Lower Resting and Exercise-Induced Circulating Angiogenic Progenitors and Angiogenic T-Cells in Older Men

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Running Head:

Lower resting and exercise-induced CACs in older adults

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Abstract

Ageing is associated with a dysfunctional endothelial phenotype, as well as reduced angiogenic capabilities. Exercise exerts beneficial effects on the cardiovascular system, possibly by increasing/maintaining the number and/or function of circulating angiogenic cells (CACs) that are known to decline with age. However, the relationship between cardiorespiratory fitness (CRF) and age related changes in frequency of CACs, as well as the exercise-induced responsiveness of CACs in older individuals has not yet been determined. One hundred and seven healthy male volunteers, aged 18-75 years, participated in the study 1. CRF was estimated using submaximal cycling ergometer test. Circulating endothelial progenitor cells (EPCs), angiogenic T-cells (T_{ANG}) and their CXCR4 cell surface receptor expression were enumerated by flow cytometry using peripheral blood samples obtained under resting conditions prior to the exercise test. Study 2 recruited 17 healthy males (8 young, 18-25 yrs; 9 older, 60-75yrs) and these participants undertook a 30-minute cycling exercise bout at 70% $\dot{V}O_2$max, with CACs enumerated pre- and immediately post-exercise. Age was inversely associated with both $CD34^+$ progenitor cells (r^2=-0.140, p=0.000) and T_{ANG} (r^2=-0.176, p=0.000) cells, as well as CXCR4-expressing CACs ($CD34^+$, r^2=-0.167, p=0.000; EPCs: r^2=-0.098, p=0.001; T_{ANG}, r^2=-0.053, p=0.015). However, after correcting for age, CRF had no relationship with either CAC subset. In addition, older individuals displayed attenuated exercise-induced increases in $CD34^+$ progenitor cells, T_{ANG}, $CD4^+$ T_{ANG}, and $CD8^+CXCR4^+$ T_{ANG} cells. Older men display lower CAC levels, which may contribute to increased CVD risk, and older adults display an impaired exercise-induced responsiveness of these cells.

New and Noteworthy:

Older adults display lower circulating progenitor cell and angiogenic T-cell counts compared to younger individuals, independent of cardiometabolic risk factors and cardiorespiratory fitness.

Older adults also display impaired exercise-induced mobilization of these vasculogenic cells.

Key Words

Age, fitness, exercise, progenitor cells, angiogenesis, T-cells

Introduction

Cardiovascular disease (CVD) has been estimated to contribute to nearly 30% of all deaths worldwide (22). Risk factors include smoking, hypertension, dyslipidemia, diabetes, physical inactivity and ageing (8, 21). As a result of medical advancements, the death rate from CVD has fallen in comparison to the 1970s (22); however, as a population we are becoming older and are living longer. Therefore age is becoming a more significant risk factor for developing CVD.

Endothelial dysfunction is an important step in the development of CVD. Endothelial function is impaired in those with CVD compared to healthy controls (11) with increased oxidative stress purported to be a possible mechanism, through reducing nitric oxide bioavailability (38). Advancing age is often characterized with a dysfunctional endothelium (4), leading us to believe that the age-related decline in

endothelial function may be an important mechanism in the age-related increase in CVD risk.

Circulating angiogenic cells (CACs) play a role in the maintenance of a healthy endothelium. CACs include endothelial progenitor cells (EPCs; $CD34^+$, $CD34^+CD45^{dim}VEGFR2^+$), which can promote endothelial regeneration and maintenance of endothelial function through replacing damaged or dysfunctional endothelial cells, or by secreting proangiogenic factors which support the proliferation of resident endothelial cells (16). These cells are also independent predictors of endothelial function (6) and have been demonstrated to be reduced in the circulation or have impaired function in those with CVD or those with risk factors for CVD compared to healthy controls (17, 41). There have been observations that circulating EPC counts are lower in older vs younger individuals (39) and that progenitor cell function is impaired with age (44). Therefore, maintaining high levels of EPCs later in life may offer protection against the onset and/or progression of CVD by helping to maintain a healthy endothelium.

Recently, a new subset of CACs, $CD3^+$ T-cells that co-express CD31, have been identified (15). These T-cells were found to be required for optimal *in vitro* growth of EPCs (15) and have been termed 'angiogenic T-cells', or T_{ANG}. T_{ANG} are able to secrete significantly higher levels of proangiogenic factors (e.g. VEGF, IL-8 and G-CSF) than their $CD31^-$ counterparts (15). These T_{ANG} cells are inversely correlated with Framingham Risk Score (FRS), as well as age (15, 20) and are also reduced in those with cerebral small vessel disease (34), indicating that the reduction of these T-cells may play a role in onset of CVD.

Exercise and physical activity have been consistently shown to be protective against CVD (14, 25). The observed risk reduction may be due, in part, to the improved endothelial function observed with exercise training and increased levels of physical activity (4). Acute (33, 40), and chronic exercise training (43) have been shown to lead to increased circulating number and/or function of EPCs in humans, as well as some recent data from our lab showing large increases in circulating T_{ANG} cells in response to acute exercise (32). However, there is no research to date to show the effects of age on the acute exercise response of both CAC populations, and thus this warrants investigation.

Both CAC subsets reportedly express C-X-C chemokine receptor 4 (CXCR4) (15, 32), which is involved in cell migration and neovascularization capacity of EPCs (42). Blocking of CXCR4 on such cells results in reduced ability of EPCs to migrate to both SDF-1α and VEGF *in vitro* via disrupted intracellular signalling between CXCR4 and downstream target, Janus Kinase 2 (42), which suggests CXCR4 expression on CACs may confer functional benefits. $CXCR4^+$ bone marrow-derived cells have been shown to be lower in aged animal models (45), however data in humans are lacking. We have shown that a single bout of exercise preferentially mobilized CXCR4-expressing T_{ANG} cells in healthy young males (32), but there is no study to date investigating age-related differences in exercise-induced mobilization of $CXCR4^+$ CACs.

The aim of study 1 was to investigate the effects of age on both EPCs and T_{ANG} populations and the cell surface receptor expression of C-X-C chemokine receptor 4

(CXCR4), which is involved in regulation of migration of CACs (42, 43), in addition to the effects of cardiorespiratory fitness (CRF) on these cell populations in a cross-sectional study in healthy men aged 18-75yrs. The aim of study 2 was to investigate the effects of an acute bout of exercise on mobilization of CACs in young and older men (18-25yrs, and 60-75yrs). It was hypothesized that age would be negatively associated with both CAC subsets, and that CRF would be positively associated with CAC subsets independent of age. It was also hypothesized that older individuals will display an attenuated exercise-induced increase in CAC populations in comparison vs. younger individuals.

Materials and Methods

Study 1

Subjects

One hundred and seven healthy, non-obese (body mass index [BMI] <30), non-smoking, male participants aged 18-75yrs (Table 1), were recruited to the cross-sectional study. The study was approved by Edinburgh Napier University's Research Ethics and Governance Committee. All subjects gave written informed consent prior to data collection.

Subjects reported to the Human Performance Laboratory after an overnight fast, having not exercised for at least 24 hours prior to the visit, having refrained from alcohol consumption the night before and having not ingested caffeine the morning of

the visit. Subjects were measured for height, body mass (from this BMI was calculated) and waist and hip circumference measures were taken to calculate waist-to-hip ratio. Blood pressure (BP) was measured using an automated BP cuff after 5-minute supine rest.

