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

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Article

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3   **Post-exercise endothelial function is not associated with extracellular**

4   **vesicle release in healthy young males.**

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14   **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

48   **Introduction**

49   The vascular response to acute exercise is varied with increases (Atkinson et al.

50   2015a; Hallmark et al. 2014; Johnson et al. 2012), decreases (Bond et al. 2015;

51   Dawson et al. 2008; Llewellyn et al. 2012) and no changes (McClean et al. 2015;

52   Rognmo et al. 2008; Shenouda et al. 2018) in flow-mediated dilation (FMD) reported.

53   There are several factors which may somehow explain this heterogeneous response

54   including methodological factors (timing of post-exercise measurements), exercise

55   parameters (modality, intensity or volume) or subjects' characteristics (fitness level,

56   cardiovascular risk factors) (Adams 2018; Dawson et al. 2013). In particular, it has

57   been suggested that strenuous exercise, especially in untrained individuals, is more

58   likely to result in early acute transient impairment of endothelial function (Atkinson et

59   al. 2015b; Birk et al. 2013; Bond et al. 2015; Yoo et al. 2017). Although there is

60   adequate number of studies to support the above, not a lot of studies had incorporated

61   other assessments as an attempt to explain the mechanisms behind the reduction in

62   endothelial function following strenuous exercise. Given the growing trend of high-

63   intensity interval exercise not only in general public (Santos et al. 2020) but also in

64   cardiac rehabilitation schemes (Dun et al. 2019) and the uncertainty if this transient

65   decrease in endothelial function (Dawson et al. 2013) is associated with an elevated

66   risk for cardiovascular events (Roffi et al. 2003), further work is needed to characterise

67   the post-exercise response to these commonly used exercise protocols.

68

69   Indeed, transient endothelial impairment following strenuous exercise may reflect

70   underpinning mechanisms including elevated oxidative stress, inflammation and

71   platelet aggregation (Dawson et al. 2013; Tryfonos et al. 2019). Extracellular vesicles

72   (EVs) in the circulation have been also implicated as they increase in response to

73　exercise stimuli (Ayers et al. 2015; Han et al. 2021; Wilhelm et al. 2018). In support of

74　a potential association between FMD and EV, a biphasic response has been proposed

75　for post-exercise EV release, similar to that seen with FMD (Dawson et al. 2013); with

76　an increase or decrease immediately post-exercise, followed by normalisation (Di

77　Credico et al. 2020; Wilhelm et al. 2018). Furthermore, similar to FMD, exercise

78　parameters (modality, intensity, volume) and/or individual characteristics (fitness level)

79　have also been shown to affect exercise-induced EV release (Han et al. 2021; Wilhelm

80　et al. 2018). As such, this transient increase of EVs may represent a pre-cursor

81　mechanism resulting in decrease in endothelial function following acute exercise and

82　partly explain the variability in responses seen between subjects and interventions.

83

84　This study aimed to a) determine the time-course of FMD and EV release following

85　moderate-intensity continuous exercise (MICE) and high-intensity interval exercise

86　(HIIE) in young healthy males, and b) examine whether FMD changes are associated

87　with changes in endothelial cell- and platelet-derived EV release. We hypothesised

88　that acute exercise would result in an immediate decrease in endothelial function, with

89　a greater decrease in FMD and a larger change in EVs in HIIE compared to MICE and

90　a return to baseline by 2 hours. The decrease in FMD would be associated with an

91　increase in platelet-derived EVs, whereas endothelial cell-derived EVs will remain

92　unchanged.

93

94　**Materials and Methods**

95　**Participants**

96　Eighteen males aged 18-35 years were recruited. Participants were free of

97　cardiovascular disease (CVD) or risk factors and were not taking medications that

98   could impact cardiovascular function. All participants provided written informed

99   consent, and the study was approved by the Liverpool John Moores Ethics Committee

100   (17/SPS/022) and adhered to the Declaration of Helsinki (Harriss et al. 2017).

