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Post-exercise endothelial function is not associated with extracellular vesicle release in healthy young males.

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Running Title. FMD and extracellular vesicles after exercise

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ABSTRACT

Acute exercise can result in temporary decrease in endothelial function, which may represent a transient period of risk. Numerous mechanisms underpin these responses included release of extracellular vesicles (EVs) derived from apoptotic or activated endothelial cells and platelets. This study aimed to compare the time-course of endothelial responses to moderate-intensity-continuous-exercise (MICE) and high-intensity-interval-exercise (HIIE) and the associations with EV release. Eighteen young healthy males (age: 22.6±3.7y, BMI: 25.6±2.5m^2/kg, VO_{2peak}: 38.6±6.5ml/kg/min) completed two randomly assigned exercises; HIIE (10x1min-@-90% heart rate reserve (HRR), 1min passive recovery) or MICE (30min-@-70% HRR) on a cycle ergometer. Flow-mediated dilation (FMD) was used to assess endothelial function and blood samples were collected to evaluate endothelial cell-derived EV ($CD62E^+$) and platelet-derived EV ($CD41a^+$), prior- and 10, 60, and 120min post-exercise. There were similar increases, but different time-courses (P=0.017) in FMD (increased 10min post-HIIE, P<0.0001 and 60min post-MICE, P=0.038). $CD62E^+$ remained unchanged (P=0.530), whereas overall $CD41a^+$ release was reduced 60min post-exercise (P=0.040). FMD was not associated with EV absolute release or change (P>0.05). Acute exercise resulted in similar improvements, but different time-course in FMD following either exercise. Whilst EVs were not associated with FMD, the reduction in platelet-derived EVs may represent a protective mechanism following acute exercise.

Keywords. acute exercise; endothelial function; FMD, time-course; extracellular vesicles; microparticles, young healthy males

Introduction

The vascular response to acute exercise is varied with increases (Atkinson et al. 2015a; Hallmark et al. 2014; Johnson et al. 2012), decreases (Bond et al. 2015; Dawson et al. 2008; Llewellyn et al. 2012) and no changes (McClean et al. 2015; Rognmo et al. 2008; Shenouda et al. 2018) in flow-mediated dilation (FMD) reported. There are several factors which may somehow explain this heterogeneous response including methodological factors (timing of post-exercise measurements), exercise parameters (modality, intensity or volume) or subjects' characteristics (fitness level, cardiovascular risk factors) (Adams 2018; Dawson et al. 2013). In particular, it has been suggested that strenuous exercise, especially in untrained individuals, is more likely to result in early acute transient impairment of endothelial function (Atkinson et al. 2015b; Birk et al. 2013; Bond et al. 2015; Yoo et al. 2017). Although there is adequate number of studies to support the above, not a lot of studies had incorporated other assessments as an attempt to explain the mechanisms behind the reduction in endothelial function following strenuous exercise. Given the growing trend of high-intensity interval exercise not only in general public (Santos et al. 2020) but also in cardiac rehabilitation schemes (Dun et al. 2019) and the uncertainty if this transient decrease in endothelial function (Dawson et al. 2013) is associated with an elevated risk for cardiovascular events (Roffi et al. 2003), further work is needed to characterise the post-exercise response to these commonly used exercise protocols.

Indeed, transient endothelial impairment following strenuous exercise may reflect underpinning mechanisms including elevated oxidative stress, inflammation and platelet aggregation (Dawson et al. 2013; Tryfonos et al. 2019). Extracellular vesicles (EVs) in the circulation have been also implicated as they increase in response to

exercise stimuli (Ayers et al. 2015; Han et al. 2021; Wilhelm et al. 2018). In support of a potential association between FMD and EV, a biphasic response has been proposed for post-exercise EV release, similar to that seen with FMD (Dawson et al. 2013); with an increase or decrease immediately post-exercise, followed by normalisation (Di Credico et al. 2020; Wilhelm et al. 2018). Furthermore, similar to FMD, exercise parameters (modality, intensity, volume) and/or individual characteristics (fitness level) have also been shown to affect exercise-induced EV release (Han et al. 2021; Wilhelm et al. 2018). As such, this transient increase of EVs may represent a pre-cursor mechanism resulting in decrease in endothelial function following acute exercise and partly explain the variability in responses seen between subjects and interventions.

