Microparticle responses to aerobic exercise and meal consumption in healthy men

Microparticle Responses to Aerobic Exercise and Meal Consumption in Healthy Men

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Abstract

Purpose: Microparticles (MPs) are shed extracellular vesicles that express the pro-thrombotic tissue factor (TF). Aerobic exercise may reduce MP count and TF expression. This study investigated the impact of acute running or rest followed by standardised meal consumption on MP phenotypes and TF expression.

Methods: 15 males (age: 22.9 ± 3.3 years; body mass: 81.9 ± 11.4 kg; $\dot{V}O_2$ max 54.9 ± 6.5 $mL \cdot kg \cdot min^{-1}$; mean ± SD) completed 1h of running (70% $\dot{V}O_2$max) or rest at 9am, and consumed a standardised meal (1170 kcal, 43% CHO, 17% PRO, 40% fat) at 10:45am. Venous blood samples were taken at 9am, 10am and 11:30am. MP concentration, diameter, phenotypes and TF-expression were assessed using nanoparticle tracking analysis (NTA) and flow cytometry.

Results: NTA identified no changes in MP concentration or diameter in response to time or trial. Flow cytometry revealed total MP count increased from 9am to 10am (1.62 ± 2.28 to 1.74 ± 2.61 $x10^{10}$/L, p = .016, effect size (η^2) = .105), but was unaffected by trial. TF^+ platelet-derived MP % reduced from 9am to 10am (44.0 ± 21.2 to 21.5 ± 9.3%, p = .001, η^2 = .582) after exercise only (control: 36.8 ± 18.2 to 34.9 ± 11.9%, p = .972). TF^+ neutrophil-derived MP % reduced from 9am to 11:30am (42.3 ± 17.2 to 25.1 ± 14.9%, p = 0.048, η^2 = .801) in the exercise trial only (control: 28.5 ± 15.7 to 32.2 ± 9.6%, p = .508).

Conclusion: Running induced a significant reduction in $\%TF^+$ platelet and neutrophil MP, suggesting a transient reduction in cardiovascular risk via reduced TF-stimulated thrombosis. This requires further investigation over longer time periods in cardiovascular disease populations.

Key Words: Microparticle phenotypes, thrombotic potential, cardiovascular risk, running.

Introduction

Microparticles (MPs) are small (0.1-1.0 μm) extracellular vesicles shed by a variety of cell types during activation or apoptosis. They express similar surface antigens to their parent cell, which allows phenotype-specific MP detection using laboratory techniques. MPs can also act as biomarkers of cell dysfunction, as well as influence inflammation, oxidative stress and thrombosis (e.g. by promoting immune cell cytokine secretion, reducing endothelial nitric oxide release, increasing tissue factor (TF) bioavailability to drive thrombus formation) (1). However, aerobic exercise has been suggested as a strategy which can positively influence MP concentrations and characteristics.

Previous research has investigated the impact of both acute and regular aerobic exercise on MP kinetics in healthy participants, with conflicting findings. Some studies report increased post-exercise circulating platelet-MP numbers after strenuous or high-intensity exercise (2–4), most likely caused by haemodynamic stress-induced platelet activation which promotes MP shedding. This suggests a pro-thrombotic effect as platelet-derived MPs can express high levels of TF (5). Conversely, other studies have found either no change in endothelial cell or platelet-derived MP numbers following high-intensity (100% peak power output) cycling (6), or even a reduction in circulating endothelial cell-derived MP numbers after cycling of various intensities (55-100% peak power output) (7). Further investigation is required to fully elucidate these effects.

Dietary intake can also influence phenotype-specific MP circulating counts. Postprandial hypertriglyceridaemia and hyperglycaemia can induce vascular dysfunction (impaired vasodilatation), and as such endothelial cell-derived and total MP numbers are elevated in response to high fat and carbohydrate meals (8,9). However, previous research has shown that pre-prandial aerobic exercise (cycling at 60-75% $\dot{V}O_2$ max until 4-6 kcal/kg energy

expended) completed 1 hour before meal ingestion (1070 kcal, 40% fat) can blunt the postprandial increase in endothelial-cell derived MP levels observed in the absence of pre-prandial exercise (10). However, less is known about the effects of more 'balanced' macronutrient meal consumption and how this is influenced by pre-prandial aerobic exercise.

Therefore, this study aimed to investigate the effect of 1 hour of moderate intensity running (versus 1 hour of rest) and post-exercise meal consumption on phenotype-specific MP circulating count and TF expression in healthy participants. This will provide data on which to base similar investigations in at-risk clinical populations in need of aerobic exercise participation. It is hypothesised that running will increase circulating MP numbers but reduce their TF expression, which will persist following the subsequent meal consumption and recovery period.

Methods

Favourable ethical opinion was given by the Loughborough University Human Participants Ethics Sub-Committee (R15-P128). Participants were recruited from the local community. All participants gave written informed consent.

Participants

Fifteen healthy male volunteers (age 22.9 ± 3.3 years, height 181.7 ± 6.1 cm, body mass 81.9 ± 11.4 kg) participated. Inclusion criteria included male gender, aged 18 years or over and able to run continuously for 60 minutes at a moderate intensity (70% of maximum oxygen uptake ($\dot{V}O_2$ max)). Exclusion criteria included unstable weight (> 3kg change in body mass over the last 3 months), current smoker, current cardio-metabolic disease, currently dieting, presence of food allergies, and currently taking any prescribed medications. Sample size calculations (GPower 3.1) suggested that 15 participants would provide sufficient statistical power (80%, alpha level .05) based on total MP number results in response to similar exercise from previous research (11).

Preliminary assessments

Prior to the main trials, participants completed a preliminary assessment visit. Percentage body fat was calculated using the sum of seven skinfold thickness measurements technique (12,13).

