1 Exercise intensity modulates the appearance of circulating microvesicles with pro-angiogenic
2 potential upon endothelial cells

3 Eurico N. Wilhelm[1], José González-Alonso[1], Christopher Parris[2], Mark Rakobowchuk[3]

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5 [1]Centre for Human Performance, Exercise and Rehabilitation, College of Health and Life Sciences,
6 Brunel University London, Uxbridge, UK

7 [2]Institute for the Environment, Health and Societies, Brunel University London, Uxbridge, UK

8 [3]Department of Biological Sciences, Faculty of Science, Thompson Rivers University Kamloops, British
9 Columbia, Canada

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12 Running title: Circulating microvesicles, exercise and endothelial cells

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14 Page number: 26

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16 Corresponding Author:

17 Mark Rakobowchuk, PhD

18 Department of Biological Sciences

19 Faculty of Science

20 Thompson Rivers University

21 900 McGill Road, Kamloops, British Columbia, Canada, V2C 0C3

22 mrakobowchuk@tru.ca

Abstract

The effect of endurance exercise on circulating microvesicle dynamics and their impact upon surrounding endothelial cells is unclear. Here we tested the hypothesis that exercise intensity modulates the time-course of platelet (PMV) and endothelial-derived (EMV) microvesicle appearance in the circulation through haemodynamic and biochemical-related mechanisms, and that microvesicles formed during exercise would stimulate endothelial angiogenesis *in vitro*. Nine healthy young men had venous blood samples taken prior, during and throughout the recovery period after 1 h of moderate (46 ± 2% $\dot{V}O_2$max) or heavy (67 ± 2% $\dot{V}O_2$max) intensity semi-recumbent cycling and a time matched resting control trial. *In vitro* experiments were performed by incubating endothelial cells with rest and exercise-derived microvesicles to examine their effects on cell angiogenic capacities. PMVs ($CD41^+$) increased from baseline only during heavy exercise (from 21 ± 1 x 10^3 to 55 ± 8 x 10^3 and 48 ± 6 x 10^3 PMV / µl at 30 and 60 min, respectively; $P < 0.05$), returning to baseline early in post-exercise recovery ($P > 0.05$), whereas EMVs ($CD62E^+$) were unchanged ($P > 0.05$). PMVs were related to brachial artery shear rate ($r^2 = 0.43$) and plasma noradrenaline concentrations ($r^2 = 0.21$) during exercise ($P < 0.05$). Exercise-derived microvesicles enhanced endothelial proliferation, migration and tubule formation compared to rest microvesicles ($P < 0.05$). These results demonstrate substantial increases in circulating PMVs during heavy exercise and that exercise-derived microvesicles stimulate human endothelial cells by enhancing angiogenesis and proliferation. This involvement of microvesicles may be considered a novel mechanism through which exercise mediates vascular healing and adaptation.

Keywords: platelet microvesicles; microparticles; shear stress; human umbilical vein endothelial cell; angiogenesis

45 **News and Noteworthy**

46 Increases in intravascular [PMV] occur during exercise but this depends on exercise intensity, and
47 correlates with elevations in vascular shear stress and plasma [noradrenaline]. Circulating
48 microvesicles isolated from exercising humans display pro-angiogenic potential upon cultured
49 endothelial cells. Thus, it is possible that microvesicles are involved in vascular responses to exercise.

50 **List of abbreviations**

51 CON, rest control trial; EMV, endothelial-derived microvesicle; eNOS, endothelial nitric oxide
52 synthase; exMVs, circulating exercise microvesicles; HI, heavy intensity trial; HUVECs, human
53 umbilical vein endothelial cells; MI, moderate intensity trial; MTT, thiazol blue tetrazolium bromide;
54 PMV, platelet-derived microvesicle; PPP, platelet poor plasma; rMVs, circulating rest microvesicles;
55 $\dot{Q}$, cardiac output; SR, shear rate; SV, stroke volume; $\dot{V}CO_2$, carbon dioxide output; $\dot{V}O_2$, oxygen
56 uptake; $\dot{V}O_2max$, maximal oxygen uptake; VT1, first ventilatory threshold.

57 **Introduction**

58 Intraluminal shear stress is a key modulator of endothelial nitric oxide synthase (eNOS) activity and
59 expression in arteries (45, 61), and has been identified as a major stimulus leading to the exercise-
60 induced improvement in vascular function (4, 13, 52, 53), but endothelial adaptations also take place
61 at sites not directly exposed to increased shear forces and acute changes in circulating factors are
62 believed to be involved in such adaptations (43). However, the mechanisms by which exercise bring
63 about endothelial adaptations not directly dependent on shear stress are not fully understood and
64 may relate to a variety of still unidentified factors.

65 Cell-derived microvesicles have been identified as potential biomarkers linked to obesity (19),
66 dyslipidaemia (1), and coronary artery disease (3, 25). In addition, increased concentrations of
67 several microvesicle varieties are found in the blood post exercise (11, 31, 34, 48, 49). As small
68 membrane-derived vesicles (diameter < 1 μm), their phenotype depends on their cell of origin and
69 stimulus of formation (29, 47). Platelet-derived microvesicles (PMVs) are the most abundant
70 circulating microvesicle population (24, 48, 49), and *in vitro* stimulation of platelets with thrombin
71 (7), noradrenaline (54), IL-6 (42), and shear stress (39, 44) induce shedding of PMVs, whereas high
72 shear stress downregulates the shedding of endothelial microvesicles (EMVs) from cultured
73 endothelial cells (56).

74 Plasma PMV appearance occurs during recovery from maximal (11) and submaximal (34, 36, 48, 49)
75 exercise protocols, whereas a less pronounced increase or completely absent alteration in plasma
76 EMV (31, 34, 48, 49), erythrocyte (11) and monocyte microvesicles (11, 48, 49) has been observed.
77 The dynamics of PMV during exercise, however, is unknown and the impact of exercise intensity on
78 circulating microvesicle appearance has not been explored. Several intravascular factors known to
79 stimulate *in vitro* PMV and EMV production (29, 39, 44, 54) are modulated by exercise intensity.
80 Because vascular shear stress is expected to increase with workrate, and elevated shear forces are
81 strong agonists stimulating *ex vivo* microvesicle blebbing from platelets, we anticipated that PMVs
82 would increase during exercise according to exercise intensity, whereas EMV release would be
83 restricted by elevated shear stress (56).

84 Beyond being simple inert plasma membrane fragments, microvesicles are now recognised as
85 bioactive blood constituents capable of interacting with diverse cell populations, including the
86 vascular endothelium (47). Certain EMVs isolated from cell culture exhibit paracrine effects;
87 stimulating inflammation, increasing superoxide anion production while decreasing nitric oxide
88 bioavailability in recipient endothelial cells (8, 14). Depressed endothelial function has also been

89 reported in arteries exposed to circulating microvesicles from cardiac patients and pre-eclamptic
90 women, but not from apparently healthy donors (6, 55). In contrast, PMVs may protect endothelial
91 cells from apoptosis (30) and stimulate angiogenesis *in vitro* (7, 30) and *in vivo* (7). The physiological
92 relevance of microvesicles produced with exercise upon the vascular endothelium, however, has not
93 been explored but based on previous research we postulated that microvesicles formed during
94 exercise bouts could be involved in vascular adaptations, leading to a proliferative and angiogenic
95 endothelial phenotype.

96 Therefore, we aimed (1) to characterize the impact of modulating exercise intensity on the time-
97 course of PMV and EMV concentrations during exercise and recovery; (2) to examine putative
98 relationships between shear stress and microvesicle formation during exercise *in vivo*; (3) and to
99 investigate the potential impact of circulating microvesicles produced during exercise on
100 angiogenesis, proliferation, migration, of human endothelial cells. We hypothesised that PMVs, but
101 not EMVs, would increase during exercise with intensity, and remain elevated up to 1 h post-
102 exercise; that the increase in PMVs would be associated with shear stress during exercise; and that
103 exercise-derived circulating microvesicles would stimulate endothelial cell proliferation, migration
104 and angiogenesis *in vitro* to a greater extent than those isolated at rest.

105

106 **Methods**

107 *Ethical approval*

108 Written informed consent was obtained from all participants prior taking part in the experiments.
109 Human and cell culture experimental procedures were approved by Brunel University London
110 Research Ethics Committee (RE55-12 and RE39-13), and experiments were performed in accordance
111 with the Declaration of Helsinki.

112 *Participants*

113 Nine healthy young participants (25 ± 1 yr, 1.79 ± 0.03 m, 80.5 ± 4 kg, $\dot{V}O_2$max 3.3 ± 0.2 l / min[1])
114 completed all five visits of this study. Volunteers were young lean male non-smokers, who were free
115 from cardiorespiratory and metabolic diseases, and mostly involved in recreational physical activities
116 (self-reported). Microvesicles used in the cell culture experiments were obtained from samples
117 collected from a subset of this group (n = 6; 24 ± 1 years, 1.79 ± 0.04 m, 82.5 ± 5.9 kg, $\dot{V}O_2$max 3.3 ±
118 0.3 l / min).

119 *Experimental design*

120 Two experiments were performed to (1) elucidate the time-course of microvesicle appearance in the
121 circulation in response two intensities of dynamic exercise, while gaining insights about the potential
122 mechanisms involved with this response *in vivo*; and to (2) investigate the impact of circulating
123 microvesicles produced during exercise upon endothelial proliferation, chemotaxis, and
124 angiogenesis *in vitro*.

125 All participants attended the laboratory for 2 preliminary visits, followed by 3 experimental trials
126 during which exercise or resting control experiments were performed in a thermoneutral
127 environment. Participants were familiarised with the experimental procedures, the semi-recumbent
128 cycle ergometer (Angio, Lode, Netherlands) and the maximal incremental exercise test at the first
129 visit to minimise possible learning effects. After at least 48 h of recovery, participants had their
130 oxygen uptake ($\dot{V}O_2$), CO_2 output ($\dot{V}CO_2$) and derived variables determined using a ramp incremental
131 test with continuous collection of expired gases using open-circuit spirometry (Quark B^2, Cosmed,
132 Italy). Briefly, the test started with participants cycling at 80 rpm at a power output of 25 W with a
133 ramp slope of 25 W / min. They cycled continuously until the limit of exercise tolerance, and a
134 confirmatory step test to exhaustion was performed after 5 min of recovery at a relative intensity 5%
135 above their ramp peak power output (46). Data were analysed using 10 s moving average windows
136 and the maximal oxygen uptake ($\dot{V}O_2max$) was determined as the highest $\dot{V}O_2$ obtained during the
137 incremental test. The first ventilatory threshold (VT1) was determined using a two criteria
138 confirmation method, comprising the first deflection point in the $\dot{V}O_2$–$\dot{V}CO_2$ curve, and the point
139 when the ventilatory equivalent of O_2 increased without a concomitant rise in the respective CO_2
140 equivalent (9).

141 The last three visits were the experimental trials. Participants arrived at the laboratory in the
142 morning, approximately 1.5 h after a light breakfast and rested for approximately 30 min during
143 instrumentation. The first measurement took place approximately 20 min after cannulation. During
144 the resting control trial (CON) participants remained in a semi-recumbent position throughout the
145 protocol (240 min) while blood samples were obtained at baseline, 30, 60, 80, 100, 120, 150, 180
146 and 240 min of the protocol. Haemodynamic measurements were obtained at similar time-points.
147 Exercise trials were performed in random order. For the moderate intensity exercise trial (MI), 60
148 min of cycling at 80% of the workrate relative to VT1 (*i.e.* 46 ± 2% of $\dot{V}O_2max$) was performed
149 followed by 180 min of rest (recovery period), whereas during the heavy exercise intensity trial (HI)
150 participants cycled at 30% of the difference between the workrate relative to their individual VT1
151 and $\dot{V}O_2max$ (*i.e.* 67 ± 2% of their $\dot{V}O_2max$). The delta method was selected for the HI protocol
152 because it reduces the variability of inter individual physiological responses (35), and the

153 classification of exercise domains as moderate and heavy intensity was based on previous work (60).
154 The three experimental trials were separated by at least one week to reduce potential cumulative
155 effects of blood sampling.

