UNIVERSITY^{OF} BIRMINGHAM University of Birmingham Research at Birmingham

Higher levels of physical activity are associated with reduced tethering and migration of proinflammatory monocytes in males with central obesity

Wadley, Alex; Roberts, Matthew J.; Creighton , Jade; Thackray, Alice E.; Stensel, David J.; Bishop, Nicolette C.

License: None: All rights reserved

Document Version Peer reviewed version

Citation for published version (Harvard):

Wadley, A, Roberts, MJ, Creighton , J, Thackray, AE, Stensel, DJ & Bishop, NC 2021, 'Higher levels of physical activity are associated with reduced tethering and migration of pro-inflammatory monocytes in males with central obesity', *Exercise Immunology Review*, vol. 27, pp. 54-66.

<https://repository.lboro.ac.uk/articles/journal_contribution/Higher_levels_of_physical_activity_are_associated_w ith_reduced_tethering_and_migration_of_pro-

inflammatory_monocytes_in_males_with_central_obesity/14717157>

Link to publication on Research at Birmingham portal

Publisher Rights Statement:

This document is the Author Accepted Manuscript version of a published work which appears in its final form in Exercise Immunology Review. The final Version of Record can be found at: ttp://eir-isei.de/2021/eir-2021-054-article.pdf

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.

•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.

•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?) •Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Higher levels of physical activity are associated with reduced tethering and migration of pro-inflammatory monocytes in males with central obesity

Alex J. Wadley^{1*} Matthew J. Roberts¹ Jade Creighton^{1*} Alice E. Thackray¹ David J. Stensel¹ & Nicolette C. Bishop¹

¹ National Centre for Sport and Exercise Medicine, School of Sport, Exercise and Health Sciences, Loughborough University, Epinal Way, Loughborough LE11 3TU See current author affiliations in acknowledgements *

Keywords: Migration; obesity; leukocytes; exercise; immunity

Running Title: Physical activity, adiposity and monocyte migration

Word Count: 5015

Corresponding author: Professor Nicolette Bishop National Centre for Sport and Exercise Medicine School of Sport, Exercise and Health Loughborough University Loughborough Leicestershire LE11 3TU

Email: <u>n.c.bishop@lboro.ac.uk</u> Phone: 01509 226385

Abstract

Despite evidence that monocyte migration is accentuated by central adiposity, the impact of physical activity (PA) and exercise, particularly in the post-prandial state, on limiting migration are not established. We hypothesised that PA and a single bout of walking exercise would be associated with reduced ex vivo monocyte tethering and migration in middle-aged males with central obesity (CO). Objective levels of PA were measured for 7 days in lean males (LE, N=12, mean (SD) age 39 (10) years, waist circumference 81.0 (6.3) cm) and males with CO (N=12, mean (SD) age 40 (9) years, waist circumference 115.3 (13.9) cm), followed by donation of a fasted blood sample. On the same day, CO undertook a bout of walking exercise, before donation of a second fasted blood sample. An ex vivo assay, coupled to flow cytometry, determined tethering and migration of classical, intermediate, and non-classical monocytes. C-C and CXC chemokine receptor (CCR2, CCR5 and CX3CR1) expression were also determined on total and classical monocytes. Monocyte subsets (total, classical, intermediate and CCR2+ monocytes), metabolic (glucose and lipids) and inflammatory (C-reactive protein) markers were greater in CO vs. LE (lower high-density lipoprotein); however, adjustments for PA mitigated group differences for glucose, lipids, and monocyte subsets. Ex vivo tethering and migration (absolute and relative) of most monocyte subsets was greater in CO vs LE. Relative monocyte tethering and migration was largely not influenced by PA; however, higher PA was associated with reduced absolute migration and tethering of CD16 expressing monocytes in CO. Prior walking had no impact on these variables. These results highlight that regular PA, not single exercise bouts may limit the migration of pro-inflammatory monocytes in CO. These changes may relate to physiological parameters in blood (i.e. number of cells and their adhesion), rather than differences in chemokine receptor expression.

1 Introduction

2 Cardiovascular, metabolic, and neurological diseases are threatening to reach epidemic proportions, presenting an enormous health, economic and societal challenge. Common to all 3 these conditions is their chronic inflammatory aetiology (40). Increased central adiposity is 4 known to significantly elevate the risk of developing these diseases, with a heightened 5 6 migration of immune cells from blood into various tissues, namely monocytes into adipose 7 tissue (44). An increase in adipose tissue-resident macrophages can initiate a perpetual inflammatory cycle whereby proteins that tether (e.g. cellular adhesion molecules) (34), attract 8 9 (e.g. chemokines) (32), and subsequently cause damage (e.g. pro-inflammatory cytokines) (35) are chronically elevated, thus drawing more cells into tissues. Over time, excessive monocyte 10 migration in individuals with central obesity (CO) drives a dysfunctional interaction that 11 promotes metabolic dysfunction and an increased risk of chronic disease (44). 12

This risk of developing cardiovascular, metabolic, and neurological diseases is exaggerated 13 by multiple factors, including physical inactivity and diet (41). For the latter, prolonged 14 elevations of triglycerides in the bloodstream after high fat meals, termed postprandial lipaemia 15 (PPL), is a known independent predictor of cardiovascular disease (41). There is evidence that 16 PPL is also associated with higher systemic inflammation (8, 41), with monocyte activation 17 (29), adhesion and propensity for foam cell formation higher following a high fat meal (28). 18 19 Given the central role of monocytes in the aetiology of chronic inflammatory disease, monocyte blood profiles have been used to predict current and future cardiovascular (22), 20 metabolic (37), and neurological health (38). Recent evidence has documented that individuals 21 22 with obesity have a more pro-inflammatory and pro-migratory monocyte profile than individuals who are lean (15, 33). This is reflected by higher concentrations, and an altered 23 composition of the three monocyte sub-populations; e.g. decreased classical (CM: 24 CD14++CD16-), higher intermediate (IM: CD14++CD16+), and higher non-classical (NCM: 25

CD14+CD16++) monocytes (15, 20, 33). A reduced percentage of CM occurs as a result of 26 preferential tissue migration in response to cues from inflamed tissues, whereas IM and NCM 27 proportions increase due to their role in patrolling the circulation by adhering to the 28 29 endothelium (31). These functions are governed by higher expression of C-C and CXC chemokine receptors (e.g. CCR2, CCR5 and CXC3R1), which positively correlate with body 30 weight, body mass index, fat mass, and insulin insensitivity on total monocytes ⁶. Chemokine 31 32 receptors have high affinity for complementary chemokine ligands (e.g. CCL2, CCL5 and CX3CL1), which are released from cells or tissues under metabolic and inflammatory stress. 33 34 Obesity-associated inflammation also causes an increase in circulating cellular adhesion molecule concentration (34), which enhances the potential for monocytes to tether to the 35 surface of these cells and tissues. A higher number of circulating monocytes with a higher 36 37 capacity to tether and migrate therefore generates a favourable gradient for the progressive 38 movement of monocytes towards metabolically active tissues or the endothelial layer in individuals with CO, increasing the risk of cardiovascular diseases (14). Given the substantial 39 economic and social impact of managing and treating these diseases, cost-effective 40 interventions to prevent monocyte tethering and migration are urgently required. 41