101

102   **Study design**

103   Three visits were separated by at least 72h. During the first visit, participants

104   performed a maximal graded exercise test on a cycle ergometer (Lode Excalibur Sport

105   Cycle Ergometer, The Netherlands) to determine the peak oxygen consumption

106   (VO_{2peak}), using a gas analysis system (MOXUS Metabolic Cart (AEI Technology, USA)

107   (Tryfonos et al. 2020). Briefly, participants started cycling at 60W for 3min, then the

108   workload was increased by 35W every 3 min until volitional fatigue. VO_{2peak}

109   corresponded to the highest value achieved over a 15s recording period.

110

111   The second and third visits consisted of a randomly assigned HIIE or MICE bout. HIIE

112   consisted of 10x1min intervals on a cycle ergometer at a workload equivalent to 90%

113   heart rate reserve (HRR) using this equation ($HRR=HR_{max}-HR_{rest}$), interspersed with

114   1min passive recovery periods. MICE was 30 min of continuous cycling at a workload

115   equivalent to 70% HRR. MICE and HIIE protocols were selected as both have been

116   recommended by American College of Sport Medicine (ACSM) for aerobic training

117   (Garber et al. 2011) and adopted by World Health Organization (WHO) (Bull et al.

118   2020) in both healthy and clinical population. All experimental procedures were

119   conducted between 7am and 1pm, in a quiet temperature-controlled room, and

120   participants were fasted overnight and instructed to abstain from caffeine (>8h),

121   alcohol and vigorous exercise (>24h) before each visit (Thijssen et al. 2019).

122 Estimation of energy expenditure using the average HR, average workload, age and

123 measured VO_{2peak} was calculated retrospectively (Keytel et al. 2005).

124

Experimental procedures

126 Following ≥10 min supine rest, blood pressure and heart rate (HR) were measured

127 using an automated sphygmomanometer (GE Pro 300V2, Dinamap, Tampa, FL, USA).

128 Subsequently, brachial artery FMD was assessed, and a venous catheter was inserted

129 into an antecubital vein (contralateral arm) for blood sampling. Participants were

130 supervised during exercise and HR was continuously monitored using telemetry (Polar

131 FT1 Heart Rate Monitor and Sports Watch, USA). Following exercise, participants

132 returned immediately to a supine position, where FMD and blood samples were taken

133 10, 60 and 120 min post-exercise.

134

Brachial Artery FMD

136 Brachial artery FMD was measured as described previously (Thijssen et al. 2019).

137 Briefly, an optimal B-mode image of the brachial artery was acquired, using a 12-MHz

138 multi-frequency linear array probe, attached to a high-resolution ultrasound machine

139 (T3000; Terason, Burlington, MA), using the lowest possible insonation angle

140 (always < 60°) to image the artery proximal to olecranon (distal 1/3 of the upper arm).

141 Relative diameter change, time to peak, and shear rate area under the curve (SRAUC)

142 were analysed by the same observer, using custom-designed edge-detection and

143 wall-tracking software (Thijssen et al. 2019). FMD was reported as the maximum

144 percentage change in artery diameter from baseline to peak when the cuff was

145 released, as described in detail previously (Thijssen et al. 2019). The same ultrasound

146 and sonographer were used within visits, and between participants.

Blood Sample Collection

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

EVs Isolation and Staining

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

172   a previous methodological paper (Dey-Hazra et al. 2010), demonstrated no significant

173   loss of EVs comparing 2 or 20 min spin for this second wash stage. After removal of

174   the supernatant (450µl), 150µl of dfPBS was added and the EV pellet was re-

175   suspended. 20µl of the EV suspension was diluted in 100µl sterile filtered Annexin

176   Binding buffer containing 0.1M Hepes (pH 7.4, 1.4M NaCl and 25mM CaCl (Becton

177   Dickinson Biosciences, USA) for staining.

178

179   The total mid to large size (500-1500nm) population of EVs was assessed using size

180   gating and phosphatidylserine (PS) expression (Annexin V+) to determine the number

181   of AnnexinV positive MPs. Positive MPs were further identified, and their origin