156 The second set of experiments investigated the impact of exercise-derived microvesicles on
157 endothelial cells. Culture studies were performed by incubating human umbilical vein endothelial
158 cells (HUVECs) with circulating microvesicles obtained from a subset of the previous experimental
159 group at rest (rMVs) or during heavy exercise (exMVs). Endothelial cell capacity to migrate,
160 proliferate, repair a disturbed monolayer, and form tubule-like structures *in vitro* was assessed.
161 Experiments were performed by supplementing the experimental media with microvesicles. FBS or
162 VEGF was used as positive control, and the supernatant of microvesicle pellets (microvesicle free
163 plasma) was used as an internal control.

164 *Human experiments*

165 *Catheter placement and blood sample storage*

166 Venous blood samples were taken during the CON, MI and HI trials. Upon arrival, participants rested
167 in the supine position in a quiet room and an 18 gauge cannula (BD Venflon, Becton, Dickson and
168 Company, USA) was inserted into a superficial antecubital vein of the arm. A 0.9% NaCl solution (BD
169 PosiFlush, Becton, Dickson and Company, USA) was flushed through the cannula to maintain patency
170 following each blood draw. Samples were obtained without venous stasis and the first 3 ml of blood
171 were discarded. Collected blood was immediately mixed in tubes containing 0.129 mol / l sodium
172 citrate (Sigma-Aldrich, USA) or 8 mg of EDTA (Sarstedt, Germany). Hct, Hb, and lactate were
173 determined in whole blood samples. Platelet rich plasma was obtained by centrifugation at 300 x g
174 at 4°C for 10 min. EDTA samples were aliquoted in tubes and stored at -80 °C. Sodium citrate plasma
175 underwent a second centrifugation step at 15,000 x g at 4°C for 10 min to obtain platelet poor
176 plasma (PPP). Aliquots were stored at -80 °C for microvesicle quantification or further cell culture
177 experiments.

178 *Systemic and Limb Haemodynamics*

179 Arterial blood pressure and heart rate (HR) were recorded continuously using a Finometer® Pro
180 device (Finapress Medical Systems, Netherlands) throughout the 3 experimental trials. Participant's
181 stroke volume (SV) was determined from apical four chamber echocardiography (phased array
182 probe, Vivid 7, GE, UK). Measurements were obtained with participants in the semidecubitus
183 position on their left side, which was accomplished by tilting the recumbent cycle ergometer. End
184 diastolic and end systolic left ventricular areas were determined using EchoPac software (version

185 112, GE, UK) and volumes calculated using the single plane Simpson's method (33). At least three
186 heart cycles were selected for analysis. Cardiac output ($\dot{Q}$) was calculated as SV multiplied by the
187 corresponding HR. $\dot{Q}$ as well as SV were estimated from the Finometer® Pro in situations where the
188 echocardiographic recordings could not be obtained or were of low quality. $\dot{Q}$ of 5 participants
189 obtained during diverse resting and exercise conditions displayed a moderate agreement between
190 echocardiographic and Modelflow methods, with the mean difference of -0.5 l / min not being
191 different from 0 (single sample T test $P > 0.05$; 95% confidence intervals: 4.1 to -5.2), and no
192 proportional bias ($P > 0.05$).

193 Upper and lower limb haemodynamics were assessed by vascular ultrasonography with a multi-
194 frequency linear array transducer (Vivid 7 ultrasound, GE, UK) to examine the relationship between
195 haemodynamic changes and circulating microvesicle concentrations. The left brachial artery was
196 assessed at rest and during leg exercise. Blood flow was calculated as the product of $V_{mean} \times \pi \times (D^2$
197 $/ 4) \times 60$; and vascular shear rate (SR) was calculated as $4 \times V_{mean} / D$, where Vmean refers to
198 weighted time averaged mean Doppler velocity, and D refers to vessel diameter (4, 53). The
199 common femoral artery was assessed at baseline and throughout recovery, but it could not be
200 assessed during exercise due to the dynamic nature of cycling. Hence, leg blood flow during exercise
201 was estimated assuming two leg blood flow = exercise $\dot{Q}$ - (resting $\dot{Q}$ + exercise two arm blood flow).

202 *Microvesicle quantification*

203 Platelet poor plasma samples were thawed at room temperature and Fc receptors were blocked
204 with Human TruStain FcX™ (BioLegend, USA) for 10 min. Samples were incubated in the dark for 25
205 min with PE conjugated anti-human CD62E (E-selectin; BioLegend, USA; at 18 µg / ml), and PE/Cy5
206 anti-human CD41 (integrin α2b; BioLegend, USA; at 2.04 µg / ml) fluorescent antibodies. Samples
207 were diluted 1:40 in 0.2 µm filtered PBS and centrifuged for 15 min at 17,960 x g and 4°C. The
208 microvesicle pellet was resuspended in PBS. Microvesicle concentration was quantified using an
209 ImageStream^{x}® Mark II imaging flow cytometer (Amnis Corporation, USA) using INSPIRE software
210 (Amnis Corporation, USA), with 60x magnification. All events were collected and sample acquisition
211 was limited to no less than 1,000 events in the expected PMV gate. The *a priori* choice for PMV gate
212 relied on previous evidence indicating that PMVs are more responsive to an exercise stimulus than
213 EMVs. Single-stained samples were used to create a multi-staining compensation matrix.

214 Data were analysed off-line with IDEAS software (version 6.1, Amnis Corporation, USA) using an
215 adapted microvesicle gating strategy described by others (24). Briefly, after compensation,
216 microvesicles were determined as events below 1 µm diameter using calibration beads (Fluoresbrite,

217 Polysciences, USA), and displayed very low side scatter and moderate mean fluorescence intensity
218 for PE/Cy5 (PMVs), or PE (EMVs) (Figure 1) (24). A threshold for positive events was set as adapted
219 from the fluorescence minus one procedure.

220 *Plasma noradrenaline and interleukin-6 quantification*

221 Plasma noradrenaline and IL-6 concentration were measured in EDTA samples by commercial ELISA
222 kits (Noradrenalin ELISA, IBL International, Germany; Human IL-6 Quantikine, R&D Systems, USA).
223 Samples were analysed in duplicate according to the manufacturer's instructions. The mean
224 coefficient of variation between duplicates was 9.7% for noradrenaline and 6% for IL-6.

225 *Whole blood haematocrit, haemoglobin, lactate, and plasma volume corrections*

226 One ml of blood was immediately separated to determine Hct, Hb, and whole blood lactate
227 concentrations. Hct was determined by packed cell volume method after standard centrifugation of
228 sodium-heparinized capillary tubes (micro-haematocrit tubes, HaematoSpin 1400 centrifuge,
229 Hawksley, UK). Blood Hb concentration was obtained by photometric analysis (HemoCue® Hb 201+
230 System, HemoCue AB, Sweden), and lactate concentration was determined using a Biosen C-line
231 analyser (EKF Diagnostics, UK) after daily calibration. Plasma volume shifts were calculated as
232 described previously (17).

233 *Cell culture experiments*

234 *Culture of human umbilical vein endothelial cells*

235 HUVECs were cultivated in culture flasks (T75, Sarstedt, Germany) in growth medium (medium 199
236 supplemented with 20% FBS, 15 µg / ml endothelial cell growth supplement, 5 U / ml of heparin, 100
237 U / ml - 100 µg / ml of penicillin-streptomycin, 0.6 mg / ml pyruvate, and 20 mM HEPES), at 37°C in
238 5% CO_2, with half medium changes every 48 – 72 h. Cells were removed from wells with trypsin
239 when monolayers became 60 – 80% confluent for passaging or experiments. Experiments were
240 performed with cells between passage 3 – 6.

241 *Microvesicle experimental medium preparation*

242 On the day of the experiments, PPP vials were thawed at room temperature, diluted 1:1 in PBS, and
243 isolated by centrifugation for 1 h at 17,500 g at 4 °C as adapted from others (6, 55). Experimental
244 media were prepared by suspending the microvesicle pellet to its original concentration in FBS free
245 growth medium for migration and wound-healing assays, or to double the original microvesicle
246 concentration in medium 199 supplemented with penicillin-streptomycin and heparin for

247 proliferation or tubule formation assays (diluted to the original concentration in subsequent steps of
248 those corresponding assays). Thus, microvesicles were administered at their physiological
249 concentrations determined in plasma samples at baseline (*e.g.* 24.1 x 10^3 PMV / µl (25.9, 20.0), and
250 12.4 x 10^3 EMV / µl (14.3, 10.6); median (96% confidence intervals), n = 5 per assay) or during
251 exercise (*e.g.* 49.9 x 10^3 PMV / µl (63.7, 41.8), and 12.7 x 10^3 EMV / µl (14.3, 10.5); median (96%
252 confidence intervals), n = 5 per assay). The plasma supernatant obtained after centrifugation was
253 used to make separate aliquots of microvesicle free experimental media. Results from a pilot study
254 confirmed very low concentrations of microvesicles in these plasma supernatant samples (*i.e.* 9 ± 2%
255 positive microvesicle events in comparison to the microvesicle pellet suspension; n = 4). Hence,
256 microvesicle free plasma experimental medium was used as an internal control in the current
257 experiments.

258 *Proliferation assay*

259 Proliferation of HUVECs was quantified using the thiazol blue tetrazolium bromide (MTT) assay in
260 0.5% gelatin pre-coated 96 well plates. A total of 50 µl of the cell suspension (2,000 cells) was
261 seeded in each well followed by the addition of 50 µl of experimental medium. Experimental
262 medium with 20% FBS served as positive controls. After 48 h each well was incubated with the MTT
263 solution for a further 4 h. All but 30 µl was removed and the wells were incubated for 10 min in
264 DMSO before absorbance analyses were obtained at 540 nm using a micro plate reader (ELx808,
265 BioTek Instruments, USA). The average of quadruplicates was calculated and expressed as a
266 percentage of the negative control condition.

267 *Migration assay*

268 Migration of HUVECs toward microvesicle containing wells was determined using a modified 48 well
269 Boyden chamber (AP48, Neuro Probe, USA). Experimental medium (rMVs, exMVs, or microvesicle
270 free supernatant) was loaded into lower chamber wells to form a slight meniscus, then a pre-coated
271 gelatin polycarbonate filter was positioned over the lower chamber. Wells loaded with 10% FBS
272 served as positive controls. The upper chamber was assembled and loaded with 50 µl of a
273 suspension of serum starved cells in microvesicle free medium (25,000 cells), and incubated for 4 h.
274 Adherent cells on the underside of the filter were fixed with methanol and stained with Giemsa.
275 Three images of each well acquired at random locations were obtained with an Axioskop 2
276 microscope (Zeiss, Germany) at 20x objective magnification, and the number of migrated cells were
277 counted with ImageJ software (version 1.48, National Institutes of Health, USA). The average of
278 triplicates was expressed as a percentage of positive controls.

279 *Scratch wound-healing assay*

280 The scratch wound-healing assay was performed to investigate the impact of microvesicles on
281 HUVECs undergoing the repair process. Cells were seeded on gelatin pre-coated 96 well plates and
282 incubated at 37 °C in 5% CO_2 until cells became almost confluent. On the experimental day and after
283 4 h of serum starvation, a scratch was made by disturbing the cell monolayer with a 200 µl pipette
284 tip. All medium was aspirated and replaced with experimental medium. Medium supplemented with
285 20% FBS served as positive control. Images were obtained at baseline and after 3.5 h of incubation
286 using an inverted microscope (Axiovert 200M, Zeiss, Germany) at 10x objective magnification. The
287 cell free area was measured using ImageJ software (version 1.48, National Institutes of Health, USA),
288 and the wound closure was calculated by subtracting the cell free area obtained post incubation
289 from the baseline measurement. The average of quadruplicates for each condition was expressed as
290 a percentage of the wound closure observed in the negative control condition.

