ABSTRACT

Title of Document:	THE EFFECT OF THROMBIN ON ENDOTHELIAL PROGENITOR CELLS WITH EXERCISE AND EXERCISE TRAINING.
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BACKGROUND: Circulating number of endothelial progenitor cells (EPCs) and EPC colony forming units (EPC-CFUs) have emerged as valuable predictors of cardiovascular health. Improvements in EPC number and EPC-CFUs have been associated with exercise and exercise training, although the mechanism for these improvements has not been identified. PURPOSE: This study examined the association of exercise-induced thrombin production with changes in circulating EPC number and EPC-CFUs as well as investigated the effects of exercise and thrombin supplementation on EPC differentiation and proliferation *in vitro* through gene expression analysis. METHODS: Subjects included healthy male Masters athletes (n = 12) and sedentary matched controls (n = 11) aged 55 – 80 years. Circulating EPC number, EPC-CFUs, plasma prothrombin fragment (F1+2) concentration and plasma thrombin-antithrombin III (TAT) concentration were measured at rest and after 30 minutes of vigorous treadmill exercise. Gene expression was assessed on resting

EPC samples treated with 0U, 1U, 5U, or 10U of thrombin, as well as on EPC samples obtained after exercise. Gene expression analysis was performed for cell cycle genes cyclin A2, cyclin D1, and p27, and for cell surface markers VE-Cadherin and VEGFR2. To investigate the association of long-term exercise training, all outcomes were compared between Masters athletes and sedentary controls. RESULTS: Plasma concentrations of F1+2 and TAT increased significantly after exercise, however, EPC number and EPC-CFUs did not change. Changes in plasma F1+2 concentration with exercise correlated with changes in EPC number and EPC-CFUs in Masters athletes and with EPC number in control subjects Expression of the cell cycle genes cyclin A2 and cyclin D1 increased with thrombin treatment, while expression of the cell cycle inhibitor p27 decreased, peaking at 5U of thrombin. No change in VE-Cadherin or VEGFR2 expression was observed in control subjects, however, expression increased at 1U thrombin treatment in Masters athletes. Similarly, EPCs isolated after exercise demonstrated increased expression in cyclin A2 and cyclin D1, decreased in p27, and showed no change in VE-Cadherin or VEGFR2. CONCLUSION: Elevated thrombin production during vigorous exercise may play an important role in the regulation of EPCs, specifically cellular proliferation through changes in expression of cell cycle genes.

THE EFFECT OF THROMBIN ON ENDOTHELIAL PROGENITOR CELLS
WITH EXERCISE AND EXERCISE TRAINING.

By

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BMI – body mass index

BSA – bovine serum albumin

CFU – colony-forming units

CAD – coronary artery disease

CPC – circulating progenitor cell

CVD – cardiovascular disease

EPC – endothelial progenitor cell

F1+2 – prothrombin Fragment 1+2

FACS – fluorescence activated flow cytometry

FBS – fetal bovine serum

NO – nitric oxide

PAR-1 – protease activated receptor-1

PAOD – peripheral arterial occlusive disease

PBS – phosphate buffered solution

RT-PCR – real time polymerase chain reaction

SEM – standard error of the mean

TAT – thrombin antithrombin III

VE-cadherin – vascular endothelial cadherin

VEGF – vascular endothelial growth factor

VEGFR2 – vascular endothelial growth factor receptor-2

Chapter 1: Introduction

Background

Regular physical activity and improved physical fitness are associated with enhanced cardiovascular health and decreased risk for the development of cardiovascular disease (CVD) (Wei et al., 1999). While exercise training has been repeatedly associated with improvement in those risk factors associated with CVD, it appears that training results in an additional reduction in risk, independent of these risk factors (Blair et al., 1996). This association implies a direct causal link between regular physical activity and improvement in cardiovascular health. To date, a specific mechanism for the independent effect of regular physical activity on cardiovascular health has not been clearly defined.

It has been suggested by several authors that a direct causal link between regular physical activity and reduced CVD risk is through the improvement of endothelial health and associated vascular function (Green, Maiorana, O'Driscoll, & Taylor, 2004; Niebauer & Cooke, 1996). Impairment of the functional health of the endothelium, termed endothelial dysfunction, describes the imbalanced activity of endothelium-derived relaxing and contracting factors (Drexler et al., 1993). As assessed in terms of vasomotor dysfunction, endothelial dysfunction can occur long before evidence of structural atherosclerosis and is interpreted as an early and powerful predictor of CVD risk (Duprez & Cohn, 2007).

While the relationship between exercise training and improved endothelial function has been well documented, the direct mechanism linking the two has not been established. Bone marrow derived endothelial progenitor cells (EPCs) represent what may be a link between exercise training, improved vascular function, and decreased risk for CVD. Depressed levels of circulating EPCs have been shown to be an independent risk factor for CVD (Hill et al., 2003) and are directly involved in post-natal angiogenesis and vascular remodeling (Asahara et al., 1997). Recent evidence has shown that both an acute bout of exercise (Rehman et al., 2004) and exercise training (Laufs et al., 2004) are associated with an increase in the level of circulating EPCs. The mechanism by which exercise and exercise training increase the level of EPCs, however, remains unclear.

Endothelial Progenitor Cells and Cardiovascular Health

The endothelium, once viewed as simply a passive barrier lining the lumen of the vasculature, is now known to be an active regulatory tissue, participating in autocrine and endocrine signaling. The endothelium is central to the maintenance of homeostasis and proper blood flow throughout the vascular system. In this role, the endothelium must respond to environmental stimuli by adjusting vasomotor tone, maintaining hemostatic balance, maintaining a selectively permeable barrier, and responding to injury and/or ischemia. Imbalanced regulation of the endothelium, termed endothelial dysfunction, can occur long before evidence of structural atherosclerosis, and has been found to be an independent predictor of cardiovascular events. Endothelial dysfunction directly results in an increased likelihood of a cardiovascular event through impaired regulation of nitric oxide and

vascular tone (Schram & Stehouwer, 2005), increased susceptibility to vascular shear and oxidative stress (Sun et al., 2004), and impaired maintenance of blood coagulation, fibrinolysis, and platelet adhesion (Anderson, 2003).

Recent evidence has shown a strong relationship between EPCs, endothelial function, and CVD risk. Depressed levels of circulating EPCs and impaired EPC function have been observed in individuals with documented heart disease (Vasa et al., 2001), and endothelial dysfunction (Heiss et al., 2005). Furthermore, differences in EPC number and function have been shown to correlate strongly with conventional risk factors for CVD including blood pressure (Imanishi, Moriwaki, Hano, & Nishio, 2005), serum cholesterol (Vasa et al., 2001), diabetes (Tepper et al., 2002), smoking (Wang, Zhu, Chen, & Shang, 2004) and obesity (Wolk, Deb, Caplice, & Somers, 2005), as well as the novel CVD risk factors C-reactive protein (Verma et al., 2004) and homocysteine (Tan et al., 2006). Interestingly, Hill et al. (2003) found that compared to the Framingham Risk Score for predicted relative risk of developing coronary artery disease (CAD) in healthy men, circulating levels of EPCs not only correlated inversely with predicted CAD risk, but were in fact a better predictor of endothelial function as measured by flow mediated vasodilatation.

Mechanistically, EPCs are believed to be a vital component for both endothelial maintenance and repair. Under conditions of normal cellular turnover and recovery from injury, circulating EPCs have been found to actively incorporate into the endothelial layer (Hunting, Noort, and Zwaginga, 2005). Additionally, EPCs have been observed to be a required component of proper blood clot resolution through the process of recanalization (Moldovan & Asahara, 2003).

It appears that not only exercise training, but also acute bouts of exercise influence circulating EPCs. Among the limited number of studies published thus far, results have consistently shown a relationship between physical activity and circulating number and function of EPCs. Laufs, et al (2004) initially demonstrated the association between EPCs and exercise in mice. After only one week of voluntary wheel running, there was a significant increase in circulating EPCs. The functional consequence of elevated EPCs in these mice was confirmed by inducing injury in the carotid artery of a subset of mice. Mice in the training group demonstrated reduced neointimal formation, increased lumen circumference and increased area of neoangiogenesis. To relate these results to humans, the authors then exercise trained patients with stable CAD for 28 days and found similar increases in circulating EPCs, suggesting similar implications for vascular health in humans (Laufs et al., 2004). Steiner, et al (2005) later reported similar findings in men with a history of CAD and men with multiple risk factors who were exercise trained for 12-weeks. They found an increase in circulating EPCs after training that was significantly correlated with an increase in nitric oxide (NO) activity and endothelial function, as determined by flow-mediated vasodilation (Steiner et al., 2005). In healthy subjects, Hoetzer, et al. (2007) demonstrated an improvement in EPC function *in vitro*, as determined by an increase in colony forming units and migratory capacity, with three months of regular aerobic exercise in middle aged and older men. Hoetzer et al., however, did not measure circulating EPC number.

Similar results have been observed in patients with peripheral artery occlusive disease (PAOD). Sandri, et al (2005) examined the effect of four weeks of exercise training on PAOD patients with clinically active PAOD and those that were successfully revascularized, in comparison to patients with stable CAD. In active PAOD patients who experienced ischemia during exercise there was a significant increase in circulating EPCs with an associated improvement in treadmill walking time and ankle-brachial pressure index. There was no such increase in EPCs in non-ischemic PAOD and CAD patients, suggesting a potential role of ischemia in the exercise response of EPCs (Sandri et al., 2005).

These studies present a clear association between EPCs and exercise training. It remains unclear, however, if changes in number and function of EPCs are the result of a long-term adaptation or the accumulation of acute responses to exercise. Laufs, et al (2005) investigated the response of circulating EPC levels and *in vitro* function to varying intensities and durations of acute exercise in healthy physically active young men. They found that EPC levels increased as a result of 30 minutes of acute exercise in both the intensive running (100% lactate threshold) and moderate running protocols (80% lactate threshold), but not the short duration protocol (80% lactate threshold, 10 minutes) (Laufs et al., 2005). Similar increases in circulating EPCs have been found in patients with a history of vascular disease in response to symptom limited treadmill and bicycle exercise (Rehman et al., 2004), as well as ischemia inducing maximal bicycle exercise (Adams et al., 2004).

Mechanism for EPC Response to Exercise and Exercise Training

It can be hypothesized that the improvement in circulating EPC levels and function, along with improved endothelial function after exercise training, could be the additive effect of regular individual bouts of exercise. While the studies discussed above have consistently demonstrated a change in circulating EPC numbers with acute exercise, a mechanism for these changes remains elusive. Despite the known association between vascular endothelial growth factor (VEGF) and EPCs, VEGF was found to increase only in ischemic patients (Adams et al., 2004), with no changes observed in non-ischemic CAD patients (Rehman et al., 2004) or healthy subjects (Laufs et al., 2005). The increase in EPCs was also found to be independent of serum cortisol levels (Laufs et al., 2005) and serum levels of hepatocyte growth factor (Rehman et al., 2004).

A novel hypothesis for regulation of EPCs with acute exercise involves signaling by the blood coagulation protein thrombin. During acute exercise, thrombin production increases significantly *in vivo* due to the increased vascular shear stress and associated endothelial injury (Womack, Nagelkirk, & Coughlin, 2003). The role of thrombin, however, is not limited to that of blood clot formation. Thrombin and its receptor, protease activated receptor-1 (PAR-1), are required participants in endothelial regulation in adults as well as angiogenesis during fetal development. EPCs, which are believed to direct both adult and fetal endothelial maintenance and vascular genesis, also express PAR-1 on their cellular surface. There is increasing evidence that this g-protein coupled receptor is directly involved in the regulation of EPC function.

The involvement of thrombin and PAR-1 receptors in vascular regulation and angiogenesis was first observed in fetal development. Griffin et al (2001) found that thrombin signaling through PAR-1 is required in the building and stabilization of new blood vessels in mouse embryos (Griffin, Srinivasan, Zheng, Huang, & Coughlin, 2001). Smadja, et al (2006) further characterized the involvement of PAR-1 in post-natal angiogenesis. EPCs were isolated from human blood and stimulated *in vitro* with a PAR-1 agonist. When compared to untreated cells, EPCs treated with the PAR-1 agonist increased proliferation and migration, indicating a clear role of PAR-1 in EPC signaling and regulation (Smadja et al., 2006).

To further characterize the role of PAR-1 in EPC regulation, and specifically the role of thrombin, Tarzami, et al (2006) investigated the regulation of EPCs by thrombin stimulation *in vitro*. Bone marrow derived mononuclear cells were isolated from mice and grown *in vitro* in the presence or absence of thrombin. Thrombin treatment increased expression of EPC and mature endothelial cell characteristics in mononuclear cells as well as stimulating an increase in proliferation of EPCs. These effects were attenuated with the addition of a thrombin inhibitor. Furthermore, the effects of thrombin were mirrored by the addition of a PAR-1 agonist without the presence of thrombin, indicating that thrombin exerts its effect through the PAR-1 receptor (Tarzami, Wang, Li, Green, & Singh, 2006) . The results of Tarzami et al. (2006) clearly demonstrate thrombin's involvement in EPC regulation in mice, implicating a direct role in cellular differentiation and proliferation.

Given the evidence of EPC regulation by thrombin, it is possible that exercise-induced thrombin production may provide a direct link to the changes in EPCs

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previously reported with acute exercise. Due to elevated blood pressure, increased blood flow, and increased vascular shear stress, thrombin production is known to increase as a result of a single bout of exercise, specifically with higher intensity and longer duration exercise (Womack et al., 2003). This response is similar to the response of EPCs to exercise (Laufs et al., 2005). Additionally, thrombin production after acute exercise may be further elevated after exercise training. van den Burg, et al (2000) found that men aged 50 – 60 years had an increased level of thrombin production in response to maximal exercise after 12 weeks of endurance exercise training compared to baseline (van den Burg, Hospers, Mosterd, Bouma, & Huisveld, 2000). In the context of hemostasis alone, the increase in thrombin production after exercise training appears to contradict the reduced thrombus formation and improved endothelial health that is also known to occur with training. However, if thrombin is viewed in its role as a signaling molecule, specifically in its role of signaling EPCs, this seemingly paradoxical increase in thrombin may actually be beneficial. Thrombin may be the signal by which acute exercise affects EPC differentiation and proliferation. This represents a potential major pathway of exercise-induced regulation of EPCs that has yet to be investigated.

It was, therefore, the purpose of this study to examine the response of circulating EPC number and colony forming units to an acute bout of vigorous exercise. We further investigated the role that thrombin plays as a signaling molecule in EPC regulation, specifically cellular differentiation and proliferation, through gene expression analysis of endothelial cell surface markers and cell cycle proteins. Finally, in a cross-sectional design, we investigated any differences that may exist in

these relationships with long-term endurance exercise training by comparison of Masters athletes to matched low-active controls.

Endothelial progenitor cells have emerged as a central component in endothelial maintenance, post-natal angiogenesis, and thrombus resolution. Increased number and function of circulating EPCs are associated with improved endothelial function, decreased CVD risk, and enhanced vascular recovery from injury. With acute exercise and exercise training there is an increase in circulating level and function of EPCs, although a mechanism has not been determined. Plasma levels of thrombin, a central component to the coagulation cascade, increase with acute exercise in response to elevated vascular shear stress. Additionally, exercise-induced thrombin production has been observed to increase even further after exercise training. Thrombin has been observed to act as a primary regulator of EPC proliferation and differentiation. These facts raise the possibility that thrombin may represent a mechanistic link between exercise, circulating EPCs, and endothelial health.