42 Increased levels of physical activity are associated with reduced blood markers of chronic inflammation (e.g. inflammatory cytokines and adhesion molecules), and a reduced risk of 43 44 chronic inflammatory disease, compared with sedentary individuals (16, 24). Studies in adults 45 with obesity indicate that regular structured exercise can reduce the pro-inflammatory monocyte phenotype in blood (13, 42), as well as the expression of chemokine receptors on 46 total monocytes (2). It is well documented that single sessions of exercise result in acute 47 elevations in chemokine concentrations in blood plasma (12, 17, 39). It has been suggested that 48 49 this response may result in the internalisation of chemokine receptors on the surface of immune cells that with regular physical activity, drive a reduction in expression level (2, 6, 24). Indeed, 50

51 recent evidence has highlighted that moderate intensity exercise lowered the expression of CCR2 on intermediate monocytes within an hour of exercise cessation in individuals with CO 52 53 (6). When considering how this transient internalisation of CCR2 on monocytes may translate 54 into chronic changes in expression that have been reported separately (2), the impact of PPLinduced inflammation must also be considered, particularly in individuals with CO as this is 55 associated with unfavourable metabolic and inflammatory profiles (14). A large body of 56 57 evidence supports a role for single sessions of exercise being an effective strategy to attenuate PPL within 24 hours of a high-fat meal (23). Despite this, there is limited available data to 58 59 support a reduction in PPL-associated inflammation over this 24 hour period with prior exercise. At present, our collective understanding of how regular physical activity and single 60 sessions of exercise, particularly in the PPL state, independently impact chemokine expression 61 62 on monocyte populations in individuals with CO is lacking. Furthermore, how these changes then relate to the tethering and migration of monocytes is yet to be established. 63

We, and others, have previously quantified the ex vivo migratory capacity of peripheral 64 blood mononuclear cells (PBMCs) towards a fixed chemokine gradient over time (3, 33). Using 65 this method, there is evidence that monocyte migration is greater in individuals who are obese 66 67 than those who are lean (33). Evidence from our group indicates that a single session of exercise can reduce the ex vivo migration of T-helper lymphocytes towards chemokine-rich supernatants 68 69 in healthy individuals (3). To our knowledge, there is no data indicating that regular physical 70 activity or single sessions of exercise in a state of PPL, can reduce monocyte migration in individuals with CO. Importantly, previous investigations into monocyte migration are limited 71 by only quantifying the number of cells that migrate in response to a fixed chemokine gradient 72 (33), rather than their phenotype, as done in our laboratory previously with lymphocytes (3). 73 74 Adopting this approach would indicate more about the inflammatory characteristics of monocytes and potential to cause damage within metabolically active tissues (i.e. CM) or the 75

vasculature (i.e. IM and NCM). Furthermore, it has been highlighted that mimicking conditions
of physiological blood flow better maintains monocyte phenotype when implementing *ex vivo*models (45), and this is often overlooked (3, 33).

By validating a dynamic experimental platform, which coupled *ex vivo* monocyte tethering and migration under conditions of physiological blood flow to digital flow cytometry, the aims of this study were to: 1) cross-sectionally determine the association of central adiposity and physical activity levels with monocyte tethering and migration in middle-aged males and 2) determine whether a single session of exercise can impact the tethering and migration of monocytes in middle-aged males with CO under conditions of PPL.

85

86 Materials and Methods

87 Participants

This project involved cross-sectional assessments in males who were lean (LE, N=12) 88 and males with central obesity (CO, N=12), followed by awalking intervention in CO only. 89 Using our preliminary data, we based our sample size calculation on mean resting (and SD) 90 differences in total monocytes between CO and LE. Using GPower 3.1.9.7, we calculated we 91 would need 12 participants in each group to detect similar differences in the present study, with 92 an effect size of 1.1, 80% power and a of 5%. Age and ethnicity-matched participants (White 93 European (WE), N=6 and South Asian (SA), N=6 in both LE and CO) gave their informed 94 written consent and the investigation was approved by the ethical review committee at 95 96 Loughborough University (ethics code: R18-P120) in accordance with the Declaration of Helsinki. Central obesity was defined as a waist circumference \geq 94cm in White European men 97 and \geq 90cm in South Asian men according to the International Diabetes Federation cut-off 98 points (1). Males who were lean were classified based on a waist circumference under the 99

aforementioned boundaries. All participants had stable weight for the preceding 3 months, were non-smokers and had not taken any anti-inflammatory drugs (e.g. NSAIDs) for 4 weeks prior to taking part. Participants were screened for diabetes using an HbA1c test, and a health screen questionnaire was used to screen for other underlying health conditions, plus lifestyle factors that may influence the results such as smoking. In addition, participants were required to refrain from any strenuous physical activity or consumption of alcoholic or caffeine-based beverages in the 48 hours prior to or during the experimental session.

107

108 Experimental Procedures

All participants first visited the laboratory for screening of height and weight using a 109 110 fixed wall stadiometer with a digital weighing scale built in (Seca Ltd, Hamburg, Germany). Hip and waist circumference were measured using a flexible, non-elastic tape (Hokanson, 111 Washington, USA) whilst adhering to established measurement guidelines (1). An assessment 112 of peak oxygen uptake (\dot{VO}_2 peak) was undertaken in CO only using the modified Bruce 113 114 treadmill test (43). Heart rate was continuously measured using short-range telemetry (Polar T31; Polar Electro, Kempele, Finland) and subjective effort measured using the rating of 115 perceived exertion scale (7). At the end of the first visit, participants were fitted with an 116 accelerometer (ActiGraph GT3X, ActiGraph corporation, Florida, USA) to quantify levels of 117 habitual physical activity for a period of 7 days. Data were analysed over 15 second epochs 118 using specialised software (Actilife, Actigraph corporation, Florida, USA). Accelerometer data 119 were screened for wear time using standard methods (10). Time spent in a defined intensity of 120 activity was determined by summing together counts per minute and categorising this based on 121 122 widely used cut points (19).

Prior to the experimental period, all participants were asked to maintain their normal 123 habitual diet for a period of 7 days. The day before, both groups undertook an overnight fast 124 from 22:00 (except plain water). The next morning (08:00), both groups returned to the 125 laboratory and consumed 250mL of water prior to bioelectrical impedance analysis (Seca 126 mBCA 515, Seca Ltd, Hamburg, Germany) to measure body fat percentage. Participants then 127 donated a blood sample via venepuncture to an antecubital vein. After this, CO remained rested 128 129 in the laboratory throughout the day, with standardised meals (57% fat, 32% carbohydrate, 11% protein, 14.2 kcal per kg of body mass) provided, before undertaking a 60-minute session 130 of walking exercise at 60-65% of their $\dot{V}O_2$ peak (15:00-16:00). Walking intensity was 131 confirmed using a portable metabolic cart which analysed breath-by-breath gases (Metalyzer 132 3B, Cortex, Leipzig, Germany) and subjective measures of perceived exertion were obtained 133 using the Borg Scale (6-20) (7). Participants were then free to leave the laboratory and were 134 provided with a standardised evening meal to consume before 22:00. After this, participants 135 fasted overnight (except plain water) and returned to the laboratory the next morning (08:00) 136 to donate a second rested blood sample. 137

138

139 Blood collection and analysis

Whole blood (40.9mL) was collected via venepuncture into EDTA (4.9mL) and sodium heparin-coated (36mL) monovettes. Heparinised blood was used for the isolation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation. Briefly, whole blood was diluted 1:1 with D-PBS and layered on top of Histopaque 1077 (2:1). Blood was centrifuged at 400g for 30 minutes at 21 degrees (brake off) and the PBMC layer aspirated and washed in PBS and RPMI. EDTA monovettes were centrifuged at 3500g for 10 minutes at 4 degrees and plasma isolated for future analysis of triacylglycerol (TAG), total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), non-esterified fatty acids(NEFA), glucose, and C-Reactive Protein (CRP).