291 *Tubule formation assay*

292 The angiogenic potential of microvesicles was assessed by a tubule formation assay. Briefly, 96 well
293 plates were coated with basement membrane protein gel (Geltrex®, Thermo Fisher Scientific, UK) on
294 ice at 78 µl per cm^2. The gel was gently spread with combitip inserts, and the plate was incubated at
295 37 °C to allow the gel to solidify (20). Serum starved cells were suspended in experimental medium
296 199 (with penicillin-streptomycin and 0.5% FBS), and 50 µl of cell suspension (10,000 cells) were
297 incubated in Geltrex® pre-coated wells with 50 µl of experimental medium. VEGF (50 ng / ml) was
298 used as positive control. Wells were imaged after 24 h of incubation using an AxioVert microscope
299 (Zeiss, Germany) at 5x objective magnification. The number of tubule-like structures, and branching
300 points of 3 fields of view per well were averaged using ImageJ software (version 1.48, National
301 Institutes of Health, USA), and the average of three wells per condition was expressed as a
302 percentage of the results observed in the negative control condition.

303 *List of materials*

304 Medium 199 (Hyclone; CAT# SH30024.01), modified medium 199 (phenol red, l-glutamine, and
305 sodium hydrogen carbonate free; CAT# M3769), MTT (CAT# M5655), VEGF (CAT# RP-75746) were
306 from Fisher Scientific (UK). Geltrex® (CAT# A1413202) and FBS (CAT# 10270-106) were from Life
307 Technologies (UK). Fc receptor blocking solution (Human Trustain FcX; CAT# 422302), PE anti-human
308 CD62E (CAT#322606) and PE/Cy5 anti-human CD41 antibodies (CAT#303708) were from BioLegend
309 (USA); and combitips used for coating plates for angiogenesis assay were from Eppendorf (0.5 ml,
310 CAT# 12674587, Eppendorf, Germany).

311 *Statistical analysis*

312 All data are presented as mean ± SEM. For the first study, dependent variables over time and
313 conditions were compared by two-way repeated measures ANOVA. If a significant F-ratio was
314 observed for time x condition interactions the Dunnett's test for multiple comparisons was
315 performed to compare within-condition results vs baseline values. Between-condition comparisons
316 across time were performed with repeated measure ANOVA with Bonferroni correction. A within
317 subject repeated measures multiple regression was performed to examine the relationship between
318 circulating microvesicles, haemodynamics, and biochemical variables (5). *In vitro* experiments were
319 compared with one-way repeated measures ANOVA, and differences between conditions were
320 identified by the least significant difference when a significant F ratio was observed.

321 Due to technical issues, data of one individual had to be acquired using a different ultrasound
322 system (ProSound SSD5500, Aloka, Japan) during the exercise trials, with no data recorded on the
323 control day. Due to the completely random nature of the missing data, a mean substitution
324 treatment was applied for the missing data (*i.e.* CON trial), instead of the listwise deletion
325 procedure. In addition, Hb results could not be collected in the MI protocol for one participant
326 because of technical limitations, so a similar mean substitution data treatment was used.

327 General statistical analyses were performed using statistical software (SPSS version 20, IBM, USA),
328 with Dunnett's test derived from GraphPad Prism software (version 5.03, GraphPad Software, USA),
329 and the within subject repeated measures multiple regression calculated with SigmaPlot (version 13,
330 Systat Software, UK), always setting the significance level at $\alpha < 0.05$ for all analyses.

331

332 **Results**

333 *Limb and systemic haemodynamics*

334 Estimated leg blood flow increased during cycling as a function of exercise intensity ($P < 0.05$), and
335 measured leg blood flow remained elevated at 5 min of recovery with more pronounced values after
336 HI exercise ($P < 0.05$) (Figure 2C). Accordingly, early recovery femoral artery mean SR was higher
337 than baseline, with greater values after heavy cycling compared to moderate exercise ($P < 0.05$)
338 (Table 1). This blood flow and SR response occurred mostly through a rise in blood velocity to the
339 lower limbs ($P < 0.05$), with arterial vasodilation (increases in conduit vessel diameter) playing a
340 smaller role (Table 1).

341 During exercise, the brachial artery dilated and mean blood velocity increased ($P < 0.05$) (Table 1),
342 with a resultant rise in arm blood flow during exercise and into early recovery ($P < 0.05$) (Figure 2D).
343 The higher blood flow to upper limbs during heavy compared to moderate exercise was mostly
344 driven by changes in blood velocity, so mean brachial artery SR was augmented during cycling as a
345 function of exercise intensity ($P < 0.05$), and remained elevated at 5 min of heavy exercise recovery
346 ($P < 0.05$), returning to resting values by 20 min.

347 Cardiac output was elevated throughout cycling in proportion with exercise intensity ($P < 0.05$)
348 (Figure 2A) as a result of increases in HR and SV, with corresponding adjustments in left ventricular
349 end-diastolic and end-systolic volume (Table 2). In the first 5 min of recovery after HI exercise, $\dot{Q}$
350 remained higher than baseline and CON ($P < 0.05$), whereas it returned to values similar to rest
351 following the MI protocol ($P > 0.05$). Mean arterial pressure increased during exercise reaching
352 higher values during the HI compared to the MI protocol at 40 min ($P < 0.05$) but quickly returned to
353 baseline at the end of exercise. Mean arterial pressure at 5 min of recovery, however, was lower
354 following the HI compared to MI protocol reflecting a tendency for post-exercise hypotension after
355 prolonged heavy exercise (Figure 2B).

356 *Circulating platelet and endothelial-derived microvesicles at rest and during exercise*

357 PMV and EMV concentrations were stable throughout the 4 h of resting control and did not change
358 with moderate exercise ($P > 0.05$). Similarly, EMV concentrations were unaltered by heavy exercise
359 (Figure 3B). Yet, PMV concentrations increased more than 2-fold by 30 and 60 min of heavy exercise
360 ($P < 0.05$, Figure 3A) after which they decreased. However, a second rise above baseline was evident
361 at 60 min of recovery ($P < 0.05$). The PMV area under the time-concentration curve was larger during
362 HI compared to CON and MI trials (154 ± 16 *vs.* 89 ± 6 and 108 ± 9 x 10^3 PMV x h / µl, respectively) (P
363 < 0.05). There were no differences in the EMV area under the curve between CON (46 ± 3 x 10^3 EMV
364 x h / µl), MI (46 ± 1 x 10^3 EMV x h / µl) and HI trials (48 ± 3 x 10^3 EMV x h / µl). Changes in plasma
365 volume did not affect the increases in PMV during and after heavy exercise (Supplementary data 1),
366 and thus the aforementioned results represent uncorrected values.

367 *Blood variables*

368 Plasma volume was reduced by between 10-13% during exercise ($P < 0.05$), but returned to resting
369 values early in recovery after moderate exercise ($P > 0.05$) (Table 3), with the change in plasma
370 volume still evident 20 min after heavy exercise ($P < 0.05$). Since changes in plasma volume did not
371 affect the overall microvesicle results (Supplementary data 1), data of the remaining blood-derived
372 variables are also presented without correction. Plasma noradrenaline concentration rose from

373 baseline levels during heavy cycling ($P < 0.05$), with higher values observed throughout the heavy
374 exercise period compared to resting control, and at 30 min of exercise compared to moderate
375 cycling ($P < 0.05$) (Table 3). Noradrenaline concentration returned to baseline within the first hour of
376 recovery after heavy exercise remaining stable until the end of recovery ($P > 0.05$). No changes from
377 baseline were observed during resting control or moderate exercise ($P > 0.05$). By the end of 1 h of
378 heavy cycling IL-6 concentration increased compared to moderate exercise and resting control ($P <$
379 0.05) (Table 3) and values remained elevated above baseline throughout recovery. IL-6
380 concentrations also increased from baseline in the CON and MI trials during the 3rd and 4th h of the
381 protocol ($P < 0.05$), so that at the end of the recovery period no differences were observed between
382 protocols.

383 *Relationship between microvesicles, shear rate, and blood-derived variables in vivo*

384 Modest yet significant correlations were found between PMVs, vascular SR and noradrenaline
385 concentrations when all time-points were investigated ($P < 0.05$, Figure 4A to C). Exploratory analysis
386 revealed that PMV concentrations moderately correlate with BA SR ($R^2 = 0.43$; $P < 0.05$; Figure 4E),
387 estimated femoral artery SR ($R^2 = 0.48$; $P < 0.05$; Figure 4F) and, to a lesser degree, noradrenaline
388 levels ($R^2 = 0.21$; $P < 0.05$; Figure 4G) prior and during exercise, but not during baseline and recovery
389 time-points only ($P > 0.05$). PMVs displayed no significant correlation to IL-6 within these conditions
390 ($P > 0.05$).

391 *Circulating microvesicles increase endothelial cell proliferation and migration*

392 HUVECs proliferated almost 50% quicker than the negative control condition when treated with
393 rMVs, and proliferation almost doubled from control with exMVs (Figure 5A), with smaller effects
394 observed with microvesicle free supernatant conditions. The exMV treatment increased HUVEC
395 proliferation in comparison to the rMV and both the resting and exercise supernatants ($P < 0.05$).
396 rMVs also increased HUVEC proliferation in comparison to resting supernatant, but no more than
397 the exercise supernatant condition ($P > 0.05$). No differences in cell proliferation were observed
398 between resting and exercise supernatants ($P > 0.05$). Both microvesicle and supernatant conditions
399 stimulated HUVEC migration as depicted in Figure 5B. The microvesicle treatments, however,
400 induced substantially more endothelial migration when compared with their respective
401 supernatants ($P < 0.05$). A greater number of cells migrated towards wells loaded with exMVs in
402 comparison to rMVs ($P < 0.05$), but no difference was observed between the two microvesicle free
403 supernatant conditions.

404 *Microvesicles improve endothelial scratch wound-healing*

405 HUVECs treated with supernatants displayed an almost 2-fold increase in the rate of repair
406 compared to negative control wells, but no difference was observed between resting and exercise
407 supernatants ($P > 0.05$, Figure 5C). Both microvesicle conditions enhanced the closure rate
408 compared to the supernatant conditions, with rMVs stimulating wound healing to a similar extent to
409 that observed in the FBS positive control wells (*i.e.* nearly 3-fold quicker than the negative control),
410 and this process was even more rapid in the presence of exMVs ($P < 0.05$).

411 *Exercise microvesicles stimulate angiogenesis in vitro*

412 Treatment with exMVs induced greater formation of tubule-like structures in comparison to all other
413 conditions ($P < 0.05$, Figure 6A), with no differences observed amongst rMVs and supernatant
414 treatments ($P > 0.05$). The number of branching points was also increased in endothelial cells treated
415 with exMVs compared to those incubated with microvesicle free supernatant ($P < 0.05$, Figure 6B),
416 although exMVs treatment did not increase the number of branching points in comparison to rMVs
417 ($P > 0.5$).

418 **Discussion**

419 This study investigated the time-course of plasma microvesicles appearance in the venous
420 circulation during and after prolonged cycling to gain insights into the relationships between
421 exercise intensity and potential physiological stimuli inducing microvesicle formation *in vivo,* and to
422 establish the physiological relevance of intravascular microvesicles produced during exercise upon
423 human endothelial cells. We have shown, for the first time, that the increase in circulating PMV
424 concentrations occur not only after, but also during exercise within the heavy intensity exercise
425 domain, with a second peak observed at 1 h of recovery suggesting a biphasic response. In addition,
426 exercise within the moderate exercise domain did not elicit a response. The PMV dynamics during
427 exercise moderately correlate with haemodynamic measurements, suggesting that augmented
428 vascular shear stress may be involved in PMV formation in exercising humans. Moreover, we have
429 taken the first step in understanding the effect of microvesicles produced during exercise on the
430 vascular endothelium by demonstrating that exMVs display a stimulatory effect in cultured
431 endothelial cells, revealing a potential link between intravascular microvesicles and vascular
432 adaptation with exercise.