This study aimed to a) determine the time-course of FMD and EV release following moderate-intensity continuous exercise (MICE) and high-intensity interval exercise (HIIE) in young healthy males, and b) examine whether FMD changes are associated with changes in endothelial cell- and platelet-derived EV release. We hypothesised that acute exercise would result in an immediate decrease in endothelial function, with a greater decrease in FMD and a larger change in EVs in HIIE compared to MICE and a return to baseline by 2 hours. The decrease in FMD would be associated with an increase in platelet-derived EVs, whereas endothelial cell-derived EVs will remain unchanged.

Materials and Methods

Participants

Eighteen males aged 18-35 years were recruited. Participants were free of cardiovascular disease (CVD) or risk factors and were not taking medications that

could impact cardiovascular function. All participants provided written informed consent, and the study was approved by the Liverpool John Moores Ethics Committee (17/SPS/022) and adhered to the Declaration of Helsinki (Harriss et al. 2017).

Study design

Three visits were separated by at least 72h. During the first visit, participants performed a maximal graded exercise test on a cycle ergometer (Lode Excalibur Sport Cycle Ergometer, The Netherlands) to determine the peak oxygen consumption (VO_{2peak}), using a gas analysis system (MOXUS Metabolic Cart (AEI Technology, USA) (Tryfonos et al. 2020). Briefly, participants started cycling at 60W for 3min, then the workload was increased by 35W every 3 min until volitional fatigue. VO_{2peak} corresponded to the highest value achieved over a 15s recording period.

The second and third visits consisted of a randomly assigned HIIE or MICE bout. HIIE consisted of 10x1min intervals on a cycle ergometer at a workload equivalent to 90% heart rate reserve (HRR) using this equation ($HRR=HR_{max}-HR_{rest}$), interspersed with 1min passive recovery periods. MICE was 30 min of continuous cycling at a workload equivalent to 70% HRR. MICE and HIIE protocols were selected as both have been recommended by American College of Sport Medicine (ACSM) for aerobic training (Garber et al. 2011) and adopted by World Health Organization (WHO) (Bull et al. 2020) in both healthy and clinical population. All experimental procedures were conducted between 7am and 1pm, in a quiet temperature-controlled room, and participants were fasted overnight and instructed to abstain from caffeine (>8h), alcohol and vigorous exercise (>24h) before each visit (Thijssen et al. 2019).

Estimation of energy expenditure using the average HR, average workload, age and measured VO_{2peak} was calculated retrospectively (Keytel et al. 2005).

Experimental procedures

Following ≥10 min supine rest, blood pressure and heart rate (HR) were measured using an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL, USA). Subsequently, brachial artery FMD was assessed, and a venous catheter was inserted into an antecubital vein (contralateral arm) for blood sampling. Participants were supervised during exercise and HR was continuously monitored using telemetry (Polar FT1 Heart Rate Monitor and Sports Watch, USA). Following exercise, participants returned immediately to a supine position, where FMD and blood samples were taken 10, 60 and 120 min post-exercise.

Brachial Artery FMD

Brachial artery FMD was measured as described previously (Thijssen et al. 2019). Briefly, an optimal B-mode image of the brachial artery was acquired, using a 12-MHz multi-frequency linear array probe, attached to a high-resolution ultrasound machine (T3000; Terason, Burlington, MA), using the lowest possible insonation angle (always < 60°) to image the artery proximal to olecranon (distal 1/3 of the upper arm). Relative diameter change, time to peak, and shear rate area under the curve (SRAUC) were analysed by the same observer, using custom-designed edge-detection and wall-tracking software (Thijssen et al. 2019). FMD was reported as the maximum percentage change in artery diameter from baseline to peak when the cuff was released, as described in detail previously (Thijssen et al. 2019). The same ultrasound and sonographer were used within visits, and between participants.

