ABSTRACT

Title of Document:	EFFECT OF LONG-TERM EXERCISE ON ENDOTHELIAL PROGENITOR CELLS IN HEALTHY HUMANS
	Sarah Witkowski
Dissertation directed by:	Professor James Hagberg Department of Kinesiology

The maintenance of vascular function has emerged as an important factor in keeping rates of cardiovascular disease (CVD) low. Endothelial progenitor cells (EPCs) are derived from the bone marrow and have been found to play a role in postnatal neovascularization (6) and re-endothelialization (106; 130). Reduced EPC number and function, including colony formation and migration, and increased senescence have been associated with death from cardiovascular diseases (133), CVD risk factors (126), future coronary events (103), and endothelial dysfunction (51). Oxidative stress has been proposed as a mechanism that decreases EPC number and function *in vitro*. In particular, oxidized LDL (OxLDL) has been shown to decrease EPC number and function (132), and increase EPC senescence (63) *in vitro*. Regular physical activity is related to lower rates of CVD; however the mechanisms underlying the benefits of exercise in the prevention of CVD are not fully clear. Exercise may improve the number (77; 77; 78), and function (54) of EPCs while improving oxidative stress status (127; 129).

The primary purpose of this study was to compare endothelial progenitor cell (EPC) number, EPC clonogenic capacity, and senescence, in healthy men that have participated in greater than 20 years of moderate- to high-intensity exercise with age-matched control subjects. To assess the effect of physical inactivity on these markers, a subset of exercisers (n=10) stopped exercising for 10 days after which, measures of EPC number, colony forming units, and senescence, endothelial function and oxidative stress were re-evaluated.

Results showed that $CD34^+/KDR^+$ cell number, CFU-Hill colonies, and EPC senescence were not statistically different between athlete and control groups. $CD34^+/KDR^+$ cell number was closely related to endothelial function. Specifically, the forearm blood flow response to reactive hyperemia was correlated with $CD34^+/KDR^+$ number in sedentary participants (AUC_{1min}; r=-0.78, p=0.005). Additionally, 10 days of physical inactivity revealed 5 athletes who either had no change or an increase in $CD34^+/KDR^+$ number and 5 athletes significantly decreased their $CD34^+/KDR^+$ number (70 ± 30% vs. -86 ± 7%, p=0.006). In athletes that decreased $CD34^+/KDR^+$ number with exercise training, the change in EPC number was significantly related to a decline in endothelial function (-25.7 ± 15.7% change in peak flow), indicating that regular physical activity is important for some athletes to maintain healthy endothelial function, perhaps through the maintenance of elevated number of circulating $CD34^+/KDR^+$ cells. CFU-Hill colony number was strongly correlated with hyperemic blood flow response in control participants, such that individuals with better endothelial function had enhanced EPC clonogenic capacity (r=0.84, p=0.001). CFU-Hill colony forming capacity was related to oxidized LDL independent of physical activity status; however, athletes who participated

in 10-days of exercise detraining demonstrated a trend for a significant decrease in EPC senescence (22.8 ± 3.3 before vs. 16.4 ± 3.1% after training cessation, p=0.06), which was related to improved total antioxidant capacity (r=-0.66, p=0.04). Overall, these results show that while $CD34^+/KDR^+$ number is closely related to endothelial function and may be affected by exercise and inactivity, it is not influenced by oxidative stress whereas the function of EPCs appears to be affected by oxidative stress and antioxidant availability. These results may contribute to the understanding of the mechanisms involved in the maintenance of healthy endothelial function and prevention of cardiovascular disease.

EFFECT OF LONG-TERM EXERCISE ON ENDOTHELIAL PROGENITOR CELLS IN HEALTHY HUMANS

By

Sarah Witkowski

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2008

Advisory Committee:
James M Hagberg, Ph.D., Chair
Espen E. Spangenburg, Ph.D.
Stephen M. Roth, Ph.D.
Richard B Horenstein, M.D.
David M. Mosser, Ph.D.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Drs. Spangenburg, Roth, Horenstein, and Mosser for their service on this committee, review of this manuscript, and overall support on this project. Specifically, I would like to express my deep appreciation to my advisor, Dr. Hagberg, for allowing me to pursue my interest in this new area and your unyielding support and encouragement during the entire process.

My sincere appreciation goes to the participants of this research project. Your enthusiastic dedication to the project allowed this work to be possible. To my fellow graduate students, Andy Ludlow, Nathan Jenkins, Jenny McKenzie, and Erik Hansen, thank you for creating a fun and cooperative research environment where ideas, insights, and learning are shared and enjoyed. Thank you to the students who assisted with various aspects of recruiting, testing and data collection, including Chris Parsons, Sean Sweeney, Neha Agarwal, Tim Naugle, Britney Tsui, and Anne Chlebowski. I would like to thank Dr. Hurley for his generous assistance with DEXA body composition assessment. Also, I would like to acknowledge Keith Tanner from the University of Maryland at Baltimore Medical School who assisted in the optimization of EPC isolation and labeling and Regina Harley and Kathy Storrer from the Flow Cytometry Core Lab for their assistance with EPC quantification.

Thank you to Josef Brandauer and Jen Sterling, for your friendship and support. Lastly, I would like to express gratitude to my family for their encouragement throughout my academic career. In particular, to my partner Kerri, I am deeply grateful for your steadfast support and love.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACE – angiotensin converting enzyme
AUC – area under the curve
BMI – body mass index
CAD – coronary artery disease
CFU – colony-forming unit
CHD – coronary heart disease
CO_2 – carbon dioxide
CVD – cardiovascular disease
DEXA - dual energy X-ray absorptiometry
DNA – deoxyribonucleic acid
ECG – echocardiogram
ELISA – enzyme-linked immunosorbent assay
eNOS – endothelial nitric oxide synthase
EPC – endothelial progenitor cell
FBF – forearm blood flow
FBS – fetal bovine serum
Flk-1 - fetal liver kinase-1
FVR – forearm vascular resistance
GPX-1 – glutathione peroxidase – 1
HDL – high-density lipoprotein
HPC – hematopoietic progenitor cell
HUVECS – human umbilical vein endothelial cells
H_2O_2 – hydrogen peroxide
ICAM-1 – intracellular adhesion molecule – 1
KDR - Kinase insert domain receptor
LDL – low density lipoprotein
mnSOD – manganese superoxide dismutase
NSAID – non-steroidal anti-inflammatory drug
NO – nitric oxide
NOx – nitrates/nitrites
O_2- - superoxide
OxLDL – oxidized low-density lipoprotein
RER – respiratory exchange ratio
ROS – reactive oxygen species
SDF-1 – stromal cell-derived factor-1
TAC – total antioxidant capacity
TG – triglyceride
VEGF – vascular endothelial growth factor
VLDL – very low-density lipoprotein
VO_{2max} – maximal oxygen consumption

INTRODUCTION

Endothelial Progenitor Cells and CVD

Disruption of endothelial integrity and function occupies a central role in the development of atherosclerosis and cardiovascular disease. Endothelial damage and dysfunction are evident prior to the development of atherosclerotic lesions. Historically, it was believed that the growth of new blood vessels and repair of the endothelial layer occurred via existing vascular and endothelial cells. In 1997, Asahara, et al characterized a novel group of cells by cell surface antigens ($CD34^+/Flk\text{-}1^+$) found in circulation that differentiated into endothelial cells and were incorporated into sites of angiogenesis (6); these new cells were termed endothelial progenitor cells (EPCs). Soon thereafter, Shi, et al (106) demonstrated that these cells colonized a graft placed in the canine aorta and later, Walter, et al (130) reported that EPCs home, or migrate, to balloon-injured arterial segments and re-endothelialize the denuded areas.

More recently, the factors that stimulate EPC mobilization, homing and incorporation have been investigated. EPCs are released from bone marrow stromal cells in response to various stimuli, including vascular endothelial growth factor (VEGF), stromal cell-derived factor-1 (SDF-1), erythropoietin (Epo), HMG CoA reductase inhibitors (statins), PPARγ agonists, exercise, estrogen, and eNOS (4; 60; 123). EPCs home to sites of vascular injury via signals that include shear stress, (145), VEGF and SDF-1 as well as platelets (75), thrombin (116) and apoptotic bodies (58). The mechanisms of incorporation of EPCs into the endothelium are less clear, however, this process likely involves adhesion molecules (i.e., ICAM-1) and integrins (70; 70; 144).

Endothelial progenitor cell number and function have been associated with cardiovascular disease, cardiovascular disease risk factors, atherosclerosis progression and future occurrence of CVD events (51; 103; 126; 133). In 2001, Vasa, et al (126) were the first to report an association between circulating EPC number and EPC functional characteristics with coronary artery disease family history and number of atherosclerotic risk factors. They found lower EPC number in patients with a positive family history for coronary artery disease (CAD) and impaired EPC migratory response in patients with a greater number of atherosclerotic risk factors. EPC function (assessed by colony-forming assay) have also been found to be associated with endothelium-dependent flow-mediated vasodilation, a measure of endothelial function (51). This study also found that EPCs from patients with a higher risk for cardiovascular events demonstrated greater *in vitro* β-galactosidase activity, an indicator of cellular senescence. Interestingly, the number of EPCs was found to be a better predictor of vascular reactivity than the Framingham risk factor score. More recently, the number of circulating EPCs has been found to independently predict atherosclerotic disease progression (103) and cardiovascular events including death from cardiovascular causes, revascularization surgery, and hospitalization due to cardiovascular events, which included angina, congestive heart failure, stroke, myocardial infarction, and arrhythmia (133).

Cardiovascular disease risk factors such as hypertension and diabetes that are related to decreases in endothelial integrity and function have also been associated with decreased EPC function. For example, Imanishi, et al (64) reported that EPCs from both spontaneously hypertensive rats and human patients with essential hypertension demonstrated increased cell senescence and decreased telomerase activity, an indicator of

increased cell turnover. Tepper, et al (117) found that EPCs from type II diabetes patients had a 48% decrease in proliferation in culture compared to controls. In addition, EPCs from diabetics had decreased adhesion to HUVECS and were 2.5 times less likely to participate in tubule formation compared to controls. These data suggest that patients with type II diabetes have impaired EPC function, which may explain the vascular complications and increased incidence of peripheral vascular disease, atherosclerosis, and cerebrovascular disease in diabetics (17). Therefore, EPCs appear to be a biological marker of endothelial health and function, and EPC dysfunction may lead to an impaired vascular and endothelial regenerative capacity, increased atherosclerosis, and CVD.

Endothelial Progenitor Cells and Oxidative Stress

Oxidative stress has emerged as an important mechanism in the development of endothelial dysfunction, atherosclerosis and progression of CVD. Sources of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are the oxidative burst of immune cells, the electron transport chain and cellular respiration, and in the endothelium, the action of the enzymes xanthine oxidase and nitric oxide synthase (97). ROS have more recently been recognized as cell signaling factors for growth, survival, and apoptosis (67). Oxidative stress occurs with disruptions of the redox balance leading to damage to DNA and lipids, and cellular senescence and death.

Oxidative stress may be an important factor in the determination of EPC number and function. Although EPCs, similar to other types of stem cells, exhibit higher expression of the antioxidant enzymes catalase, glutathione peroxidase and manganese superoxide (MnSOD) than mature endothelial cells (25; 49), disruptions in redox state

have been shown to affect EPC function. Although Dernbach, et al (25) found that inhibition of any one of these enzymes did not increase EPC reactive oxygen species (H_2O_2 and O_2^-), the inhibition of all three enzymes together increased EPC ROS and decreased EPC survival and migration in response to hydrogen peroxide. In addition, Galasso, et al (36) found that mice deficient in GPX-1, the gene that encodes glutathione peroxidase, had decreased EPC function, decreased angiogenesis in response to hindlimb injury, and increased sensitivity to oxidative stress-inducing peroxide to induce EPC apoptosis. Therefore, it appears that EPCs can be vulnerable to ROS when their redox status is disrupted by inhibition of their antioxidant enzymes.

One consequence of increased oxidative stress is the oxidation of LDL. In the endothelium, LDL oxidation leads to endothelial cell dysfunction, decreased nitric oxide (NO) bioavailability, the expression of adhesion molecules on monocytes, and migration of monocytes to the subendothelial space. Therefore, oxidized LDL (OxLDL) is an important factor in the pathogenesis of cardiovascular disease. For example, circulating oxidized LDL has been found to be an independent predictor for future cardiovascular events in patients with coronary artery disease and diabetes (107; 108). OxLDL increases endothelial dysfunction by decreasing NO bioavailability, increasing proliferation of smooth muscle cells, promoting platelet accumulation, and thrombus formation. OxLDL also increases damage to the endothelium by impairing the regenerative capacity of EPCs. Recently, ex vivo studies have shown that OxLDL leads to decreased EPC number and function and increased senescence. Wang, et al (132) reported that incubation of cultured EPCs with increasing concentrations of oxidized LDL decreased EPC number, proliferative capacity, migration, and adhesion in a dose-dependent

manner. Oxidized low-density lipoprotein has been shown to induce EPC senescence, decrease telomerase activity and decrease the proliferative capacity of EPCs *in vitro* via increases in oxidative stress (63). The same group found that in the presence of VEGF, OxLDL may contribute to lower EPC function via dephosphorylation of Akt, a protein kinase that is activated by growth factors and cytokines (62). Recently, Ma, et al (82) found that OxLDL inhibits EPC survival and impairs their function via inhibition of eNOS. Therefore, the highly-atherogenic OxLDL may directly affect EPCs to further decrease endothelial health.

Endothelial Progenitor Cells and Physical Activity

Regular physical activity is associated with decreased risk of development of atherosclerosis and cardiovascular disease and improvement in cardiovascular disease risk factors. In addition, physical activity is associated with improved endothelial health and vascular function and has been shown to be an effective therapy to improve vascular collateralization in patients with coronary artery disease (43). In general, exercise, both acute and exercise training, has been found to stimulate increases in circulating EPCs in healthy subjects and patients with ischemic coronary artery disease and cardiovascular disease risk, although there are few studies that lend insight into the mechanisms and signaling that connect exercise with EPC release and action.

Exercise training has been shown to increase EPC number and function in mice, healthy humans, and some patient populations. In 2004 Laufs, et al (78) showed that 3 weeks of voluntary wheel running in mice and 4 weeks of exercise training (60-80% peak VO_2) in humans resulted in a significant persistent increase in EPC number in mice after

7 days of exercise and EPC number in humans increased 78 ± 34% compared to before exercise training. In this study, both mice and humans had decreased EPC apoptosis following exercise. Steiner, et al (111) demonstrated that 12 weeks of exercise training for patients with asymptomatic coronary artery disease (CAD) and/or cardiovascular disease risk factors resulted in a 2.9 ± 0.4-fold increase in circulating EPCs, which was correlated with an increase in endothelial function. Exercise training (4 weeks) has also been shown to increase EPC incorporation into vascular networks (98).

In healthy moderately-trained subjects, an acute bout of moderate to hard-intensity endurance exercise has also been shown to increase EPC number, EPC migration and colony-forming units (77). In this study, researchers found that moderate (~68% VO_{2max}) and intense (~82% VO_{2max}) running for 30 minutes increased EPC number to 235 ± 93% and 263 ± 106%, respectively, but moderate, short term running (10 minutes) did not significantly increase EPC number following exercise. In this study, researchers also characterized the time course of EPC number and function in response to an acute bout of intense exercise. Following exercise, EPC number was increased at 10 minutes to a maximum at 30 minutes then decreased to pre-exercise levels by 24 hours. Intensive running increased EPC migration in a similar fashion. Colony-forming units increased as soon as 10 minutes following exercise and remained significantly elevated up to 6 hours following exercise. These data demonstrate that there appears to be both an acute exercise and exercise training effect with regard to EPC number and function. What remains unknown is the effect of long-term endurance exercise training independent of the contribution of an acute bout of exercise.

In conclusion, recent data have shown that endothelial progenitor cells serve as a source of cells for the regeneration and repair of the vascular endothelium. Decreases in EPC number and function are associated with CVD and CVD-related conditions and risk factors. Oxidative stress and OxLDL may not only damage endothelial cells, leading to endothelial dysfunction and atherosclerosis progression, but may also damage EPC antioxidant capacity, decrease EPC telomere length, telomerase, and viability, thereby damaging a source of endothelial regeneration. However, evidence supporting the interaction of OxLDL and EPCs is limited to *in vitro* studies on cultured EPCs. Physical activity has been shown to increase EPC number both following an acute bout of exercise and exercise training, although few studies have been performed on healthy humans.

PURPOSE OF THE STUDY

Endothelial dysfunction has been shown to be one of the earliest events in the development of atherosclerosis. Therefore, maintenance of a functional endothelial layer has emerged as an important factor in the prevention of cardiovascular disease. Circulating endothelial progenitor cells from the bone marrow have been shown to play a role in the repair of the endothelium, however their efficacy may be influenced by oxidative stress.

Regular physical activity is related to lower rates of cardiovascular disease. Exercise training has been demonstrated to improve endothelial function; however, the mechanisms of this adaptation are not clear. Exercise may improve the mobilization of circulating endothelial progenitor cells and preserve their function by decreasing the influence of oxidative stress.

Therefore, the purpose of this study was to 1) evaluate differences in endothelial function and EPC number and function between low-active individuals and those with a long-term physical activity history, 2) to evaluate the relationship between EPC number and function and oxidative stress in these two groups, and 3) to analyze the effects of 10 days of training cessation in long-term exercisers on EPC number, function, and oxidative stress.

HYPOTHESES

The central hypothesis of this study is that healthy individuals with chronic long-term endurance physical activity will have better endothelial function and greater EPC number, colony-forming capacity, and decreased EPC senescence and that these factors will be related to a favorable oxidative stress profile compared to low-active control individuals.

Hypothesis #1

Long-term physical activity is associated with improved endothelial function which is related to greater EPC number, EPC-Colony Forming Units (CFU-Hill), and lower EPC senescence compared to age and BMI-matched healthy low-active control individuals.

Specific Aims

1.1 Determine whether long-term exercisers have greater peak forearm blood flow, total hyperemic forearm blood flow, and NOx compared to low-active control subjects.

1.2 Determine whether long-term exercisers have greater EPC number, greater CFU-Hill count, and lower EPC senescence compared to low-active control subjects.

1.3 Determine whether greater EPC number and CFU-Hill count and decreased EPC senescence are related to increased peak forearm blood flow, total hyperemic forearm blood flow, and NOx.

Hypothesis #2

Oxidative stress is lower in long-term exercisers compared to controls and is associated with greater EPC number and CFU-Hill count and lower EPC senescence.

Specific Aims

2.1 Determine whether long-term exercisers have lower oxidized LDL and higher total antioxidant capacity compared to low-active control subjects.

2.1 Determine whether greater EPC number and CFU-Hill count and decreased EPC senescence are related to oxidized LDL and total antioxidant capacity.

Hypothesis #3

EPC number and CFU-Hill count will decrease and EPC senescence will increase following 10-days of training cessation and these changes will be associated with decreased endothelial function and increased oxidative stress.

Specific Aims

2.1 Determine whether EPC number and CFU-Hill count decrease and EPC senescence increases after 10 days of training cessation.

2.1 Determine whether changes in EPC number, CFU-Hill and EPC senescence are related to changes in oxidized LDL, total antioxidant capacity, forearm blood flow measures and NOx after 10 days of training cessation.

METHODS

Participants

Twelve healthy male long-term exercisers (athletes) aged 55 to 80 years old with a training history of at least 20 years were recruited for this study. Athletes had to participate in moderate to intense endurance exercise greater than 3 days/week. Healthy low-active male age- and BMI-matched participants (n=11) were recruited as controls. Control subjects could not be participating in or have a recent history of moderate to intense regular endurance exercise. Participants were screened over the phone and asked to fill out health history and physical activity history questionnaires. All participants were non-smokers, non-diabetic and free of cardiovascular, lung, liver disease, and cancer. Participants did not qualify if they were taking medications that have been demonstrated to affect EPC number and/or function. Briefly, these include HMG-CoA reductase inhibitors (statins), angiotensin II receptor antagonists, ACE inhibitors, peroxisome proliferator-activated receptor gamma agonists, and EPO.

Testing Procedures

Written informed consent was obtained for all subjects and the study was approved by the University of Maryland College Park Institutional Review Board.

All participants engaged in at least two laboratory visits; 1) a preliminary screening visit to evaluate resting blood pressure and fasting blood chemistry profile, and to rule out the presence of cardiovascular disease and 2) a second visit to assess endothelial function, obtain blood samples for analyses, and body composition testing.

Athletes who agreed to undergo training cessation for 10 days, returned to the laboratory for a third visit after the 10 days for repeated measures of endothelial function, blood-derived parameters, and body composition. Athletes were instructed to maintain a stable weight for the 10 days of training cessation. To accomplish this, athletes were given a weight data log and instructed to weigh themselves in the morning and evening and record their weight in the log.

For visits 1 and 2, data collection occurred 24-hours following the athletes' last exercise session. For all visits, participants were tested following an overnight (12-14 hour) fast in which they refrained from alcohol and caffeine for at least 24 hours prior to the test and any vitamins or medications (antihistamines, NSAIDs, etc) 48 hours prior to the test. As plasma nitrate/nitrite concentration has been shown to be highly influenced by dietary nitrate/nitrite intake, and nitrite is an intrinsic vasodilator (76), prior to visit 2 and 3 for athletes, participants were asked to adhere to a low-nitrate diet for the 3 days prior to testing for forearm blood flow and NOx measures. Participants were educated about foods/fluids high in nitrates and given a list of foods/fluids to avoid as well as samples of low-nitrate meals. Participants were instructed to record their intake for the three days on a log supplied to them.

Maximum Oxygen Uptake (VO_{2max})

VO_{2max} testing was performed on a treadmill with physician monitoring. Heart rate, ECG, and blood pressure were monitored continuously throughout the test. For long-term exercisers, the treadmill testing was a modification of the Bruce protocol

which consisted of the first two 3 minute stages of the Bruce Protocol (1.7 mph, 10% grade and 2.5mph, 12% grade, respectively), followed by 2 minute incremental stages after which the treadmill grade was increased by 2% until the subject reached exhaustion. Treadmill speed for stages three to maximal were determined by the investigator based on subject experience, typical run speed, and heart rate such that VO_{2max} was achieved between 8-12 minutes. For low-active participants, the treadmill protocol consisted of 2 minute stages in which the treadmill was elevated by 2% each stage. Similar to the exercisers, treadmill speed was determined by the investigator based on subject experience, typical walking speed, and heart rate such that VO_{2max} was achieved between 8-12 minutes. Expired air was sampled using indirect calorimetry. Briefly, data were collected with an online computer system (Oxycon Pro, Viasys) in the mixing chamber mode. Expired volume was recorded via a digital volume transducer and expired gas concentrations were analyzed by O_2 and CO_2 analyzers. Frequency of sample analysis was 30 seconds for VO_2 and respiratory exchange ratio (RER). For all tests, three of four criteria were met for a valid VO_{2max} test: RER $\geq$ 1.15, maximal heart rate (age-predicted) was reached, a plateau in the VO_{2max} increase with an increase in work rate (< 250mL VO_{2max} increase) was observed, or the subject indicated exhaustion.

Endothelial-Dependent Flow-Mediated Dilation

Endothelial-dependent dilation was measured via forearm blood flow (FBF, $ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue volume) in the non-dominant arm using strain gauge plethysmography at baseline and during reactive hyperemia. Following 15-20 minutes of

supine rest in a quiet, dim, temperature-controlled room (22-25ºC), the subject was instrumented with a small wrist pressure cuff, an upper arm pressure cuff on the non-dominant arm, and a standard automated blood pressure cuff on the opposite side. A mercury-filled strain gauge was fitted to the subject's forearm which was positioned at an angle slightly above the atrium and connected to the Hokanson EC 5R plethysmograph (Hokanson Inc.) and chart recorder. For the baseline measurement, the wrist cuff was inflated to 200mmHg to eliminate any artifact from hand blood flow. The forearm cuff was inflated via a rapid cuff inflator (Hokanson E20) to 55mmHg for 7 seconds then released for 8 seconds. Baseline FBF was determined as the average of 3 resting measures.

For the reactive hyperemic measurement, a baseline blood pressure was recorded to determine the inflation pressure in the occlusion cuff (50mmHg above resting systolic blood pressure). Forearm ischemia was achieved by a 5 minute occlusion with the upper arm pressure cuff. Following forearm ischemia, forearm blood flow was measured as described above (every 15 seconds) continuously for 3 minutes.

Reactive hyperemic measures included peak FBF, minimum forearm vascular resistance (FVR, $mmHg \cdot ml^{-1} \cdot min^{-1} \cdot 100ml^{-1}$), and duration of hyperemia which was calculated as the time (seconds) between cuff release and the return of FBF to +5% of baseline. Area under the FBF curve (AUC, ml/100ml) was calculated according to the trapezoidal rule as the area from release of the cuff to the end of the 3 minutes of recovery. Since the maximal hyperemic response has been demonstrated to occur within the first minute of occlusion release (20), the AUC for the first minute of the hyperemic

response (AUC_{1min}, ml/100ml) was also calculated. Flow debt (ml/100ml, baseline FBF x duration of occlusion) excess flow (ml/100ml, AUC – (baseline FBF x duration of hyperemia) and hyperemic repayment (%, flow debt/excess flow) were also calculated to characterize the hyperemic response.

Nitrate/Nitrite (NOx) Assay

Plasma was filtered via centrifugation with 10 kD cut-off centrifugal ultrafilters (Millipore Corporation) at 9,000*g* and 4°C for 50 minutes. Nitrate and nitrite concentration (NOx) of the filtered plasma was analyzed using a colorimetric assay modification of the Griess reaction as described elsewhere (34). Briefly, to convert plasma nitrate to nitrite, 40μL of filtered sample was incubated with 20μL nitrate reductase solution (1050U/L) and 10μL NADPH (175 μM) for 60 minutes. To remove excess NADPH, samples were incubated for 60 minutes in 20μL NH_4Cl (250U/L), 10μL glutamate dehydrogenase, and 20μL α-ketogluterate (10mM). The Griess reaction was started with the addition of 40μL of sulfanilamide solution (1.0% sulfanilamide in 5.0% phosphoric acid) followed by 30 minutes of incubation. Finally, 40μL of N-1-napthyethylenediamine dihydrochloride solution (0.1%) was added and samples were incubated for 30 minutes. All incubations occurred at room temperature with the samples protected from light. Absorbance was read at 541nm by a microplate reader (Emax, Molecular Devices). Samples were assayed in duplicate. The inter-assay and intra-assay coefficient of variation was 9.94% and 3.37%, respectively.