433 *Time-course of microvesicle appearance with moderate and heavy exercise*

434 Earlier attempts to characterize PMV dynamics to exercise generally agree that platelets introduce
435 microvesicles into the circulation during the recovery period, with peak plasma concentrations
436 occurring somewhere within 1 h after the session (11, 36, 48, 49). However, little attention has been
437 placed upon microvesicle temporal kinetics and no study has explored the PMV dynamics during
438 exercise. Our results show for the first time that elevations in PMV concentrations are not limited to
439 the post-exercise recovery period as elevations occurred as early as 30 min into heavy exercise. The
440 concentrations of PMVs increased ~2-fold from baseline during heavy exercise in the present study,
441 which tended to be higher than the 20 and 40 min post-exercise values but similar to values after 1
442 hour of recovery. The present 60 min recovery data agree with findings of previous studies using
443 traditional flow cytometry in which 2- to 3-fold increases in [PMV] were observed after 1 hour of
444 recovery (11, 48, 49). This increase in PMVs seemed to be related to exercise intensity rather than
445 being a function of the total work done, since PMV concentrations did not increase during moderate
446 exercise even though a greater amount of mechanical work was accomplished by the end of this
447 condition compared to 30 min of heavy cycling (see Supplementary data 2). Moreover, the fact that
448 PMVs increased from baseline and then remained stable from 30 to 60 min during exercise probably
449 indicates a rapid microvesicle turnover, which ought to be confirmed using tracer techniques.
450 Several processes may account for PMV removal as suggested by *in vitro* experiments. Specifically,

451 evidence for endothelial and renal epithelial cell uptake exists (10, 50). Infused endothelial
452 progenitor cell-derived microvesicles were localised in renal endothelial and tubular cells of rats
453 during recovery from renal injury, suggesting the kidney as a microvesicle extractor organ within
454 these conditions (10). Besides the fact that microvesicles, including PMVs and EMVs, have been
455 identified in the urine of healthy humans (57), it is still difficult to assess the fate of microvesicles
456 with exercise from these experiments, even more so when one considers that renal blood flow (and
457 thus microvesicle delivery) is reduced during intense exercise (51).

458 The second rise in PMV concentration observed 1 h after the heavy intensity bout is intriguing whilst
459 still in agreement with authors who note peak PMV values between the end of exercise and 1 h (36,
460 48, 49) or 2 h (11, 48) of recovery. Our biphasic response is a function of the improved temporal
461 resolution of our sampling protocol since previous works did not measure the PMV response during
462 early exercise recovery. Our observations imply a mechanism unrelated to shear rate induces
463 platelet activation after 1 h of exercise and causes an accumulation of PMVs in the circulation
464 comparable to the exercise response. This secondary PMV peak might be related to late platelet
465 recruitment, as exercise may lead not only to platelet activation (12, 15) but also acutely increase
466 platelet concentrations after exercise sessions (15, 27) recruited from various platelet pools
467 including the spleen and bone marrow (18), that then serve as a source of new microvesicles.

468 EMV concentrations remained similar to baseline across all experimental protocols, implying that
469 the two submaximal exercise stimuli had little impact on endothelial vesiculation. Differing from
470 PMVs, microvesicles derived from endothelial cells have already been studied during exercise, with
471 no changes observed during moderate intensity cycling (40). Inconsistent results exist regarding
472 EMV kinetics during recovery, with researchers reporting increases (31, 34, 48), no change (11, 23,
473 40, 49), or even decreases (59) after exercise. For instance, Kirk et al. (2013) reported elevated
474 plasma $CD105^+$ and $CD106^+$ EMVs 1.5 h after supramaximal interval cycling. Endoglin (CD105)
475 expression is known to increase in endothelial cells undergoing angiogenesis (41), whereas the
476 expression of vascular adhesion molecule-1 (CD106) is upregulated in the endothelium under
477 inflammatory stimulation (62), which indicates that the strenuous protocol used by Kirk *et al.* (2013)
478 may have caused endothelial activation. The fact that $CD62E^+$ EMVs were elevated in men in a
479 previous study (34) but not in the current experiment is intriguing and may be related to their
480 slightly higher intensity protocol (~70% $\dot{V}O_2$max on an upright cycle ergometer). In agreement,
481 results from our laboratory indicate that EMVs expressing the same markers increase during
482 strenuous exercise conditions (*i.e.* 80% of peak power output under heat stress - EN Wilhelm, J

483 González-Alonso, ST Chiesa, SJ Trangmar, M Rakobowchuk; unpublished observations), suggesting
484 that higher exercise intensities may be required to induce endothelial vesiculation.

485 *Relationship between platelet microvesicles and putative agonists*

486 The physiological mechanisms eliciting PMV formation during exercise have not been elucidated,
487 and based on previous *in vitro* studies we thought that shear stress might be a potential candidate
488 (39, 44). Hence, a novel finding of this study was the correlation between PMV concentrations and
489 vascular SRs during exercise coupled with a weaker relationship with noradrenaline in these
490 conditions. The latter relationship also seems logical, since sympathetic nervous system activity has
491 been proposed to play a role in haemostasis and catecholamines have been reported to activate
492 platelets (58), with noradrenaline reported to stimulate PMV formation *in vitro* (54). The major role
493 of shear stress on PMV formation *in vivo* still has to be fully elucidated in experiments where shear
494 stress is the single variable manipulated, for example by independently increasing blood flow with
495 the infusion of vasodilators, but the positive associations observed in the current study provides
496 some support for shear stress as a candidate mediator of PMV formation during exercise, with
497 noradrenaline playing a synergistic role within these experimental conditions. The fact that PMVs did
498 not increase markedly during moderate exercise despite substantial increases in shear is interesting
499 and indicates a shear threshold may be required to stimulate PMV formation. Additional
500 mechanisms may involve the purinergic activation of platelets, since increased concentrations of
501 intravascular nucleotides during heavy exercise stimulate platelet activation *ex vivo* (63), but the
502 actual link between circulating PMVs and nucleotides ought to be determined.

503 *Effect of circulating microvesicles on cultured endothelial cells*

504 Evidence from *in vitro* studies outside the context of exercise suggests that microvesicles may be
505 involved in vascular adaptations. Endurance training induces angiogenesis in active tissues in animal
506 (26) and human models (22, 28) which may be stimulated through many mechanisms including
507 hypoxia (16) and local vascular shear stress (21), with VEGF suggested as the major, albeit not the
508 only growth factor implicated (22, 26, 28). Because high intensity training enhances skeletal muscle
509 capillarization (28), and acutely elevates the concentrations of circulating PMVs in humans (as
510 demonstrated in the present study), it seems plausible that microvesicles could play a role in
511 exercise-induced angiogenesis. A unique finding of this study was that exMVs increased the number
512 of endothelial tubule-like formations in comparison to rMVs and their respective microvesicle free
513 supernatants, suggesting that microvesicles produced during exercise have pro-angiogenic potential.
514 The pathways through which exMVs bring about their endothelial effects are unknown, but may

515 relate to the delivery of VEGF (2, 7) and biologically active lipids (30) to recipient endothelial cells.
516 Furthermore microvesicles obtained from thrombin-stimulated platelets have been shown to display
517 angiogenic potential *in vitro* (7, 30), and *in vivo* (7), which may indicate that PMV could be the
518 microvesicle population stimulating cultured endothelial cells in the present experiments.

519 Angiogenesis depends on orchestrated endothelial cell migratory and mitogenic events, which in
520 turn are stimulated by growth factors released where new capillaries will infiltrate (22, 28). By
521 staining the proliferation-related protein Ki-67, Jensen et al. (2004) demonstrated that the increased
522 skeletal muscle capillarization observed after training is indeed the result of migration and
523 proliferation of endothelial cells to form new capillaries. Our data indicate that microvesicles may be
524 involved in this intricate process, since exMVs enhanced both endothelial migration and
525 proliferation in individual assays. Endothelial scratch wound-healing (a complex process highly
526 dependent on cell migratory and proliferative capacities) was also elevated with exMV treatment,
527 supporting the findings of individual assays. Previous work outside the context of exercise
528 demonstrates that PMVs induce a dose-dependent proliferation and migration of endothelial cells
529 (30), and may substantiate endothelial wound repair through direct and indirect stimulatory
530 mechanisms (30, 37). It is worth noting that exercise increased the concentration of PMVs in the
531 intravascular space, which might seem counterproductive since chemotactic stimulation on the
532 endothelial apical side would stimulate migration into the vessel lumen. PMVs, however, have been
533 reported to adhere to proteins of the extracellular matrix, serving as a binding site for platelets on
534 the vessel wall at regions of extracellular matrix exposure (38), and platelets have been shown to
535 transmigrate to the subendothelial space under certain conditions (32) where they could deliver
536 MVs to the basal surface of endothelial cells, but the existence of similar mechanisms during
537 exercise are unknown.

538 *Methodological considerations*

539 The current series of studies do have some limitations. It is important to note that correlations do
540 not necessarily represent causation, and the present data only provide indirect evidence of shear
541 stress as a potential stimulus for PMV formation during exercise. Further studies that manipulate
542 shear stress whilst sampling arterial and venous microvesicles across exercising limbs ought to be
543 performed in order to directly evaluate the relevance of shear stress in PMV release with exercise, as
544 well as to address the source of microvesicles (*e.g.* organs or exercising limbs). Current methods for
545 microvesicle isolation do not enable separation of specific populations from human plasma without
546 *ex vivo* stimulation, and even though it seems logical that PMVs were the primary mediators of our
547 findings, we cannot rule out the possibility that other microvesicle populations may be involved.

548 Furthermore, it remains unknown whether the influence of exMVs on endothelial cells resulted
549 simply from their greater concentration, or distinct intrinsic characteristics (*e.g.* different cargo).
550 Finally, it is important to keep in mind that observations taken from static cell culture experiments
551 are not necessarily transferable to other cell lines and to complex whole organism models.

552 *Conclusion*

553 In conclusion, submaximal exercise is a potent physiological stimulus that triggers PMVs formation
554 during and 1 h post exercise, with no impact on EMVs. This phenomenon, however, is exercise
555 intensity dependent and requires a substantial stimulus. The rise in PMVs during exercise was
556 coupled with changes in vascular shear stress, and plasma noradrenaline concentration, with both
557 variables explaining part of microvesicle dynamics during exercise. Moreover, human circulating
558 microvesicles produced during exercise increased the angiogenic potential in cultured endothelial
559 cells, which is supported by their mitogenic and chemotactic enhancements. Together, this set of
560 integrative physiology experiments in healthy young humans characterised the physiological time-
561 course of PMV and EMV appearance during and after submaximal exercise, and provides the first
562 evidence that exercise-derived microvesicles play an important biological role within the human
563 vasculature, suggesting a novel mechanism that may help us to further understand how exercise
564 mediates vascular adaptation.

565 **Competing interests**

566 All authors declare no conflict of interests in relation to this work.

567

568 **Acknowledgements**

569 The authors would like to thank all the participants for taking part in this study, Dr. Adrian Chester
570 from Imperial College at the Heart Science Centre Harefield Hospital for providing the endothelial
571 cell isolated from human umbilical cords and Dr. Kameljit Kalsi for her technical assistance with the
572 cell work at the start of the project. M.R. received a Physiological Society Research Grant. E.N.W.
573 was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Bolsista
574 CAPES – Proc. n° BEX 134/13-6; CAPES Foundation, Ministry of Education of Brazil, Brasília – DF
575 70040-020, Brazil) and is grateful for the financial support during his studies.

576

577 **Author contributions**

578 Experiments were performed in the Centre for Sport, Exercise and Rehabilitation, Brunel University
579 London, UK. E.N.W., M.R. and J.G.-A. were involved in the design and conception of the studies.
580 E.W.N. and M.R. were involved in data collection and analysis of *in vivo* and *in vitro* experiments.
581 E.W.N, M.R. and C.P. optimized the methods to quantify the microvesicles. E.W.N, M.R. and J.G-A
582 were involved with interpretation of results, writing, as well as reviewing the manuscript. The final
583 version of the manuscript was approved by all authors before submission.

