exercise, although this was not measured. Additionally, by interspersing high work rates with brief recovery periods during interval exercise, allowing both VO_2 and HR to fall in the recovery periods, ensured that (despite the higher work rate during IT) the exercise remained remains tolerable, such that oxidative stress may have not become a factor in reducing post-exercise FMD.

In-exercise characterization of shear rate during CON and IT exercise

At the onset of exercise, across all exercise protocols, we observed small increases in anterograde SR accompanied by large increases in retrograde SR in the inactive limb, consistent with previous literature (15). This pattern of response relates to brief changes in upstream and downstream blood pressure gradients impacting SR during each cardiac cycle (30, 35). Indeed, increased vascular tone in downstream resistance vessels may also induce retrograde SR (17), whilst blood pressure changes during exercise increase sympathetic outflow in inactive muscle which has previously been seen to correlate with increased brachial artery retrograde and oscillatory SR following sympatho-excitatory maneuvers (35).

Subsequently, continuation of exercise induces a thermoregulatory response whereby the microcirculation dilates leading to a reduction in downstream total peripheral resistance, altering upstream blood flow and SR patterns (18, 34, 42). This thermoregulatory response reduces volumes of retrograde SR, whilst further increasing anterograde SR (34, 42) leading to the more laminar SR demonstrated in the present study. CON produced the greatest mean anterograde SR compared to the other protocols, except Long IT 70, and the greatest total volume of anterograde SR compared only to Long IT. The Short IT protocol, comprising of a high number of repetitions alternating between short durations of exercise at a higher work rate and active recovery, affected the pattern of SR resulting in lower mean volume of anterograde SR compared to CON. Increasing the work rate during active recovery in the Long IT 70 protocol, increased peak and mean anterograde SR, compared to Long IT, and more closely matched the CON protocol. Indeed, when assessing the cardiorespiratory responses to CON and Long IT 70 it is evident that the protocols are closely matched in terms of mean VO_2 (CON 72 ± 8 vs. Long IT 70 72 ± 9 %VO_{2peak}), mean HR (CON 79 ± 7 vs. Long IT 70 83 ± 7 %HR_{peak}) and energy expenditure (CON 193 ± 38 vs. Long IT 70 185 ± 37 KJ). This suggests that when interval exercise is manipulated to produce similar cardiorespiratory responses to CON exercise, volume of SR becomes equivalent irrespective of the variable mean and pattern, thus producing similar FMD responses. Thus, as the increase in FMD following an acute bout of intensity-matched exercise did not differ as a result of variations in (i) SR pattern or (ii) the subsequent mean in exercise SR, the similar post exercise FMD response appears to be resultant of matched total SR.

The differences in volumes of anterograde SR between the exercise protocols resulted in differing mean OSI, with CON demonstrating the lowest OSI (**Figure 4**). Purely oscillatory SR occurred during the first half of each exercise protocol, consistent with previous research where the greatest OSI occurred within the first 5-10 min of a 60 min CON cycling protocol (34). This has been attributed to constriction of resistance vasculature in the forearm via sympathetic neural mechanisms, or other circulating vasoconstrictors at the onset of exercise (34). These short time periods above an OSI of 0.5 in our study were not sufficient to adversely affect endothelial function, as evidenced by the increase in post-exercise FMD with all exercise protocols. However, chronic exposure to episodes of purely oscillatory SR may promote endothelial dysfunction and atherosclerotic lesion development (10), suggesting further investigation of these exercise protocols in an exercise training setting is required.

Circulating microRNA-21 expression

MiR-21 was chosen as a potential biomarker as it is reportedly flow sensitive and has a role in regulation of target genes involved in endothelial function. In previous studies by others, human umbilical vein endothelial cells (HUVECs) exposed to unidirectional SR for 24 hours demonstrated a 5 fold increase in miR-21 expression (48). This was associated with decreased EC apoptosis and increased eNOS phosphorylation and NO production, via a 83% reduction in PTEN gene expression (a negative regulator of the PI3K/Akt/eNOS signaling pathway (48)). In another study, when HUVECs were exposed to oscillatory SR for 24 hours there was a 3 fold upregulation in miR-21 expression, with peak expression (10 fold increase) occurring following 6 hours of exposure, leading to the promotion of a pro-atherogenic EC phenotype reportedly via inhibition of PPARα (50). Short duration (30 min) exposure of HUVECS to oscillatory and pulsatile SR resulted in no change or a decrease in miR-21 expression, respectively (50). Studies using short duration exercise as a stimulus have reported an approximately 2 fold upregulation in circulating miR-21 following a maximal exercise test (<20 min duration)(2), reductions in circulating miR-21 expression 30 min following 4 x

4min interval exercise (26) and no effect immediately, 1 hour and 3 hours following a period of 60 min continuous aerobic exercise (32). The differing responses observed in these studies may be due to different sites used for blood sampling, lack of standardized participant preparation i.e. fasted or not, diverse intensities and/or differing shear patterns produced from the varying exercise regimes. The present study attempted to establish the miR-21 response to acute exercise by controlling exercise intensity and manipulating the shear rate patterns. However, despite evidence from previous studies for divergent SR mediated effects upon cultured ECs, the differing patterns of exercise-induced SR in our current study did not result in any changes in expression of circulating miR-21, at least at the time point we selected based on these previous studies (39).