Blood Sampling

Venepuncture was performed with the subjects in a supine position after 5-minutes rest. A 21-gauge needle and collection kit (BD Biosciences, USA) was used for collection of peripheral blood samples. Blood samples were evacuated into 6ml tubes spray-coated with Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulants using the BD Vacutainer Safety-Lok^{TM} system (BD Biosciences, USA). In addition, 6ml serum tubes (BD Biosciences) were used for the collection of blood for quantification of cardiovascular risk factors (fasting glucose, triglycerides, LDL-cholesterol and HDL-cholesterol). Peripheral blood in EDTA vacutainers were also centrifuged at 1500g x 15 minutes at 22°C, and subsequent plasma aliquoted for analysis of associated mobilizing factors. Plasma was frozen at -80°C until analysis.

Peripheral Blood Mononuclear Cell Separation

Mononuclear cells (MNC) were isolated using density gradient centrifugation using Lymphoprep^{TM} (Axis-Shield plc, United Kingdom), as previously described (32)

Flow Cytometric Quantification of CD31^{+} T-Cells

Briefly, 0.5 x 10^6 MNCs were incubated with 1μL anti-CD3-APC, anti-CD31-FITC and anti-CXCR4-PE-Cy5 (BD Biosciences, USA) for 45 minutes at 4˚C in the dark. Immediately prior to flow cytometric enumeration, 500μL PBS-BSA was added. T_{ANG} cells and CXCR4 cell surface expression were quantified on a flow cytometer (BD FACS Calibur, BD Biosciences, USA). Lymphocyte gate was identified using a forward scatter and side-scatter plot. A minimum of 100,000 lymphocyte events were collected per sample. Isotypes for both CD31 (FITC Anti-Mouse Isotype; BD Biosciences, USA) and CXCR4 (PE-Cy5 Anti-Mouse Isotype; BD Biosciences, USA) were used in matched concentrations as controls to distinguish between positive and negative events. Following data acquisition, data was analyzed using FCS Express v3.0 (De Novo, Los Angeles, USA). The percentage of all lymphocytes and lymphocyte subsets expressing CD3, CD31 and CXCR4 were analyzed, and total T_{ANG} cells were calculated by multiplying the percentage of lymphocytes expressing the cell surface antigens of interest by total lymphocyte count as quantified by semi-automated haematology analyser (XS-1000i, Sysmex, Japan). All flow cytometry T-cell data were measured in duplicate and averaged.

Flow Cytometric Quantification of Endothelial Progenitor Cells

EPCs were quantified using peripheral whole blood using a BD FACS Calibur (BD Biosciences, USA). Briefly, 200μL of EDTA whole blood was incubated with 10μL of Fc Receptor Blocking Reagent (Miltenyi Biotec, Germany) for 15 minutes in the dark at 4°C, followed by incubation with 10μL anti-CD34-FITC, 10μL anti-CD45-APC, 15μL anti-VEGFR2-PE and 10μL anti-CXCR4-PE-Cy5 (all BD Biosciences USA) for 45 minutes at 4°C in the dark. Samples containing no antibody for VEGFR2

and CXCR4 were used as negative controls. Subsequently, 2mL Pharm Lyse^{TM} (BD Biosciences, USA) was added and left to incubate for 20 minutes prior to flow cytometric quantification of the EPCs. For each sample, 500,000 $CD45^{+}$ events were collected for analysis. Flow cytometric data for EPCs was analyzed using FCS Express v3.0 (De Novo, Los Angeles, USA), and expressed as % MNCs.

Gating strategies for both T_{ANG} and EPCs are shown in Figure 1.

Analysis of Circulating SDF-1α, Lipids, Cholesterol and Fasting Glucose

Aliquots of plasma (peripheral blood centrifuged at 1500g x 15 minutes) and platelet-free plasma (PFP; double centrifugation at 1500g x 15 minutes followed by centrifugation at 13000g x 2 minutes) were prepared and stored -80˚C. Circulating SDF-1α was analyzed by enzyme-linked immunosorbent assay (ELISA) in PFP (R&D Systems Inc., USA). Fasting glucose, triglycerides, total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured in human serum by semi-automated spectrophotometry (RX Monza Clinical Chemistry Analyzer, Randox, UK). All samples were measured in duplicates and average values used for analysis.

Submaximal Exercise Test Protocol for Estimation of Maximal Oxygen Consumption ($\dot{V}O_2max$)

All participants completed a submaximal cycling exercise test (YMCA) as described by Golding et al. (12), to estimate maximal oxygen uptake ($\dot{V}O_2max$). The YMCA

submaximal cycling test consisted of 3-4 x 3 minute incremental stages, starting at 50W at 50rpm. The exercise test was completed when the participants reached their predicted 80% maximum heart rate. Heart rate was measured continuously throughout the test (Polar, Finland). Using HR and $\dot{V}O_2$ values measured using breath-by-breath gas analysis (MasterScreen™ CPX, Jaeger®, CareFusion, USA), $\dot{V}O_2$max was estimated using the equations provided by Adams and Beam (1) .

Study 2

Subjects

Eight young (18-25yrs) and nine older (60-75yrs) physically active, healthy males took part in the second study. Subject characteristics for study 2 are shown in Table 2. The study was approved by Edinburgh Napier University's Research Ethics and Governance Committee. All subjects gave written informed consent prior to data collection.

Subjects were required to attend the Human Performance Laboratory on 2 occasions. The first visit was used to ascertain subjects' $\dot{V}O_2$max, from which the workload at 70% $\dot{V}O_2$max was calculated for use for the 2nd visit (30-minute cycling bout).

Visit 1

Subjects reported to the Human Performance Laboratory after an overnight fast, having not exercised for at least 24 hours prior to each visit, having refrained from

alcohol consumption the night before and having not ingested caffeine the morning of the visit. Subjects were measured for height, and body mass (from this BMI was calculated). Blood pressure (BP) was measured using an automated BP cuff after 5-minute supine rest.

Subjects underwent a graded cycling exercise test to volitional exhaustion. Breath-by-breath measures were made to quantify $\dot{V}O_2$max. Heart rate (Polar, Finland) and rating of perceived exhaustion (RPE) (5) was monitored throughout the test. Regression analyses were performed to calculate workload at 70% $\dot{V}O_2$max.

Visit 2

After an overnight fast, participants undertook a 30 minute cycling ergometer bout at 70% $\dot{V}O_2$max, with blood samples taken pre- and immediately post-exercise. Blood samples were used for the quantification of circulating EPCs (33) and T_{ANG} cells by flow cytometry as previously described (32). Both cell populations were expressed as absolute counts (cells·ml^{-1} or cells·µl^{-1} using % of gated events against differential leukocyte count). Blood was also processed for plasma for quantification of circulating mobilizing factors (VEGF, granulocyte colony stimulating factor [G-CSF], SDF-1α) by enzyme-linked immunosorbent assay (ELISA).