584 **References**

585 1. **Amabile N, Cheng S, Renard JM, Larson MG, Ghorbani A, McCabe E, Griffin G, Guerin C, Ho
586 JE, Shaw SY, Cohen KS, Vasan RS, Tedgui A, Boulanger CM, and Wang TJ**. Association of circulating
587 endothelial microparticles with cardiometabolic risk factors in the Framingham Heart Study. *Eur
588 Heart J* 2014.
589 2. **Benameur T, Soleti R, Porro C, Andriantsitohaina R, and Martínez MC**. Microparticles
590 carrying Sonic hedgehog favor neovascularization through the activation of nitric oxide pathway in
591 mice. *PLoS One* 5: e12688, 2010.
592 3. **Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, de Marchena E, and
593 Ahn YS**. High levels of circulating endothelial microparticles in patients with acute coronary
594 syndromes. *Am Heart J* 145: 962-970, 2003.
595 4. **Birk GK, Dawson EA, Atkinson C, Haynes A, Cable NT, Thijssen DH, and Green DJ**. Brachial
596 artery adaptation to lower limb exercise training: role of shear stress. *J Appl Physiol* 112: 1653-1658,
597 2012.
598 5. **Bland MJ, and Altman DG**. Calculating correlation coefficients with repeated observations:
599 Part 1 - correlation within subjects. *British Medical Journal* 310: 446, 1995.
600 6. **Boulanger CM, Scoazec A, Ebrahimian T, Henry P, Mathieu E, Tedgui A, and Mallat Z**.
601 Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction.
602 *Circulation* 104: 2649-2652, 2001.
603 7. **Brill A, Dashevsky O, Rivo J, Gozal Y, and Varon D**. Platelet-derived microparticles induce
604 angiogenesis and stimulate post-ischemic revascularization. *Cardiovasc Res* 67: 30-38, 2005.
605 8. **Brodsky SV, Zhang F, Nasjletti A, and Goligorsky MS**. Endothelium-derived microparticles
606 impair endothelial function in vitro. *Am J Physiol Heart Circ Physiol* 286: H1910-1915, 2004.
607 9. **Caiozzo VJ, Davis JA, Ellis JF, Azus JL, Vandagriff R, Prietto CA, and McMaster WC**. A
608 comparison of gas exchange indices used to detect the anaerobic threshold. *J Appl Physiol Respir
609 Environ Exerc Physiol* 53: 1184-1189, 1982.
610 10. **Cantaluppi V, Gatti S, Medica D, Figliolini F, Bruno S, Deregibus MC, Sordi A, Biancone L,
611 Tetta C, and Camussi G**. Microvesicles derived from endothelial progenitor cells protect the kidney
612 from ischemia-reperfusion injury by microRNA-dependent reprogramming of resident renal cells.
613 *Kidney Int* 82: 412-427, 2012.
614 11. **Chaar V, Romana M, Tripette J, Broquere C, Huisse MG, Hue O, Hardy-Dessources MD, and
615 Connes P**. Effect of strenuous physical exercise on circulating cell-derived microparticles. *Clin
616 Hemorheol Microcirc* 47: 15-25, 2011.
617 12. **Coppola A, Coppola L, dalla Mora L, Limongelli FM, Grassia A, Mastrolorenzo L, Gombos G,
618 and Lucivero G**. Vigorous exercise acutely changes platelet and B-lymphocyte CD39 expression. *J
619 Appl Physiol (1985)* 98: 1414-1419, 2005.
620 13. **Credeur DP, Hollis BC, and Welsch MA**. Effects of handgrip training with venous restriction
621 on brachial artery vasodilation. *Med Sci Sports Exerc* 42: 1296-1302, 2010.
622 14. **Curtis AM, Wilkinson PF, Gui M, Gales TL, Hu E, and Edelberg JM**. p38 mitogen-activated
623 protein kinase targets the production of proinflammatory endothelial microparticles. *J Thromb
624 Haemost* 7: 701-709, 2009.
625 15. **Davis RB, Boyd DG, McKinney ME, and Jones CC**. Effects of exercise and exercise
626 conditioning on blood platelet function. *Med Sci Sports Exerc* 22: 49-53, 1990.
627 16. **Deveci D, Marshall JM, and Egginton S**. Chronic hypoxia induces prolonged angiogenesis in
628 skeletal muscles of rat. *Exp Physiol* 87: 287-291, 2002.
629 17. **Dill DB, and Costill DL**. Calculation of percentage changes in volumes of blood, plasma, and
630 red cells in dehydration. *J Appl Physiol* 37: 247-248, 1974.
631 18. **El-Sayed MS, Ali N, and El-Sayed Ali Z**. Aggregation and activation of blood platelets in
632 exercise and training. *Sports Med* 35: 11-22, 2005.

633 19. **Esposito K, Ciotola M, Schisano B, Gualdiero R, Sardelli L, Misso L, Giannetti G, and**
634 **Giugliano D**. Endothelial microparticles correlate with endothelial dysfunction in obese women. *J*
635 *Clin Endocrinol Metab* 91: 3676-3679, 2006.
636 20. **Faulkner A, Purcell R, Hibbert A, Latham S, Thomson S, Hall WL, Wheeler-Jones C, and**
637 **Bishop-Bailey D**. A thin layer angiogenesis assay: a modified basement matrix assay for assessment
638 of endothelial cell differentiation. *BMC Cell Biol* 15: 41, 2014.
639 21. **Galie PA, Nguyen DH, Choi CK, Cohen DM, Janmey PA, and Chen CS**. Fluid shear stress
640 threshold regulates angiogenic sprouting. *Proc Natl Acad Sci U S A* 111: 7968-7973, 2014.
641 22. **Gavin TP, Kraus RM, Carrithers JA, Garry JP, and Hickner RC**. Aging and the Skeletal Muscle
642 Angiogenic Response to Exercise in Women. *J Gerontol A Biol Sci Med Sci* 70: 1189-1197, 2015.
643 23. **Guiraud T, Gayda M, Juneau M, Bosquet L, Meyer P, Theberge-Julien G, Galinier M, Nozza**
644 **A, Lambert J, Rheaume E, Tardif JC, and Nigam A**. A single bout of high-intensity interval exercise
645 does not increase endothelial or platelet microparticles in stable, physically fit men with coronary
646 heart disease. *Can J Cardiol* 29: 1285-1291, 2013.
647 24. **Headland SE, Jones HR, D'Sa AS, Perretti M, and Norling LV**. Cutting-edge analysis of
648 extracellular microparticles using ImageStream(X) imaging flow cytometry. *Sci Rep* 4: 5237, 2014.
649 25. **Horn P, Cortese-Krott MM, Amabile N, Hundsdörfer C, Kröncke KD, Kelm M, and Heiss C**.
650 Circulating microparticles carry a functional endothelial nitric oxide synthase that is decreased in
651 patients with endothelial dysfunction. *J Am Heart Assoc* 2: e003764, 2013.
652 26. **Iemitsu M, Maeda S, Jesmin S, Otsuki T, and Miyauchi T**. Exercise training improves aging-
653 induced downregulation of VEGF angiogenic signaling cascade in hearts. *Am J Physiol Heart Circ*
654 *Physiol* 291: H1290-1298, 2006.
655 27. **Ikarugi H, Taka T, Nakajima S, Noguchi T, Watanabe S, Sasaki Y, Haga S, Ueda T, Seki J, and**
656 **Yamamoto J**. Norepinephrine, but not epinephrine, enhances platelet reactivity and coagulation
657 after exercise in humans. *J Appl Physiol (1985)* 86: 133-138, 1999.
658 28. **Jensen L, Bangsbo J, and Hellsten Y**. Effect of high intensity training on capillarization and
659 presence of angiogenic factors in human skeletal muscle. *J Physiol* 557: 571-582, 2004.
660 29. **Jimenez JJ, Jy W, Mauro LM, Soderland C, Horstman LL, and Ahn YS**. Endothelial cells
661 release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb*
662 *Res* 109: 175-180, 2003.
663 30. **Kim HK, Song KS, Chung JH, Lee KR, and Lee SN**. Platelet microparticles induce angiogenesis
664 in vitro. *Br J Haematol* 124: 376-384, 2004.
665 31. **Kirk RJ, Peart DJ, Madden LA, and Vince RV**. Repeated supra-maximal sprint cycling with
666 and without sodium bicarbonate supplementation induces endothelial microparticle release. *Eur J*
667 *Sport Sci* S (n): 2013.
668 32. **Kraemer BF, Borst O, Gehring EM, Schoenberger T, Urban B, Ninci E, Seizer P, Schmidt C,**
669 **Bigalke B, Koch M, Martinovic I, Daub K, Merz T, Schwanitz L, Stellos K, Fiesel F, Schaller M, Lang F,**
670 **Gawaz M, and Lindemann S**. PI3 kinase-dependent stimulation of platelet migration by stromal cell-
671 derived factor 1 (SDF-1). *J Mol Med (Berl)* 88: 1277-1288, 2010.
672 33. **Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, Picard MH, Roman**
673 **MJ, Seward J, Shanewise J, Solomon S, Spencer KT, St John Sutton M, Stewart W, Committee**
674 **ASoEsNaS, Quantification TFoC, Committee ACoCE, Association AH, and European Association of**
675 **Echocardiography ErSoC**. Recommendations for chamber quantification. *Eur J Echocardiogr* 7: 79-
676 108, 2006.
677 34. **Lansford KA, Shill DD, Dicks AB, Marshburn MP, Southern WM, and Jenkins NT**. Effect of
678 acute exercise on circulating angiogenic cell and microparticle populations. *Exp Physiol* 2015.
679 35. **Lansley KE, Dimenna FJ, Bailey SJ, and Jones AM**. A 'new' method to normalise exercise
680 intensity. *Int J Sports Med* 32: 535-541, 2011.
681 36. **Maruyama K, Kadono T, and Morishita E**. Plasma levels of platelet-derived microparticles
682 are increased after anaerobic exercise in healthy subjects. *J Atheroscler Thromb* 19: 585-587, 2012.

683 37. **Mause SF, Ritzel E, Liehn EA, Hristov M, Bidzhekov K, Muller-Newen G, Soehnlein O, and Weber C**. Platelet microparticles enhance the vasoregenerative potential of angiogenic early outgrowth cells after vascular injury. *Circulation* 122: 495-506, 2010.
686 38. **Merten M, Pakala R, Thiagarajan P, and Benedict CR**. Platelet microparticles promote platelet interaction with subendothelial matrix in a glycoprotein IIb/IIIa-dependent mechanism. *Circulation* 99: 2577-2582, 1999.
689 39. **Miyazaki Y, Nomura S, Miyake T, Kagawa H, Kitada C, Taniguchi H, Komiyama Y, Fujimura Y, Ikeda Y, and Fukuhara S**. High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles. *Blood* 88: 3456-3464, 1996.
692 40. **Mobius-Winkler S, Hilberg T, Menzel K, Golla E, Burman A, Schuler G, and Adams V**. Time-dependent mobilization of circulating progenitor cells during strenuous exercise in healthy individuals. *J Appl Physiol (1985)* 107: 1943-1950, 2009.
695 41. **Nassiri F, Cusimano MD, Scheithauer BW, Rotondo F, Fazio A, Yousef GM, Syro LV, Kovacs K, and Lloyd RV**. Endoglin (CD105): a review of its role in angiogenesis and tumor diagnosis, progression and therapy. *Anticancer Res* 31: 2283-2290, 2011.
698 42. **Nomura S, Imamura A, Okuno M, Kamiyama Y, Fujimura Y, Ikeda Y, and Fukuhara S**. Platelet-derived microparticles in patients with arteriosclerosis obliterans: enhancement of high shear-induced microparticle generation by cytokines. *Thromb Res* 98: 257-268, 2000.
701 43. **Padilla J, Simmons GH, Bender SB, Arce-Esquivel AA, Whyte JJ, and Laughlin MH**. Vascular effects of exercise: endothelial adaptations beyond active muscle beds. *Physiology (Bethesda)* 26: 132-145, 2011.
704 44. **Reininger AJ, Heijnen HF, Schumann H, Specht HM, Schramm W, and Ruggeri ZM**. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood* 107: 3537-3545, 2006.
707 45. **Rizzo V, McIntosh DP, Oh P, and Schnitzer JE**. In situ flow activates endothelial nitric oxide synthase in luminal caveolae of endothelium with rapid caveolin dissociation and calmodulin association. *J Biol Chem* 273: 34724-34729, 1998.
710 46. **Rossiter HB, Kowalchuk JM, and Whipp BJ**. A test to establish maximum O2 uptake despite no plateau in the O2 uptake response to ramp incremental exercise. *J Appl Physiol (1985)* 100: 764-770, 2006.
713 47. **Shantsila E, Kamphuisen PW, and Lip GY**. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. *J Thromb Haemost* 8: 2358-2368, 2010.
716 48. **Sossdorf M, Otto GP, Claus RA, Gabriel HH, and Lösche W**. Cell-derived microparticles promote coagulation after moderate exercise. *Med Sci Sports Exerc* 43: 1169-1176, 2011.
718 49. **Sossdorf M, Otto GP, Claus RA, Gabriel HH, and Lösche W**. Release of pro-coagulant microparticles after moderate endurance exercise. *Platelets* 21: 389-391, 2010.
720 50. **Terrisse AD, Puech N, Allart S, Gourdy P, Xuereb JM, Payrastre B, and Sie P**. Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow. *J Thromb Haemost* 8: 2810-2819, 2010.
723 51. **Tidgren B, Hjemdahl P, Theodorsson E, and Nussberger J**. Renal neurohormonal and vascular responses to dynamic exercise in humans. *J Appl Physiol (1985)* 70: 2279-2286, 1991.
725 52. **Tinken TM, Thijssen DH, Hopkins N, Black MA, Dawson EA, Minson CT, Newcomer SC, Laughlin MH, Cable NT, and Green DJ**. Impact of shear rate modulation on vascular function in humans. *Hypertension* 54: 278-285, 2009.
728 53. **Tinken TM, Thijssen DHJ, Hopkins N, Dawson EA, Cable NT, and Green DJ**. Shear stress mediates endothelial adaptations to exercise training in humans. *Hypertension* 55: 312-318, 2010.
730 54. **Tschuor C, Asmis LM, Lenzlinger PM, Tanner M, Härter L, Keel M, Stocker R, and Stover JF**. In vitro norepinephrine significantly activates isolated platelets from healthy volunteers and critically ill patients following severe traumatic brain injury. *Crit Care* 12: R80, 2008.

