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Effect of a 10 Day Decrease in Physical Activity on Circulating Angiogenic Cells

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Effect of a 10 day Decrease in Physical Activity on Circulating Angiogenic Cells

A Thesis Presented

By

GAYATRI GUHANARAYAN

Submitted to the Graduate School of the
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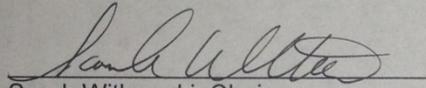
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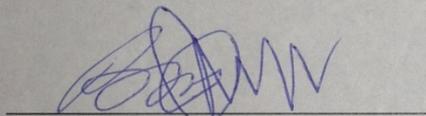
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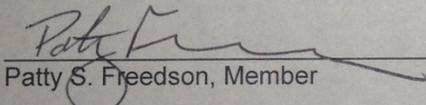
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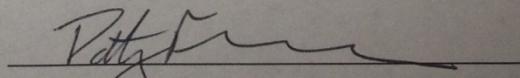
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ABSTRACT

EFFECT OF A 10 DAY DECREASE IN PHYSICAL ACTIVITY ON CIRCULATING ANGIOGENIC CELLS

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Circulating angiogenic cells (CACs) are early predictors of cardiovascular health and are inversely proportional to related outcomes. Increased number and function of CACs is seen in healthy individuals compared with individuals with cardiovascular disease (CVD). Exercise increases CAC number and function in CVD populations, through a nitric oxide-mediated mechanism. Inactivity is a growing concern in industrialized nations; it is an independent risk factor for CVD and is linked to increased mortality. The purpose of this study was to understand the effect of reduced physical activity (rPA) on two CAC populations (CFU-Hill and CD34⁺) in highly active individuals. We examined the mechanisms underlying changes in CAC function as a result of rPA with maintained energy balance. The two sub-populations of CACs responded differently to rPA. CFU-Hill CACs, decreased in number and amount of intracellular nitric oxide while CD34⁺ cells, did not change. Gene expression analyses indicated that oxidative stress-related genes did not change in CFU-Hill cells with rPA. However, correlations between CFU-Hill cell numbers, intracellular nitric oxide, and genes that are related to nitric oxide were observed. We concluded that rPA caused the observed decrease in CFU-Hill number and intracellular nitric oxide through a decrease in nitric oxide cellular availability, not oxidative stress.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death in the United States and worldwide.⁴⁴ Traditional risk factors for CVD include age, sex, hyperglycemia, high cholesterol, inactivity, tobacco smoke and diabetes mellitus. Despite the identification of these risk factors, CVD can go undiagnosed in individuals with early signs of the disease and it remains the dominant health problem worldwide.²⁸ There is a need to identify novel biomarkers that indicate disease progression early on. Detecting early stages of CVD will permit early intervention and reversal of disease by lifestyle intervention may be easier than with later stages of disease.

Endothelial dysfunction underlies the pathophysiology of a number of cardiovascular diseases including diabetes and renal failure.¹¹ The endothelial cell layer that acts as the interface between blood and underlying tissue has several functions including regulation of vascular tone and maintenance of local homeostasis. Damage to the endothelium decreases its ability to perform these roles. However, if there is an efficient repair mechanism that helps maintain endothelial function the endothelium can remain in a state of homeostasis and perform its roles optimally.

Bone marrow derived vaso-regenerative cells have been found to aid in the maintenance and repair of the endothelium. These cells have the capacity to differentiate into mature endothelial cells and incorporate themselves into pre-existing vasculature or form new

blood vessels. They have also been found to support vascular repair via a paracrine effect by which these cells secrete factors to assist repair of damaged endothelium.⁶⁴ The most highly studied of these cell types are circulating angiogenic cells (CACs).

Interest in CACs has been directed towards understanding their relationship to CVD and mechanisms that diminish their regenerative potential. The number of CACs are inversely correlated to the Framingham Risk Score for CVD and directly related to endothelial function.²¹ Further, circulating CAC numbers are lower in patients with cardiovascular events and these low numbers are a significant predictor of future CVD events.⁵⁵ Similar to mature endothelial cells, CAC function appears to be related to intracellular nitric oxide (NO). Decreased CAC NO leads to diminished CAC differentiation and incorporation into vascular networks.⁶⁰ Therefore, interventions to improve circulating CAC NO, and CAC function are likely important to decrease CVD. Exercise interventions in patients with CVD have been found to be successful in increasing circulating CAC number and function. A few studies have also shown that these cell numbers are higher in individuals who are chronically active compared to age-matched sedentary individuals.⁵ Importantly, exercise may increase CAC NO.^{27,67} Therefore, improvement of circulating CAC and maintenance of CAC NO may be key mechanisms through which physical activity improves vascular function and decreases rates of CVD.

Inactivity is one of the established risk factors for CVD and it is also associated with several other risk factors and disease states including diabetes mellitus, high cholesterol and hyperglycemia.³⁷ Models of inactivity that have been used to investigate changes in the endothelium include spinal cord injury, bed-rest and limb immobilization studies. They have

provided us with information on the drastic cardiorespiratory , vessel structure and FMD changes that can occur as a result of inactivity.⁵⁸ However, most of the models that have been used are extreme situations, very often confounded by underlying disease processes. It becomes difficult to make any direct associations between endothelial health and inactivity when using such models of inactivity.

We proposed that the CAC NO system is important to the maintenance of proper CAC function and that it is vulnerable to reduced physical activity. Therefore, the aim of this study was to examine the impact of reduced physical activity on CAC NO and CAC number. We investigated this by recruiting healthy, highly active, endurance trained runners. They completed a 10-day period of reduced physical activity where they did not perform their regular structured exercise and maintained energy balance. Blood was drawn before and after the 10 days to evaluate CAC number, CAC NO and CAC gene expression for a potential mechanism of reduced CAC NO production.

The specific aims and hypotheses for this study:

Aim 1: To study the effects of reduced physical activity (rPA) on circulating angiogenic cell (CAC) number.

Hypothesis 1: The rPA will reduce CAC number.

Aim 2: To evaluate the effects of rPA on CAC intracellular nitric oxide and genes that are regulators of nitric oxide.

Hypothesis 2: The rPA will cause a decrease in CAC intracellular nitric oxide, and genes that are regulators of nitric oxide.

Aim 3: To evaluate the relationship between changes in CAC number and nitric oxide-related CAC factors.

Hypothesis 3: There will be a positive correlation between the change in CAC number and the changes in nitric oxide related CAC factors with rPA.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review will cover the importance of studying cardiovascular disease in the United States and the relevance of studying endothelial function to prevent it. Circulating angiogenic cells (CACs), a novel biomarker for CVD will be introduced with regard to their role in endothelial maintenance. The role of both exercise and the impact of inactivity will be reviewed in reference to the number and function of CACs. Lastly, the mechanistic role of nitric oxide and its mediators in maintaining a healthy endothelium will also be reviewed.

2.2 Cardiovascular Disease and Endothelial Function

I. Cardiovascular disease:

Cardiovascular disease (CVD) is the leading cause of death worldwide.⁴⁴ In 2007, CVD accounted for 1 of every 3 deaths in the United States and the estimated direct and indirect costs associated with CVD were \$286.6 billion.⁵² Cardiovascular disease comprises of a group of conditions which include high blood pressure (HBP), coronary heart disease (CHD), heart failure (HF), stroke, angina pectoris and congenital cardiovascular defects. Coronary heart disease accounts for about half the CVD-related deaths while the others account for anywhere between 4-16% of deaths from CVD.⁵² Traditional risk factors for CVD include age, gender, heredity, smoking, high blood pressure, cholesterol, physical inactivity, overweight, obesity and diabetes mellitus. However, people without CVD risk factors still have coronary events and in fact 1/5 of

all CV events occur in individuals without traditional CVD risk factors.²⁸ Therefore novel pathways of risk detection such as markers for inflammation, oxidative stress, endothelial function, thrombosis and coagulation need to be further studied for their potential to identify individuals at risk.