Circulating Progenitor Cell Number

$CD34^+$ (HPC, hematopoietic progenitor cell) and $CD34^+/KDR^+$ (EPC) number was determined by flow cytometry. For this assay, 4mL of blood was collected into an EDTA-coated tube. Mononuclear cells were separated via density centrifugation (Ficoll-Paque Plus, GE Healthcare). Cells were washed twice in PBS and counted with a hemocytometer. $1x10^6$ mononuclear cells were immunostained with monoclonal antibodies against human CD34 (BD Biosciences) and against human VEGFR2 (R&D Systems). Isotype-identical antibodies (mouse IgG2a-FITC, BD Biosciences, and mouse IgG1-PE, R&D Systems) served as controls. For each group of analyses, one set of control tubes for machine calibration was generated. In this case, at least $5x10^5$ cells were labeled with CD3-FITC-conjugated mouse anti human mAb (BD Biosciences) and $5x10^5$ cells were labeled with CD3-R-PE-conjugated mouse anti-human mAb (BD Biosciences). All cells were FcR blocked (Miltenyi Biotech Inc.). After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde. Flow cytometry was performed at the University of Maryland School of Medicine Flow Cytometry/Cell Sorting CORE Laboratory with a Beckman Coulter Epics Elite ESP flow cytometer and cell sorter. The forward- side-scatter plot was used to identify the lymphocyte gate. 100,000 events per sample were acquired. To address reproducibility, in a subset of individuals, two samples were analyzed from the same subject (n=30). The correlation between the two measures was $r=0.90$, $p < 0.001$.

Colony Formation Assay (CFU-Hill)

The colony forming unit (CFU-Hill) assay is an *in vitro* assay for the colony-forming potential of endothelial progenitor cells (51). 14 mL blood was collected directly into K_3EDTA tubes. Mononuclear cells were isolated by density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare), washed two times with PBS and 2% FBS and resuspended in EndoCult® Medium (Stem Cell Technologies). Cells were counted with a hemocytometer and 5 x 10^6 cells/well were plated on fibronectin-coated 6-well plates (BD Pharmingen). After 48 hours, non-adherent cells were harvested and counted. 1 x 10^6 cells/well were re-plated in 4 wells of fibronectin-coated 24-well plates (BD Pharmingen). CFUs were evaluated and counted three days later via inverted light microscopy. A colony was defined as a central core of "round" cells with more elongated "sprouting cells" at the periphery. A central core of cells without cells emanating was not counted as a colony. Colonies were counted manually by individuals trained in the identification of colonies. To address reproducibility, colonies were counted by two independent observers. The correlation between observers was $r=0.98$, $p < 0.001$. The endothelial lineage of these cells has been confirmed previously via immunocytochemical staining for von Willebrand factor, vascular endothelial growth factor receptor 2, and CD31 (51).

Endothelial Progenitor Cell Senescence

EPC senescence was determined using β-galactosidase Cellular Senescence Assay Kit (#KAA002, Chemicon International). β-galactosidase is a marker expressed by senescent but not pre-senescent cells at a pH of 6 and has been used to quantify EPC senescence by Hill, et al (21). For this assay, cultures from the colony-forming assay (CFU-Hill) were used. On day 5 of the EPC-CFU culture following colony counting, EndoCult® Medium was changed and cells were incubated for another 48 hours. Following 7 days of culture, culture medium was removed and EPCs were washed in phosphate-buffered saline (PBS). Cells were incubated in fixation solution for 15 minutes (room temperature). Fixation solution was removed, cells were washed, and senescence-associated β-Gal stain solution (SA-β-gal) was added to the wells. Cells were incubated protected from light for 4h at 37ºC (no CO_2). Cells distant from central colonies from 4 randomly selected fields that contained 100-200 cells each were analyzed with an inverted light microscope (VistaVision, VWR). Cells with a distinctly blue cytoplasm were counted as senescent (β-gal-positive). Total cell number and number of senescent-positive cells in each field were counted twice. If counts were >10% different from one another, a third count was performed. Total cell count and number of senescent-positive cells were each averaged. Senescence was quantified by the percentage of β-gal-positive cells in each of the 4 wells and averaged for the total percentage.

Plasma Oxidized LDL

Plasma was obtained from blood that was drawn from each subject directly into a K_3EDTA tube. Plasma oxidized LDL levels (OxLDL) were measured using a commercially-available competitive ELISA kit (Mercodia, Uppsala, Sweden) that utilizes the murine monoclonal antibody mAb-4E6. This technique was developed by Holvoet, et al (55) and has been used to relate OxLDL levels with subclinical atherosclerosis (61) and coronary artery disease (56). Briefly, OxLDL in the sample competes with well-bound OxLDL for biotin-labeled antibody (mouse monocolonal anti-oxidized LDL). Biotin-labeled antibody is detected with HRP-conjugated streptavidin and the bound conjugate is determined by a reaction with 3, 3', 5, 5'-tetramethylbenzidine (TMB). The addition of a stop solution (0.5M H_2SO_4) yields a colorimetric endpoint. The plate was read with a plate reader (Emax, Molecular Devices). All samples were assayed in duplicate. Two-level control samples (Mercodia Oxidized LDL Control Kit) were assayed to confirm assay performance. These controls were within the manufacturer's specified range. All samples, including controls, were analyzed in a single assay to eliminate inter-assay variability. The intra-assay coefficient of variation was 6.8%.

Total Antioxidant Capacity

Total antioxidant activity (aqueous and lipid-soluble antioxidants, including vitamins, proteins, lipids, glutathione, and uric acid) in plasma was analyzed via a commercial kit (Cayman Chemical) developed from the protocol of Miller, et al (83).

Briefly, this assay measures the ability of antioxidants in the sample to prevent the oxidation of ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS $^{\bullet+}$, a radical cation, by metmyoglobin. ABTS $^{\bullet+}$ is a green chromogen which was analyzed with spectrophotometry at 750nm. This reaction was compared to that of the control antioxidant, Trolox, a water-soluble vitamin E analog. Samples were assayed in duplicate. All samples were analyzed in a single assay to eliminate inter-assay variability. The intra-assay coefficient of variation was 4.1%.

Body Composition

Height and weight were recorded and body mass index, BMI (kg/m^2) was calculated for all subjects. To quantify percentages of lean and fat mass, body composition was assessed via dual energy x-ray absorptiometry (Hologic, Discovery A, software version 12.7.1). Scanner calibration was performed daily with the manufacturer's quality assurance standard. Those long-time exercisers who choose to stop exercise training underwent a second DEXA scan following the 10 days of training cessation.

Framingham Risk Score

Hill, et al (51) reported a strong correlation between endothelial progenitor colony forming units and Framingham risk score. To assess conventional CHD risk factors, risk

scores for CHD were calculated based on equations from the Framingham study (136). Points were assigned according to age, LDL cholesterol, HDL cholesterol, blood pressure, diabetes and smoking status. 10-year heart disease risk was also assigned according to the total point value.

Statistical Analysis

Analyses were performed with SAS version 9.1. Data were analyzed for normality and heterogeneity of variance. Student's t-tests were used to test for differences between athletes and control groups. Where data were found to not meet the assumption of normality, the nonparametric Mann-Whitney *U*-test (Wilcoxon rank sum test) was used to compare difference between groups. In these cases, for descriptive data the median (lowest value-highest value) are displayed; for EPC data, means ± SE are displayed. Paired t-tests were used to test for differences between athletes before and after detraining. Where paired data were found to not meet the assumption of normality, the nonparametric Wilcoxon signed rank test was used to compare athletes before and after detraining. Differences between groups for reactive hyperemic FBF response were tested using a repeated measures analysis of variance (ANOVA). Pearson correlation coefficients were used to examine the relationships between normally-distributed variables. For parameters with non-normal distributions, nonparametric Spearman correlation coefficients were used. Regression analysis was used to determine the best predictors of $CD34^+/KDR^+$ endothelial progenitor cells, CFU-Hill colony number, and EPC senescence. Data transformation was performed to satisfy test assumptions where

appropriate. For directional hypotheses, 1-sided *p*-values are reported and noted when used. All other *p*-values are two-sided. An α-level of 0.05 was used to indicate statistical significance.

RESULTS

Subject Characteristics

Twelve long-term exercisers and 11 low-active control males participated in the study. Groups were matched for age (62 ± 2 vs. 64 ± 2 years, p = 0.23) and BMI (22 ± 0.8 vs. 23.5 ± 0.6, p = 0.15). The percentage of body fat and diastolic blood pressure for the low-active group was significantly greater (Table 1, p = 0.02, and p = 0.04, respectively) compared to the athletes. Likewise, long-term exercisers had better blood chemistry profiles (Table 1). Although the Framingham risk score was not significantly different between groups, there was a significant difference between the groups for 10-year risk for developing CHD (p = 0.007). As expected, athletes had a higher VO_{2max} compared to low-active controls. Physical activity questionnaire data revealed that this group of athletes, composed of 11 runners and 1 triathlete, exercised an average of 5 ± 0.4 days a week and had been exercising continuously for 32 ± 3 years. Low-active control participants were not engaging in regular endurance exercise, nor did they have a recent history of physical activity. Low-active participants did engage in activities of daily living, such as walking, climbing stairs and gardening, however, these activities were neither regular nor intense.

Ten athletes agreed to participate in 10-days of training cessation. Participants were instructed to not engage in regular exercise training sessions during this period. Activities of daily living (i.e. walking, climbing stairs) were permitted provided the intensity and duration were minimal. Participants were also instructed to not lose or gain

weight during the interval. Therefore, each maintained a body weight log in which they recorded their body weights in the morning and evening. Body weight and BMI did not change following training cessation (67.2 ± 2.3 vs. 67.6 ± 2.3 kg and 22.0 ± 0.7 vs. 22.1 ± 0.7 kg/m^2, respectively), however body fat (%) was slightly greater (17.3 ± 1.2 vs. 18.1 ± 1.1, $p<0.001$) and SBP was slightly lower (121 ± 4 vs. 114 ± 3, $p=0.006$) following the 10 days of training cessation.

Table 1. Descriptive data

Variable		Athletes (n=12)	Control (n=11)	p-value
Characteristic				
	Age (yr)	62 ± 2	64 ± 2	0.23
	Height (m)	1.78 ± 0.03	1.76 ± 0.02	0.55
	Weight (kg)	70.1 ± 2.9	73.0 ± 2.4	0.45
	BMI	22.0 ± 0.8	23.5 ± 0.6	0.15
	Body Fat (%)	18.0 ± 1.3	23.5 ± 1.8	0.02
	SBP (mmHg)	122 ± 3	129 ± 3	0.17
	DBP (mmHg)	79 ± 2	85 ± 2	0.04
Blood Chemistry				
	Glucose (mg/dl)	94.5 (83-104)	99 (91-122)	0.09
	Cholesterol (mg/dl)	199.1 ± 8.9	194.2 ± 10.6	0.72
	Triglycerides (mg/dl)	66.2 ± 8.4	103.0 ± 13.5	0.03
	HDL-C (mg/dl)	71.2 ± 3.3	51.0 ± 4.6	0.002
	LDL-C (mg/dl)	114.8 ± 8.4	122.5 ± 11.4	0.59
	VLDL-C (mg/dl)	13.2 ± 1.7	20.6 ± 2.7	0.03
	TC/HDL	2.9 ± 0.2	4.2 ± 0.5	0.02
Framingham Risk				
	Score	4.0 (0-7)	7.0 (1-9)	0.13
	10-year CHD risk (%)	7.67 ± 0.86	12.82 ± 2.2	0.007
Physical Activity				
	VO_{2max} (ml/kg)	50.0 ± 1.9	28.1± 1.7	<0.0001
	VO_{2max} (L/min)	3.51 ± 0.17	2.07 0.12	<0.0001
	days/week running	5 ± 0.4		
	miles/week running	36 ± 3		
	years exercising	32 ± 3		

Values are means ± SE. For data that were not normally distributed, median (lowest value-highest value) is displayed and p value represents a Mann-Whitney *U*-test; n, number of subjects; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; TC, total cholesterol; VO_{2max}, maximal oxygen uptake.

Endothelial Function

Table 2 displays the results from resting and reactive hyperemia blood flow responses in athletes, athletes who detrained and controls. Resting forearm blood flow ranged from 0.95 to 3.23 $ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue in the total group and was not different between athletes and controls. Following 5 minutes of cuff occlusion, forearm blood flow increased in the athlete ($p < 0.001$) and control ($p < 0.001$) groups (837.6 ± 90.1 % and 608.9 ± 73.6 %, respectively). Peak forearm blood flow was significantly greater in athletes compared to controls ($p = 0.03$) which corresponded to a lower minimum FVR in athletes ($p = 0.03$). Athletes also had a shorter hyperemic duration ($p = 0.04$). Area under the curve for the first minute (AUC_{1min}) was calculated as it has been shown that the greatest hyperemic response occurs within the first minute following cuff occlusion (21). This parameter displayed a trend to be lower in controls compared to athletes ($p = 0.08$).

There were no significant differences in baseline or reactive hyperemic blood flow parameters between the 12 athletes at baseline and the 10 athletes who underwent detraining. Following 10 days of training cessation, there were no significant changes in any of the forearm blood flow measures. Although peak forearm blood flow and minimum forearm vascular resistance remained significantly higher than controls (Table 2), there was a downward shift in the reactive hyperemic response to the 5-minute cuff occlusion following detraining (Figure 1) toward that of the control group.

Table 2. Summary of reactive hyperemic forearm blood flow response.

Resting	**Athletes**	**Controls**
FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue)		
before	2.23 ± 0.23 (2.26± 0.22)	2.01 ± 0.20
after	2.00 ± 0.28	
FVR ($mmHg \cdot ml^{-1} \cdot min^{-1} \cdot 100ml^{-1}$)		
before	45.48 ± 5.67 (42.11 ± 4.4)	50.8 ± 5.42
after	51.5 ± 7.2	
Reactive Hyperemia		
Peak FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$)		
before	20.4 ± 2.7 (21.7 ± 3.1)	13.2 ± 1.0[a,b]
after	19.1 ± 1.6	
% Change in FBF		
before	837.6 ± 90.1 (864.0 ± 100.5)	608.9 ± 73.6[b]
after	1003.4 ± 167.6	
Minimum FVR ($mmHg \cdot ml^{-1} \cdot min^{-1} \cdot 100ml^{-1}$)		
before	5.3 ± 0.7 (5.0 ± 0.8)	7.4 ± 0.6[a,b]
after	4.9 ± 0.5	
% Change in FVR		
before	-88.0 ± 1.4 (-88.2 ± 1.5)	-84.4 ± 1.3[b]
after	-89.3 ± 1.2	
Total AUC (ml/100ml)		
before	17.7 ± 2.1 (18.6 ± 2.2)	15.2 ± 1.5
after	16.6 ± 1.9	
AUC_{1min} (ml/100ml)		
before	11.0 ± 1.3 (11.6 ± 1.4)	8.13 ± 0.8
after	10.3 ± 1.0	
Flow debt (ml/100ml)		
before	11.2 ± 1.1 (11.3 ±1.1)	10.1 ± 1.0
after	10.0 ± 1.4	
Excess flow (ml/100ml)		
before	12.5 ± 1.6 (13.1 ± 1.7)	9.7 ± 1.0
after	11.6 ± 1.2	
Repayment (%)		
before	115.8 ± 12.6 (119.6 ± 14.8)	100.3 ± 8.7
after	124.3 ± 9.96	
Duration (seconds)		
before	137.5 ± 9.2 (142.5 ± 10.3)	165.0 ± 8.1[a]
after	150.0 ± 9.5	

Data are means ± SE. Sample sizes for the cross-sectional comparison were athletes (n=12), controls (n=11). Values in parentheses represent mean ± SE for athletes (n=10) who underwent detraining. % change, percent change from resting; FBF, forearm blood flow; FVR, forearm vascular resistance; AUC, area under curve; AUC_{1min}, area under curve for the first minute of reactive hyperemia. a, $p \leq 0.05$, athletes baseline vs. controls; b, $p \leq 0.05$, athletes after detraining vs. controls.

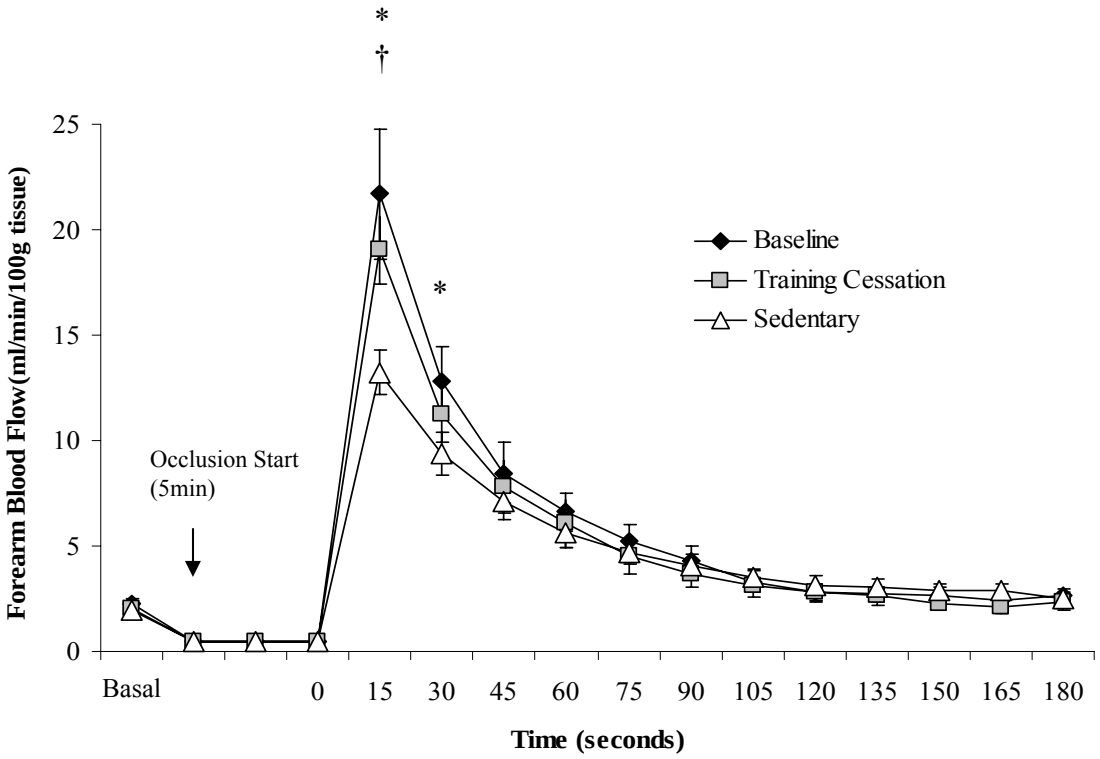

Figure 1. Reactive hyperemic response in athletes before and after training cessation and control subjects. Data are means ± SE. Baseline and Training Cessation represent data for 10 athletes who underwent 10-days of training cessation. Sedentary (n=11). *, $p<0.05$ Baseline vs. Sedentary groups, †, $p<0.05$ Training Cessation vs. Sedentary group.

There was no difference in plasma nitrate/nitrite concentration (NOx) between athlete and control groups (11.88 ± 0.94 vs. 11.99 ± 1.39µmol/L, $p = 0.95$) or in athletes before and after training cessation (11.32 ± 1.0 1 vs. 11.26 ± 0.95µmol/L, $p = 0.96$).

Oxidative Stress

To determine whether oxidative stress status was related to EPC number and colony-forming capacity, plasma oxidized LDL (OxLDL) and total antioxidant capacity (TAC) were measured. There were no differences between athlete and control groups in mean plasma OxLDL or in OxLDL ratio. The control group was found to have greater TAC compared with athletes.

Table 3. Oxidative stress data in athletes and controls.

Variable	Athletes	Controls	p-value
OxLDL (U/L)	61.35 ± 4.66	61.77 ± 3.79	0.94
OxLDL/LDL-C	0.55 ± 0.03	0.53 ± 0.03	0.68
TAC (mmol/L)	2.25 ± 0.05	2.54 ± 0.06	0.002

Values are means ± SE; athletes n=12, controls n=11. OxLDL, plasma oxidized LDL concentration; TAC, plasma total antioxidant capacity.

Plasma OxLDL and TAC did not change with 10 days of training cessation in athletes (baseline vs. detraining; 58.99 ± 4.59 U/L vs. 57.82 ± 3.26 U/L, p=0.63, and 2.25 ± 0.06 vs. 2.21 ± 0.08, p = 0.40, respectively).

Endothelial Progenitor Cells

Data for $CD34^+$ and $CD34^+/KDR^+$ number were not normally distributed; therefore, Mann-Whitney *U*-tests were used to compare differences between athlete and control groups. There were no significant differences between athletes and controls for

either $CD34^+$ HSCs (Figure 2a) or any of the EPC measures including $CD34^+/KDR^+$ cell number (Figure 2b), EPC-CFU count (Figure 2c), and EPC senescence percent (Figure 2d) although means were lower for all of these variables in the control group compared to the athletes (Table 4).

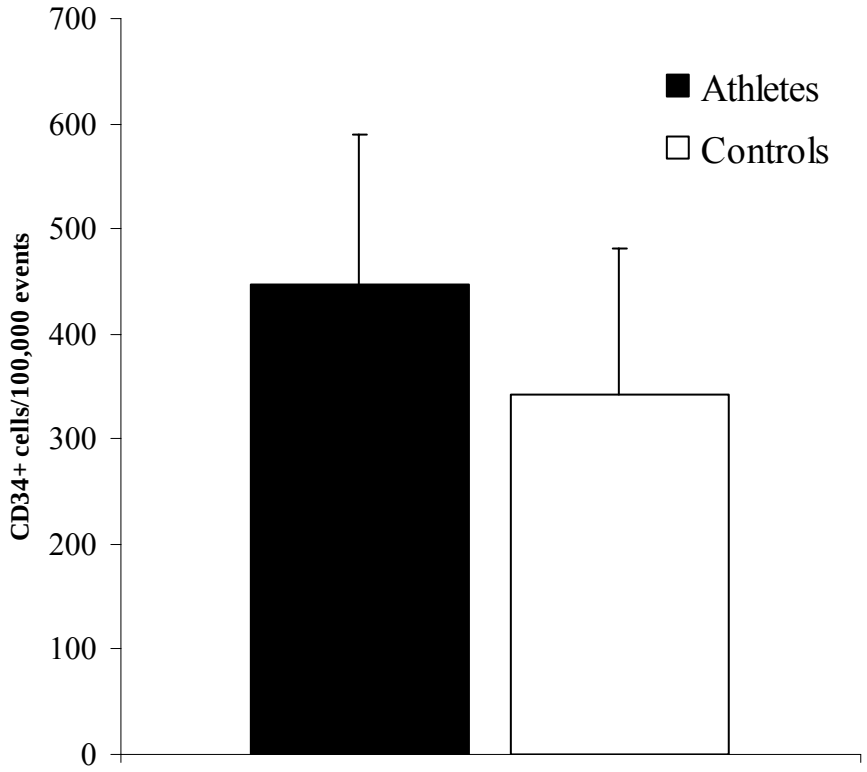

Figure 2a. $CD34^+$ (HPC) cells in athlete and control groups. Values are means ± SE; athletes n=12, controls n=11.

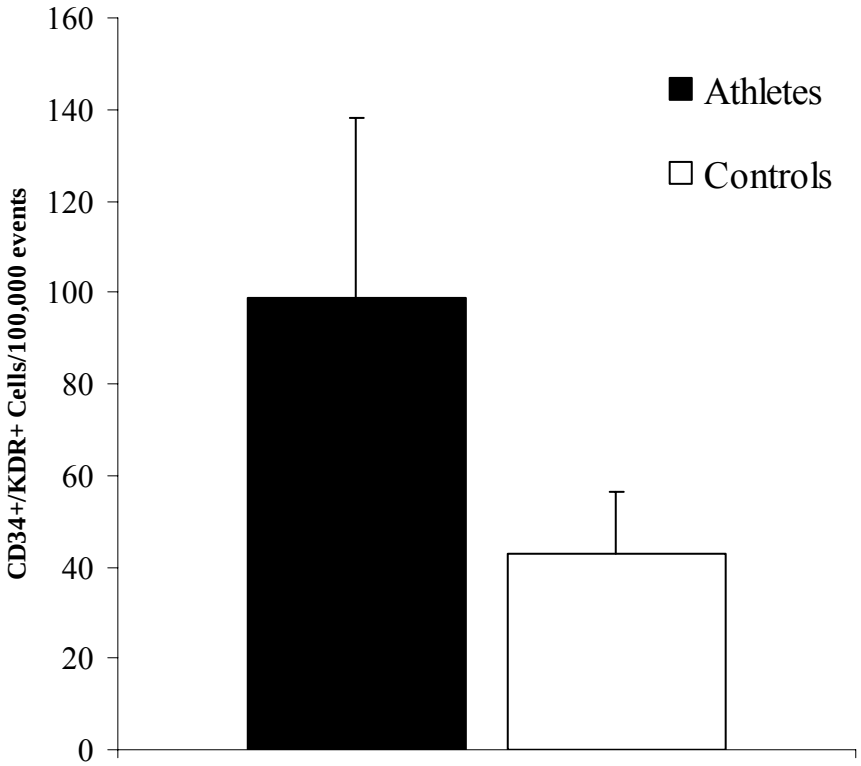

Figure 2b. CD34$^+$/KDR$^+$ cells in athlete and control groups. Values are means ± SE; athletes n=12, controls n=11.