Therefore, the primary purpose of this cross-sectional study was to investigate the link between physical activity, EPCs, and endothelial function. Specifically, we investigated the role of thrombin as a signaling molecule in relation to changes in EPC number and colony forming units (CFUs) with vigorous exercise, as well as thrombin's role in *in vitro* EPC differentiation and proliferation. Furthermore, we investigated the influence of long-term exercise training on these variables.

Hypothesis 1: Thirty minutes of vigorous exercise will induce an increase in thrombin production, circulating EPC number and EPC-CFUs. Furthermore, Masters athletes will have greater exercise-induced thrombin

production, circulating EPC number and CFUs than low-active controls.

Hypothesis 2: Exercise-induced thrombin production will positively correlate with exercise-induced changes in circulating EPC number and CFUs.

Hypothesis 3: Masters athletes will have greater endothelial function (endothelial-dependent flow-mediated dilation) than low-active controls. The difference in endothelial function between groups will be associated with the difference in circulating EPC number and CFUs between groups.

Hypothesis 4: EPCs isolated from human peripheral blood and supplemented with thrombin *in vitro* will have altered mRNA expression of cell cycle proteins and increased mRNA expression of mature endothelial cellular markers compared to untreated cells in a dose-dependent manner. Furthermore, the change in gene expression due to thrombin treatment in EPCs isolated from Masters athletes will be greater than that in the low-active group.

Hypothesis 5: EPCs isolated from human peripheral blood after 30-minutes of treadmill exercise will have altered mRNA expression of cell cycle proteins and mature endothelial cellular markers compared to cells isolated at rest. Furthermore, the relative change in gene expression will be greater in Masters athletes than low-active controls.

Chapter 2: Methods

Overview

Subjects who qualified for the study after the initial telephone screening were invited to participate in two to three testing sessions. During the initial session, informed consent was obtained as approved by the University of Maryland Institutional Review Board (Appendix A). Health history of each subject was reviewed followed by a resting blood pressure measurement and a blood draw for a general lipid panel. A maximal graded exercise test was then performed to screen for signs or symptoms of CVD and electrocardiogram (ECG) arrhythmias, as well as to measure maximal oxygen consumption (VO_2max). The second session was comprised of three components: 1) forearm blood flow test, 2) 30-minute vigorous treadmill exercise test, and 3) measurement of body composition. Alternatively, subjects were given the option to complete the 30-minute treadmill test during a separate third session. Each testing session took place ~24 hours after the most recent bout of exercise in Masters atheletes. Low-active controls were instructed to maintain their normal routine, exercising no less than 24 hours prior to testing. Subjects were fasted for 12 hours, refrained from using antihistamines and non-steroidal anti-inflammatory drugs (NSAIDs) for 48 hours, and refrained from alcohol consumption for 24 hours prior to testing. Blood samples for the hemostatic and EPC

variables were collected prior to, and after the completion of, the 30-minute vigorous exercise test.

Subjects

Subjects included twelve healthy male Masters athletes aged 55 to 80 years and eleven healthy low-active age- and BMI-matched controls. All subjects were healthy, nonsmoking, and had no history of CVD, bleeding disorders, diabetes, or cancer. Subjects were excluded if they were undergoing anti-coagulant therapy (Warfarin or similar) or were taking medications known to affect EPCs (Appendix B). Masters athletes had maintained an aerobic training history of at least 20 years, greater than 3 times per week at a moderate to vigorous intensity. Low-active controls exercised aerobically less than two times per week for less than 20 minutes per session for at least the previous 5 years.

Study volunteers were recruited from the Washington DC Metropolitan area. Masters athletes were recruited by collection of names from the results of recent race results in the area, through local running clubs, running organizations, and the University community. Low-active subjects were recruited through newspaper advertisements, word-of-mouth, community groups, the University community, and from a pool of prior research participants in the Department of Kinesiology at the University of Maryland who had previously consented to be contacted. Subjects were contacted via telephone for initial screening of exercise training and health history.

Qualification for participation in the study was determined through a brief telephone screening as well as health history and exercise training questionnaires. During the telephone screening, prospective subjects were provided with an overview of the study qualifications, testing procedures, requirements, risks, and benefits. After providing oral consent, the prospective subjects were questioned about health and physical activity status to determine qualification for the study. In addition to the above mentioned criteria, individuals were excluded if any of the following exclusion criteria were evident: orthopedic condition prohibiting treadmill exercise, history of uncontrolled hypertension (systolic > 160 mmHg or diastolic greater than 99 mmHg) or elevated cholesterol (>240 mg/dl). Those remaining qualified were asked to complete mail-in health history and physical activity questionnaires (Appendix C) to confirm qualification.

Eligible individuals who agreed to further participation were scheduled for the initial testing session. During this time subjects were again provided with a detailed overview of the study and given the opportunity to ask all remaining questions prior to signing the informed consent form. During this visit resting blood pressure was measured and blood was drawn to screen for lipid abnormalities and assess other basic blood chemistries. To screen for signs or symptoms of CVD, including ECG abnormalities, subjects underwent a brief physical examination and maximal graded exercise test under the supervision of a physician.

Blood Pressure

Blood pressure was measured manually using a mercury sphygmomanometer and stethoscope using the auscultatory method according to the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC VII) guidelines. Subjects arrived in the morning after fasting overnight for 12 hours. Blood pressure was measured after approximately 15 minutes of quiet seated rest. Measurements were taken at least twice or until both systolic and diastolic values were within 4 mmHg.

Maximal Graded Exercise Test

After a general physical examination by a physician, subjects were instrumented with a 12-lead chest electrocardiogram (ECG) to be monitored at rest, during exercise, and during recovery. After a brief warm-up and treadmill familiarization period, the treadmill test was completed following a modified Bruce protocol. Briefly, this protocol utilizes two- to three-minute stages of increasing intensity. Stage One included three minutes at 1.7 mph and 10% grade. Stage Two included three minutes at 2.5 mph and 12% grade. If the subject remained negative for signs or symptoms through the second stage, the testing protocol continued with two-minute stages that included a progressive increase in grade by two to three degrees per stage. Treadmill speed from stage three to completion of the test was determined by the investigator as a speed which elicited approximately 70 – 80% of predicted maximal heart rate at 0% grade and would elicit a maximal effort within 8 – 12 minutes of testing.

Heart rate and blood pressure were monitored at rest, during each stage of the treadmill test, and during recovery. Termination criteria included abnormal ECG or physiological responses to exercise as defined by the ACSM (2000), at the request of the subject, or by the indication of the attending physician. The test was considered to be a valid maximal exercise test if three of the following four criteria were met: the subject indicated exhaustion, respiratory exchange ratio (RER) was greater than 1.15, there was a plateau of VO_2 with increasing workload, or there was a plateau of heart rate with increasing workload in agreement with the guidelines described by the ACSM (2000).

Metabolic variables were measured using indirect calorimetry. Gas fractions of oxygen and carbon dioxide in the ambient and expired air as well as volume of expired air were measured by an Oxycon Pro gas analyzer and turbine flow meter (Viasys Healthcare, Yorba Linda, CA). Data were collected by an online computer system and analyzed in 30-second intervals.

Endothelium Dependent Flow Mediated Vasodilation

To assess endothelial dysfunction, endothelium dependent vasodilation was assessed by reactive hyperemia and measured via strain gauge plethysmography. Prior to testing, the subject rested supine in a quiet comfortable room for 15-20 minutes with low ambient lighting. Two pressure cuffs were placed on the subject's arm, one cuff on the upper arm and one on the wrist. A mercury filled strain gauge was placed around the widest part of the forearm and strain was measured by Hokanson EC-5R plethysmograph (Hokanson Inc, WA). A standard automated blood pressure cuff was fit to the opposite arm to monitor

blood pressure during the test. For the initial baseline measurement, the wrist cuff was inflated to a supra-systolic pressure (200 mmHg) to stop arterial hand circulation in order to eliminate inter-subject variability in hand blood flow. The upper arm cuff, controlled via a rapid cuff inflator (E-20, Hokanson Inc), was then inflated to 55mmHg for seven seconds and released for 8 seconds. This 15-second cycle was repeated for three to five cycles. Peak blood flow was measured at the time of upper arm cuff release and a return to baseline follows.

Reactive hyperemia was determined by occluding the brachial artery for five minutes, then repeating the measurement of flow mediated vasodilation. The upper arm cuff was inflated to 50 mmHg above systolic blood pressure for five minutes. After the five-minute occlusion, forearm blood flow was measured continuously in 15-second cycles for three minutes as described above. Reactive hyperemia was calculated as the percent change in forearm blood flow from rest to post-ischemic peak blood flow. To reduce variability associated with dietary nitrates, participants ingested a low-nitrate diet for the 3 days prior to forearm blood flow (Larsen, Ekblom, Sahlin, Lundberg, & Weitzberg, 2006).

30-minute Vigorous Exercise Test

To assess the response of circulating EPC number, EPC-CFUs, and thrombin production to an acute bout of exercise, subjects exercised on a treadmill for 30 minutes at $75\% \pm 5\%$ VO_2max as determined during the maximal graded exercise test. Similar to the maximal graded exercise test, gas fractions of the ambient and expired air as well as volume of expired air were measured by an Oxycon Pro gas

analyzer and turbine flow meter (Viasys Healthcare, Yorba Linda, CA). Data were collected by an online computer system and analyzed in 30-second intervals. Subjects were also instrumented with a Polar heart rate monitor. Speed and grade of the treadmill were adjusted to elicit the prescribed relative intensity. The test included a 5-minute ramp-up period and subsequent 25 minutes at the prescribed intensity. At the conclusion of the 30-minute exercise period, subjects entered a low-intensity active cool-down period for 3 minutes.

Body Composition

Height and weight were measured and body mass index (BMI, kg/m^2) was calculated for all subjects. Body composition analysis, including percentage of total body weight consisting of fat mass and lean mass, was determined via dual-energy x-ray absorptiometry (DEXA) (Hologic, Discovery A, software version 12.7.1).

Blood Collection

All blood samples were obtained via clean venipuncture, using standard aseptic laboratory techniques. Blood samples for the screening of blood lipids and basic blood chemistries were drawn at rest with the subject in the seated position. Approximately 10 mL blood were obtained by using 10-mL SST gel & clot activator tubes. Samples were analyzed by Quest Diagnostics (Baltimore, MD), a commercial diagnostic laboratory service.

To assess the acute response of EPCs and thrombin production to exercise, blood samples were collected before and after the 30-minute treadmill exercise test with the subject in the supine position. Blood sampling before the exercise test took

place after the subjects were resting quietly for $\geq$ 15 minutes. Blood was drawn for thrombin analysis five minutes after the completion of the 30-minute exercise test. Blood samples for EPC isolation were collected 30 minutes after the completion of the 30-minute exercise test. Hematocrit was measured in duplicate before exercise and five minutes after exercise using standard methods.

Blood Coagulation

To assess thrombin production, blood was collected at rest and after 30 minutes of vigorous exercise. Plasma levels of thrombin-antithrombin III (TAT) and prothrombin fragment F1+2 (F1+2) were assessed as markers indicative of thrombin production. Plasma TAT level was measured using Enzygnost TAT micro enzyme linked immunoassay (ELISA) (Dade Behring, Deerfield, IL) and F1+2 was measured using Enzygnost F_{1+2} micro ELISA (Dade Behring, Deerfield, IL) according to manufacturers' instructions.

Assays for TAT and F1+2 followed similar protocols. Briefly, plasma samples were incubated initially to bind to antigen specific monoclonal antibodies previously bound to the surface of the 96-well plate, washed to remove unbound protein and then incubated again with peroxidase conjugated antibodies. Plasma levels of F1+2 and TAT were determined by a microplate photometer (Multiscan EX, Thermo Electron Corp.). Protein concentrations were determined by comparison to a standard curve line-of-best-fit generated using standards of known protein concentration. Samples were run in duplicate and values were adjusted for changes in hematocrit with exercise. Assay validity was confirmed using samples of known protein concentration provided by the

manufacturer. The intra-assay coefficient of variation was 12.0 and 11.1% for TAT and F1+2 respectively.

Endothelial Progenitor Cells

Circulating Endothelial Progenitor Cells: The level of EPCs in the circulating blood was assessed by flow cytometry. Peripheral blood mononuclear cells were isolated by addition of lymphocyte separation media (Ficoll Paque PLUS) and density centrifugation. At least $5x10^5$ cells were immunostained with monoclonal antibodies against human cell surface markers CD34 and VEGFR2, followed by a phycoetherin (PE)-conjugated secondary antibody. Isotype-identical antibodies served as controls (Human CD34 FITC Mouse-IgG1, Mouse IgG2a FITC, CD3-FITC Ms-IgG1, and CD3-R-PE Mouse-IgG1: BD Pharmingen, BD Biosciences, NJ; Human VEGFR-2 PE biotinylated, and PE Mouse-IGg1: R&D Systems, MN). After a brief incubation period at room temperature, cells were lysed, washed with phosphate buffered solution (PBS), and fixed in a 4% paraformaldehyde fixation buffer before analysis by flow cytometry. All analyses were performed in the Flow Cytometry/Cell Sorting CORE Laboratory at the University of Maryland Baltimore School of Medicine using a Beckman Coulter Epics Elite ESP flow cytometer. Data are presented as the number of detections per 100,000 events. For a subset of subjects, samples were processed and analyzed in duplicate to assess reproducibility. The correlation between the two measures was $r = 0.90$ ($p < 0.001$) ($n = 30$).

EPC Isolation and Colony Forming Units: To assess the functional capacity of the isolated EPCs *in vitro*, a colony forming unit (CFU) assay was performed (Hill, 2003). Mononuclear cells were isolated from peripheral blood by density gradient

centrifugation using lymphocyte separation media, washed twice with PBS and 2% fetal bovine serum (FBS) and resuspended in EndoCult® Medium (Stem Cell Technologies, Inc, CA). Cells were counted with a hemacytometer using standard methods. 5×10^6 mononuclear cells were plated in duplicate wells in fibronectin-coated 6-well plates (BD Pharmingen) and incubated for two days at 37^0 C and 5% CO_2. After 48 hours, non-adherent cells from duplicate wells were collected and counted. 1×10^6 of those non-adherent cells were re-plated in quadruplicate wells of fibronectin-coated 24-well plates (BD Pharmingen) and incubated at 37^0 C and 5% CO_2 for three days. After 3 days, the number of CFUs per well were counted by a trained independent observer using inverted light microscopy. CFUs were defined as "multiple thin, flat cells emanating from a central cluster of rounded cells" (Hill et al., 2003). A central cluster without emanating cells was not counted as a colony. To assess inter-observer variability EPC-CFU counts by two independent observers were correlated in a subsample of wells (r = 0.98, p < 0.001, n = 36 wells).

Gene Expression

In a subsample of six Masters athletes and six control subjects, endothelial progenitor cells were isolated from blood samples drawn at rest and 30 minutes after vigorous treadmill exercise. Day 2 non-adherent cells were incubated in-vitro as described above. To investigate the dose response relationship of thrombin treatment in EPCs, the incubation medium of the resting samples was supplemented in duplicate wells with 0 U, 1 U, 5 U, or 10 U of thrombin (Human α-thrombin, Enzyme Research Laboratory, IN) in a 100μl solution with PBS + 5% bovine serum albumin

(BSA) in each well. Cells that were isolated from blood samples drawn 30 minutes after exercise were assessed without thrombin supplementation.