II. Endothelial function:

a. CVD and endothelium

Coronary heart disease which accounts for about half the deaths due to CVD in the United States is a condition which causes changes in both the structure and function of blood vessels.³¹ Blood vessels are lined by endothelial cells which are responsible not just for maintenance of vascular tone and permeability but also in regulating leukocyte traffic and modulating vascular homeostasis and thrombosis.⁴⁸ As with any physiologically responsive tissue, the endothelium is susceptible to injury and will try to reestablish homeostasis when injured. Ross characterized atherosclerosis as part of the response to (endothelial) injury.⁵³ Early in the disease process, the endothelial cells become dysfunctional. The endothelial dysfunction leads to migration of cells under the surface of the endothelium where monocytes become macrophages, accumulate lipid, become foam cells and with the accompanying lymphocytes lead to fatty streak formation. While fatty streaks are evident in children and are fairly reversible, repeated injury leads to further accumulation of cells in these 'hot spots' and ultimately leads to fibrous plaque formation.⁵³ The progression of this atherosclerotic state is indicative of the endothelium being unable to perform one of its primary functions- protecting the vasculature.

b. Nitric oxide

Endothelial dysfunction has been shown to be the underlying cause of several cardiovascular diseases, renal failure and diabetes mellitus.¹¹ Studies have found flow mediated dilation (FMD) to be as effective as traditional risk factors as an independent predictor of endothelial dysfunction and cardiovascular events.⁵⁷ Changes in FMD are primarily nitric-oxide mediated.^{10,57} Nitric oxide is one of the most important substances released by the endothelium; it acts as a vasodilator, inhibits growth and inflammation, and has anti-aggregant effects on platelets. It helps maintain a healthy, non-atherogenic milieu in the vasculature. Decreased nitric oxide levels on the other hand have been directly linked to endothelial dysfunction in various disease states.¹¹

c. Lifestyle

Shear stress that accompanies regular exercise increases the synthesis and release of nitric oxide and thus improves endothelial function.⁴³ Healthy lifestyle factors such as regular participation in endurance exercise have been shown to attenuate the ageing-related decline in endothelial function.⁵¹ Rinder et al⁵¹ have found that endothelial-dependent dilation was greater in older athletes compared with age-matched healthy sedentary men. While sedentary lifestyle is associated with endothelial dysfunction and increased cardiovascular events³³ it seems that regular exercise confers some amount of protection to the endothelium.

d. Maintenance of endothelial function

Maintaining endothelial function is important in order to avoid the onset of all the cardiovascular diseases that begin with disruptions at the level of the endothelium. Maintaining a lifestyle that promotes vascular health is an essential part of endothelial maintenance. Exercise induced increases in shear stress and consequent increase in nitric oxide consist of only a part of the mechanism involved in maintaining a healthy endothelium. Circulating angiogenic cells, a group of adult stem cells that respond to NO release (among several other factors) have been found to play an integral role in the maintenance and repair of the endothelium.⁶⁴ They do so by forming new blood vessels or by repairing pre-existing vasculature. They mediate these effects by either directly becoming incorporated into the endothelium or via paracrine signals. The numbers and function of CACs are directly related to endothelial function^{35,21} and they are considered a biomarker for cardiovascular disease.^{56,13} CACs have been found to be dysfunctional in individuals with cardiovascular disease^{21,66} chronic kidney disease¹⁷ and diabetes.²⁰ Studies have been done to harness the regenerative potential of CACs to repair the vasculature in these patient populations. However, very little is known about the mechanisms that lead CACs to become dysfunctional in healthy individuals.

2.3 Circulating angiogenic cells (CACs)

I. Overview:

All blood cells arise from a type of cell called the hematopoietic stem cell (HSC) and they have the potential to differentiate into other cell types.²⁹ After birth, the bone marrow becomes the major factor in hematopoiesis where differentiation of the HSCs takes place. There are normally fewer than one HSC per 5×10^4 cells in the bone marrow and this makes them difficult to culture *in vitro*. However, when there is an increased demand for hematopoiesis, HSCs will display an enormous proliferative capacity. Circulating angiogenic cells (CACs) are one of the many cell types that emerge from this lineage. CACs were discovered in 1997 by Asahara and colleagues.³ They are a population of hematopoietic cells that can be isolated from circulating mononuclear cells, bone marrow and cord blood.

II. Function of CACs:

Circulating angiogenic cells play an important role in the maintenance and repair of vasculature. They do so by neo-vascularization- formation of new blood vessels or by re-endothelialization- repair of pre-existing vasculature.⁶⁴ They mediate these effects by either directly becoming incorporated into the endothelium or by a paracrine effect. Given the appropriate stimulus, such as exercise, they are first mobilized from their stem-cell niche in the bone marrow; from there, they migrate to the site of injury in the vasculature. With the aid of several other signaling molecules from the damaged vessel wall they either become incorporated at the site of injury or they enhance the repair process through the release of signaling factors such as growth factors, cytokines, and chemokines.²⁶ It is primarily the early

outgrowth CACs such as CFU-ECs that work via this paracrine mechanism by providing a potent mixture of growth factors to facilitate endothelial repair.^{13,22} The paracrine effect of transplanted marrow-derived cells has proved to be a critical mechanism underlying the angiogenic effect of cell therapy.³⁰ The paracrine mechanism has also been shown to restore the endothelial function of restored carotid arteries.¹⁸

While acute injury will cause an increase in CACs, it is hypothesized that repeated injury will eventually decrease the number and function of CACs. When CAC number is reduced or when the function of CACs is impaired, the endothelium is affected. The endothelium is the first site to be damaged before an atherosclerotic plaque is formed.⁵³ If CAC number is decreased or function is impaired, they will be unable to mitigate the consequences of endothelial damage. Repeated insult of the endothelial and sub-endothelial layers can lead to fibrous plaque formations.⁵³ This ultimately contributes to cardiovascular diseases such as atherosclerosis and hypertension.

III. Types of CACs:

The identification and definition of CACs has been controversial. The population of HSCs found to aid in the maintenance and repair of the endothelium has broadly been termed CACs but with a lack of a unified definition of CAC to date, there is a heterogeneous nature of this population. A few prominent markers and characteristics have been used to identify these cells and they include: CD34, CD133, CD31, VEGFR2, CD45, acLDL uptake, UEA-1 lectin binding, eNOS and vWF factor.²⁴ However there is still no consensus about the presence of these markers for all CAC populations. *In vitro* techniques used to isolate and culture these cells have

been used to broadly classify them into either early or late outgrowth cells as shown in the figure below.

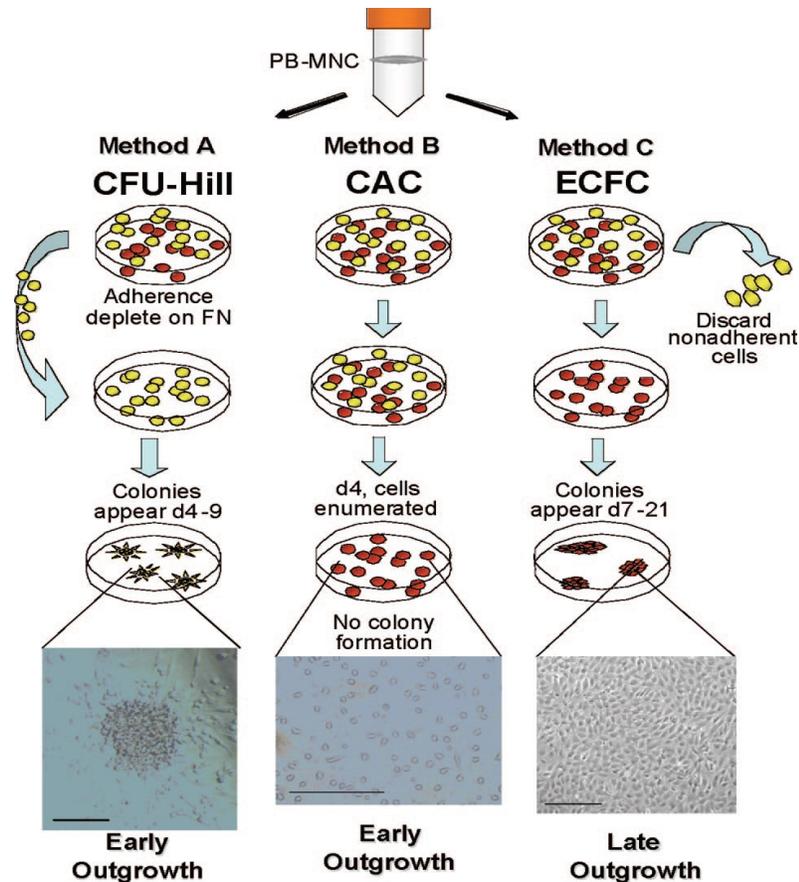


Figure 1. Different culturing techniques that lead to Early and Late Outgrowth CACs (Hirschi et al, 2008): The Early Outgrowth CACs aid in vascular recovery via paracrine effect. The Late Outgrowth CACs are more similar to endothelial cells and aid in neovascularisation.

Late CACs form colonies after about 2-4 weeks in culture, they have been shown to contribute directly to neovascularization (formation of new blood vessels) and have a robust proliferative capacity.⁷⁰ They have a cobblestone appearance and are more similar to mature endothelial cells. On the other hand, early CACs form colonies in culture after approximately 5-7 days and appear to mediate vascular repair mainly via a paracrine effect i.e. secreting proangiogenic growth factors at sites of endothelial injury.⁶⁷

The CFU-Hill assay developed by Hill et al²¹ has been commonly used to isolate and culture these early outgrowth cells from peripheral blood mononuclear cells. By the 5th day of culture, colony-forming units (CFUs) start to appear which provides us with a quantitative measure of CAC function. CAC function, which is represented by the capacity of CACs to form CFUs *in vitro* was found to be inversely related to cardiovascular Framingham risk score and a better predictor of vascular function than the presence or absence of traditional CVD risk factors²¹ (Figure. 2). This data provides us with a good rationale that cells isolated via this technique may be clinically relevant biomarkers of cardiovascular health. Studies have confirmed their importance in playing a crucial role in angiogenesis via the supportive, paracrine mechanism.¹³

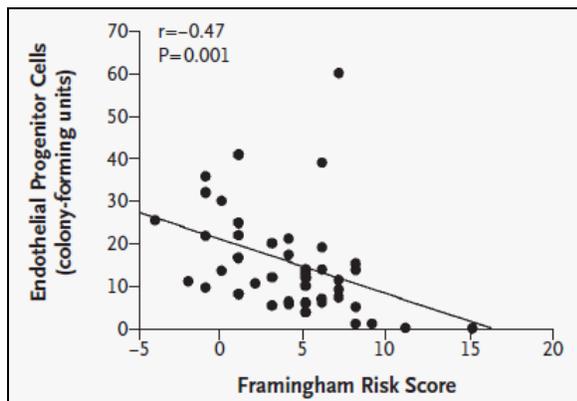


Figure. 2 CAC CFUs and Framingham Risk Score for CVD (Hill et al., 2003): Higher CAC colony forming units (CFUs) correlated with a decreased Framingham Risk Score for cardiovascular disease while a lower CFU count correlated with an increased score. CFUs are a measure of CAC function.