149

150 *Ex vivo* Migration Assay

The ex vivo migration assay was adapted from previously published protocols published 151 in our laboratory (3) and others (33), as well as internal validation experiments. Whole blood 152 cell counts were initially performed using a haematology analyser (Yumizen H500, Horiba, 153 *Northampton, UK*) to determine the circulating monocyte count ($\approx 0.2 - 1.0 \ge 10^6$ /mL). PBMC 154 counts were then determined by using CountBright[™] Absolute Counting Beads on a BD 155 C6 Accuri Flow Cytometer (Becton Dickinson, Oxford, UK). The seeding density of monocytes 156 157 for the migration assay matched the monocyte concentration in whole blood, thus mimicking physiological conditions. Peripheral blood mononuclear cells (in 2mL RPMI) were added in 158 duplicate to fibronectin-coated polyester (PET) inserts (5µm pore size) and placed into 6-well 159 non-tissue culture treated plates containing 3mL of heat-inactivated 10% fetal bovine serum 160 (FBS) or RPMI (background migration control). Monocytes were then allowed to migrate for 161 162 3 hours at 37 degrees (5% CO₂). To further generate conditions that closely mimic moderate physiological flow within the circulation (3.2 dyn/cm³) (9) and to maintain monocyte 163 phenotype, the PBMC suspension was oscillated on a 2-dimensional orbital shaker at 77 rpm 164 165 during this 3 hour period (45). Fetal bovine serum concentration and incubation times were based on in-house validation experiments from our laboratory. After 3 hours, non-adherent 166 cells were removed from the upper side of the PET insert, and this was then washed twice with 167 168 1mL of D-PBS. Tethered PBMCs were then removed from the upper side of the PET inserts by adding 1mL of enzyme free, EDTA-based dissociation media (4 degrees, 30 minutes), 169 followed by five washes with D-PBS (1ml). The underside of the PET inserts and the wells of 170

the tissue culture plate beneath were treated identically to collect cells that had *migrated* across

the PET insert. *Tethered* and *migrated* cells were collected into separate tubes and washed in

173 D-PBS ready for counting and phenotyping using flow cytometry.

174

175 Flow Cytometry

Pre-migration (baseline) and collected tethered and migrated PBMCs were counted as 176 above. For each sample, 1.75×10^5 PBMCs were then stained using fluorescently conjugated 177 antibodies for identification of monocytes subsets and chemokine receptor expression (e.g. 178 CCR2, CCR5 and CX3CR1) using four-colour flow cytometry. Cells were incubated with 179 CD14-FITC, CD16-PE, CX3CR1-APC, CCR5-APC, CCR2-Alexa Fluor-647 antibodies, and 180 181 7-AAD for 30 minutes at 4°C in the dark. Cells were then twice washed with FACS buffer (D-PBS supplemented with 0.5% bovine serum albumin and 2 mM EDTA; pH=7.2) for 5 minutes 182 at 300 x g. Compensation was adjusted weekly by using single stained controls and gates 183 established using fluorescence minus one controls. Confirmation of non-specific antibody 184 binding was determined by using isotype-matched controls. 185

186 Flow cytometry data were analysed using BD C6 Accuri software (Becton Dickinson, Oxford, UK). Briefly, monocytes were gated on forward versus side scatter. Doublets were 187 discriminated using FSC-A vs FSC-H plots, and non-viable monocytes (i.e. 7-AAD+) 188 189 excluded. CD14+ and CD16+ positive populations were then used to determine classical (CD14++CD16-), intermediate (CD14++CD16+), and non-classical (CD14+CD16++) 190 monocyte proportions (%) as previously described (18). Histogram plots of the cells in the total 191 192 monocyte, and CM regions that positively expressed CCR2, CCR5, and CX3CR1 were subsequently used to calculate the percentage of chemokine receptor positive cells. Mean 193 fluorescent intensity (MFI), an indicator of the density of chemokine receptor expression on 194

each cell, was also determined for total monocyte and CM populations. A sub-population of cells was defined as ≥ 1000 positive cells in a gate. For baseline, the percentage of monocyte subsets and CCR2+, CCR5+, and CX3CR1+ monocytes were used with whole blood cell counts to determine the circulating number of chemokine receptor positive monocytes for each sub-population. Similarly, the percentages of these cells following tethering and migration were used with the total number of tethered and migrated cells to determine absolute changes at each assay stage.

202

203 Metabolic Parameters

Concentrations of TC, HDL, LDL, TAG, glucose, CRP (Horiba Medical, Montepellier,
France), and NEFA (Randox Laboratories Ltd., County Antrim, UK) were determined
spectrophotometrically using commercially available kits (high detection for CRP) and a
benchtop analyser (Pentra 400, Horiba Medical, Montpellier, France). Concentrations of
HbA1c were determined using a bench top analyser (Quo-lab HbA1c POC, EKF Diagnostics,
Penarth, UK).

210

211 Data and Statistical Analyses

Data were analysed using the statistical package for social sciences (SPSS version 24). Residuals for the outcomes were explored using histograms. Normally distributed data are presented as the arithmetic mean (95% CIs). Skewed data underwent natural logtransformation and were back transformed which gave a similar variance to non-logged data and a reasonable estimate which is presented as the geometric mean (95% CI) (4). Logtransformed data are presented as the ratio of geometric means and 95% CIs for the ratio difference between geometric means (5). An effect size of 0.2 was considered the minimal value for a meaningful difference, 0.5 for moderate and 0.8 for large (11). Statistical
significance was accepted as P<0.05.

221 Physical and behavioural characteristics were compared using linear mixed models (LMM) with waist circumference category (CO vs LE) and ethnicity (WE vs. SA) as fixed 222 factors. Blood and ex vivo migration variables were compared using LMM with the same fixed 223 224 factors mentioned above. These variables were then compared in CO only using LMM with day (day 1 vs day 2) and ethnicity as fixed factors. Chemokine receptor expression was 225 compared before (baseline) and after the ex vivo assay (tethered and migrated) using LMM 226 with stage (baseline vs migrated vs tethered) as a fixed factor. Adjusted models were performed 227 to account for differences in moderate-vigorous PA (MVPA) and daily steps between CO and 228 LE. From here on adjustment refers to the adjusted model for daily steps and MVPA, and no 229 difference refers to a statistical difference. 230

231

232 **Results**

233 Cross-sectional comparison of CO vs. LE

Participant characteristics are displayed in Table 1. CO participants demonstrated higher BMI, waist circumference, hip circumference, waist-to-hip ratio, body fat percentage, and lean mass than the LE group (ES \geq 2.30, P \leq 0.001). CO demonstrated lower daily steps and MVPA (ES \geq 1.47, P \leq 0.001).