Statistical Analysis

All data were assessed for normal distribution. Progenitor cell data were not normally distributed and so were logarithmically or square root transformed. $CD34^+$ cell subset

comparisons between age groups (18-30yrs, 31-50yrs, and 51-75yrs) for study 1 were performed on non-transformed data using Kruskal-Wallis rank comparisons tests with Dunn's test for multiple comparisons, and T_{ANG} comparisons between age groups performed using one-way analysis of variance (ANOVA), with Bonferroni post-hoc tests performed to correct for multiple comparisons. To assess the influence of age and CRF and other circulating factors on CAC number and CXCR4 cell surface expression (Study 1, % CACs expressing CXCR4 and mean fluorescence intensity [MFI]), single linear regressions were performed using Pearson's coefficient (R^2) and F-statistics. Subsequent multiple linear regressions were performed to control for the influence of age and cardiometabolic risk factors on CACs to investigate if CRF had any independent effect on circulating number of EPCs and or T_{ANG} cells. In all models, standardized regression coefficients (Beta-values) and R^2 values are reported as measures of association between variables and cell subsets.

To analyse the influence of an acute bout of exercise on CAC levels in both young and older men (Study 2), mixed model ANOVA analyses were performed, with age group as the independent factor, and time (pre, immediately post-exercise) as fixed factor. To adjust for multiple comparisons, Bonferroni post-hoc tests were performed. Pearson correlations were performed to assess the relationship between changes in CACs and known chemoattractants for these cells. Independent T-tests were performed to determine significant differences between age groups in baseline characteristics and trial data.

Data was analyzed using SPSS for Windows (IBM, USA) and GraphPad Prism 7 for Windows (GraphPad Software, Inc, USA). Significance was set at alpha (p) ≤ 0.05.

Data are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

Results

Chronological Age and Circulating Angiogenic Cells

Circulating $CD34^{+}CD45^{dim}$ cells were significantly lower in the 51-75yrs group compared to the 18-30yrs group (0.106 ± 0.010% vs. 0.143 ± 0.011%, $p<0.01$), and lower in the 31-50yrs group compared to the 18-30yrs group (0.113 ± 0.009% vs. 0.143 ± 0.011%, $p<0.05$). Circulating EPCs ($CD34^{+}CD45^{dim}VEGFR2^{+}$) only significantly differed between the 18-30yrs and 31-50yrs group (0.015 ± 0.006% vs. 0.004 ± 0.001%, $p<0.001$). There was a trend for a difference for circulating EPCs between 18-30yrs and 51-70yrs (0.015 ± 0.006% vs. 0.009 ± 0.001%), but this difference was not significant. There were significant differences observed for $CXCR4^{+}$ HPCs between 51-75yrs compared to 18-30yrs (0.040 ± 0.006% vs. 0.089 ± 0.009, $p<0.005$) and 31-50yrs compared with 18-30yrs (0.048 ± 0.006% vs. 0.089 ± 0.009%, $p<0.005$), but no significant differences between 31-50yrs and 51-75yrs in $CXCR4^{+}$ HPC count. CXCR4-positive EPCs were only significantly different between 18-30yrs and 31-50yrs (0.011 ± 0.002% vs. 0.004 ± 0.001%, $p<0.001$), with no other significant differences found, despite a trend for lower circulating cells in 51-75yrs compared to 18-30yrs group (0.006 ± 0.001% vs. 0.011 ± 0.002%). Circulating T_{ANG} cells were significantly lower in the 51-75yrs group compared to the 18-30yrs (539 ± 32 cells.μl^{-1} vs. 751 ± 42 cells.μl^{-1}, respectively, $p<0.001$). In addition, T_{ANG} cells were lower in the 31-50yrs group compared to the 18-30yrs group (631 ±34

cells.μl^{-1} vs. 751 ± 42 cells.μl^{-1}, p<0.05). There was no significant difference in T_{ANG} cells between 31-50yrs and 51-75yrs groups. There were no significant differences in these age groups for CXCR4^{+} T_{ANG} cell number. CAC differences between age groups are shown in Figure 2.

Advancing age was associated with a lower number of circulating CD34^{+} progenitor cells (r= -0.374, r^2=0.140, p=0.000) as well as a significantly lower number of circulating T_{ANG} cells (CD3^{+}CD31^{+} cells·μL^{-1}: r = -0.420, r^2 = 0.176, p = 0.000; % of total CD3^{+} cells: r = -0.510, r^2 = 0.260, p = 0.000). Additionally, the number of circulating CXCR4-expressing CD34^{+} progenitors, and CXCR4-expressing EPCs (CD34^{+}CD45^{dim}VEGFR2^{+}) were also inversely related to chronological age (CD34^{+}CXCR4^{+}: r = -0.408, r^2 = 0.167, p = 0.000; CXCR4^{+} EPCs: r = -0.313, r^2 = 0.098, p = 0.001), however total circulating EPCs was not found to be significantly associated with chronological age (r = -0.153, r^2 = 0.023, p = 0.058). CXCR4-expressing T_{ANG} cells were inversely associated with age (r = -0.230, r^2 = 0.053, p = 0.008). CXCR4 cell surface expression intensity, as quantified as mean fluorescence intensity (MFI) of CXCR4-expressing EPCs was significantly lower with advancing age (r = -0.177, r^2 = 0.031, p = 0.036), but no such observation was made for CXCR4^{+} CD34 progenitor cells, or CXCR4-expressing T_{ANG} cells. Data is shown in Supplementary Tables 1, 2 and 3.

Influence of Cardiorespiratory Fitness on the Age-Associated Decline in Circulating Angiogenic Cells

To assess the potential for CRF to attenuate the advancing age associated lower number in CAC numbers, submaximal exercise tests were performed to quantify CRF, and estimated $\dot{V}O_2$max was used as a marker of CRF. These values in study 1 ranged from 16.89ml·kg·min^{-1} to 66.78ml·kg·min^{-1}. Stepwise multiple regression analyses were performed to assess the influence of CRF on CAC subsets after correcting for age. After including age in the predictive model, there was no impact of CRF on the basal levels of these CACs or CXCR4-expressing CACs (Supplementary Table 1: CD34$^+$ progenitors; Supplementary Table 2: EPCs, Supplementary Table 3: T_{ANG} cells), with age remaining a significant independent predictor of resting CD34$^+$/CD34$^+$CXCR4$^+$CXCR4$^+$ EPCs/T_{ANG}/CXCR4$^+$ T_{ANG} cells in males aged 18-75 years.

Influence of Cardiometabolic Risk Factors and Circulating Angiogenic Cell Mobilizing Factors on EPCs and T_{ANG} Cells

The association of other cardiometabolic risk factors, such as BMI, blood pressure, waist-to-hip ratio, fasting glucose, and lipid profile (LDL-C, HDL-C, total cholesterol), as well as SDF-1α, a known mobilizing factor for progenitor cells, with these various CACs were quantified using several multiple level regression analyses after correcting for age. Of note, after controlling for age, systolic pressure was positively associated with T_{ANG} cells (r^2-change = 0.038, F-change = 5.205, p = 0.024) and CXCR4$^+$ T_{ANG} cells (r^2-change = 0.036, F-change = 4.232, p = 0.042). Total cholesterol was positively associated with CXCR4 cell surface expression on CD34$^+$ progenitor cells (CXCR4 MFI, r^2-change = 0.133, F-change = 15.010, p = 0.000), but inversely associated with percentage of T_{ANG} cells expressing CXCR4 (r^2-

change=0.066, F change=6.918, p=0.010), and CXCR4 expression intensity on T_{ANG} cells (r^2-change = 0.051, F-change = 5.331, p = 0.023). In addition, LDL-C was positively associated with CXCR4 MFI on $CD34^+$ progenitors (r^2-change = 0.112, F-change = 12.389, p = 0.001), but negatively associated with circulating $CXCR4^+$ T_{ANG} cells (r^2-change = 0.044, F-change = 4.614, p = 0.034), and intensity of CXCR4 expression on T_{ANG} cells (r^2-change = 0.058, F-change = 6.165, p = 0.015).