Cells were incubated at 37^0 C and 5% CO_2 for approximately 24 hours. Cells were washed with sterile PBS and detached from the plate using 250µl TRIzol per well, combining duplicate wells into the same sample and incubating for 10 minutes. 20ul chloroform per 100 µl of TRIzol was added, inverted for 10 minutes and centrifuged for 12 minutes at 4^0 at 12,000 x g. The upper clear layer containing RNA was then removed and combined with 50ul isopropyl alcohol for every 100ul trizol, inverted for 10 minutes, then centrifuged for 8 minutes at 4^0 C at 12,000 x g to pellet the sample. The supernatant was removed and 150ul ethanol for every 100 ul of trizol was added. The sample was incubated for 5 minutes at room temperature then centrifuged for 5 minutes at 4^0 C @ 7500 x g. The ethanol was removed. Once dry, the pellet was suspended in 25µl TE buffer per sample, incubated at $55 – 60^0$ C for 10 minutes and stored at -80^0 C. RNA concentrations were determined by optical density at 260 nm. RNA quality was confirmed by ethidium bromide staining of 18S and 28S visualized by gel electrophoresis under ultraviolet light. .

Real Time-PCR: Total RNA was reverse transcribed to cDNA using a cDNA reverse transcription kit (Applied Biosystems, CA). cDNA was amplified and gene expression assessed via pre-designed TaqMan RT-PCR gene expression kits using an Applied Biosystems 7500 RT-PCR System (Applied Biosystems, CA). EPCs within each treatment group were incubated in duplicate. Duplicate wells were pooled and samples were amplified in triplicate using RT-PCR. Gene expression was analyzed using Applied Biosystems Sequence Detection Software (Applied Biosystems, CA).

To avoid amplifying genomic DNA, all primer sequences spanned exons. The thermal cycling conditions included an initial denaturation cycle at 95^0 C for 10 min followed by 40 cycles at 95^0 C for 15 seconds and 65^0 C for 1 min.

Proliferation: To assess the role of thrombin as a signaling molecule in the proliferation of EPCs, the following genes were assessed for changes in gene expression: Cyclin A2, Cyclin D1, and p27 (cyclin dependent kinase inhibitor-1) (Applied Biosystems pre-designed RT-PCR kits Hs00153138_m1, Hs00277039_m1, and Hs00271467_m1 respectively, Applied Biosystems, CA). Cyclin A2 is a cell cycle protein required for the progress of the cell though the S-Phase of the cell cycle and has been shown to be elevated in concentration during cell proliferation. Cyclin D1 is a cell cycle protein required for the G1/S phase transition in the cell cycle and its expression is elevated during the cell proliferation cycle. p27 is known to be an inhibitor of cell proliferation and has been shown to be decreased in concentration during cell proliferation.

Differentiation: To assess the role of thrombin in signaling the differentiation of EPCs, the following genes were assessed for changes in gene expression: VE-Cadherin and VEGFR2 (aka: KDR, flk-1) (Applied Biosystems pre-designed RT-PCR kits Hs00901463_m1 and Hs00176676_m1, Applied Biosystems, CA). These membrane-bound cell surface markers identify these cells as being of endothelial lineage and progressing towards differentiation into mature endothelial cells.

Analysis of Gene Expression: Changes in mRNA gene expression were assessed though relative quantification by comparing expression of each gene to that of an endogenous control known to be stably expressed across treatment conditions.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1, Applied Biosystems, CA), a commonly used reference gene in the literature, was found to be stably expressed across treatment conditions in our samples. No significant difference was found in gene expression of GAPDH between treatment conditions (p = 0.494) and it was therefore used as the reference gene.

Quantification of gene expression in each treatment group was analyzed according to manufacturer's specifications and is expressed as $\log_{10}$ relative quantification (RQ) indicating the fold difference in gene expression compared to the control sample. Briefly, the threshold cycle of amplification (Ct) for each sample is compared to that of the endogenous control GAPDH. The difference in Ct between the sample and GADPH is expressed at ΔCt. Within each gene assay, the difference in ΔCt between each treatment level (1U, 5U, 10U thrombin, and 30 min sample) and the control sample (0U thrombin) is expressed as $\Delta\Delta$Ct. RQ of each sample is calculated as $2^{-\Delta\Delta Ct}$. The fold difference of each sample compared to the control is then expressed as $\log_{10}$ RQ.

All gene expression analyses of a given sample were performed on a single plate to eliminate inter-assay variation. Intra-assay coefficient of variation for gene expression was 0.65%.

Statistical Analysis

Hypothesis 1: **Thirty minutes of vigorous exercise will induce an increase in thrombin production, circulating EPC number and EPC-CFUs. Furthermore, Masters athletes will have greater exercise-induced thrombin production, circulating EPC number and CFUs than low-active controls.**

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Comparisons were made for each variable between values before and after maximal exercise using repeated measures ANOVA. The exercise-induced change in F1+2 levels, TAT levels, EPC number, and EPC-CFUs were compared between Masters athletes and low-active controls by univariate ANOVA.

Hypothesis 2**: Exercise-induced thrombin production will positively correlate with exercise-induced changes in circulating EPC number and CFUs.** The association between exercise-induced changes in the levels of F1+2 and TAT and the change in EPC number and EPC-CFUs was analyzed by Spearman's correlation coefficient (r).

Hypothesis 3**: Masters athletes will have greater endothelial function (endothelial-dependent flow-mediated dilation) than low-active controls. The difference in endothelial function between groups will be associated with the difference in circulating EPC number and CFUs between groups.** Comparison of endothelial function between Masters athletes and controls was performed using an univariate ANOVA. The relationships of endothelial function with EPC number and EPC-CFUs were analyzed by Spearman's correlation coefficient (r).

Hypothesis 4: **EPCs isolated from human peripheral blood and supplemented with thrombin *in vitro* will have altered mRNA expression of cell cycle proteins and increased mRNA expression of mature endothelial cellular markers compared to untreated cells in a dose-dependent manner. Furthermore, the change in gene expression due to thrombin treatment in EPCs isolated from Masters athletes will be greater than that in the low-active group.** The differences in proliferation among four levels of thrombin treatment (0U, 1U,

5U, 10U) by mRNA gene expression of the Cyclin A2, Cyclin D1, and p27 genes were analyzed by univariate ANOVA using a simple pairwise comparison by treatment. The differences in differentiation among thrombin treatment groups were assessed by the difference in mRNA gene expression of VE-cadherin and VEGFR2 and were analyzed by univariate ANOVA using a simple pairwise comparison by treatment. Comparisons between Masters athletes and low-active controls were performed by univariate ANOVA.

Hypothesis 5: **EPCs isolated from human peripheral blood after 30-minutes of treadmill exercise will have altered mRNA expression of cell cycle proteins and mature endothelial cellular markers compared to cells isolated at rest. Furthermore, the relative change in gene expression will be greater in Masters athletes than low-active controls.** The difference in mRNA expression between cells isolated in the rested state and after 30-minutes of vigorous (75% VO$_2$max) treadmill exercise was analyzed by repeated measures ANOVA. The change in gene expression was compared between Masters athletes and low-active subjects by univariate ANOVA.

All statistical analyses were performed using SPSS statistical software (SPSS version 16.0 for Windows, SPSS Inc. IL). Data are presented as mean ± SE. Statistical significance was set at $p \leq 0.05$. Variables were tested for assumptions of normality and homogeneity of variance. Variables not included in the repeated measures ANOVA were also tested for independence. These analyses indicated that those variables measured after 30 minutes of vigorous exercise, specifically EPC number, EPC-CFU, TAT, and F1+2 had non-normal, positively skewed single modal

distributions. This was, likewise, the case for the 'change variables' that measured the difference in each of these variables from rest to after exercise. Having met the other assumptions of ANOVA, the assumption of normality was considered robust and ANOVA was used in the final analysis (Hinkle, Wiersa, & Jurs, 1998). For all correlations, Spearman's correlation was utilized. Spearman's correlation uses rank order correlation and does not require the assumption of normality, reducing the influence of outlier data (Hinkle et al., 1998).

Chapter 3: Results

Masters athletes and low-active controls were successfully matched for age and body mass index (BMI) with non-significant mean differences of 3 years and 1.5 kg/m^2, respectively, between groups (Table 1). Additionally, CVD risk factors including total cholesterol, LDL-cholesterol, and blood pressure were similar between groups. As expected, aerobic fitness, as measured by VO$_2$max, was significantly greater in Masters athletes than low-active controls ($p < 0.001$). Likewise, HDL-cholesterol was significantly higher in athletes ($p = 0.002$) while body fat percentage was significantly lower in athletes compared to controls ($p = 0.019$).

Table 1: Descriptive Data

Variables	Athletes (n=12)	Controls (n=11)
Age (years)	62 ± 1.6	65 ± 1.5
Height (m)	1.79 ± 0.04	1.76 ± 0.02
Weight (kg)	70.1 ± 2.9	72.6 ± 1.9
BMI (kg/m^2)	22.0 ± 0.9	23.5 ± 0.6
Body Composition (% fat)	18.0 ± 1.3	23.6 ±1.8*
Total Cholesterol (mg/dl)	199 ± 8.9	194 ±10.6
HDL-C (mg/dl)	71 ± 3.3	51 ± 4.6*
LDL-C (mg/dl)	115 ± 8.4	123 ± 11.4
Systolic BP (mmHg)	122 ± 3.0	129 ± 3.0
Diastolic BP (mmHg)	79 ± 2.0	85 ± 2.0
VO$_2$max (ml/kg/min)	50.0 ± 1.3	28.2 ± 1.8*

Values are means ± S.E. * Difference between groups was significant ($p \leq 0.05$). BMI: Body Mass Index; HDL-C: High Density Lipoprotein Cholesterol; LDL-C: Low Density Lipoprotein Cholesterol; BP: Blood Pressure; VO$_2$max: Maximal oxygen consumption.

Thrombin Production

To quantify the *in vivo* activation of prothrombin to thrombin in response to 30 minutes of vigorous treadmill exercise, prothrombin fragment (F1+2) and

thrombin-antithrombin III (TAT) concentrations were measured in blood samples

drawn at rest and five minutes after exercise. In both Masters athletes and low-active

controls there was a significant increase in both F1+2 and TAT following exercise

(Table 2). On average, low-active controls had an approximately 3.6 times greater

exercise-induced increase in plasma F1+2 concentration than Masters athletes (37.56

pmol/L and 10.51 pmol/L respectively) (Table 2). Statistically, however, there was

no significant difference between groups. This was likely due to the large variability

in plasma F1+2 concentrations following exercise in the control group, with

individual values ranging from 35.86 pmol/L to 134.89 pmol/L (SD = 43.6 pmol/L).

Table 2: Prothrombin fragment and thrombin-antithrombin III production in response
to 30 minutes of vigorous exercise

	Athletes (n=12)	Controls (n=7)
F1+2 (pmol/L)		
Before Exercise	35.10 ± 1.49	34.27 ± 1.12
After Exercise	45.61 ± 3.25 *	71.83 ± 16.47 *
TAT (µg/L)		
Before Exercise	2.72 ± 0.20	2.47 ± 0.15
After Exercise	3.35 ± 0.37 *	3.26 ± 0.32 *

Values are means ± (S.E.) * Significant change from baseline ($p \leq 0.05$)

Endothelial Progenitor Cells

Circulating EPCs were quantified in blood samples drawn at rest and 30

minutes after vigorous exercise. Additionally, EPC function was assessed by

counting EPC-CFUs *in-vitro* at both time points. No change was observed in either

circulating EPC number or EPC-CFUs in response to vigorous exercise within either

Masters athletes or controls. Additionally, when all subjects were pooled, no

significant change with exercise was observed (Figures 1-2). The individual response

to exercise varied considerably between subjects. The change in circulating EPC number with exercise ranged from -98 to +385 cells/100,000 events, while the change in EPC-CFUs with exercise ranged from -27 to +142 colonies. Additionally, when comparing circulating EPCs and EPC-CFUs between Masters athletes and low-active controls, there was no significant difference between groups either at rest or in response to vigorous exercise.

The correlation between exercise-induced changes in thrombin production, as determined by plasma levels of F1+2 and TAT, and the changes in circulating EPC number and EPC-CFUs were also investigated. When groups were analyzed separately, we found that in Masters athletes the changes in circulating EPC number ($r = 0.711$, $p < 0.005$) but not EPC-CFUs ($r = 0.343$, $p = 0.138$) was positively correlated with the change in plasma F1+2 with vigorous exercise. In low-active controls, a similar correlation was seen with the change in circulating EPC number ($r = 0.771$, $p = 0.036$) but not the change in EPC-CFUs ($r = -0.036$, $p = 0.47$) with vigorous exercise. When analyzed across all subjects, we found that the change in circulating EPC number ($r = 0.698$, $p = 0.001$) but not EPC-CFUs ($r = 0.307$, $p = 0.10$) was significantly correlated with the change in plasma F1+2 concentration with vigorous exercise. No correlation was evident between the change in plasma TAT concentration with exercise and the change in either circulating EPCs or EPC-CFUs.

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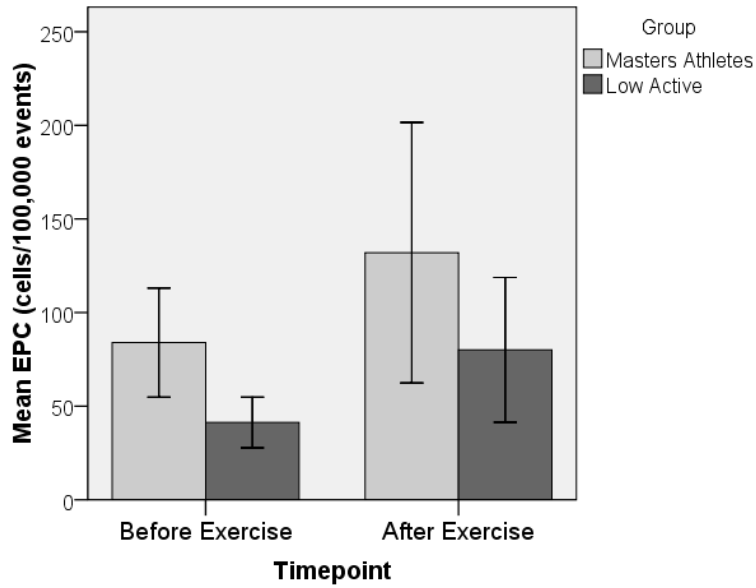**Figure 1:** Change in EPC numbers with 30 minutes of vigorous treadmill exercise. EPCs were measured as cells/100,000 events. Error bars represent S.E. Athletes n=12; Controls before exercise n=11, after exercise n=6.
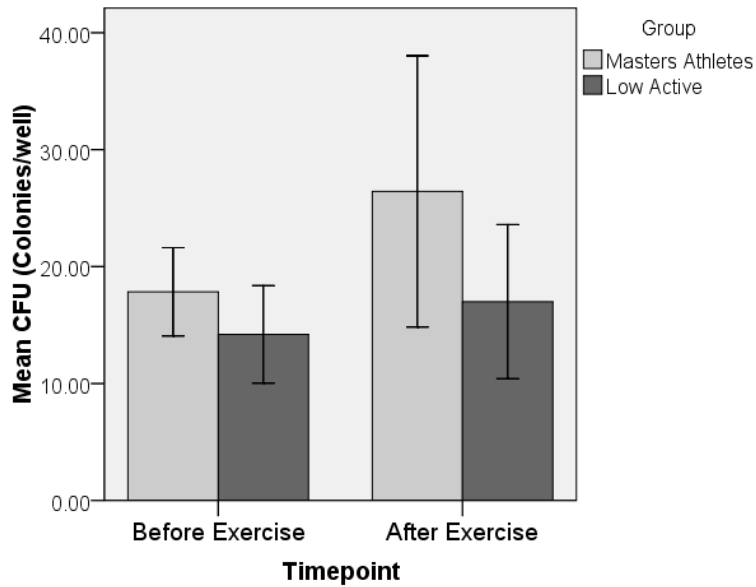
Figure 2: Change in EPC-Colony forming units with 30 minutes of vigorous exercise. Error bars represent S.E. Athletes n=12; Controls before exercise n=11, after exercise n=7.