There were no differences in participant characteristics between WE and SA within or between CO and LE groups. Due to low numbers of participants and inconclusive data on ethnic differences between CO and LE participants for the remaining variables, ethnic data are not presented throughout the rest of the manuscript. 242

243 [Insert Table 1 here]

244

245	Metabolic	markers

Unadjusted fasting metabolic marker concentrations are presented in Table 1. Concentrations of HDL were lower (ES=1.22, P=0.003), and concentrations of TC, LDL, TAG, glucose, CRP, and NEFA were higher in CO vs LE (ES \ge 0.93, P \le 0.025). There was no difference for HbA1c (ES=0.43, P=0.100). Adjustment eliminated the difference for TC, TAG, glucose, and NEFA (P \ge 0.101). No difference for HbA1c remained (P=0.265). HDL was still lower, and LDL and CRP remained higher in CO vs. LE (P \le 0.048).

252

253 <u>Monocyte phenotype in blood</u>

A representative gating strategy for monocyte subsets and their respective chemokine 254 receptor expression are presented in Figure 1. Unadjusted fasting blood monocyte subset 255 256 concentrations are presented in Table 1. Higher concentrations of monocytes, CM, IM, CCR2+ monocytes, and CCR2+CM subsets were seen in CO vs. LE (ES≥0.90, P≤0.037). No 257 differences for NCM, CCR5+, and CX3CR1+ monocytes were seen (ES≤0.77, P≥0.066). 258 Adjustment eliminated all differences for monocyte subset concentrations (P≥0.155). There 259 were no differences in the unadjusted or adjusted models for the percentages of monocyte 260 261 subsets (P≥0.154).

262

263 [Insert Figure 1 here]

264

265 <u>Absolute and relative *ex vivo* migration</u>

Unadjusted and adjusted absolute migrated monocyte subsets are presented in Figure 2 panel A. Higher absolute migration of monocytes, CM, IM, NCM, CCR2+ monocytes, CCR2+CM, and CX3CR1+monocytes were seen in CO vs. LE (ES \geq 0.90, P \leq 0.046). No difference was seen for the absolute migration of CCR5+ monocytes (ES=0.02, P=0.953). Adjustment maintained higher absolute migration of monocytes, CM, CCR2+ monocytes, and CCR2+ CM subsets (P \leq 0.023), eliminated the differences for IM, NCM, and CX3CR1+ monocytes (P \geq 0.465), and maintained no difference for CCR5+ monocytes (P=0.763).

Unadjusted and adjusted percentages of migrated monocyte subsets are presented in Figure 2 panel B. Higher relative migration of CX3CR1+ monocytes were seen in CO vs. LE (ES=1.01, P=0.017). No difference was seen for the relative migration of the other subsets (ES \leq 0.85, P \geq 0.058). Adjustment eliminated the difference for CX3CR1+ monocytes (P=0.091), but revealed higher relative migration of monocytes, CM, CCR2+ monocytes, and CCR2+CM in CO (P \leq 0.040).

279

281

282 Absolute and relative ex vivo tethering

Unadjusted and adjusted absolute tethered monocyte subsets are presented in Figure 3 panel A. Higher absolute tethering of monocytes, CM, IM, NCM, CCR2+ monocytes, and CX3CR1+ monocytes were seen in CO vs. LE (ES \ge 0.92, P \le 0.029). No difference was seen for CCR5+ monocytes (ES=0.65, P=0.125). Adjustment maintained higher absolute tethering

^{280 [}Insert Figure 2 here]

of monocytes, CM, NCM, CCR2+ monocytes, and CCR2+CM in CO vs. LE ($P \le 0.039$). The differences for IM and CX3CR1M were eliminated ($P \ge 0.070$), and there was still no statistical difference for CCR5+ monocytes (P=0.238).

Unadjusted and adjusted percentages of tethered monocyte subsets are presented in Figure 3 panel B. Higher relative tethering of IM, NCM, and CX3CR1+ was seen in CO vs. LE (ES \ge 1.11, P \le 0.008). No difference was seen for the relative tethering of other subsets (ES \le 0.78, P \ge 0.057). Adjustment maintained a higher relative tethering of IM, NCM, and CX3CR1+ monocytes (P \le 0.003), and revealed a higher relative tethering for monocytes, CM, CCR2+ monocytes, and CCR2+CM in CO vs. LE (P \le 0.019). No difference was maintained for CCR5+ monocytes (P=0.437).

297

298 [Insert Figure 3 here]

299

300 <u>Chemokine receptor expression</u>

Unadjusted expression of CCR2, CCR5, and CX3CR1 on total monocytes between CO and LE at baseline, and at stages of the *ex vivo* assay (baseline, tethered and migrated) are presented in Table 2. No difference for CCR2, CCR5, or CX3CR1 at baseline was found in the unadjusted (ES \leq 0.82, P \geq 0.060) or adjusted model (P \geq 0.081). When comparing CO vs. LE, no central obesity x stage interactions for CCR2, CCR5, or CX3CR1 were detected for the unadjusted (ES \leq 0.34, P \geq 0.743) or adjusted (P \geq 0.612) models.

To explore changes in chemokine receptor expression during different stages of the *ex* vivo assay in isolation, we evaluated changes in both groups combined. Unadjusted expression of CCR2, CCR5, and CX3CR1 on total monocytes between stages of the assay for CO and LE combined (N=24) revealed lower CCR2 receptor expression on migrated (mean difference = -610, 95% CI, -1023 to -196) and tethered (mean difference = -644, 95% CI, -1058 to -231) monocytes compared to baseline. No difference was found between the other stages for the monocyte subsets (ES \leq 0.41, P \geq 0.073). Adjustment maintained the differences for CCR2 (P \leq 0.011), CCR5 and CX3CR1 receptor expression (P \geq 0.083).

315

316 [Insert Table 2 here]

317

318 Walking exercise intervention

319 <u>Physiological Responses</u>

Participants walked at 59% (SD=3) $\dot{V}O_2$ peak and consumed 1.87 (SD=0.67) litres of O₂ per minute. Average heart rate was 126 beats per minute (SD=9) and a subjective rating of perceived exertion score of 12 (SD=2).

323

324 Metabolic and monocyte phenotype in blood

- 325 Unadjusted concentrations of TAG, NEFA, and CRP were similar between day 1 and day 2
- 326 (ES≤0.33, P≥0.481). Unadjusted concentrations of glucose were lower on day 2 (ES=1.43,
- 327 P=0.003). Adjustment maintained no difference for TAG, NEFA, and CRP between day 1 and
- day 2 ($P \ge 0.457$), and lower concentrations of glucose on day 2 (P = 0.006).
- 329 Fasting concentrations of monocyte subsets and monocytes positive or CCR2, CCR5, and
- 330 CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES≤0.21, P≥0.653) and

adjusted models (P \ge 0.615). There were no differences in the percentages of monocyte subsets for the unadjusted (ES \le 0.23, P \ge 0.600) and adjusted models (P \ge 0.533).

333

334 Absolute and relative ex vivo migration and tethering

The number of migrated monocyte subsets were similar between day 1 and day 2 for the unadjusted (ES ≤ 0.37 , P ≥ 0.446) and adjusted models (P ≥ 0.363). Percentage of migrated monocyte subsets and monocytes positive for CCR2, CCR5M, and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES ≤ 0.45 , P ≥ 0.314) and adjusted models (P ≥ 0.244).

The number of tethered monocyte subsets were similar between day 1 and day 2 for the unadjusted (ES \leq 0.49, P \geq 0.293) and adjusted models (P \geq 0.319). The percentage of tethered monocyte subsets and monocytes positive for CCR2, CCR5M and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES \leq 0.58, P \geq 0.130) and adjusted (P \geq 0.147) models.