During this visit a submaximal incremental treadmill running test and a $\dot{V}O_2$ max treadmill running test were completed, separated by 20-30 minutes of rest. Expired gas samples were collected into Douglas bags (Plysu Protection Systems, Milton Keynes, UK) to determine oxygen consumption and carbon dioxide production (Servomex 1400, East Sussex, UK) and the volume of expired air (Harvard Apparatus, Kent, UK). These measures were used to

59 determine the running speed required to elicit 70% $\dot{V}O_2$ max for each participant, which was
60 subsequently used in the exercise visit in the main trials.

61 *Main Trials*

62 Participants completed one exercise and one control trial, separated by at least 5 days, in a
63 randomised crossover design (www.randomization.com).

64 Dietary intake 24 hours prior to each main trial was standardised using weighed food diaries
65 and the provision of a standardised meal to be consumed between 7-8 pm the previous
66 evening. The meal consisted of a pepperoni pizza (1169 kcal, 48% CHO, 18% PRO, 34%
67 FAT). Caffeine, alcohol and strenuous exercise were not permitted during these 24 hours.

68 The study design is presented schematically in Figure 1. Participants arrived in the morning
69 (8 am) after fasting for a minimum of 12 hours. A cannula was inserted into the antecubital
70 vein upon arrival, 1 hour prior to the first venous blood sample collection in order to allow
71 acclimatisation and reduce needle-related anxiety.

72 In the exercise trial, following the first venous blood sample, participants ran continuously
73 for 60 minutes on a treadmill set to a speed predicted to elicit 70% of $\dot{V}O_2$ max. Expired air
74 samples were collected for one minute every 15 minutes to measure oxygen consumption and
75 if this differed from 70% of $\dot{V}O_2$ max running speed was adjusted accordingly. Heart rate and
76 rating of perceived exertion were recorded every 15 minutes. A second blood sample was
77 taken immediately post-exercise.

78 After exercise, participants rested for the remainder of the trial. A standardised breakfast was
79 provided at 10:45 am and a final venous blood sample was taken at 11:30 am (Figure 1). The
80 standardised breakfast consumed during the main trials consisted of a ham and cheese
81 sandwich, chocolate milkshake and a chocolate bar (1170 kcal, 43% CHO, 17% PRO, 40%
82 FAT). Participants were asked to consume this meal within 15 minutes. In the control trial,

this protocol was followed identically except participants rested for 1 hour in place of the running bout.

Microparticle Analysis – Nanoparticle Tracking

NTA was completed on a cohort (n = 5) of the participants as the combination of NTA and flow cytometry is the current gold-standard methodology for MP analysis (14). Immediately following sample collection, sodium citrate-treated venous blood was centrifuged at 2,500g for 15 minutes at room temperature. Subsequently, the supernatant was separated and further centrifuged identically to create platelet-free plasma, which was stored at -80^{o}C for future batch analysis. The average duration of sample storage prior to NTA analysis was 22 months. Before batch analysis (both NTA and flow cytometry), samples were thawed at room temperature and further double-centrifuged at 18,000g (bracketing a wash step where supernatant was discarded) for 30 minutes to remove unwanted debris.

Platelet-free plasma samples were suspended in PBS (dilution of 1:3, corrected post-acquisition) and visualised using a Nanosight nanoparticle analyser (Malvern, UK). This apparatus uses a laser arrangement to elicit particle scatter in suspended MPs, which can be visualised using a microscope and high-sensitivity camera to create an image of individual MPs within a sample. As an automatic syringe enters the sample into a prism, the camera completes five, 30 second recordings and uses post-acquisition analysis to track the random oscillation of particles due to Brownian Motion (camera level 12.0, camera gain 1.0, minimum track length 5). Ambient temperature was kept constant (20-21 °C). This technique produces values for mean particle concentration and mean particle diameter.

Duplicate sample analysis produced mean coefficients of variation of 5.21% and 3.55% for the particle concentration and diameter results, respectively.

This technique can visualise particles below the minimum threshold provided by flow cytometry (0.3 μm); however, it cannot provide the same qualitative information (i.e. cellular derivation or protein expression). Similarly, the flow cytometry technique explained below can provide qualitative information on particles greater than 0.3 μm but cannot be used to measure the concentration or characteristics of particles below 0.3 μm. As such, the NTA technique will be used to assess MPs in the entire size range (0.1-1.0 μm) and the flow cytometry technique used to comment on MPs over 0.3 μm in diameter.

Microparticle Analysis – Flow Cytometry (MPs > 0.3 μm*)*

The average duration of sample storage prior to flow cytometry analysis was 10 months. The total measurable MP population (0.3 – 1.0 μm) was assessed (n = 15) using size gating and phosphatidylserine expression (An-V^+) to determine the number of MPs which were Annexin-V^+. Microparticle derivations were categorised as platelet-derived ($CD42b^+$), neutrophil-derived ($CD66b^+$), monocyte-derived ($CD14^+$) and endothelial cell-derived ($CD144^+$). The pro-thrombotic potential of each MP derivation was assessed using TF ($CD142^+$) expression. Samples were incubated in the dark at room temperature for 25 minutes with the appropriate antibodies. The isotype controls used were anti-IgG1k PE, anti-IgG1k APC, anti-IgMk PE and anti-IgG2ak PE. The antibodies used for assessing MPs were anti-AnV FITC, anti-CD42b PE, anti-CD66b PE, anti-CD14 PE, anti-CD144 PE and anti-CD142 APC. All antibodies were purchased from BD Biosciences (Oxford, UK) apart from CD142-APC, which was purchased from Biolegend (London, UK).

Following incubation, samples were redistributed in an inert buffer, and then analysed on a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK). All samples were collected for

2 minutes at a 'slow' flow rate (14 $\mu L \cdot min^{-1}$) and events were collected into previously established scatter and fluorescence plots. Examples of the gating strategy and the data produced by this flow cytometry protocol are included in Figure 2.

This sample preparation and analysis procedure was based on recommendations in previous recent literature (14,15). Repeatability measurements achieved via duplicate analyses of samples from this study displayed a mean coefficient of variation of 13.88% for this technique.