147 **Blood Sample Collection**

148 A venous catheter (cannula) was inserted into antecubital vein to facilitate blood
149 sample collection at multiple time-points (baseline, and 10 min, 60 min and 120 min
150 post-exercise). Approximately 5 ml saline was used to flush the cannula following each
151 blood drawing to prevent blood clotting. Thus, the first 5 ml of blood drawings (following
152 saline flushes) were discarded, before blood samples were collected in tubes
153 containing 3.2% buffered sodium citrate solution (BD Vacutainer Glass Citrate Tube
154 4.5ml with Light Blue Hemogard Closure, 367691, USA), for further EV analysis. Blood
155 tubes were then centrifuged at 1500 x g for 10 mins at room temperature (RT) to
156 remove larger cells and platelets. Resulting platelet poor plasma (PPP) was stored at
157 -80^{o}C until further analysis, as previously described (Dey-Hazra et al. 2010; Jenkins
158 et al. 2013).

159

160 **EVs Isolation and Staining**

161 EV isolation and staining were performed based on previous methods (Highton et al.
162 2020; Jenkins et al. 2013). Briefly, PPP (500µl) were thawed rapidly at RT and
163 centrifuged at 17000 x g for 20 mins to the pellet, the medium to large (500-1000nm)
164 EV population of interest, according to the most recent guidelines (Laffan and Manning
165 2012; Théry et al. 2018). The top 450µl of supernatant, containing small EV (exosomes)
166 and larger particles greater than 1µm in size (apoptotic bodies, contaminating
167 platelets), was removed and replaced with 450µl phosphate-buffered saline (PBS)
168 (Thermo Fisher Scientific, USA) containing 10.9 mmol/L trisodium citrate, which was
169 filtered x2 with 0.22µm filter (double filtered PBS; dfPBS). The EV pellet was re-
170 suspended and centrifuged again at 17000 x g for 2 mins at RT to wash the pellet and
171 remove any contaminating platelets and cell debris. Data from our lab (not shown) and

a previous methodological paper (Dey-Hazra et al. 2010), demonstrated no significant loss of EVs comparing 2 or 20 min spin for this second wash stage. After removal of the supernatant (450µl), 150µl of dfPBS was added and the EV pellet was re-suspended. 20µl of the EV suspension was diluted in 100µl sterile filtered Annexin Binding buffer containing 0.1M Hepes (pH 7.4, 1.4M NaCl and 25mM CaCl (Becton Dickinson Biosciences, USA) for staining.

The total mid to large size (500-1500nm) population of EVs was assessed using size gating and phosphatidylserine (PS) expression (Annexin V+) to determine the number of AnnexinV positive MPs. Positive MPs were further identified, and their origin categorised, by dual staining with fluorochrome-labelled antibodies marking the EV fraction of interest. For staining, 1µl Annexin-PE (Product code: 556422, BD Biosciences, USA) was added to each tube containing diluted EV suspension (1:100) to identify all Annexin V+ extracellular vesicles and 1µl (0.5µg) CD62E-fluorescein isothiocynate (FITC) or CD41a-FITC (25µg/ml) to identify EV-derived from endothelial cells and platelets respectively (Product codes: 555648 and 340929, Becton Dickinson Biosciences, USA). Following incubation for 45 mins at RT, 400µl of dfPBS was added to each tube and analysed by a BD Accuri C6 Flow cytometer (Becton Dickinson Biosciences, USA). Samples were acquired for 180s at 35ul min^{-1}. EV populations of interest were gated on the basis of their size, density and their capacity to positively bind PE labelled Annexin V and cell specific FITC antibodies, compared to negative no antibody or no annexin binding controls (Annexin Binding buffer minus CaCl2). Fluorescence minus one antibody controls were employed to determine background non-specific staining and adjust compensation settings. 1µm non-fluorescent microspheres (Product code 89904, Sigma-Aldrich, USA) were used to identify the

upper size limit for EV detection and all events above the noise floor and below 1μm were collected. Instrument 'noise' was determined by running ultrapure water alone set with a forward scatter primary threshold of 2000. The gating strategy and example data is described in Supplementary material (Figure S1).