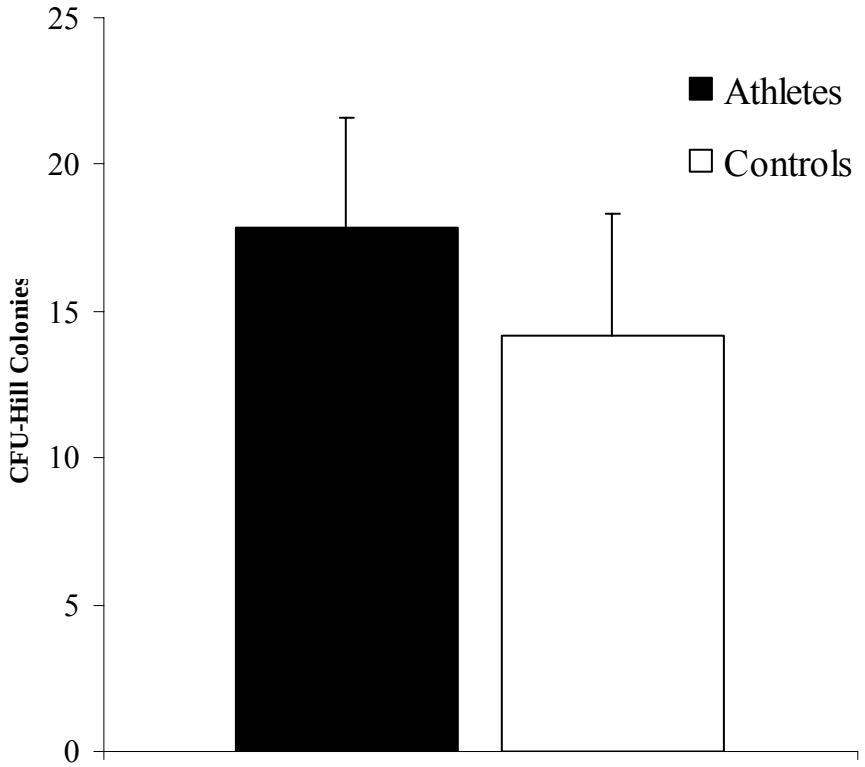

Figure 2c. Number of CFU-Hill colonies in athlete and control groups. Values are means ± SE; athletes n=12, controls n=11.

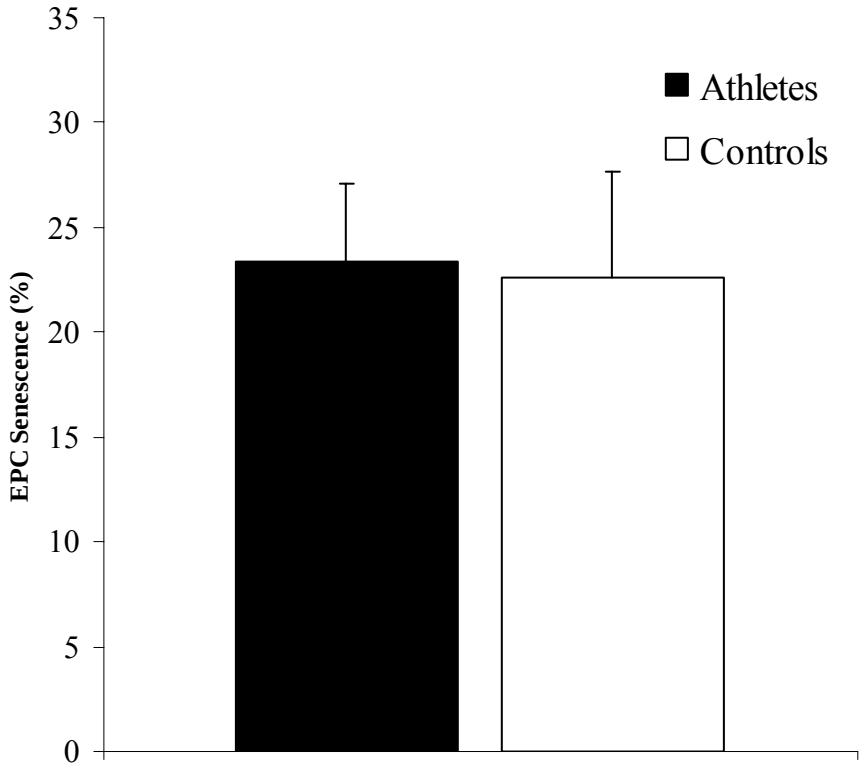

Figure 2d. Percentage of EPC senescence in athlete and control groups. Values are means ± SE; athletes n=12, controls n=11.

Table 4. Circulating progenitor cell data

Variable	Athletes (n=12)	Control (n=11)	*p*-value
$CD34^+$ cells/100,000 events	447 ± 143	342 ± 140	0.22
$CD34^+/KDR^+$ cells/100,000 events	99 ± 39	43 ± 14	0.23
CFU-Hill (count)	18 ± 4	14 ± 4	0.26
EPC Senescence (%)	23.4 ± 3.7	22.6 ± 5.1	0.45

Values are means ± SE; n, number of subjects; CFU, colony-forming unit; EPC, Endothelial Progenitor Cell. Because data for CD34+ number, $CD34^+/KDR^+$ cell number was not normally distributed, a Mann-Whitney *U*-test was used to assess differences between groups and exact test results presented. 1-sided *p*-values are displayed.

There was considerable individual variability in the response to training cessation (Figures 3a-d). Following 10 days of training cessation, athletes had a significant decrease in the number of $CD34^+$ cells (Table 5, $p = 0.02$). Although the other EPC-related variables did not approach statistical significance, the number of $CD34^+/KDR^+$ cells/100,000 events ($p = 0.11$), CFU-Hill count ($p = 0.17$), and EPC-senescence ($p = 0.06$) tended to decrease with training cessation. Following detraining, the athletes had an average of 16 ± 7 $CD34^+/KDR^+$ cells, which was lower compared to the sedentary group 43 ± 14 $CD34^+/KDR^+$ cells but not significantly different ($p = 0.14$).

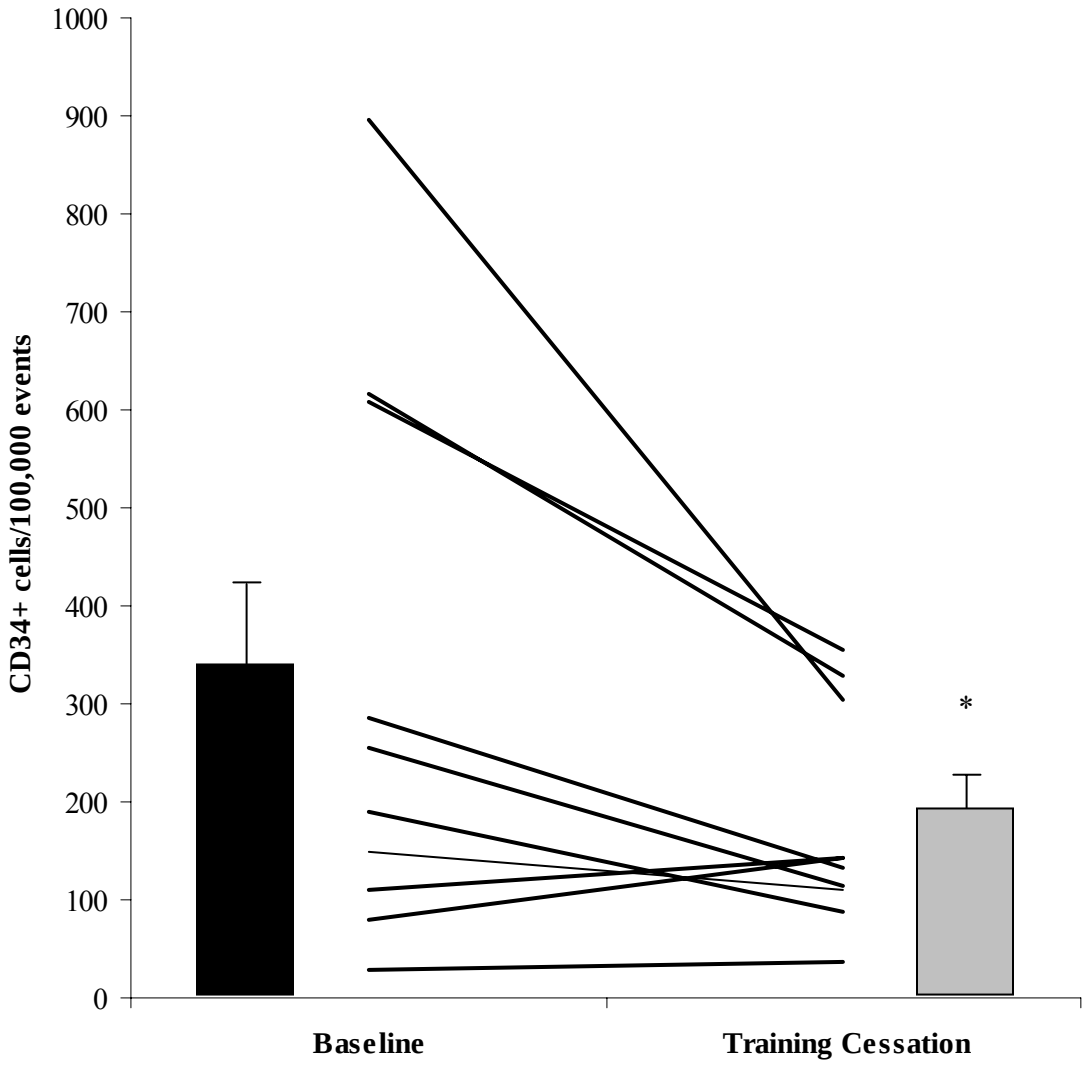

Figure 3a. CD34$^+$ cells before and after 10-days of training cessation. Data represent individual responses to training cessation and mean ± SE. n=10; *, $p<0.05$.

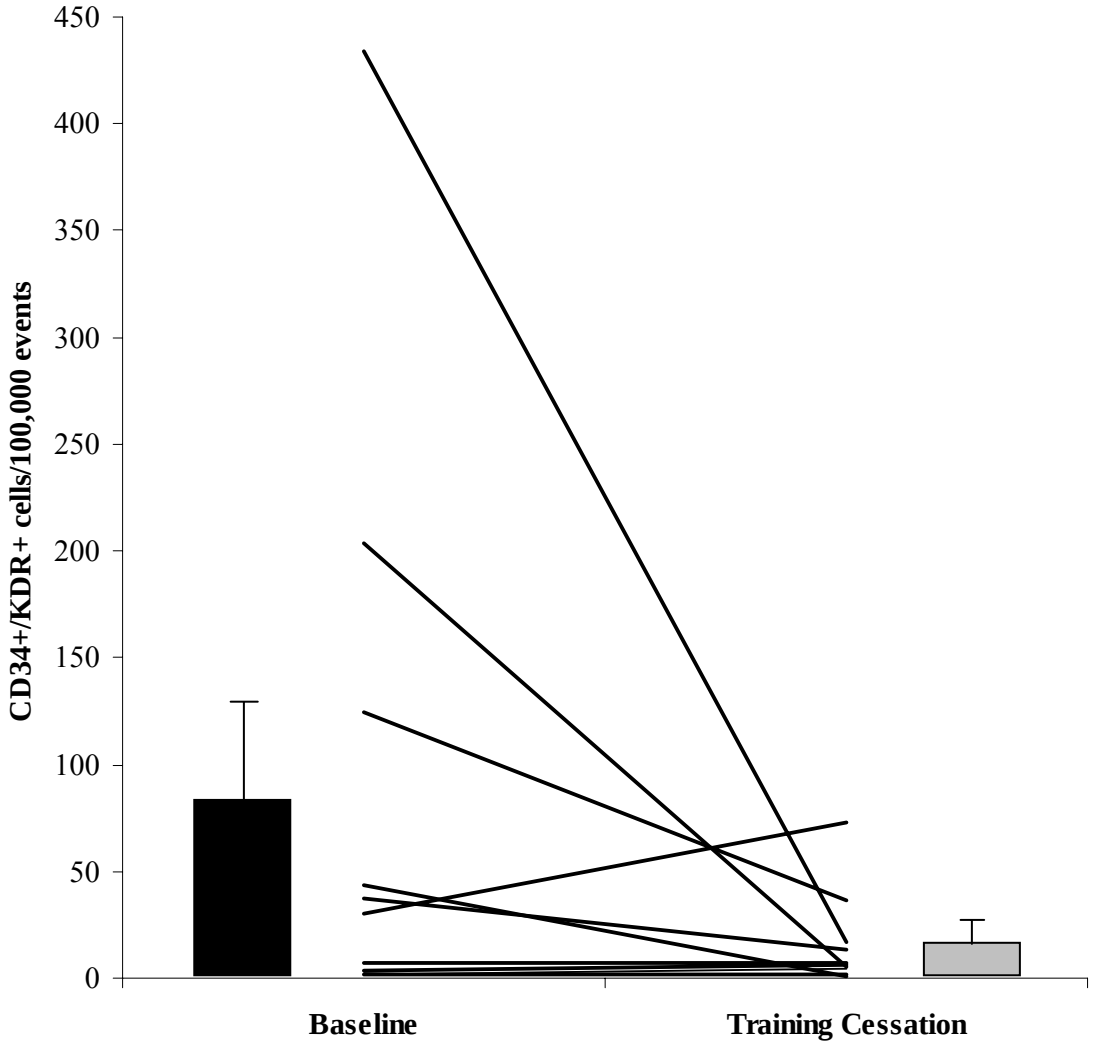

Figure 3b. $CD34^+/KDR^+$ cells before and after 10-days of training cessation. Because data for $CD34^+/KDR^+$ cell number was not normally distributed, data was analyzed via the nonparametric Wilcoxon signed rank test. Data represent individual responses to training cessation and mean ± SE. n=10; *, $p<0.05$.

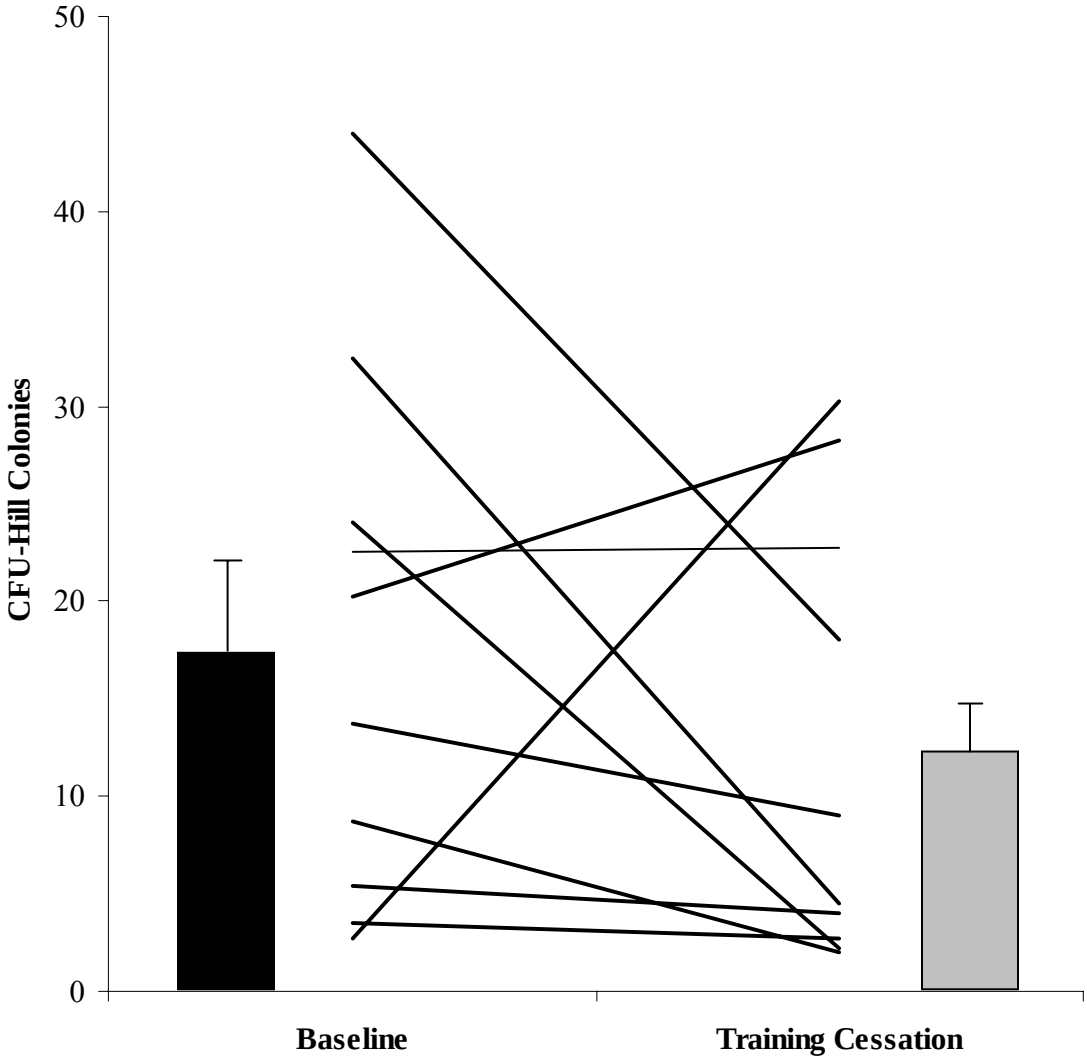

Figure 3c. Number of CFU-Hill colonies before and after 10-days of training cessation. Data represent individual responses to training cessation and mean ± SE. n=10; *, $p<0.05$.

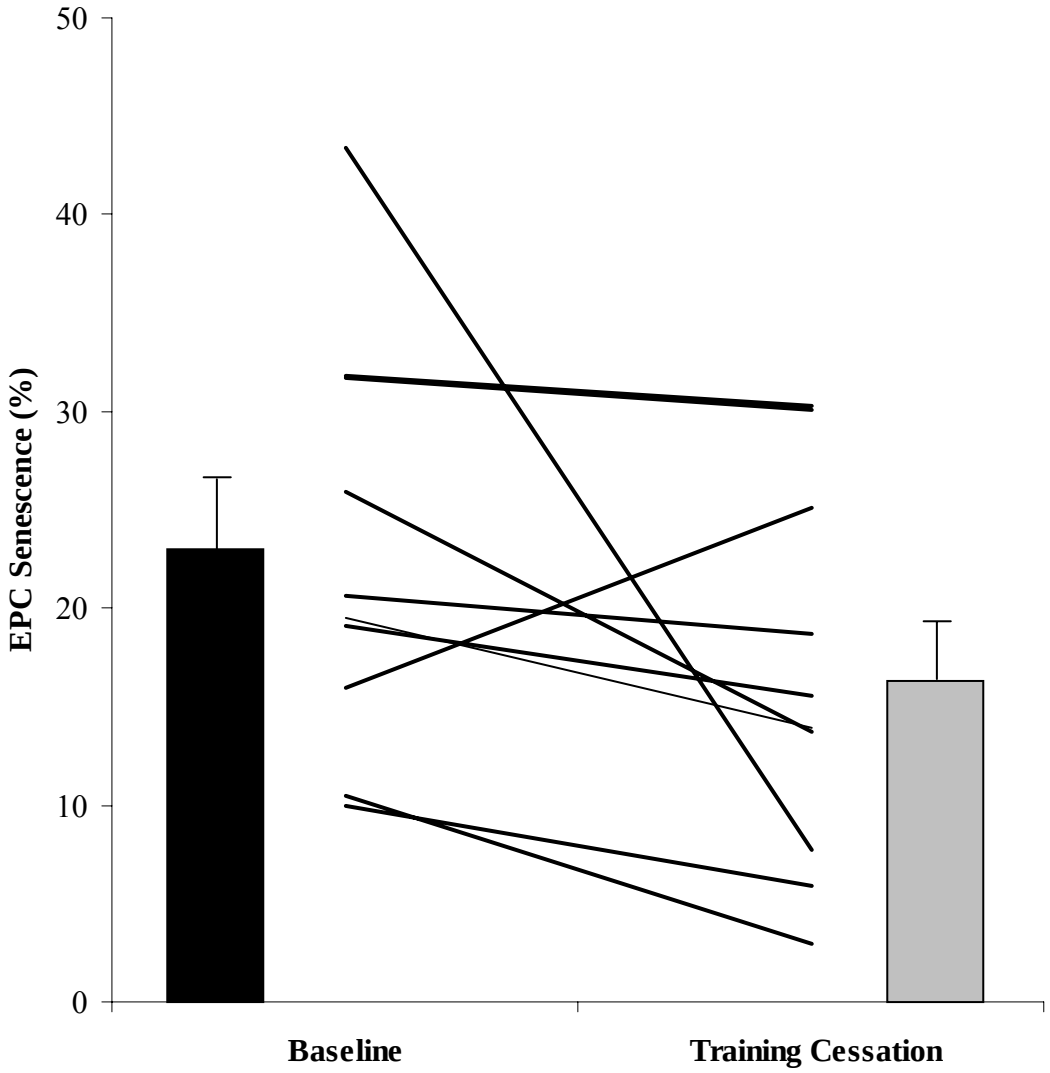

Figure 3d. EPC senescence before and after 10-days of training cessation. Data represent individual responses to training cessation and mean ± SE. n=10; *, $p<0.05$.

Table 5. Training cessation and progenitor cell data

Variable	Baseline	Training Cessation	p-value
$CD34^+$ cells/100,000 events	322 ± 91	179 ± 37	0.02
$CD34^+/KDR^+$ cells/100,000 events	89 ± 44	16 ± 7	0.11
CFU-Hill (count)	18 ± 4	12 ± 4	0.17
EPC Senescence (%)	22.8 ± 3.3	16.4 ± 3.1	0.06

Values are means ± SE; CFU, colony-forming unit; EPC, Endothelial Progenitor Cell. Because data for $CD34^+/KDR^+$ cell number was not normally distributed, data was analyzed via the nonparametric Wilcoxon signed rank test and exact test results are presented. 1-sided *p*-values are displayed.

Response to Cessation of Training

Examination of individual results from 10-days of physical inactivity revealed five athletes who had decreased $CD34^+/KDR^+$ cell number ("responders"), and five athletes who showed no change or slightly increased $CD34^+/KDR^+$ cell number ("non-responders"). Overall, percent change in $CD34^+/KDR^+$ cell number correlated significantly with baseline $CD34^+/KDR^+$ cell number ($r = -0.75915$, $p = 0.01$) in the 10 athletes who detrained. Compared with non-responders, the responders had greater $CD34^+/KDR^+$ cell number at baseline (169 ± 3 vs. 9 ± 5 cells/100,000 events, $p = 0.008$) and a greater percentage change in $CD34^+/KDR^+$ cell number with detraining (-86 ± 7 vs. 70 ± 30%, p=0.006) . There were no differences between these groups in baseline or percent change with detraining in CFU-Hill colonies or EPC senescence.

The baseline forearm blood flow and response to reactive hyperemia parameters were analyzed in the two groups (Figures 4 and 5). Baseline FBF and peak FBF prior to detraining were significantly different between the responders and non-responders (p =

0.02 and p = 0.02, respectively), but not after detraining (Table 6). Of note, peak flow for both responders and non-responders was still significantly greater than peak flow for the control group after detraining (p=0.02 for both comparisons), however, AUC_{1min} was not. There were no differences between responders and non-responders to detraining in the percent change in NOx (15.03 ± 7.42% vs. -4.05 ± 25.21%, p=0.50), OxLDL (3.18 ± 8.71% vs. -3.575 ± 0.69 %, p = 0.48), or TAC (-4.38 ± 2.93% vs. 0.4784 ± 3.37%, p=0.31). The two groups were analyzed for other cardiovascular factors that may have influenced differences in vascular reactivity and $CD34^{+}/KDR^{+}$ cell number and there were no differences between responders and non-responders to detraining in age, blood chemistry profiles, blood pressure, or Framingham risk score.

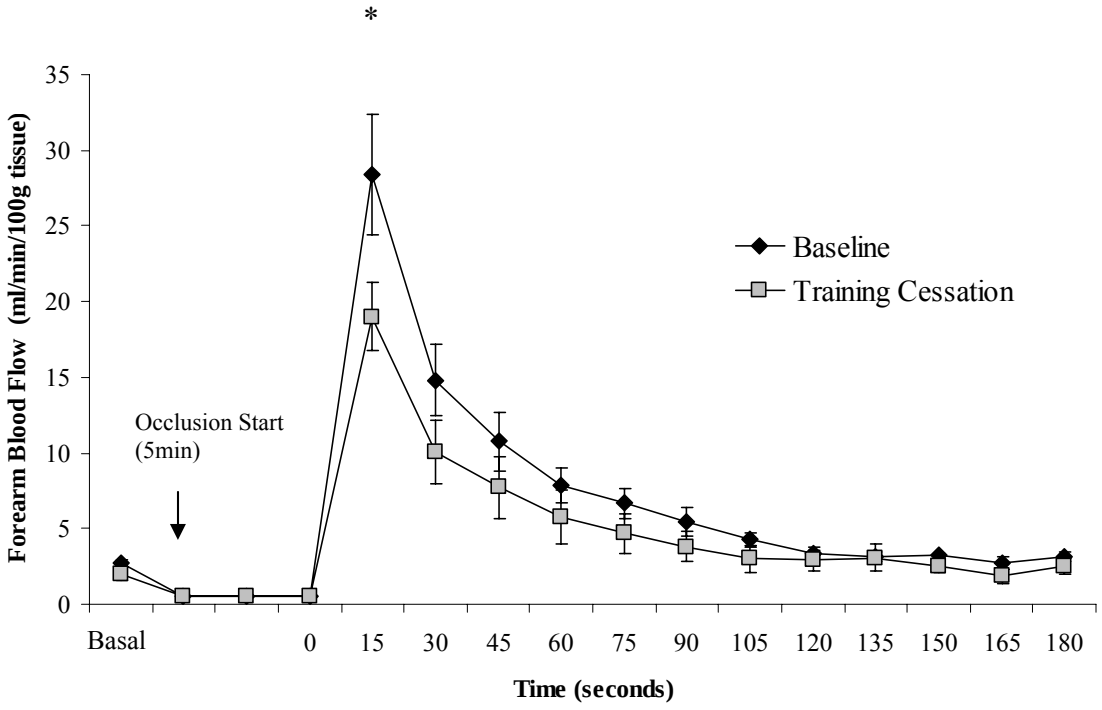

Figure 4. Reactive hyperemic blood flow in responders to training cessation. Data are means ± SE. Baseline and Training Cessation represent data for 5 "responder" athletes who underwent 10-days of training cessation. *, p<0.05 Baseline vs. Training cessation.

