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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.

Introduction

Cardiovascular, metabolic, and neurological diseases are threatening to reach epidemic proportions, presenting an enormous health, economic and societal challenge. Common to all these conditions is their chronic inflammatory aetiology (40). Increased central adiposity is known to significantly elevate the risk of developing these diseases, with a heightened migration of immune cells from blood into various tissues, namely monocytes into adipose 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 (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 migration in individuals with central obesity (CO) drives a dysfunctional interaction that promotes metabolic dysfunction and an increased risk of chronic disease (44).

This risk of developing cardiovascular, metabolic, and neurological diseases is exaggerated by multiple factors, including physical inactivity and diet (41). For the latter, prolonged elevations of triglycerides in the bloodstream after high fat meals, termed postprandial lipaemia (PPL), is a known independent predictor of cardiovascular disease (41). There is evidence that PPL is also associated with higher systemic inflammation (8, 41), with monocyte activation (29), adhesion and propensity for foam cell formation higher following a high fat meal (28). 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), metabolic (37), and neurological health (38). Recent evidence has documented that individuals 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 composition of the three monocyte sub-populations; e.g. decreased classical (CM: CD14++CD16-), higher intermediate (IM: CD14++CD16+), and higher non-classical (NCM:

CD14+CD16++) monocytes (15, 20, 33). A reduced percentage of CM occurs as a result of preferential tissue migration in response to cues from inflamed tissues, whereas IM and NCM proportions increase due to their role in patrolling the circulation by adhering to the 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 weight, body mass index, fat mass, and insulin insensitivity on total monocytes [6]. Chemokine 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. Obesity-associated inflammation also causes an increase in circulating cellular adhesion molecule concentration (34), which enhances the potential for monocytes to tether to the surface of these cells and tissues. A higher number of circulating monocytes with a higher capacity to tether and migrate therefore generates a favourable gradient for the progressive 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 economic and social impact of managing and treating these diseases, cost-effective interventions to prevent monocyte tethering and migration are urgently required.

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 chronic inflammatory disease, compared with sedentary individuals (16, 24). Studies in adults 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 total monocytes (2). It is well documented that single sessions of exercise result in acute elevations in chemokine concentrations in blood plasma (12, 17, 39).It has been suggested that 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,

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 (6). When considering how this transient internalisation of CCR2 on monocytes may translate into chronic changes in expression that have been reported separately (2), the impact of PPL-induced inflammation must also be considered, particularly in individuals with CO as this is associated with unfavourable metabolic and inflammatory profiles (14). A large body of 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 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 sessions of exercise, particularly in the PPL state, independently impact chemokine expression 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.

We, and others, have previously quantified the *ex vivo* migratory capacity of peripheral blood mononuclear cells (PBMCs) towards a fixed chemokine gradient over time (3, 33). Using this method, there is evidence that monocyte migration is greater in individuals who are obese 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 in healthy individuals (3). To our knowledge, there is no data indicating that regular physical 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 by only quantifying the number of cells that migrate in response to a fixed chemokine gradient (33), rather than their phenotype, as done in our laboratory previously with lymphocytes (3). 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

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.

Materials and Methods

Participants

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

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.

Experimental Procedures

All participants first visited the laboratory for screening of height and weight using a 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, Washington, USA) whilst adhering to established measurement guidelines (1). An assessment of peak oxygen uptake ($\dot{V}O_2$ peak) was undertaken in CO only using the modified Bruce 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 perceived exertion scale (7). At the end of the first visit, participants were fitted with an accelerometer (*ActiGraph GT3X, ActiGraph corporation, Florida, USA*) to quantify levels of habitual physical activity for a period of 7 days. Data were analysed over 15 second epochs using specialised software (*Actilife, Actigraph corporation, Florida, USA*). Accelerometer data were screened for wear time using standard methods (10). Time spent in a defined intensity of activity was determined by summing together counts per minute and categorising this based on widely used cut points (19).

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

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 400*g* for 30 minutes at 21 degrees (brake off) and the PBMC layer aspirated and washed in PBS and RPMI. EDTA monovettes were centrifuged at 3500*g* 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).

Ex vivo Migration Assay

The *ex vivo* migration assay was adapted from previously published protocols published in our laboratory (3) and others (33), as well as internal validation experiments. Whole blood cell counts were initially performed using a haematology analyser (*Yumizen H500, Horiba, Northampton, UK*) to determine the circulating monocyte count (≈ 0.2 – 1.0 x 10^6/mL). PBMC counts were then determined by using CountBright™ Absolute Counting Beads on a BD C6 Accuri Flow Cytometer (*Becton Dickinson, Oxford, UK*). The seeding density of monocytes 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 duplicate to fibronectin-coated polyester (PET) inserts (5μm pore size) and placed into 6-well non-tissue culture treated plates containing 3mL of heat-inactivated 10% fetal bovine serum (FBS) or RPMI (background migration control). Monocytes were then allowed to migrate for 3 hours at 37 degrees (5% CO_2). To further generate conditions that closely mimic moderate physiological flow within the circulation (3.2 dyn/cm^3) (9) and to maintain monocyte phenotype, the PBMC suspension was oscillated on a 2-dimensional orbital shaker at 77 rpm 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 cells were removed from the upper side of the PET insert, and this was then washed twice with 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), followed by five washes with D-PBS (1ml). The underside of the PET inserts and the wells of

171 the tissue culture plate beneath were treated identically to collect cells that had *migrated* across
172 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

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

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

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.