733 55. **Vanwijk MJ, Svedas E, Boer K, Nieuwland R, Vanbavel E, and Kublickiene KR**. Isolated
734 microparticles, but not whole plasma, from women with preeclampsia impair endothelium-
735 dependent relaxation in isolated myometrial arteries from healthy pregnant women. *Am J Obstet*
736 *Gynecol* 187: 1686-1693, 2002.
737 56. **Vion AC, Ramkhelawon B, Loyer X, Chironi G, Devue C, Loirand G, Tedgui A, Lehoux S, and**
738 **Boulanger CM**. Shear stress regulates endothelial microparticle release. *Circ Res* 112: 1323-1333,
739 2013.
740 57. **Viñuela-Berni V, Doníz-Padilla L, Figueroa-Vega N, Portillo-Salazar H, Abud-Mendoza C,**
741 **Baranda L, and González-Amaro R**. Proportions of several types of plasma and urine microparticles
742 are increased in patients with rheumatoid arthritis with active disease. *Clin Exp Immunol* 180: 442-
743 451, 2015.
744 58. **von Känel R, and Dimsdale JE**. Effects of sympathetic activation by adrenergic infusions on
745 hemostasis in vivo. *Eur J Haematol* 65: 357-369, 2000.
746 59. **Wahl P, Jansen F, Achtzehn S, Schmitz T, Bloch W, Mester J, and Werner N**. Effects of high
747 intensity training and high volume training on endothelial microparticles and angiogenic growth
748 factors. *PLoS One* 9: e96024, 2014.
749 60. **Whipp BJ, and Rossiter HB**. The kinetics of oxygen uptake: physiological inferences from the
750 parameters. In: *Oxygen uptake kinetics in sport, exercise and medicine: research and practical*
751 *applications*, edited by Jones AM, and Poole DCTaylor & Francis Ltd, 2005, p. 62 - 94.
752 61. **Woodman CR, Price EM, and Laughlin MH**. Shear stress induces eNOS mRNA expression and
753 improves endothelium-dependent dilation in senescent soleus muscle feed arteries. *J Appl Physiol*
754 *(1985)* 98: 940-946, 2005.
755 62. **Wu K, Tian S, Zhou H, and Wu Y**. Statins protect human endothelial cells from TNF-induced
756 inflammation via ERK5 activation. *Biochem Pharmacol* 85: 1753-1760, 2013.
757 63. **Yegutkin GG, Samburski SS, Mortensen SP, Jalkanen S, and González-Alonso J**. Intravascular
758 ADP and soluble nucleotidases contribute to acute prothrombotic state during vigorous exercise in
759 humans. *J Physiol* 579: 553-564, 2007.

760

Table 1. Limb haemodynamics at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition									
	Baseline	Rest or exercise		Recovery						
Time (min)	**0**	**20**	**40**	**65**	**80**	**100**	**120**	**150**	**180**	**240**
BA diameter (cm)										
Control	0.39±0.01	0.39±0.02	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.02	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Moderate exercise	0.39±0.01	0.41±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01
Heavy exercise	0.38±0.02	0.41±0.01*†	0.42±0.01*†	0.41±0.01*†	0.40±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.01	0.39±0.04
BA Vmean (cm / s)										
Control	9.4±0.9	8.8±0.9	8.8±1.1	9.3±1.0	8.3±1.0	8.7±0.6	8.4±0.8	8.4±0.7	8.3±0.9	8.3±1.1
Moderate exercise	10.1±1.2	19.4±2.6*†#	20.5±3.4*†#	16.0±2.6	10.9±2.0	9.3±1.3	8.7±1.5	8.2±1.6	7.9±1.2	7.9±1.1
Heavy exercise	10.4±1.3	30.5±2.8*†	32.3±3.3*†	23.7±3.6*†	12.9±2.5	11.6±2.0	11.5±1.9	9.3±1.6	9.9±1.4	9.0±1.3
BA mean shear rate (/ s)										
Control	98±10	92±10	93±13	97±13	88±13	91±8	88±10	88±10	87±11	87±13
Moderate exercise	106±15	194±28*†#	201±36*†#	162±29	112±21	97±16	91±18	88±19	84±15	83±14
Heavy exercise	112±18	300±32*†	309±34*†	236±40*†	135±29	122±24	120±22	97±19	106±19	97±17
FA diameter (cm)										
Control	0.90±0.02	-	-	0.89±0.02	0.90±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.89±0.02	0.90±0.02
Moderate exercise	0.90±0.02	-	-	0.96±0.02*†	0.94±0.02†	0.92±0.02	0.92±0.02	0.91±0.02	0.91±0.02	0.91±0.02
Heavy exercise	0.90±0.02	-	-	0.96±0.02*†	0.96±0.02†	0.92±0.02	0.91±0.02	0.91±0.02	0.90±0.02	0.90±0.02
FA Vmean (cm / s)										
Control	10.0±0.9	-	-	9.1±1.1	9.3±0.8	8.5±1.0	9.3±0.9	9.3±1.0	8.9±0.7	8.8±0.7
Moderate exercise	8.2±0.5	-	-	16.6±1.4*†#	9.0±0.7	8.8±0.5	8.2±0.4	9.0±0.7	8.5±0.7	9.0±0.4
Heavy exercise	9.0±0.7	-	-	29.4±2.0*†	12.1±1.0†	10.5±0.9	10.4±0.9	9.5±0.7	9.6±0.9	9.4±0.6
FA mean shear rate (/ s)										
Control	45±5	-	-	42±6	42±4	39±5	42±5	42±5	41±4	40±4
Moderate exercise	37±2	-	-	70±7*†#	39±3	39±3#	36±2	40±4	38±4	40±2
Heavy exercise	40±4	-	-	122±8*†	52±5	46±5	46±4	42±4	43±4	42±3

Data are mean±SEM for 9 participants. BA, brachial artery; Vmean, time averaged mean blood flow velocity; FA, femoral artery; * $P \le 0.05$ compared to baseline within condition; † $P \le 0.05$ from control visit at the same time-point; # $P \le 0.05$ from heavy exercise visit at the same time-point.

Table 2. Central haemodynamics at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition									
	Baseline	Rest or exercise		Recovery						
Time (min)	**0**	**20**	**40**	**65**	**80**	**100**	**120**	**150**	**180**	**240**
Heart rate (beats / min)										
Control	59±4	61±4	60±3	61±2	58±3	58±3	59±3	57±3	59±3	60±4
Moderate exercise	59±3	118±5*†#	118±6*†#	78±7*	63±7	65±3	61±3	58±3	56±2	57±2
Heavy exercise	61±3	157±5*†	158±4*†	84±3*†	73±2†	64±3	62±3	60±3	60±4	58±2
Left ventricular EDV (ml)										
Control	132±9	130±8	130±8	129±9	127±8	129±7	131±8	131±8	124±8	128±9
Moderate exercise	136±9	145±11	142±9	134±9	131±10	131±8	130±9	134±9	130±8	131±9
Heavy exercise	126±10	146±12	143±12	122±8	120±8	120±9	126±10	128±10	124±9	127±10
Left ventricular ESV (ml)										
Control	45±6	46±6	44±5	45±5	45±5	44±5	45±5	46±5	41±5	44±6
Moderate exercise	53±8	43±7	41±6*	47±6	47±6	49±6	49±7	50±6#	47±6	48±7
Heavy exercise	46±7	27±7	27±7	35±2	38±3	39±5	44±7	44±6	45±9	43±6
Stroke volume (ml)										
Control	92±4	86±3	89±4	89±4	87±4	89±3	89±4	88±3	87±3	89±4
Moderate exercise	86±3	107±6*†	107±5*†	96±5	86±3	84±2	83±3†	84±3	84±3	88±4
Heavy exercise	89±5	119±6*†	116±5*†	79±10	82±3	80±2†	82±2†	90±5	83±3†	89±5
SBP (mmHg)										
Control	130±2	127±2	127±3	127±4	129±4	129±4	128±4	124±4	123±4	126±4
Moderate exercise	130±3	160±3*†#	153±4*†#	134±3	125±2	125±4	127±4	129±3#	125±4	129±3
Heavy exercise	129±2	173±3*†	176±3*†	123±5	119±2	121±3	121±2	120±3	119±3	122±3
DBP (mmHg)										
Control	70±2	69±3	69±3	70±4	71±3	71±3	73±3	71±3	71±3	71±3
Moderate exercise	71±1	79±2*	77±2*	74±3#	73±2	72±3	72±3	73±2	70±2	74±2
Heavy exercise	68±2	80±2*	80±3*	65±2	71±1	72±2	72±2	70±2	70±2	69±2

Data are mean±SEM for 5-9 participants; EDV: end diastolic volume (n = 5); ESV: end systolic volume (n = 5); SBP: systolic blood pressure; DBP: diastolic blood pressure; *: $P \leq 0.05$ compared to baseline within condition; †: $P \leq 0.05$ from control trial at the same time-point; #: $P \leq 0.05$ from heavy exercise trial at the same time-point.

Table 3. Blood-derived parameters at baseline and throughout resting control or exercise and recovery from moderate or heavy semi-recumbent cycling.