Much of the work with CACs has been focused on individuals with CVD. However, there are a limited number of studies examining their function in healthy individuals. If we gain a better understanding of how these cells become dysfunctional in healthy people, we can come

a bit closer to understanding the pathophysiology of vascular disease caused by common lifestyle factors.

2.4 Exercise and CACs

I. Acute exercise and CACs

A single bout of exercise leads to mobilization of CACs from the bone marrow. Acute exercise is believed to mobilize CACs through the increase in shear stress and activation of endothelial nitric oxide synthase (eNOS) via the Phosphoinositide 3-kinase/ protein kinase (PI3/Akt) dependent pathway (Figure. 3)³⁵. The increased concentration of nitric oxide (NO) activates Matrix Metalloproteinase-9 (MMP-9), essential for stem cell mobilization from the bone marrow, which causes mobilization of the resident progenitor cells out to the peripheral

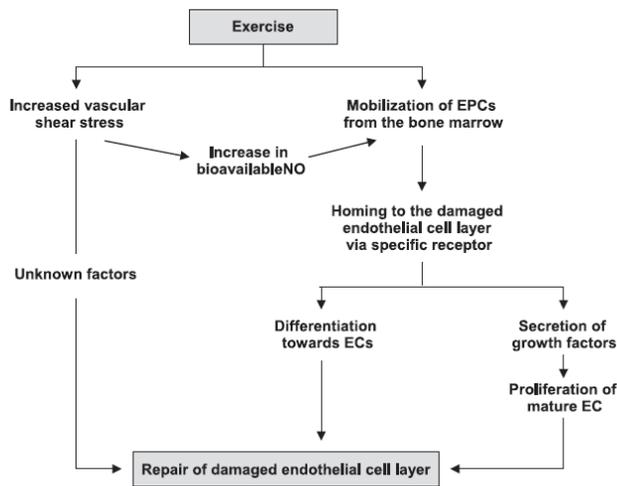


Figure 3. Diagram depicting the possible pathways by which exercise leads to repair and maintenance of an intact endothelial cell layer through CACs (Lenk, 2011):

Exercise causes an increase in vascular shear stress which increases NO. This NO in turn will stimulate the mobilization of CACs from the bone marrow. Once they are mobilized and released, they will go to the damaged endothelium where they will either differentiate into endothelial cells or

secrete growth factors that allow for proliferation of mature endothelial cells.

circulation. The exercise induced release of NO via eNOS stimulates the expression of circulating factors such as Stromal cell derived factor 1a (SDF-1a) and vascular endothelial-

growth factor (VEGF), chemokines that are considered to play an important role in trafficking of CACs.^{68,354} CXC chemokine cell receptor 4 (CX-CR4) and very late activation antigen- 4 (VLA4) are both cell surface receptors that are essential for the homing of these cells to the endothelium.⁷ Therefore, the role of eNOS appears to be critical in the liberation of CACs into circulation.³⁵

Mobius-Winkler et al.⁴⁰ conducted a study to follow the time-dependent release of CACs during and after a 4-hour bout of sub-maximal (70% anaerobic threshold) cycling exercise in healthy individuals. They found that there was a steady and significant increase in CD34⁺/KDR⁺ cells by the end of the exercise session, compared to baseline. They found a similar increase for CD133⁺/KDR⁺ and CD133⁺ cells. 24 hours after the start of the exercise the CD34⁺/KDR⁺ cell numbers returned back to baseline while the CD133⁺ cells remained elevated 2 hours after the end of the exercise and were slightly higher than baseline even at 24 hours⁴⁰. In another study that looked at the effects of acute exercise on patients with CVD risk factors, the post-exercise results were more dramatic.⁵⁰ After 5-10 minutes of having the patients do a treadmill/ bicycle exercise to exhaustion, CD133⁺/VE Cadherin⁺ cells increased by 400% while the early progenitors, cells that only have the CD133⁺ marker, increased by just 40%. The cultured angiogenic cells that express markers for monocytes/macrophages, increased by 250%. Van Craenenbroeck et al.⁸ compared the results of graded exercise testing between CHF patients and healthy subjects. Within 10 minutes of the exercise they found that the CD34⁺/KDR⁺ cells had increased in the healthy subjects and remained elevated after 2 hours. However, with the CHF patients they saw a small initial increase in these cells which was normalized within 30

minutes. These studies highlight that different cell types seem to respond to acute exercise differently in healthy individuals versus individuals with heart disease or risk factors for CVD.

II. Exercise training and CACs:

Exercise training has been shown to cause an increase in circulating CACs in individuals with cardiovascular disease.^{33,66} Exercise training can also positively affect the functional capacity of CACs.³⁵ In contrast to acute bouts of exercise where the elevation in CACs after the exercise is attenuated within minutes, regular physical activity can lead to an elevated baseline number of CACs as was observed in a group of young male runners, compared to sedentary controls.⁵

Laufs et al.³³ conducted a 28-day exercise program to measure the resting circulating CACs in patients with coronary artery disease (CAD). They found the resting CACs in these patients to be higher at the end of the program. Sarto et al.⁵⁴ investigated the specific mechanism involved in the increased CAC levels in CHF patients after exercise training. They found a 25% increase in resting CD34⁺/KDR⁺ CAC levels in CHF patients in response to 8 weeks of supervised aerobic training. They also saw a marked increase in two CAC mobilizing cytokines, namely VEGF and SDF-1.⁵⁴ Their results were accompanied by an increased potential of generating CAC colonies. Lauf et al.³⁴ also found that eNOS^{-/-} mice and wild-type mice treated with N^G-nitro-L-arginine methyl ester (L-NMMA) had lower CAC numbers at baseline and a significantly attenuated increase of CACs in response to 28 days of treadmill running. The wild-type control mice showed increases in CACs upwards of 250% after 28 days of treadmill running

while there were no significant differences seen in either the eNOS^{-/-} mice or the wild-type mice treated with L-NMMA after the training.

Jenkins et al.²⁷ found that endurance trained men had greater resting CAC intracellular NO (NO_i) compared with age-matched sedentary men. In trained men they also found greater resting CAC eNOS gene expression and lower expression of GP91phox which is a subunit of NADPH oxidase, an enzyme that produces oxidative stress in the vascular wall. Therefore, regular physical exercise appears to impart a pro-NO and anti-oxidative stress phenotype on circulating CACs.

Importantly, exercise training may alter the CAC response to acute exercise. Jenkins, et al.²⁷ showed that trained men had a significant increase in their CFU-EC counts (a measure of CAC function) with an acute bout of treadmill exercise whereas no change in CFU-EC was seen in the untrained group after this acute exercise. Further, while acute exercise and the NADPH oxidase inhibitor apocynin both increased CAC NO_i to a similar extent in the untrained group, CAC NO_i was still lower than the trained group, and the trained men had no further increase in CAC NO_i with acute exercise or apocynin. This study demonstrates differences in the CAC response to acute exercise based on training status and that while decreased CAC NO_i may be partially due to NADPH and oxidative stress, there are likely other mechanisms that alter CAC NO_i with exercise.

Diminishing CAC levels are a good predictor of disease progression in patients with CVD.²¹ Exercise interventions have been used to improve CAC levels primarily in the CVD disease population.^{33, 66} But how exercise increases CAC number and function in this disease

population or in healthy people is still unclear. The improvement in CAC number and function after exercise interventions in individuals with CVD indicates the potential of these cells to respond to stimuli in an environment where endothelial dysfunction may have already progressed to a significant extent. However, it tells us very little about why these cells started to decrease in number and function in the first place. Physical inactivity has been associated with several CVD risk factors that include- high cholesterol, high blood pressure, obesity, overweight and diabetes mellitus.³⁷ While we are starting to understand how risk factors such as diabetes and high cholesterol affect CAC function, there is limited information on the deleterious effects of inactivity on CAC number and function.

2.5 Inactivity and CACs

I. Definitions of inactivity:

Fewer than 5% of adults in the United States of America satisfy the minimum physical activity recommendations of 30 minutes per day.⁶² There has been an increase in the amount of time devoted to the sedentary activities such as TV viewing in the past few decades. Based on data from the Center for Disease Control (CDC), physical inactivity and poor diet may soon overtake tobacco as a leading cause of death.⁴¹ Until recently the health risks associated with a sedentary lifestyle were thought to be a result of insufficient physical activity but we are learning that this is different from inactivity.

The American College of Sports Medicine (ACSM, 2006) defines a sedentary lifestyle as “not participating in a regular exercise program or not meeting the minimal physical activity recommendations from the US Surgeon General”.¹⁹ However, sedentary behavior and physical activity are not opposites of each other. Owen et al.⁴⁶ was among the first to challenge this when he reported that the determinants of sedentary behavior and physical activity are distinct. Marshall et al.³⁷ argue that sedentary behavior is best defined as a distinct class of behaviors that involve sitting and low levels of energy expenditure, typically less than 1.5 METs. Finally, Tudor-Locke et al.⁶³ categorize sedentary individuals as adults with <5000 steps/day, a commonly used cut-off point.