After controlling for age, SDF-1α was positively associated with the circulating number of CXCR4-expressing $CD34^+$ progenitors (r^2-change = 0.038, F-change = 4.489, p = 0.029), but conversely was negatively associated with CXCR4 MFI on these $CD34^+$ cells (r^2-change = 0.056, F-change = 6.308, p = 0.014).

Due to the potential confounding factors systolic blood pressure (T_{ANG}, $CXCR4^+$ T_{ANG}), total cholesterol ($CD34^+CXCR4^+$ MFI, CXCR4-expressing T_{ANG}, T_{ANG} CXCR4 MFI), LDL-C ($CD34^+CXCR4^+$ MFI, $CXCR4^+$ T_{ANG}, T_{ANG} CXCR4 MFI) and SDF-1α (CXCR4-expressing $CD34^+$ cells, $CD34^+CXCR4^+$ MFI), these were again entered into the regression analyses to assess the CRF on these CAC variables after controlling for age and these cardiometabolic and mobilizing factors. After controlling for age and these factors, CRF had no association with any of the given CAC variables (data not shown).

Acute Exercise and EPC Mobilization: Influence of Age

There was a main effect of exercise on circulating $CD34^+CD45^{dim}$ (p = 0.018, F (1, 16) = 6.998) and $CD34^+CD45^{dim}VEGFR2^+$ progenitor cells (p = 0.003, F (1, 16) =

11.99). There was no main effect of exercise on CXCR4-expressing progenitor cells. There was a significant exercise x age interaction for both $CD34^{+}CD45^{dim}$ haematopoietic progenitors ($p = 0.019$, $F (1, 16) = 6.869$) and a close to significant interaction for $CD34^{+}CD45^{dim}VEGFR2^{+}$ EPCs ($p = 0.098$, $F (1, 16) = 3.123$). This was reflected by significantly greater absolute cell mobilization in younger vs. older individuals for both $CD34^{+}CD45^{dim}$ haematopoietic progenitors (1140 ± 294 cells·mL^{-1} vs. 275 ± 191 cells·mL^{-1}, respectively, $p = 0.029$) and a trend for increased EPC mobilization in young vs. older adults (212 ± 72 cells·mL^{-1} vs. 67 ± 23 cells·mL^{-1}, respectively, $p = 0.076$). There were no such exercise x age interactions, or differences between age groups for absolute cell mobilization for CXCR4-expressing progenitors. Progenitor cell data is shown in Figure 3.

Acute Exercise and T_{ANG} Changes: Influence of Age

Due to insufficient blood draw in one young participant, analysis includes 8 young, and nine older individuals. The single bout of moderate intensity exercise significantly elevated total T_{ANG} cells ($CD3^{+}CD31^{+}$: $p=0.001$, $F (1, 14) = 18.47$), $CD4^{+}$ T_{ANG} ($p = 0.011$, $F (1, 14) = 8.65$) and $CD8^{+}$ T_{ANG} cells ($p = 0.007$, $F (1, 14) = 10.25$). There was a significant exercise x age interaction for total T_{ANG} cells ($p=0.029$, $F (1, 14) = 6.07$) with younger individuals displaying greater response, but not for either $CD4^{+}$ ($p=0.058$, $F (1, 14) = 4.34$) or $CD8^{+}$ T_{ANG} cells ($p = 0.148$, $F (1, 14) = 2.37$).

CXCR4-expressing T_{ANG} cells and $CD4^{+}$ T_{ANG} cells did not significantly change with exercise in either group ($p>0.05$), but there was significant exercise and interaction

effects for $CD8^+CXCR4^+$ T_{ANG} cells (main effect of exercise: p = 0.019, F (1, 14) = 7.06; interaction exercise x age: p = 0.040, F (1, 14) = 5.11) with the younger individuals demonstrating a greater response to the exercise bout. Independent T-test analysis revealed significantly greater absolute cell changes in young individuals compared to older men for T_{ANG} (634 ± 173 cells·μl^{-1} vs. 262 ± 77 cells·μl^{-1}, p = 0.046), $CD4^+$ T_{ANG} (229 ± 84 cells·μl^{-1} vs. 59 ± 19 cells·μl^{-1}, p = 0.027), and CXCR4-expressing $CD8^+$ T_{ANG} cells (88 ± 35 cells·μl^{-1} vs. 11 ± 6 cells·μl^{-1}, p = 0.039). Data for T_{ANG} cell changes with age and exercise are shown in Figure 4.

Acute Exercise and CAC Mobilizing Factors

Exercise resulted in an increase in circulating plasma VEGF and cortisol (main effects of exercise: p = 0.012, p= 0.000, respectively). There was a significant exercise x age interaction for cortisol (p = 0.006, F (1, 15) = 10.366) but not for VEGF (p = 0.220, F (1, 15) = 1.659; Figure 5). To investigate if there is a relationship between increases in circulating VEGF, G-CSF, SDF-1α and cortisol with changes in CACs, several Pearson correlations were performed. There were no relationships evident for changes in G-CSF and cortisol for any CAC subset changes with exercise, but significant positive relationships were found for changes in SDF-1α and $CD34^+CD45^{dim}$ (r = 0.898, p = 0.015) progenitor cell changes with exercise, but only for the young individuals. Interestingly, when analysing age groups in isolation, changes in cortisol were significantly associated with changes in total T_{ANG} cells (r = 0.715, p = 0.030).

Discussion

449 The main findings of the two studies were that older age was characterized by a lower
450 number of a variety of CACs in healthy men aged 18-75 years, and CRF was
451 unsuccessful in attenuating this effect. In addition, older adults display an impaired
452 mobilization of $CD34^+$ progenitor cells and ingress of T_{ANG} cells into circulation in
453 comparison to younger individuals.
454
455 Advancing age was shown to be significantly deleterious for a $CD34^+$ progenitors,
456 and $CD31^+$ T-cells, named angiogenic T-cells (T_{ANG}). These CACs play an important
457 role in the maintenance of endothelial function (6, 18) and the associated advancing
458 age-associated lower numbers of these cells as shown in this study and others (20, 39)
459 may represent a key mechanism in the ageing decline in endothelial function (4) and
460 endothelial repair ability (43). This decline in endothelial function is a key process in
461 the development of atherosclerotic CVD. Our study is also the first study to state that
462 CXCR4-expressing CACs are also significantly lower in circulating number with age.
463 CXCR4 expression on these cells may play an important role in the migratory ability
464 of these cells (15, 42, 43), and thus the loss of CXCR4 expression on CACs may play
465 a role in CAC dysfunction, potentially subsequently leading to development of
466 endothelial dysfunction. Kushner et al. (20) found that CAC migration to SDF-1α, a
467 CAC chemokine bound by CXCR4, was associated more strongly to endothelial
468 function than T_{ANG} cell number alone. Interestingly, Xia et al. (44) observed no
469 differences in CXCR4-expressing CACs or intracellular CXCR4 content in EPCs
470 between age groups, but rather found an impaired CXCR4:JAK-2 intracellular
471 signalling under stimulation with SDF-1α in the older compared to the younger men.
472 The differences between our study and the study by Xia et al. (44) can be explained
473 by methodological differences, as we measured cell surface CXCR4 expression on