	Experimental Condition								
	Baseline	Rest or exercise		Recovery					
Time (min)	**0**	**30**	**60**	**80**	**100**	**120**	**150**	**180**	**240**
Haematocrit (%)									
Control	42.1±0.8	42.2±0.9	42.2±0.9	42.1±0.9	42.3±0.8	42.7±0.8	42.9±0.9	42.3±0.9	42.6±0.9
Moderate exercise	42.2±0.8	44.3±0.6	44.6±0.7*	43.3±0.7*	42.9±0.7	42.9±0.4	43.2±0.5	43.1±0.7	43.0±0.8
Heavy exercise	42.3±0.9	45.7±0.9*†	45.2±0.8*†	43.7±1.1†	42.9±0.9	42.6±0.9	42.6±0.9	42.8±0.9	42.9±0.8
Haemoglobin (g / l)									
Control	141±4	142±3	141±4	141±3	140±3	142±3	142±4	140±4	142±4
Moderate exercise	144±3	154±3*†	154±3*	146±3	146±3	146±3	146±3	148±3	145±3
Heavy exercise	142±3	155±4*†	154±4*†	147±4	141±3	139±4	142±3	142±3	142±3
Plasma volume change (%)									
Control	-	-0.4±0.7	-0.1±1.4	0.3±1.4	0.5±.1.7	-1.3±1.9	-1.7±1.6	0.7±1.6	-0.9±1.4
Moderate exercise	-	-10.2±1.2*	-10.7±1.4*	-3.5±1.2	-2.8±1.2	-2.4±2.5	-2.8±2.4	-4.3±2.2	-2.2±2.1
Heavy exercise	-	-13.7±1.2*	-13.3±1.5*	-5.3±1.7*	-0.3±1.9	2.0±2.5	-0.3±1.7	-0.6±1.5	-1.1±1.3
Lactate (mmol / l)									
Control	1.0±0.1	0.9±0.1	0.9±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1	0.8±0.1
Moderate exercise	1.0±0.1	1.6±0.1*#†	1.3±0.1#†	0.9±0.1#	0.8±0.1#	0.8±0.1#	0.7±0.1#	0.8±0.1	1.1±0.1
Heavy exercise	1.0±0.1	6.3±0.7*†	5.1±0.9*†	2.2±0.3*†	1.4±0.2	1.2±0.1	0.9±0.1	0.9±0.1	1.0±0.1
Noradrenaline (nmol / l)									
Control	3.2±0.3	3.2±0.5	3.6±0.4	-	-	2.9±0.4	-	2.6±0.3	3.4±0.4
Moderate exercise	3.0±0.3	4.4±0.8#	4.4±0.5	-	-	2.5±0.4	-	3.7±0.6	3.0±0.5
Heavy exercise	3.0±0.2	8.8±1.1*†	9.0±1.5*†	-	-	2.8±0.4	-	3.0±0.5	3.5±0.6
IL-6 (pg / ml)									
Control	0.8±0.1	0.8±0.2	0.7±0.2	-	-	1.1±0.3	-	2.0±0.5*	2.5±0.5*
Moderate exercise	0.8±0.2	0.7±0.1	0.9±0.1#	-	-	1.3±0.2	-	1.7±0.2*	1.9±0.5*
Heavy exercise	0.6±0.1	0.7±0.1	1.9±0.3*†	-	-	2.0±0.4*	-	2.3±0.3*	1.8±0.1*

Data are mean±SEM for 9 participants; IL-6: interleukin-6 *: P ≤ 0.05 compared to baseline within condition; †: P ≤ 0.05 from control trial at the same time-point; #: P ≤ 0.05 from heavy exercise trial at the same time-point.

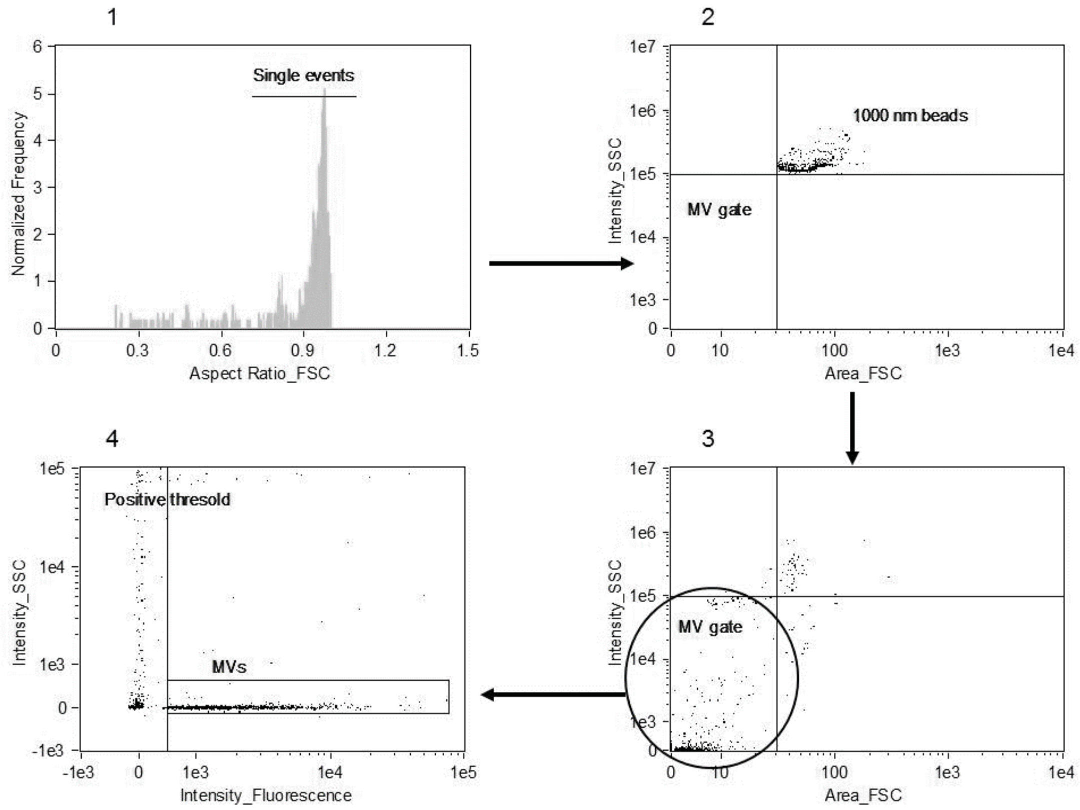

Figure 1. Microvesicle gating strategy. Single events are separated from doublets through their high aspect ratio values (1), and forward scatter (FSC) by side scatter (SSC) do-plots of calibration beads are used to determine a 1 µm size gate (2) which is applied in platelet poor plasma samples to exclude non-microvesicle large events (3). Using the ImageStream microscopy feature at 60x magnification, microvesicles tend to exhibit low SSC intensity and moderate fluorescence for the relevant conjugated antibody (4).

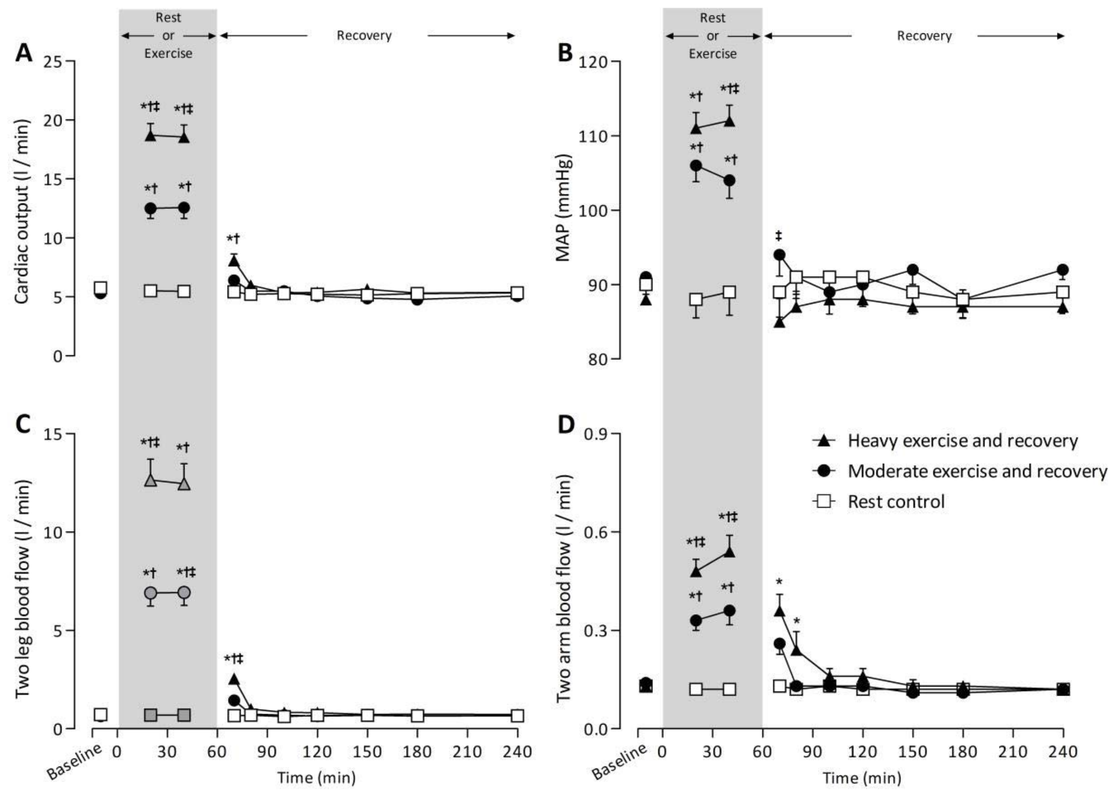

Figure 2. Systemic and limb haemodynamics during the experimental trials with significant increases during exercise and into early recovery. Leg blood flow reflecting exercise time-points (grey symbols) were estimations. Data are expressed as means ± SEM; n = 9; * Significant difference from baseline within condition (P < 0.05); † Significant difference from control visit (P < 0.05); ‡ Significant difference from moderate exercise (P < 0.05).

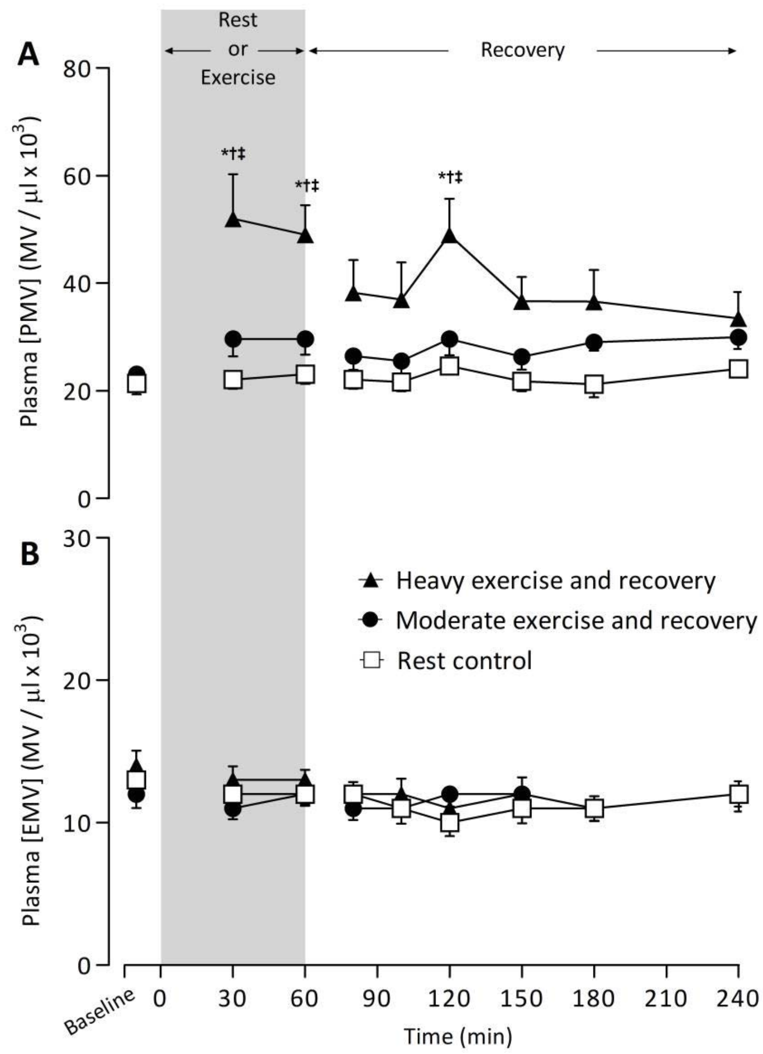

Figure 3. Circulating platelet (PMV) (A) and endothelial (EMV) (B) microvesicle concentrations during the experimental trials. Exercise had no impact on EMV concentrations, but heavy intensity cycling increased circulating PMVs during and at 1 h of post-exercise recovery. Data are expressed as means ± SEM; n = 9; * Significant difference from baseline ($P < 0.05$); † Significant difference from control visit ($P < 0.05$); ‡ Significant difference from moderate exercise ($P < 0.05$).