Statistical Analyses

Any non-normally distributed data (i.e. Shapiro-Wilks of $p < .05$) was logarithmically transformed to allow the use of parametric testing. Effects of trial and time were analysed using a repeated-measures ANOVA, allowing detection of significant main effects of trial and time, and a trial*time interaction effect. When Mauchly's Test of Sphericity was violated, the Greenhouse-Geisser p value was reported. If a significant effect of time was found, *post-hoc* testing was used to analyse multiple comparisons and the derived p-values were adjusted using the Bonferroni method (16). If a significant time*trial effect was detected, a paired-samples t-test was completed between trials at each time-point to elucidate the effect. Partial eta squared (η^2) was used to estimate effect sizes for all ANOVAs. Effect sizes were classified as small (0.2), medium (0.5) and large (0.8) (Cohen 1988). For all analyses, $p < .05$ was considered statistically significant. All data are presented as 'mean ± standard deviation'. Statistical analysis was completed on IBM SPSS version 23.0 (Chicago, Illinois, US).

Results

Participant mean body fat was 13.1 ± 5.9%, whilst mean $\dot{V}O_2$ max was 54.9 ± 6.5 $mL \cdot kg^{-1} \cdot min^{-1}$.

Nanoparticle tracking analysis

A representative graph displaying the mean particle size distribution and concentration is presented in Figure 3. The NTA data displayed a significant main effect of time for MP circulating count ($p = .040$, $\eta^2 = .331$), however further post-hoc testing did not reveal any significant effects between each time-point ($p \geq .064$, $\eta^2 \leq .217$). No significant main effect of trial or interaction effects ($p \geq .392$, $\eta^2 \leq .111$) were revealed (Figure 4a).

Similarly, no significant time, trial or interaction effects ($p \geq .170$, $\eta^2 \leq .199$) were exhibited for mean MP diameter (Figure 4b).

Microparticle phenotypes – flow cytometry (MPs > 0.3 µm)

'Total MPs' (i.e. quantified by size only – figure 4c) increased from 9am to 10am (1.62 ± 2.28 to 1.74 ± 2.61 $x10^{10}$/L, $p = .016$, $\eta^2 = .105$), but were unaffected by trial ($p = .762$, $\eta^2 = .003$). No time*trial interaction effect was revealed ($p = .127$, $\eta^2 = .071$). The number of Annexin-V positive (PS^+) MPs showed no time, trial or interaction effects ($p \geq .065$, $\eta^2 \leq .113$).

The number of all phenotype-specific MPs (i.e. platelet, neutrophil, monocyte and endothelial derived MPs) remained unaffected by time ($p \geq .187$, $\eta^2 \leq .058$) or trial ($p \geq .072$, $\eta^2 \leq .111$) and displayed no significant time*trial effect ($p \geq .160$, $\eta^2 \leq .063$). Similarly, the number of each MP phenotype as a percentage of total MP count was unaffected by time ($p \geq .051$, $\eta^2 \leq .115$) or trial ($p \geq .314$, $\eta^2 \leq .036$) and exhibited no significant time*trial interaction effect ($p \geq .395$, $\eta^2 \leq .027$).

172 *Microparticle TF expression*

173 The number of TF^+ platelet, neutrophil, monocyte and endothelial cell-derived MPs remained
174 unaffected by time ($p \geq .199$, $\eta^2 \leq .058$) or trial ($p \geq 223$, $\eta^2 \leq .054$) and displayed no
175 significant time*trial interaction effect ($p \geq .069$, $\eta^2 \leq .094$).

176 However, the number of TF^+ platelet-derived MPs as a percentage of the total platelet-
177 derived MP count decreased from 9am to 10am (44.0 ± 21.2 to 21.5 ± 9.3%, $p = .001$, $\eta^2 =$
178 .582) in the exercise trial, but remained unchanged in the control trial (36.8 ± 18.2 to 34.9 ±
179 11.9%, $p = .972$, $\eta^2 = .076$) – a significant time*trial interaction effect was revealed ($p = .001$,
180 $\eta^2 = .216$) (Figure 5a). Similarly, the number of TF^+ neutrophil-derived MPs as a percentage
181 of the total neutrophil-derived MP count decreased from 9am to 11:30am (42.3 ± 17.2 to 25.1
182 ± 14.9%, $p = 0.048$, $\eta^2 = .801$) in the exercise trial, but remained unchanged in the control
183 trial (28.5 ± 15.7 to 32.2 ± 9.6%, $p = .508$, $\eta^2 = .038$) – again a significant time*trial
184 interaction effect was observed ($p = .002$, $\eta^2 = .465$). This is displayed in Figure 5b.
185 However, the number of TF^+ monocyte-derived MPs and endothelial cell-derived MPs as a
186 percentage of their total respective phenotype count were not affected by time ($p \geq .230$, $\eta^2 \leq$
187 .071) or trial ($p \geq .974$, $\eta^2 \leq .001$) and exhibited no time*trial interaction effect ($p \geq .113$, $\eta^2 \leq$
188 .103).

189 All other phenotype and TF expression data displayed no main or interaction effects and are
190 presented in Table 1.

191 **Discussion**

192 This study aimed to investigate the impact of a 1 hour bout of moderate intensity running or
193 rest followed by standardised meal consumption on MP phenotype counts and TF expression
194 in young, healthy males. There were no exercise-dependent effects observed in total MP
195 number, and no changes were seen in any of the MP phenotype-specific numbers. However,

196 exercise induced a reduction in the percentage of platelet- and neutrophil-derived MPs that
197 express TF.

198 *Nanoparticle Tracking Analysis and Flow Cytometry*

199 NTA was completed in combination with flow cytometry in line with recent
200 recommendations (14). However, given the mean particle diameter obtained using NTA was
201 0.15-0.20 μm, it is possible that the minimum detectable threshold of the flow cytometry
202 technique (0.3 μm) may exclude a large proportion of the particle population. This is a
203 previously described limitation of flow cytometric MP analysis (17). Whilst it is accepted that
204 the flow cytometric protocol may be excluding a proportion of the intended particles, flow
205 cytometry remains the gold standard for MP analysis and still incorporates the majority of the
206 reported size range of MPs (0.1 – 1.0 μm). Future research should aim to align these two
207 analysis techniques, as these findings support the notion that there is a need for technique
208 standardisation in order to better compare results from research using various analysis
209 protocols (14,18).