II. Inactivity and Metabolic Syndrome

Recent evidence claims that the rapid increase in rates of type 2 diabetes (T2D) is primarily attributable to an increase in physically inactive lifestyle. It is true that genetic predisposition to

T2D plays a role in the degree to which inactivity impacts the individual. However, Booth et al.⁶¹ made the observation that 10 days of bed rest in healthy individuals reduced some metabolic markers of physiologic function down to the level that is witnessed in T2D offspring prior to bed rest. Changes at the level of gene expression have been seen with 9 days of bed rest where several insulin resistance and diabetes candidate genes had been altered in healthy individuals.² Even after 4 weeks of exercise training only part of the 4500 genes, that were initially altered, were partially normalized to the pre-bed rest levels. The lack of complete normalization underscores the importance of maintaining the minimum daily exercise recommendations.

III. Inactivity and the endothelium:

The 1953 study conducted by Morris et al.⁴² found that there was an increased incidence of coronary thrombosis for the drivers of London's double-decker buses compared to the conductors.⁴² A study by the same group in 1958 found that ischemic myocardial fibrosis was more common in light (sedentary) occupations and it appeared earlier in this group.⁴² It also seemed to be more severe among these sedentary workers, especially at younger ages. A 30 year follow-up on the widely cited 1966 Dallas Bed Rest study presented data on changes in VO₂max, CO and SV that emphasized the detrimental role of inactivity on cardiovascular health.³⁹ The most remarkable finding was that 3 weeks of bed rest in 1966 caused a greater deterioration in cardiovascular and physical work capacity than did 30 years of aging in the 5 men that were tested in this follow-up study.

Physical inactivity is also associated with changes in hemodynamic stimuli which exert direct effects on the vasculature, leading to remodeling and a proatherogenic phenotype.⁵⁸ It can have a marked effect on conduit arterial lumen dimension. There are dose and time dependent decreases in arterial size in response to physical inactivity. Studies that looked at the effect of inactivity of the lower limbs and femoral artery diameter via FMD found that the number of days that the subjects' lower limb was inactive was directly related to the percentage decrease in femoral artery diameter.⁵⁸ Physical inactivity leads to arterial stiffness in subjects with spinal cord injury and there is a general consensus in the literature that that basal vascular resistance increases with increased physical inactivity. Increased plasma levels of ET1 (a potent vasoconstrictor) have been reported after 4-8 weeks of detraining in healthy individuals.³⁶ However, most of the work in this area has been done on individuals with spinal cord injury, bed-rest and limb immobilization.

IV. Models of Inactivity:

In the spinal cord injury and limb immobilization (where the limb is in a cast) models, there is the possibility that the disease state or recovery processes are causing systemic changes in the endothelium. The inactivity studies that have traditionally involved complete bed-rest are also extreme cases because these models lead to changes in the cardiac, respiratory, renal systems and on the endothelium itself.³² While these inactivity models have provided invaluable information about the detrimental effects of inactivity on the endothelium, the confounding variables that influence the outcomes and circumstances in which the subjects

were placed cannot be disregarded. In order to truly assess the effect of inactivity on the endothelium a healthy group of adults without pre-existing diseases needs to be studied.

V. Inactivity and Nitric oxide:

a. Importance of NO for the endothelium

Vascular endothelial cells produce nitric oxide (NO) by the enzyme NO synthase (NOS). eNOS, the predominant NOS isoform in the vasculature is responsible for most of the NO produced in this tissue.¹⁴ This basal NO production can be enhanced by agonists such as Ach, increased shearing forces (e.g. exercise where there is an increase in blood flow) and under the influence of certain cytokines during inflammation and infection.³¹ NO relaxes smooth muscle, inhibits platelet function and inhibits inflammatory responses. It represents the most important anti-atherogenic defense component of the vasculature.¹⁴

Endothelial dysfunction is frequently encountered when cardiovascular risk factors are present.¹⁵ Cardiovascular risk factors increase the expression and activity of NADPH oxidases (NOX) in the vascular wall which enhances the production of reactive oxygen species (ROS). Hypertensive rats, hypercholesterolemia and models of diabetes mellitus have been associated with increased activation of NOX.^{23,65} There is a growing body of evidence that vascular NOX plays a crucial role in the phenomenon of eNOS uncoupling in humans. NOS uncoupling has been observed in patients with endothelial dysfunction resulting from hypercholesterolemia, diabetes and hypertension.¹⁵

Endothelial nitric oxide is essential for the proper functioning and signaling processes for CACs, which play an important role in protecting the endothelium. Mice deficient in eNOS showed reduced basal CAC mobilization from the bone marrow¹ as well as CAC mobilization in response to exercise training.³⁴ This may contribute to the reduced reparative capacity of these cells in individuals with CVD who have a reduced NO bioavailability. While reduced numbers and function of CACs have been linked to eNOS uncoupling in diabetic individuals, differentiation and adhesion into pre-existing vasculature is found to be impaired with eNOS inhibition in others.⁶⁰ Therefore, decreased NO bioavailability may affect CACs mobilization and function.

b. Sirt1- regulator of NO:

Sirt1, also known as Sirtuin1 is an NAD⁺ dependent class III histone deacetylase and a key regulator of vascular endothelial homeostasis.⁴⁹ Recently, it was shown that Sirt1 expression and activity control eNOS activity via deacetylation of eNOS in endothelial cells.⁹ Blocking Sirt1 function via transduction of endothelial cells with a deacetylase defective Sirt1 mutant decreased NO-bioavailability and decreased endothelial-dependent vasodilation.³⁸ Since most studies related to Sirt1 have been related to caloric restriction, its response to exercise is not well understood.

Shear stress on endothelial cells has shown an increase in Sirt1 expression which correlated with an increase in NO bioavailability.^{72,38} Shear stress, such as that which can be generated by blood flow during exercise, has shown to accelerate the proliferation, differentiation and capillary-like tube formation of CACs.⁶⁹ Since we know that eNOS is a key

player in the migration, differentiation and adhesion of CACs, it would be interesting to understand the role of Sirt1 in its regulation in healthy individuals independent of disease.

c. Inactivity , Sirt1, eNOS:

Endothelial nitric oxide synthase (eNOS) function and consequently NO production is impaired with physical inactivity.³³ As mentioned above, while decreased NADPH oxidase may in part be responsible for increased CAC NOi with exercise training²⁷, there are likely other unexplored factors that contribute to changes in CAC NOi related to exercise and inactivity. If that is the case, it would be beneficial for us to start by investigating the effects of reduced physical activity on Sirt1, which ultimately controls vascular homeostasis via the deacetylation of eNOS in CAC. This will not only inform us about the effects of detraining on a key marker of endothelial health but it might also elucidate a novel mechanism by which it is regulated.