CACs by flow cytometry, whereas Xia et al. (44) quantified total cell CXCR4 expression using RT-PCR and western blotting techniques, which may be more representative of functional responses

The exact cause for the lower number in resting CAC number and function in older adults are yet to be fully elucidated. We did observe an inverse relationship in circulating SDF-1α with age, which may contribute to the lower progenitor cell number (data not shown), however, it is likely that this is not the single causative factor. Ageing-associated increases in oxidative stress may play a significant role in CAC number and function reduction with advancing age (24), via reduced EPC SIRT1 content (24), reduced CXCR4 gene expression (28), or increased susceptibility to apoptosis (19). Bone marrow-resident progenitor cells appear unchanged with advancing age (29), whereas the mobilization of progenitors in older populations are significantly impaired compared with younger counterparts (17, 47). However, the mechanisms for the lower circulating number of progenitor cells and T_{ANG} cells are likely to be very different. T_{ANG} cells represent a vasculogenic subpopulation of T-cells (15, 18), and age-associated differences in T-cell populations will differ to that of bone marrow-derived progenitors. Thymic involution occurs with advancing age, resulting in a decrease in thymic output of naïve T-cells (36). Studies have shown distinct T-cell population changes with age, such that the proportion of total T-cells displaying markers of senescence (e.g. CD28) are elevated in comparison to naïve T-cells (3, 37). In addition, Zehnder et al. (46) found that T-cells lose the expression of CD31 upon activation, with T-cells differentiating from a naïve to an effector-type T-cell. As we age we encounter many viral antigens, and thus ageing, through the increased occurrence of these antigen-T-cell encounters, is likely to be associated with

loss of CD31 expression on effector-type T-cell populations. Therefore, ageing is potentially promoting the loss of vasculogenic function in the T-cell population.

To evaluate the effect of CRF on these CACs, we performed multiple level regression analyses, controlling for age, and when required, confounders (systolic blood pressure, total cholesterol, LDL-C and SDF-1α). We surprisingly found no association between CRF on any CAC subset. This was confirmed by no significant difference found between age-adjusted $\dot{V}O_2$max categories when analyzed by one-way ANOVA for the CAC subsets (data not shown). This is in contrast to previous studies which have shown the beneficial impact of regular exercise and CRF on resting number and/or function of EPCs (43). However, our data is in line with several studies which demonstrate no effect of a regular exercise training program, or increasing levels of CRF on these cells (39). The differences between studies may be due to the differences in phenotype of CAC quantified. This is indeed the first study to evaluate the influence of CRF independent of age on $CD34^{+}CD45^{dim}VEGFR2^{+}$ population of EPCs, reported to have endothelial differentiation properties (7), whereas the $CD45^{bright}$ population do not (7) and are reported to exert beneficial effects on the endothelium by secreting proangiogenic growth factors and cytokines (16). Therefore, our study is specifically measuring precursor endothelial cells.

This is the first study to investigate the influence of CRF, independent of age, on circulating T_{ANG} cells. Previous T-cell studies have reported significant impact of CRF on T-cell populations, independent of age, reporting an inverse relationship between proportion of cytotoxic and senescent T-cells with increasing $\dot{V}O_2$max (37). It was expected that since CD31 expression may be lost on effector-memory

phenotypes, that we would observe similar findings, with increased levels of $CD31^+$ T-cells with increasing levels of $\dot{V}O_2max$, independent of age. However, no such effect was observed. Further studies are required to quantify CD31 expression on both naïve and effector-memory T-cell phenotypes, which may partly explain the effects of advancing age and potential CRF influences on these vasculogenic T-cells. From our data we cannot discount that CRF may impact on functional capacities of these cells, and so, further studies along these lines are required also.

Acute exercise has been consistently shown to acutely increase circulating progenitor cells in healthy and diseased populations (33, 40), as well as some functional improvements in the post-exercise recovery period (41). We have also recently shown that T_{ANG} cells are also redistributed into the circulation immediately post-exercise in trained men (32). Since advancing age is associated with lower number of basal levels of CACs, we sought to investigate whether age was also characterized by an exercise-induced impairment in the mobilization of these cells. Our results show that older individuals display an attenuated circulating progenitor cell increase in response to an exercise stressor. This response was specifically for $CD34^+CD45^{dim}$ haematopoietic progenitors, and $CD34^+CD45^{dim}VEGFR2^+$ EPC ($p=0.076$) and not for CXCR4-expressing progenitors, despite some differences in absolute cell changes. Previously, we (33) and others (40) have demonstrated that a single bout of exercise is successful in increasing the number of progenitor cells in peripheral blood, and some have also demonstrated that this response is attenuated in diseased populations, such as heart failure (40) and type 2 diabetes mellitus (23). Thijssen et al. (39) were the first to demonstrate that there were differences in the haematopoietic progenitor cell response to a single bout of exercise between young and older men which our study supports.

549 However, they did not observe such changes with EPCs, and so this is the first study
550 to demonstrate age-related changes in exercise-induced circulating EPC levels. These
551 changes could not be explained by differences in circulating chemoattractants such as
552 VEGF, SDF-1α, or G-CSF as there were no age x exercise interactions, despite an
553 association between circulating changes in $CD34^{+}CD45^{dim}$ cells and SDF-1α only
554 being present in young individuals. SDF-1α (31) and G-CSF (30) are known to
555 stimulate the release of progenitor cells from the bone marrow into the circulation,
556 and increases in VEGF with acute exercise accompanies increases in circulating EPCs
557 (33). We did observe significant changes in VEGF with exercise in both groups, but
558 the change in circulating VEGF did not correlate with changes in either progenitor
559 cell population. Differences in these acute exercise-induced progenitor cell changes
560 may be attributable to other known chemoattractants, such as stem cell factor (SCF)
561 (26), which we did not quantify in this study. Therefore future studies should quantify
562 a host of known chemoattractants to determine what the factors are that may explain
563 the age-associated differences in progenitor cell mobilization with acute exercise.
564 Additionally, some data suggest ageing is linked with reduced bone marrow resident
565 progenitors (9) therefore a reduced pool from which to mobilize these cells from in
566 response an acute stressors.
567
568 Our results also show that older adults display a blunted movement of T_{ANG} cells into
569 the blood in response to an acute exercise stressor compared to younger counterparts.
570 We did observe main effects of exercise for most of our T_{ANG} subsets, but
571 interestingly, the impaired response with advancing age was specific for total T_{ANG}
572 cells, and $CD8^{+}CXCR4^{+}$ T_{ANG} cells. Acute exercise stimulating an increase in
573 circulating numbers of these cells have also been reported by our lab previously (32),

as well as others reporting significant increases in circulating T-cell subsets with a single bout of moderate and intense exercise (3, 27). The age-related attenuation of this response, in addition to lower basal levels may partly explain the increased risk of vascular dysfunction in ageing populations observed elsewhere (4). Other studies have demonstrated different exercise-induced T-cell changes in older vs. younger counterparts (3, 27) with greater absolute T-cells entering circulating in response to exercise in younger individuals in a variety of subsets, namely γδ T-cells (27), and $CD8^+$ naïve subsets (3). We observed greater responsiveness of $CD3^+CD31^+$ and $CD8^+CXCR4^+$ T_{ANG} cells in younger compared to older adults in response to the 30-minute exercise bout. It is unknown the differentiation status of these cells, but these cells may be naïve or low differentiated cells and may partly explain this response. The full differentiated status of T_{ANG} cells needs to be quantified in order to investigate this. The impaired response of $CXCR4^+$ T_{ANG} cells may be of clinical importance, as these cells possess high migratory capacity to ischaemic tissue. Previous studies have found that it may be that age-associated impairment in CAC mobilization/migration is due to altered intracellular signalling of the SDF-1:CXCR4:JAK-2 pathway (43, 44). This has yet to be investigated in T-cells, and thus is an area of future research.