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).

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 log-transformation 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). Log-transformed 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 $P<0.05$.

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 factors. Blood and *ex vivo* migration variables were compared using LMM with the same fixed 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 compared before (baseline) and after the *ex vivo* assay (tethered and migrated) using LMM with stage (baseline vs migrated vs tethered) as a fixed factor. Adjusted models were performed to account for differences in moderate-vigorous PA (MVPA) and daily steps between CO and LE. From here on adjustment refers to the adjusted model for daily steps and MVPA, and no difference refers to a statistical difference.

Results

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

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

252

253 Monocyte phenotype in blood

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

262

263 *[Insert Figure 1 here]*

264

265 Absolute and relative *ex vivo* migration

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

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

279

280 *[Insert Figure 2 here]*

281

282 Absolute and relative *ex vivo* tethering

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

of monocytes, CM, NCM, CCR2+ monocytes, and CCR2+CM in CO vs. LE (P≤0.039). The differences for IM and CX3CR1M were eliminated (P≥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≥1.11, P≤0.008). No difference was seen for the relative tethering of other subsets (ES≤0.78, P≥0.057). Adjustment maintained a higher relative tethering of IM, NCM, and CX3CR1+ monocytes (P≤0.003), and revealed a higher relative tethering for monocytes, CM, CCR2+ monocytes, and CCR2+CM in CO vs. LE (P≤0.019). No difference was maintained for CCR5+ monocytes (P=0.437).

[Insert Figure 3 here]

Chemokine receptor expression

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≤0.82, P≥0.060) or adjusted model (P≥0.081). When comparing CO vs. LE, no central obesity x stage interactions for CCR2, CCR5, or CX3CR1 were detected for the unadjusted (ES≤0.34, P≥0.743) or adjusted (P≥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≤0.41, P≥0.073). Adjustment maintained the differences for CCR2 (P≤0.011), CCR5 and CX3CR1 receptor expression (P≥0.083).

[Insert Table 2 here]

Walking exercise intervention

Physiological Responses

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

Metabolic and monocyte phenotype in blood

Unadjusted concentrations of TAG, NEFA, and CRP were similar between day 1 and day 2 (ES≤0.33, P≥0.481). Unadjusted concentrations of glucose were lower on day 2 (ES=1.43, P=0.003). Adjustment maintained no difference for TAG, NEFA, and CRP between day 1 and day 2 (P≥0.457), and lower concentrations of glucose on day 2 (P=0.006).

Fasting concentrations of monocyte subsets and monocytes positive or CCR2, CCR5, and CX3CR1 were similar between day 1 and day 2 for the unadjusted (ES≤0.21, P≥0.653) and

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

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≤0.49, P≥0.293) and adjusted models (P≥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≤0.58, P≥0.130) and adjusted (P≥0.147) models.

Chemokine receptor expression

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≤0.17, P≥0.718). Adjustment maintained no difference (P≥0.749). No day x stage interactions were detected for the unadjusted (ES≤0.15, P≥927) or adjusted (P≥878) models.

[Insert Table 3 here]

Discussion

The results of the current study provide novel evidence for the independent associations of adiposity, physical activity levels, and single sessions of exercise on aspects of obesity-driven monocyte tethering and migration. By validating a dynamic methodological approach that quantified the movement of monocytes towards chemokine-rich serum under conditions 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. Higher levels of physical activity (i.e. daily MVPA and step count) were associated with reduced absolute tethering and migration of CD16 expressing monocytes, but not classical monocytes. Under controlled laboratory conditions, a single bout of walking exercise had no impact on monocyte tethering and migration in males with CO 16 hours after the exercise bout. Taken together, these data indicate that regular physical activity was associated with reduced movement of pro-inflammatory monocytes towards chemokines in males with CO. These findings have important implications for the potential anti-inflammatory effects of physical activity independent of weight status.

The movement of immune cells (e.g. monocytes, lymphocytes, and dendritic cells) from blood into vascular walls and metabolically active tissues is enhanced by increased adiposity (44). In particular, monocytes are metabolically plastic cells that can migrate into tissues (i.e. CM) and transendothelial sites (i.e. IM and NCM) to form macrophages in individuals with CO, which increases the risk of several chronic diseases (25, 31). The enhanced movement of monocytes stimulates haematopoiesis in the bone marrow, resulting in higher numbers of these monocytes in the circulation (30). In the present study, most 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* change), our method examined how monocytes at this concentration tethered and migrated