182   categorised, by dual staining with fluorochrome-labelled antibodies marking the EV

183   fraction of interest. For staining, 1µl Annexin-PE (Product code: 556422, BD

184   Biosciences, USA) was added to each tube containing diluted EV suspension (1:100)

185   to identify all Annexin V+ extracellular vesicles and 1µl (0.5µg) CD62E-fluorescein

186   isothiocynate (FITC) or CD41a-FITC (25µg/ml) to identify EV-derived from endothelial

187   cells and platelets respectively (Product codes: 555648 and 340929, Becton Dickinson

188   Biosciences, USA). Following incubation for 45 mins at RT, 400µl of dfPBS was added

189   to each tube and analysed by a BD Accuri C6 Flow cytometer (Becton Dickinson

190   Biosciences, USA). Samples were acquired for 180s at 35ul min^{-1}. EV populations of

191   interest were gated on the basis of their size, density and their capacity to positively

192   bind PE labelled Annexin V and cell specific FITC antibodies, compared to negative

193   no antibody or no annexin binding controls (Annexin Binding buffer minus CaCl2).

194   Fluorescence minus one antibody controls were employed to determine background

195   non-specific staining and adjust compensation settings. 1µm non-fluorescent

196   microspheres (Product code 89904, Sigma-Aldrich, USA) were used to identify the

197   upper size limit for EV detection and all events above the noise floor and below 1µm

198   were collected. Instrument 'noise' was determined by running ultrapure water alone

199   set with a forward scatter primary threshold of 2000. The gating strategy and example

200   data is described in Supplementary material (Figure S1).

201

202   The number of EVs per µl of plasma was calculated using the flow rate method as

203   described by Lok et al. (2009) using the formula: Number/µl plasma= N x (200/20) x

204     $(102/35)$ x $(10^6/500)$ (N= number of events that stained positive for both Annexin V

205   and a cell-specific antibody). EVs from both visits and all time-points of each

206   participant were analysed on the same day to avoid methodological errors. However,

207   different orders between visits and time-points were followed for each participant to

208   prevent an impact of order on the results.

209

210   **Statistics**

211   All analyses were performed using IBM SPSS statistics for Windows, version 26.0.

212   (Armonk, NY: IBM Corp). Allometric scaling was performed to control for differences

213   in baseline diameter (Atkinson and Batterham 2013), and a mixed-linear model with

214   covariate control for scaled baseline diameter, was used to determine the main effect

215   of time and exercise protocol. A mixed-linear model was also used to analyze the

216   differences in EVs and sub-measurements of FMD including baseline diameter, peak

217   diameter, time to peak and SRAUC. Pairwise comparisons were performed when

218   significant main or interaction effects were detected, using Bonferroni correction.

219   Pearson correlations were also used to examine associations between FMD and EV

220   release at different time-points and exercise protocols. Results are presented as

221   mean±SD, and significance was set at $P<0.05$.

222　**Results**

223　Subjects' characteristics are shown in Table 1. Targeted HR (HIIE: 90% HRR, MICE:

224　70% HRR), and rate of perceived exertion (RPE) were achieved during all exercise

225　bouts (Table 1).

226

227　**Impact of exercise intensity on FMD**

228　There was no difference in baseline FMD between exercise protocols (P=0.497)

229　(Figure 1). There was a significant main effect of time (P=0.003), showing increased

230　FMD at 10 (P=0.001) and 60 min (P=0.014) but not at 120 min post-exercise. There

231　was a significant interaction (time*exercise protocol, P=0.017), indicating a different

232　time-course between exercise protocols (Figure 1). FMD was higher at 10 min post-

233　HIIE (P<0.001) returning to baseline by 60 min. Following MICE, FMD increased at 60

234　min (P=0.038) and then return to baseline (P=0.929). However, the magnitude of the

235　FMD increase was not different between the exercise protocols (P=0.598).

236

237　There was a significant main effect of time for baseline artery diameter (P=0.015),

238　peak artery diameter (P=0.001), SRAUC (P<0.001) and time to peak (P<0.001), with

239　all reporting significantly higher values at 10 min post-exercise, when compared to

240　baseline, 60 and 120 min post-exercise (P<0.05). However, these were not

241　significantly different between exercise protocols and there was no interaction (Table

242　2).