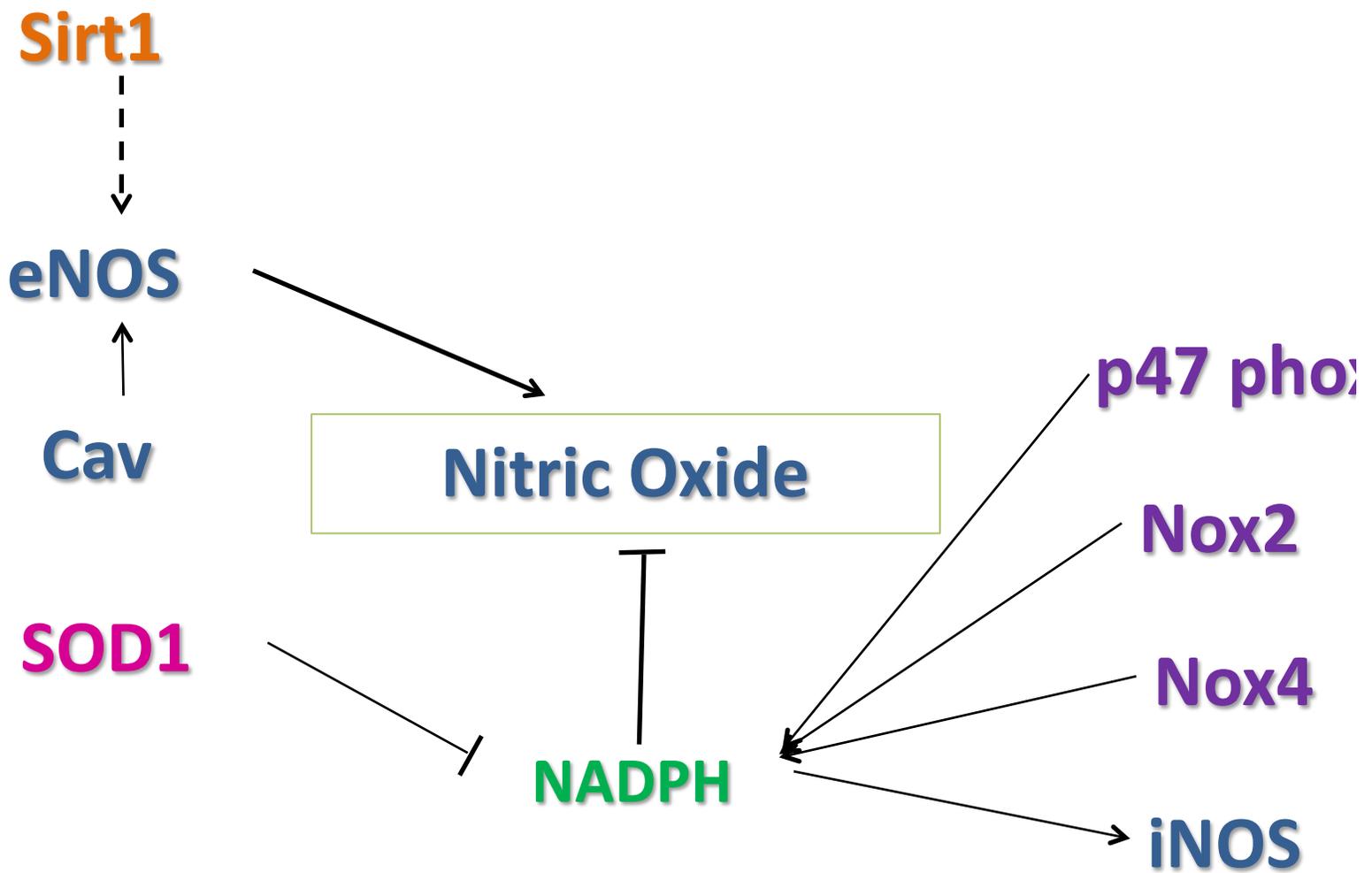


Figure 4. Genes that are direct and indirect regulators of nitric oxide in CACs. eNOS, Sirt1, Cav1, SOD1 can lead to increase in nitric oxide production whereas the different NADPH (activated by its subunits p47phox, nox2, nox4) can inhibit nitric oxide production while causing an increase in iNOS.

2.6 Summary of literature review

Traditional risk factors for CVD do not inform us about changes in the vasculature although damage to the vascular endothelium is one of the first signs of CVD progression. Circulating angiogenic cells (CACs) are responsible for the maintenance and repair of the vasculature and are a novel biomarker that may allow for the early detection of CVD. CAC function is strongly correlated with the Framingham Risk Score for CVD and CAC dysfunction found in individuals with CVD.

Few studies on healthy, active individuals suggest that exercise plays an important role in maintaining CAC function when compared to their sedentary counterparts. The causes for decreases in CAC function in sedentary, healthy individuals compared to active, healthy individuals are not fully known. However, data suggests a cardio-protective effect of exercise on CACs. In contrast, inactivity is linked to CVD and CVD risk factors and is a growing problem in industrialized and developing nations. Inactivity studies involving bed-rest, limb immobilization and spinal cord injury have shown that inactivity can have deleterious effects on the vascular endothelium. However, these models represent extreme situations and have confounding factors (such as the disease state) inherent in them. In order to study the effects of inactivity on the vascular endothelium and on CACs, which may be an early biomarker of vascular damage, we propose to study active, healthy individuals and reducing their physical structured activity.

In this study, we will explore a potential mechanism for changes in CAC function with reduced physical activity. Cross-sectional data comparing sedentary versus active, healthy individuals suggests that the functional differences seen in these two populations may be

related to nitric oxide (NO) bioavailability and signaling in CACs. Nitric oxide is not only important for the functioning of a healthy endothelium but also for the normal functioning of CACs. There are several regulators of NO and their up-regulation or down-regulation can provide us with information regarding the effect of the inactivity perturbation on NO and CAC function. By using a model where healthy, active individuals are asked not to perform their regular structured exercise for a short period of time, we hope to elucidate the early cellular pathways responsible for CAC dysfunction observed in individuals with CVD.

CHAPTER 3

METHODS

3.1 Introduction

The primary aim of this study was to examine the effect of reduced physical activity (rPA) which we defined as 10 days of reduced physical activity, on two populations of CAC (circulating angiogenic cells). This chapter outlines the methods employed to answer these questions.

3.2 Subjects

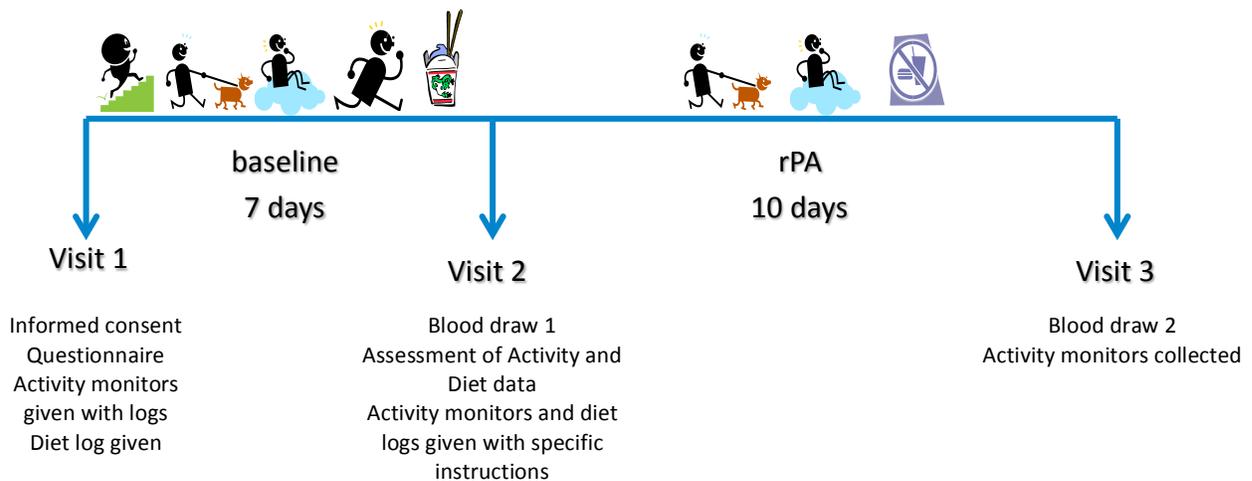
The participants for this study were healthy, non-smoking male runners between the ages of 18-50 with a training history of at least 5 years. Participants who exercised above 3 days a week with moderate to intense endurance exercise were recruited for this study. Participants were screened for adequate training history including miles run per week and competition history. They were further screened for history of cardiovascular, lung, liver disease, diabetes or cancer, and they could not be on any medications that could influence CAC number or function. Participants were asked to stop taking any vitamins and other over the counter medications for the duration of this study. We accounted for these factors with the health history and physical activity questionnaires. (see 6.2, 6.3 in Appendix) We did not include women in this study due to the gender differences associated with the number and function of CACs and the influences of the uterine cycle on CACs.^{12,25}

3.3 Experimental Set-up

Physical activity monitors (ActiGraph GT3X and Omron pedometer) were used to procure a baseline physical activity level and for the 10 days of reduced physical activity.

Activity logs and diet recalls were used to further evaluate energy expenditure and energy intake respectively. (see 6.4-6.8 in Appendix). Standard blood draw procedures were used for the venipuncture. Blood for CACs was collected into 6 EDTA-coated 10ml tubes. We collected 60 mls per draw. Lipid profile was also measured at baseline and rPA. Baseline lipid profile results were also used as an additional screening measure to verify that participants did not have any CV risk factors. Heart rate, blood pressure and weight were recorded at every visit.

3.4 Protocols



Physical activity measurements and energy expenditure:

During the 1st visit the participants filled out and handed in the questionnaires (physical activity and health history), and their signed informed consents. (see 6.1-6.3 in Appendix) We measured their weight, HR, stride length and BP during this time. We also fit them with an Omron pedometer and initialized the ActiGraph GT3X activity monitor for them to wear for a week, with specific instructions of when to wear them and how to use the monitor logs. (see 6.4 in Appendix) ActiGraph and the pedometer were worn on their left hip, secured with an elastic belt. Before coming in for the 2nd visit, their activity data was analyzed from the

ActiGraph GT3X monitor using their monitor logs, to confirm their physical activity levels were consistent with those in their physical activity questionnaires. If the subjects qualified in terms of their physical activity requirement and their lipid profile results were all within the normal range, then they were asked to come in for their 2nd visit. The Actigraph data from their baseline week was further analyzed using the NeuralNetwork through a website developed by Staudenmayer et al.⁷⁹ to calculate their EE (energy expenditure) in kcals for all activities over 3 METs. The output from the NeuralNetwork utilized for kcal calculations was the METs (kcal/kg/hr). This was used to approximate each individual's EE based on their body weight and time spent in activities above 3 METs (approximated as average kcals/day).

The ActiGraph measured acceleration in 3 axes and over a specified time period (epoch). The acceleration signal outputs helped us distinguish between activity intensity (sedentary, moderate, vigorous and very vigorous) based on Freedson cut-points⁸⁰ using the ActiLife software. Data were analyzed as average number of minutes spent at each activity level per day. In addition to this, participants were asked to wear the Omron pedometer at all times, except for when they were exercising, during their baseline week. They were also asked to note their step count at the end of each day in the activity monitor log. From their baseline week, the average number of steps/day were calculated and participants were asked to stay at this number during rPA.