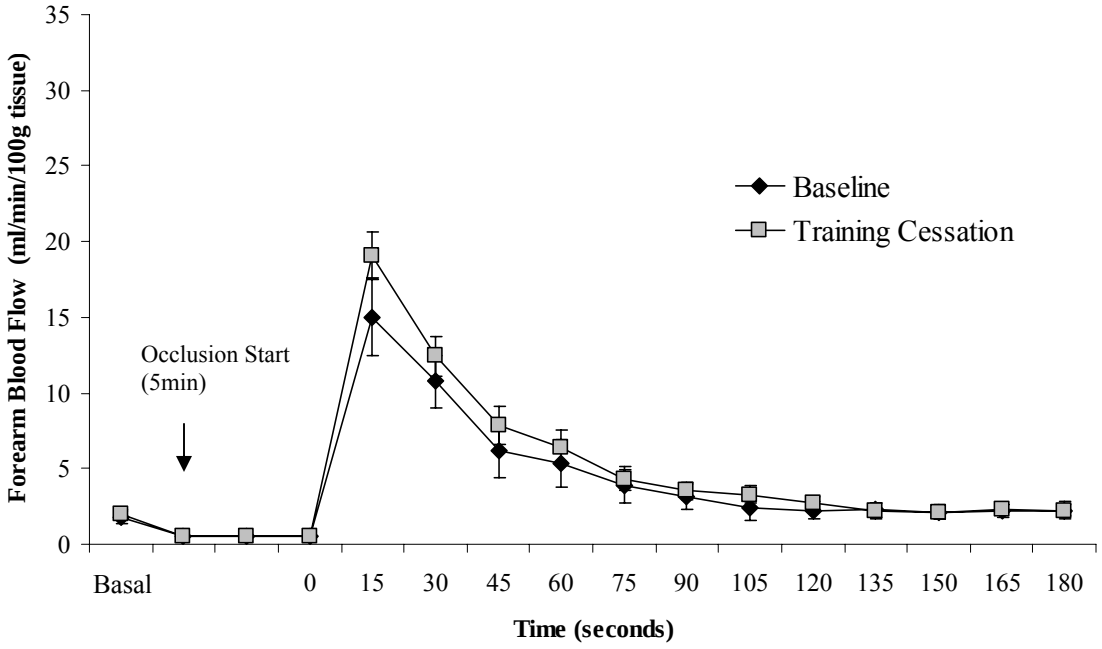

Figure 5. Reactive hyperemic blood flow in non-responders to training cessation. Data are means ± SE. Baseline and Training Cessation represent data for 5 "non-responder" athletes who underwent 10-days of training cessation.

Table 6. Forearm blood flow in responders and non-responders with training cessation.

Variable	Responders	Non-Responders	p-value
Baseline			
Resting FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue)	2.7 ± 0.2	1.8 ± 0.3	0.02
Peak FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue)	28.4 ± 4.0	15.0 ± 2.2	0.02
Training Cessation			
Resting FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue)	2.0 ± 0.4	2.0 ± 0.4	0.96
Peak FBF ($ml \cdot min^{-1} \cdot 100ml^{-1}$ tissue)	19.0 ± 2.2	19.1 ± 2.6	0.99
Change in peak FBF (%)	-25.7 ± 15.7	32.1 ± 13.3	0.02
Change in AUC (%)	-24.8 ± 16.4	24.1 ± 16.3	0.07
Change in AUC_{1min} (%)	-25.6 ± 15.9	32.0 ± 17.2	0.04

Data are means ± SE. Sample sizes for the group comparison were "responders" (n=5), "non-responders" (n=5). Change (%), percent change from baseline to 10-days of training cessation; FBF, forearm blood flow; FVR, forearm vascular resistance; AUC, area under curve; AUC_{1min}, area under curve for the first minute of reactive hyperemia.

Endothelial Progenitor Cells and Endothelial Function

Correlation analysis was performed on the entire group (athletes and controls, n=23) to analyze the relationship between EPC variables (CFU-Hill colonies, $CD34^+/KDR^+$ cells, and EPC senescence) and forearm blood flow parameters. There were no significant correlations found for number of CFU-Hill colonies, number of $CD34^+/KDR^+$ cells or EPC senescence variables with endothelial function variables in the combined groups. Likewise, there were no significant relationships between EPC variables and resting NOx.

The athlete and control groups were also analyzed separately for relationships between EPCs and forearm blood flow variables. The athlete group demonstrated a significant relationship between the number of $CD34^+/KDR^+$ cells and forearm blood flow at rest (Figure 6, $r = 0.70$, $p = 0.01$). There were no other significant relationships between $CD34^+/KDR^+$ number, CFU-Hill colonies, EPC-senescence and forearm blood flow variables or NOx in the athlete group.

There were several relationships between EPC variables and forearm blood flow parameters in the control group. $CD34^+/KDR^+$ number correlated with AUC_{1min} (Figure 7, $r = -0.78$, $p = 0.005$) and excess flow ($r = -0.70$, $p = 0.02$). CFU-Hill colonies correlated with the percentage change in FBF (Figure 8, $r = 0.84$, $p = 0.001$) and percentage change in FVR ($r = -0.73$, $p=0.002$) with reactive hyperemia.

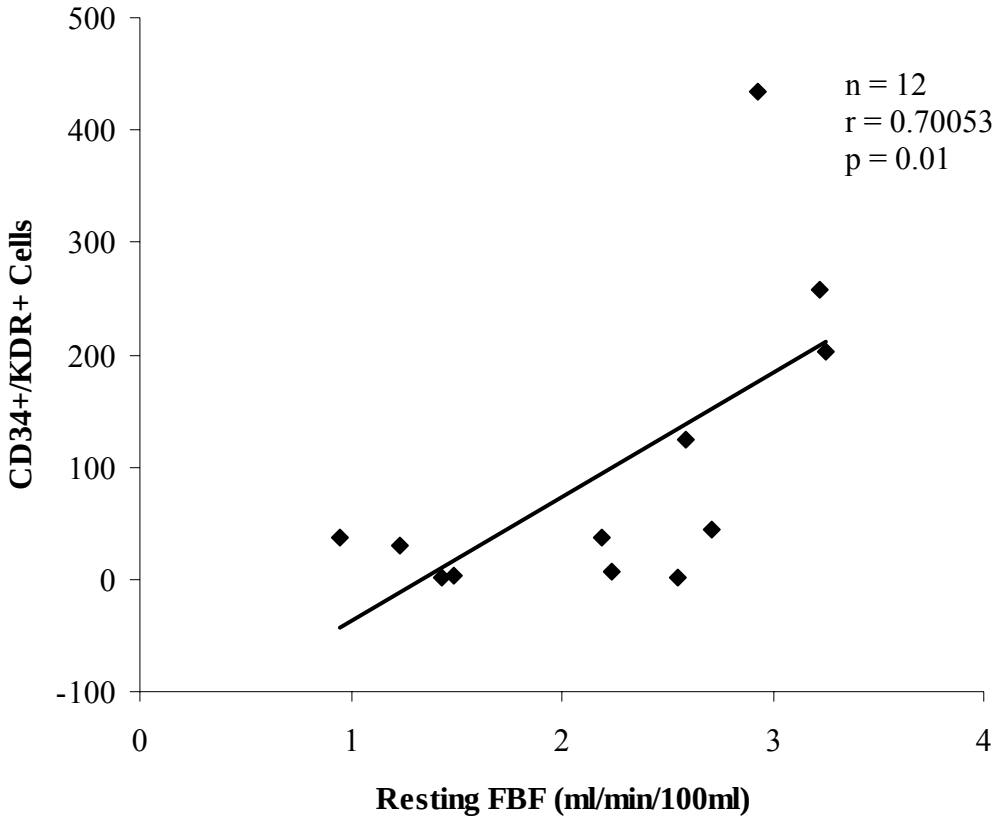

Figure 6. Correlation between $CD34^{+}/KDR^{+}$ cells and resting forearm blood flow (FBF) in athletes.

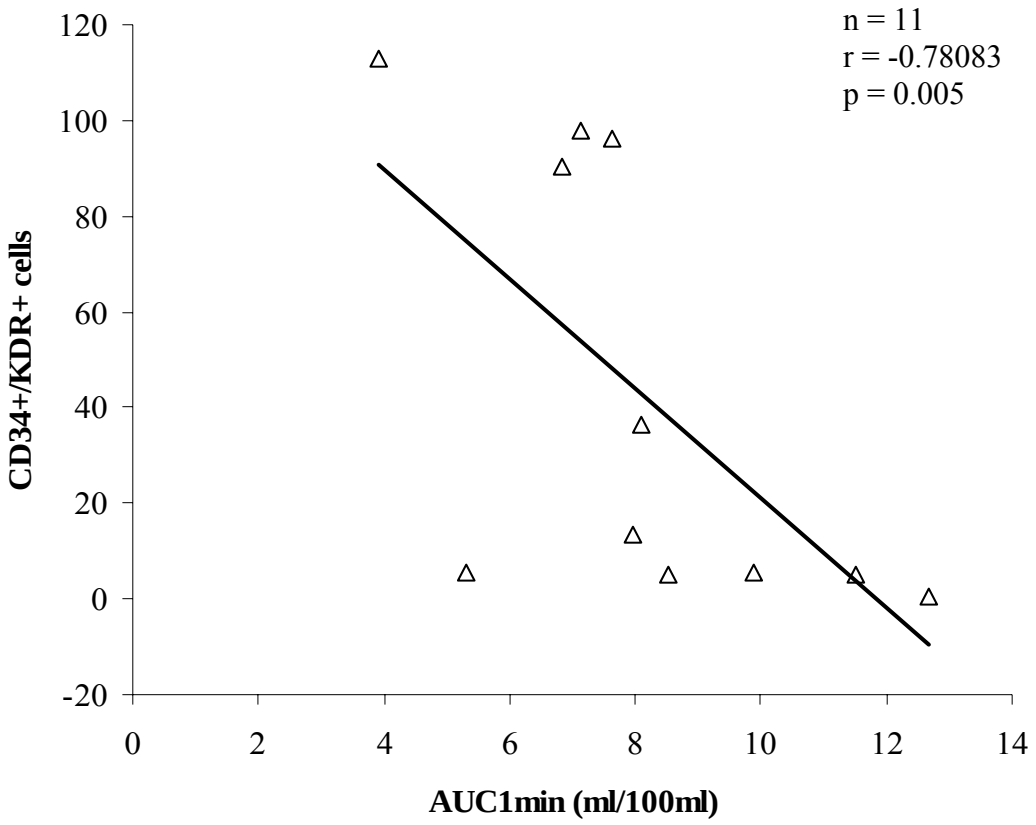

Figure 7. Correlation between $CD34^{+}/KDR^{+}$ cells and AUC_{1min} in low-active control participants.

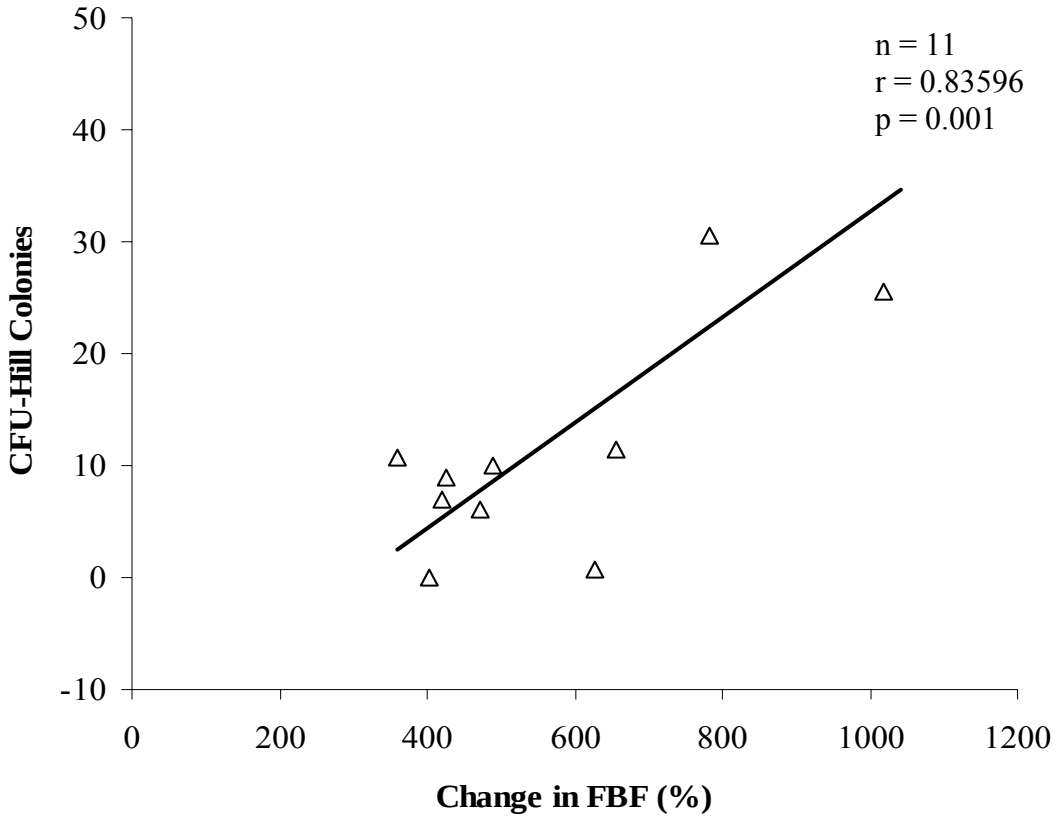

Figure 8. Correlation between CFU-Hill colonies and the change in forearm blood flow in low-active control participants.

Changes in EPC variables were analyzed for relationships with changes in forearm blood flow parameters with 10-days of detraining in athletes. The percent change in $CD34^+/KDR^+$ cells from before to after detraining was significantly correlated with the percentage change in area under the forearm blood flow curve (minute 1) in response to reactive hyperemia (Figure 9, r = 0.70, p = 0.02). The change in $CD34^+/KDR^+$ cells with detraining also displayed a trend for a relationship with the percentage change in resting forearm blood flow (r = 0.62, p = 0.06). The percent change in $CD34^+$ cells was related to the percent change in hyperemic duration with detraining (r = 0.64, p = 0.05).

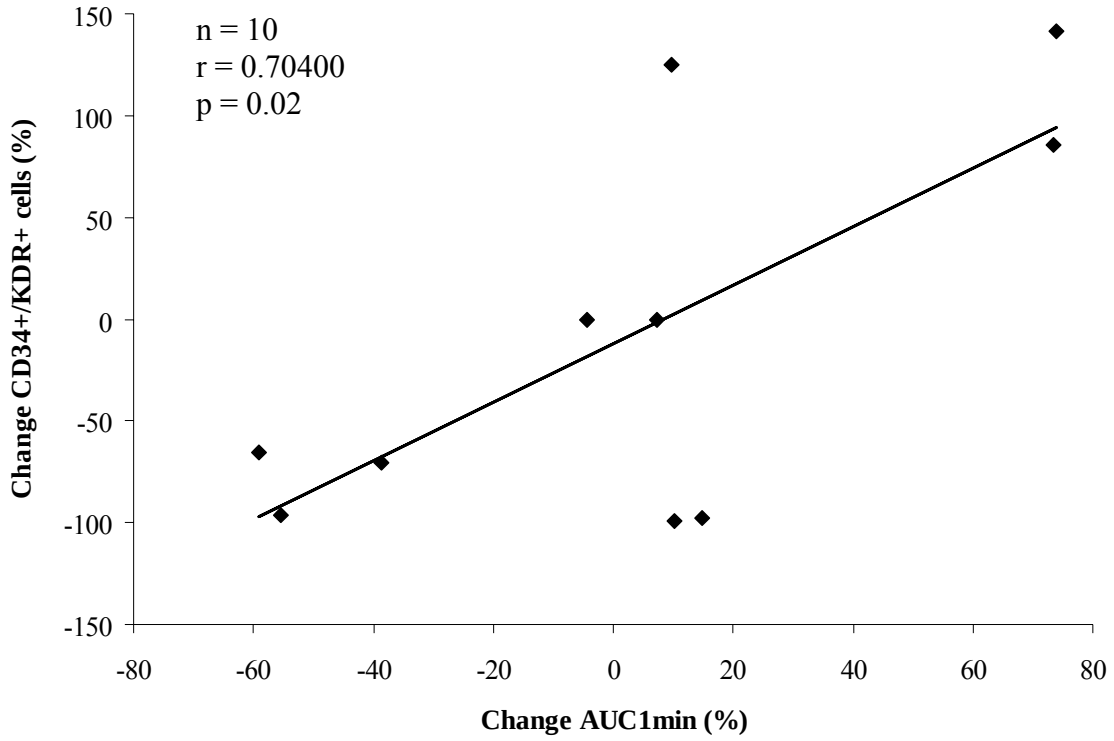

Figure 9. Correlation between the percentage change in $CD34^+/KDR^+$ cells and the percentage change in AUC_{1min} in athletes with 10-days of training cessation.

Endothelial Progenitor Cells and Oxidative Stress

Relationships between EPC and oxidative stress variables (OxLDL and TAC) were examined for athlete and control groups separately and combined via correlation analysis. Neither oxidized LDL nor TAC was significantly related to $CD34^+/KDR^+$ cells, CFU-Hill colonies, or EPC senescence in the entire group. However, CFU-Hill colony number showed a tendency to be correlated with OxLDL (Figure 10, $r = -.33$, $p = 0.12$). This relationship was not evident when groups were analyzed separately. None of the EPC variables were related to TAC in either athlete or control groups.

In athletes who underwent 10-days of exercise cessation, the percentage change in EPC senescence was significantly related to the percentage change in TAC from before to after detraining (Figure 11, $r = 0.66$, $p = 0.04$). The percent change in $CD34^+/KDR^+$ cells tended to be related to the percentage change in TAC from before to after detraining ($r = 0.62$, $p = 0.06$). There were no relationships between changes in EPC variables with detraining and oxidized LDL.

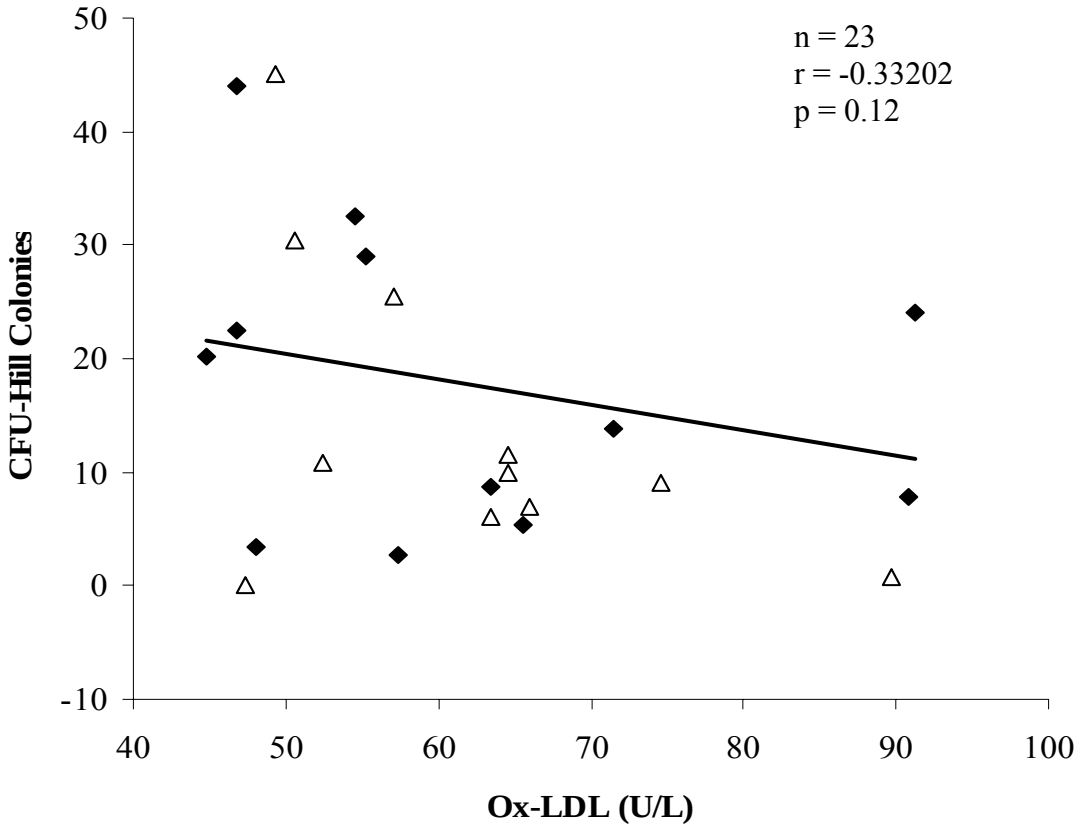

Figure 10. Correlation between CFU-Hill colony number and Ox-LDL in athlete (diamonds) and control (triangles) participants.

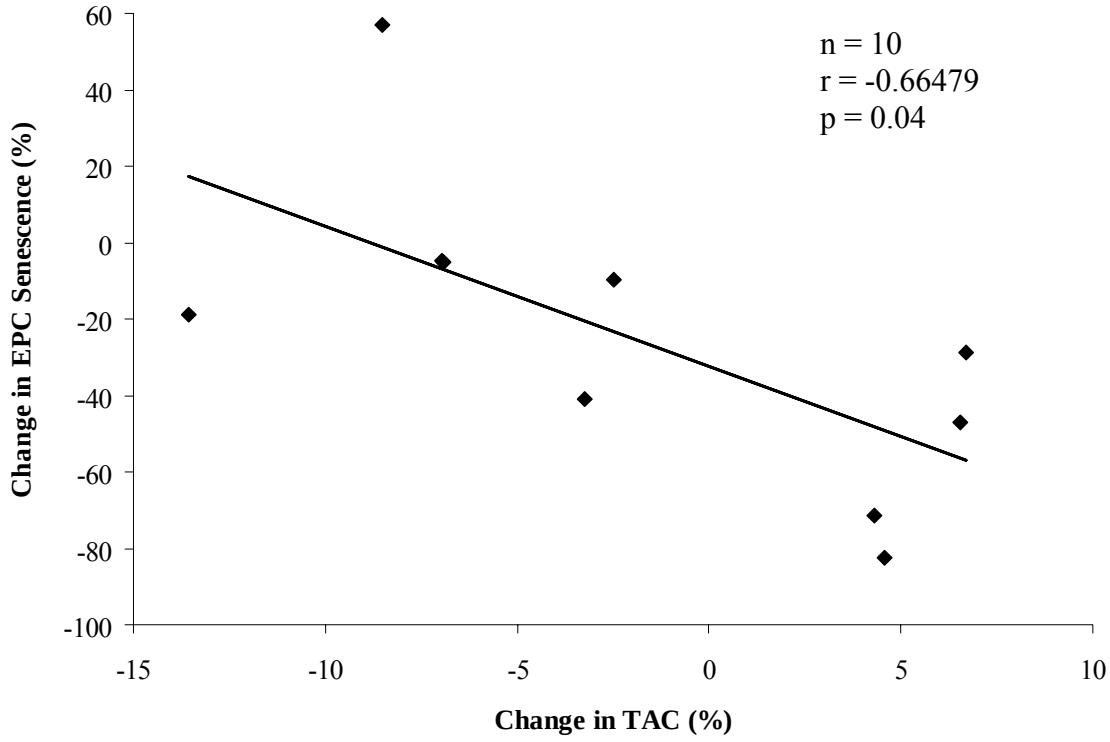

Figure 11. Correlation between the percentage change in EPC senescence and the percentage change in total antioxidant capacity in athletes with 10-days of training cessation.

Predictors of Endothelial Progenitor Cell Variables

Multiple regression analysis was used to determine the best predictors of $CD34^+/KDR^+$ number, number of CFU-Hill colonies, and EPC senescence in the entire cohort, separately for the athlete and control groups, and for the change with detraining in athletes. Variables were transformed to meet the testing assumptions when appropriate and noted when used.

For the combined groups, as Hill, et al (51) reported, greater number of CFU-Hill colonies was related to lower Framingham 10-year CHD risk score ($p = 0.02$) and risk percentage (Figure 12, $r = -0.49$, $p = 0.02$). Regression analysis also revealed a

relationship between $\log_{10}$CFU-Hill colonies and $\log_{10}$OxLDL ($p = 0.03$) in both groups combined.

When the control group was analyzed alone, multiple regression analysis revealed significant relationships between $\log_{10}CD34^+/KDR^+$ number and plasma cholesterol ($p = 0.04$) and excess forearm blood flow ($p = 0.01$). A strong relationship between OxLDL and $\log_{10}$CFU-Hill colonies ($p < 0.001$) was also found. There were no significant predictors of EPC senescence in the control group.

For the athlete group, regression analysis revealed only a relationship between $\log_{10}CD34^+/KDR^+$ cells and resting FBF ($p = 0.05$).

In the multivariate analysis to determine the best model to predict the change in EPC parameters with detraining, $CD34^+/KDR^+$ number had two independent variables in the model; percent change in AUC ($p = 0.02$) and percentage change in TAC ($p = 0.10$). Additionally, EPC senescence was significantly related to the percentage change in TAC from before to after detraining ($p = 0.04$).

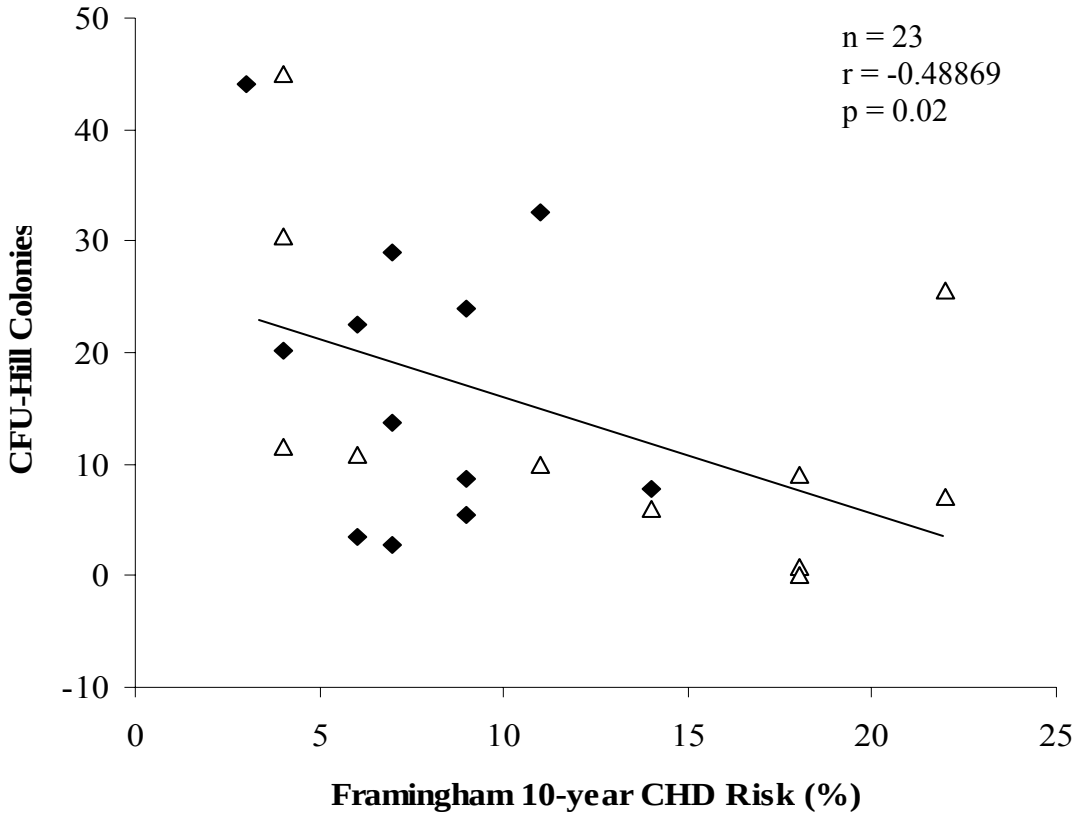

Figure 12. Correlation between CFU-Hill colonies and CHD risk. Spearman's correlation coefficient revealed that number of CFU-Hill colonies were strongly correlated with Framingham risk score (r= -0.47, p=0.02) and coronary heart disease risk percent.