The number of EVs per μl of plasma was calculated using the flow rate method as described by Lok et al. (2009) using the formula: Number/μl plasma= N x (200/20) x (102/35) x (10^6/500) (N= number of events that stained positive for both Annexin V and a cell-specific antibody). EVs from both visits and all time-points of each participant were analysed on the same day to avoid methodological errors. However, different orders between visits and time-points were followed for each participant to prevent an impact of order on the results.

Statistics

All analyses were performed using IBM SPSS statistics for Windows, version 26.0. (Armonk, NY: IBM Corp). Allometric scaling was performed to control for differences in baseline diameter (Atkinson and Batterham 2013), and a mixed-linear model with covariate control for scaled baseline diameter, was used to determine the main effect of time and exercise protocol. A mixed-linear model was also used to analyze the differences in EVs and sub-measurements of FMD including baseline diameter, peak diameter, time to peak and SRAUC. Pairwise comparisons were performed when significant main or interaction effects were detected, using Bonferroni correction. Pearson correlations were also used to examine associations between FMD and EV release at different time-points and exercise protocols. Results are presented as mean±SD, and significance was set at $P<0.05$.

Results

Subjects' characteristics are shown in Table 1. Targeted HR (HIIE: 90% HRR, MICE: 70% HRR), and rate of perceived exertion (RPE) were achieved during all exercise bouts (Table 1).

Impact of exercise intensity on FMD

There was no difference in baseline FMD between exercise protocols (P=0.497) (Figure 1). There was a significant main effect of time (P=0.003), showing increased FMD at 10 (P=0.001) and 60 min (P=0.014) but not at 120 min post-exercise. There was a significant interaction (time*exercise protocol, P=0.017), indicating a different time-course between exercise protocols (Figure 1). FMD was higher at 10 min post-HIIE (P<0.001) returning to baseline by 60 min. Following MICE, FMD increased at 60 min (P=0.038) and then return to baseline (P=0.929). However, the magnitude of the FMD increase was not different between the exercise protocols (P=0.598).

There was a significant main effect of time for baseline artery diameter (P=0.015), peak artery diameter (P=0.001), SRAUC (P<0.001) and time to peak (P<0.001), with all reporting significantly higher values at 10 min post-exercise, when compared to baseline, 60 and 120 min post-exercise (P<0.05). However, these were not significantly different between exercise protocols and there was no interaction (Table 2).

Impact of exercise intensity on EVs

EVs were analysed in 14 participants (4 participants: no blood samples were available).

Endothelial cell-derived EVs ($CD62E^+$): Whilst there was no significant difference in baseline $CD62E^+$ between HIIE and MICE (P=0.069), there was a main effect of exercise protocol (P=0.047), suggesting overall higher endothelial cell-derived EVs release in MICE when compared to HIIE. However, there was no main effect for time or interaction suggesting that there was no change in endothelial cell-derived EVs following HIIE and MICE (Figure 1b).

Platelet-derived EVs ($CD41a^+$): There was no difference in baseline $CD41a^+$ between HIIE and MICE (P=0.081). There was a main effect of time (P=0.013). Post-hoc analysis revealed a lower concentration at 60 min post-exercise compared to baseline (P=0.040) and 10 min post-exercise (P=0.003), with further decreases from 10 min at 120 min (P=0.023). A main effect of exercise protocol was observed with a higher concentration in MICE compared to HIIE (P=0.017). However, there was no interaction (time*exercise protocol; P=0.635), suggesting that platelet-derived EVs follow the same pattern following both exercise protocols (Figure 1c).

Associations between FMD and EVs

FMD was not related to either endothelial cell- ($CD62E^+$) or platelet-derived ($CD41a^+$) EV release when compared to the same time-point post-HIIE or post-MICE, including baseline (P>0.05). Similarly, FMD change (Dchange; percentage change from Baseline), following HIIE or MICE was not associated with the Dchange in either endothelial cell- or platelet-derived EV release, in either of time-points (P>0.05) (Table 3).