345

346 <u>Chemokine receptor expression</u>

Unadjusted expression of CCR2, CCR5, and CX3CR1 on day 1 and day 2, and at different stages of the *ex vivo* assay (baseline, tethered and migrated) are presented in Table 3. No difference between days was seen for CCR2, CCR5, or CX3CR1 expression (ES \leq 0.17, P \geq 0.718). Adjustment maintained no difference (P \geq 0.749). No day x stage interactions were detected for the unadjusted (ES \leq 0.15, P \geq 927) or adjusted (P \geq 878) models.

352

353 [Insert Table 3 here]

354 **Discussion**

The results of the current study provide novel evidence for the independent associations 355 of adiposity, physical activity levels, and single sessions of exercise on aspects of obesity-356 driven monocyte tethering and migration. By validating a dynamic methodological approach 357 that quantified the movement of monocytes towards chemokine-rich serum under conditions 358 359 of physiological blood flow, for the first time we established that both *absolute* and *relative* monocyte tethering and migration were greater in CO vs. LE for most monocyte subsets. 360 Higher levels of physical activity (i.e. daily MVPA and step count) were associated with 361 reduced absolute tethering and migration of CD16 expressing monocytes, but not classical 362 monocytes. Under controlled laboratory conditions, a single bout of walking exercise had no 363 impact on monocyte tethering and migration in males with CO 16 hours after the exercise bout. 364 Taken together, these data indicate that regular physical activity was associated with reduced 365 movement of pro-inflammatory monocytes towards chemokines in males with CO. These 366 findings have important implications for the potential anti-inflammatory effects of physical 367 activity independent of weight status. 368

The movement of immune cells (e.g. monocytes, lymphocytes, and dendritic cells) 369 from blood into vascular walls and metabolically active tissues is enhanced by increased 370 adiposity (44). In particular, monocytes are metabolically plastic cells that can migrate into 371 tissues (i.e. CM) and transendothelial sites (i.e. IM and NCM) to form macrophages in 372 individuals with CO, which increases the risk of several chronic diseases (25, 31). The 373 enhanced movement of monocytes stimulates haematopoiesis in the bone marrow, resulting in 374 higher numbers of these monocytes in the circulation (30). In the present study, most 375 376 circulating monocyte counts were higher in CO vs. LE (Table 1). To subsequently determine physiologically relevant differences in ex vivo monocyte tethering and migration (absolute 377 change), our method examined how monocytes at this concentration tethered and migrated 378

towards a fixed chemokine stimulus under conditions mimicking physiological blood flow 379 (45). This approach controlled for important systemic variables that are known to differ 380 between individuals who are lean and obese, e.g. the number of monocyte subsets and receptors 381 that govern their adhesion and subsequent migration. Our data demonstrate that absolute total 382 monocyte tethering and migration were greater in CO vs LE, independent of levels of physical 383 activity (Figures 2A and 3A). Within the monocyte pool, CM and those expressing CCR2 384 385 appeared to be the main subsets driving these group differences. This supports previous work indicating that monocytes expressing CCR2 have the greatest propensity to migrate via 386 387 chemokine gradients towards inflamed tissues (31), and specifically adipose tissue in individuals with obesity (36). Importantly, adjustments for MVPA and step count removed 388 group differences for the absolute migration of IM, NCM and monocyte expressing CX3CR1. 389 390 Group differences for the absolute tethering of IM and CX3CR1+ monocytes were also lost. 391 CD16 expressing monocytes, such as IM primarily migrate towards transendothelial sites and are a major source of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α), 392 interleukin (IL)-1β, IL-6, and IL-8 (46). These results therefore highlight an association 393 between higher physical activity levels and reduced IM tethering and migration. Adjustments 394 for PA also mitigated group differences in the blood concentration of IM, suggesting that 395 reduced tethering and migration may be driven by a reduced number of cells within the 396 circulation. This provides some support for other work highlighting reduced percentages of IM 397 398 and NCM after structured exercise training in individuals who are lean (42) and obese (13). In addition, an impact of PA on the number of CX3CR1+ monocytes that tethered and migrated 399 also indicates reduced monocyte adhesion. These results therefore highlight that although PA 400 does not impact the migration of CM with high tissue homing-potential, it is associated with 401 reduced transendothelial migration of IM and NCM populations, a key step in the development 402 of chronic systemic inflammation. 403

In addition to the notable *absolute* differences between groups, *relative* tethering and 404 migration of most monocyte subsets were also higher in CO vs. LE (Figures 2B and 3B). This 405 406 suggests that differences at the cellular level are also important in driving monocyte migration. With regards to chemokine receptors however, we observed no difference in the protein 407 expression of CCR2, CCR5, and CX3CR1 between LE and CO. Previous studies conducted in 408 females with obesity have reported higher expression of CCR2, CCR5, and CX3CR1 on 409 410 monocytes at both the mRNA (15) and protein level (15, 33). Despite similar screening (i.e. no metabolic disease) and anthropometric measures in these studies, serum CRP levels were 411 412 markedly higher (mean: 7.0 (33) and 9.8 (15) mg/L) when compared to the present study (mean: 1.8 mg/L), indicating a much more heightened state of systemic inflammation. 413 Furthermore, these studies noted differences in the relative numbers of blood monocyte subsets 414 between obese vs. lean (i.e. higher percentage of IM), also indicative of systemic inflammation. 415 416 To further interrogate our data, we explored changes in chemokine receptor expression after ex vivo tethering and migration between CO and LE (Table 2). After binding to their cognate 417 chemokines, chemokine receptors are typically desensitized and internalized via endocytosis 418 to limit the magnitude and duration of the stimulus (21). In support of this, CCR2 receptor 419 expression was lower after ex vivo tethering and migration. This indicates that receptor 420 internalisation may have played a role in driving monocyte migration in our ex vivo model; 421 however, no statistical differences were noted between CO and LE. Collectively, we can 422 423 therefore speculate that in individuals with CO that have relatively low levels of systemic inflammation, chemokine receptor expression and internalisation does not explain the higher 424 relative rates of tethering and migration vs. lean individuals. Although not measured in our 425 study, this again suggests that properties of monocyte adhesion could explain these differences. 426 Importantly, there were no associations between physical activity and chemokine receptor 427 expression and internalisation. 428

On the same day as the cross-sectional analysis, CO undertook a singlebout of walking 429 exercise under controlled lifestyle and dietary conditions, with ex vivo monocyte tethering and 430 migration measured the morning afterwards (i.e. 24 hours after the first sample). This 431 experimental model has been previously used in obese populations to demonstrate that prior 432 brisk walking can lower postprandial lipaemia (PPL) after intake of a high fat meal (23). Acute 433 elevations in soluble inflammatory markers (i.e. IL-6 and TNF- α) also accompany PPL; 434 435 however there is limited evidence to support the notion that prior walking reduces PPLassociated inflammation (8, 41). By applying our ex vivo assay to this experimental model, we 436 437 explored the impact of prior walking on changes in functional immunity for the first time. We report no differences in circulating monocyte counts, ex vivo monocyte tethering and migration, 438 or chemokine receptor expression (absolute or migration mediated loss, Table 3) after prior 439 440 brisk walking. Previous studies have reported elevated chemokine concentrations in the 441 circulation after exercise (12, 17, 39). It has been suggested that these changes may drive internalisation of chemokine receptors that lower their surface expression over time (24), in 442 turn reducing migration. Despite a recent study reported lower expression of CCR2 on IM 443 immediately after (but not 1h and 2h after) a bout of cycling exercise (6), our data importantly 444 highlight that any acute loss of monocyte chemokine receptor expression was not maintained 445 the morning afterwards. 446

The current results support an association between higher levels of physical activity and reduced migration of specific pro-inflammatory monocytes, as well as important markers of metabolic health (TC, TAG, glucose and NEFA, Table 1). Epidemiological studies report that being more physically active is associated with reduced blood markers of inflammation (26, 27); however it is unclear whether this is directly related to changes in adiposity (16, 24). Our data provide further support for the notion that the movement of specific populations of monocytes (i.e. IM) may be reduced independently of adiposity (2, 13). Given that our data reveals no short-term impact of walking exercise on monocyte migration, future studies should
examine the impact of regular PA on immune cell migration, in the context of energy balance
and weight loss.