Summary

Older men display reduced number of CACs, as well as an impaired ability to mobilize and increase circulating number of these cells in response to an acute exercise stressor. This may partly contribute to age-associated decline in endothelial function and thus an increased CVD risk. Future studies are required to augment the

acute exercise response in older men via manipulating the exercise stressor, or via dietary interventions designed to do as such.

Limitations

For study 1, we were limited to the use of a submaximal exercise test to estimate CRF in 107 individuals aged 18-75yrs. We were able to perform pilot studies prior to study 1 which determined that the YMCA submaximal exercise test was reliable for our population cohort. Other published studies support this for our age group (2, 13). Additionally, we would have liked to include functional measures of CACs in our cohorts which would add significantly to the strength of this study and is a line of future work.

This study reflects the changes in CACs with exercise and in different age groups in men, and so care must be taken to extrapolate these findings to women. There are some data to suggest that women display higher levels of circulating EPCs compared to men, potentially due to estrogen availability (10), and that exercise-induced changes in CAC subsets are affected by the phase of the menstrual cycle (35). Yet more work is to be done to determine the influence of age and menopause on these cellular populations.

CAC subsets expressing CXCR4 displayed large inter-individual variability, and thus study 2 may be underpowered to fully explain any age-related exercise-induced response of these cells, and thus further studies should be performed to elucidate the influence of age on exercise-induced changes in these CAC subsets.

Conflicts of Interest

The authors declare that there are no conflict of interests regarding the publication of this article.

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Figures

Figure 1. Gating strategies for flow cytometric quantification of CACs. 1A-1E displays gating strategy for EPC quantification, firstly identification of $CD45^+$ PBMCs (1A), subsequent detection of CD34 population vs. side-scatter (1B), identification of $CD45^{dim}$ population (1C) and VEGFR2 (1D) to quantify $CD34^+CD45^{dim}VEGFR2^+$ EPCs. Finally, $CD34^+$ progenitors and EPCs were gated for expression of CXCR4 (1E). 1F-1K identifies T_{ANG} gating strategy, with identification of lymphocytic gate (1F), gating on $CD3^+$ T-cells (1G), and co-expression of CD31 (1H). T_{ANG} cells were further gated for subset gating of CD4 (1I) and CD8 (1J), and finally expression of CXCR4 (1K).

Figure 2. Age group differences in CAC subpopulations. 2A- Age groups and $CD34^+$ progenitor cell subsets. 2B- Age groups and T_{ANG} cell subsets. *$p<0.05$ vs. 18-30yrs group. *Values shown are mean ± SEM.*

820

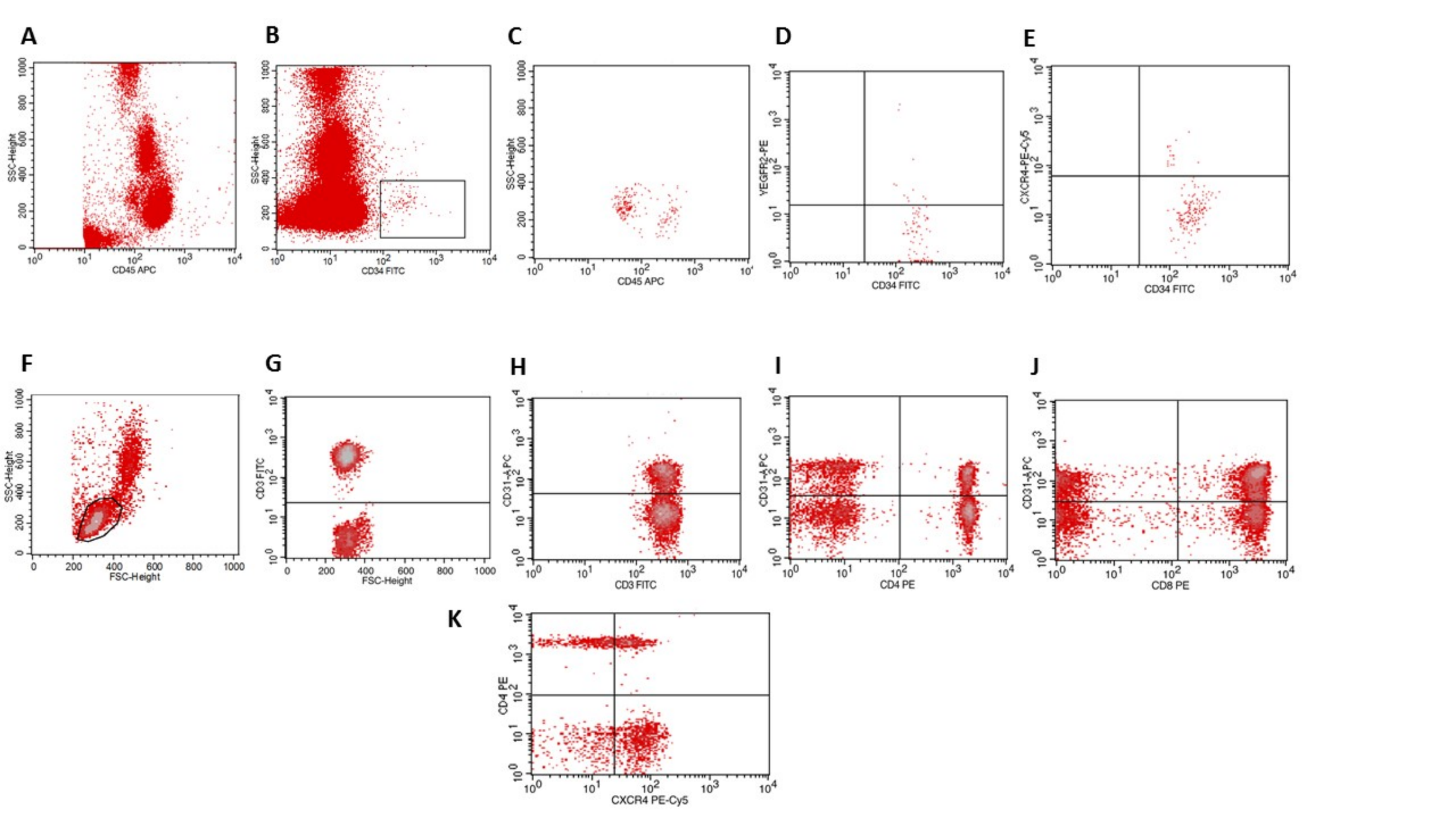
821 **Figure 3.** Circulating progenitor cell changes in response to acute moderate exercise
822 in young and older healthy men. 3A- $CD34^{+}CD45^{dim}$ progenitor cells; 3B - CXCR4-
823 expressing $CD34^{+}$ progenitor cell changes; 3C - EPC changes; 3D- CXCR4-
824 expressing EPC changes. *$p<0.05$ main effect of exercise, δ $p<0.05$ exercise x age
825 interaction. *Values shown are mean ± SEM*.