Discussion

The primary aim of this study was to determine if 'typical' exercise sessions of HIIE or MICE result in altered vascular function and if the change was associated with endothelial- and platelet-derived EVs in healthy young males. Contrary to our hypothesis, both MICE and HIIE resulted in transient increases in endothelial function which were unrelated to endothelial- or platelet-derived EV. Our data suggest that both HIIE and MICE can be undertaken safely, at least in apparently healthy yet inactive individuals, without a detriment to vascular function.

Endothelial function following different exercise protocols

Both MICE and HIIE resulted in similar increases in FMD, but with an earlier increase with HIIE. Previous work demonstrating a transient decrease in FMD with high-intensity exercise may be driven by higher retrograde shear rate (Tinken et al. 2009) and/or oxidative stress, both of which reduce NO-bioavailability and thus cause endothelial dysfunction (Green et al. 2017). Furthermore, the multiple transitions between 'work' and 'recovery' during HIIE increase retrograde shear stress when compared to moderate intensity exercise (Lyall et al. 2019; McManus et al. 2019). However, whilst retrograde shear rate is typically associated with decreased FMD, this periodic high-and-low retrograde shear stress during HIIE may offer some prophylactic effects on the vasculature (Cheng et al. 2019; Ghardashi Afousi et al. 2018; Holder et al. 2019; Iwamoto et al. 2018). In addition, whilst retrograde flow increases, there is also a large increase in antegrade shear rate compared to MICE (Iwamoto et al. 2018; McManus et al. 2019), which has been associated with increased FMD (Green et al. 2017; Tinken et al. 2009). In the current study the increased, as opposed to decreased, endothelial function post-exercise would suggest that this exercise mode may induce

only low levels of oxidative stress and/or that the beneficial effect of anterograde shear rate and endothelial nitric oxide synthase (eNOS) activation (Casey et al. 2017; Cocks et al. 2013; Tryfonos et al. 2022) overcomes oxidative stress and retrograde shear resulting in improved FMD in healthy young males.

The magnitude of increases in FMD in this study are similar to previous work comparing HIIE vs MICE (Currie et al. 2012; Lyall et al. 2019). However, both studies reported a similar time-course in FMD following HIIE and MICE, which is in contrast of our evidence. Difference may be due to matching exercise intensities and durations between HIIE and MICE (Lyall et al. 2019) or differences in clinical versus healthy populations (Currie et al. 2012). As such, this delayed response in MICE observed in the current study may be related to a larger exercise dose and associated oxidative stress in MICE (Johnson et al. 2012), opposing the early shear-mediated improvements (Tinken et al. 2009).

Exercise and EV release

Although exercise-induced vascular adaptions are largely mediated by hemodynamic forces (i.e. elevated anterograde shear stress) (Green et al. 2017), systemic circulating factors (Padilla et al. 2011), including EV release (Han et al. 2021; Wilhelm et al. 2018), may be involved, predominantly due to their role in intracellular communication. In line with previous studies in healthy males after low intensity exercise (Rakobowchuk et al. 2017), MICE (HIGHTON et al. 2019; Wilhelm et al. 2016), or HIIE (Sapp et al. 2019), endothelial cell-derived EVs ($CD62E^+$) remained unchanged. However, earlier work reported increases in endothelial cell-derived EV release ($CD62E^+$ or $CD105^+$ following MICE (Lansford et al. 2016) or HIIE (Kirk et al. 2014). Of particular interest was Shill

et al., 2018 (Shill et al. 2018) study that compared endothelial cell-derived EV release ($CD62E^+$) in the same cohort (young healthy males and females) following similar exercise protocols as our study and reported a decrease following MICE (65% VO_{2max}, matched time and energy expenditure of HIIE) but not HIIE (10x1min intervals-@-90% and 100% VO_{2max}). Unchanged or decreased endothelial cell-derived EV release in response to exercise may be a consequence of the beneficial effects of exercise-induced shear stress counteracting other factors such as cytokines (Jimenez et al. 2003). Alternatively, endothelial cells may uptake endothelial cell-derived EVs (Ayers et al. 2015) or release and clearance may occur simultaneously during exercise, masking any increased production (Jimenez et al. 2003; Sapp et al. 2019). Whilst endothelial-derived EV release is typically unchanged/decreased and likely unaffected by exercise intensity, there is large variability within and between studies so further work is needed to elucidate responses to acute exercise.