210 *Total MP Number – Flow Cytometry*

211 Total MP number (those < 0.3 μm in diameter) rose significantly throughout the morning in
212 both trials and showed no trial-dependent response, representing a possible diurnal variation
213 effect as MP number, size and TF expression can undergo significant diurnal fluctuations
214 (19). Whilst this study was completed over a morning period only, the MP count mimics that
215 seen in the morning period (9am – 1pm) of a previous 24 hour investigation (20). An
216 explanation for the mechanism behind this diurnal variation is fluctuations in the parent cell
217 number, however the circadian rhythm of each cell type is not uniform (21,22). Accordingly,
218 phenotype-dependent disparity in diurnal variation has been demonstrated in coronary artery
219 disease patients (19); however, the total MP response was altered depending on the marker of

220 determination used ($CD14^+$ monocyte MPs showed no change, whilst $CD62P^+$ platelet MPs
221 decreased throughout the day). The unspecific nature of MP release stimuli (i.e. anything that
222 induces cellular activation or apoptosis) makes identifying the mechanisms responsible very
223 challenging. For instance, as each trial was preceded by an overnight fast, hypoglycaemia
224 and/or hypotriglyceridaemia may have also influenced MP numbers. However, the effects of
225 fasting (versus normal feeding) on MP numbers has not yet been examined and therefore
226 deserves investigation.

227 It should be noted that MP characterisation based solely on diameter is potentially unreliable
228 as it is more susceptible to influence by sample debris (15). Whilst all samples were treated
229 identically, it cannot be ruled out that debris of 0.3 – 1.0 μm in diameter influenced the total
230 event number. Therefore, size-only gating should be considered more as a necessary step to
231 subsequently characterise MPs in combination with a specific marker, rather than an outcome
232 measure *per se*.

233 *Phosphatidylserine Expression*

234 Phosphatidylserine (PS) is the membrane phospholipid which is externalised during the
235 process of MP formation and present in the MP membrane. Annexin-V binds with this
236 externalised PS, and thus has been considered as a pan-cell marker for 'all MPs' in previous
237 studies. However, recent research has suggested that only a small proportion of MPs exhibit
238 PS-Annexin-V binding, with as many as 80% of all MPs not showing Annexin-V positivity
239 (23). Accordingly, in this study only a very small number of MPs were found to be Annexin-
240 V positive. Additionally, it is unsurprising that Annexin-V expression did not change in
241 response to exercise as the moderate intensity would not be expected to induce significant
242 parent cell apoptosis (24) and, therefore, would not be expected to alter MP phospholipid
243 membrane composition (25). It remains unclear why such a small number of MPs would

express PS, or how PS^+ and PS^- MPs differ in formation and function. This line of enquiry warrants further investigation.

MP Derivations and Proportions

MP phenotype number and proportion remained unchanged in response to acute exercise. Previous results regarding the impact of aerobic exercise on MP phenotypes are conflicting (2,6,7) and may depend on training status. The participants included here were relatively aerobically fit (mean $\dot{V}O_2$ max of 54.9 $mL \cdot kg^{-1} \cdot min^{-1}$) and therefore the results should more closely mimic those found in trained populations, which primarily observed either no change (6) or a reduction (7) in endothelial-derived MPs after acute aerobic exercise. The increased haemodynamic demand of the working muscles during aerobic exercise increases blood flow which elevates shear stress, thus causing mechanical activation of leukocytes, platelets and endothelial cells, leading to reduced membrane quiescence and increased MP formation (26). However, in aerobically trained individuals improved endothelial function and increased NO availability (27) may dampen the aerobic exercise-induced shear stress increase and thus prevent subsequent increased endothelial MP formation. However, it is unclear how this adaptation would affect other MP phenotypes. Similarly, the aerobic exercise-induced catecholamine response should also activate leukocytes and thus increase MP release (28) – it is possible that MP release was increased but that MPs were sequestered, for instance via endothelial cell-adhesion. Additionally, a recent study found that, following 20 min of aerobic exercise (65% of $\dot{V}O_2$ max), a significant reduction in the number of $CD62E^+$ MPs was observed in female participants only, with no change in the male participants (29). This suggests a sex-dependent aerobic exercise response and may explain why no changes were seen here in this all-male study sample. However, another simple explanation may be that the large variations seen in baseline values precluded the detection of significant exercise-dependent changes. Lastly, no changes were observed in the control trial, which suggests that

balanced meal consumption alone does not influence MP phenotype distribution. This is in contrast to previous research that has investigated high-fat or high-carbohydrate meal intake, which have both elicited increases in endothelial cell-derived and total MP counts (8,9).

MP Tissue Factor Expression

MPs have been proposed to provide a catalytic surface within the circulation for the initiation and promotion of coagulation and thrombosis (30). Additionally, TF externalised on MPs promotes coagulation via stimulation of the coagulation factor FVII/FVIIa and P-Selectin (31), thus eliciting platelet aggregation (5). Increased TF^+ MP numbers are hypothesised to increase cardiovascular risk via the promotion of thrombotic events, which are the primary cause for many cardiac events such as myocardial infarction (32). In this study, whilst TF^+ MP number was unaffected by exercise, the proportion of TF^+ platelet and neutrophil-derived MPs were significantly reduced by the acute exercise bout whilst remaining unaffected during the corresponding rest control trial. Acute aerobic exercise and regular aerobic training have previously been shown to elicit favourable changes in thrombotic potential via mechanisms such as reduced fibrinogen concentration (33) and decreased tissue-type plasminogen-activator (34). To the authors' knowledge, this is the first study to propose another mechanism, namely a reduced proportion of circulating TF^+ MPs. However, it remains to be seen whether the absolute number of TF^+ MPs or the relative proportions are more significant with regards to thrombotic potential. Similarly, the nature of this relationship in response to regular aerobic exercise training is unknown, as well as the response in patient populations. Further research is required to investigate the effect of regular exercise training in at-risk populations for thrombotic events to determine if reduced MP TF expression may be another mechanism via which cardiovascular disease risk can be decreased. Preliminary research investigating moderate intensity intradialytic cycling in haemodialysis patients (a population at a higher risk of cardiovascular disease) suggests that exercise may impact the

294 oxidative stress influence of MPs and that this may govern the anti-inflammatory effect of
295 exercise (35), though further research is required. Lastly, as with the MP phenotype findings,
296 no changes were observed in the meal-only control trial, suggesting that balanced meal
297 consumption does not impact MP TF expression.