We must acknowledge some limitations to the current study. Our ex vivo method when 457 coupled to flow cytometry permits much more detailed phenotypic analysis of monocyte sub-458 populations; however, analysis of rarer cell populations was challenging. For example, we did 459 460 not present data on chemokine receptor positive cells for IM and NCM due to low event counts during acquisition (< 500). This was a limitation of the number of monocytes that tethered and 461 migrated, which was lower in LE vs. CO. Repeated blood measures after the bout of walking 462 463 exercise collected before 16 hours post-exercise may have been beneficial, but given assay logistics and ethical considerations of blood volume, this was not possible. Finally, we must 464 acknowledge that the walking intervention was only carried out in CO, and therefore a direct 465 comparison with LE was not made; however, this would not have influenced the interpretations 466 of the present study. 467

468

469 Conclusion

The current data adds to the growing body of evidence highlighting that central 470 adiposity is a major driver of monocyte migration in individuals with CO. By exploring both 471 absolute and relative changes, we established that the concentration of monocytes in the 472 circulation of middle-aged males with CO may drive their heightened tethering and migration 473 towards an ex vivo chemokine stimulus, compared to lean individuals. Cellular level changes 474 475 likely also contribute (e.g. adhesion); however, we highlight that this was not chemokine receptor-mediated in individuals with a relatively low level of systemic inflammation. Regular 476 physical activity did not impact the capacity of classical monocytes to tether and migrate; 477 478 however, specific pro-inflammatory subsets were reduced by MVPA and step count. Further research is needed to establish the significance of these changes by monitoring individuals whoare lean and centrally obese over time and under controlled lifestyle interventions.

481

482 Funding

This research was supported by the National Institute for Health Research (NIHR) Leicester Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

486

487 Author Contribution Statement

AJW, MR, AET, DJS, and NCB were involved in the conception and design of the study. AW and NCB developed the laboratory methods. MR undertook recruitment and participant testing. AJW, JC and MR carried out all data acquisition. MR, AJW, and NCB carried out statistical analysis and data presentation. Drafting of the article for important intellectual content was undertaken by AJW and MR and all authors undertook revision and final approval of the manuscript.

494

495 **Conflict of Interest**

496 None of the authors declare a conflict of interest.

497

498 Current Author Affiliations (*)

499 AW: School of Sport, Exercise & Rehabilitation Sciences, College of Life & Environmental

500 Sciences, University of Birmingham, B15 2TT

501 JC: Department of Health, University of Bath, BAth, BA2 7AY

502

503

504 **Table Legends**

Table 1. Groups were ethnicity matched, with a 1:1 ratio of WE and SA males. Data were analysed using linear mixed models with the ethnic specific waist circumference category (CO vs LE) as a fixed factor. Values are mean and standard deviations. 95% CI, 95% confidence interval of the difference between the groups. *Main effect of central obesity (P \leq 0.032). ¹Body fat % determined by bioelectrical impedance. ^aFor HbA1c, n=10 for the LE group.

510 MVPA, moderate to vigorous PA; TC, total cholesterol; HDL, high-density lipoprotein 511 cholesterol; LDL, low-density lipoprotein cholesterol; TAG, triacylglycerol; HbA1c, glycated 512 haemoglobin; CRP, C-reactive protein; NEFA, non-esterified fatty acids; CM, classical 513 monocytes; IM, intermediate monocytes; NCM, non-classical monocytes; CCR, C-C 514 chemokine receptor; CX3CR1, CX3C chemokine receptor-1

515

Table 2. Values for CCR2 and CX3CR1 are arithmetic means (95% confidence intervals) and
between group differences are absolute differences (95% confidence intervals). Values for
CCR5 are geometric means (95% confidence intervals) and between group differences are 95%
confidence limits of the ratio difference between geometric means.

520

Table 3: Values for CCR2 and CX3CR1 are arithmetic means (95% confidence intervals) and
between group differences are absolute differences (95% confidence intervals). Values for
CCR5 are geometric means (95% confidence intervals) and between group differences are 95%
confidence limits of the ratio difference between geometric means.

525

526

527 Figure Legends

Figure 1: Representative gating strategy for monocyte subsets and their respective chemokine 528 receptor expression levels. The example presented includes data from a male with central 529 obesity on Day 1 of the study. The example data indicate how monocyte subsets and then 530 CCR2+ classical monocytes were determined: (A) Monocytes were gated on forward light 531 532 scatter (FSC) vs. side light scatter (SSC); (B) doublets were discriminated using FSC-Area vs. FSC-Height; (C) non-viable cells were excluded using a 7-AAD; (D) monocyte subsets were 533 identified using a CD14-Area vs. CD16-Area bivariate plot; (E) the positive gate for CCR2+ 534 classical monocytes was determined using a fluorescence minus one control; (F) CCR2+ 535 classical monocytes (%) were determined on CCR2-Area vs. SSC-Area. 536

7-AAD, 7-Aminoactinomycin D; CM, classical monocytes; IM, intermediate monocytes;
NCM, non-classical monocytes; CCR2+ CM, C-C chemokine receptor-2+ classical
monocytes.

540

Figure 2. Unadjusted and adjusted absolute (Panel A) and relative (Panel B) monocyte subset migration between men with central obesity (CO, N=12) and men who were lean (LE, N=12). Linear mixed models were used with the ethnic specific waist circumference category (CO vs LE) as a fixed factor (unadjusted). The adjusted model accounted for group differences in moderate-vigorous physical activity (MVPA) and daily steps. Data are presented as the arithmetic / geometric mean (95% confidence intervals).

CM, classical monocytes; CCR2+M, C-C chemokine receptor-2+ monocytes; CCR2+CM, CC chemokine receptor-2+ classical monocytes; IM, intermediate monocytes; NCM, nonclassical monocytes; CCR5+M, C-C chemokine receptor-5+ monocytes; CX3CR1+M, CX3C
chemokine receptor-1+ monocytes.

551 *Main effect of central obesity ($P \le 0.046$).