Platelet-derived EVs ($CD41a^+$) were significantly reduced 60 min post-exercise, independent of exercise mode. This reduction following exercise was unexpected, as previous research demonstrates an increase at 5-120 mins (Chaar et al. 2011; Hilberg et al. 2008; Maruyama et al. 2012; Rakobowchuk et al. 2017; Sossdorf et al. 2010; Wilhelm et al. 2016), or no change (Durrer et al. 2015; Lansford et al. 2016; Shill et al. 2018). Exercise-induced platelet-derived EVs may represent an intercellular communication mechanism in the vascular wall, with the reduction representing a cellular signal between platelet-derived EVs and endothelial cells to initiate angiogenesis or vascular remodelling, contributing to exercise-induced vascular adaptations (Ayers et al. 2015; Di Credico et al. 2020). Further work is needed to

characterize the response to exercise and determine the functional implications of these findings.

Associations between exercise-induced changes in FMD and EV release

In line with exercise training responses (Dawson et al. 2021; Green et al. 2014), individual FMD responses to acute exercise were variable. We hypothesised that this may relate to underpinning mechanisms, such as EV release. Only one previous study has explored the relationship between EV release and change in endothelial function in response to acute exercise. Sapp et al., (2019) observed a positive association between the immediate change in endothelial cell-derived EVs (10 min) and change in FMD 1h post-MICE but not HIIE (MICE;30 min continuous exercise-@-60%W_{max} and HIIE;6min-@-40% W_{max} followed by 3min interval-@-85% W_{max} interspersed with 4min-@-40 W_{max} matched for total time and workload) in active young males. In contrast, we report no association between change in FMD and EV change following either MICE or HIIE. Given the limited, yet inconsistent evidence regarding the direct relationship between FMD and EV release in response to exercise, further work is required. It is worth noting that standardization of the methods for evaluating EV release is required, including markers, isolation/staining protocols and time-course to allow comparison between studies and to elucidate if it is a key factor in the individual FMD responses following acute exercise (Ayers et al. 2015; Wilhelm et al. 2018).

Limitations

This study assessed endothelial function and EV release following exercise in young healthy males. The results cannot therefore be compared to other groups including women, older individuals and those with diseases. Although the MICE intensity is

'moderate' compared to 90% HRR in our HIIE bout, the majority of previous data is at lower intensities (40-65% VO_{2max}), in order to match exercise dose. Whilst we have not directly measured exercise dose, we estimated the energy expenditure during both exercise bouts, using the known VO_{2peak} (Table 1) demonstrating that MICE may result in larger overall dose than HIIE. However, it is worth noting that the purpose of the study was to examine the acute vascular responses following two commonly used exercise protocols of MICE and HIIE, in the same individuals. For that reason, we used HRR and not $\%HR_{max}$ or $\%VO_{2peak}$, to better capture real-world scenarios. However, given that exercise dose may affect both FMD and EVs, further studies with real-time measurement of energy expenditure during exercise, should evaluate the inter and intra relationships between FMD time-course response, EVs and exercise dose. In regards to the PPP production, although we have used similar techniques as previously descripted at the time of our date collection, a recent paper (Rikkert et al. 2021) suggest that a single higher spin (5000g for 20 minutes) may be more effective in reducing platelets compared to lower spins. Although flow cytometry is one of the most common methods of detection, quantification and size evaluation of larger extracellular vesicles, the sensitively of the most standard flow cytometers (BD Accuri system) (also employed in our study) cannot accurately detect particles below 500nm based on size alone. This raises the possibility that we may have significantly excluded a large number of particles, thus influencing the final result. A further limitation is the use of polystyrene beads for determining the upper limit of detection, as studies have shown that a 1000nm polystyrene particle is not truly reflective of a 1000nm EV, and that a silica bead with a lower refractive index may be more accurate. As shown in the supplementary data online (Figure S2), we compared the FSC SSC parameters of a mixture of polystyrene and silica beads and demonstrated that our upper size limit,