826

827 **Figure 4.** Circulating T_{ANG} cell changes in response to acute moderate exercise in
828 young and older healthy men. 4A - $CD3^{+}CD31^{+}$ T-cell changes; 4B- CXCR4-
829 expressing T_{ANG} changes; 4C- $CD4^{+}$ T_{ANG} changes; 4D- CXCR4-expressing $CD4^{+}$
830 T_{ANG} changes; 4E- $CD8^{+}$ T_{ANG} changes; 4F- CXCR4-expressing $CD8^{+}$ T_{ANG} changes.
831 *$p<0.05$ main effect of exercise, δ $p<0.05$ exercise x age interaction. *Values shown*
832 *are mean ± SEM*.

833

834 **Figure 5.** Circulating CAC mobilizing factors in response to acute moderate exercise
835 in young and older healthy men. 5A – SDF-1α changes, 5B – VEGF changes, 5C – G-
836 CSF changes, 5D- Cortisol changes. *$p<0.05$ main effect of exercise, δ $p<0.05$
837 exercise x age interaction. *Values shown are mean ± SEM*.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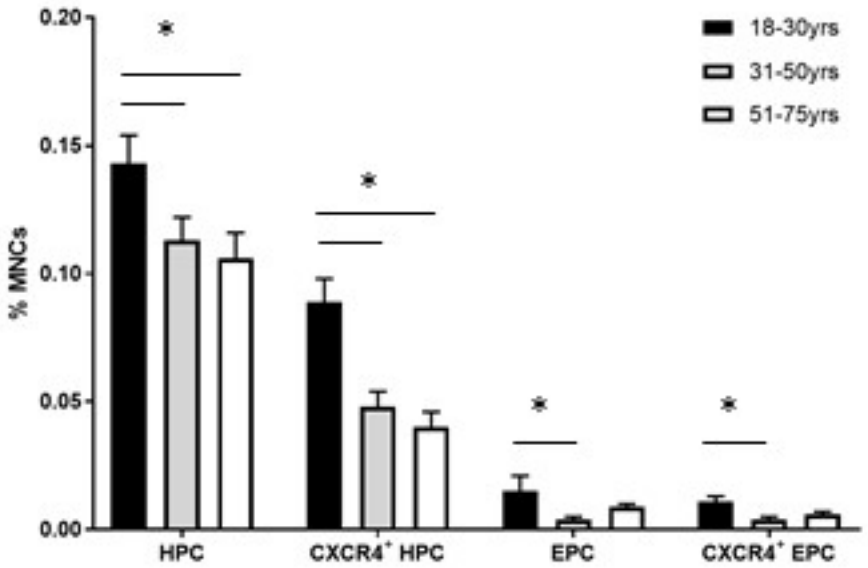
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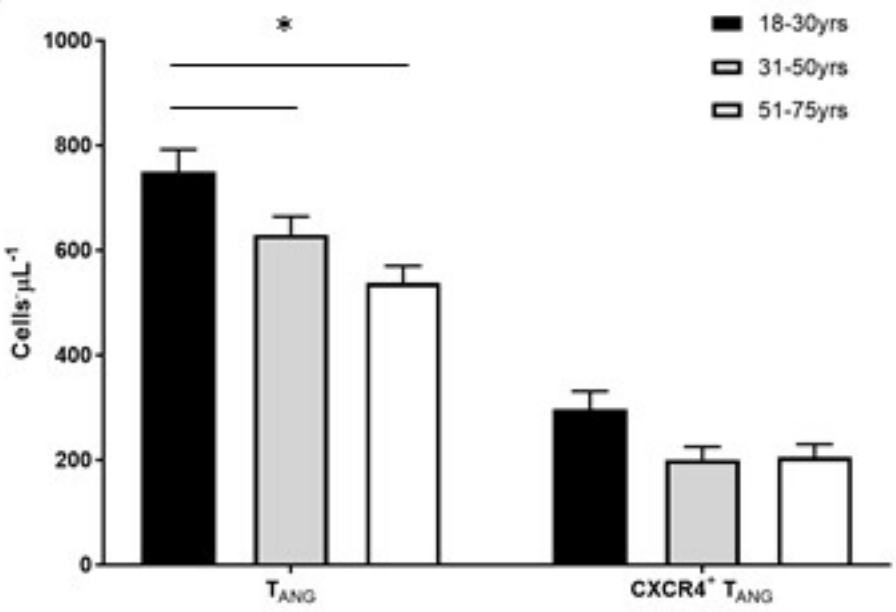
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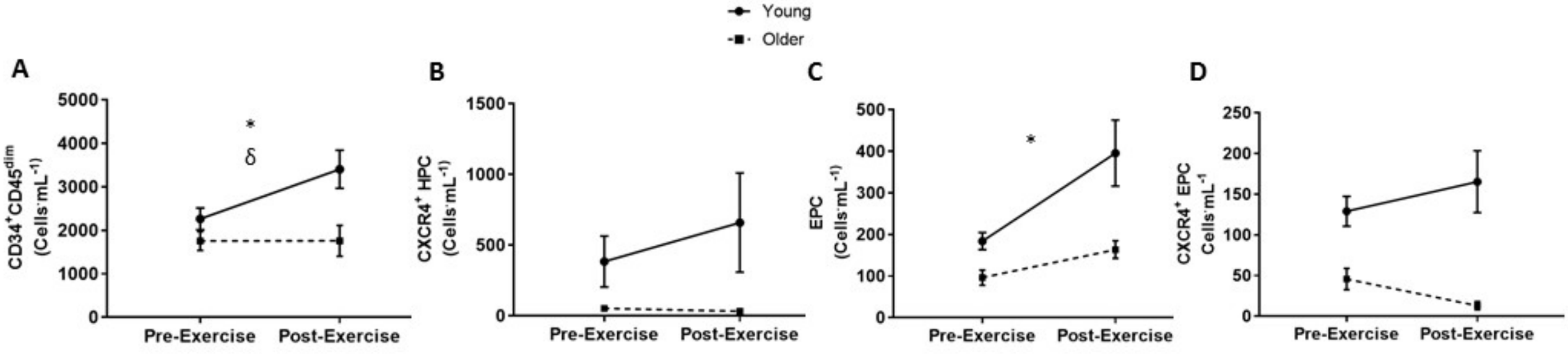
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Pre-Exercise
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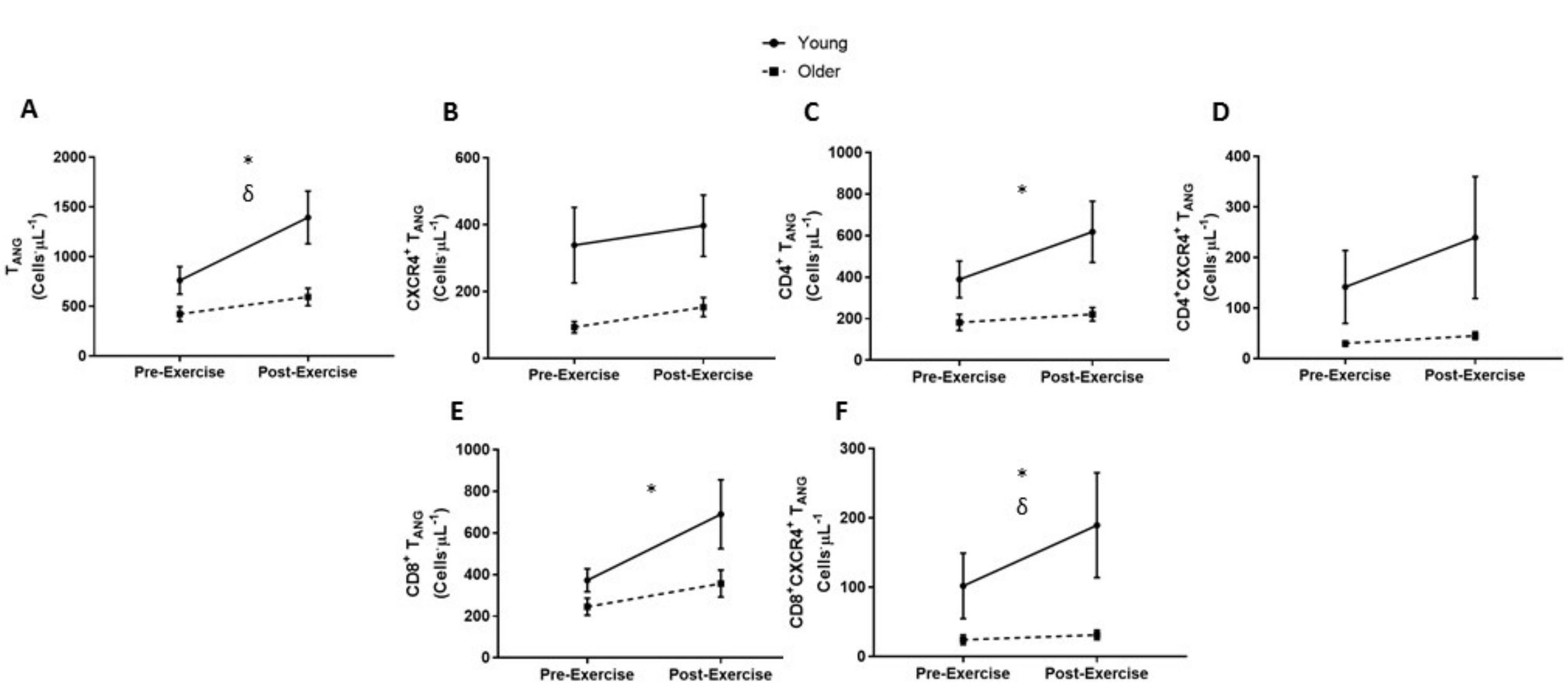
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Post-Exercise
*
δ