Forearm Blood Flow

As an assessment of endothelial health and vascular function, forearm blood flow was measured at rest and after five minutes of ischemia. While no difference was found between groups at rest, peak forearm blood flow was significantly greater in the Masters athletes after five minutes of ischemia ($p = 0.028$) (Figure 3). When groups were analyzed separately, we found that forearm blood flow at rest was positively correlated with circulating EPC number ($r = 0.705$, $p = 0.01$) in Masters athletes but not low-active controls ($r = -0.479$, $p = 0.136$). Resting forearm blood flow was not related to EPC-CFUs in either Masters athletes ($r = -0.112$, $p = 0.729$) or low-active controls ($r = -0.409$, $p = 0.212$). The peak change in forearm blood flow after five minutes of ischemia did not correlate with circulating EPC number in either group. Additionally, the peak change in forearm blood flow after ischemia did not correlate with EPC-CFUs in Masters athletes ($r = -0.098$, $p = 0.762$) but was positively correlated in low-active controls ($r = 0.682$, $p = 0.021$).

When all subjects were pooled, circulating EPC number there was no correlation between circulating EPC number or EPC-CFUs with forearm blood flow. The change in peak forearm blood flow after five minutes of ischemia was not related to either circulating EPC number or EPC-CFUs.
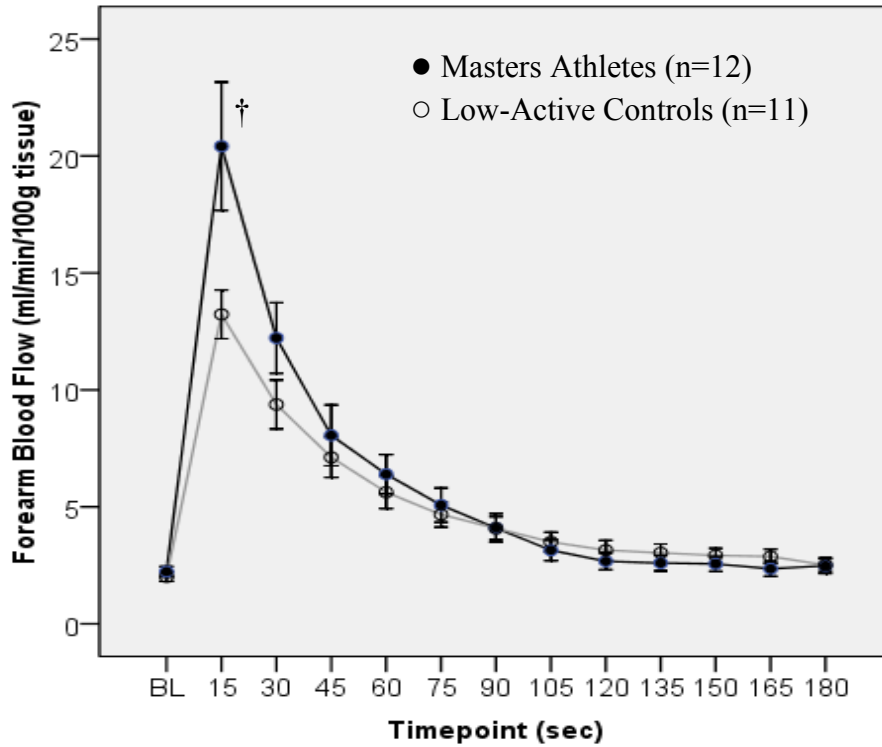
Figure 3: Forearm blood flow response to 5 minutes of ischemia.
BL: Baseline. † Significant difference between groups ($p \le 0.05$).

Gene Expression

Gene expression was assessed in EPCs in response to increasing levels of thrombin supplementation *in vitro*. Additionally, gene expression was assessed in EPCs obtained after 30 minutes of vigorous exercise. Pooling gene expression results from all subjects revealed a dose-response increase in cell proliferation genes cyclin A2 and cyclin D1 peaking at 5U thrombin (50% and 78% increase respectively) when compared to control cells receiving no thrombin (Figure 4). This change was mirrored by a similar decrease in the gene expression of the cell proliferation inhibitor p27 at 5U compared to control cells (90% decrease). After 30 minutes of vigorous exercise, a similar gene expression profile was seen compared to the thrombin treatment. The exercise stimulus elicited an increase in expression in the

cyclin A2, cyclin D1 (20% and 58% respectively) and a decrease in expression of p27 genes (58%) compared to control cells. Gene expression of VE-Cadherin and VEGFR2, indicators of cell proliferation, increased by 87% and 66% respectively with 1U thrombin compared to control cells, but did not change significantly from control under all other conditions. The 30-minute exercise stimulus did not significantly change gene expression of VE-Cadherin or VEGFR2 compared to control cells.

When analyzing Masters athletes and low-active controls separately, similar trends were observed in cell cycle genes (Figure 5). There was a 2.97 fold greater peak decrease in p27 expression in Masters athletes with 5U thrombin compared to low-active controls. Additionally, the spike in gene expression of VE-Cadherin and VEGFR2 at 1U thrombin observed across groups is only apparent in the athletes (183% and 120% respectively) with no significant change in expression in the low-active controls. The changes in gene expression in response to 30 minutes of vigorous exercise were not significantly different between groups.
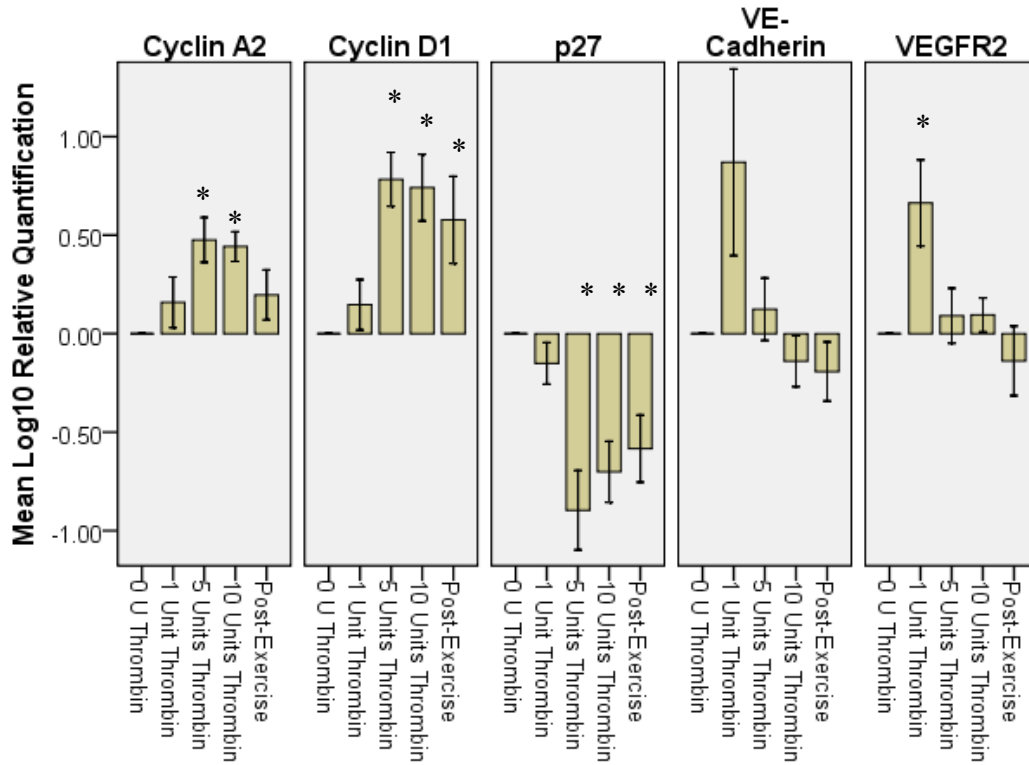
Figure 4: Gene expression in thrombin supplemented EPCs sampled at rest and EPCs obtained after 30 minutes of vigorous exercise. Day 2 non-adherent cells were replated and the growth medium was supplemented with 0U, 1U, 5U, or 10U of thrombin for 24 hours. All comparisons were made to the control, 0U thrombin supplementation, set at 0.0. * Significant difference from control ($p \leq 0.05$). Athletes n=6; Control subjects n=5.
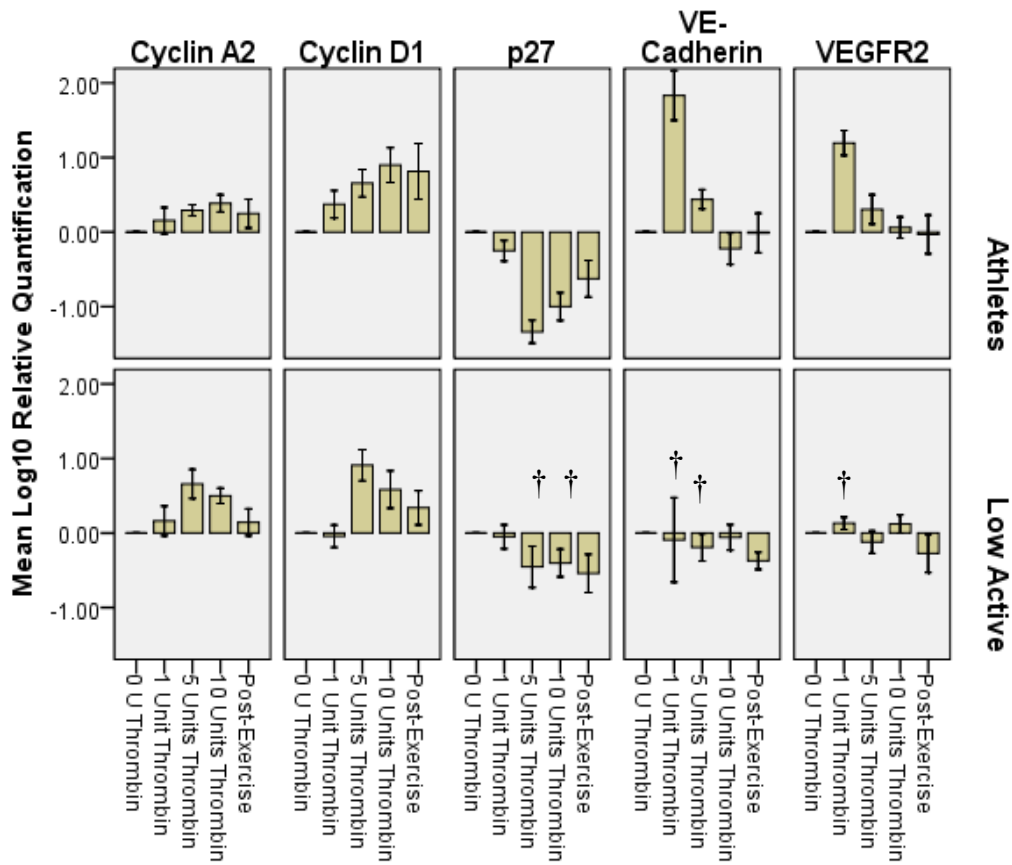
Figure 5: Comparison of gene expression between Masters athletes and low-active controls. All comparisons were made to the control, 0U thrombin supplementation, set at 0.0.   † Significant difference between groups (p ≤ 0.05). Athletes n=6; Control subjects n=5.

Chapter 4: Discussion

The purpose of this study was to examine the link between physical activity, EPCs, and endothelial function. We sought to investigate the relationship between exercise-induced thrombin production and changes in circulating EPC (CD34+/VEGFR2+) number and colony forming units (CFUs) with vigorous exercise. We then investigated the role of thrombin as a signaling molecule in *in vitro* EPC differentiation and proliferation through gene expression analysis. Furthermore, in a cross-sectional design, we investigated the influence of long-term exercise training on these variables.

Exercise-Induced Thrombin Production

It is believed that vigorous exercise gives rise to elevated thrombin production as a result of endothelial damage and the resultant activation of the coagulation cascade. Endothelial damage during exercise is the consequence of turbulent blood flow and the resulting shear stress along the vessel walls (Padilla, Harris, Rink, & Wallace, 2008). The initial component of this investigation was to confirm elevated thrombin production in response to the vigorous exercise stimulus within our subject population. To this end, the prothrombin activation product, F1+2, and the product of free thrombin inactivation, TAT, were measured at rest and after vigorous treadmill exercise. Our results indicated a significant increase in both markers of thrombin production. These results have been previously reported in the literature by other laboratories for both F1+2 and TAT (Womack et al., 2003).

We further examined whether exercise-induced thrombin production varied between Masters athletes and low-active controls. We found no significant difference between groups at rest or in response to vigorous exercise. Interestingly, the mean change in plasma F1+2 concentration with exercise in low-active controls was ~3.6 times greater than that of Masters athletes. However, due to the large standard deviation in plasma F1+2 concentration among low-active controls after exercise (SD = 43.6 pmol/L) this difference between groups did not reach statistical significance. Given the cross-sectional nature of our study design, it is difficult to pinpoint the cause of such variability within this group of low-active individuals.

Thus far, there has been little published evidence on the association of exercise training with exercise-induced changes in F1+2 or TAT. van den Burg et al. (2000) investigated the effect of 12 weeks of exercise training on the exercise-induced changes in several hemostatic markers, including F1+2 and TAT, in healthy sedentary men aged 50 – 60 years. Similar to our results, they found no difference in exercise-induced changes in plasma TAT concentration between sedentary and trained individuals. They did, however, find that after 12 weeks of training, these men demonstrated a greater increase in plasma F1+2 with maximal exercise. To our knowledge, no other published study has investigated this relationship between exercise training and exercise-induced changes in F1+2 in healthy adults. The paradoxical nature of greater exercise-induced thrombin production after exercise training, as observed by van den Burg et al., suggests greater endothelial damage and/or plaque rupture in the trained versus sedentary state, presumably because of greater shear stress resulting from higher blood flow in trained individuals exercising

at the same relative exercise intensity. The design of our present study allows us to cross-sectionally examine the association of long-term exercise training with these hemostatic variables in contrast to the relatively short-term training over 12 weeks utilized by van den Burg, et al. The disparity between studies may be indicative of a training threshold, such that elevated plasma F1+2 observed after 12-weeks of training is alleviated with longer-term training. The difference between studies may also be more simply due to the difference in exercise intensity. We utilized a submaximal protocol in our study compared to the maximal protocol used in the study by van den Burg et al. (2000). We cannot discount the notion that the disparity may be due to the difference in cross-sectional versus longitudinal study design. Without further investigation it is difficult to conclude on the source of this disagreement.

Endothelial Progenitor Cells

Several studies have recently investigated the relationship of both acute exercise and exercise training with EPCs. The majority of these studies have focused on recovery from, and secondary prevention of, CVD. Participants in these studies were generally individuals with pre-existing atherosclerotic disorders including coronary heart disease and peripheral vascular disease. Of those studies that have examined the association of exercise training with EPCs in CVD patients, the majority have demonstrated an increase in circulating EPC concentration (Laufs et al., 2004; Steiner et al., 2005; Sandri et al., 2005; Sarto et al., 2007), as well as an improvement in EPC-CFUs (Sarto et al., 2007), and enhanced *in vitro* network formation (Sandri et al., 2005). Similarly, among CVD patients, maximal acute

exercise elicited an increase in circulating EPCs (Rehman et al., 2004; Adams et al., 2004).

When considering healthy sedentary participants, Hoetzer et al. (2007) demonstrated an increase in circulating EPC-CFUs and *in vitro* migratory capacity with three months of exercise training in middle aged and older men. In response to acute exercise Laufs et al. (2005) found an increase in both circulating EPCs and EPC-CFUs with 30 minutes of submaximal exercise at anaerobic threshold, while VanCraenenbroek et al. (2008) found an increase in circulating EPCs, but not EPC-CFUs with maximal exercise in healthy individuals.