previously established as 1000nm based on polystyrene bead, may indeed by more like 1500nm silica bead/EV. Hence, potentially counting particles greater than 1000nm in the analysis. In addition to size, we also used phosphatidylserine (PS) positivity to define our EV population of interest, as large EVs generally expose PS on their outer leaflet. However, there is also evidence to suggest that EVs can also be PS negative, which may have been omitted from our analysis, and if evaluated, may have produced different findings. Finally, there was a high day-to-day variability in regards to baseline EV levels. Biomarker baseline variability can be attributed to many factors, either biological or technical. With this in mind, biomarker analysis is often presented as a fold change above baseline for each time point to account for this rather than absolute levels. As such, in our study we have examined the time course rather than a snapshot and we calculated the exercise response as fold change from the baseline (as measured on each day) to overcome this limitation. To conclude, given that EV release may significantly varied due to the techniques used, we believe that efforts should be made to develop a consensus guideline in regards to EV analysis, including PPP production.

Conclusion

Acute exercise resulted in similar improvements in endothelial function, but with different time-course following MICE or HIIE exercise bouts in young healthy males. The individual changes in FMD were not associated with platelet- or endothelial cell-EV release. Although several studies have previously investigated the effect of acute exercise in endothelial function in healthy individuals, the conflicting data necessitates the need for more ‘mechanistic’ studies to develop our understanding in regards to the different mechanisms and potentially individual characteristics (e.g. fitness, sex or *a*

419 *priori* endothelial dysfunction) leading to the large diverse and individualised
420 responses of post-exercise endothelial responses. Finally, further work is needed to
421 determine the physiological relevance, if any, of these changes on either transient risk
422 or as a stimulus for long-term adaptations.

Conflict of interest statement

The authors declare no conflicts of interest.

Authors contribution statement

A.T., N.B., and E.A.D.: conceptualization. A.T., M.C., N.B., and E.A.D.: investigation, methodology and formal analysis. M.C. and E.A.D.: supervision. A.T.: writing - original draft. A.T., M.C., N.B., and E.A.D.: writing - review and editing. All authors approved the final version of the manuscript.

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Data availability statement

Data generated or analyzed during this study are available from the corresponding author upon reasonable request.

Figures legends

Figure 1. Endothelial function assessed via flow-mediated dilation (FMD) and EV release prior to exercise (Baseline) and 10, 60 and 120 min post-exercise.

FMD **(a)** following high-intensity-interval exercise (HIIE) and moderate-intensity-continuous exercise (MICE) (mean±SD; n=18). **(b)** Endothelial cell-derived extracellular vesicles **($CD62E^+$)** in HIIE and MICE (mean±SD; n=14). **(c)** Platelet-derived extracellular vesicles (**$CD41a^+$**) in HIIE MICE (mean±SD; n=14). A mixed-linear model (time*exercise protocol), covariating baseline diameter (FMD only), and Bonferroni correction for post-hoc pairwise comparisons was used. *Significantly different from Baseline ($P<0.05$), $^{\#}$Significantly different from HIIE ($P<0.05$).

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Tables

Table 1. Participant characteristics and mean HR and power (watts) during high-intensity-interval exercise (HIIE) and moderate-intensity-continuous exercise (MICE). Mean percentage of heart rate reserve (%HRR), maximal power (%W_{max}), rating of perceived exertion (RPE) (1: no effort to 10: maximal effort), and estimation of energy expenditure using equation for known VO_{2peak}. Participants exercised at an intensity of ~90% HRR in HIIE and ~70% HRR in MICE, workload was adjusted by the researchers in order to reach the targeted HR. Results are presented as mean±SD, n=18.