Dietary monitoring and energy intake:

Participants were carefully counseled on the requirements for the study including dietary habits, supplements and medications. They were given detailed instructions to follow a low-

nitrate diet (see 6.6 in Appendix) 3 days prior to coming in for their 2nd visit as well as a checklist of activities, foods and medications to avoid for the 24 hours prior to coming in. (see 6.5 in Appendix) They were also told that they would have to respond to 3 dietary recalls (on 2 weekdays and 1 weekend day) over the course of the baseline week, which was administered by us through the ASA24 (Automated self administered 24-hour dietary recall) developed by the National Cancer Institute (url: <http://appliedresearch.cancer.gov/tools/instruments/asa24/>). The data from their dietary recalls were used to calculate their average EI (energy intake) in kcals/day. Based on this, we calculated EI – EE and this number was recommended to them as their goal for daily kcal intake for their 10 days in rPA. We created an account for each subject on myfitnesspal (url: <http://www.myfitnesspal.com>), a website to help them keep track of their daily energy intake based on our EI – EE kcal recommendation. Since this was a shared account, we could also log into the participant's account to help them maintain their goal.

Cell culture protocols

a. Colony forming units (CFU-Hill) assay:

CFUs were cultured after the baseline and final visit. CFU stands for colony forming units. The CFU-Hill assay is a 5-day culture method developed by Hill et al. used to assess CACs based on CFU growth.²¹ The CFU-Hill kit was purchased from StemCell technologies. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples via density centrifugation (Ficoll-Paque), then washed. PBMCs were placed at a density of 5×10^6 cells per well in 2 fibronectin coated 6-well plates with growth medium. Cells were cultured for 2 days

(37°, 5% CO₂, ≥95% humidity). After 2 days, non-adherent cells were removed and counted with a hemocytometer. 1×10^6 of the non adherent cells were re-plated on Day 2 into wells of a 24-well fibronectin-coated plate with fresh CFU-Hill media. Cells were cultured under the same conditions (37°, 5% CO₂, ≥95% humidity) for 3 more days. On Day 5, the number CFUs were counted. CFU was characterized as a cluster of cells that form a round center with outward radiating cells and quantified as average CFU/well. (see 6.9 in Appendix)

b. CD34⁺ cell isolation

PBMCs were isolated as described above and used for isolation of CD34⁺ cells. These cells were isolated with magnetic bead separation, using an EasySep Magnet (StemCell Technologies, Vancouver, BC). PBMCs were suspended in PBS + 2% FBS with EDTA at a density of 1.5×10^5 . The CD34⁺ selection antibody cocktail was added at 100 uL/ml. The cell/antibody suspension was incubated at room temperature for 15 min. Magnetic nanoparticles specifically designed to select CD34⁺ cells were added to this mixture (100uL/ml) and incubated for 10 min. PBS + 2% FBS with EDTA was added to a volume of 2.5ml then the tube was placed in the magnet for 10 min. CD34⁺ cells remain magnetically attached to the side of the tube while the supernatant was poured into a second tube and put into the magnet for another 10 minutes. After the 10 minutes, the cells that were poured out went towards the CD34⁻ cells. 2.5 ml of PBS + 2% FBS with EDTA was added to the 1st tube which was put into the magnet again for 10 minute. The cells that were poured out were added to the CD34⁻ cells. The CD34⁺ cells that were attached to the walls of the tubes were collected in 300uL of 1xPBS and the number of cells were counted using a hemocytometer. (see 6.10 in Appendix)

We assessed CD34⁺ cell yield with this protocol via flow cytometry analysis. CD34⁺ cells are found in less than 0.01% of PBMCs. We found that our protocol enriched cells 60% (0.6%, see 6.15 in Appendix). Cell purity was assessed from a similar protocol by Jenkins, et al.⁷⁷ who reported 59% purity in the positively selected fraction.

c. Intracellular Nitric Oxide (NOi):

Intracellular Nitric Oxide (NOi) was measured in freshly-isolated CD34⁺ cells and CFU-Hill cultured cells using 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate fluorescent dye (Invitrogen, Carlsband, CA).²⁷ DAF-FM diacetate emits fluorescence when reacting with an intermediate of NO as NO is oxidized to NO₂⁻. 10uM DAF-FM in PBS was added to the cells and incubated for 30 minutes at 37°C. NOi was measured with a fluorescence plate reader (PolarStar Optima, BMG Labtech) with excitation and emission wavelengths of 488 and 535nm, respectively. Wells including unloaded cells, no cells and DAF-FM dye, and PBS were used as fluorescence controls. We added an additional positive control SIN-1 (nitric oxide donor) on each plate, at known concentrations (see 6.12, 6.13 in Appendix). Several pilot tests were conducted to test for the appropriate gain settings, DAF-FM concentrations, SIN-1 concentrations and later, to account for intra-assay and inter-assay variability 6.5% and 1.1% respectively.

d. Gene expression:

RNA was isolated from freshly-isolated CD34⁺ cells and the CFU-Hill cells at the 5th day of the culture assay, utilizing Trizol Reagent. The isolated RNA was quantified using a Nanodrop spectrophotometer and was used to make 0.2ug of cDNA using 5X iScript reverse transcriptase

master mix (BioRad). All 8 primers used for the gene expression were designed and purchased from Integrated DNA Technologies (Coralville, IA). Each primer pair was optimized to determine the most optimal temperature and concentration for an efficiency of >90%, using EvaSsofast PCR mastermix (BioRad, Hercules, CA). All gene expression analyses were run in triplicate on the CFX96 RT-PCR machine (Biorad) with GAPDH as the control gene. The genes that we analyzed were: *Enos*, *Nox2*, *Nox4*, *Cav1*, *Sirt1*, *P47*, *Sod1* and *Inos* in order to evaluate changes in nitric oxide and its regulators after rPA. The results were analyzed and presented based on Δ Ct values and fold change, respectively. The full names, functions, and primer pairs of the analyzed genes are in Table 1.

Table 1. Names and regulatory roles of genes related to nitric oxide

Gene name, HGCN approved name	Full gene name	Regulatory role w.r.t. nitric oxide	F (forward) and R (reverse) primer sequence (5-3' direction)
Enos ,Nos3	endothelial nitric oxide synthase	Controls availability of nitric oxide ³¹	F: GAGGGGAGCTGTTGTAGGG R: GTGGTAACCAGCACATTTGG
Cav1	Caveolin 1	eNOS related membrane protein ¹⁶	F: AATACTGGTTTTACCGCTTGCT R: ATGCCGTCAAAACTGTGTGTC
Ssirt1	Sirtuin 1	Regulation of eNOS via deacetylation ⁹	F: CAGTGGCTGGAACAGTGAGA R: TCTGGCATGTCCCACTATCA
Sod1	Superoxide dismutase	Antioxidant that metabolizes superoxide radicals and prevents oxygen toxicity ⁴⁵	F: ATG ACT TGG GCA AAG GTG GAA ATG R: GTT AAG GGG CCT CAG ACT ACA TCC
Nox2, Cybb	NADPH oxidase 2 (also known as gp91 ^{phox})	Subunit of NADPH oxidase contributing to ROS formation ⁴⁷	F: AGG ATT GCC TGA AGG GTT CT R: AGG GCT AGC TGG AGA AGA CC
Nox4	NADPH oxidase 4	Subunit of NADPH oxidase contributing to ROS formation ⁴⁷	F: CAG AAG GTT CCA AGC AGG AG R: GTT GAG GGC ATT CAC CAG AT
P47 phox, Ncf1	Phagocytic NADPH oxidase subunit	Phosphorylation of p47(phox) subunit marks NADPH activation ⁷¹	F: CAC GGA CAA CCA GAC AAA AA R: AGA ACC ACC AAC CGC TCT C
Inos, Nos2	Phagocytic NADPH oxidase subunit	Produces NO in large quantities in hematopoietic cells ⁵⁹	F: GGC CGC AGA GAA CTC AGC CTC A R: CTC AAA ACA GCC GCT TCC CCA GAA

3.5 Statistical Analyses

All statistical analyses were performed using Minitab statistical software. Analyses consisted of paired Student T-test to determine differences in CFU-Hill colonies, NOi and gene expression from before to after 10 days of rPA. Correlations were used to determine the relationship between the change in CAC function with rPA and the change in CAC NO gene regulators. Reverse stepwise multiple regression analysis was performed to determine significant predictors of changes in CFU-Hill, and NOi. Statistical significance was accepted at an alpha level of less than 0.05. From the three variables analyzed, that with the largest estimated sample size (NOi) was used.²⁷ To obtain appropriate statistical power (beta = 0.8; alpha 0.05), 10 participants were needed. Calculations were based on an effect size of 0.6 Relative fluorescence units (RFU), with a standard deviation of 0.51RFU.

CHAPTER 4

RESULTS

All subjects recruited for the study were healthy and physically active. Individuals on medication and who had injuries within the past 6 months were not included in the study. Subjects were primarily runners, with greater than 5 years of running history. The Actigraph data from their baseline week was used to verify that they were currently active. 10 subjects were recruited and completed this study. However, 2 subjects were excluded from all analyses because one subject did not adhere to the no-caffeine and no-alcohol requirements during the 24 hours prior to coming in for the final visit. The other subject was excluded from all analyses due to their non-adherence of decreasing physical activity during the 10 days of inactivity as shown by their Actigraph data (physical activity section below).