553	Figure 3. Unadjusted and adjusted absolute (Panel A) and relative (Panel B) monocyte subset
554	tethering between men with central obesity (CO, N=12) and men who were lean (LE, N=12).
555	Linear mixed models were used with the ethnic specific waist circumference category (CO vs
556	LE) as a fixed factor (unadjusted). The adjusted model accounted for group differences in
557	moderate-vigorous physical activity (MVPA) and daily steps. Data are presented as the
558	arithmetic / geometric mean (95% confidence intervals).
559	CM, classical monocytes; CCR2+M, C-C chemokine receptor-2+ monocytes; CCR2+CM, C-
560	C chemokine receptor-2+ classical monocytes; IM, intermediate monocytes; NCM, non-
561	classical monocytes; CCR5+M, C-C chemokine receptor-5+ monocytes; CX3CR1+M, CX3C
562	chemokine receptor-1+ monocytes.
563	*Main effect of central obesity ($P \le 0.039$).
564	
565	
566	
567	
568	
569	
570	
571	
572	

573 **References**

574

- Alberti KGMM, Zimmet P, Shaw J. International Diabetes Federation: a consensus
 on Type 2 diabetes prevention. *Diabet Med* 24: 451–63, 2007. doi: 10.1111/j.1464 5491.2007.02157.x.
- Barry JC, Simtchouk S, Durrer C, Jung ME, Little JP. Short-Term Exercise
 Training Alters Leukocyte Chemokine Receptors in Obese Adults. *Med Sci Sports Exerc* 49: 1631–1640, 2017. doi: 10.1249/MSS.00000000001261.
- 3. Bishop NC, Walker GJ, Gleeson M, Wallace F a, Hewitt CR a. 7. Human T
 lymphocyte migration towards the supernatants of human rhinovirus infected airway
 epithelial cells: influence of exercise and carbohydrate intake. *Exerc Immunol Rev* 15:
 127–44, 2009.
- Bland JM, Altman DG. Transformations, means, and confidence intervals. *BMJ* 312:
 1079, 1996. doi: 10.1136/bmj.312.7038.1079.
- 5. Bland JM, Altman DG. The use of transformation when comparing two means. *BMJ*312: 1153, 1996. doi: 10.1136/bmj.312.7039.1153.
- 589 6. Blanks AM, Wagamon TT, Lafratta L, Sisk MG, Senter MB, Pedersen LN,
 590 Bohmke N, Shah A, Mihalick VL, Franco RL. Impact of physical activity on
 591 monocyte subset CCR2 expression and macrophage polarization following moderate
 592 intensity exercise. *Brain, Behav Immun Heal* 2: 100033, 2020. doi:
 593 10.1016/j.bbih.2019.100033.
- 594 7. Borg G. The psychphysical bases of peceived exertion. *Med Sci Sport Exerc* 14: 377–
 595 381, 1982. doi: 10.1249/00005768-198205000-00012.
- Brown M, McClean CM, Davison GW, Brown JCW, Murphy MH. Preceding
 exercise and postprandial hypertriglyceridemia: Effects on lymphocyte cell DNA
 damage and vascular inflammation. *Lipids Health Dis* 18: 1–12, 2019. doi:
 10.1186/s12944-019-1071-y.
- Buchanan CF, Verbridge SS, Vlachos PP, Rylander MN. Flow shear stress
 regulates endothelial barrier function and expression of angiogenic factors in a 3D
 microfluidic tumor vascular model. *Cell Adh Migr* 8: 517–524, 2014.
- Chomistek AK, Yuan C, Matthews CE, Troiano RP, Bowles HR, Rood J, Barnett
 JB, Willet WC, Rimm EB, D.R. BJ. Physical Activity Assessment with the
 ActiGraph GT3X and Doubly Labeled Water. *Med Sci Sport Exerc* 49: 1935–1944,
 2017.
- 607 11. Cohen J. Statistical Power Analysis for the Behavioural Sciences, 2nd Edition. 1988.
- 12. Crabb EB, Franco RL, Caslin HL, Blanks AM, Bowen MK, Acevedo EO. The

609 610		effect of acute physical and mental stress on soluble cellular adhesion molecule concentration. <i>Life Sci</i> 157: 91–96, 2016. doi: 10.1016/j.lfs.2016.05.042.
611 612 613 614	13.	de Matos MA, Garcia BCC, Vieira DV, de Oliveira MFA, Costa KB, Aguiar PF, Magalhães F de C, Brito-Melo GA, Amorim FT, Rocha-Vieira E. High-intensity interval training reduces monocyte activation in obese adults. <i>Brain Behav Immun</i> 80: 818–824, 2019. doi: 10.1016/j.bbi.2019.05.030.
615 616	14.	Després J-P, Lemieux I . Abdominal obesity and metabolic syndrome. <i>Nature</i> 444: 881–7, 2006. doi: 10.1038/nature05488.
617 618 619	15.	Devêvre EF, Renovato-Martins M, Clément K, Sautès-Fridman C, Cremer I, Poitou C . Profiling of the Three Circulating Monocyte Subpopulations in Human Obesity. <i>J Immunol</i> 194: 3917–3923, 2015. doi: 10.4049/jimmunol.1402655.
620 621 622	16.	Dungey M, Young HML, Churchward DR, Burton JO, Smith AC, Bishop NC. Regular exercise during haemodialysis promotes an anti-inflammatory leucocyte profile. <i>Clin Kidney J</i> 10: 813–821, 2017. doi: 10.1093/ckj/sfx015.
623 624 625 626	17.	Fico BG, Whitehurst M, Slusher AL, Mock JT, Maharaj A, Dodge KM, Huang C-J . The comparison of acute high-intensity interval exercise vs. continuous moderate- intensity exercise on plasma calprotectin and associated inflammatory mediators. <i>Physiol Behav</i> 183: 27–32, 2018. doi: 10.1016/j.physbeh.2017.10.015.
627 628 629 630	18.	Figueroa-Vega N, Marín-Aragón CI, López-Aguilar I, Ibarra-Reynoso L, Pérez- Luque E, Manuel Malacara J . Analysis of the percentages of monocyte subsets and ILC2s, their relationships with metabolic variables and response to hypocaloric restriction in obesity. <i>PLoS One</i> 15: 1–19, 2020. doi: 10.1371/journal.pone.0228637.
631 632	19.	Freedson PS, Melanson E, Sirard J. Calibration of the Computer Science and Applications, Inc. Accelerometer. <i>Med Sci Sport Exerc</i> 30: 777–781, 1998.
633 634 635	20.	Friedrich K, Sommer M, Strobel S, Thrum S, Blüher M, Wagner U, Rossol M . Perturbation of the monocyte compartment in human obesity. <i>Front Immunol</i> 10: 1–10, 2019. doi: 10.3389/fimmu.2019.01874.
636 637 638	21.	García Lopez MA , Aguado Martínez A , Lamaze C , Martínez-A. C , Fischer T . Inhibition of dynamin prevents CCL2-mediated endocytosis of CCR2 and activation of ERK1/2. <i>Cell Signal</i> 21: 1748–1757, 2009. doi: 10.1016/j.cellsig.2009.07.010.
639 640 641 642	22.	Garofallo SB, Portal VL, Markoski MM, Dias LD, Quadrosa AS De, Marcadenti A. Correlations between traditional and nontraditional indicators of adiposity, inflammation, and monocyte subtypes in patients with stable coronary artery disease. <i>J Obes</i> 2019, 2019. doi: 10.1155/2019/3139278.
643 644 645	23.	Gill JMR, Al-Mamari A, Ferrell WR, Cleland SJ, Packard CJ, Sattar N, Petrie JR, Caslake MJ. Effects of prior moderate exercise on postprandial metabolism and vascular function in lean and centrally obese men. <i>J Am Coll Cardiol</i> 44: 2375–82,