	Overall	
Age (years)	23±4	
Height (m)	1.77±0.06	
Weight (kg)	80.6±12.6	
BMI (m_2/kg)	25.6±2.5	
VO_{2peak} (ml/kg/min)	38.6±6.5	
SBP rest (mmHg)	122±9	
DBP rest (mmHg)	61±6	
HR rest (beats/min)	63±11	
HR max (beats/min)	192±4	
Workload max (watts)	219±36	
RER (VCO_2/VO_2)	1.19±0.05	
	During Exercise Visits	
	HIIE	**MICE**
Mean HR (beats/min)	171±8 (85±5% HRR)	154±12 (67±6% HRR)

Mean workload (watts)	197±50 (92±16% W_{max})	138±34 (63±7%W_{max})
RPE (1-10)	8.9±0.6	6.7±0.5
Energy expenditure (kcal)	480±78	519+112

Table 2. Baseline diameter, peak diameter, time to peak and shear rate under the curve (SRAUC), before (Baseline) and at 10, 60 and 120 min following high-intensity-interval exercise (HIIE) and moderate-intensity-continuous exercise (MICE). Results are presented as mean±SD, n=18, *Significant difference from Baseline (main time effect)

		Baseline diameter (mm)	Peak diameter (mm)	Time to peak (s)	SRAUC (s^{-1} 10^3)
Baseline	HIIE	4.00±0.47	4.27±0.47	61.0±31.7	19.4±8.4
	MICE	4.05±0.51	4.34±0.54	52.3±24.4	19.1±9.0
10 min*	HIIE	4.21±0.31	4.58±0.34	80.9±19.2	29.0±15.3
	MICE	4.15±0.55	4.44±0.55	78.9±22.5	28.8±9.5
60 min	HIIE	4.14±0.54	4.45±0.53	58.1±18.0	20.3±8.2
	MICE	4.00±0.48	4.34±0.49	51.6±17.2	19.4±7.1
120 min	HIIE	4.09±0.48	4.39±0.48	52.6±19.5	16.7±5.9
	MICE	4.00±0.42	4.30±0.41	52.0±18.6	17.8±6.2

Table 3. Associations between values obtained at the same timepoints (a), and as a percentage change from Baseline (Dchange) (b). Data collected at Baseline (prior), 10, 60 and 120 min following high-intensity-interval exercise (HIIE) and moderate-intensity-continuous exercise (MICE).

	Baseline	10 min	60 min	120 min
HIIE				
a) Absolute				
FMD - $CD62E^+$	R=-0.447, P=0.109	R=0.122, P=0.737	R=-0.102, P=0.779	R=-0.216, P=0.549
FMD - $CD41a^+$	R=-0.032, P=0.918	R=0.415, P=0.233	R=-0.249, P=0.371	R=0.009, P=0.979
b) Dchange				
FMD - $CD62E^+$		R=-0.94, P=0.795	R=-0.263, P=0.462	R=0.123, P=0.752
FMD - $CD41a^+$		R=-0.404, P=0.247	R=-0.321, P=0.360	R=0.050, P=0.898
MICE				
a) Absolute				
FMD - $CD62E^+$	R=0.098, P=0.750	R=-0.382, P=0.198	R=-0.032, P=0.926	R=-0.358, P=0.276
FMD - $CD41a^+$	R=0.098, P=0.750	R=-0.204, P=0.503	R=-0.313, P=0.276	R=0.092, P=0.800
b) Dchange				

FMD - $CD62E^+$	R=-0.053, P=0.871	R=-0.046, P=0.899	R=-0.211, P=0.559
FMD - $CD41a^+$	R=-0.323, P=0.282	R=-0.354, P=0.236	R=-0.064, P=0.851

FMD; flow mediated dilatation, endothelial cell-derived vesicles; $CD62E^+$, platelet-derived extracellular vesicles; $CD41a^+$, R=Pearson correlation coefficient, P=Statistical significance, n=14