243

244　**Impact of exercise intensity on EVs**

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

246   _Endothelial cell-derived EVs (CD62E⁺):_ Whilst there was no significant difference in

247   baseline CD62E$^+$ between HIIE and MICE (P=0.069), there was a main effect of

248   exercise protocol (P=0.047), suggesting overall higher endothelial cell-derived EVs

249   release in MICE when compared to HIIE. However, there was no main effect for time

250   or interaction suggesting that there was no change in endothelial cell-derived EVs

251   following HIIE and MICE (Figure 1b).

252

253   _Platelet-derived EVs (CD41a⁺):_ There was no difference in baseline CD41a$^+$ between

254   HIIE and MICE (P=0.081). There was a main effect of time (P=0.013). Post-hoc

255   analysis revealed a lower concentration at 60 min post-exercise compared to baseline

256   (P=0.040) and 10 min post-exercise (P=0.003), with further decreases from 10 min at

257   120 min (P=0.023). A main effect of exercise protocol was observed with a higher

258   concentration in MICE compared to HIIE (P=0.017). However, there was no interaction

259   (time*exercise protocol; P=0.635), suggesting that platelet-derived EVs follow the

260   same pattern following both exercise protocols (Figure 1c).

261

262   **Associations between FMD and EVs**

263   FMD was not related to either endothelial cell- (CD62E$^+$) or platelet-derived (CD41a$^+$)

264   EV release when compared to the same time-point post-HIIE or post-MICE, including

265   baseline (P>0.05). Similarly, FMD change (Dchange; percentage change from

266   Baseline), following HIIE or MICE was not associated with the Dchange in either

267   endothelial cell- or platelet-derived EV release, in either of time-points (P>0.05) (Table

268   3).

269

270   **Discussion**

271   The primary aim of this study was to determine if 'typical' exercise sessions of HIIE or

272   MICE result in altered vascular function and if the change was associated with

273   endothelial- and platelet-derived EVs in healthy young males. Contrary to our

274   hypothesis, both MICE and HIIE resulted in transient increases in endothelial function

275   which were unrelated to endothelial- or platelet-derived EV. Our data suggest that both

276   HIIE and MICE can be undertaken safely, at least in apparently healthy yet inactive

277   individuals, without a detriment to vascular function.

278

279   **<u>Endothelial function following different exercise protocols</u>**

280   Both MICE and HIIE resulted in similar increases in FMD, but with an earlier increase

281   with HIIE. Previous work demonstrating a transient decrease in FMD with high-

282   intensity exercise may be driven by higher retrograde shear rate (Tinken et al. 2009)

283   and/or oxidative stress, both of which reduce NO-bioavailability and thus cause

284   endothelial dysfunction (Green et al. 2017). Furthermore, the multiple transitions

285   between 'work' and 'recovery' during HIIE increase retrograde shear stress when

286   compared to moderate intensity exercise (Lyall et al. 2019; McManus et al. 2019).

287   However, whilst retrograde shear rate is typically associated with decreased FMD, this

288   periodic high-and-low retrograde shear stress during HIIE may offer some prophylactic

289   effects on the vasculature (Cheng et al. 2019; Ghardashi Afousi et al. 2018; Holder et

290   al. 2019; Iwamoto et al. 2018). In addition, whilst retrograde flow increases, there is

291   also a large increase in antegrade shear rate compared to MICE (Iwamoto et al. 2018;

292   McManus et al. 2019), which has been associated with increased FMD (Green et al.

293   2017; Tinken et al. 2009). In the current study the increased, as opposed to decreased,

294   endothelial function post-exercise would suggest that this exercise mode may induce

295   only low levels of oxidative stress and/or that the beneficial effect of anterograde shear

296   rate and endothelial nitric oxide synthase (eNOS) activation (Casey et al. 2017; Cocks

297   et al. 2013; Tryfonos et al. 2022) overcomes oxidative stress and retrograde shear

298   resulting in improved FMD in healthy young males.