DISCUSSION

Regular endurance physical activity is associated with enhanced endothelial function which has been related to lower incidence of cardiovascular disease (22; 23; 45; 46). Evidence indicates that bone-derived progenitor cells targeted toward the vascular endothelium (EPCs) may aid in the maintenance of endothelial integrity via endothelial repair. However oxidative stress, which has been implicated in the pathogenesis of CVD, has been shown *in vitro* to impair EPCs (25; 62; 63; 66; 82; 132). Chronic exercise training may improve anti-oxidant capacity and may be one mechanism by which exercise improves EPC function. In the present study, we hypothesized that individuals who participated in long-term endurance exercise training would have greater hyperemic forearm blood flow response, which would be related to greater number of $CD34^+/KDR^+$ EPCs and better EPC function (measured by CFU-Hill colony forming capacity and EPC senescence) compared to low-active control participants. Additionally, we proposed that repetitive bouts of acute exercise were important to protect athletes from deterioration of EPC number and function therefore, 10-days of training cessation would lead to decreased $CD34^+/KDR^+$ cell number and EPC function and that these changes would be related to diminished forearm blood flow response to reactive hyperemia and increased oxidative stress. The principal findings of this study were: 1) $CD34^+/KDR^+$ cell number, CFU-Hill colonies, and EPC senescence were not significantly different between athlete and control groups, 2) $CD34^+/KDR^+$ cell number was closely related to endothelial function in low-active controls subjects and athletes who participated in 10-day cessation of training, 3) CFU-Hill colony-forming capacity was related to plasma oxidized LDL levels independent of physical activity status, 4) athletes who participated in 10-days of

exercise cessation demonstrated a decrease in EPC senescence, which was related to improved antioxidant capacity.

EPCs and Endothelial Function

Endothelial dysfunction has emerged as a hallmark of early cardiovascular disease (96) and predicts cardiovascular disease progression and future cardiovascular events (102). Improvement in endothelial function with exercise training has been demonstrated in the coronary (38; 43) and peripheral vasculature (79) and is associated with an increase in eNOS expression, increased phosphorylation of eNOS by Akt (44), and decreased NO inactivation by reactive oxygen species (2). Our data revealed that better endothelial function, assessed by the forearm blood flow response to reactive hyperemia, was related to increased CFU-Hill colonies and decreased $CD34^{+}/KDR^{+}$ number in low-active controls. Therefore, healthy endothelial function may occur due to improved EPC function despite low EPC number. This effect could be indicative of a shift toward the regenerative capacity of EPCs at rest from the liberation of EPCs from the bone marrow that has been shown to occur with acute exercise (1; 77). Our results are similar to those reported by Hill, et al (51) who found a relationship between greater EPC function (CFU-Hill colonies) and better brachial artery reactivity in individuals who had various degrees of CHD risk. Interestingly, with our groups combined, we found an equivalent relationship between CFU-Hill colonies and Framingham risk as Hill, et al reported. The lack of an association between CFU-Hill colonies and endothelial function in athletes may indicate that endothelial function is influenced by factors other than resting EPC function.

The long-term exercisers in our study showed superior endothelial function compared with low-active controls as expected. However, athletes who had the highest reactive hyperemic FBF response had the greatest number of $CD34^+/KDR^+$ cells. Furthermore, when these athletes stopped exercising, they had a dramatic decrease in reactive hyperemic FBF response. Therefore, in this group of athletes, the repeated bouts of acute exercise appears to be essential to maintain high levels of circulating $CD34^+/KDR^+$ EPCs and endothelial function. For the other half of the athletes, both $CD34^+/KDR^+$ cells and endothelial function were not affected by 10 days of exercise cessation, indicating that for this group, the chronic nature of their exercise training led to an adaptation of enhanced endothelial function that persisted despite the cessation of exercise and may be less dependent upon the presence of circulating $CD34^+/KDR^+$ cells. Interestingly, Werner, et al (135) recently reported increased coronary endothelial function in patients with CAD who had greater $CD34^+/KDR^+$. Through the use of training cessation, we were able to show a similar relationship in the peripheral vasculature in healthy individuals. It is important to note that the relationship between endothelial function and $CD34^+/KDR^+$ cells reported herein was seen without differences in plasma NOx, which may indicate that there may be other factors involved. The molecular mechanisms involved in EPC regulation and their relationship with endothelial function have yet to be elucidated.

EPCs and Exercise Training

Previous studies have shown that an acute bout of exercise increases the number of bone marrow-derived endothelial-targeted cells in the blood (77; 95; 98; 105; 125).

However, results from studies evaluating resting EPC number following exercise training in humans are not consistent; specifically, groups have reported that exercise training improves resting $CD34^+/KDR^+$ cells in patients with CAD (78) and chronic heart failure (99) and $CD34^+/KDR^+/CD133^+$ cells in patients with CAD and CAD risk factors (111) but not in healthy young and older men (118) trained for 8 weeks. It appears that exercise training, either short term (8 weeks) or long term (>30 years in our study), does not affect resting $CD34^+/KDR^+$ cells in healthy individuals, whereas exercise training may increase resting circulating EPCs in individuals with cardiovascular disease and therefore, greater endothelial damage. We also found no difference between our groups in resting plasma NOx. Nitric oxide has been implicated as an important factor for the release of EPCs from the bone marrow (3; 4; 78). Steiner, et al (111) demonstrated a strong relationship between plasma NOx and change in EPC number with exercise training in their patients with CAD and CAD risk factors, however this group did not control for nitrate intake prior to testing. Given that we did not see a difference in plasma NOx, our lack of a difference in $CD34^+/KDR^+$ cells is not surprising. Therefore, as previous data have shown, EPCs may be increased with acute exercise, but in healthy individuals, resting EPC number is independent of training status.

The number of circulating EPCs likely represents the balance between liberation of EPCs from the bone marrow and incorporation at the level of the vessel or differentiation. Laufs, et al (77) demonstrated that $CD34^+/KDR^+$ cells increase after 30 minutes of high intensity running in healthy participants, but returned to resting levels by 24 hours following exercise. Our results can be explained with this finding as we obtained our samples 24 hours following the athletes' last exercise session and we

defined EPCs ($CD34^+/KDR^+$) equivalently. We can speculate that in healthy regularly-exercising individuals, by 24 hours following exercise, EPCs released from the bone marrow with exercise have either been incorporated for endothelial repair, neovascularization, or have undergone differentiation.

With regard to training cessation, we found that $CD34^+$ cells decreased following the 10 days of no exercise. There is little information regarding resting $CD34^+$ cells and exercise; specifically, these myeloid cells have been found to be both higher at rest in middle-aged runners compared to sedentary controls (13) and no different in older trained men compared to older untrained men (118). To our knowledge, this is the first report of a decrease in these cells in long-term trained runners with cessation of exercise. As bone-marrow derived hematopoietic progenitors, these cells have the capacity to differentiate into endothelial marker-expressing cells that may contribute to angiogenesis (6), have been shown to be mobilized after acute myocardial infarction (109), and are also emerging as a potential therapy for CAD (71). Our data reveal that regular bouts of exercise are necessary to maintain heightened levels of $CD34^+$ progenitors, which have the potential to differentiate toward the vascular endothelium to participate in neovascularization or re-endothelialization (106).

The functional capabilities of EPCs can be assessed with clonogenic assays, such as the CFU-Hill assay, and EPC senescence. To date, there are few reports regarding exercise and EPC function. Our results showed no difference in CFU-Hill colonies or EPC senescence between long-term exercisers and low-active healthy controls. Contrary to our results, EPC-CFUs have been found to increase following 3 months of aerobic exercise training in healthy men (54) and following 8 weeks of supervised training in

patients with heart failure (99). Patients with disease, as those studied by Sarto, et al (99) may show greater benefit in EPC colony-forming capacity from exercise training due to greater disease-related endothelial damage. That our study did not find increased EPC clonogenic capacity in healthy trained men as Hoetzer, et al (54) reported, may be accounted for by differences in the length and intensity of training. Hoetzer, et al reported no increase in VO_{2max} with an increase in treadmill walk time following their home-based exercise program, indicating that it was a lower intensity program. Since our group had been exercising regularly for over 30 years at moderate to high intensity, including regular participation in competitive events, it is possible that any adaptation in clonogenic capacity may have occurred earlier in their training history. These two studies also used different assays to assess clonogenic capacity which may explain the different results between the two studies, although endothelial cell lineage of the cultured cells from both assays have been confirmed (51; 54).

EPCs and Oxidative Stress

Oxidative stress in a system is the balance between the pro-oxidant and anti-oxidant forces in that system. Antioxidants have been shown to be protective to both the vascular endothelium and EPCs by countering insult by ROS. Both EPCs and mature endothelial cells express glutathione peroxidase (GPX-1), manganese superoxide dismutase (mnSOD), and catalase although EPC have higher levels of such enzymes (25; 49); a characteristic of stem and progenitor cells. Despite heightened defenses, EPC function may be influenced by oxidative stress.

Elevated plasma oxidized LDL levels have been shown in patients with CAD and predicts future cardiovascular events in these patients (107; 108). Oxidized LDL is associated with activation of monocytes and increased trans-endothelial migration which promotes atherosclerosis (131). OxLDL decreases EPC clonogenic capacity (66), increases senescence (63; 82), and decreases VEGF-stimulated differentiation (62) of cultured EPCs. We found that oxidized LDL was a significant predictor of EPC function in healthy men such that greater OxLDL was predictive of lower CFU-Hill colonies. To our knowledge, this is the first report of *in vivo* evidence that OxLDL has a significant effect on EPC function in healthy individuals. OxLDL has been shown to decrease Akt phosphorylation, eNOS mRNA and protein expression, and EPC nitrite concentration in a dose-dependent manner (82). Therefore, although we did not observe any differences in plasma nitrite concentration, it is possible we may have seen a relationship with OxLDL and CFU-Hill levels if nitrite was measured directly from EPCs.

Cellular senescence is an indicator of exhaustion of the replicative potential of a cell (30) and higher EPC senescence has been related to increased cardiovascular risk (51). This is the first study to our knowledge to assess EPC senescence, oxidative stress and exercise. We found that plasma TAC was a significant predictor of the change in EPC senescence in athletes who stopped exercising for 10 days, such that decreased EPC senescence with detraining was related to increasing TAC. Intense exercise training has been shown to decrease antioxidants in the blood (12) and 10 days of physical inactivity may have allowed TAC to recover in these athletes. Although these results are intriguing, the measure of TAC only gives an overview of antioxidant status. Future

studies are needed to elucidate the relationship of specific antioxidant enzymes in EPCs such as GPX-1, mnSOD, and catalase with exercise training and inactivity.

Summary

We found evidence that $CD34^+/KDR^+$ EPC number is closely associated with the forearm blood flow response to reactive hyperemia in healthy men. Further, EPC function was influenced by plasma oxidized LDL and the change in EPC senescence was related to plasma TAC with cessation of training. Since EPCs have been shown to be related to endothelial function and cardiovascular disease, these results are important to the understanding of factors that influence their number and function in healthy men. The results of this study highlight the individual variability in factors that may influence cardiovascular disease and the need to understand mechanisms that may be responsible for these individual differences in disease risk. Such understanding will enhance personalized prevention and therapy.

CONCLUSIONS

Hypothesis #1 : Long-term physical activity is associated with improved endothelial function which is related to greater EPC number, EPC-Colony Forming Units (CFU-Hill), and lower EPC senescence compared to age and gender-matched low-active healthy individuals.

Contrary to our hypothesis, there was no difference between long-term exercisers and age- and BMI-matched low-active control participants for EPC number, CFU-Hill colonies, or EPC senescence. However, EPC number was related to resting FBF in athletes and AUC_{1min}, and excess flow in control participants and CFU-Hill colony number was correlated with the % change in FBF and % change in FVR with reactive hyperemia.

Hypothesis #2 : Oxidative stress is lower in long-term exercisers compared to low-active controls and is associated with greater EPC number and CFU-Hill count and lower EPC senescence.

Contrary to our hypothesis, low-active control participants exhibited higher total antioxidant capacity compared with long-term exercisers. Regression analysis revealed that CFU-Hill count was related to OxLDL in control subjects and entire group combined.

Hypothesis #3: EPC number, CFU-Hill count and EPC senescence will deteriorate following 10-days of training cessation and these changes will be associated with deterioration of endothelial function and oxidative stress.

$CD34^+$ (hematopoietic progenitor cells) decreased and EPC senescence showed a tendency to improve with 10 days of training cessation. $CD34^+/KDR^+$ cells decreased in 5 of the athletes. This response was related to decreasing FBF response to reactive hyperemia. Five athletes did not have a change in $CD34^+/KDR^+$ cells with cessation of training which corresponded with no change in FBF response to reactive hyperemia.

REVIEW OF LITERATURE

Endothelial Progenitor Cells: Definition and Function

The development of new blood vessels, or angiogenesis, and re-endothelialization was long believed to occur via existing vascular and endothelial cells. On the other hand vasculogenesis is the formation of new vessels from stem cells or progenitors. In 1997, Asahara, et al (6) was the first to identify a population of endothelial progenitors by cell surface antigens that differentiated into endothelial cells and incorporated into sites of angiogenesis. These cells, endothelial progenitor cells (EPCs), were later found to also participate in endothelial regeneration and repair. In 1998, Shi, et al (106) discovered that these cells rapidly colonized a graft placed in the canine aorta and Walter, et al (130) found that EPCs home or migrate to balloon-injured arterial segments and re-endothelialize the denuded areas. In addition to neovascularization and re-endothelialization, these bone marrow-derived cells have been implicated in tumor vascularization, wound repair, and recovery from cardiac and limb ischemia.

Stem cells occur as both embryonic stem cells and postnatal stem cells. Postnatal stem cells are quiescent until activated by physiological or pathological stimuli for tissue regeneration. Pluripotent stem cells differentiate into ectodermal, mesodermal, and endodermal stem cells which give rise to tissue-specific stem cells. In the bone marrow, mesodermal stem cells differentiate into hemangioblasts which differentiate into hematopoietic stem cells (HSCs), which are the precursors for lymphoid cells (T-lymphocytes, B-lymphocytes), myeloid cells (i.e. monocytes, erythrocytes, granulocytes), and vascular stem cells. The vascular stem cells evolve into EPCs. HSCs and EPCs

share several common cell surface antigens, such as Flk-1, Tie-2, c-Kit, CD133 and CD34, evidence that supports a common precursor, the hemangioblast (5).

Characterization and differentiation

The phenotypic characterization of these cells has been and remains controversial as there are several possible sources of endothelial cells that can be isolated from the blood. These include hematopoietic stem cells, myeloid cells, circulating mature endothelial cells that have sloughed off the vessel wall, and other circulating progenitor cells or "side population" cells (123), which possess stem cell characteristics and may function to maintain tumors (40; 52). Identification via surface proteins or antigens can be used to characterize EPCs; however, many of the above mentioned cell types have similar expression of surface proteins, including CD31, CD34, and KDR (VEGFR-2). Difficulty in defining EPCs also comes from the changes in cell surface markers that occur on EPCs as they mature. For example, the antigen AC133 (aka CD133), a five-transmembrane glycoprotein, is expressed on early bone marrow EPCs but is lost once they differentiate into mature endothelial cells (5).

EPCs can be isolated from either bone marrow or peripheral blood via surface markers and fluorescence-activated cell sorting (FACS); however as mentioned above, they likely will have different surface marker phenotypes depending on their origin and stage of development. Immature EPCs isolated from the bone marrow have the general phenotype $CD133^{+}/CD34^{+}/VEGFR\text{-}2^{+}/VE\text{-}cadherin^{-}$. EPCs isolated from peripheral blood have the general phenotype $CD133^{+/-}/CD34^{+/-}/VEGFR\text{-}2^{+}/CD14^{-}/VE\text{-}cadherin^{-}/eNOS^{-}$. After several weeks in culture, these EPCs adopt the phenotype $CD133^{-}/$

$CD34^{+/-}$/ VEGFR-2^{+}/ $CD14^{-}$/ VE-cadherin^{+}/ $eNOS^{+}$/ von Willebrand factor^{+}. Currently, the timing and location of the maturation process is not clear. EPCs appear to lose CD133 in systemic circulation, however, this may also occur prior to release from the bone marrow. In addition, the definition of when an EPC becomes a mature EC is also not clear. Some regard the presence of von Willebrand factor and other EC-like characteristics as the determining factor; however this has not been standardized. Also, this phenotype may occur after homing or after incorporation into the existing endothelium (59).

Further difficulty in isolating EPCs arises from another monocytic-like endothelial progenitor cell population that has been found to have the capacity to re-endothelialize following balloon vessel injury (35; 47). These cells were isolated from bone marrow, had a $CD14^{+}/CD34^{-}$ phenotype, and developed into endothelial cells (ECs) although it is reported that they can differentiate into macrophages, dendritic cells, or EC depending on environmental cues. Awad, et al (11) reported that $CD14^{+}/CD34^{-}$ cells may have a better capacity to promote vascular growth and healing *in vivo* than $CD34^{+}$ cells in diabetic patients, a patient population that has decreased function of $CD34^{+}$ progenitors. Therefore, defining EPCs remains controversial and crucial given that there appears to be more than one population of cells that can differentiate into endothelial cells and not all stem cells or stem cell lineages may have been characterized.

EPCs can also be defined using cell culture and assessing functional characteristics. For example, EPCs can form colony forming units (CFUs) and capillary tubes and secrete nitric oxide, typical of ECs. EPCs also form monolayers with a "cobblestone" appearance after several weeks of culture (60). More recently, the

utilization of proliferative potential has become another way to characterize EPCs, which has led to the conclusion that cells formerly classified as EPCs represent a heterogeneous group of cells that have various *in vitro* characteristics (65; 93; 143). Therefore, both physiological and functional characteristics have been used to define EPCs and phenotypic characterization has emerged as an important method to verify conclusions regarding cell populations.

Mobilization and factors influencing EPC mobilization

EPCs are released from the bone marrow in response to a variety of growth factors, enzymes, ligands and surface receptors. Stem cells reside in the bone marrow stromal cells or stem cell niche. Generally, activation of the protease matrix metalloproteinase-9 (MMP-9) cleaves membrane-bound Kit ligand (mKitL) to a soluble Kit ligand (AKA stem cell factor). This allows $cKit^+$ hemangioblast cells to move into the vascular zone of the bone marrow, where further proliferation occurs and early EPCs enter the blood stream.

Currently, there are several stimuli that have been associated with the induction of EPC mobilization in that they have been shown to increase circulating EPCs. These factors include VEGF, stromal cell -derived factor-1 (SDF-1), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), erythropoietin (Epo), HMG CoA reductase inhibitors (statins), PPARγ agonists, angiopoietin-1, exercise, estrogen, and eNOS (4; 60; 123). Ischemia has been recognized as a major factor that increases neovascularization and circulating EPCs and may stimulate the release of many factors that lead to both EPC mobilization and homing to

the site of injury. Certainly, VEGF, SDF-1, exercise, and Epo may all stimulate the release of EPCs via an ischemic response pathway.

VEGF and SDF-1 are released by cells in areas of ischemia and have been identified as the major regulators of EPC mobilization in response to ischemia. Asahara et al (7) found that VEGF administration resulted in increases in EPC number and enhanced neovascularization. SDF-1 was found to increase EPC mobilization (85) and recently a possible mechanism has been uncovered involving SDF-1 and the interaction with its receptor CXCR-4 (69). In particular, Sbaa, et al (100) showed that caveolin deficient mice, that is mice with cells that could not form caveolae used for receptor internalization and signal trasduction, did not release EPCs from the bone marrow in response to SDF-1. Therefore, one way that EPCs may be mobilized through SDF-1 stimulation is via CXCR-4 receptor internalization. There is evidence that calveolin may act via eNOS, which appears to be an important factor in EPC mobilization in response to VEGF (reviewed below). Brouet, et al (16) reported that statins down-regulated the expression of caveolin in ECs and promoted NO-dependent angiogenesis *in vitro*. The same group, (110) later found that EC caveolin activity and the ability to compartmentalize VEGFR-2 receptors in caveolae was critical to eNOS stimulation via VEGF and neovascularization. Therefore, caveolin may play a role in ischemia-induced EPC mobilization via VEGF and SDF-1.

eNOS has been recently found to be an important regulator of EPC mobilization. eNOS deficient mice have impaired ischemia-induced neovascularization and decreased EPC mobilization in response to VEGF (88). Dimmeler's group (3) reported that deficient eNOS in the bone marrow microenvironment caused a decrease in the activity

in proMMP-9, which led to a decrease in soluble Kit ligand and release of Kit^+ cells from the stromal cell niche. eNOS is also likely involved in EPC mobilization in response to estrogen. Two separate groups, Iwakura, et al (68) and Strehlow, et al (112), showed that estrogen increases circulating EPC number, however, Iwakura, et al also reported that estradiol enhances recovery after MI by activating eNOS and MMP-9, thereby increasing EPC mobilization. Statins have been demonstrated to induce the release of EPCs (26; 80) as well as up-regulate the expression of eNOS. Dimmeler, et al showed that EPC mobilization occurs via the PI-3 kinase/Akt pathway, a pathway that plays a role in endothelial cell survival by inhibiting apoptosis and stimulating NO synthesis (26). Lastly, exercise may also work to increase circulating EPCs through the increased nitric oxide production and bioavailability that accompanies exercise training (78). Therefore, eNOS appears to play an important role in EPC mobilization in response to ischemia-induced VEGF production, estrogen, and statins.

Angiopoietin-1 (Ang-1) is a growth factor expressed in endothelial and developing vascular cells which works via the Tie-2 receptor tyrosine kinase. Angiopoietin-1 enhances endothelial cell survival, capillary development, and also limits vascular permeability. Hattori, et al (48) found that Ang-1 promoted the mobilization of HSCs and circulating endothelial progenitors. In this study, when Ang-1 and VEGF were given together, more cells were mobilized. This group also demonstrated that while VEGF induced a rapid release of EPCs, Ang-1 caused a delayed mobilization of EPCs, which in combination produced a prolonged EPC release (85). Although the researchers hypothesized that Ang-1 may alter the adhesion of progenitors to the bone marrow stromal cells, resulting in enhanced mobilization, the mechanism has not been clarified.

G-CSF and GM-CSF are some of the most powerful stimulants of EPC mobilization. G-CSF is released by endothelial cells and macrophages and induces the bone marrow to release granulocytes, a type of white blood cell which plays a role in atherosclerotic fatty streak formation and progression to a complex fibrous lesion. Natori, et al (89) found that G-CSF increased tumor neovascularization and that bone marrow-derived EPCs participated in this effect in mice. GM-CSF is a protein released by macrophages that stimulates stem cell differentiation of granulocytes and macrophages and therefore is part of the inflammatory cascade. Asahara's group (114) found that cytokine therapy with GM-CSF mobilized EPCs and improved vascularization to ischemic areas in mice and rabbits. In addition, cytokine therapy with G-CSF was shown to increase the release of $kit^{+}/flk1^{+}$ cells by 9-fold in rats and these cells were found to directly contribute to endothelial regeneration (115). However, in a recent study, Dimmeler's group found that although G-CSF therapy increased circulating EPCs and the number of colony-forming units produced in patients with ischemic heart disease, the functional activity of the EPCs (assessed by migration to VEGF and SDF-1) was reduced (57). In the same study, the group found similar results in treated mice in addition to reduced *in vivo* capacity to augment blood flow recovery and to prevent necrosis by 27%. Therefore, although some have considered the use of G-CSF a potential therapy for re-endothelialization following vascular injury, others have recognized that due to the association with inflammation and other potential negative consequences, these factors may not be an optimal in the induction of EPC release as a future therapy (4).

Many of the other factors identified as increasing EPC mobilization may work via the mechanisms mentioned, however, some have not had mechanisms of action

investigated and clarified. For example, PPARγ agonists have been shown to increase EPC number and migratory activity in type 2 diabetic patients (91) and PPARγ agonists have been shown to attenuate the negative effects of CRP on EPC survival differentiation and function (128). Although PPARγ activation has been associated with cellular differentiation and inhibition of endothelial dysfunction, atherosclerosis, and restenosis, the mechanisms behind these effects are not clear. Recently, it was found that the PPARγ agonist, thiazolidinedione (TZD) increased EPC number and migration in patients with CAD and normal glucose tolerance. Cell culture experiments revealed that TZD treatment decreased NADPH oxidase, a producer of endothelial superoxide free radicals and decreased EPC apoptosis (134). Although this study showed that oxidative stress may influence EPC function, a connection between oxidative stress and EPC mobilization is not yet clear. Therefore, there may be mechanisms that increase the mobilization of EPCs from the bone marrow that are still unknown.

Several pathological factors have been found that lead to increases in number and function of putative EPCs. For example, acute myocardial infarction (109) and class I-II congestive heart failure (124) have been shown to increase EPC number. Associated CVD conditions found to decrease EPC number and function are hypertension, increased number of CVD risk factors, smoking, family history of CVD (126), cumulative risk factor score (51), and class III-IV CHF (124). Certainly, many of these pathological factors may work via similar mechanisms stated above (VEGF and NOS) to increase EPC mobilization. Currently, there are few studies that have investigated the mechanisms underlying the disease-related decreases in EPC mobilization; however, it is

possible that the pathology of diseases associated with reductions in EPC numbers (such as hypertension) may be due to decreased eNOS or other unidentified factors.