SR patterns in the brachial artery differed between the exercise protocols in the present study, with much greater increases in anterograde SR observed compared to retrograde SR. We have observed a similar SR response in the femoral artery during acute 125 % LT CON and 60:60s INT exercise, where no time spent in pure oscillatory shear was seen (unpublished data). It may thus be that for acute short duration exercise at submaximal intensity the magnitude of oscillatory shear is not able to differentiate miR-21 expression. Furthermore, as miR-21 is not only a regulator of EC phenotype but is also highly expressed in many other cardiovascular tissues (as reviewed in detail by Cheng and Zhang (8)), for example vascular smooth muscle cells, cardiomyocytes and cardiac fibroblasts (8), therefore in the present study we cannot determine the cell source of miR-21. It thus appears that for varying acute exercise protocols circulating miR-21 is not a viable biomarker of exercise-induced SR.

Limitations

Pressure influences EC growth and alignment via cyclic circumferential strain, which might be further increased with increased blood pressure associated with exercise (18, 28). Blood pressure was not continually assessed during exercise in the current study. This should be assessed continuously during exercise in future work, to yield further understanding of the mechanisms governing SR patterns.

The assessment of miR-21 only may oversimplify the complex regulation of EC phenotype and NO production pathways, particularly when assessed in the circulation. In future, a broader screen of additional miRs as known regulators of EC phenotype should be assessed as potential biomarkers, in combination with potential gene targets which are known to be associated with endothelial function. Additionally, further work on circulating miR expression and determination of the time-course of detecting circulating miR at the point of greatest expression and relevance is required before it can become a viable option as a circulating biomarker of SR and endothelial function.

Conclusion

In conclusion, acute CON and different IT exercise protocols, matched for intensity and duration, produced distinct protocol specific SR patterns. By manipulating the exercise protocols through changes in work rate and the duration of the work and recovery periods, we were able to produce high intensity IT exercise protocols which demonstrated acute improvements in endothelial function, independent of any detectable change in circulating miR-21. This finding has implications for chronic exercise training interventions, where a targeted approach to IT exercise could be used for specific physiological adaptations, whilst not detrimentally impacting endothelial function. The acute exercise and resultant SR patterns utilized in the present study did not affect circulating miR-21 levels suggesting that in the current scenario, this miR is not a suitable biomarker of exercise induced SR.

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Author Contributions

G.L. and K.B. designed these experiments. The article was written by G.L. with editorial input from K.B., M.D., K.P. and C.F. Experimental research was carried out by G.L. and M.D. and analyzed by G.L. and K.B. G.L. performed all of the ultrasound analysis. K.P. advised on all miR experiments.

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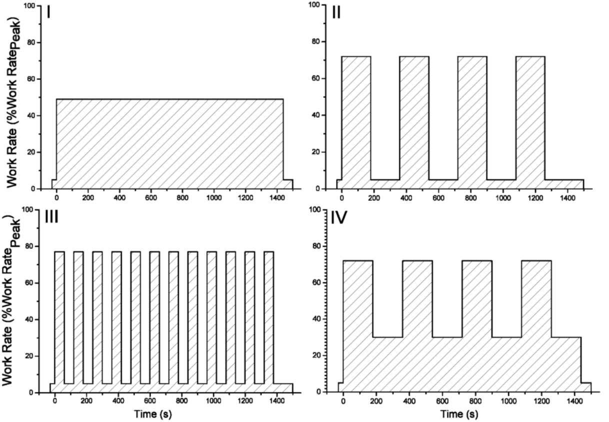
Figure Legends

Figure 1: The four exercise protocols utilized in the current study (I) CON (II) Long IT (III) Short IT and (IV) Long IT 70, with relative mean work rates displayed as a percentage of the work rate peak achieved during the initial ramp incremental test.