298 The exercise-induced reduction in the proportion of TF^+ platelet and neutrophil-derived MPs
299 in this study points to the importance of assessing MP functional characteristics rather than
300 only phenotype numbers. Future research should include measures of MP functional
301 characteristics (or even *in vitro* MP function) and how they respond to exercise as this may be
302 more relevant to health than purely enumeration.

303 *Limitations*

304 This study is limited by the reported mean MP diameter obtained by NTA, and the
305 subsequent discrepancy with the size thresholds used in the flow cytometry technique.
306 Furthermore, NTA was only completed on a cohort of 5 participants due to logistical and
307 resource constraints. However, there are certain limitations to NTA when compared to flow
308 cytometry. Firstly, NTA does not provide comparable detail with regards to phenotypic
309 characterisation (e.g. multiple concurrent markers) compared to flow cytometry. Secondly,
310 subjective alterations in the instrument settings (e.g. camera level and gain) can affect
311 quantification of MPs using NTA. Additionally, the relatively low NTA minimum tracking
312 duration may have discounted some of the slower moving, larger particles (36), and thus
313 artificially lowered the mean particle diameter. Current guidelines propose that flow
314 cytometry remains the most commonly used technique with the best capability to determine
315 the cellular origin of MPs (37). However, combining this technique with NTA provides the
316 greatest amount of information regarding the entire reported MP diameter range. However,
317 current recommendations suggest that optimal results are obtained when MPs are analysed

within one year of initial storage at -80°C (38); therefore the prolonged storage of samples here (~22 months) may limit the reliability of the NTA findings.

Other study protocol limitations also exist. Obviously, the morning period assessed here only reflects a small proportion of diurnal variation and multiple samples collected over a 24 hour period would be necessary to fully investigate this. Also, as the standardised meal was consumed after exercise the response to exercise recovery alone could not be assessed. Due to the time-intensive nature and expense of flow cytometry MP analysis, only MP TF expression was assessed with no further functional assays. Additionally, the large variations seen in baseline MP values suggest that the inclusion criteria may not have been specific enough, suggesting that other variables that may impact inflammatory status or MP characteristics, such as dietary habits or anti-oxidant supplement usage, should be accounted for in future research. Lastly, only the most commonly assessed MP phenotypes were assessed here but given that almost all types of mammalian cells can release MPs (39), other relevant phenotypes may have been excluded.

Conclusions, implications and further research

In summary, a 1 hour bout of pre-prandial moderate intensity running did not affect MP phenotype number or proportion, however it did reduce the proportion of platelet- and neutrophil-derived MPs that positively expressed TF, which may elicit a transient reduction in thrombotic potential and thus cardiovascular risk. This effect was not impacted by post-exercise meal consumption. This finding requires further investigation in populations at risk of cardiovascular disease, and the longevity of this effect must also be examined to guide the prescription of exercise frequency. The functional assessment of the pro-thrombotic potential of MPs in response to aerobic exercise training in clinical populations could provide another

method to assess intervention efficacy, and ultimately could be used to tailor individual exercise programmes to reduce cardiovascular disease risk.

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Conflict of Interest

The authors declare no conflict of interest. The results of this study are presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation. The results of the study do not constitute an endorsement by the American College of Sports Medicine.

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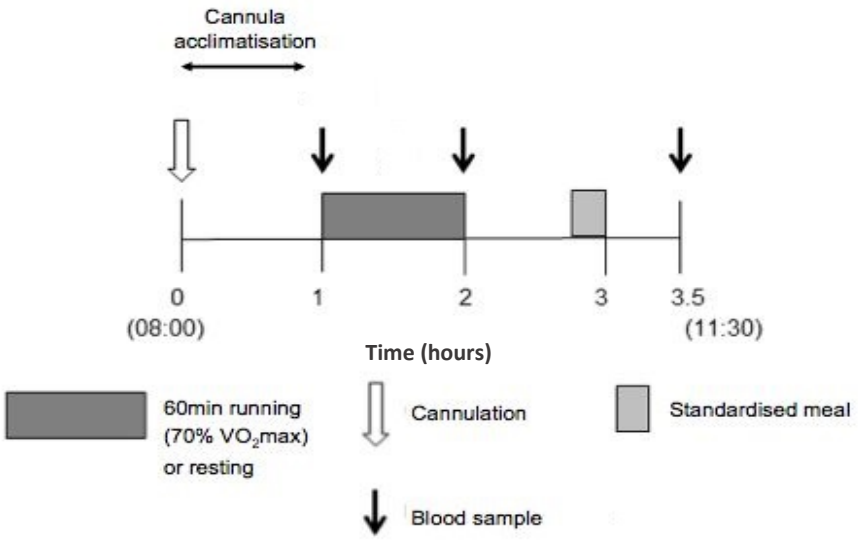
Figure 1. Trial schematic demonstrating the timings of cannulation, blood sampling, exercise and meal consumption.