Recently, reactive oxygen species have been hypothesized as having an effect on mobilization, homing, incorporation and survival of EPCs. Like all stem cells, EPCs have been shown to have an increased capacity to resist ROS as they have high levels of antioxidant enzymes. Dernbach, et al (25) showed that EPCs have increased expression of catalase, glutathione peroxidase and manganese superoxide dismutase (MnSOD), compared to HUVECs or human microvascular endothelial cells. However, some disease states such as diabetes, hypertension and CVD, which are associated with increased ROS production, have been associated with decreased number, function, or survival of EPCs. For example, EPC senescence is accelerated in rats and human patients with hypertension (64). Galasso, et al (36), found that glutathione peroxidase (GPx-1) deficient mice had impaired mobilization, and migration of EPCs, as well as a reduced capacity to neutralize ROS, increased ROS-induced apoptosis, and decreased angiogenesis in ischemic tissue. Additionally, Ma, et al (82) recently reported that OxLDL, which is oxidized by ROS in the endothelium during the process of atherosclerosis, impairs EPC survival via the inhibition of eNOS. Therefore, EPC function in CVD may be closely related to increases in ROS associated with CVD.

Homing and endothelial incorporation

Currently, the mechanisms of how EPCs function in neovascularization and re-endothelialization are not completely understood. It has been established that EPCs colonize denuded vascular graphs or injured endothelium *in vivo* (106). However, the

signals that stimulate EPCs to home to the site of injury and incorporate into the endothelium are not well characterized as most of the available literature provides only indirect evidence of mechanisms. In the case of re-endothelialization, homing includes signals that localize EPCs to the site of injury or chemotaxis, adhesion of EPCs to the endothelial surface, transmigration and incorporation into the endothelial wall and differentiation into endothelial cells or other vascular cells. Recently some factors have been identified that may be involved in the homing and incorporation of EPCs into the endothelium. Interestingly, many of these factors are similar to those that induce mobilization.

Stromal cell-derived factor-1 (SDF-1) has emerged as one of the major signals that attract EPCs to the site of injury, or chemotaxis. SDF-1 and its receptor, CXCR4, are known to be required for stem cell homing to bone marrow. Recently, the CXCR4 receptor has been shown to also be important in EPC homing to vasculature in ischemic tissue. In this study, Sbaa, et al (100) found that inhibiting the sequestration of CXCR4 receptors into calveolae improved homing to ischemic tissues. Not only do SDF-1 and its receptor appear to be important to EPC homing, but SDF-1 has been shown to attract EPCs. Yamaguchi, et al (140) found that greater numbers of labeled EPCs migrated to areas of ischemia when SDF-1 was administered locally. EPC incorporation at day 3 was 1.8-fold higher than the control group, but similar between days 3-7. In addition, SDF-1-administered mice had greater capillary density at day 28, which demonstrated that improvements in angiogenesis were a result of early SDF-1 exposure. They were also able to demonstrate a strong dose-dependent migratory response of EPCs toward SDF-1 via the Boydon chamber assay; however, Western blot analysis did not show SDF-1

protein in ischemic muscles. They did, however, show increases in VEGF mRNA in ischemic tissue in the SDF-1 treated mice. SDF-1 is released in response to ischemia as Askari, et al (8) reported an increase in SDF-1 immediately following (1h-24h) MI in mice. Therefore, it is not clear how SDF-1 works to home EPCs to the site of injury or ischemia, however, it appears to be an important factor in the acute response to damage.

VEGF appears to be another factor that may be a chemoattractant for EPCs to home to a site of injury/ischemia. Asahara, et al (7) demonstrated that bone marrow mononuclear cells, cells considered upstream to differentiated EPCs and cultured EPCs, displayed chemotaxis toward VEGF *in vitro*. In addition VEGF administration improved neovascularization of the cornea. As mentioned above, VEGF mRNA was increased in ischemic tissue of SDF-1-treated mice, which may indicate that the two factors work together to attract EPCs to injury. Further, VEGF increases acutely (up to day 7) following MI, which is accompanied by an increase in circulating EPCs (109). EPCs migrate toward VEGF *in vitro* and the migratory capacity of isolated EPCs to VEGF or SDF-1 was found to be related to the functional improvement in MI patients following stem cell therapy (15).

Apoptosis, thrombin and platelets appear to be other factors that may stimulate EPC homing. In 2004 Hristov, et al (58) found that apoptotic bodies from endothelial cells stimulated the proliferation and differentiation of EPCs *in vitro*. Thus, endothelial cells that go through apoptosis may serve as a signal to circulating EPCs to the location of injury. Thrombin, the coagulation factor that has been shown to play a role in post injury inflammatory responses, has recently been found to promote EPC differentiation into endothelial cells (116). Additionally, EPCs have been found to be necessary for

thrombus resolution. Therefore, in the case of thrombosis, EPCs may locate the injury via thrombin. More recent evidence shows that platelets may play a role in the homing of EPCs to sites of vascular lesions (75). Therefore, it appears that signals associated with vascular damage, such as EC apoptosis, thrombin and platelets are signals for EPC homing to that site of damage.

Following homing to the site of injury, EPCs must incorporate into the vascular wall and differentiate into mature EPCs. Integrins, adhesion molecules, and possibly vascular endothelial cadherin (VE-cadherin) have been recently found to participate in the incorporation of EPCs into an existing endothelium. In their study showing the effect of platelets on EPC homing, Langer, et al (75) also reported that adhesion of EPCs to platelets is inhibited by neutralizing β1 integrins. Other recent findings have shown that the integrin α4β1, which is expressed on EPCs, binds to the endothelial cell ligands VCAM and fibronectin, which are expressed on actively remodeling new vasculature (70). Similarly, Yoon, et al (144) found that ischemia induced intracellular adhesion molecule – 1 (ICAM-1) expression on endothelial cells, which bound EPCs via ICAM-1/β-2 integrins *in vitro*. In this study, *in vivo* results showed that EPCs preferentially homed to ischemic muscle and blocking ICAM-1 expression decreased this effect with accompanying decreases in neovascularization. VE-cadherin is expressed on endothelial cells and is required for EC-cell interaction as well as maintenance of EC integrity and barrier function. Kogata, et al (72) found that ischemia induced VE-cadherin expression in existing ECs and bone-derived cells and that VE-cadherin-activated bone-derived cells were found surrounding the ischemic area. These researchers hypothesized that this may be a potential mechanism of EPC homing to sites of ischemia and vascular damage.

Therefore, more information is needed regarding the stimuli that effect EPC homing and incorporation into the existing endothelium.

Recent evidence appears to show that homing and incorporation of EPCs may not simply be a function of signal molecules released from the area of damage, but EPCs themselves have also been shown to express several growth factors, such as VEGF, SDF-1, and insulin-like growth factor-1 (IGF-1), which may not only work in the migration and homing of EPCs, but may also supply the surrounding existing differentiated cells with factors to support their survival and function (122). Other recent data show that progenitor cells may not only be derived from the bone marrow, but from a "vasculogenic zone" that lies between the smooth muscle and adventitia. These cells were $CD34^+$ but $CD31^-$ and termed vascular wall EPCs or VW-EPCs (146). These cells were capable of forming capillary sprouts and became positive for endothelial markers VEGFR-2, Tie-2, and VE-cadherin and had the capacity to differentiate into ECs.

Therefore, as more information is gained regarding EPCs, it appears that these cells have multiple strategies to aid in successful neovascularization and re-endothelialization. Not only are several redundant factors involved in their stimulation of mobilization and homing, but they have increased capacity to neutralize ROS, may have more than one source, and may themselves release factors that aid in their survival.

Endothelial Progenitor Cells and Cardiovascular Disease

In 2001, Vasa, et al (126) were the first to report an association between the number and function of endothelial progenitor cells with coronary artery disease family history and number of atherosclerotic risk factors. They reported lower EPC number in

patients with a positive family history for CAD and impaired EPC migratory response in patients with a greater number of atherosclerotic risk factors. EPC number and function have also been found to be associated with endothelium-dependent flow-mediated vasodilation, a measure of endothelial function (50; 51; 135). Interestingly, the number of EPCs was found to be a better predictor of vascular reactivity than the Framingham risk factor score (51). The number of circulating EPCs was found to independently predict atherosclerotic disease progression (103) and cardiovascular events including death from cardiovascular causes, revascularization surgery, and hospitalization due to cardiovascular events, which included angina, congestive heart failure, stroke, myocardial infarction, and arrhythmia (133).

Mechanisms relating EPCs to CVD

Atherosclerosis is a hallmark of CVD. It is commonly accepted that atherosclerosis begins as dysfunction and damage of the endothelial layer. Endothelial damage leads to a cascade of proinflammatory events, infiltration of monocytes, adhesion molecule expression and smooth muscle cell proliferation which result in atherosclerotic lesions (96). Current risk factors for CVD share a common mechanism, which is endothelial damage. The endothelium is constantly repaired by two mechanisms; from pre-existing, fully-differentiated endothelial cells and bone-derived endothelial progenitor cells. The existing mature cells may have a limited proliferative potential and their capacity to repair a damaged endothelium may be restricted. EPCs, on the other hand, are "new" cells with a greater capacity for proliferation and differentiation.

EPCs are released from the bone marrow by a variety of signals, including VEGF, SDF-1, G-CSF, GM-CSF, and eNOS (as discussed above). Ischemic tissues release VEGF and SDF-1, which likely explains the increase in EPCs in response to myocardial infarction (109). In this case, the angiogenic capacity of EPCs is valuable in working to revascularize the damaged tissue. In fact, exogenous EPCs have been used in humans for both clinical cardiac repair resulting in improved ejection fraction, end systolic LV volume (10) and enhanced contractile motion and neovascularization of occlusive arterial disease, improving transcutaneous oxygen pressure, and decreasing resting and walking pain (81).

EPCs are also mobilized by factors that are present in the process of atherosclerosis and associated inflammation. In addition, thrombin (116) and platelets (75) have also been shown to be involved in the differentiation and homing of EPCs to a site of injury. This is further evidence that endogenous EPCs function in the repair of vascular damage of atherosclerosis progression. Schmidt-Lucke, et al, (103) reported that the number of circulating EPCs independently predicted atherosclerotic disease progression in patients with CVD, which was determined by the need for coronary revascularization of de novo lesions due to ischemia. In this study, patients with lower EPC number had a 4-fold increased risk of suffering a CVD event during the follow-up period. Therefore, the number of circulating EPCs is correlated with event-free survival in CVD.

In the examples of MI and atherosclerosis, EPCs are important in vascular repair and regeneration following a CVD event or overt vascular disease. Impaired endothelial function is closely related to the development of atherosclerosis as changes in endothelial

function, such as the decrease in nitric oxide and eNOS, have been shown to precede atherosclerosis and reflects both potential and existing atherosclerosis. Endothelial function has also been shown to be a prognostic indicator of atherosclerosis and clinical cardiovascular events (113). EPCs likely are involved in ongoing endothelial repair as they may serve as a pool of cells that home to a site of injury. As mentioned above, EPCs home to an endothelial cell-denuded arterial section and re-endothelialize the region (130). Over time, endothelial cells undergo replicative senescence as they are exposed to stressors. Endothelial dysfunction has been related to senescent changes in endothelial cells such as decreases in telomere length and telomerase (141) and increases in the cell-senescence indicator, β galactosidase. Minamino, et al (84) showed that endothelial cells that overlay atherosclerotic plaques show an enhanced senescence-associated phenotype (increased β galactosidase staining). Injury to endothelial cells is often followed by apoptosis which has been regarded as a critical step in the progression of atherosclerosis (96). Recently, Hristov, et al (58) found that apoptotic bodies from endothelial cells stimulated EPC differentiation *in vitro*. Hill, et al (51), reported that number of circulating EPCs were related to flow-mediated brachial reactivity, an assessment of endothelial function. In this study, lower numbers of EPCs was related to lower endothelial function. Therefore, overall endothelial damage may be represented by the balance between the magnitude of injury and the capacity for repair and EPCs may be important for the maintenance of a healthy, functional endothelium.

Currently, it is unknown why EPCs appear to be decreased in response to excessive or continuous damage or stress. There may be a few possible explanations. First, excessive damage to the endothelium may lead to depletion in the pool of

progenitor cells in the bone marrow. There has been an association of age with decreased EPC number in that increased age is correlated with decreased EPC number (28; 139) which may led evidence to this theory. However, recently, Chen, et al (18) reported that there was no association of age and basal EPC number in healthy individuals who did not have any CVD risk factors, although this does not preclude the possibility that EPC mobilization to a stimulus such as VEGF is not attenuated with age. Therefore, it is unclear whether chronic stress decreases EPC number by depletion of the EPC pool. Second, chronic stress on the endothelium may decrease EPC mobilization. Decreased NO is a hallmark of endothelial dysfunction and eNOS is an important regulator of EPC release from the bone marrow. eNOS deficient mice show reduced EPC mobilization in response to ischemia (3) or exercise (78). Lastly, chronic stress and CVD risk factors may affect the differentiation and/or incorporation of EPCs into the endothelium by damaging EPCs directly. For example, Hill, et al (51) found that EPCs from patients with a higher risk for cardiovascular events demonstrated greater *in vitro* β-galactosidase activity, an indicator of cellular senescence. It was recently reported that EPCs from both spontaneously hypertensive rats and human patients with essential hypertension demonstrated increased EPC senescence and decreased EPC telomerase activity (64). Senescent EPCs would not have the proliferative capacity of normal EPCs and may not have the ability to differentiate and incorporate into the endothelium as effectively. Oxidative stress produced from CVD risk factors could also damage EPCs. Galasso, et al (36) reported that glutathione peroxidase (GPx-1) deficient mice had impaired mobilization and migration of EPCs, as well as a reduced capacity to neutralize ROS, increased ROS-induced apoptosis, and decreased angiogenesis in ischemic tissue.

Certainly, decreases in the ability of EPCs to repair an injured endothelium may be caused by all three of these factors, or even perhaps factors that have not yet been identified.

Therefore, although not all details of mechanisms regarding the way in which EPCs work in the cardiovascular system to maintain vascular function, most data show that EPCs not only are mobilized to neovascularize and re-endothelialize damaged tissue, but that they may be an important coomponent of maintaining a healthy endothelial layer, which may become damaged over time and lead to endothelial dysfunction, atherosclerosis and CVD.

Studies relating EPCs to CVD

Currently, there are several studies that have associated EPC number with cardiovascular diseases. Some CVD-related factors have been shown to be associated with an *increase* EPC number. Acute myocardial infarction (109; 137), vascular injury (68), and class I-II congestive heart failure (124) have all been shown to increase EPC number. MI and vascular injury represent an acute response to injury in which EPCs would be mobilized. Associated CVD conditions found to decrease EPC number and function are hypertension, increased number of CVD risk factors, smoking, family history of CVD (126), cumulative risk factor score (51), and class III-IV CHF (124). In the case of CHF, Valgimigli, et al (124) attributed the increase in EPCs in the early stages and decrease in later stages to TNF-α, which is an inflammatory cytokine that is increased in the later stages of CHF but not in the early stages. TNF-α suppresses

hematopoiesis which may overwhelm the mobilization signal seen in the early stages of the condition.

There are a few studies that include individuals with CVD or various degrees of CVD risk that show a relationship of EPCs and CVD risk factors, endothelial function, and/or CVD progression and prognosis. In a prospective study in 2001, Vasa, et al (126) were the first to report an association between the number and function of these cells with coronary artery disease family history and number of atherosclerotic risk factors. In a study with 45 patients with CAD and age-matched controls, they reported ~40% lower EPC number in patients compared to controls. In only the patient group, the number of risk factors was correlated with decreased EPC number. Impaired EPC migratory response was correlated with a greater number of atherosclerotic risk factors. Hill, et al (51), studied 45 men with various CVD risk factors but no history of CVD and found EPC function (assessed by colony-forming assay) was associated with endothelium-dependent flow-mediated vasodilation, a measure of endothelial function and precursor to CVD. Interestingly, in this study, the number of EPCs was found to be a better predictor of vascular reactivity than the Framingham risk factor score (51). In a study by Schmidt-Lucke, et al (103), 120 individuals (43 controls, 44 patients with stable CAD, and 33 with acute coronary syndromes) were followed for atherosclerotic disease progression and acute coronary events. The number of circulating EPCs was found to independently predict atherosclerotic disease progression assessed by the need for coronary revascularization of de novo lesions, and future cardiovascular events including death from cardiovascular causes, unstable angina, or myocardial infarction. In the same year, Werner, et al (133) published results of a prospective analysis of 507 patients with CAD

and the relationship between EPC number and colony-forming units and outcomes over a 12 month period. Patients with the lowest EPC number had significantly higher incidence of death from CV causes. Low EPC numbers did not predict death from all causes. In addition, this group found similar results of Hill, et al (51), in that EPC function, assessed by colony-forming units, was impaired in a subgroup of patients. Therefore, not only EPC number, but EPC function influences cardiovascular outcomes. In a study on cerebrovascular disease, Ghani, et al (37) found that EPC number was significantly lower in patients with stroke compared to control subjects. In addition, they reported a significant inverse relationship between EPC number and Framingham risk factor score. This study supports the data that EPCs function as a factor that works to maintain the endothelial monolayer.

EPCs (and other progenitors) have become an experimental novel therapy to improve cardiac function following myocardial infarction (MI) and stroke. In this case, EPCs likely improve neovascularization in an ischemic region, but also have been shown to differentiate into cardiac myocytes (27). The BOOST trial was the first randomized-controlled trial using bone-derived progenitor ($CD34^+$) cells. This group reported improved left ventricular function in myocardial infarction patients (measured via left-ventricular ejection fraction) 6 months following bone marrow cell transplantation (138). In the TOPCARE-AMI trial patients with acute MI underwent progenitor cell transplantation. Cells were either bone marrow- ($CD34^+/CD45^+/CD133^+$) or blood-derived ($VEGFR2^+$/von Willebrand $factor^+$/ $PECAM\text{-}1^+/VE\text{-}Cadherin^+$). Regardless of the type of cells transplanted, 4 months post AMI, treated patients had a significant increase in global LV ejection fraction, improvement in wall motion in the infarct area,

and a significant reduction in end-systolic LV volumes (9). The one-year follow-up of this study showed increased ejection fraction, decreased infarct size, and no indication of reactive hypertrophy (101). Although the means by which the administered progenitor cells function has not been directly measured in these cases, exogenous progenitor cell administration appears to improve prognosis following acute MI. The optimal cell population to use, method to mobilize and isolate the cells, and methods to optimize downstream cell function has yet to be elucidated.

These studies relate EPC number and function to CVD disease progression and prognosis in patients with established CVD. Currently, there are no long-term studies investigating EPC number or function in healthy patients with CVD development. These large population-based studies are crucial in the determination of factors as those that cause CVD. As EPCs are not routinely measured, and the nature of measuring EPC number and migration require a fresh blood sample, retrospective studies are not possible to relate aspects of patient history to disease development. Therefore, although the above studies provide strong evidence, data from longitudinal studies will be needed to convincingly regard EPCs as a CVD risk factor.

Interrelationships of EPCs and CVD risk factors

EPCs have been related to many of the established CVD risk factors and have shown various degrees of correlation, but overall, an inverse significant relationship between the risk factors and EPCs persists. In the article published by Schmidt-Lucke, et al (103), the number of EPCs was correlated with CVD risk factors by univariate analysis. They demonstrated that age, blood pressure, smoking, and family history are all

significantly correlated with EPC number. Lipids were not included in the analysis. Diabetes and hsCRP, however, were not correlated with EPC number. By multivariate analysis, age and family history of CVD remained significant independent predictors of reduced EPC number. In the results from the study by Vasa, et al, (126), using a similar analysis researchers reported that smoking was associated with lower EPC number. This group also reported that migratory capacity of EPCs was influenced mainly by hypertension. LDL cholesterol, family history of CVD, and age were all shown to determine the number of circulating EPCs and migration. In the Bruneck study, a large population-based study, EPC number (evaluated via cell-culture assay) and EPC-CFUs were measured in over 500 participants (139). Contrary to expectations, EPC number was positively correlated with Framingham risk score although a negative relationship was found between EPC number and extent of carotid atherosclerosis. Researchers also demonstrated decreasing EPC number with increasing age, a finding that has not been consistently found. A possible explanation for these results could be the technique used to quantify EPC number, as EPCs derived from culture have not been found to correlate with those quantified via flow cytometry (31). However, increased circulating EPCs with increased Framingham CVD risk may be indicative of cardiovascular stress or vascular damage that signaled the mobilization of EPCs.

There are several studies that have investigated the relationship of individual risk factors including smoking, diabetes, hypertension, and age with EPC number or function. Kondo, et al (73) also reported that the number of EPCs was reduced in smokers and that smoking cessation led to a rapid restoration of EPC numbers that was greater in light smokers compared to heavy smokers. This link of smoking and EPC number could

potentially explain a mechanism for the increased incidence of CVD in smokers. Alterations in EPC function have also been associated with diabetes. Tepper, et al (117) found that EPCs from type II diabetes patients had a 48% decrease in proliferation in culture compared to controls. In addition, EPCs from diabetics had decreased adhesion to HUVECS and were 2.5 times less likely to participate in tubule formation compared to EPCs from non-diabetic subjects. These data suggest that patients with type II diabetes have impaired EPC function, which may explain the increased incidence of CVD in diabetics. Imanishi's group also found that hypertension in rats and human patients is associated with EPC senescence (64). The relationship of age with EPCs is not clear. In the study by Vasa, (126) which included 45 patients with CVD and 9 controls, they showed an inverse relationship of EPC number with age. However, recently, Chen, et al (18) reported that in healthy patients with no CVD risk factors, there was no relationship between CVD and age. Physical activity is known to stimulate EPC release (77). In this study there is no mention of physical activity level or participation prior to sample collection. Therefore, it is possible that PA level may attenuate any age-related changes observed in EPC number.

Plasma lipids, another CVD risk factor, have also been related to EPC number and function, although there are not much data in these areas. Tso, et al (121) reported that HDL enhanced repair of the endothelium by EPCs in mice. In humans, Pellegatta, et al (90) reported that EPC colony-forming capacity was associated with total cholesterol, HDL, and the HDL/TC ratio. These data suggest a synergistic effect of EPCs and HDL, although the mechanism is not clear.

In conclusion, the interrelationships between EPCs and other established CVD risk factors, the mechanistic evidence, and population-based study evidence mentioned above show that EPCs may be a good candidate for inclusion in the list of CVD risk factors. Currently, the data with EPCs in humans demonstrate that lower EPC number and function are correlated with decreased prognosis and outcome in patients with CVD. There is, however, a lack of larger, longitudinal studies with healthy individuals demonstrating relationships between EPC number and development of CVD. In the future, primary prevention trials which utilize methods to increase EPCs may also help to demonstrate that EPC number and function are useful in assessing risk for CVD.

Endothelial Integrity and Oxidative Stress

The integrity of the vascular endothelium is determined by the balance of injury and repair. Oxidative stress results when pro-oxidants are greater than antioxidants and has been shown to be a significant contributor to CVD via the damaging effects on the vascular endothelium. EPCs work to repair the endothelium and despite the characteristic of heightened antioxidant levels, EPCs have been shown to be vulnerable to damage induced by oxidative stress. Oxidative stress-induced damage to EPCs may lead to decreased capacity of these cells for repair, thereby resulting in accumulating endothelial damage and progression of atherosclerosis and heart disease.

Reactive oxygen species

Oxidative stress is cellular damage that is caused by reactive oxygen species (ROS). ROS include (but are not limited to) free radicals, which are oxygen or nitrogen-

based substances with an unpaired electron (i.e. hydroxyl radical, OH.) superoxide (O2.-), peroxynitrite (ONOO.-), nitric oxide (NO), and hydrogen peroxide (H2O2) (94). With an unpaired electron, these molecules are highly reactive and can participate in several biochemical reactions. ROS are produced by several mechanisms. The three mechanisms that are most prevalent in endothelial cells are NADPH oxidase (NOX), NO synthase (NOS), and xanthine oxidase (97). ROS production also comes from mitochondrial respiration and the electron transport chain (ETC). The reduction of oxygen to water takes 4 electrons in the ETC. With each addition of 1 electron, a different ROS can be generated (39). ROS can also be produced by cycloxygenases, lipoxygenases, and cytochrome p450 monooxygenases. Factors that stimulate ROS pathways which are important in endothelial cells include homocysteine, ANGII, TNF-α, platelet-derived growth factor (PDGF) and glucose.

ROS can exert cellular damage in many ways, including DNA damage, cellular senescence, and apoptosis. Recently, Demirbag, et al (24) found that patients with CAD had decreased plasma total antioxidant capacity and increased leukocyte DNA damage. ROS can oxidize nuclear and mitochondrial DNA (mtDNA). Involvement of ROS in mtDNA damage has been considered the most relevant as mitochondria continuously produce O2- and mtDNA is continuously exposed to oxidative stress throughout the lifespan (14). Oxidative mtDNA damage leads to mitochondrial dysfunction, decreased numbers of mitochondria, and the formation of dysfunctional mitochondrial proteins, which can lead to increased production of ROS and more mtDNA damage (32).

Another consequence of ROS-induced DNA damage is cell senescence. Endothelial senescence has been associated with atherosclerosis and senescent ECs have

been found to overlay atherosclerotic plaques (84). Human vascular endothelial cells (HUVECs) that have been treated with H_2O_2 enter senescence earlier than control cells. The treated cells also had decreased telomere length, which is an indicator of DNA damage and replicative senescence (74). Evidence for the involvement of ROS in this telomere-mediated cell senescence and dysfunction via TERT (the catalytic component of telomerase) comes from a study by Haendeler, et al (41). Haendeler and her group have identified that oxidative stress leads to nuclear export of TERT through the activation of the tyrosine kinase Src, which phosphorylates TERT, which signals the transport of the phosphorylated TERT from the nucleus and leaves the cell without the ability to attenuate telomere attrition with replication.

Cellular apoptosis, or programmed cell death, can also occur when cells are damaged by ROS. Apoptosis refers to the morphological alterations exhibited by "actively" dying cells, including cell shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation (29). ROS have been found to induce endothelial cell apoptosis. Dimmeler et al (29), showed that oxLDL induces apoptosis in HUVECs, which was completely prevented by exposure of cells to antioxidants. Induction of apoptosis by ROS appears to be dependent on the species generated and the concentration of ROS. For example, high concentrations of hydrogen peroxide and peroxynitrite promote apoptosis and cell detachment and shedding (anoikis) (120). On the other hand, low doses of ROS protect ECs from apoptosis (42). In 2005, Muakami, et al (86) found that hydrogen peroxide stimulated endothelial cell apoptosis via JNK phosphorylation. Interestingly, in this study, they also observed that angiopoetin-1 (Ang-1), a hormone involved in vascular remodeling, stabilization, and maturation, inhibited the H2O2-

induced apoptosis by Akt phosphorylation through PI3 kinase. Therefore, ROS appear to affect endothelial integrity by promoting EC apoptosis, however, this effect may depend on the species generated and the concentration.