299

300   The magnitude of increases in FMD in this study are similar to previous work

301   comparing HIIE vs MICE (Currie et al. 2012; Lyall et al. 2019). However, both studies

302   reported a similar time-course in FMD following HIIE and MICE, which is in contrast of

303   our evidence. Difference may be due to matching exercise intensities and durations

304   between HIIE and MICE (Lyall et al. 2019) or differences in clinical versus healthy

305   populations (Currie et al. 2012). As such, this delayed response in MICE observed in

306   the current study may be related to a larger exercise dose and associated oxidative

307   stress in MICE (Johnson et al. 2012), opposing the early shear-mediated

308   improvements (Tinken et al. 2009).

309

310   **Exercise and EV release**

311   Although exercise-induced vascular adaptions are largely mediated by hemodynamic

312   forces (i.e. elevated anterograde shear stress) (Green et al. 2017), systemic circulating

313   factors (Padilla et al. 2011), including EV release (Han et al. 2021; Wilhelm et al. 2018),

314   may be involved, predominantly due to their role in intracellular communication. In line

315   with previous studies in healthy males after low intensity exercise (Rakobowchuk et

316   al. 2017), MICE (HIGHTON et al. 2019; Wilhelm et al. 2016), or HIIE (Sapp et al. 2019),

317   endothelial cell-derived EVs (CD62E^{+}) remained unchanged. However, earlier work

318   reported increases in endothelial cell-derived EV release (CD62E^{+} or CD105^{+} following

319   MICE (Lansford et al. 2016) or HIIE (Kirk et al. 2014). Of particular interest was Shill

320 et al., 2018 (Shill et al. 2018) study that compared endothelial cell-derived EV release

321 (CD62E$^+$) in the same cohort (young healthy males and females) following similar

322 exercise protocols as our study and reported a decrease following MICE (65% VO$_{2max}$,

323 matched time and energy expenditure of HIIE) but not HIIE (10x1min intervals-@-90%

324 and 100% VO$_{2max}$). Unchanged or decreased endothelial cell-derived EV release in

325 response to exercise may be a consequence of the beneficial effects of exercise-

326 induced shear stress counteracting other factors such as cytokines (Jimenez et al.

327 2003). Alternatively, endothelial cells may uptake endothelial cell-derived EVs (Ayers

328 et al. 2015) or release and clearance may occur simultaneously during exercise,

329 masking any increased production (Jimenez et al. 2003; Sapp et al. 2019). Whilst

330 endothelial-derived EV release is typically unchanged/decreased and likely unaffected

331 by exercise intensity, there is large variability within and between studies so further

332 work is needed to elucidate responses to acute exercise.

333

334 Platelet-derived EVs (CD41a$^+$) were significantly reduced 60 min post-exercise,

335 independent of exercise mode. This reduction following exercise was unexpected, as

336 previous research demonstrates an increase at 5-120 mins (Chaar et al. 2011; Hilberg

337 et al. 2008; Maruyama et al. 2012; Rakobowchuk et al. 2017; Sossdorf et al. 2010;

338 Wilhelm et al. 2016), or no change (Durrer et al. 2015; Lansford et al. 2016; Shill et al.

339 2018). Exercise-induced platelet-derived EVs may represent an intercellular

340 communication mechanism in the vascular wall, with the reduction representing a

341 cellular signal between platelet-derived EVs and endothelial cells to initiate

342 angiogenesis or vascular remodelling, contributing to exercise-induced vascular

343 adaptations (Ayers et al. 2015; Di Credico et al. 2020). Further work is needed to

344 characterize the response to exercise and determine the functional implications of

345 these findings.