Given the limited published information available, we sought to investigate the response of healthy individuals to acute exercise as well as to investigate the association with exercise training. Within our results, despite the ~80% average increase in EPC number from rest to after 30 minutes of vigorous treadmill exercise, the wide variability in individual responses led us to conclude that there was no significant change within our subject population. Similarly, there was no significant change in EPC-CFUs despite the ~40% mean increase in EPC-CFUs from rest to after 30-minutes of vigorous exercise. This was also attributed to the wide variability between participants. In comparison to the current literature investigating healthy individuals, our observation that EPC-CFUs did not change with exercise is in concurrence with results of VanCraenenbroeck et al. (2008). However, Laufs et al (2005) found a significant exercise-induced increase in circulating EPC concentration (VEGFR2+/CD34+) of ~125% as well as a significant increase in EPC-CFUs of ~66%. While the magnitude of change for each variable with acute exercise is similar

to that observed in our study, the inter-individual variability in our results led us to conclude that there was no effect of vigorous exercise on circulating EPC concentration or EPC-CFUs.

We also found no differences between Masters athletes and low-active controls in the responses of circulating EPC concentration or EPC-CFUs to vigorous exercise. Masters athletes demonstrated a non-significant increase of 48 ± 46.7 cells per 100,000 events in circulating EPCs compared to a non-significant increase of 24.3 ± 31.2 cells per 100,000 events in low-active controls. Likewise, when examining EPC-CFUs, Masters athletes demonstrated a non-significant increase of 8.6 ± 12.4 colonies per well with exercise compared to a non-significant increase of 2.5 ± 5.9 colonies per well in low-active controls. As evidenced by our results and discussed above, there was wide variability in individual responses to exercise within both groups. The results of our cross-sectional design indicate that the response to vigorous treadmill exercise is independent of exercise training status.

The wide variability in responses of both circulating EPC concentration and EPC-CFUs to vigorous treadmill exercise led us to conclude that there is an as yet unidentified factor that influences the response of these variables to exercise. We observed a correlation between exercise-induced changes in F1+2 and exercise-induced changes in circulating EPC concentration in both Masters athletes and low active controls. These correlations imply two possibilities about the relationship between thrombin and EPCs. One possible explanation is that the changes seen in these variables with exercise may be the result of the same physiological stimulus. As previously discussed, prothrombin is activated to thrombin in response to elevated

shear stress and resulting endothelial damage during exercise. It follows that greater damage results in greater thrombin production. Given the correlation between exercise-induced changes in thrombin production and these EPC variables, the magnitude of changes seen in circulating EPC concentration within individuals may be the direct result of the magnitude of endothelial damage induced by exercise. A second possibility suggests a causal relationship between these variables, such that thrombin may directly or indirectly act to enhance EPC mobilization.

Gene Expression

To directly investigate the role of thrombin as a cell signaling molecule in EPC proliferation and differentiation, we measured changes in gene expression in the cell cycle genes cyclin A2, cyclin D1, and p27 as well as genes for the endothelial cell surface markers VEGFR2 and VE-Cadherin. We supplemented Day 2 non-adherent cells with 0U, 1U, 5U, or 10U of thrombin for 24 hours and simultaneously cultured Day 2 non-adherent cells isolated 30 minutes after vigorous exercise. We found that EPCs supplemented with thrombin increased gene expression of the cell cycle genes cyclin A2 and cyclin D1 in a dose-dependent manner, with a plateau in gene expression between 5U and 10U thrombin. Concurrently, there was a dose-dependent decrease in expression of the cell cycle inhibitor p27, which also showed a plateau in gene expression between 5U and 10U. This pattern of gene expression supports the role of thrombin as a signal for the proliferation of EPCs. This conclusion is supported by the recent evidence by Smadja et al. (2008) who demonstrated that within a fibrin matrix simulating *in vivo* activity, the presence of thrombin significantly increased proliferation of EPCs mediated through the PAR-1

receptor. Tarzami et al. (2006) similarly found that EPCs isolated from the bone marrow of mice increased cellular proliferation in response to *in vitro* supplementation with 10U of thrombin.

Gene expression of both cell surface markers, VEGFR2 and VE-Cadherin, was significantly elevated in the Masters athletes with 1U thrombin supplementation, but returned to baseline at all other concentrations. Among the low-active controls, there was no significant effect of thrombin on expression of these cell surface markers. The elevated gene expression seen in Masters athletes, but not low-active controls, with 1U thrombin supplementation may be indicative of a training effect on EPCs. This effect was seen only at the lowest level of supplementation, and was absent at 5U and 10U supplementation. At present there are no other data in the literature examining such a relationship between gene expression or EPC differentiation with a low thrombin supplementation level. Further investigation is necessary to draw definitive conclusions.

At higher thrombin supplementation levels of 5U and 10U, our results indicate no effect on gene expression in VEGFR2 and VE-Cadherin in both Masters athletes and low-active controls. From this we conclude that thrombin is not directly involved in signaling of EPC differentiation at higher concentrations. These results challenge previous evidence by Tarzami et al. (2006) who found increased cellular differentiation of bone marrow-derived murine EPCs *in vitro* when supplemented with 10U thrombin under similar conditions to our study. These differences may highlight a disparity between EPC regulation in mice and humans.

We further investigated Day 2 non-adherent cells isolated from blood drawn 30 minutes after vigorous exercise. A significant increase in gene expression of cyclin D1 and decreased expression of p27, without changes in expression of VEGFR2 or VE-Cadherin, mirrors the gene expression profile of cells that received thrombin supplementation. These results indicate that during vigorous exercise there is apparent signaling to circulating EPCs, possibly by thrombin, that results in increased gene expression of cell cycle genes up to 72 hours after exercise and subsequent *in vitro* incubation. We conclude, therefore, that there is a direct physiological link between acute exercise and EPC activity, specifically increased cellular proliferation.

Limitations and Future Research

The cross-sectional design of this study limits the degree to which definitive conclusions can be drawn about the influence of exercise training on endothelial progenitor cells and the *in vitro* response to thrombin supplementation. Specifically, we observed large variability in our results for changes in circulating EPC concentration, EPC-CFUs, and F1+2 in response to acute exercise. We prescribed an exercise intensity of 75 ± 5% VO$_2$max to elicit a vigorous exercise stimulus. This prescription was based on previous evidence by Laufs et al. (2005), who observed an increase in circulating EPCs with exercise at anaerobic threshold but not at the lower intensity of 80% anaerobic threshold. Given the wide variability in responses among our participants, it is possible that there exists a threshold intensity for inducing EPC mobilization and cellular activation in the blood. While the physiological stimuli that determine this threshold have not been clearly defined, it is possible that the

prescribed intensity of 75% VO$_2$max used in the present study was a sufficient stimulus for some, but not all, of the subjects. Future research may more precisely investigate whether there exists a threshold intensity that elicits changes in EPC mobilization and EPC-CFU capacity with acute exercise, and furthermore, characterize the physiological parameters associated with such a threshold.

To examine the association of long-term exercise training with endothelial progenitor cells we recruited male Masters athletes and control subjects that were similar age and BMI, but differed dramatically in their levels of physical activity and physical fitness. While this distinction in subject groups enabled our cross-sectional analysis, it also limits the external validity of our results. We investigated a specific population of healthy, non-smoking, 55 – 80 year old men with no history of CVD or related metabolic disorders. Specifically, the Masters athletes were men that had maintained a high level of training for at least the last 20 years, potentially limiting the implications of our results to women, other age groups, and the general population. Two approaches may be used to overcome this limitation. To directly investigate the influence of training on EPC variables, a longitudinal exercise training study that includes both sedentary men and women from the general population should be undertaken. To further investigate the acute effects of exercise on EPC variables, future investigations may include a larger random sampling of subjects, both men and women, across a range of physical fitness levels.

The results of our gene expression analysis, in combination with the previous literature, demonstrate a clear role for thrombin in EPC regulation, specifically cellular proliferation. It remains unclear, however, if the changes in gene expression

of cell cycle genes with both thrombin supplementation and vigorous exercise will translate directly into increased proliferation of EPCs or the timeframe in which such a change in proliferation would be measurable. We observed correlations between exercise-induced thrombin production and changes in circulating EPC concentration. We further demonstrated the role of thrombin as a signaling molecule in EPC proliferation. While this implies a connection between exercise-induced endothelial damage and the accumulated improvement to endothelial health with exercise training, it does not directly demonstrate the proliferation and incorporation of circulating EPCs at the sites of exercise-induced endothelial dammage.   An important next step in this line of research would be to establish that there is incorporation of EPCs along the endothelium following vigorous endurance exercise. Furthermore, it must be established that, over time, the accumulation of these small changes along the endothelium result in improved endothelial health and function.

Implications and Conclusions

Endothelial dysfunction is a key determinant in the progression and ultimate manifestation of CVD. Exercise training is associated with the decreased occurrence of cardiovascular events and the improved profile of several known CVD risk factors including endothelial function. The relationship between thrombin and EPC gene expression suggests a mechanism linking exercise and exercise training with improved cardiovascular health.

EPCs have been well established as the primary regulator of endothelial maintenance and repair (Asahara et al., 1997; Walter & Dimmeler, 2002) as well as for the resolution of a fibrin blood clot through clot recanalization (Moldovan &

Asahara, 2003; Modarai, Burnand, Sawyer, & Smith, 2005) and vascular remodeling (Smadja et al., 2008; Li, Meng, & Wu, 2007). Elevated circulating levels of EPCs, furthermore, have been linked to improved endothelial health as determined by forearm blood flow reactive hyperemia (Hill et al., 2003), a correlation supported by our results. As described above, previous studies have demonstrated an increase in circulating EPCs and EPC-CFUs with both acute exercise and exercise training, though the direct mechanism has not been determined.

As confirmed in our results, acute vigorous exercise results in a distinct increase in thrombin production. Elevated thrombin production is the direct result of endothelial damage and the resultant activation of the coagulation cascade (Mann, 1999) and concomitant platelet activation (Kahn, Nakanishi-Matsui, Shapiro, Ishihara, & Coughlin, 1999). Thrombin-inducing vascular damage is the result of increased shear stress along the endothelium during exercise (Padilla et al., 2008). Upon activation of prothrombin, free thrombin is quickly bound and inactivated, limiting the activity of free thrombin to the area immediately surrounding the lesion. Therefore, thrombin activity *in vivo* is localized to sites of vascular damage, including the endothelium and fibrin clot lesions (Mann, 1999).

The results of our study indicate that EPCs respond to direct stimulation by thrombin. We have further demonstrated similar gene expression profiles among EPCs supplemented with thrombin *in vitro* and those isolated after 30 minutes of vigorous treadmill exercise.

Taken together, the results of our study, along with previously published data, suggest a novel mechanism for improved endothelial health and reduced

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cardiovascular risk with exercise training. Elevated thrombin production in response to exercise-induced vascular shear stress provides a localized signal for EPC proliferation at the site of endothelial injury. This implies a direct mechanism for endothelial repair following acute exercise. Repeated bouts of regular acute exercise, defined as exercise training, result in the repeated cycle of damage and repair along the endothelium. Exercise training will, therefore, exaggerate the mechanism of EPC mediated endothelial maintenance and repair. In essence, recurring endothelial damage, caused by the repetitive shear stress of acute exercise bouts, is followed by subsequent repair and maintenance of the endothelium by circulating endothelial progenitor cells. Our results suggest that thrombin, known to be activated from prothrombin in direct response to the exercise-induced endothelial damage, acts as a signaling molecule to alter gene expression of cell cycle genes. With an increased expression of cell cycle genes Cyclin A2 and Cyclin D1 and suppressed expression of the cell cycle inhibitor p27, EPC proliferation is promoted at the site of endothelial damage. Ultimately, through this mechanism, regular exercise leads to more frequent cellular turnover along the endothelium, enhanced fibrin clot resolution, and improved endothelial function.

Chapter 5: Review of the Literature

Background

Regular physical activity and improved physical fitness are associated with enhanced cardiovascular health and decreased risk for the development of cardiovascular disease (CVD) (Wei et al., 1999). While the effect of physical activity on the conventional risk factors for CVD is well established, it appears that regular physical activity is able to reduce CVD risk through additional, as yet unidentified mechanisms (Blair et al., 1996).

A likely candidate, which has been suggested by several authors, involves the improvement of endothelial health and associated vascular function with exercise training (Green et al., 2004; Niebauer & Cooke, 1996). Endothelial dysfunction is defined as a disruption in normal vasomotor activity. Specifically, dysfunction is an impairment of the endothelial directed, NO mediated, flow-mediated dilatation (Duprez & Cohn, 2007). It is believed that the first step in the progression of atherosclerosis is endothelial dysfunction. Furthermore, a positive correlation has been found between endothelial dysfunction and the total number of heart disease risk factors in an individual (Hashimoto, Miyamoto, Matsuda, & Akita, 2003).

Endurance exercise training has been repeatedly associated with an improvement in endothelial function as measured by flow-mediated vasodilatation (Green et al., 2004). Furthermore, the improvement in endothelial function with training may represent a direct mechanism by which exercise training decreases CVD risk (Niebauer & Cooke, 1996). Niebauer and Cooke (1996) have suggested that the

repeated increase in vascular flow with individual bouts of exercise results in chronic structural and functional changes along the endothelium. The mechanism for these changes, however, has not been defined.

Endothelial Progenitor Cells and Cardiovascular Health

Where the endothelium was once viewed as a passive barrier lining the lumen of the vasculature, it is now known to be an active regulatory tissue, participating in autocrine and endocrine signaling. The endothelium is central to the maintenance of cardiovascular homeostasis by regulating proper blood flow, hemostasis, vascular maintenance, repair, and angiogenesis. In this role, the endothelium must respond to both acute and chronic environmental stimuli and adjust vasomotor tone, maintain a hemostatic balance, maintain a selectively permeable barrier, and respond to injury and/or ischemia. To maintain structural and functional integrity, there must be a basal cellular turnover of endothelial cells that are aged, injured, or apoptotic. In addition to a basal cellular turnover, the sprouting of new blood vessels, termed neovascularization, is also known to occur. This endothelium-directed action has been observed in response to ischemia in animals and humans (Walter & Dimmeler, 2002).

Until recently, the paradigm was that endothelial cell turnover and post natal neovascularization was the result of proliferation, migration, and remodeling of mature endothelial cells derived from pre-existing endothelium (Folkman & Shing, 1992). Asahara, et al. (1997) investigated the possibility of post-natal neovascularization mediated by circulating hematopoietic stem cells, and first characterized EPCs in circulation. Using a model of unilateral hind limb ischemia in

rabbits, DiI-labeled CD34+ and KDR+ cells derived from hematopoietic stem cells in the bone marrow were found to differentiate and incorporate into areas of active angiogenesis *in vivo*. These observations established a new paradigm of post-natal neovascularization (Asahara et al., 1997). EPCs have since been observed to function in several different capacities including endothelial maintenance and remodeling (Hunting, Noort, & Zwaginga, 2005), and blood clot recanalization (Moldovan & Asahara, 2003). Asahara et al. (1999) investigated the role of EPCs in both physiological and pathological vascular maintenance and repair. Recipient mice received transgenic donor bone marrow designed for overexpression of lacZ by an endothelial promotor. After transplantation endothelial cells were found to overexpress lacZ throughout the vascular system via RT-PCR gene expression analysis. As predicted by the prior literature, a large proportion of lacZ expressing endothelial cells were located in areas of active repair, including mice that had undergone hind limb ischemia and those that had undergone myocardial ischemia. LacZ expressing endothelial cells were additionally found to be incorporated into healthy undamaged vessels as part of physiological cell turn-over.