Cells have systems, chemicals and enzymes or antioxidants, to combat the accumulation of ROS. Chemical antioxidants are glutathione and other thiols and enzymatic antioxidants are cu/zn SOD (SOD-1) located in the cytosol, MnSOD (SOD-2) located in the mitochondria, ecSOD (SOD-3) located in the exracellular matrix, glutathione peroxidase (GPx), and catalase. However, oxidative stress occurs when there is an imbalance between the two - when prooxidants are greater the antioxidants. Many diseases have been attributed to increases in ROS-induced cellular damage. In particular, cardiovascular disease has been associated with ROS accumulation as ROS promote atherosclerosis by factors such as endothelial dysfunction, VSMC migration, monocyte migration, and oxidation of LDL.

Oxidative stress and endothelial dysfunction

The vascular endothelium has several important functions. Not only does it provide a nonthrombogenic layer for laminar blood flow, but it also senses and transduces signals from the circulation. The endothelial layer is important in the maintenance of vascular tone. Endothelial cells maintain the balance between vasodilating factors (ie NO) and vasoconstricting factors (ie. ET-1, ATII). The endothelium also maintains the coagulation state by regulating platelet activation, fibrinolytic system and the clotting cascade. Endothelial cells also regulate the inflammatory and adhesion process via release of cytokines, CRP, interleukins, TNF-α

and adhesion molecules (97). The myriad of functions and regulatory processes performed by the endothelium reflect a system that is imperative to maintain homeostatsis and efficiently repair damage. Endothelial dysfunction is closely related to the development of atherosclerosis as changes in endothelial function, such as the decrease in eNOS, have been shown to precede atherosclerosis and reflects both potential and existing atherosclerosis.

Endothelial damage from ROS can occur via direct and indirect mechanisms. Primarily, the endothelium can be damaged such that the bioavailability of NO is decreased resulting in endothelial dysfunction. Endothelial dysfunction is characterized by a decrease in NO. ROS affects the bioavailability of NO in ECs in several ways. First, NO synthase, the enzyme that forms NO, can experience "uncoupling". NO uncoupling was defined by Pritchard et al in 1995 and it occurs when NO reacts with O2- to form peroxynitrite (ONOO-). ONOO- can inactivate mnSOD by tyrosine nitration, leading to a lower antioxidant capacity in the cell. ONOO- can also switch NOS from an NO- to an O2- generating enzyme by oxidation of BH4. BH4 is an essential cofactor that maintains eNOS dimer stability. The net result is a decrease in NO bioavailability, an increase in ROS, and a decrease in the antioxidant capacity. A second mechanism that decreases NO in ECs is direct scavenging of NO by oxygen radicals. Endothelial dysfunction can also be caused by tyrosine nitration of prostacyclin synthase by ONOO- and decreased prostacyclin availability (14). Endothelin-1 (ET-1) is a vasoconstricting substance in ECs. ET-1 has recently been found to increase ROS production (92). ET-1 was found to increase superoxide by NADPH oxidase and ET-1 activity led to NOS uncoupling.

Loss of endothelial function and integrity via ROS can also occur by stimulating adhesion molecule expression which can attract leukocytes and inflammatory substances. In endothelial cells, ROS can directly stimulate vascular cell adhesion molecule (VCAM-1), monocyte chemoattractant protein (MCP-1) and granulocyte-macrophage colony stimulatory factor (GM-CSF). VCAM-1 is responsible for the adhesion of monocytes to the endothelial cell surface. Once bound, MCP-1 stimulates the transmigration of the monocyte through the endothelium to the subendothelial layer. GM-CSF is a cytokine that targets bone marrow progenitors signaling growth and differentiation.

There are several indirect mechanisms by which ROS can induce the expression of inflammation-related proteins. ROS can oxidize LDL, which then can stimulate gene expression of VCAM-1 and intercellular adhesion molecule (ICAM-1), another molecule responsible for the adhesion of leukocytes to the endothelium. The oxidation of LDL by ROS has several effects on endothelial function and integrity. In addition to the function of the stimulation of VCAM-1 and the adhesion of leukocytes to the endothelial surface, oxidized LDL induces endothelial cell toxicity, inactivates NO and impairs receptor-induced endothelial NO production (104). Another indirect mechanism involves angiotensin II-activated NADPH oxidase which produces superoxide that can also induce expression of these adhesion molecules. A third indirect mechanism is hyperglycemia. Increased glucose levels lead to glycation of lipids and proteins, which in turn produces advanced glycation end products (AGEs). Interaction of AGEs with their receptors (RAGEs) produces ROS and promotes expression of ICAM-1, VCAM-1, MCP-1, and GM-CSF (33).

ROS and EPC function

Endothelial progenitor cells function in angiogenesis and re-endothelialization. In these roles, EPCs home to areas of injury that have high levels of associated oxidative stress. Progenitor and stem cells characteristically have a large antioxidant capacity. In EPCs, it is believed that this higher antioxidant capacity helps to maintain a self-renewing state and afford them with defenses for the environments to which they are targeted. Dernbach, et al (25) first reported heightened gene expression of antioxidant enzymes such as catalase, glutathione peroxidase and manganese superoxide dismutase (MnSOD) in EPCs compared to HUVECs. Further, inhibition of any one of these enzymes alone was not enough to increase ROS in EPCs as the inhibition had to be combined to decrease EPC function. The same year, He, et al (49) found that EPCs cultured from humans had 3-4-fold greater expression and activity of manganese superoxide dismutase compared to HUVECs or human coronary artery endothelial cells, when exposed to oxidative stress, although copper zinc superoxide dismutase (CuZnSOD) and catalase were not different.

Since these reports, oxidative stress has been implicated in decreases in EPC function and the effects of certain medications on EPCs have been related to oxidative stress. Galasso, et al (36) found that GPX-1 deficient mice had decreased angiogenesis, decreased EPC function, and increased sensitivity to H_2O_2 to induce EPC apoptosis. More recently, Ingram, et al (66) also reported that oxidant treatment decreased the clonogenic capacity, increased apoptosis, and decreased the tube-forming capacity of EPCs in culture. This effect was mirrored by activation of the stress-induced kinase pathway. Another possible mechanistic link between oxidative stress and EPC

dysfunction is telomere-associated senescence. Imanishi et al (62) showed that EPCs cultured with the highly atherogenic oxidized LDL had increased EPC senescence, decreased proliferation and network formation, and decreased telomerase activity. Indeed, in mature endothelial cells, antioxidants have been shown to decrease the export of telomerase reverse transcriptase (TERT) from the nucleus, thereby preserving DNA telomere integrity (42). In EPCs, human TERT gene transfer enhanced postnatal neovascularization of an ischemic limb (87). In another study, oxidized LDL decreased EPC function, which was paralleled by decreased Akt phosphorylation and eNOS protein expression (82). Therefore, the mechanism of oxidative stress EPC dysfunction has yet to be clarified.

Oxidative stress has been implicated in diabetes-related EPC dysfunction (117) with decreased proliferation, adhesion and tube formation. Thum, et al (119) recently reported that EPCs from diabetic patients produced large amounts of superoxide anion and had lower migratory capacity. Further, this effect appeared to be mediated by eNOS uncoupling. However, in another recent study, hyperglycemia was shown to decrease EPC function, but was related to NO rather than oxidative stress (19). Therefore, the role of oxidative stress on EPCs in diabetes-related vascular dysfunction is not clear.

Endothelial Progenitor Cells and Physical Activity

Endothelial progenitor cells are released from the bone marrow by several stimuli. In 2004, the first evidence was published that exercise was another stimulus that increased the number of circulating EPCs. There are data regarding the response to both an acute exercise bout and exercise training, however most studies include subjects with

CVD or CVD risk factors. Although the mechanisms are not clear how exercise increases EPCs, there are several hypotheses based on what is known about EPCs. These hypotheses will be presented following a review of the available literature on EPCs and physical activity.

Acute exercise

The effect of an acute bout of exercise on EPCs was investigated in 2004 in 22 middle-aged volunteers with various CVD risk factors. Rehman, et al (95) found that EPCs ($AC133^{+}$/$VE\text{-}Cadherin^{+}$) increased nearly 4-fold compared to baseline values when measured 5-10 minutes following exhaustive treadmill or bicycle exercise. In this study, investigators measured another EPC-related cell population; cells that were positive for AC133 and negative for VE Cadherin, which are early progenitors. This population of cells increased 40%. These results together show a shift toward VE-cadherin positive cells with an acute bout of exercise. This group also did not find an increase in G-CSF or GM-CSF, two factors known to be powerful stimulants of EPC release. Therefore, other factors associated with exercise, such as VEGF or eNOS, are likely to be the stimulus of release in response to an acute bout of exercise.

In a study on the effect of an acute bout of maximal exercise in patients with CAD, Adams, et al (1) tested subjects with 1) CAD with exercise-induced ischemia, 2) asymptomatic patients with no exercise-induced ischemia, and 3) healthy age-matched controls. Researchers collected samples prior to and 2, 4, 6, 8, 24, and 48 hours following the acute exercise test. They found that EPC number in response to acute maximal bicycle exercise in CAD patients with exercise-induced myocardial ischemia increased

maximally 48-hours following exercise, 3.1 ± 0.6-fold via FACS analysis and 3.3 ± 0.5-fold via cell culture assay. However, CAD patients without exercise-induced myocardial ischemia and normal subjects in this study did not display any significant increase in EPCs. Plasma VEGF levels were increased in the patient group with exercise-induced ischemia but not in the other 2 groups. Plasma G-CSF and TNF-α levels were not elevated in any group. Since patients with exercise-induced ischemia were the only group to show a change in EPCs and VEGF in response to the exercise stimulus, it appears that EPCs were increased in this group as a response to ischemia and VEGF.

The other study that investigated EPC response to an acute bout of exercise was performed in healthy subjects to assess the link between extent of physical exercise and changes in EPCs (77). In the prior two studies, the stimulus was a maximal exercise test, which although it may be at a higher intensity, typically lasts only about 10 minutes. In this study, subjects performed 3 exercise tests: 1) intensive running for 30 minutes at ~ 82% VO_{2max}, 2) moderate running for 30 min at ~68% VO_{2max} and 3) short term running for 10 minutes at ~68% VO_{2max}. Samples were drawn before and 10 minutes following exercise and in a subset of the participants 10min, 30min, 2h, 6h, and 24h following the exercise session. In addition, three cell populations were evaluated, including $CD34^+/KDR^+$, $CD34^+/CD133^+$, and $CD34^+/CD117^+$ cells. Migratory capacity and colony-forming capacity were also measured. Researchers found that moderate (~68% VO_{2max}) and intensive (~82% VO_{2max}) running for 30 minutes increased all of the cell populations significantly but moderate, short term running (10 minutes) did not significantly increase EPC number following exercise. In addition, plasma VEGF level did not change with any exercise stimulus. Following exercise, EPC number was increased to a maximum

between 30 minutes and 2 hours. Analysis of EPC function found that intensive running increased EPC migration and colony-forming units as soon as 10 minutes following exercise. EPC migration remained significantly elevated up to 30 minutes after exercise and then decreased between 2 and 24 hours after the exercise. Colony forming units remained significantly elevated up to 6 hours following exercise. The number of colony forming units reflects the proliferative potential of EPC and this remained elevated following exercise longer than EPC number, which may indicate improved endothelial regeneration and maintenance induced by exercise.

In comparing the studies by Adams and Laufs, they present different findings with EPC number and exercise. Adams, et al (1) found that EPC number in response to acute maximal bicycle exercise in CAD patients with exercise-induced myocardial ischemia increased 3.1 ± 0.6-fold (FACS analysis) and 3.3 ± 0.5-fold (cell culture analysis) 48-hours following exercise. However, in the same study, CAD patients without exercise-induced myocardial ischemia and normal subjects did not display a significant increase in EPCs. Comparing these results to those of Laufs, et al (77), it is possible that Adams et al (1) did not detect an increase in EPCs in the other two patient groups because the sampling window was too large. Laufs et al (77) found a significant increase in EPC number 30 and 60 minutes following the bout of exercise whereas Adams, et al (1) took the first sample at 2 hours following exercise. This however, does not rule out the possibility that a different response and time course of EPC release may be present in patients with exercise-induced ischemia.

There are other more recent studies highlighting the EPC response to acute physical activity. Shaffer, et al (105) reported that EPCs tended to increase following

acute treadmill exercise in young and older healthy participants, and patients with peripheral artery disease (PAD) and that the changes in EPCs were more similar between the young and patients with PAD. Yang, et al (142) found that EPCs increased following Bruce treadmill testing in healthy males and that plasma nitric oxide (NO) increased in parallel but there was no change in plasma VEGF or CM-CSF. Van Craenenbroeck et al (125) reported this year that $CD34^+/KDR^+$ EPCs, measured by two separate techniques, increased following symptom-limited cardiopulmonary bicycle exercise testing. Opposite to Yang's data, this group reported no change in plasma NO and a trend for an increase in VEGF. They also did not find differences in EPC-CFU with acute exercise. In opposition to these studies, Thijssen, et al (118) did not find a change in $CD34^+/KDR^+$ cells following acute maximal cycle exercise in either young or older sedentary men. This group utilized magnetic microbeads to quantify EPC number, which may have accounted for differences between this study and the others.

Overall, it appears that an acute bout of exercise has the capacity to increase EPC number in patients with CAD, individuals with various CVD risk factors, and healthy subjects. Data on EPC function are equivocal. These studies on acute exercise also give some insight to the time course of release, with an initial rise in EPC number from 30 min to 2h following exercise, an increase in EPC migration at 30 min with a subsequent decrease, and an increase in CFUs that lasts for 6 hr following exercise. This effect is similar to that seen in the Rehman study in which EPC number increased following exercise in the proliferative type and only moderately in early EPCs. This indicates that an initial increase in EPCs appears to be followed by a more sustained increase in function, although more studies are needed to characterize the nature of this response. In

addition, VEGF and NO have been implicated as substances that increase EPC mobilization from the bone marrow; however, it is still not clear how and whether these factors function with acute exercise. It is possible that healthy and diseased populations may have different stimuli for EPC release, proliferation, and/or function as well as different time-courses for release. The largest limitation in comparing the response to acute exercise is that the exercise stimulus varies between studies; however, the lack of a uniform definition of EPCs and methodology to quantify them also likely leads to problems drawing conclusions from this body of data.

Exercise training

There have been a few studies published regarding the effect of exercise training on EPCs. In 2004, Laufs, et al (78) measured EPCs in mice and patients with stable CAD. Mice engaged in 3 weeks of voluntary wheel running and humans underwent a 4 week training program of bicycle ergometer endurance exercise (60-80% peak VO2), strength exercises, and walking. In mice, EPC number was measured 1, 7, 14 and 28 days following the start of wheel running exercise. EPC number was not significantly increased after only one day of wheel running, however, EPC number was significantly increased in the blood, bone marrow and spleen of mice after 7 days of exercise, which persisted for the 28 days of the training program. The up regulation of EPCs was accompanied by an increase in NO and VEGF but not SDF-1. EPC apoptosis was also found to be lower in the exercise group versus the control group after 28 days of exercise. A subgroup of mice underwent vascular injury. The exercising mice had decreased neointima formation and increased angiogenesis following injury. In humans, EPC

number increased 78 ± 34% compared to before the 4 week training program. EPC apoptosis was also found to decrease 41 ± 11% after training.

In another study investigating EPCs and exercise training, Steiner, et al (111) utilized a 12 week exercise training program in patients with asymptomatic coronary artery disease (CAD) and/or cardiovascular disease risk factors. Samples were collected before and at least 24-hours after the last training session. Endothelial-dependent dilation was measured in subjects. Results showed a 2.9 ± 0.4-fold increase in circulating EPCs in the exercise group. This increase was correlated with an increase in flow-mediated dilation and NO synthesis. These results show exercise training to be beneficial to improve endothelial function and EPC number in patients with CAD and support the data found by Laufs, et al (78) mentioned above.

In another training study, Sandri, et al (98) analyzed the responses of circulating progenitor cell ($CD34^+/KDR^+$) number and function in three patient groups; 1) those with ischemic PAOD, 2) patients with PAOD who had revascularization surgery and performed non-ischemic training, and 3) patients with some disease who performed sub-ischemic ergometer training. Training occurred for 4 weeks and consisted of several bouts of exercise/ day (6) at 3.5 km/h in the first two groups and 6 times per day for 10 minutes at 70% max HR for the sub-ischemic group. Researchers found that the group that performed sub-ischemic exercise training was the only group to increase CPC number following training. Increases in CPC function (incorporation into endothelial networks) was increased in all three groups accompanied by an increase in the CXCR4, the receptor of SDF-1 which has been found to be important in EPC homing. Plasma VEGF levels were increased in the ischemic exercise group, but not in the other two

groups. Therefore, although EPC function, measured via incorporation of cells into endothelial networks, was increased in all three groups with exercise training, only the ischemic exercise group appeared to increase VEGF, which may have stimulated the increase in CPC numbers. Thijssen et al (118) reported no change in $CD34^+/KDR^+$ cells in healthy young and older participants following 8 weeks of cycle exercise training for 20 minutes 3 times per week at 65% of heart rate reserve. Therefore, exercise training may not increase EPC number in healthy individuals.

Hoetzer, et al (54) utilized assays for clonogenic and migratory capacity to assess EPC function in middle-aged and older men before and after 3 months of home-based exercise training. At baseline, the clonogenic capacity in these two groups was lower than a group of young sedentary individuals. Migratory capacity was lower in older compared to younger men. Both colony-forming and migratory capacity increased in middle-aged and older men after the training program.

Overall, exercise training appears to increase resting EPC number in mice and patients with CAD or CAD risk factors, and patients with PAOD, however it is not clear whether training increases EPC number in healthy individuals. The EPC increase in patients was accompanied by an increase in endothelial function measured by flow-mediated dilation (FMD) and NO. As in the studies on acute exercise, VEGF appeared to increase with exercise training in patients with ischemic disease, although, VEGF increased in mice in the Laufs, et al (2004) study. There does exist the possibility that there are two different mechanisms of action between the ischemic patient group and healthy subjects. Exercise training also appears to increase EPC clonogenic capacity, migration, incorporation into vascular networks and the CXCR4 receptor, the receptor for

SDF-1, which has been implicated in EPC release. Interestingly, none of the exercise training studies or acute exercise studies showed an increase in SDF-1. Therefore, the receptor may work in homing without changes in SDF-1 concentration but the mechanisms are still not clear.

APPENDIX A

Limitations of the Study

1) One of the limitations to the present study was the small sample size. The sample size for this study was calculated based on many factors that were unknown at the time the study was designed. Given the large amount of variability in many of the EPC measures, the inclusion of more subjects may have revealed more significant relationships, differences and effects.

2) We proposed to match our research groups by age and BMI, which we performed successfully. Despite our efforts, other factors differed between the groups other than VO_{2max} and training status. We did not observe any direct relationship between these factors and EPCs specifically; however, we can not disregard the possibility that some of these factors may have influenced the results of the present study.

3) There is evidence that EPCs may be influenced by gender (53). Since we only used men in the present study, we can only relate our results to this specific population.

4) The interpretation of our data should be confined to our definitions of EPC number and clonogenic capacity. We defined EPCs with commonly accepted markers (CD34 and KDR), and used a commercially available kit for EPC clonogenic capacity for which endothelial cell lineage has been verified (51). The field of EPC research is relatively new and currently there is not an accepted definition of EPCs nor are there

consistent methods to quantify them or evaluate their functional characteristics. We can not ignore the possibility that we may have seen different results if we defined EPCs with different cell markers or cultured EPCs with a different method.

5) We can not eliminate the possibility that the repeated measures results following the 10-day training cessation were an effect of regression to the mean. To evaluate whether the changes observed following the training cessation were an actual effect, the study design would have to have included repeated measures in the control group 10 days following their second visit.

APPENDIX B

Definition of Terms

Angiogenesis: The growth of new blood vessels from pre-existing vessels.

Antioxidant: A molecule that can prevent the oxidation of other molecules, thereby decreasing free radicals that lead to cellular damage.

Atherosclerosis: The development of plaque-like lesions on the interior of the vessel wall via a cascade that begins with damage to the endothelial layer, subsequent infiltration of monocytes and macrophages and inflammatory response.

Bioavailability: The amount of substance or drug that is present in a usable, bioactive form at the site of action. Bioavailability is dependent upon the rate of production and degradation.

CD34: A cell surface glycoprotein that is found on cells from the umbilical cord and bone marrow. They are pluripotent hematopoietic progenitors.

Endothelial-dependent dilation: The relaxation of the smooth muscle layer of a blood vessel due to the production of nitric oxide from the endothelial layer.

Endothelial function: the capacity of the endothelium to regulate vascular tone, immune cell filtration, and angiogenesis. Endothelial dysfunction is the loss of this capacity and is an early hallmark of cardiovascular disease. In vascular biology, endothelial function refers to the endothelial-dependent dilation that is mediated by nitric oxide production and bioavailability.

Kinase insert domain receptor (KDR, VEGFR-2, CD309): A tyrosine kinase receptor found mainly on vascular endothelial cells that serves as the receptor for vascular endothelial growth factor (VEGF).

Maximal oxygen uptake (VO_{2max}): An indicator of cardiorespiratory fitness, the maximum capacity of an individual's body to transport and utilize oxygen during incremental exercise.

Monoclonal antibody (mAb): Monospecific antibodies that are identical because they are produced by one type of immune cell that are all clones of a single parent cell.

Nitric oxide (NO): The primary vasodilator in vascular beds. It is a transient signal molecule produced from L-arginine by endothelial nitric oxide synthase (eNOS).

Oxidative stress: The state of imbalance between the production of reactive oxygen and a biological system's ability to detoxify the reactive intermediates or easily repair the resulting damage.

Oxidized low-density lipoprotein (OxLDL): the oxidative modification of LDL that is involved in the development of atherosclerotic lesions via uptake by scavenger receptors and foam cell formation.

Reactive hyperemia: increase in blood flow following a transient period of ischemia.

Vasculogenesis: is the process of blood vessel formation occurring by a *de novo* production of endothelial cells.

BIBLIOGRAPHY

1. **Adams V, Lenk K, Linke A, Lenz D, Erbs S, Sandri M, Tarnok A, Gielen S, Emmrich F, Schuler G and Hambrecht R**. Increase of circulating endothelial progenitor cells in patients with coronary artery disease after exercise-induced ischemia. *Arterioscler Thromb Vasc Biol* 24: 684-690, 2004.

2. **Adams V, Linke A, Krankel N, Erbs S, Gielen S, Mobius-Winkler S, Gummert JF, Mohr FW, Schuler G and Hambrecht R**. Impact of regular physical activity on the NAD(P)H oxidase and angiotensin receptor system in patients with coronary artery disease. *Circulation* 111: 555-562, 2005.

3. **Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM and Dimmeler S**. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 9: 1370-1376, 2003.

4. **Aicher A, Zeiher AM and Dimmeler S**. Mobilizing endothelial progenitor cells. *Hypertension* 45: 321-325, 2005.

5. **Asahara T and Kawamoto A**. Endothelial progenitor cells for postnatal vasculogenesis. *Am J Physiol Cell Physiol* 287: C572-C579, 2004.

6. **Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G and Isner JM.** Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964-966, 1997.

7. **Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M and Isner JM**. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 18: 3964-3972, 1999.

8. **Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, Rovner A, Ellis SG, Thomas JD, DiCorleto PE, Topol EJ and Penn MS**. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 362: 697-703, 2003.

9. **Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S and Zeiher AM**. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 106: 3009-3017, 2002.

10. **Assmus B, Honold J, Schachinger V, Britten MB, Fischer-Rasokat U, Lehmann R, Teupe C, Pistorius K, Martin H, Abolmaali ND, Tonn T,**

Dimmeler S and Zeiher AM. Transcoronary transplantation of progenitor cells after myocardial infarction. *N Engl J Med* 355: 1222-1232, 2006.

11. **Awad O, Dedkov EI, Jiao C, Bloomer S, Tomanek RJ and Schatteman GC**. Differential healing activities of CD34+ and CD14+ endothelial cell progenitors. *Arterioscler Thromb Vasc Biol* 26: 758-764, 2006.

12. **Bergholm R, Mäkimattila S, Valkonen M, Liu ML, Lahdenperä S, Taskinen MR, Sovijärvi A, Malmberg P and Yki-Järvinen H**. Intense physical training decreases circulating antioxidants and endothelium-dependent vasodilatation *in vivo*. *Atherosclerosis* 145: 341-349, 1999.

13. **Bonsignore MR, Morici G, Santoro A, Pagano M, Cascio L, Bonanno A, Abate P, Mirabella F, Profita M, Insalaco G, Gioia M, Vignola AM, Majolino I, Testa U and Hogg JC**. Circulating hematopoietic progenitor cells in runners. *J Appl Physiol* 93: 1691-1697, 2002.

14. **Brandes RP, Fleming I and Busse R**. Endothelial aging. *Cardiovascular Research* 66: 286-294, 2005.

15. **Britten MB, Abolmaali ND, Assmus B, Lehmann R, Honold J, Schmitt J, Vogl TJ, Martin H, Schachinger V, Dimmeler S and Zeiher AM**. Infarct remodeling after intracoronary progenitor cell treatment in patients with acute myocardial infarction (TOPCARE-AMI): Mechanistic insights from serial contrast-enhanced magnetic resonance imaging. *Circulation* 108: 2212-2218, 2003.

16. **Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL and Feron O**. Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statins. *Circ Res* 89: 866-873, 2001.

17. **Callaghan MJ, Ceradini DJ and Gurtner GC**. Hyperglycemia-induced reactive oxygen species and impaired endothelial progenitor cell function. *Antioxidants & Redox Signaling* 7: 1476-1482, 2005.

18. **Chen MC, Yip HK, Chen CJ, Yang CH, Wu CJ, Cheng CI, Chen YH, Chai HT, Lee CP and Chang HW**. No age-related change in circulating endothelial progenitor cells in healthy subjects. *Int Heart J* 47: 95-105, 2006.

19. **Chen YH, Lin SJ, Lin FY, Wu TC, Tsao CR, Huang PH, Liu PL, Chen YL and Chen JW**. High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms. *Diabetes* 56: 1559-1568, 2007.

20. **Corretti MC, Plotnick GD and Vogel RA**. Technical aspects of evaluating brachial artery vasodilatation using high-frequency ultrasound. *Am J Physiol Heart Circ Physiol* 268: H1397-H1404, 1995.