346

Associations between exercise-induced changes in FMD and EV release

348 In line with exercise training responses (Dawson et al. 2021; Green et al. 2014),

349 individual FMD responses to acute exercise were variable. We hypothesised that this

350 may relate to underpinning mechanisms, such as EV release. Only one previous study

351 has explored the relationship between EV release and change in endothelial function

352 in response to acute exercise. Sapp et al., (2019) observed a positive association

353 between the immediate change in endothelial cell-derived EVs (10 min) and change

354 in FMD 1h post-MICE but not HIIE (MICE;30 min continuous exercise-@-60%W_{max}

355 and HIIE;6min-@-40% W_{max} followed by 3min interval-@-85% W_{max} interspersed with

356 4min-@-40 W_{max} matched for total time and workload) in active young males. In

357 contrast, we report no association between change in FMD and EV change following

358 either MICE or HIIE. Given the limited, yet inconsistent evidence regarding the direct

359 relationship between FMD and EV release in response to exercise, further work is

360 required. It is worth noting that standardization of the methods for evaluating EV

361 release is required, including markers, isolation/staining protocols and time-course to

362 allow comparison between studies and to elucidate if it is a key factor in the individual

363 FMD responses following acute exercise (Ayers et al. 2015; Wilhelm et al. 2018).

364

Limitations

366 This study assessed endothelial function and EV release following exercise in young

367 healthy males. The results cannot therefore be compared to other groups including

368 women, older individuals and those with diseases. Although the MICE intensity is

369   'moderate' compared to 90% HRR in our HIIE bout, the majority of previous data is at

370   lower intensities (40-65% VO_{2max}), in order to match exercise dose. Whilst we have

371   not directly measured exercise dose, we estimated the energy expenditure during both

372   exercise bouts, using the known VO_{2peak} (Table 1) demonstrating that MICE may result

373   in larger overall dose than HIIE. However, it is worth noting that the purpose of the

374   study was to examine the acute vascular responses following two commonly used

375   exercise protocols of MICE and HIIE, in the same individuals. For that reason, we used

376   HRR and not $\%HR_{max}$ or $\%VO_{2peak}$, to better capture real-world scenarios. However,

377   given that exercise dose may affect both FMD and EVs, further studies with real-time

378   measurement of energy expenditure during exercise, should evaluate the inter and

379   intra relationships between FMD time-course response, EVs and exercise dose. In

380   regards to the PPP production, although we have used similar techniques as

381   previously described at the time of our date collection, a recent paper (Rikkert et al.

382   2021) suggest that a single higher spin (5000g for 20 minutes) may be more effective

383   in reducing platelets compared to lower spins. Although flow cytometry is one of the

384   most common methods of detection, quantification and size evaluation of larger

385   extracellular vesicles, the sensitively of the most standard flow cytometers (BD Accuri

386   system) (also employed in our study) cannot accurately detect particles below 500nm

387   based on size alone. This raises the possibility that we may have significantly excluded

388   a large number of particles, thus influencing the final result. A further limitation is the

389   use of polystyrene beads for determining the upper limit of detection, as studies have

390   shown that a 1000nm polystyrene particle is not truly reflective of a 1000nm EV, and

391   that a silica bead with a lower refractive index may be more accurate. As shown in the

392   supplementary data online (Figure S2), we compared the FSC SSC parameters of a

393   mixture of polystyrene and silica beads and demonstrated that our upper size limit,

394   previously established as 1000nm based on polystyrene bead, may indeed by more

395   like 1500nm silica bead/EV. Hence, potentially counting particles greater than 1000nm

396   in the analysis. In addition to size, we also used phosphatidylserine (PS) positivity to

397   define our EV population of interest, as large EVs generally expose PS on their outer

398   leaflet. However, there is also evidence to suggest that EVs can also be PS negative,

399   which may have been omitted from our analysis, and if evaluated, may have produced

400   different findings. Finally, there was a high day-to-day variability in regards to baseline

401   EV levels. Biomarker baseline variability can be attributed to many factors, either

402   biological or technical. With this in mind, biomarker analysis is often presented as a

403   fold change above baseline for each time point to account for this rather than absolute

404   levels. As such, in our study we have examined the time course rather than a snapshot

405   and we calculated the exercise response as fold change from the baseline (as

406   measured on each day) to overcome this limitation. To conclude, given that EV release

407   may significantly varied due to the techniques used, we believe that efforts should be

408   made to develop a consensus guideline in regards to EV analysis, including PPP

409   production.