4.1 Subject characteristics

Subject height, weight, blood pressure and heart rate was measured at every visit. In addition to this, a lipid panel was evaluated by the lab at UHS during their screening visit and at their final visit (Table 1). Results from the lipid panel at the screening visit, was used to determine the subjects' health status. As shown in Table 1, cholesterol, triglycerides, HDL and LDL for all subjects were within the normal range. Weight, blood pressure, resting heart rate and lipid profile did not change significantly with rPA, compared to baseline.

Each subject's energy expenditure was calculated in terms of kCals for activities > 3 METs. Using the Neural Network, the average EE (energy expenditure) for each subject, was

found to be 360 kcals/day. Their average EI (energy intake) based on data through their dietary recalls (2 week days, 1 weekend day) was 1980 kcals/day. Each subject's average EE was subtracted from their EI and that kcal number was recommended to them in order to maintain their weight, which was accomplished as evidenced by no weight change from before to after rPA (Table 1, p=0.98). In addition to this, their pedometer data indicated that their average steps/day did not differ greatly between baseline and rPA either (Table 1, p=0.67).

Table 2. Subject Characteristics

	Baseline			rPA			p-value
	Mean	±	SE	Mean	±	SE	
Age (yrs)	25	±	3		-		-
Height (in)	70.4	±	0.8		-		-
Weight (kg)	74.4	±	2.6	74.3	±	2.6	0.98
Steps/day	7431	±	793	6757	±	1339	0.68
SBP (mmHg)	119	±	5	122	±	5	0.92
DBP (mmHg)	71	±	4	69	±	2	0.92
HR (bpm)	63	±	5	61	±	5	0.76
TC (mg/dL)	146	±	10	152	±	11	0.69
TG (mg/dL)	58	±	8	52	±	7	0.57
HDL (mg/dL)	53	±	13	52	±	16	0.87
LDL (mg/dL)	80	±	5	89	±	7	0.44

4.2 Physical activity

The physical activity data are represented as the average number of minutes spent at each activity level per day. Figure 4. represents all activities above 3 METs, termed as MVPA (moderate-vigorous physical activity) for baseline and rPA. The average amount of time in MVPA at baseline was 94.5 minutes/day and this decreased by 29% after rPA to 67.1 minutes/day ($p=0.11$). The subject who was excluded from this study due to non-adherence to the study protocol had *increased* their MVPA by 21% after rPA, relative to their baseline. The activity monitor data for one of the subjects could not be downloaded thus the physical activity analyses were run on the other 7 subjects.

While the MVPA was not significantly different between baseline and rPA, it is a combined measure of physical activity. The individual components within MVPA provided more information on how much time was spent by each subject at the moderate, vigorous and very vigorous activity levels. Subjects spent an average of 64.7 minutes/day at moderate activity (Figure 8) which decreased by 15.8% after rPA to 54.5 minutes/day; although, this decrease was not significant ($p=0.42$). The average time spent in vigorous physical activity (Figure 9) was 20.42 minutes/day at baseline, which trended towards significance with a 48.61% decrease after rPA, to 10.49 minutes/day ($p=0.08$). Very vigorous activity (Figure 4) was 9.32 minutes/day at baseline, this also trended towards a significant decrease by 77.46% after rPA to 2.10 minutes/day ($p=0.06$). Combined vigorous and very vigorous activity (Figure 8) was 29.74 minutes/day at baseline, which was significantly decreased by 57.65% after rPA to 12.59

minutes/day ($p=0.01$). Average sedentary time at baseline increased by 9.18%, from 707.70 minutes/day to 772.65 minutes/day after rPA ($p=0.18$) (Figure 9).

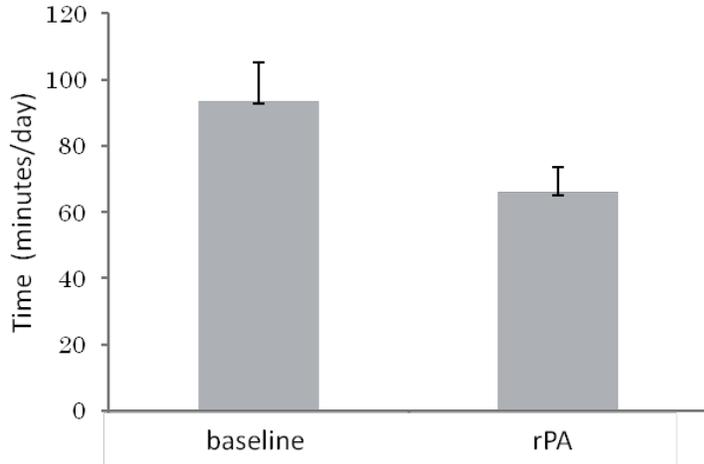


Figure 4. Moderate, vigorous and very vigorous physical activity (MVPA) in minutes per day for baseline and rPA ($p=0.42$, $n=7$)

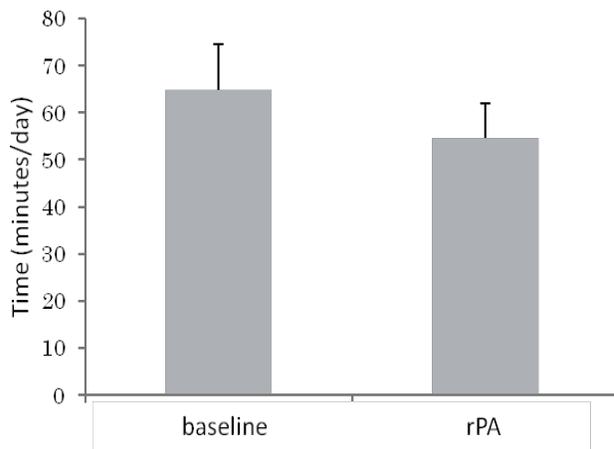


Figure 5. Moderate physical activity in minutes per day for baseline and rPA ($p=0.42$, $n=7$)

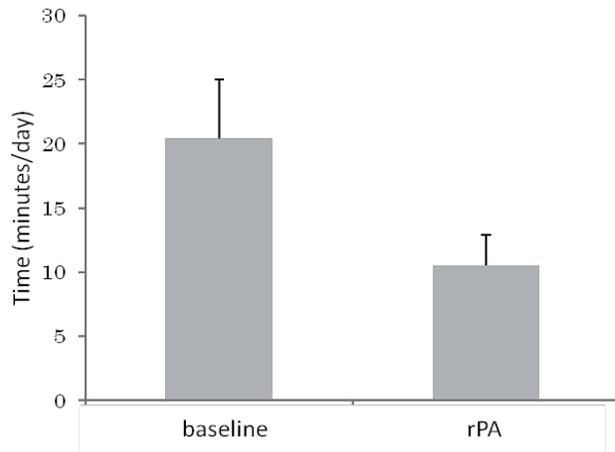


Figure 6. Vigorous physical activity in minutes per day for baseline and rPA ($p=0.08$, $n=7$)

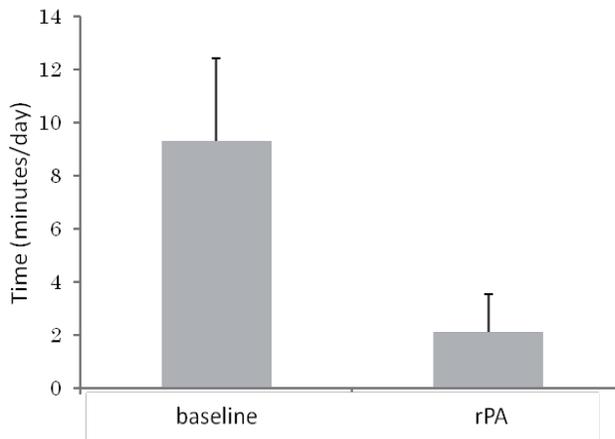


Figure 7. Very vigorous physical activity in minutes per day for baseline and rPA ($p=0.06$, $n=7$)

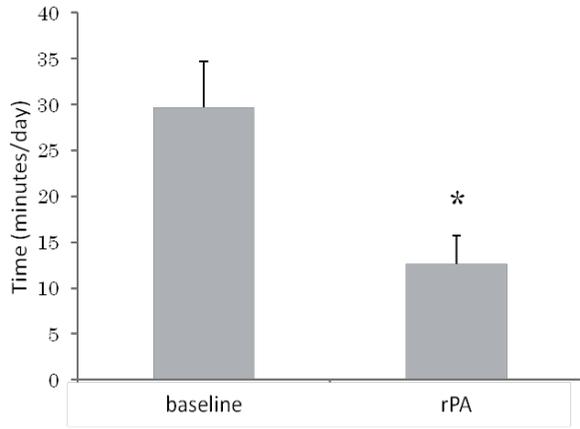


Figure 8. Vigorous and very vigorous physical activity in minutes per day for baseline and rPA

($p= 0.01$, $n=7$)

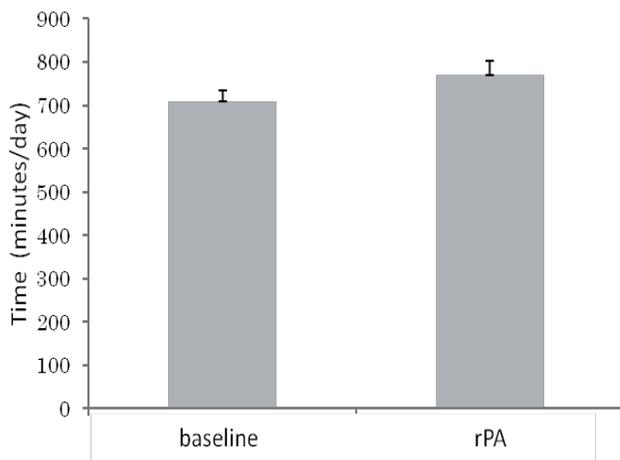


Figure 9. Sedentary time in minutes per day for baseline and rPA ($p=0.19$, $n=7$)

4.3 Cell number

The average number of CD34⁺ enriched cells per subject (obtained from 45mls of blood for each condition) was 6.59×10^6 cells at baseline (Figure 7). The number of CD34⁺ enriched cells increased by 9.5% after rPA to 7.22×10^6 cells ($p=0.68$).