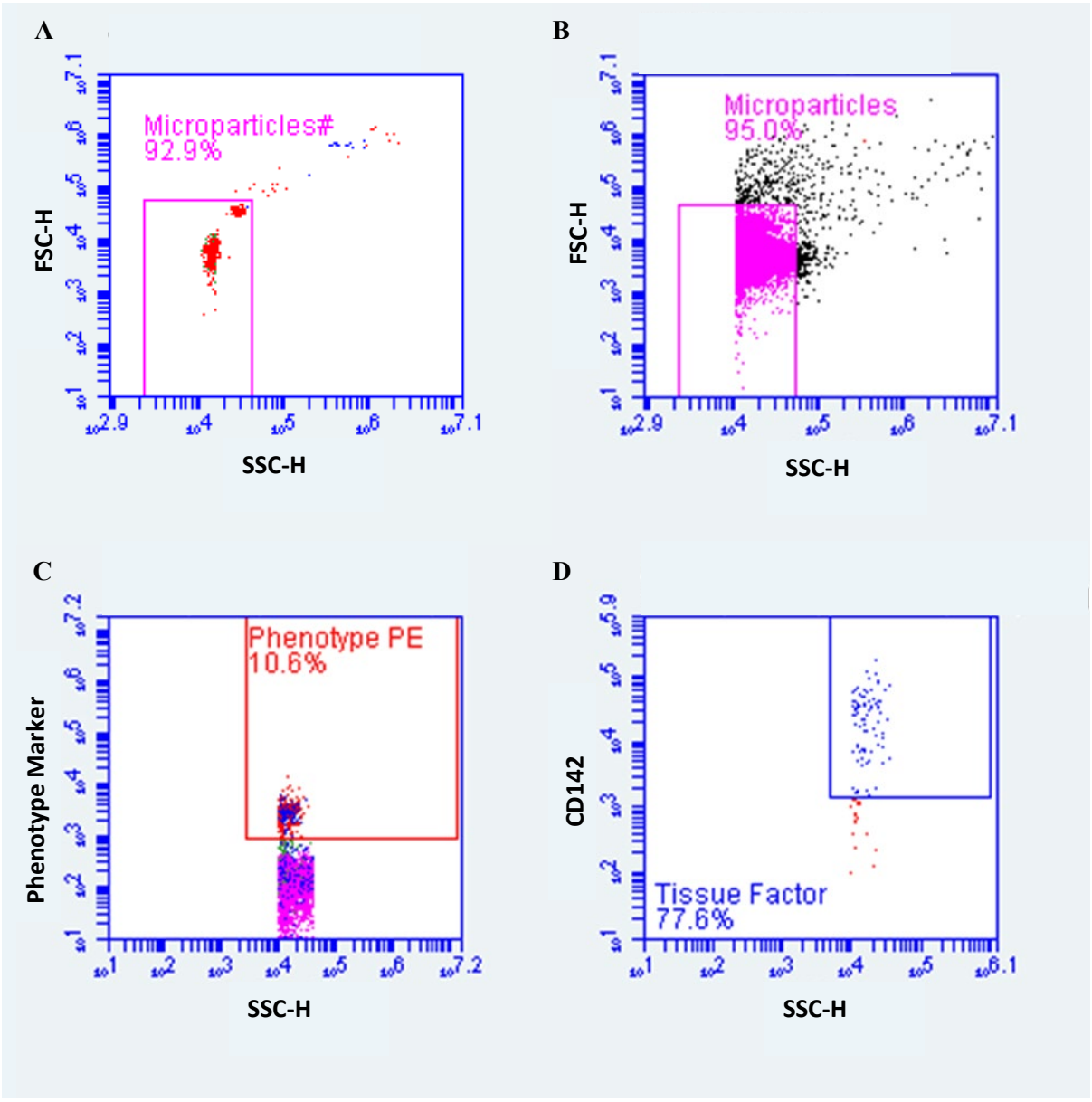
Figure 2. MP flow cytometry gating strategy. A: Mega-mix beads (BioCytex, Theale, UK, product reference 7801) of known size (0.5 µm, 0.9 µm, 3.0 µm) to determine subsequent size gating. Two distinct populations (0.5 and 0.9 µm beads, shown in red) are visible, and thus the gate can be set to include these populations whilst excluding the 3.0 µm population (not visible here). B: 'All MPs' based on size, set using previous mega-mix beads. The minimum trigger threshold of 0.3 µm is necessary to exclude the noise floor inherent in all cytometers, hence why the visible MP population (known to be comprised of particles ranging from 0.1 to 1.0 µm in diameter) is cut off at around 10^4 on the X axis. C: Phenotype marker expression (CD14, CD42b, CD66b, CD144 – all four phenotypes employed the same gating strategy), used to quantify MPs of different cellular sources. D: Tissue Factor expression, back-gated onto the previous graph (C) in order to identify those particles presenting as positive for both their phenotype marker and TF (CD142).