410

411   **<u>Conclusion</u>**

412   Acute exercise resulted in similar improvements in endothelial function, but with

413   different time-course following MICE or HIIE exercise bouts in young healthy males.

414   The individual changes in FMD were not associated with platelet- or endothelial cell-

415   EV release. Although several studies have previously investigated the effect of acute

416   exercise in endothelial function in healthy individuals, the conflicting data necessitates

417   the need for more 'mechanistic' studies to develop our understanding in regards to the

418   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.

Data availability statement

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

439   **Figures legends**

440   **Figure 1. Endothelial function assessed via flow-mediated dilation (FMD) and EV**

441   **release prior to exercise (Baseline) and 10, 60 and 120 min post-exercise.**

442   FMD **(a)** following high-intensity-interval exercise (HIIE) and moderate-intensity-

443   continuous exercise (MICE) (mean±SD; n=18). **(b)** Endothelial cell-derived

444   extracellular vesicles **(CD62E⁺)** in HIIE and MICE (mean±SD; n=14). **(c)** Platelet-

445   derived extracellular vesicles (**CD41a⁺)** in HIIE MICE (mean±SD; n=14). A mixed-

446   linear model (time*exercise protocol), covariating baseline diameter (FMD only), and

447   Bonferroni correction for post-hoc pairwise comparisons was used. *Significantly

448   different from Baseline (*P*<0.05), #Significantly different from HIIE (P<0.05).

449

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672

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)

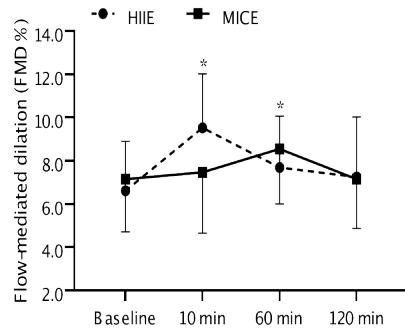|		Baseline diameter (mm)	Peak diameter (mm)	Time to peak (s)	SRAUC $(s^{-1}\ 10^3)$
Baseline	HIIE	4.00±0.47	4.27±0.47	61.0±31.7	19.4±8.4
	MICE	4.05±0.51	4.34±0.54	52.3±24.4	19.1±9.0
10 min*	HIIE	4.21±0.31	4.58±0.34	80.9±19.2	29.0±15.3
	MICE	4.15±0.55	4.44±0.55	78.9±22.5	28.8±9.5
60 min	HIIE	4.14±0.54	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).

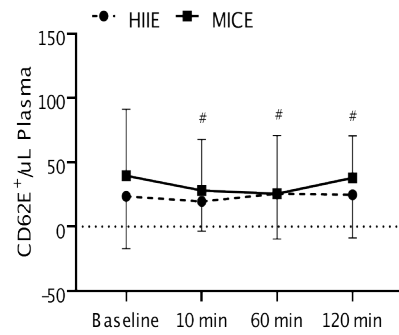|	Baseline	10 min	60 min	120 min
HIIE				
a) Absolute				
FMD - CD62E$^+$	R=-0.447, P=0.109	R=0.122, P=0.737	R=-0.102, P=0.779	R=-0.216, P=0.549
FMD - CD41a$^+$	R=-0.032, P=0.918	R=0.415, P=0.233	R=-0.249, P=0.371	R=0.009, P=0.979
b) Dchange				
FMD - CD62E$^+$		R=-0.94, P=0.795	R=-0.263, P=0.462	R=0.123, 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,	R=-0.046,	R=-0.211,
	P=0.871	P=0.899	P=0.559
FMD - CD41a$^+$	R=-0.323,	R=-0.354,	R=-0.064,
	P=0.282	P=0.236	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

(a)

(b)

(c)