Moldovan and Asahara (2003) further suggested that in the case of damaged or atherosclerotic blood vessels, EPCs play a larger role than simple replacement of existing damaged endothelial cells. EPCs appear to be responsible for resolution of a blood clot through the process of recanalization. Clot recanalization is characterized by moderate degradation of the fibrin mesh on the luminal side of the clot and through the body of the clot, thus creating a low density pocket. Endothelial cells then form a covering over the luminal side of the clot and directly through the clot in

these low density pockets creating a non-thrombogenic surface. These endothelial cells form a lining, thereby vascularizing the clot both perpendicular and parallel to the direction of blood flow. Those channels forming perpendicular to the blood flow and vessel wall had previously been assumed to "sprout" from the vessel wall; however this can not explain the early formation of the parallel channels. According to the authors, there appeared to be "no detectable reaction in the arterial wall" (Moldovan and Asahara, 2003), implying that it must be a blood borne regulatory cells. Lending support to the involvement of EPCs in this process is the fact that venous thrombi recanalize more regularly than arterial thrombi, apparently as a result of the type of blood, venous or arterial, rather than the type of vessel (Moldovan & Asahara, 2003).

Given their role in vascular maintenance, EPCs are an obvious candidate to be a risk factor for the development of CVD. Werner et al (2005) found that in coronary artery disease (CAD) patients, elevated circulating levels of EPCs significantly correlated with the increased likelihood of being free of any cardiovascular event and survival after 12 months. Hill et al (2003) further characterized the risk associated with levels of circulating EPCs by comparing them to the Framingham risk score. In a group of middle-aged men with no history of CVD, Framingham risk scores were calculated using conventional risk factors. Risk scores had a significant inverse correlation with levels of circulating progenitor cells indicating that those with the highest levels of circulating EPCs had the lowest risk score. The researchers further addressed the functional effect of circulating EPC levels by measuring flow-mediated vasodilation as a measure of endothelial function. Elevated numbers of EPCs were

significantly correlated with improved vascular function and, in fact, were a better predictor of vascular function than the Framingham risk score (Hill et al., 2003). This study indicates that the level of circulating EPCs alone is as valuable a marker for predicting CVD risk as the combination of several conventional risk factors in the Framingham score.

This association between EPCs and CVD risk has been supported by the depressed levels and function of circulating EPCs in patients with documented atherosclerosis. Vasa, et al (2001) found that aside from having lower levels of circulating EPCs, CAD patients with low circulating EPC levels also had an elevated number of conventional risk factors. Additionally, the functional activity of the EPCs, determined by migratory capacity, was significantly inversely correlated with the total number of risk factors in patients and was significantly reduced in patients compared to healthy controls. The authors further investigated the relationship between individual risk factors and EPC number and function. Risk factors that were associated with impaired circulating EPC concentration included smoking, family history of CAD, age, and LDL-C. Several risk factors were correlated with impaired EPC migratory capacity including age, LDL-C, and hypertension (Vasa et al., 2001).

Identification of Endothelial Progenitor Cells

To investigate the role of EPCs in human physiology, these cells must first be distinguished from other circulating cells in the blood. As with all cell types, EPCs express cell surface markers, transmembrane glycoproteins, on the outer surface of the cell membrane. EPCs express a combination of cell surface markers shared by endothelial cells and hematopoietic cells. Two markers commonly expressed by all

three cell lines are CD34 and KDR. Used together, these two markers are frequently utilized to distinguish the endothelial cell lineage of circulating EPCs among a pool of circulating mononuclear cells. In common with hematopoietic cells, EPCs also express CD133 and CD117. Used in combination with CD34 and KDR, these cell markers will add specificity to the identification of circulating EPCs, distinguishing them from the relatively small population of circulating endothelial cells that do not express these glycoproteins (Hunting et al., 2005).

Endothelial Progenitor Cells and Physical Activity

In addition to the association of EPCs with many of the conventional and non-conventional risk factors for CVD, there also appears to be a strong relationship between circulating number and function of EPCs and physical activity. Several recent studies have indicated that there is a relationship between circulating EPCs and both exercise training and acute bouts of exercise. While the bulk of currently published studies involved CVD patients, some limited evidence has demonstrated a relationship in healthy subjects as well. Among the limited number of studies published thus far, results have consistently shown a relationship between physical activity and circulating number and function of EPCs, although a mechanism for this interaction has yet to be determined.

In an early study Laufs, et al (2004) investigated the effect of exercise training on EPCs in mice. After only one week of voluntary wheel running, there was a significant increase in the number circulating EPCs. The circulating EPCs remained elevated through the end of the 28-day training period. To confirm the functional implications of this elevation on vascular health, carotid artery injury was induced in

a subset of mice. Mice in the training group demonstrated the beneficial outcomes of reduced neointimal formation, increased lumen circumference and increased area of neoangiogenesis. The authors further attempted to translate the results observed in the mouse model to humans. Patients with stable CAD were exercise trained for 28 days resulting in similar increases in circulating EPCs, suggesting similar implications for vascular health in humans (Laufs et al., 2004).

Building on the results of Laufs et al. (2004), Steiner, et al (2005) investigated the clinical significance of changes in circulating EPCs in humans using forearm flow-mediated vasodilation. Subjects were healthy patients with a history of CAD and patients with several cardiovascular risk factors. The subjects underwent a 12-week supervised running program, five days per week. Blood samples were collected at baseline and 48 hours after the last training session. In agreement with Laufs, et al (2004), there was an increase in circulating EPCs after training.

Steiner et al (2005) additionally attempted to narrow down a signaling mechanism for changes in circulating EPC concentration by measuring nitric oxide (NO) activity and vascular endothelial growth factor (VEGF) in the blood. The increase in circulating EPC concentration was significantly correlated with an increase in NO activity and endothelial function, as determined by flow-mediated vasodilation measured by ultrasound. There was no change in vascular endothelial growth factor (VEGF) levels with training (Steiner et al., 2005). This study confirmed the effect of exercise training on circulating EPC levels in CAD patients. Additionally, it was demonstrated that there was a relationship between circulating EPC concentration, NO activity and flow mediated vasodilation, but not VEGF.

To further characterize the clinical implications of the increase in progenitor cells after training in CVD patients, Sandri, et al (2005) examined the effect of four weeks of exercise training on patients with ischemic peripheral arterial occlusive disease (PAOD), successfully revascularized PAOD, or stable CAD. Each group of patients was randomized into either training or control groups. Patients underwent four weeks of standardized exercise training protocols specific to their clinical diagnoses. In ischemic PAOD patients and revascularized PAOD patients the training resulted in a significant increase in treadmill walking time and in PAOD patients this was accompanied by an improvement in ankle-brachial pressure index. CAD patients demonstrated a significant increase in VO2max and an increase in ischemic threshold. Circulating progenitor cells (CPC) (CD34+/KDR+) were measured 72 hours after the last exercise bout. There was an increase in CPCs in only the exercise trained patients with ischemic PAOD and not in other groups. This increase in CPCs was accompanied by an increase in plasma VEGF in PAOD patients, but no other group. Taken in context with the results of Steiner et al. (2005), VEGF is implicated as a possible mechanism only in the case of exercise induced ischemia. Sandri et al. further investigated the effect of training on these progenitor cells *in vitro*. The ability of the CPCs to participate in network formation *in vitro* was enhanced in all subject groups. Therefore, despite the lack of increase in the circulating concentration, there appeared to be enhanced cellular function among progenitor cells in these patients (Sandri et al., 2005).

Sarto et al. (2007) investigated not only the effect of exercise training on EPCs in CAD patients, but the effect of detraining as well. CAD patients underwent

eight weeks of endurance exercise training followed by another eight weeks of detraining. In agreement with the previous literature, the authors found an improvement in both circulating EPC concentration and EPC-CFUs with training in CAD patients. After eight weeks of detraining, however, all beneficial changes returned to baseline, suggesting the need for continued exercise training to maintain elevated EPC concentration and improved EPC function (Sarto et al., 2007).

Few studies have investigated the effect of endurance exercise training on EPCs in healthy individuals. Hoetzer et al. (2007) investigated the association between aging, physical activity and EPC function in healthy sedentary men. In a cross-sectional analysis, EPC-CFUs and EPC migratory capacity were compared between young (22-35 years), middle-aged (36-55 years), and older (56-75) healthy men. They found that there were ~70% fewer EPC-CFUs in middle-aged and older men compared to young men. Furthermore, migratory capacity was significantly impaired in older men compared to middle-aged and younger men. The authors then examined the effect of exercise training on EPCs in 10 middle-aged and older men. Subjects underwent a home-based moderate intensity exercise protocol for three months. After training, EPC-CFUs increased by ~120% while migratory capacity improved by ~50% (Hoetzer et al., 2007). This was the first study to clearly demonstrate that exercise training is associated with changes in EPC function in healthy individuals.

There is clearly an association between exercise training and EPCs in both CVD patients and healthy subjects. These studies do not, however, address the question of whether the changes seen in EPC number and function are the result of

exercise training over time, or simply the acute response to a recent bout of exercise. Laufs, et al (2005) advanced the question of whether there is a training effect on circulating EPC number and function or simply the accumulation of acute responses. The focus of this study was on the response of EPC levels to an acute bout of physical activity in humans. The subjects for this study were physically active, healthy young men. The protocol included exercise on a treadmill under three separate conditions including 30 minutes at lactate threshold (intensive running), 30 minutes at 80% lactate threshold (moderate running), and 10 minutes at 80% lactate threshold (moderate running short duration). Blood samples were taken prior to exercise and at several time points up to 24 hours during recovery. Samples were used to measure circulating EPCs, serum cortisol and serum VEGF levels. They found that EPC levels increased as a result of acute exercise in both the intensive running and moderate running protocols, but not the short duration protocol. Unlike the results of the previous training study from this same lab, the increase in EPCs was independent of VEGF and cortisol as serum levels did not change with any of the exercise conditions. The increase in circulating EPC levels was significant at 10 and 30 minutes after intensive and moderate exercise and had returned to baseline by two hours after exercise. This study additionally examined the migratory capacity and colony forming units of the isolated EPCs *in vitro* to determine the effect of acute exercise. The results indicated that for up to six hours after exercise the functional ability of the EPC's was enhanced (Laufs et al., 2005). These results have several implications. The subjects were physically active healthy young men, excluding the possibility of other causes of EPC elevation such as ischemia or medication.

Additionally, the results indicated that moderate intensity exercise for 30 minutes was sufficient to induce improvement in EPC levels and function, suggesting an enhanced period of endothelial maintenance after exercise (Laufs et al., 2005).

The acute increase in EPCs after exercise has been confirmed in two other studies. Rehman, et al (2004) examined the effect of a symptom-limited treadmill or bicycle ergometer test on circulating EPCs concentration (AC133+/VE-Cadherin+). Subjects were patients who had blood samples collected prior to, and shortly after, exercise. Similar to the previously discussed study, patients demonstrated an increase in EPCs with exercise. There was not an increase in VEGF or hepatocyte growth factor levels. This study confirmed the increase in circulating progenitor cells with exercise and further supports the lack of association with serum VEGF levels. Additionally, this study demonstrated these changes in a patient population (Rehman et al., 2004).

Adams, et al (2004) investigated whether exercise-induced ischemia plays a role in the regulation of circulating EPC levels in CAD patients. Previous research has shown that prolonged ischemia, such as a myocardial infarction, will trigger an increase in the circulating number of EPCs (Takahashi et al., 1999). The aim of this study was to determine if an acute reversible episode of ischemia would affect circulating EPC levels. The subjects included CAD patients with exercise-induced myocardial ischemia, CAD patients without exercise-induced ischemia, and healthy age-matched controls. After a maximal cycle ergometer exercise test, blood samples were collected periodically for up to 48 hours after the exercise with a subset of subjects analyzed up to 144 hours. At baseline there was no difference in progenitor

cell levels between groups, however, at 24 and 48 hours after the exercise the ischemic group had significantly higher levels of EPCs than both the non-ischemic and healthy groups. This suggests that in CAD patients the primary regulator of EPC release is ischemia for as long as 48 hours after exercise. Contrary to previously discussed studies, this increase was accompanied by an increase in plasma VEGF concentration in the ischemic group. There was no change in VEGF in the non-ischemic and healthy groups, confirming previous studies (Adams et al., 2004).

Taken together, the results of the acute exercise studies suggest that changes seen in EPC number and function may be the result of accumulated changes observed during acute exercise. The changes in EPCs appear to require moderate to vigorous intensity exercise for a duration of at least 30 minutes. While regulation of these changes may involve VEGF in ischemic conditions, the mechanism for regulation in healthy individuals remains unclear. It remains to be determined by what pathway EPCs are regulated during exercise, and what the effect will be in other subject populations. From the current body of research, we may conclude that in ischemic patients the primary stimulus for EPC elevation appears to be the release of VEGF. Healthy, non-ischemic individuals, however, demonstrate an increase in circulating EPC levels as a result of acute exercise, given that the exercise is of sufficient duration (at least 30 min) and intensity (at least moderate). These changes in healthy subjects are most likely not associated with changes in plasma VEGF. Although the exact mechanism for increased circulating EPCs with exercise has not been determined, hypotheses have included NO release and β-adrenergic stimulation. Given the role of EPCs in vascular repair, it is likely that the true mechanism involves

regulation through some signaling molecule generated as a result of exercise induced shear stress.

Acute Exercise and the Vascular Response to Injury

At the onset of acute endurance exercise there is a corresponding increase in vascular blood flow and endothelial shear stress. As intensity of the exercise increases, stress along the endothelium, logically, also increases. Tanaka et al. (2006) demonstrated that during graded exercise, blood flow and shear stress increased in response to increasing levels of exercise intensity. Blood flow and shear stress were assessed using Doppler transducers in brachial and femoral arteries of healthy female subjects in response to both upper- and lower-body graded exercise. The results indicated that blood flow and shear stress increased not only in the working limbs, but in the non-working limbs as well, indicating a systemic increase in vascular shear stress with increasing intensity of exercise (Tanaka et al., 2006).

It is believed that shear stress and resulting endothelial damage due to exercise gives rise to the activation of the coagulation cascade. As a direct result of vascular shear stress, injury to the endothelial cells can occur. This injury will disrupt normal cellular activity, and in particular, disrupt the normal anticoagulant and profibrinolytic properties of the endothelial cells (Wu & Thiagarajan, 1996). At the site of injury along the endothelium, coagulation and platelet adhesion are allowed to progress due to the impaired expression of anticoagulants Protein C, tissue factor pathway inhibitor, and thrombomodulin, as well as impaired expression of the fibrinolytic tPA and the anti-platelet binding molecule prostacyclin (Wu & Thiagarajan, 1996; van Hinsbergh, 2001). With more severe injury, exposure of the

subendothelial matrix to the lumen of the vessel will allow direct activation of the coagulation cascade via the tissue factor pathway as well as platelet activation and adhesion (Mann, 1999).

As the central component to the coagulation cascade, the level of thrombin activation from prothrombin is indicative of overall coagulation activity. Upon activation, thrombin molecules are directly involved in one of several pathways, including activation of fibrinogen to fibrin, activation of the intrinsic coagulation cascade, inactivation by the anticoagulant antithrombin III, binding to endothelial bound thormbomodulin, or binding to the thrombin receptor PAR-1 found on platelets and several cell types including EPCs and mature endothelial cells (Mann, 1999; Coughlin, 2000). The short half-life of thrombin within the plasma, therefore, does not allow for direct measurement to confirm its presence. Thrombin production must, therefore, be measured by its free floating activation and inactivation products. The activation of prothrombin by the prothrombinase enzyme results in complete cleavage of prothrombin and the production of thrombin and prothrombin fragment F1+2 (F1+2). As a stable activation product of thrombin, F1+2 may be measured as a direct indicator of thrombin activation (Boisclair, Philippou, & Lane, 1993). A secondary indication of thrombin production is the inactivation product thrombin-antithrombin III (TAT). Antithrombin III is a free floating anticoagulant that will bind and inactivate free thrombin within the plasma. The inactivation of thrombin creates the stable and measurable molecule TAT. The presence of TAT in the plasma is, therefore, proportional to thrombin production (Boisclair et al., 1993).