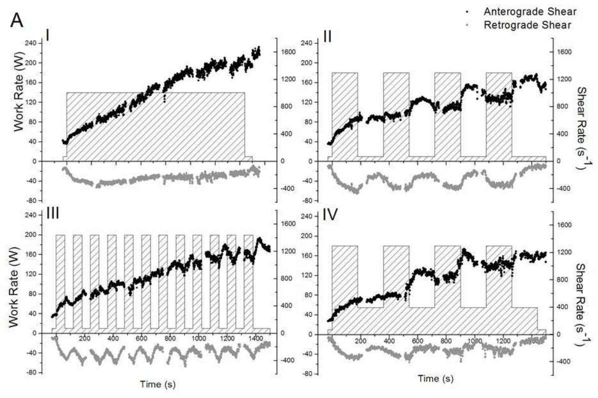
Figure 2: **Panel A)** Mean anterograde (black) and retrograde (grey) SR patterns for all participants during the four exercise protocols (shaded area): (I) CON (II) Long IT (III) Short IT and (IV) Long IT 70. **Panel B)** Mean retrograde SR (white columns) was not different between protocols ($P>0.05$). However, mean (shaded columns) and maximum (▪) anterograde SR was greater in CON than in both Long IT and Short IT ($P<0.05$), additionally Long IT 70 was greater than Long IT ($P<0.05$). Maximum retrograde SR (▫) and minimum anterograde (●) and retrograde (●) SR did not differ between protocols. All data are presented as group mean ± SD. * denotes a significant effect of exercise protocol for mean and maximum anterograde SR ($P<0.05$; n=13).

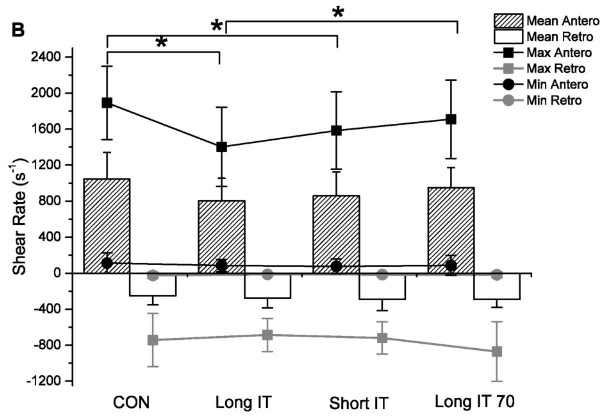
Figure 3: Panel A) Heart rate for a representative participant recorded throughout the four exercise protocols. Panel B) VO_2 data for the same representative participant recorded during the four exercise protocols demonstrating that the exercise was within the heavy intensity exercise domain (47). Heart rate peak (HR_{peak}), lactate threshold (LT) and $\dot{V}O_{2peak}$ were determined from the initial ramp incremental test.

Figure 4*:* Mean OSI (grey columns) was lower in CON compared to both Long IT and Short IT ($P<0.05$). Maximum (▪) and minimum (●) OSI were not different between protocols ($P>0.05$). In all protocols periods of purely oscillatory shear were achieved as indicated by maximum OSI > 0.5 but did not differ between exercise protocols ($P>0.05$). All data are presented as group mean ± SD. * denotes a significant effect of exercise protocol for mean OSI ($P<0.05$; n=13).

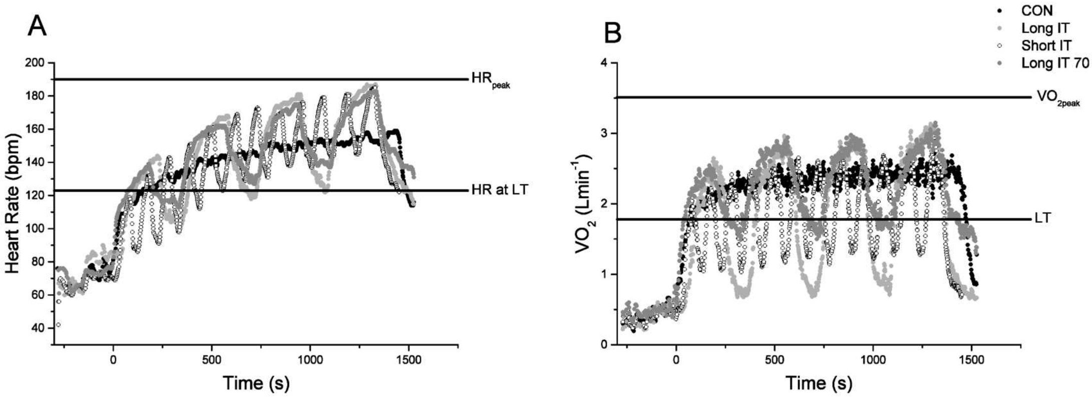


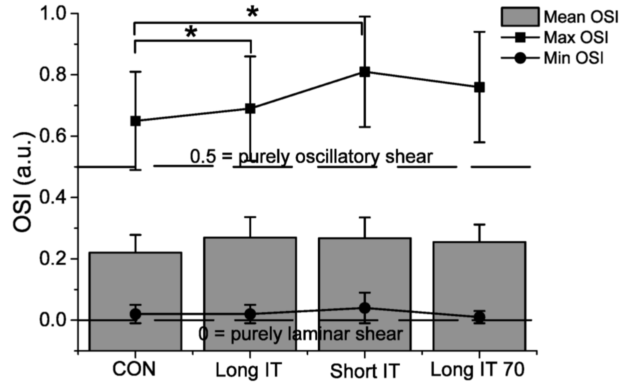
I
II
III
IV
Work Rate (%Work Rate Peak)
Time (s)
0
20
40
60
80
100
0
200
400
600
800
1000
1200
1400