2004. doi: 10.1016/j.jacc.2004.09.035. 646 Gleeson M, Bishop NC, Stensel DJ, Lindley MR, Mastana SS, Nimmo MA. The 647 24. anti-inflammatory effects of exercise: mechanisms and implications for the prevention 648 and treatment of disease. Nat Rev Immunol 11: 607-615, 2011. 649 Gómez-Olarte S, Bolaños NI, Echeverry M, Rodríguez AN, Cuéllar A, Puerta CJ, 25. 650 Mariño A, González JM. Intermediate Monocytes and Cytokine Production 651 Associated With Severe Forms of Chagas Disease. Front Immunol 10: 1671, 2019. 652 doi: 10.3389/fimmu.2019.01671. 653 26. Hamer M, Hackett RA, Bostock S, Lazzarino AI, Carvalho LA, Steptoe A. 654 Objectively assessed physical activity, adiposity, and inflammatory markers in people 655 with type 2 diabetes. BMJ Open Diabetes Res Care 2: e000030, 2014. doi: 656 10.1136/bmjdrc-2014-000030. 657 Hamer M, Sabia S, Batty GD, Shipley MJ, Tabak AG, Singh-Manoux A, 27. 658 Kivimaki M. Physical activity and inflammatory markers over 10 years follow up in 659 men and women from the Whitehall II cohort study. Circulation 126: 928-933, 2012. 660 doi: 10.1038/jid.2014.371. 661 662 28. Henning AL, Venable AS, Vingren JL, Hill DW, McFarlin BK. Consumption of a high-fat meal was associated with an increase in monocyte adhesion molecules, 663 scavenger receptors, and Propensity to Form Foam Cells. Cytometry B Clin Cytom 94: 664 606-612, 2018. doi: 10.1002/cyto.b.21478. 665 666 29. Hyson DA, Paglieroni TG, Wun T, Rutledge JC. Postprandial lipemia is associated with platelet and monocyte activation and increased monocyte cytokine expression in 667 normolipemic men. Clin Appl Thromb Hemost 8: 147-55, 2002. doi: 668 10.1177/107602960200800211. 669 30. Idzkowska E, Eljaszewicz A, Miklasz P, Musial WJ, Tycinska AM, Moniuszko M. 670 The Role of Different Monocyte Subsets in the Pathogenesis of Atherosclerosis and 671 Acute Coronary Syndromes. Scand J Immunol 82: 163-173, 2015. doi: 672 10.1111/sji.12314. 673 Kapellos TS, Bonaguro L, Gemünd I, Reusch N, Saglam A, Hinkley ER, Schultze 31. 674 JL. Human monocyte subsets and phenotypes in major chronic inflammatory diseases. 675 Front Immunol 10: 1-13, 2019. doi: 10.3389/fimmu.2019.02035. 676 32. Kim CS, Park HS, Kawada T, Kim JH, Lim D, Hubbard NE, Kwon BS, Erickson 677 KL, Yu R. Circulating levels of MCP-1 and IL-8 are elevated in human obese subjects 678 and associated with obesity-related parameters. Int J Obes 30: 1347-1355, 2006. doi: 679 680 10.1038/sj.ijo.0803259. 33. Krinninger P, Ensenauer R, Ehlers K, Rauh K, Stoll J, Krauss-Etschmann S, 681 Hauner H, Laumen H. Peripheral Monocytes of Obese Women Display Increased 682 Chemokine Receptor Expression and Migration Capacity. J Clin Endocrinol Metab 99: 683

2500-2509, 2014. doi: 10.1210/jc.2013-2611. 684 Miller MA, Cappuccio FP. Cellular adhesion molecules and their relationship with 685 34. measures of obesity and metabolic syndrome in a multiethnic population. Int J Obes 686 30: 1176–1182, 2006. doi: 10.1038/sj.ijo.0803264. 687 Park HS, Park JY, Yu R. Relationship of obesity and visceral adiposity with serum 35. 688 concentrations of CRP, TNF-alpha and IL-6. Diabetes Res Clin Pract 69: 29-35, 2005. 689 doi: 10.1016/j.diabres.2004.11.007. 690 36. Pecht T, Haim Y, Bashan N, Shapiro H, Harman-Boehm I, Kirshtein B, Clément 691 K, Shai I, Rudich A. Circulating Blood Monocyte Subclasses and Lipid-Laden 692 Adipose Tissue Macrophages in Human Obesity. PLoS One 11: 1-14, 2016. doi: 693 694 10.1371/journal.pone.0159350. 695 37. Ratter JM, Rooijackers HMM, Jacobs CWM, de Galan BE, Tack CJ, Stienstra R. Hypoglycaemia induces recruitment of non-classical monocytes and cytotoxic 696 lymphocyte subsets in type 1 diabetes. *Diabetologia* 61: 2069–2071, 2018. doi: 697 10.1007/s00125-018-4683-2. 698 38. Saresella M, Marventano I, Calabrese E, Piancone F, Rainone V, Gatti A, 699 700 Alberoni M, Nemni R, Clerici M. A Complex Proinflammatory Role for Peripheral Monocytes in Alzheimer's Disease. J Alzheimer's Dis 38: 403-413, 2013. doi: 701 10.3233/JAD-131160. 702 703 39. Serviente C, Troy LM, de Jonge M, Shill DD, Jenkins NT, Witkowski S. Endothelial and inflammatory responses to acute exercise in perimenopausal and late 704 postmenopausal women. Am J Physiol Regul Integr Comp Physiol 311: R841-R850, 705 706 2016. doi: 10.1152/ajpregu.00189.2016. 40. Szczepanska-Sadowska E, Cudnoch-Jedrzejewska A, Ufnal M, Zera T. Brain and 707 cardiovascular diseases: common neurogenic background of cardiovascular, metabolic 708 and inflammatory diseases. J Physiol Pharmacol 61: 509-21, 2010. 709 41. Teeman CS, Kurti SP, Cull BJ, Emerson SR, Haub MD, Rosenkranz SK. 710 Postprandial lipemic and inflammatory responses to high-fat meals: A review of the 711 roles of acute and chronic exercise. Nutr Metab 13: 1-14, 2016. doi: 10.1186/s12986-712 016-0142-6. 713 42. Timmerman KL, Flynn MG, Coen PM, Markofski MM, Pence BD. Exercise 714 training-induced lowering of inflammatory (CD14+CD16+) monocytes: a role in the 715 anti-inflammatory influence of exercise? J Leukoc Biol 84: 1271-8, 2008. doi: 716 10.1189/jlb.0408244. 717 43. Trabulo M, Mendes M, Mesquita A, Seabra-Gomes R. Does the Modified Bruce 718 Protocol Induce Physiological Stress Equal to That of the Bruce Protocol? Rev Port 719 Cardiol 13: 753-60, 1994. 720

721 44. Trim W, Turner JE, Thompson D. Parallels in immunometabolic adipose tissue dysfunction with ageing and obesity. Front Immunol 9, 2018. doi: 722 10.3389/fimmu.2018.00169. 723 45. Tsubota Y, Frey JM, Raines EW. Technical Advance: Novel ex vivo culture method 724 for human monocytes uses shear flow to prevent total loss of transendothelial 725 diapedesis function. J Leukoc Biol 95: 191-195, 2014. doi: 10.1189/jlb.0513272. 726 46. Wong KL, Tai JJ-Y, Wong W-C, Han H, Sem X, Yeap W-H, Kourilsky P, Wong 727 S-C. Gene expression profiling reveals the defining features of the classical, 728 729 intermediate, and nonclassical human monocyte subsets. Blood 118: e16-e31, 2011. doi: 10.1182/blood-2010-12-326355. 730 731

732