Endothelial damage during exercise is the consequence of turbulent blood flow and the resulting shear stress along the vessel walls (Padilla et al., 2008; Tanaka et al., 2006). This effect of exercise is evidenced by the elevated concentration of F1+2 and TAT after acute exercise, specifically during vigorous exercise (Womack et al., 2003). Weiss et al. (1998) investigated the hemostatic response to exercise in 12 healthy young men resulting from moderate intensity, 68% VO_2max, and vigorous intensity, 83% VO_2max, treadmill exercise. After the higher intensity exercise, but not the lower intensity exercise, there was a significant increase in both F1+2 and TAT. These results in young healthy men have also been observed across older age groups. van den Burg et al. (2000) investigated the hemostatic response to submaximal and maximal exercise in healthy men. Men were divided into three age groups that included 20 – 30 yrs, 35 – 45 yrs, and 50 – 60 yrs. Markers of thrombin production, TAT and F1+2, were found to increase in response to cycle ergometer exercise at both 70% VO_2max and 100% VO_2max in all age groups. Maximal exercise appeared to elicit the greatest response in all groups (van den Burg et al., 2000).

These changes in thrombin production are not limited to the exercise period, rather they have been observed to persist after the cessation of exercise for up to 24 hours. Prisco et al. (1998) examined changes in hemostatic parameters before and immediately after a marathon in 12 trained men. They further examined the hemostatic response at 24 and 48 hours after the marathon. Consistent with other studies, TAT and F1+2 were elevated immediately after the high intensity exercise. The elevation in TAT and F1+2 continued at least 24 hours after exercise and had

returned baseline by 48 hours after exercise (Prisco et al., 1998). From these studies we can conclude that in healthy individuals there is a significant increase in markers of thrombin production in response to exercise. This increase is most pronounced at higher intensity and maximal exercise. With long duration, high intensity exercise, such as marathon running, this elevation in thrombin production may persist for up to 24 hours after exercise.

Exercise-induced thrombin and EPC regulation

EPCs are known to express protease activated receptor-1 (PAR-1) on their surface. This g-protein coupled receptor is the primary receptor for thrombin, whose production is central to the regulation of blood coagulation, fibrinolysis, and endothelial signaling. Thrombin and PAR-1 receptors were previously established to have a vital role in fetal vascular development. Griffin et al (2001) found that thrombin signaling through PAR-1 is required in the building and stabilization of new blood vessels in mouse embryos. In the absence of the PAR-1 receptor, the mice embryos were not viable (Griffin et al., 2001). While this study established the importance of thrombin and PAR-1 in vascular development, the link to regulation of EPCs during neovascularization had not yet been made.

Tarzami, et al (2006) investigated the role of thrombin and PAR-1 in the regulation of EPCs. Bone marrow derived mononuclear cells from mice were grown in vitro in the presence or absence of thrombin. They found that thrombin enhanced the differentiation of the cells to express EPC and mature endothelial cell characteristics and enhanced proliferation of EPCs. These effects were attenuated with the addition of a thrombin inhibitor. Furthermore, the effects of thrombin were

mirrored by the addition of a PAR-1 agonist without the presence of thrombin, indicating that thrombin exerts its effect though the PAR-1 receptor on the cell surface of the EPCs (Tarzami et al., 2006).

Smadja, et al (2006) further characterized the involvement of PAR-1 in post-natal angiogenesis *in vitro.* EPCs isolated from human blood were cultured and treated with a PAR-1 agonist. It was found that PAR-1 stimulation promoted EPC proliferation, migration, and differentiation compared to the untreated cells (Smadja et al., 2006).

Thrombin's involvement in the differentiation and proliferation of EPCs may provide a direct link between an acute bout of physical activity and EPCs. Thrombin production is known to increase as a result of a single bout of exercise, specifically with higher intensity and longer duration exercise (Womack et al., 2003). This is similar to the response of EPCs to acute exercise as seen in the study by Laufs et al (2005). Additionally, thrombin production after acute exercise is further elevated after exercise training. van den Burg, et al (2000) found that men aged $50 - 60$ years had an increased level of thrombin production in response to maximal exercise after 12 weeks of endurance exercise training (van den Burg et al., 2000). In the context of hemostasis alone, the increase in thrombin production after exercise training appears to contradict the reduced thrombus formation and improved endothelial health that are also known to occur with training. However, if thrombin is viewed in its role as a signaling molecule, specifically in its role of signaling EPCs, this seemingly paradoxical increase in thrombin may actually be beneficial. Thrombin may be the signal by which acute exercise affects EPC differentiation and proliferation. This

represents a major pathway of exercise-induced regulation of EPCs that has yet to be investigated.

Elevated thrombin production in response to exercise-induced vascular shear stress could provide a localized signal for EPC activation at the site of endothelial injury. As discussed above, EPCs are believed to be responsible for endothelial repair and maintenance, and would have a clear role in repairing the damage incurred by acute exercise. Therefore, exercise training may exacerbate the normal mechanism of EPC mediated endothelial maintenance and repair by accelerating the rate of endothelial cell damage, thereby accelerating the rate of endothelial cell turnover. In essence, recurring endothelial damage, caused by the repetitive shear stress of acute exercise bouts, is followed by subsequent repair and maintenance of the endothelium by circulating endothelial progenitor cells. With accelerated turnover, we may presume that the endothelium will be comprised of a greater proportion of younger endothelial cells, thereby improving cellular function along the endothelium. Ultimately, through this mechanism, regular exercise may lead to more rapid cellular turnover along the endothelium, resulting in both enhanced fibrin clot resolution and improved endothelial function, thereby reducing the risk of cardiovascular disease.

Appendix A

CONSENT FORM

Project Title: **<u>Life-Long Exercise and Novel Cardiovascular Disease</u> <u>and Aging Risk Factors – Life-Long Exercisers</u>**

This is a research project being conducted by Dr. James Hagberg in the Department of Kinesiology at the University of Maryland College Park. We are inviting you to participate in this research project because you are 55 – 80 years of age, are generally healthy, and have a long history of exercise/physical activity. The purpose of this study is to determine whether life-long exercise is associated with a better profile of novel risk factors related to cardiovascular disease and aging compared to sedentary individuals of your age. In this case, "novel" risk factors mean newer risk factors beyond the standard risk factors such as cholesterol levels, blood pressure, diabetes, and obesity. In a subset of life-long exercisers we will determine the degree to which these risk factors change when they stop exercising for 10 days. The specific tests, their requirements, and time commitments are described below.

Subject Characteristics: You have already completed a telephone or personal interview that you verbally consented to that determined that you are 55- 80 years of age, have been physically active for over 20 years, are not a diabetic, have no evidence of cardiovascular or lung disease, and have no other medical problems that keep you from exercising vigorously. Furthermore, if you are a woman, you must be postmenopausal, defined as no menstrual cycles for at least the last 2 years and are not on hormone replacement therapy. We have also discussed with you that certain medications may exclude you from taking part in this study.

Study Procedures: If you qualify and complete the phase of this study where you stop exercising for 10 days, your total involvement in this study will consist of 5 visits and will last 2 – 3 wks. If you do not undergo the 10 days of stopping exercise, your total involvement will consist of 3 visits over approximately 1-2 weeks.

All life-long exercisers will first undergo 1 Screening and 2 Testing Visits. For the Screening Visit, you will report to the laboratory the morning after an overnight fast. The study will be explained to you, your medical history will be reviewed, and you will be asked to provide your written informed consent. A physical examination will then take place and about 2 tablespoons of blood will be drawn from a vein in your arm. Your resting blood pressure will be measured. You will then undergo a treadmill exercise test supervised by a physician to determine if you have heart disease. This test will be done on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until you cannot continue or symptoms of heart heart disease develop.

Page One of Five   Date _____   Initials _____

Blood pressure, heart rate, and electrocardiogram (electrical activity of the heart) will be recorded before, during, and after the test. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. This visit will last about 1 hour and you will be excluded from the study at this point if you have evidence of heart disease.

For the first Testing Visit, you will report to the laboratory in the morning after an overnight fast (only taking in water from 8 PM the night before) and will consist of a forearm blood flow test and 30 minutes of vigorous exercise. About 8 tablespoons of blood will be drawn from a vein in your arm prior to the forearm blood flow test. Then the blood flow in your forearm will be measured noninvasively in the non-dominant arm. You will rest while laying down for 20 minutes prior to the testing. An elastic measurement gauge will be placed around your non-dominant forearm. Two inflatable cuffs also will be placed on the same arm; one on the upper arm above the elbow and another around the wrist. Another cuff will be placed on the opposite arm to measure blood pressure. The cuff around the wrist then will be inflated to prevent blood flow from the hand. Next, the cuff around the upper arm will be inflated slightly for 7 seconds and then released for 8 seconds. This cycle will continue for 3 minutes. Blood pressure will be measured in the opposite arm at the same time. The procedure then will be repeated following the stoppage of blood flow in the non-dominant arm for 5 minutes.

You will then undergo treadmill exercise for 30 minutes at a moderate to vigorous intensity. Heart rate and blood pressure will be monitored before, during, and after the exercise. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. At 5 and 30 minutes after the exercise approximately 2 tablespoons of blood will be drawn from a vein in your arm. Prior to this visit you will record all items that you eat over a 7 day period. This visit will last about 2.5 hours.

For the second Testing Visit, you will be transported to the USDA in Beltsville, MD to undergo an x-ray scan (DEXA) to measure your total body fat mass and total body muscle mass while you lie quietly on a table. You will need to fast overnight prior to this visit. This visit may take 1-2 hours including travel time.

If you are not undergoing the 10 days without exercise, this is all the testing that is required of you. If you are undergoing the 10 days without exercise phase of the study, you will undergo 2 additional visits to the laboratory.

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Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Life-Long Exercisers**

Following 10 days of training cessation, you will report to the laboratory for a blood draw and forearm blood flow measurement and a second visit to the USDA for an X-ray scan. During the period of training cessation, we ask that you monitor your weight to ensure that you do not gain or lose any weight when you are not exercising. If you change any of your medications during this phase of the study, please notify one of the researchers.

The maximum total amount of blood that will be drawn during the testing is about 12 tablespoons if you are not undergoing the 10 days without exercise and about 18 tablespoons over the space of 2 – 3 weeks if you are undergoing the 10 days without exercise. This is approximately 1/3 and 1/2, respectively, of the amount of blood that is typically drawn during a single blood donation. These samples will be used to measure a number of chemicals and cell numbers in the blood that may be related to a person's risk of cardiovascular disease and other age-related diseases.

☐ Yes, I consent to having my blood drawn and saved for future analyses
☐ No, I do not consent to having my blood drawn and saved for future analyses and my samples must be destroyed after the completion of this study.

Confidentiality: We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a locked office with access available only to study personnel. Furthermore, all computer data bases containing results from this study will not have any names attached to them. If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland College Park or government authorities if you or someone else is in danger or if we are required to do so by law.

Samples of your blood will be sent to other collaborating laboratories for other blood measurements. Your blood samples sent to these laboratories will be identified only by a numeric code and only investigators at the University of Maryland College Park will know whose name is associated with each coded number. The list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

Risks: The following risks are associated with your participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. You will be screened

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Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Life-Long Exercisers**

with a resting electrocardiogram and a physical examination prior to this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is risk of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk of the body composition testing is the exposure to X-rays. The amount of X-ray exposure for each test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) The risk of stopping your exercise for 10 days, if you complete this portion of the study, is that your risk factors for cardiovascular disease and aging may deteriorate. It is unlikely that substantial changes will take place over the space of the 10 days without exercise. Furthermore, you will be able to start exercising again immediately after these 10 days without exercise. (5) Measurement of Forearm Blood Flow: This procedure causes substantial discomfort that ceases shortly after the cuff is deflated. If you can not tolerate this discomfort, the test will be terminated immediately on your request. (6) Dietary Records: There are no known risks associated completing 7 day dietary records. (7) Your involvement will require some amount of time, travel, and effort. Your parking costs will be covered.

Benefits: This study may not help you personally, but may help the investigators to determine whether life-long exercise results in better levels of new cardiovascular disease and aging risk factors in older individuals and the degree to which they deteriorate when life-long exercisers stop their exercise programs for 10 days. However, you might benefit in that you will be told of any abnormalities found during testing and will be advised to consult your personal physician. You will also be told of your results of the different risk factors that we measure and will be provided an explanation of these results. Your participation in this research is completely voluntary and you may choose not to take part at all. You are free to ask questions at any time without penalty. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this project or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify.

In the event of a physical injury resulting from participation in this study, immediate medical attention is available at the Washington Adventist Hospital. The University of Maryland does not provide any medical, hospitalization, or other insurance for participants in this research study nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study except as required by law.

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Project Title: **<u>Life-Long Exercise and Novel Cardiovascular Disease</u>**
<u>and Aging Risk Factors – Life-Long Exercisers</u>

In the event of a physical injury resulting from participation in this study, immediate medical attention is available at the Washington Adventist Hospital. The University of Maryland does not provide any medical, hospitalization, or other insurance for participants in this research study nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study except as required by law.

This research is being conducted by James Hagberg PhD of the Department of Kinesiology at the University of Maryland. If you have any questions about the research study itself, please contact Dr. Hagberg at 301-405-2487 (office) or via email at hagberg@umd.edu. If you have questions about your rights as a research subject or wish to report a research-related injury, please contact: **Institutional Review Board Office, University of Maryland, College Park, MD 20742; email: irb@deans.umd.edu; telephone: 301-405-0678**.

This research has been reviewed according to the University of Maryland College Park IRB procedures for research involving human subjects.

Your signature below indicates that you are at least 18 years of age, the research has been explained to you, your questions have been fully answered, and you freely and voluntarily choose to participate in this research program.

_____
Subject's printed name

_____   _____
Subject's signature   Date

_____   _____
Witness Signature   Date

_____   _____
Investigator Signature   Date

CONSENT FORM

Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Sedentary Participants**

This is a research project being conducted by Dr. James Hagberg in the Department of Kinesiology at the University of Maryland College Park. We are inviting you to participate in this research project because you are 55 – 80 years of age, are generally healthy, and are generally sedentary. The purpose of this study is to determine whether life-long exercise is associated with a better profile of novel risk factors related to cardiovascular disease and aging compared to sedentary individuals of the same age. In this case "novel" risk factors mean newer risk factors beyond the standard risk factors of cholesterol levels, blood pressure, diabetes, and obesity. The specific tests, their requirements, and time commitments are described below.

Subject Characteristics: You have already completed a telephone or personal interview that you verbally consented to that determined that you are 55 - 80 years of age, have been generally sedentary for at least 5 years, are not a diabetic, have no evidence of cardiovascular or lung disease, and have no other medical problems that keep you from exercising vigorously. Furthermore, if you are a woman, you must be postmenopausal, defined as no menstrual cycles for at least the last 2 years, and not on hormone replacement therapy. We have also discussed with you that certain medications may exclude you from taking part in this study.