21. **Data SA, Roltsch MH, Hand B, Ferrell RE, Park JJ and Brown MD**. eNOS T-786C genotype, physical activity, and peak forearm blood flow in females. *Med Sci Sports Exerc* 35: 1991-1997, 2003.

22. **Delp MD**. Effects of exercise training on endothelium-dependent peripheral vascular responsiveness. *Med Sci Sports Exerc* 27: 1152-1157, 1995.

23. **Delp MD, McAllister RM and Laughlin MH**. Exercise training alters endothelium-dependent vasoreactivity of rat abdominal aorta. *J Appl Physiol* 75: 1354-1363, 1993.

24. **Demirbag R, Yilmaz R and Kocyigit A**. Relationship between DNA damage, total antioxidant capacity and coronary artery disease. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* 570: 197-203, 2005.

25. **Dernbach E, Urbich C, Brandes RP, Hofmann WK., Zeiher AM. and Dimmeler S**. Antioxidative stress-associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress. *Blood* 104: 3591-3597, 2004.

26. **Dimmeler S, Aicher A, Vasa M, Mildner-Rihm C, Adler K, Tiemann M, Rutten H, Fichtlscherer S, Martin H and Zeiher AM**. HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway. *J Clin Invest* 108: 391-397, 2001.

27. **Dimmeler S, Zeiher AM and Schneider MD**. Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 115: 572-583, 2005.

28. **Dimmeler S and Vasa-Nicotera M**. Aging of progenitor cells: limitation for regenerative capacity? *J Am Coll Cardiol* 42: 2081-2082, 2003.

29. **Dimmeler S, Haendeler J, Galle J and Zeiher AM**. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases : A mechanistic clue to the `Response to Injury' hypothesis. *Circulation* 95: 1760-1763, 1997.

30. **Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, Peacocke M and Campisi J**. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA* 92: 9363-9367, 1995.

31. **Fadini GP, Baesso I, Albiero M, Sartore S, Agostini C and Avogaro A**. Technical notes on endothelial progenitor cells: Ways to escape from the knowledge plateau. *Atherosclerosis* 197: 496-503, 2008.

32. **Finkel T and Holbrook NJ**. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247, 2000.

33. **Foncea R, Carvajal C, Almarza C and Leighton F**. Endothelial cell oxidative stress and signal transduction. *Biol Res* 33: 89-96, 2000.

34. **Fryburg DA**. NG-monomethyl-L-arginine inhibits the blood flow but not the insulin-like response of forearm muscle to IGF- I: Possible role of nitric oxide in muscle protein synthesis. *J Clin Invest* 97: 1319-1328, 1996.

35. **Fujiyama S, Amano K, Uehira K, Yoshida M, Nishiwaki Y, Nozawa Y, Jin D, Takai S, Miyazaki M, Egashira K, Imada T, Iwasaka T and Matsubara H**. Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells. *Circ Res* 93: 980-989, 2003.

36. **Galasso G, Schiekofer S, Sato K, Shibata R, Handy DE, Ouchi N, Leopold JA, Loscalzo J and Walsh K**. Impaired angiogenesis in glutathione peroxidase-1-deficient mice is associated with endothelial progenitor cell dysfunction. *Circ Res* 98: 254-261, 2006.

37. **Ghani U, Shuaib A, Salam A, Nasir A, Shuaib U, Jeerakathil T, Sher F, O'Rourke F, Nasser AM, Schwindt B and Todd K**. Endothelial progenitor cells during cerebrovascular disease. *Stroke* 36: 151-153, 2005.

38. **Gielen S, Erbs S, Linke A, Mobius-Winkler S, Schuler G and Hambrecht R**. Home-based versus hospital-based exercise programs in patients with coronary artery disease: effects on coronary vasomotion. *Am Heart J* 145: E3, 2003.

39. **Giordano FJ**. Oxygen, oxidative stress, hypoxia, and heart failure. *J Clin Invest* 115: 500-508, 2005.

40. **Goodell MA, Kinney-Freeman S and Camargo FD**. Isolation and characterization of side population cells. *Methods Mol Biol* 290: 343-352, 2005.

41. **Haendeler J, Hoffmann J, Brandes RP, Zeiher AM and Dimmeler S**. Hydrogen peroxide triggers nuclear export of telomerase reverse transcriptase via Src kinase family-dependent phosphorylation of tyrosine 707. *Mol Cell Biol* 23: 4598-4610, 2003.

42. **Haendeler J, Hoffmann J, Diehl JF, Vasa M, Spyridopoulos I, Zeiher AM and Dimmeler S**. Antioxidants inhibit nuclear export of telomerase reverse transcriptase and delay replicative senescence of endothelial Cells. *Circ Res* 94: 768-775, 2004.

43. **Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, Schoene N and Schuler G**. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *N Engl J Med* 342: 454-460, 2000.

44. **Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW and Schuler G**. Regular physical

activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation* 107: 3152-3158, 2003.

45. **Haram PM, Adams V, Kemi OJ, Brubakk AO, Hambrecht R, Ellingsen O and Wisloff U**. Time-course of endothelial adaptation following acute and regular exercise. *Eur J Cardiovasc Prev Rehabil* 13: 585-591, 2006.

46. **Haram PM, Kemi OJ and Wisloff U**. Adaptation of endothelium to exercise training: insights from experimental studies. *Front Biosci* 13: 336-346, 2008.

47. **Harraz M, Jiao C, Hanlon HD, Hartley RS and Schatteman GC**. CD34-blood-derived human endothelial cell progenitors. *Stem Cells* 19: 304-312, 2001.

48. **Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA and Rafii S**. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 193: 1005-1014, 2001.

49. **He T, Peterson TE, Holmuhamedov EL, Terzic A, Caplice NM, Oberley LW and Katusic ZS**. Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. *Arterioscler Thromb Vasc Biol* 24: 2021-2027, 2004.

50. **Heiss C, Keymel S, Niesler U, Ziemann J, Kelm M and Kalka C**. Impaired progenitor cell activity in age-related endothelial dysfunction. *Journal of the American College of Cardiology* 45: 1441-1448, 2005.

51. **Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA and Finkel T**. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 348: 593-600, 2003.

52. **Hirschmann-Jax C, Foster AE, Wulf GG, Goodell MA and Brenner MK**. A distinct "side population" of cells in human tumor cells: implications for tumor biology and therapy. *Cell Cycle* 4: 203-205, 2005.

53. **Hoetzer GL, MacEneaney OJ, Irmiger HM, Keith R, Van Guilder GP, Stauffer BL and Desouza CA**. Gender differences in circulating endothelial progenitor cell colony-forming capacity and migratory activity in middle-aged adults. *Am J Cardiol* 99: 46-48, 2007.

54. **Hoetzer GL, Van Guilder GP, Irmiger H, Keith RS, Stauffer BL and Desouza CA**. Aging, exercise and endothelial progenitor cell clonogenic and migratory capacity in men. *J Appl Physiol* 2006.

55. **Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, Collen D, Muls E and Van de Werf F**. Circulating oxidized LDL is a useful

marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol* 21: 844-848, 2001.

56. **Holvoet P, Vanhaecke J, Janssens S, Van de Werf F and Collen D**. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 98: 1487-1494, 1998.

57. **Honold J, Lehmann R, Heeschen C, Walter DH, Assmus B, Sasaki K, Martin H, Haendeler J, Zeiher AM and Dimmeler S**. Effects of granulocyte colony simulating factor on functional activities of endothelial progenitor cells in patients with chronic ischemic heart disease. *Arterioscler Thromb Vasc Biol* 26: 2238-2243, 2006.

58. **Hristov M, Erl W, Linder S and Weber PC**. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells *in vitro*. *Blood* 104: 2761-2766, 2004.

59. **Hristov M, Erl W and Weber PC**. Endothelial progenitor cells: Mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol* 23: 1185-1189, 2003.

60. **Hristov M and Weber C**. Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. *J Cell Mol Med* 8: 498-508, 2004.

61. **Hulthe J and Fagerberg B**. Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study). *Arterioscler Thromb Vasc Biol* 22: 1162-1167, 2002.

62. **Imanishi T, Hano T, Matsuo Y and Nishio I**. Oxidized low-density lipoprotein inhibits vascular endothelial growth factor-induced endothelial progenitor cell differentiation. *Clinical and Experimental Pharmacology and Physiology* 30: 665-670, 2003.

63. **Imanishi T, Hano T, Sawamura T and Nishio I**. Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clinical and Experimental Pharmacology and Physiology* 31: 407-413, 2004.

64. **Imanishi T, Moriwaki C, Hano T and Nishio I**. Endothelial progenitor cell senescence is accelerated in both experimental hypertensive rats and patients with essential hypertension. *J Hypertens* 23: 1831-1837, 2005.

65. **Ingram DA, Caplice NM and Yoder MC**. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. *Blood* 106: 1525-1531, 2005.

66. **Ingram DA, Krier TR, Mead LE, McGuire C, Prater DN, Bhavsar J, Saadatzadeh MR, Bijangi-Vishehsaraei K, Li F, Yoder MC and Haneline LS**.

Clonogenic endothelial progenitor cells are sensitive to oxidative stress. *Stem Cells* 25: 297-304, 2007.

67. **Irani K**. Oxidant signaling in vascular cell growth, death, and survival : A review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. *Circ Res* 87: 179-183, 2000.

68. **Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T and Losordo DW**. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. *Circulation* 108: 3115-3121, 2003.

69. **Jin DK, Shido K, Kopp HG, Petit I, Shmelkov SV, Young LM, Hooper AT, Amano H, Avecilla ST, Heissig B, Hattori K, Zhang F, Hicklin DJ, Wu Y, Zhu Z, Dunn A, Salari H, Werb Z, Hackett NR, Crystal RG, Lyden D and Rafii S**. Cytokine-mediated deployment of SDF-1 induces revascularization through recruitment of CXCR4+ hemangiocytes. *Nat Med* 12: 557-567, 2006.

70. **Jin H, Aiyer A, Su J, Borgstrom P, Stupack D, Friedlander M and Varner J**. A homing mechanism for bone marrow-derived progenitor cell recruitment to the neovasculature. *J Clin Invest* 116: 652-662, 2006.

71. **Kawamoto A. and Asahara T**. Role of progenitor endothelial cells in cardiovascular disease and upcoming therapies. *Catheterazation and Cardiovascular Interventions* 70: 477-484, 7 A.D.

72. **Kogata N, Arai Y, Pearson JT, Hashimoto K, Hidaka K, Koyama T, Somekawa S, Nakaoka Y, Ogawa M, Adams RH, Okada M and Mochizuki N**. Cardiac ischemia activates vascular endothelial cadherin promoter in both preexisting vascular cells and bone marrow cells involved in neovascularization. *Circ Res* 98: 897-904, 2006.

73. **Kondo T, Hayashi M, Takeshita K, Numaguchi Y, Kobayashi K, Iino S, Inden Y and Murohara T**. Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers. *Arterioscler Thromb Vasc Biol* 24: 1442-1447, 2004.

74. **Kurz DJ, Decary S, Hong Y, Trivier E, Akhmedov A and Erusalimsky JD**. Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *J Cell Sci* 117: 2417-2426, 2004.

75. **Langer H, May AE, Daub K, Heinzmann U, Lang P, Schumm M, Vestweber D, Massberg S, Schonberger T, Pfisterer I, Hatzopoulos AK and Gawaz M**. Adherent platelets recruit and induce differentiation of murine embryonic endothelial progenitor cells to mature endothelial cells *in vitro*. *Circ Res* 98: e2-10, 2006.

76. **Larsen FJ, Ekblom B, Sahlin K, Lundberg JO and Weitzberg E**. Effects of dietary nitrate on blood pressure in healthy volunteers. *N Engl J Med* 355: 2792-2793, 2006.

77. **Laufs U, Urhausen A, Werner N, Scharhag J, Heitz A, Kissner G, Bohn M, Kindermann W and Nickening G**. Running exercise of different duration and intensity: effect on endothelial progenitor cells in healthy subjects. *Eur J Cardiovasc Prev Rehabil* 12: 407-414, 2005.

78. **Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M and Nickenig G**. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation* 109: 220-226, 2004.

79. **Linke A, Schoene N, Gielen S, Hofer J, Erbs S, Schuler G and Hambrecht R**. Endothelial dysfunction in patients with chronic heart failure: systemic effects of lower-limb exercise training. *J Am Coll Cardiol* 37: 392-397, 2001.

80. **Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, Walsh K, Isner JM and Asahara T**. HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells. *J Clin Invest* 108: 399-405, 2001.

81. **Losordo DW and Dimmeler S**. Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. *Circulation* 109: 2692-2697, 2004.

82. **Ma FX, Zhou B, Chen Z, Ren Q, Lu SH, Sawamura T and Han ZC**. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *J Lipid Res* 47: 1227-1237, 2006.

83. **MIller NJ and Rice-Evans CA**. Factors influencing the antioxidant activity determined by the ABTS.+ radical cation assay. *Free Radical Research* 26: 195-199, 1997.

84. **Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H and Komuro I**. Endothelial cell senescence in human atherosclerosis: Role of telomere in endothelial dysfunction. *Circulation* 105: 1541-1544, 2002.

85. **Moore MA, Hattori K, Heissig B, Shieh JH, Dias S, Crystal RG and Rafii S**. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Ann N Y Acad Sci* 938: 36-45, 2001.

86. **Murakami T, Takagi H, Suzuma K, Suzuma I, Ohashi H, Watanabe D, Ojima T, Suganami E, Kurimoto M, Kaneto H, Honda Y and Yoshimura N**. Angiopoietin-1 attenuates H2O2-induced SEK1/JNK phosphorylation through the phosphatidylinositol 3-Kinase/Akt pathway in vascular endothelial cells. *J Biol Chem* 280: 31841-31849, 2005.

87. **Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW and Asahara T**. Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. *Circulation* 106: 1133-1139, 2002.

88. **Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes JF, Fishman MC, Huang PL and Isner JM**. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101: 2567-2578, 1998.

89. **Natori T, Sata M, Washida M, Hirata Y, Nagai R and Makuuchi M**. G-CSF stimulates angiogenesis and promotes tumor growth: potential contribution of bone marrow-derived endothelial progenitor cells. *Biochem Biophys Res Commun* 297: 1058-1061, 2002.

90. **Pellegatta F, Bragheri M, Grigore L, Raselli S, Maggi FM, Brambilla C, Reduzzi A, Pirillo A, Norata GD and Catapano AL**. *In vitro* isolation of circulating endothelial progenitor cells is related to the high density lipoprotein plasma levels. *Int J Mol Med* 17: 203-208, 2006.

91. **Pistrosch F, Herbrig K, Oelschlaegel U, Richter S, Passauer J, Fischer S and Gross P**. PPARgamma-agonist rosiglitazone increases number and migratory activity of cultured endothelial progenitor cells. *Atherosclerosis* 183: 163-167, 2005.

92. **Pollock DM and Pollock JS**. Endothelin and oxidative stress in the vascular system. *Curr Vasc Pharmacol* 3: 365-367, 2005.

93. **Prater DN, Case J, Ingram DA and Yoder MC**. Working hypothesis to redefine endothelial progenitor cells. *Leukemia* 21: 1141-1149, 2007.

94. **Ray R and Shah AM**. NADPH oxidase and endothelial cell function. *Clin Sci* 109: 217-226, 2005.

95. **Rehman J, Li J, Parvathaneni L, Karlsson G, Panchal VR, Temm CJ, Mahenthiran J and March KL**. Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells. *J Am Coll Cardiol* 43: 2314-2318, 2004.

96. **Ross R**. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362: 801-809, 1993.

97. **Rush JW, Denniss SG and Graham DA**. Vascular nitric oxide and oxidative stress: determinants of endothelial adaptations to cardiovascular disease and to physical activity. *Can J Appl Physiol* 30: 442-474, 2005.

98. **Sandri M, Adams V, Gielen S, Linke A, Lenk K, Krankel N, Lenz D, Erbs S, Scheinert D, Mohr FW, Schuler G and Hambrecht R**. Effects of exercise and

ischemia on mobilization and functional activation of blood-derived progenitor cells in patients with ischemic syndromes: Results of 3 randomized studies. *Circulation* 111: 3391-3399, 2005.

99. **Sarto P, Balducci E, Balconi G, Fiordaliso F, Merlo L, Tuzzato G, Pappagallo GL, Frigato N, Zanocco A, Forestieri C, Azzarello G, Mazzucco A, Valenti MT, Alborino F, Noventa D, Vinante O, Pascotto P, Sartore S, Dejana E and Latini R**. Effects of exercise training on endothelial progenitor cells in patients with chronic heart failure. *Journal of Cardiac Failure* 13: 701-708, 2007.

100. **Sbaa E, Dewever J, Martinive P, Bouzin C, Frerart F, Balligand JL, Dessy C and Feron O**. Caveolin plays a central role in endothelial progenitor cell mobilization and homing in SDF-1-driven postischemic vasculogenesis. *Circ Res* 98: 1219-1227, 2006.

101. **Schachinger V, Assmus B, Britten MB, Honold J, Lehmann R, Teupe C, Abolmaali ND, Vogl TJ, Hofmann WK, Martin H, Dimmeler S and Zeiher AM**. Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. *J Am Coll Cardiol* 44: 1690-1699, 2004.

102. **Schachinger V, Britten MB and Zeiher AM**. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation* 101: 1899-1906, 2000.

103. **Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S and Zeiher AM**. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: Proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 111: 2981-2987, 2005.

104. **Schulz R and Triggle CR**. Role of NO in vascular smooth muscle and cardiac muscle function. *Trends in Pharmacological Sciences* 15: 255-259, 1994.

105. **Shaffer RG, Greene S, Arshi A, Supple G, Bantly A, Moores JS, Parmacek MS and Mohler ER, III**. Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease. *Vascular Medicine* 11: 219-226, 2006.

106. **Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF and Hammond WP**. Evidence for circulating bone marrow-derived endothelial cells. *Blood* 92: 362-367, 1998.

107. **Shimada K, Mokuno H, Matsunaga E, Miyazaki T, Sumiyoshi K, Kume A, Miyauchi K and Daida H**. Predictive value of circulating oxidized LDL for cardiac events in type 2 diabetic patients with coronary artery disease. *Diabetes Care* 27: 843-844, 2004.

108. **Shimada K, Mokuno H, Matsunaga E, Miyazaki T, Sumiyoshi K, Miyauchi K and Daida H**. Circulating oxidized low-density lipoprotein is an independent predictor for cardiac event in patients with coronary artery disease. *Atherosclerosis* 174: 343-347, 2004.

109. **Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, Sasaki K, Shimada T, Oike Y and Imaizumi T**. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* 103: 2776-2779, 2001.

110. **Sonveaux P, Martinive P, Dewever J, Batova Z, Daneau G, Pelat M, Ghisdal P, Gregoire V, Dessy C, Balligand JL and Feron O**. Caveolin-1 expression is critical for vascular endothelial growth factor-induced ischemic hindlimb collateralization and nitric oxide-mediated angiogenesis. *Circ Res* 95: 154-161, 2004.

111. **Steiner S, Niessner A, Ziegler S, Richter B, Seidinger D, Pleiner J, Penka M, Wolzt M, Huber K, Wojita J, Minar E and Kopp CW**. Endurance training increases the number of endothelial progenitor cells in patients with cardiovascular risk and coronary artery disease. *Atherosclerosis* 181: 305-310, 2005.

112. **Strehlow K, Werner N, Berweiler J, Link A, Dirnagl U, Priller J, Laufs K, Ghaeni L, Milosevic M, Bohm M and Nickenig G**. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation* 107: 3059-3065, 2003.

113. **Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Jr. and Lerman A**. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. *Circulation* 101: 948-954, 2000.

114. **Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM and Asahara T**. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 5: 434-438, 1999.

115. **Takamiya M, Okigaki M, Jin D, Takai S, Nozawa Y, Adachi Y, Urao N, Tateishi K, Nomura T, Zen K, Ashihara E, Miyazaki M, Tatsumi T, Takahashi T and Matsubara H**. Granulocyte colony-stimulating factor-mobilized circulating c-Kit+/Flk-1+ progenitor cells regenerate endothelium and inhibit neointimal hyperplasia after vascular injury. *Arterioscler Thromb Vasc Biol* 26: 751-757, 2006.

116. **Tarzami ST, Wang G, Li W, Green L and Singh JP**. Thrombin and PAR-1 stimulate differentiation of bone marrow-derived endothelial progenitor cells. *J Thromb Haemost* 4: 656-663, 2006.

117. **Tepper OM, Galiano RD, Capla JM, Kalka C, Gagne PJ, Jacobowitz GR, Levine JP and Gurtner GC.** Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 106: 2781-2786, 2002.

118. **Thijssen DH, Vos JB, Verseyden C, van Zonneveld AJ, Smits P, Sweep FC, Hopman MT and de Boer HC**. Haematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. *Aging Cell* 5: 495-503, 2006.

119. **Thum T, Fraccarollo D, Schultheiss M, Froese S, Galuppo P, Widder JD, Tsikas D, Ertl G and Bauersachs J**. Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes. *Diabetes* 56: 666-674, 2007.

120. **Touyz RM and Schiffrin EL**. Reactive oxygen species in vascular biology: implications in hypertension. *Histochem Cell Biol* 122: 339-352, 2004.

121. **Tso C, Martinic G, Fan WH, Rogers C, Rye KA and Barter PJ**. High-density lipoproteins enhance progenitor-mediated endothelium repair in mice. *Arterioscler Thromb Vasc Biol* 26: 1144-1149, 2006.

122. **Urbich C, Aicher A, Heeschen C, Dernbach E, Hofmann WK, Zeiher AM and Dimmeler S**. Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells. *J Mol Cell Cardiol* 39: 733-742, 2005.

123. **Urbich C and Dimmeler S**. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res* 95: 343-353, 2004.

124. **Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G and Ferrari R**. CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. *Circulation* 110: 1209-1212, 2004.

125. **Van Craenenbroeck EM, Vrints CJ, Haine SE, Vermeulen K, Goovaerts I, Van Tendeloo VF, Hoymans VY and Conraads VM**. A maximal exercise bout increases the number of circulating CD34+/KDR+ endothelial progenitor cells in healthy subjects. Relation with lipid profile. *J Appl Physiol* 01210, 2008.

126. **Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM and Dimmeler S**. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 89: 1e-7, 2001.

127. **Vasankari TJ, Kujala UM, Vasankari TM and Ahotupa M.** Reduced oxidized LDL levels after a 10-month exercise program. *Med Sci Sports Exerc* 30: 1496-1501, 1998.

128. **Verma NK, Singh J and Dey CS**. PPAR-gamma expression modulates insulin sensitivity in C2C12 skeletal muscle cells. *Br J Pharmacol* 143: 1006-1013, 2004.

129. **Vuorimaa T, Ahotupa M, Irjala K and Vasankari T**. Acute prolonged exercise reduces moderately oxidized LDL in healthy men. *Int J Sports Med* 26: 420-425, 2005.

130. **Walter DH, Rittig K, Bahlmann FH, Kirchmair R, Silver M, Murayama T, Nishimura H, Losordo DW, Asahara T and Isner JM.** Statin therapy accelerates reendothelialization: A novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells. *Circulation* 105: 3017-3024, 2002.

131. **Wang JS, Chen YW, Chow SE, Ou HC and Sheu WH**. Exercise paradoxically modulates oxidized low density lipoprotein-induced adhesion molecules expression and treans-endothelial migration of monocytes in men. *Thromb Haemost* 94: 846-852, 5 A.D.

132. **Wang X, Chen J, Tao Q, Zhu J and Shang Y**. Effects of ox-LDL on number and activity of circulating endothelial progenitor cells. *Drug Chem Toxicol* 27: 243-255, 2004.

133. **Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M and Nickenig G**. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med* 353: 999-1007, 2005.

134. **Werner C, Kamani CH, Gensch C, Bohm M and Laufs U**. The peroxisome proliferator activated receptor-{gamma} agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance. *Diabetes* 56: 2609-2615, 2007.

135. **Werner N, Wassmann S, Ahlers P, Schiegl T, Kosiol S, Link A, Walenta K and Nickenig G**. Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease. *Basic Res Cardiol* 102: 565-571, 2007.

136. **Wilson PW, D'Agostino RB, Levy D, Belanger AM, Silbershatz H and Kannel WB**. Prediction of coronary heart disease using risk factor categories. *Circulation* 97: 1837-1847, 1998.

137. **Wojakowski W and Tendera M**. Mobilization of bone marrow-derived progenitor cells in acute coronary syndromes. *Folia Histochem Cytobiol* 43: 229-232, 2005.

138. **Wollert KC, Meyer GP, Lotz J, Ringes Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A and Drexler H**. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *The Lancet* 364: 141-148.

139. **Xiao Q, Kiechl S, Patel S, Oberhollenzer F, Weger S, Mayr A, Metzler B, Reindl M, Hu Y, Willeit J and Xu Q**. Endothelial progenitor cells, cardiovascular risk factors, cytokine levels and atherosclerosis – Results from a large population-based study. *PLoS ONE* 2: e975, 2007.

140. **Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch-Marce M, Masuda H, Losordo DW, Isner JM and Asahara T**. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 107: 1322-1328, 2003.

141. **Yang J, Chang E, Cherry AM, Bangs CD, Oei Y, Bodnar A, Bronstein A, Chiu CP and Herron GS**. Human endothelial cell life extension by telomerase expression. *J Biol Chem* 274: 26141-26148, 1999.

142. **Yang Z, Wang JM, Chen L, Luo CF, Tang AL and Tao J**. Acute exercise-induced nitric oxide production contributes to upregulation of circulating endothelial progenitor cells in healthy subjects. *J Hum Hypertens* 21: 452-460, 2007.

143. **Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT and Ingram DA**. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood* 109: 1801-1809, 2007.

144. **Yoon CH, Hur J, Oh IY, Park KW, Kim TY, Shin JH, Kim JH, Lee CS, Chung JK, Park YB and Kim HS**. Intercellular adhesion molecule-1 is upregulated in ischemic muscle, which mediates trafficking of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* 26: 1066-1072, 2006.

145. **Zeng L, Xiao Q, Margariti A, Zhang Z, Zampetaki A, Patel S, Capogrossi MC, Hu Y and Xu Q**. HDAC3 is crucial in shear- and VEGF-induced stem cell differentiation toward endothelial cells. *J Cell Biol* jcb, 2006.

146. **Zengin E, Chalajour F, Gehling UM, Ito WD, Treede H, Lauke H, Weil J, Reichenspurner H, Kilic N and Ergun S**. Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133: 1543-1551, 2006.