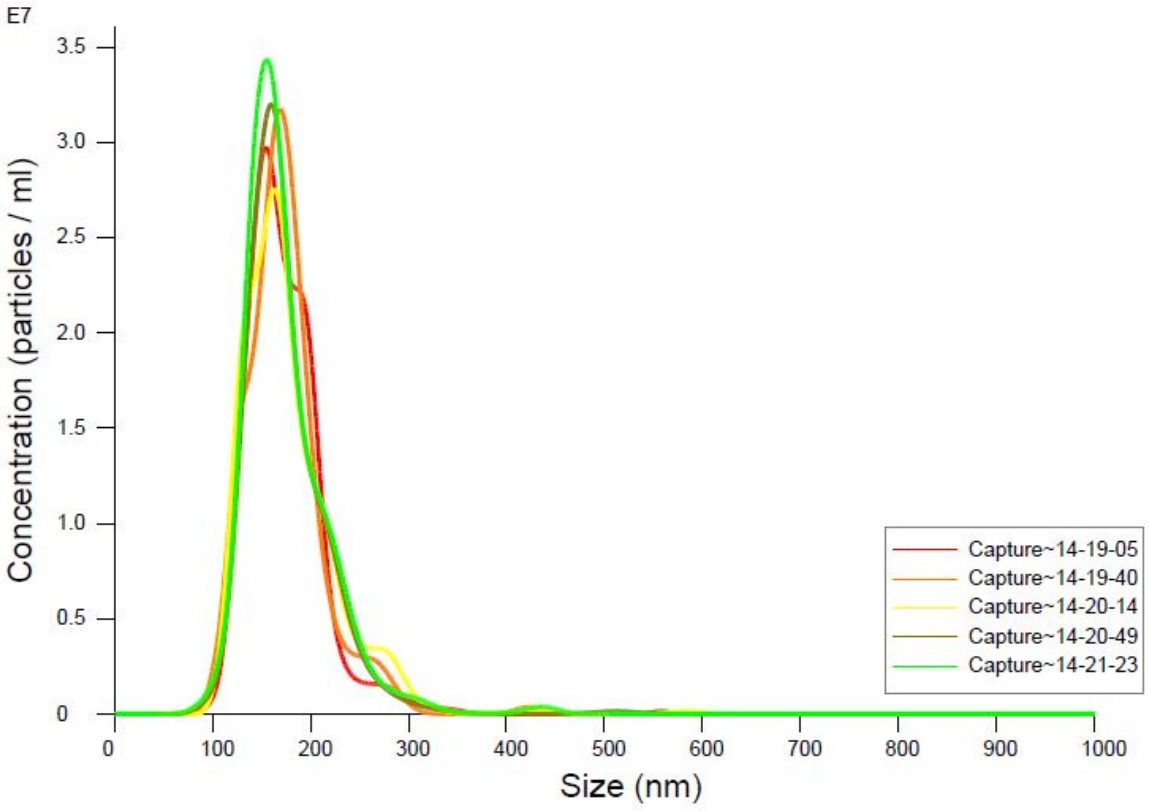
Figure 3. Representative mean particle diameter distribution and concentration obtained by NTA.

Figure 4. Mean MP concentration (A) and diameter (B) obtained using NTA (n = 5), and total MP count obtained using flow cytometry (C). Data are plotted as 'mean ± SD' with individual data points shown. * = significantly different from baseline (p = .016).

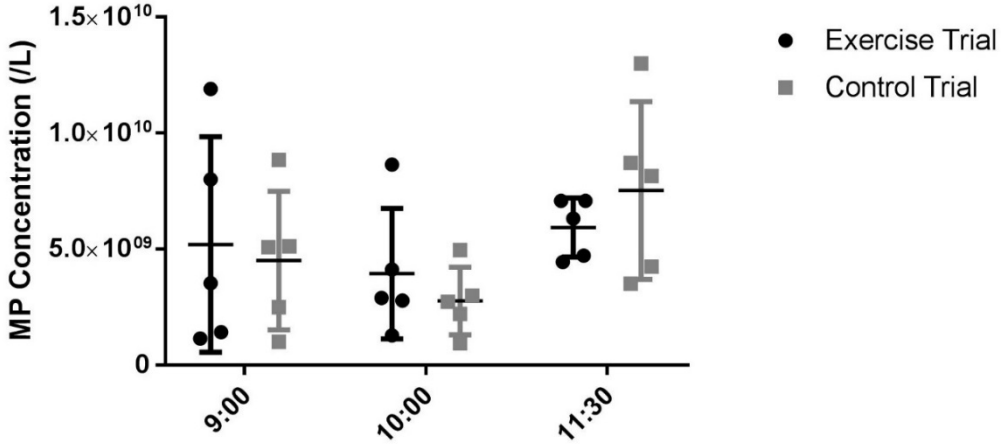
Figure 5. TF^+ platelet-derived MP (PMP) number as a % of total PMP number (A: n = 15) and TF^+ neutrophil-derived MP (NMP) number as a % of total NMP number (B: n = 8) (MPs > 0.3 µm). In some cases, the total number of NMPs was zero and therefore it is not possible or to generate a TF^+ %, hence the difference in sample sizes between the two MP phenotypes. * = significantly different from baseline (9:00) (A: p = .001, B: p = .048) (n = 15). Data are plotted as 'mean ± SD' with individual data points shown.


Cannula
acclimatisation
0
(08:00)
1
2
3
3.5
(11:30)
Time (hours)
60min running
(70% VO2max)
or resting
Cannulation
Standardised meal
Blood sample

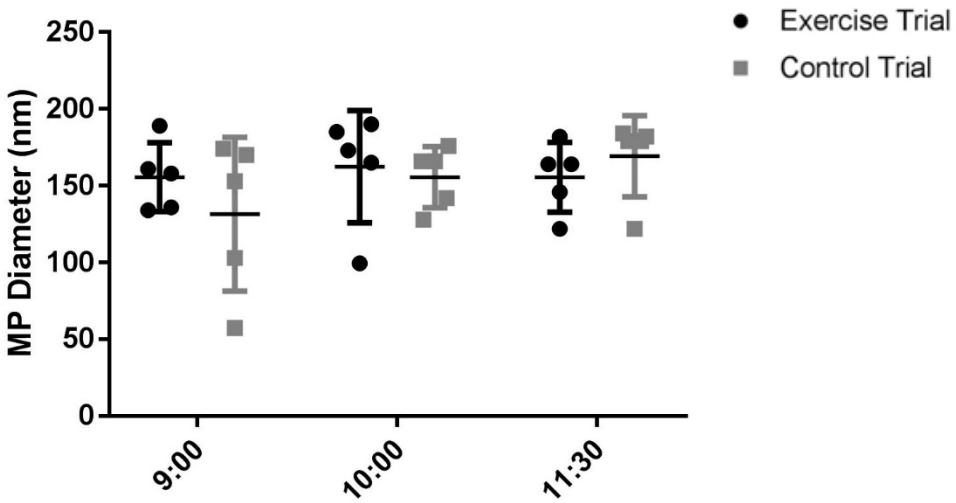

A
Microparticles#
92.9%
FSC-H
SSC-H
B
Microparticles
95.0%
FSC-H
SSC-H
C
Phenotype PE
10.6%
Phenotype Marker
SSC-H
D
Tissue Factor
77.6%
CD142
SSC-H