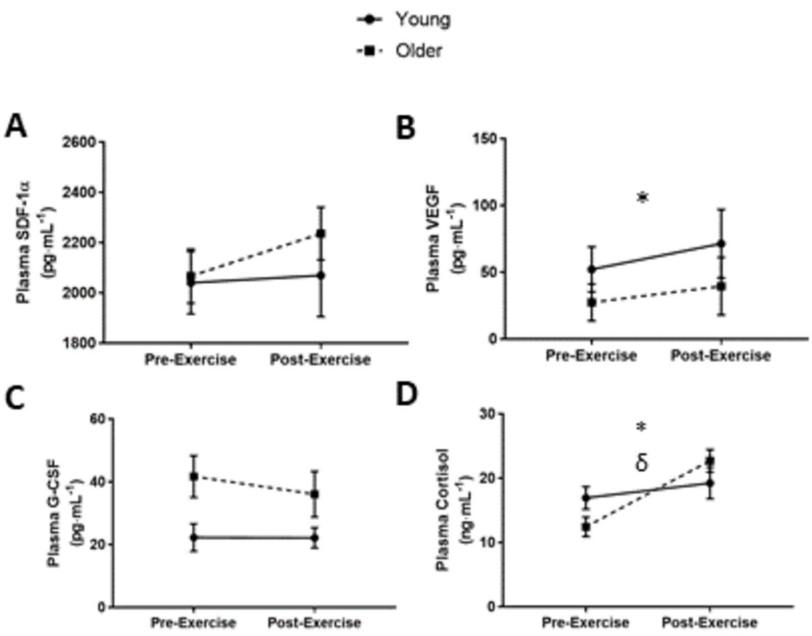
Young
Older
A
Plasma SDF-1α (pg·mL⁻¹)
2600
2400
2200
2000
1800
Pre-Exercise
Post-Exercise
B
Plasma VEGF (pg·mL⁻¹)
150
100
50
0
*
Pre-Exercise
Post-Exercise
C
Plasma G-CSF (pg·mL⁻¹)
60
40
20
0
Pre-Exercise
Post-Exercise
D
Plasma Cortisol (ng·mL⁻¹)
30
20
10
0
*
δ
Pre-Exercise
Post-Exercise

Table 1. Study 1 Participant Characteristics (n=107).

	All (n=107)	**18-30 years (n=36)**	**31-50 years (n=42)**	**51-75 years (n=25)**	**p-value**
Age (years)	39 ± 14	24 ± 3	41 ± 6*	58 ± 5*$^{\#}$	0.000
BMI ($kg \cdot m^2$)	25.83 ± 2.60	26.06 ± 2.37	26.05 ± 2.45	25.09 ± 3.12	NS
SBP (mmHg)	130 ± 15	126 ± 10	129 ± 13	140 ± 19*	0.000
DBP (mmHg)	78 ± 9	73 ± 8	80 ± 8	84 ± 9*	0.000
MAP (mmHg)	96 ± 10	90 ± 7	96 ± 9*	103 ± 11*	0.000
Estimated $\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$) [Range]	43.60 ± 9.48 [16.89-66.78]	44.19 ± 7.99 [31.55-57.59]	47.03 ± 9.62 [26.05-66.78]	36.70 ± 7.82*$^{\#}$ [16.89-52.78]	0.000

*Values are mean ± Standard Deviation. BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure. * $p<0.05$ vs. 18-30 years, $^{\#}$ $p<0.05$ vs. 31-50 years. NS- not significant.*

*Values shown are mean ± standard deviation. *p<0.05, **p<0.005*

Table 2. Acute Exercise Study Participant Characteristics and Acute Exercise Data (n=17).

	Young (n=8)	Older (n=9)	p-value
Age (years)	23 ± 2	65 ± 3	0.000**
Systolic Blood Pressure (mmHg)	126 ± 13	124 ± 13	0.771
Diastolic Blood Pressure (mmHg)	65 ± 8	74 ± 6	0.012*
Body Mass Index (kg·m^2)	25.5 ± 3.5	26.1 ± 3.5	0.755
Resting Heart Rate (bpm)	66 ± 12	57 ± 6	0.057
$\dot{V}O_2$max (ml·kg·min^{-1})	48.8 ± 8.2	35.1 ± 6.7	0.002**
Workload at $\dot{V}O_2$max (Watts)	325 ± 38	219 ± 27	0.000**
Trial Data			
Workload (Watts)	230 ± 27	156 ± 23	0.000**
Average Heart Rate (bpm)	155 ± 14	127 ± 15	0.001**
% of Maximal Heart Rate	79 ± 7	82 ± 10	0.461

*Values shown are mean ± standard deviation. *p<0.05, **p<0.005*