towards a fixed chemokine stimulus under conditions mimicking physiological blood flow (45). This approach controlled for important systemic variables that are known to differ between individuals who are lean and obese, e.g. the number of monocyte subsets and receptors that govern their adhesion and subsequent migration. Our data demonstrate that *absolute* total monocyte tethering and migration were greater in CO vs LE, independent of levels of physical activity (Figures 2A and 3A). Within the monocyte pool, CM and those expressing CCR2 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 chemokine gradients towards inflamed tissues (31), and specifically adipose tissue in individuals with obesity (36). Importantly, adjustments for MVPA and step count removed group differences for the *absolute* migration of IM, NCM and monocyte expressing CX3CR1. Group differences for the *absolute* tethering of IM and CX3CR1+ monocytes were also lost. 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), interleukin (IL)-1β, IL-6, and IL-8 (46). These results therefore highlight an association between higher physical activity levels and reduced IM tethering and migration. Adjustments for PA also mitigated group differences in the blood concentration of IM, suggesting that reduced tethering and migration may be driven by a reduced number of cells within the circulation. This provides some support for other work highlighting reduced percentages of IM 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 also indicates reduced monocyte adhesion. These results therefore highlight that although PA does not impact the migration of CM with high tissue homing-potential, it is associated with reduced transendothelial migration of IM and NCM populations, a key step in the development of chronic systemic inflammation.

In addition to the notable *absolute* differences between groups, *relative* tethering and migration of most monocyte subsets were also higher in CO vs. LE (Figures 2B and 3B). This 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 expression of CCR2, CCR5, and CX3CR1 between LE and CO. Previous studies conducted in females with obesity have reported higher expression of CCR2, CCR5, and CX3CR1 on 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 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. Furthermore, these studies noted differences in the relative numbers of blood monocyte subsets between obese vs. lean (i.e. higher percentage of IM), also indicative of systemic inflammation. 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 chemokines, chemokine receptors are typically desensitized and internalized via endocytosis to limit the magnitude and duration of the stimulus (21). In support of this, CCR2 receptor expression was lower after *ex vivo* tethering and migration. This indicates that receptor internalisation may have played a role in driving monocyte migration in our *ex vivo* model; however, no statistical differences were noted between CO and LE. Collectively, we can 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 *relative* rates of tethering and migration vs. lean individuals. Although not measured in our study, this again suggests that properties of monocyte adhesion could explain these differences. Importantly, there were no associations between physical activity and chemokine receptor expression and internalisation.

On the same day as the cross-sectional analysis, CO undertook a singlebout of walking exercise under controlled lifestyle and dietary conditions, with *ex vivo* monocyte tethering and migration measured the morning afterwards (i.e. 24 hours after the first sample). This experimental model has been previously used in obese populations to demonstrate that prior brisk walking can lower postprandial lipaemia (PPL) after intake of a high fat meal (23). Acute elevations in soluble inflammatory markers (i.e. IL-6 and TNF-α) also accompany PPL; however there is limited evidence to support the notion that prior walking reduces PPL-associated inflammation (8, 41). By applying our *ex vivo* assay to this experimental model, we 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, or chemokine receptor expression (absolute or migration mediated loss, Table 3) after prior brisk walking. Previous studies have reported elevated chemokine concentrations in the 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 turn reducing migration. Despite a recent study reported lower expression of CCR2 on IM immediately after (but not 1h and 2h after) a bout of cycling exercise (6), our data importantly highlight that any acute loss of monocyte chemokine receptor expression was not maintained the morning afterwards.

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 coupled to flow cytometry permits much more detailed phenotypic analysis of monocyte sub-populations; however, analysis of rarer cell populations was challenging. For example, we did 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 migrated, which was lower in LE vs. CO. Repeated blood measures after the bout of walking 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 acknowledge that the walking intervention was only carried out in CO, and therefore a direct comparison with LE was not made; however, this would not have influenced the interpretations of the present study.

Conclusion

The current data adds to the growing body of evidence highlighting that central adiposity is a major driver of monocyte migration in individuals with CO. By exploring both absolute and relative changes, we established that the concentration of monocytes in the circulation of middle-aged males with CO may drive their heightened tethering and migration towards an *ex vivo* chemokine stimulus, compared to lean individuals. Cellular level changes 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 physical activity did not impact the capacity of classical monocytes to tether and migrate; 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 who are lean and centrally obese over time and under controlled lifestyle interventions.

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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.

Conflict of Interest

None of the authors declare a conflict of interest.

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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 ($P \leq 0.032$). [1]Body fat % determined by bioelectrical impedance. [a]For HbA1c, n=10 for the LE group.

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

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.

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.

Figure Legends

Figure 1: Representative gating strategy for monocyte subsets and their respective chemokine receptor expression levels. The example presented includes data from a male with central obesity on *Day 1* of the study. The example data indicate how monocyte subsets and then CCR2+ classical monocytes were determined: **(A)** Monocytes were gated on forward light 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 identified using a CD14-Area vs. CD16-Area bivariate plot; **(E)** the positive gate for CCR2+ classical monocytes was determined using a fluorescence minus one control; **(F)** CCR2+ classical monocytes (%) were determined on CCR2-Area vs. SSC-Area.

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

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

551 *Main effect of central obesity (P≤0.046).

552

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≤0.039).

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