Study Procedures: If you qualify and complete this study, your total involvement will consist of 3 visits over approximately 1 week.
You will undergo 1 Screening and 2 Testing Visits. For the Screening Visit, the study will be explained to you, your medical history will be reviewed, and you will be asked to provide your written informed consent. A physical examination will then take place and about 2 tablespoons of blood will be drawn from a vein in your arm. Your resting blood pressure will be measured. You will then undergo a treadmill exercise test supervised by a physician to determine if you have heart disease. This test will be done on an exercise treadmill where the treadmill speed and grade will increase every 3 minutes until you cannot continue or symptoms of heart disease develop. Blood pressure, heart rate, and electrocardiogram (electrical activity of the heart) will be recorded before, during, and after the test. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you

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breathe out can be analyzed. This visit will last about 1 hour and you will be excluded from the study at this point if you have evidence of heart disease.

For the first Testing Visit, you will report to the laboratory in the morning after an overnight fast (only taking in water from 8 PM the night before) and will consist of a forearm blood flow test and 30 minutes of vigorous exercise. About 8 tablespoons of blood will be drawn from a vein in your arm prior to the forearm blood flow test. Then the blood flow in your forearm will be measured noninvasively in the non-dominant arm. You will rest while laying down for 20 minutes prior to the testing. An elastic measurement gauge will be placed around your non-dominant forearm. Two inflatable cuffs also will be placed on the same arm; one on the upper arm above the elbow and another around the wrist. Another cuff will be placed on the opposite arm to measure blood pressure. The cuff around the wrist then will be inflated to prevent blood flow from the hand. Next, the cuff around the upper arm will be inflated slightly for 7 seconds and then released for 8 seconds. This cycle will continue for 3 minutes. Blood pressure will be measured in the opposite arm at the same time. The procedure then will be repeated following the stoppage of blood flow in the non-dominant arm for 5 minutes.

You will then undergo treadmill exercise for 30 minutes at a moderate to vigorous intensity. Heart rate and blood pressure will be monitored before, during, and after the exercise. During this test you will have a noseclip on your nose and you will breathe through a mouthpiece so that the air that you breathe out can be analyzed. At 5 and 30 minutes after the exercise approximately 2 tablespoons of blood will be drawn from a vein in your arm. Prior to this visit you will record all items that you eat over a 7 day period. This visit will last about 2.5 hours.

For the second Testing Visit, you will be transported to the USDA in Beltsville, MD to undergo an x-ray scan (DEXA) to measure your total body fat mass and total body muscle mass while you lie quietly on a table. You will need to fast overnight prior to this visit. This test may take 1-2 hours including travel time.

The maximum total amount of blood that will be drawn during this Testing is about 12 tablespoons during a single visit. This is approximately 1/3 of the amount of blood that is typically drawn during a single blood donation. These samples will be used to measure a number of chemicals and cell numbers in the blood that may be related to a person's risk of cardiovascular disease and other age-related diseases.

☐ Yes, I consent to having my blood drawn and saved for future analyses
☐ No, I do not consent to having my blood drawn and saved for future analyses and my samples must be destroyed after the completion of this study.

Page Two of Four   Date _____   Initials _____

Project Title: **Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Sedentary Participants**

Confidentiality: We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a locked office with access available only to study personnel. Furthermore, all computer data bases containing results from this study will not have any names attached to them. If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Your information may be shared with representatives of the University of Maryland College Park or government authorities if you or someone else is in danger or if we are required to do so by law.

Samples of your blood will be sent to other collaborating laboratories for other blood measurements. Your blood samples sent to these laboratories will be identified only by a numeric code and only investigators at the University of Maryland College Park will know whose name is associated with each coded number. The list of names and codes will be retained at the University of Maryland College Park for up to 25 years.

Risks: The following risks are associated with your participation in this study. (1) The risk of maximal exercise testing is approximately 1 nonfatal event in 10,000 tests and 1 fatal cardiac event in 70,000 tests. Risks will be minimized by having the test administered by a physician and personnel trained in such tests and emergency procedures. You will be screened with a resting electrocardiogram and a physical examination prior to this test. An emergency cart with the necessary drugs and a cardiac defibrillator will be present at all testing sessions. (2) There is risk of bruising and infection associated with blood drawing. These risks will be minimized by using sterile techniques and by having experienced personnel draw all blood samples. (3) The risk of the body composition testing is the exposure to X-rays. The amount of X-ray exposure for the test is the same as that occurring during 30 minutes of any activity outside in the sun. (4) Measurement of Forearm Blood Flow: This procedure causes substantial discomfort that ceases shortly after the cuff is deflated. If you can not tolerate this discomfort, the test will be terminated immediately on your request. (5) Dietary Records: There are no known risks associated completing 7 day dietary records. (6) Your involvement will require some amount of time, travel, and effort. Your parking costs will be covered.

Benefits: This study may not help you personally, but may help the investigators to determine whether life-long exercise results in better levels of new cardiovascular disease and aging risk factors in older individuals and the degree to which they deteriorate when life-long exercisers stop their exercise programs for 10 days. However, you might benefit in that you will be told of any abnormalities found during testing and will be advised to consult your personal physician. You will also be told of your results of the different risk factors that we measure and

Page Three of Four   Date _____   Initials _____

Project Title: **<u>Life-Long Exercise and Novel Cardiovascular Disease and Aging Risk Factors – Sedentary Participants</u>**

will be provided an explanation of these results. Your participation in this research is completely voluntary and you may choose not to take part at all. You are free to ask questions at any time without penalty. If you decide to participate in this research, you may stop participating at any time. If you decide not to participate in this project or if you stop participating at any time, you will not be penalized or lose any benefits to which you otherwise qualify.

In the event of a physical injury resulting from participation in this study, immediate medical attention is available at the Washington Adventist Hospital. The University of Maryland does not provide any medical, hospitalization, or other insurance for participants in this research study nor will the University of Maryland provide any medical treatment or compensation for any injury sustained as a result of participation in this research study except as required by law.

This research is being conducted by James Hagberg PhD of the Department of Kinesiology at the University of Maryland. If you have any questions about the research study itself, please contact Dr. Hagberg at 301-405-2487 (office) or via email at <u>hagberg@umd.edu</u>. If you have questions about your rights as a research subject or wish to report a research-related injury, please contact: **Institutional Review Board Office, University of Maryland, College Park, MD 20742; email: <u>irb@deans.umd.edu</u>; telephone: 301-405-0678**.

This research has been reviewed according to the University of Maryland College Park IRB procedures for research involving human subjects.

Your signature below indicates that you are at least 18 years of age, the research has been explained to you, your questions have been fully answered, and you freely and voluntarily choose to participate in this research program.

_____
Subject's printed name

_____   _____
Subject's signature   Date

_____   _____
Witness Signature   Date

_____   _____
Investigator Signature   Date

Appendix B

Table 3: Drug therapies known to affect EPC number and/or function.

Drug common names	
Simvastatin	Irbesartan
Mevastatin	Erythropoietin
Atorvastatin	Valsartan
Ezetimibe	Vardenafil
Olmesartan	Puerarin
Rapamycin	Ginkgo biloba
Ramipril	Rosiglitazone

Adapted from Shantsila, Watson, and Lip (2007)

Appendix C

Exercise and Vascular Health Study

DEPARTMENT OF KINESIOLOGY,
255 VALLEY DR., ROOM 0117 COLLEGE PARK, MD 20742
(301) 405-2571   (301) 405-5578 FAX

Physical Activity and Performance Questionnaire

Personal Information

Name _____ Sex (circle one):   M   F

Age _____   Birth date _____ Height _____ Weight _____

Address _____ Apt # _____

City _____ State _____ Zip Code _____

Telephone number _____ Alternative telephone number_____

Occupation _____

Physical Activity Information

Mode(s) of exercise (check all that apply):
☐ Running   ☐ Cycling   ☐ Swimming   ☐ Other _____

How many days/week do you exercise at each mode?

Running _____   Cycling _____   Swimming _____   Other _____

Do you ever exercise twice a day? ☐ No   ☐ Everyday   ☐ Occasionally _____

What is the approximate duration of an average exercise session? _____ minutes

What is the approximate pace (minutes/mile) for your exercise sessions? _____

How many miles do you run/cycle/swim/etc. in an average week?

Running _____   Cycling _____   Swimming _____   Other _____

Have you been exercising *continuously* since high school or college?   ☐ Yes   ☐ No

If no, how many years elapsed until you resumed exercising?

_____

How many years have you been exercising continuously? _____

How many years have you been exercising continuously in the last 20 years? _____

    30 years? _____ 40 years? _____ 40+ years? _____

Please outline a typical week's work out schedule giving distance covered and times registered:

	EXERCISE MODE (run/cycle/etc)	MILEAGE COVERED	DURATION
SUNDAY	_____	_____	_____
MONDAY	_____	_____	_____
TUESDAY	_____	_____	_____
WEDNESDAY	_____	_____	_____
THURSDAY	_____	_____	_____
FRIDAY	_____	_____	_____
SATURDAY	_____	_____	_____

Performance History

What competitive events do you compete in regularly? _____

_____

_____

What are your best times in the past 6 months?

EVENT	TIME
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____
_____	_____

What are your best lifetime events?

EVENT	TIME	YEAR
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Did you compete in high school?   ☐ Yes   ☐ No

If yes, can you compare the sport and times to your present times?

SPORT/EVENT	HIGH SCHOOL TIME	PRESENT TIME
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Did you compete in college? ☐ Yes   ☐ No

If yes, can you compare the sport and times to your present times?

SPORT/EVENT	COLLEGE TIME	PRESENT TIME
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Please use the space below to provide any additional information regarding your exercise/training history that you feel will be helpful to us.

Exercise and Vascular Health Study

DEPARTMENT OF KINESIOLOGY,
255 VALLEY DR., ROOM 0117 COLLEGE PARK, MD 20742
(301) 405-2571   (301) 405-5578 FAX

Physical Activity and Performance Questionnaire

Personal Information

Name _____

Age _____   **Birth date** _____

Height _____ **Weight** _____

Address _____ **Apt #** _____

City _____ **State** _____ **Zip Code** _____

Telephone number _____

Alternative telephone number _____

Occupation _____

Physical Activity Information

Mode(s) of exercise (check all that apply):
☐ Walking   ☐ Running   ☐ Cycling   ☐ Swimming   ☐ Weight Lifting
☐ Gardening   ☐ Other _____

How many days/week do you exercise at each mode?

Walking _____   Running _____   Cycling _____   Swimming _____
Weight Lifting _____   Gardening _____   Other _____

Do you ever exercise twice a day? ☐ No   ☐ Everyday   ☐ Occasionally

What is the approximate duration of an average exercise session? _____ minutes

How intense is an average exercise session? (circle one) Low   Moderate   Hard

What types physical activity do you do perform in a typical week? Please indicate how many days per week you engage in these activities and the approximate duration. These include activities such as walking, gardening, cleaning, washing the car, etc.

	Type of activities	Duration of activities (minutes)
Monday	_____	_____
Tuesday	_____	_____
Wednesday	_____	_____
Thursday	_____	_____
Friday	_____	_____
Saturday	_____	_____
Sunday	_____	_____

Did you participate in athletics in high school?   □ Yes   □ No

Please Describe: _____

Did you participate in athletics in college?   □ Yes   □ No

Please Describe: _____

Please use the space below to provide any additional information regarding your physical activity history that you feel will be helpful to us.

Exercise & Vascular Health Study

DEPARTMENT OF KINESIOLOGY,
255 VALLEY DR., ROOM 0117 COLLEGE PARK, MD 20742
(301) 405-2571

Health History Questionnaire: ☐ **Exercise**   ☐ **Sedentary**

Intructions: Please complete the following health history questionnaire and return it to us during your first screening visit. If you have any questions, please do not hesitate to contact us at 301-405-2571.

Personal Information:

Last Name _____ First Name _____ MI _____

Phone (home) _____ (work)_____ (E-mail)_____

Address _____ City _____ State/Zip _____

Emergency Contact _____ Phone _____

Personal Physician _____ Phone _____

Date of Birth _____

Ethnicity (check one):

☐ Hispanic or Latino: A person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race.

☐ Not Hispanic or Latino

Race (check one):

☐ American Indian or Alaska Native: A person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliations or community attachment.

☐ Asian: A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam.

☐ Black or African American: A person having origins in any of the black racial groups of Africa.

☐ Native Hawaiian or Other Pacific Islander: A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.

☐ White: A person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

_____

GENERAL HEALTH

How would you describe your overall physical health?

____ excellent   ____ good   ____ fair   ____ poor

Have you undergone a physical examination in the last 5 years?   Yes   No

Are you on a special diet?   Yes   No
    If yes, what type:_____

Have you gained or lost more than 10 lbs in the last 6 months?
    Yes   No

Have you had any illness in the last 2 weeks?
    Yes   No
    If yes, specify: _____

Do you have documented heart disease?
    Yes   No
    If yes, how long ago was it documented?
    _____ years

Has a doctor ever told you that you have an ulcer?
    Yes   No

Has a doctor ever told you that you have any type of bleeding disorder?
    Yes   No

Have you ever had any of the following conditions/symptoms? Leave blank for "no". Indicate "yes" with a check mark and indicate year of onset.

Check for yes	Year of Onset	
_____	_____	High blood pressure
_____	_____	Heart attack/coronary problem
_____	_____	Heart murmur
_____	_____	Heart disease
_____	_____	Pain or tightness in the chest
_____	_____	Palpitations/rapid heart beat
_____	_____	Phlebitis
_____	_____	Stroke
_____	_____	Lung/respiratory problems
_____	_____	Diabetes
_____	_____	Varicose veins
_____	_____	High cholesterol
_____	_____	Anemia
_____	_____	Thyroid problems
_____	_____	Cancer

MEDICATIONS

Are you currently taking **any** medications?
    Yes   No
    If yes, list all being taken. Include daily dosage and indicate for what condition. Include over-the-counter drugs (ibuprofen, aspirin, antihistamines..)

_____

_____

_____

_____

Do you take vitamins or herbal supplements?
    Yes   No
    If yes, please list:_____

_____

Do you have any known drug allergies?
    Yes   No
    If yes, please list:

_____

FAMILY HISTORY

To your knowledge, have any immediate relatives (parent, grandparent, brother, sister) had a **heart attack** or been diagnosed with **heart disease** before the age of 60? If so, indicate relative and age:

_____

_____

Have any immediate relatives had a **high blood pressure** before the age of 60? If so, indicate relative and age:

_____

MUSCULOSKELETAL/ORTHOPEDIC HISTORY

Have you ever had any of the following:

Hernia or rupture?	Yes	No
If yes, how long ago _____ years		
Present/recurrent back injury	Yes	No
Arthritis	Yes	No
_____Osteoarthritis	_____Rheumatoid	
Osteoporosis	Yes	No
Spinal disc problem	Yes	No
Joint dislocation	Yes	No
Ligament strain	Yes	No
Cartilage tear	Yes	No
Tendon tear	Yes	No
Intermittent leg cramps	Yes	No
Swollen painful joints	Yes	No
Polio	Yes	No
Surgery	Yes	No

Specify_____

FEMALES ONLY: HORMONE STATUS

Are you postmenopausal?

        Yes   No

        How long have you been postmenopausal?

        _____

Are you currently using hormone replacement therapy (HRT)?

        Yes   No

        If yes, what medications are you on and for how long have you been on these medications?

        _____

        If no, have you ever been on HRT?

        Yes   No

        When did you stop HRT?

        _____

        Why did you stop HRT?

        _____

SMOKING HISTORY

Have you always been a *non-smoker*?

        Yes   No

        If yes, go to next section

Do you presently smoke?

        Yes   No

Are you an ex smoker?

        Yes   No

        If yes, when did you stop? _____

OTHER

Please discuss any other significant medical concerns that you consider important for us to know:

_____

_____

_____

_____

_____

_____

_____

_____

CONTACTS

Do you know of any other individuals who might be interested in our study? If so, please pass our information on to them, or you may list them with their phone number/e-mail and we will contact them.

_____

_____

_____

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