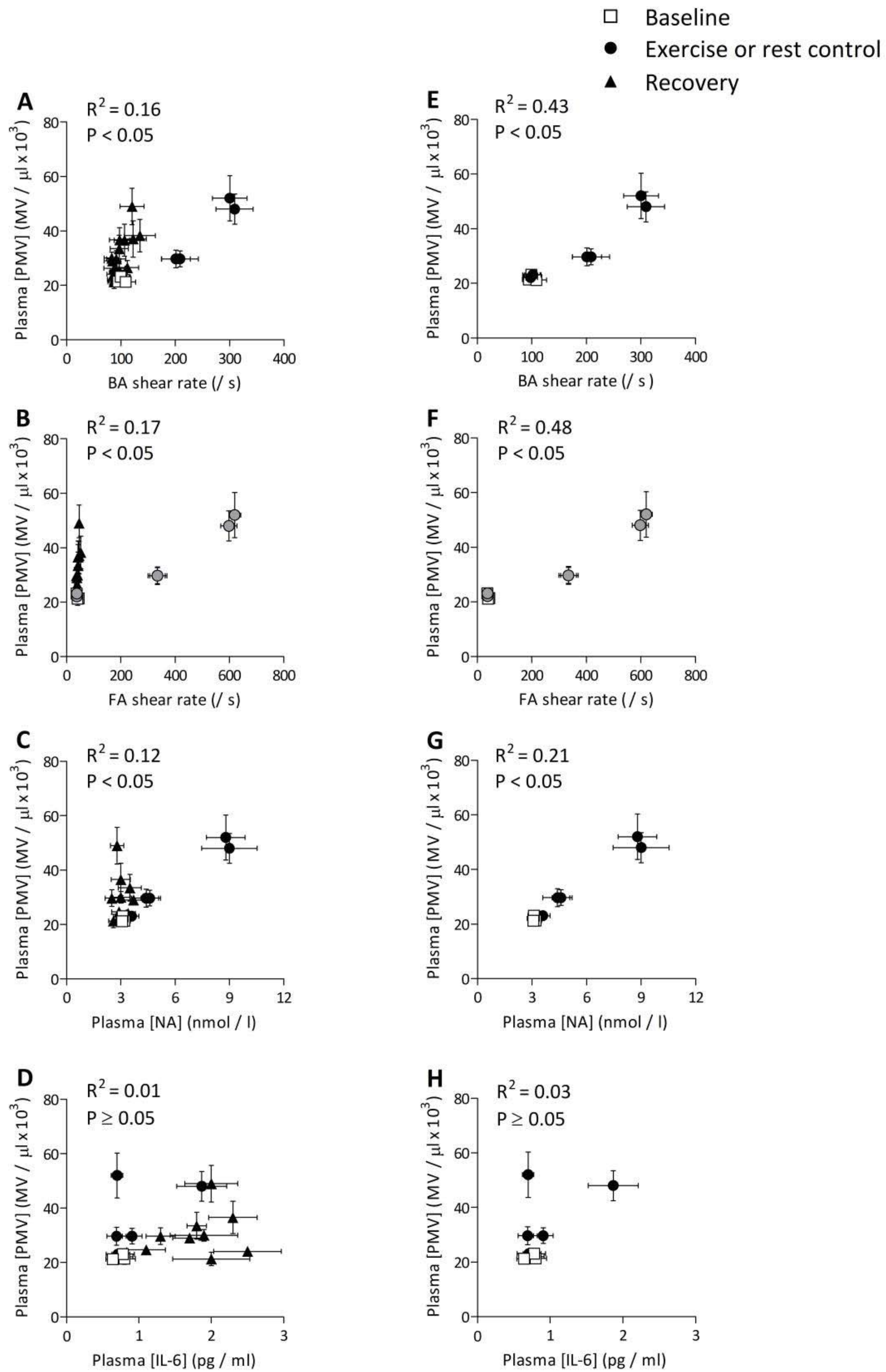

Figure 4. Relationships between platelet microvesicle (PMV), vascular shear rate, and biochemical variables measured at all time-points (A-D), and only during baseline and exercise (E-H). Exercising limb shear rate was estimated during exercise (grey symbols), yet a positive correlation was observed between exercise PMVs and FA shear rate (F). Data are expressed as means ± SEM. BA, brachial artery; FA, femoral artery; NA, noradrenaline; IL-6, interleukin-6.

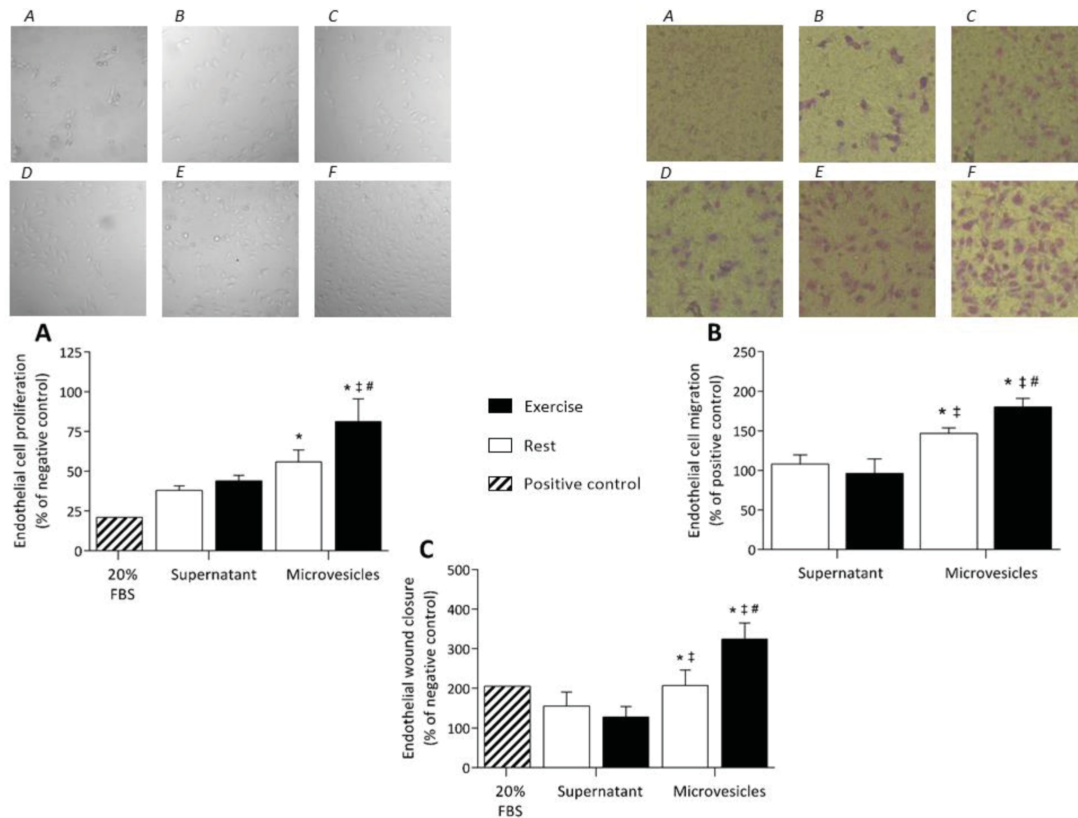

Figure 5. Human umbilical vein endothelial cell proliferation (A), migration (B), and scratch wound-healing (C) in response to resting and exercise microvesicle or microvesicle free supernatant treatment with significantly greater proliferative, migratory and wound closure rates when microvesicles were within the medium and a potentiated effect when microvesicles were derived from exercise plasma samples. Above the graphs are representative proliferation (top left) and migration (top right) images of negative control (A), positive control (B), rest microvesicle free supernatant (C), exercise microvesicle free supernatant (D), rest microvesicle (E), and exercise microvesicle (F) treatments. Data are expressed as means ± SEM; n = 5 for all assays; * Significant difference from rest supernatant ($P < 0.05$); ‡ Significant difference from exercise supernatant ($P < 0.05$); # Significant difference from rest microvesicles ($P < 0.05$).

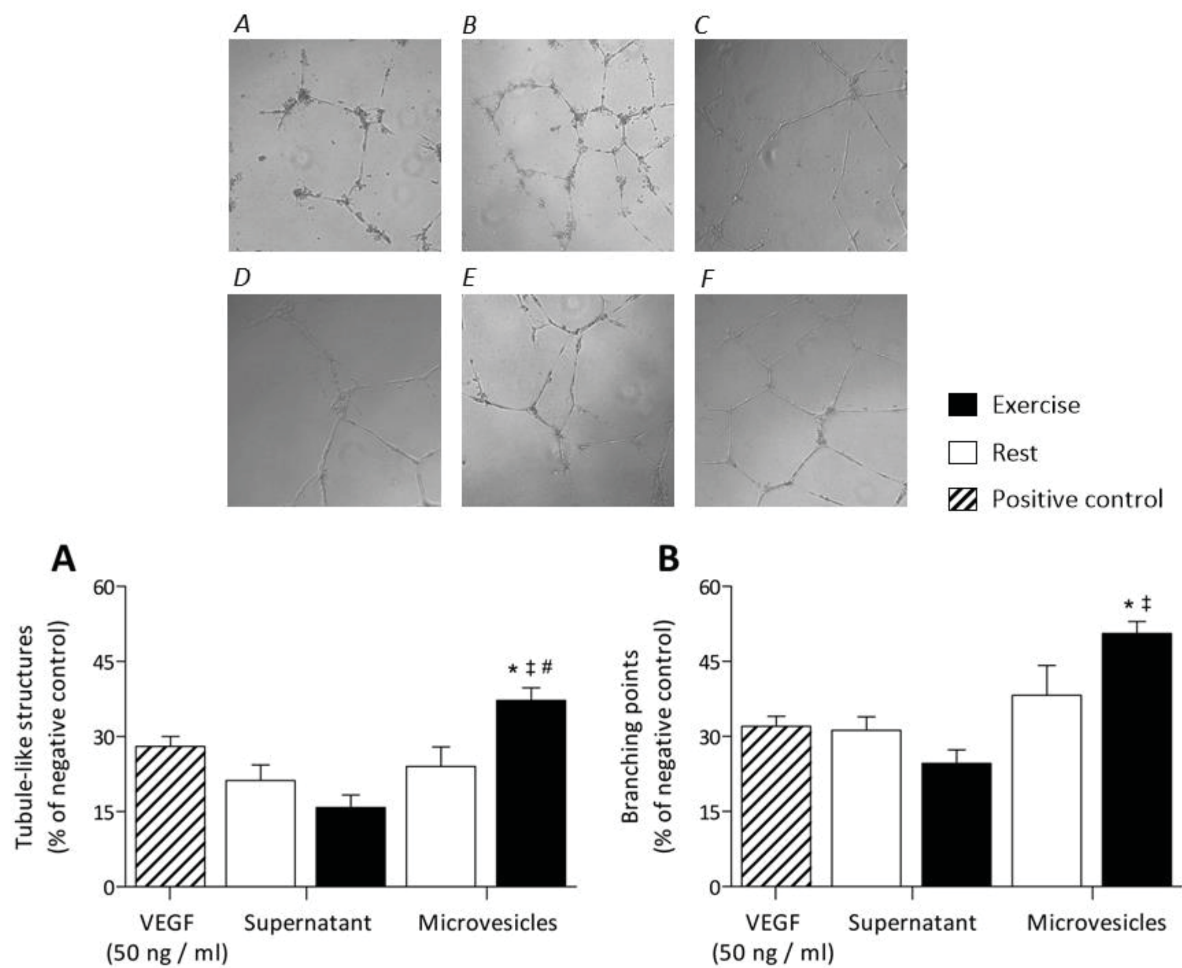

Figure 6. *In vitro* angiogenesis determined by formation of tubule-like structures (A) and branching points (B) of human umbilical vein endothelial cells in response to resting and exercise microvesicle or microvesicle free supernatant treatment. Exercise-derived microvesicles increased the formation of tubule-like structures (A) in comparison to rest microvesicles and microvesicle free supernatants; and also increased the number of branching points (B) in comparison supernatant treatments. Above the graphs are representative images of negative control (A), positive control (B), rest microvesicle free supernatant (C), exercise microvesicle free supernatant (D), rest microvesicle (E), and exercise microvesicle (F) treatments. Data are expressed as means ± SEM; n = 5; * Significant difference from rest supernatant ($P < 0.05$); ‡ Significant difference from exercise supernatant ($P < 0.05$); # Significant difference from rest microvesicles ($P < 0.05$).