A
Anterograde Shear
Retrograde Shear
I
II
III
IV
Work Rate (W)
Shear Rate (s-1)
Time (s)
240
200
160
120
80
40
0
-40
-80
1600
1200
800
400
0
-400
200
400
600
800
1000
1200
1400



B
Mean Antero
Mean Retro
Max Antero
Max Retro
Min Antero
Min Retro
*
*
*
Shear Rate (s-1)
2400
2000
1600
1200
800
400
0
-400
-800
-1200
CON
Long IT
Short IT
Long IT 70


A
B
CON
Long IT
Short IT
Long IT 70
Heart Rate (bpm)
HR$_{peak}$
HR at LT
Time (s)
VO$_2$ (Lmin^{-1})
VO$_{2peak}$
LT
200
180
160
140
120
100
80
60
40
0
500
1000
1500
4
3
2
1



Mean OSI
Max OSI
Min OSI
*
*
0.5 = purely oscillatory shear
0 = purely laminar shear
OSI (a.u.)
1.0
0.8
0.6
0.4
0.2
0.0
CON
Long IT
Short IT
Long IT 70

Table 1. Variables assessed during the FMD protocol at pre- and 15 min post each acute exercise bout are reported in the table. miR-21 was also assessed pre- and 6 hours post each acute exercise bout. Data reported as mean ± SD.

	CON		Long IT		Short IT		Long IT 70		P - Values
	Pre	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	
Baseline diameter, mm	3.98 ± 0.53	3.97 ± 0.54	3.98 ± 0.57	3.97 ± 0.54	3.96 ± 0.54	3.97 ± 0.51	3.98 ± 0.50	3.97 ± 0.54	Time P=0.80 Interaction P=0.97
Peak diameter, mm	4.24 ± 0.55	4.36 ± 0.56	4.26 ± 0.62	4.40 ± 0.53	4.24 ± 0.57	4.38 ± 0.57	4.23 ± 0.51	4.30 ± 0.58	Time P=0.001* Interaction P=0.83
Time to Peak Diameter, s	45 ± 22	74 ± 33	55 ± 27	68 ± 40	53 ± 24	68 ± 31	48 ± 14	69 ± 31	Time P=0.001* Interaction P=0.52
Absolute FMD, mm	0.25 ± 0.12	0.40 ± 0.21	0.29 ± 0.11	0.43 ± 0.16	0.28 ± 0.14	0.42 ± 0.17	0.26 ± 0.15	0.36 ± 0.16	Time P=0.001* Interaction P=0.99
Relative FMD, %	6.40 ± 3.02	10.24 ± 5.33	7.27 ± 2.46	10.98 ± 4.84	7.23 ± 3.55	10.54 ± 3.97	6.63 ± 3.05	9.24 ± 3.87	Time P=0.001* Interaction P=0.99
Peak Hyperemia, cm/s	93.1 ± 28.8	104.2 ± 14.7	88.5 ± 16.5	96.4 ± 19.8	89.2 ± 17.7	100.1 ± 21.3	95.0 ± 24.9	100.0 ± 17.7	Time P=0.002* Interaction P=0.88
Peak Shear Rate, s^{-1}	1910 ± 700	2124 ± 489	1842 ± 397	1982 ± 560	1864 ± 446	2049 ± 388	1914 ± 551	2054 ± 482	Time P=0.002* Interaction P=0.88
AUC_{60}, a.u.	41206 ± 11671	41353 ± 10824	42130 ± 8439	36899 ± 10367	44371 ± 8760	38431 ± 8016	41758 ± 13173	37081 ± 8566	Time P=0.01* Interaction P=0.48
AUC_{90}, a.u.	58894 ± 20133	61263 ± 17507	61937 ± 15001	53889 ± 15771	62840 ± 13783	56530 ± 12061	60752 ± 23066	57023 ± 12833	Time P=0.12 Interaction P=0.28
miR-21 expression (%cel-miR-39)	16.80 ± 24.14	7.88 ± 7.96	11.84 ± 23.12	11.02 ± 23.53	6.66 ± 23.06	18.16 ± 26.98	8.75 ± 3.75	6.16 ± 2.48	Time P=0.21 Interaction P=0.32

**denotes significanct time effect at P<0.05. There were no significant time by protocol interactions.*