E7
Concentration (particles / ml)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
0
100
200
300
400
500
600
700
800
900
1000
Size (nm)
Capture~14-19-05
Capture~14-19-40
Capture~14-20-14
Capture~14-20-49
Capture~14-21-23

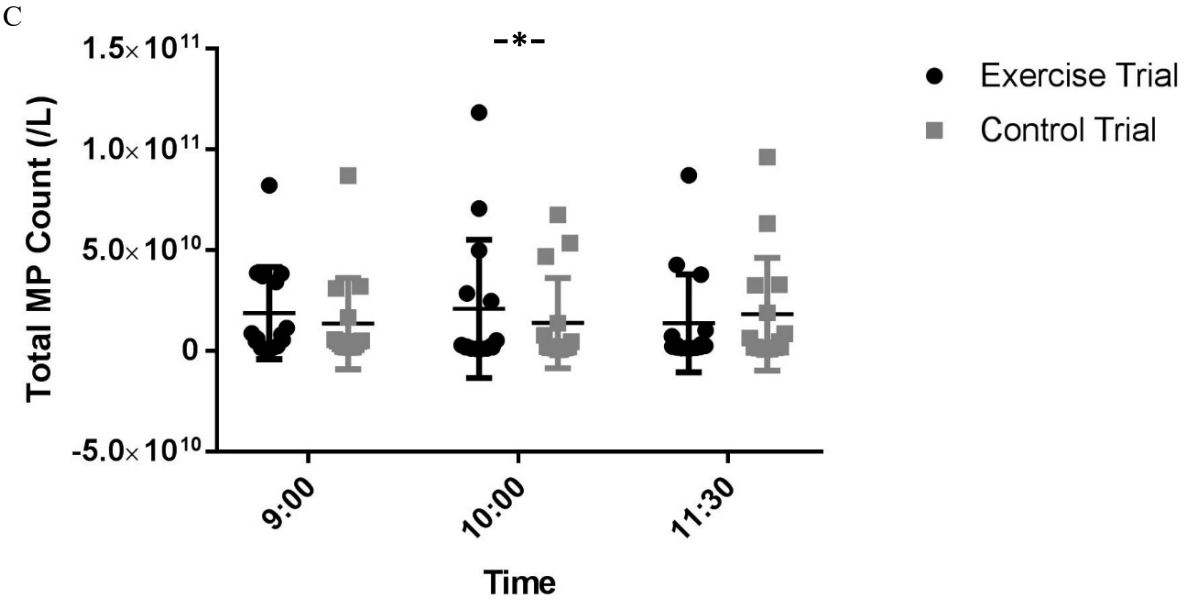
A


MP Concentration (/L)
1.5×10^10
1.0×10^10
5.0×10^09
0
9:00
10:00
11:30
Exercise Trial
Control Trial

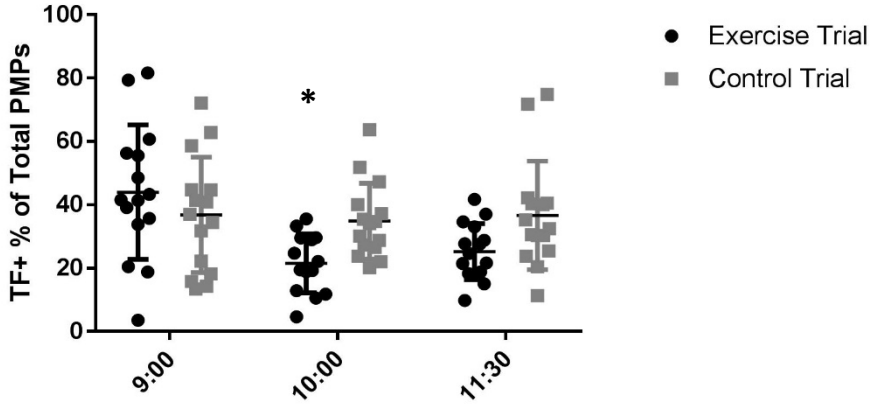
B


MP Diameter (nm)
250
200
150
100
50
0
9:00
10:00
11:30
Exercise Trial
Control Trial

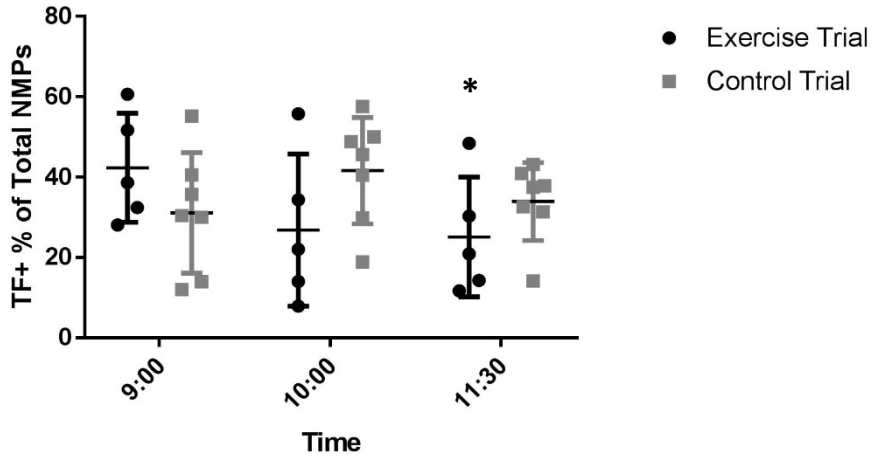
C


Total MP Count (/L)
1.5×10^11
1.0×10^11
5.0×10^10
0
-5.0×10^10
-*-
9:00
10:00
11:30
Time
Exercise Trial
Control Trial

A


TF+ % of Total PMPs
100
80
60
40
20
0
*
9:00
10:00
11:30
Exercise Trial
Control Trial

B


TF+ % of Total NMPs
80
60
40
20
0
*
9:00
10:00
11:30
Time
Exercise Trial
Control Trial