The number of CFU-Hill colonies significantly decreased after rPA, by 35.69% from baseline (Figure 14). The average number of colonies decreased from 16 colonies/well at baseline to approximately 10 colonies/well after rPA ($p=0.01$).

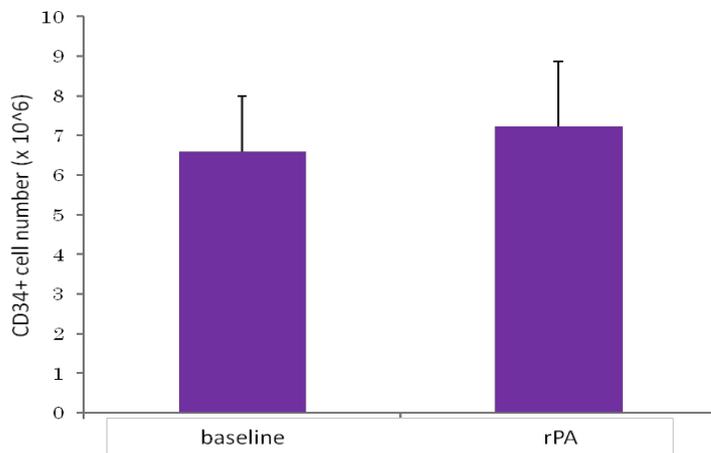


Figure 10. CD34⁺ cell numbers at baseline and rPA ($p=0.69$, $n=8$)

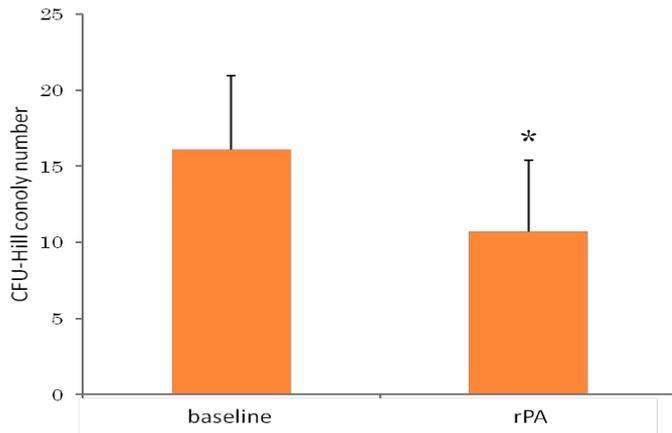


Figure 11. CFU-Hill colony numbers at baseline and rPA (p=0.01, n=8)

4.4. Intracellular Nitric Oxide

NO_i (intracellular nitric oxide) was measured for both cell types at baseline and rPA. NO_i is represented as RFUs (relative fluorescence units) by using PBS as the control to calculate the RFUs of the cells. CD34⁺ NO_i (Figure 12 A, B) at baseline was 16997.64 RFUs which decreased by 9.55% to 15374.78 RFUs after rPA (p=0.56).

CFU-Hill NO_i (Figure 13 A, B) decreased significantly by 33.84%, from a baseline value of 12251.86 RFUs to 8105.84 RFUs, after rPA (p=0.03).

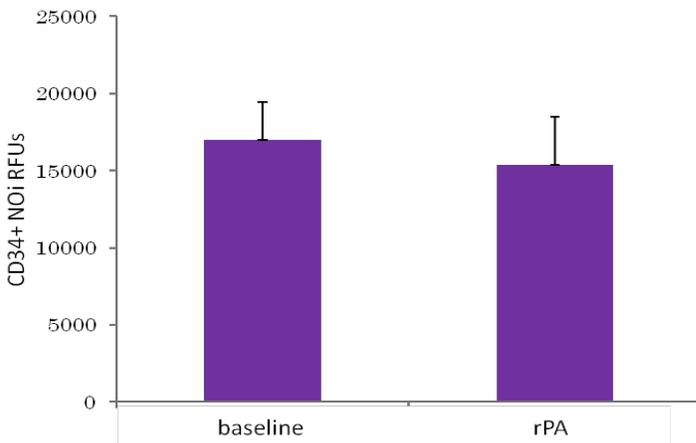


Figure 12A. CD34⁺ cells intracellular nitric oxide in relative fluorescence units RFUs between baseline and rPA (p= 0.56, n=7)

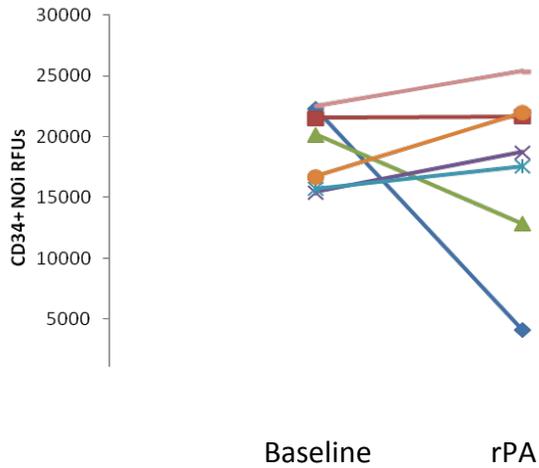


Figure 12B. Individual data CD34⁺ cells intracellular nitric oxide in relative fluorescence units RFUs between baseline and rPA (p= 0.56 , n=7)

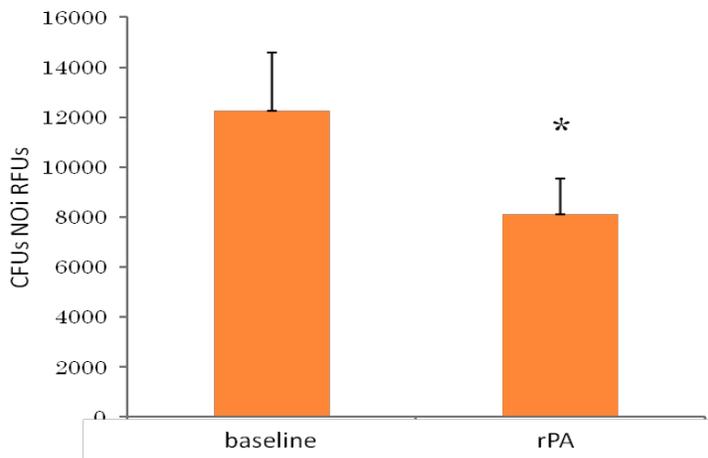


Figure 13A. CFU-Hill cells intracellular nitric oxide in relative fluorescence units RFUs between baseline and rPA (p=0.03, n=7)

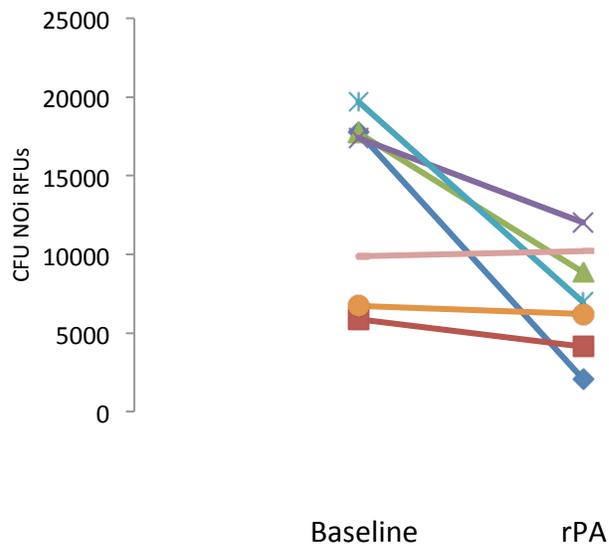


Figure 13B. Individual data for CFU-Hill cells intracellular nitric oxide in relative fluorescence units RFUs between baseline and rPA ($p=0.03$, $n=7$)

4.5 Gene expression

Gene expression is expressed in terms of fold change of a particular gene after rPA, relative to baseline. The eight genes that we proposed to investigate have been split into two broad groups for the purpose of clarity. The first group consists of genes that directly or indirectly contribute towards NO (nitric oxide) production in both cell types; they include eNOS, iNOS, Sirt1 and Cav1. The second group of genes is related to oxidative stress; these genes include Nox2, Nox4 and p47phox (components of NADPH oxidase) and SOD1.

Different trends were found between the genes that contribute towards the production of NO (eNOS, iNOS, Sirt1 and Cav1), in CD34⁺ enriched cells. However, none of the differences were significant between baseline and rPA (Figure 14A). Expression of eNOS and iNOS in CD34⁺ enriched cells were increased by less than 1 fold ($p>0.05$), while Sirt1 and Cav1 decreased, also

by less than 1 fold ($p>0.05$). The individual data (Figure 14B) demonstrates the variability in the expression of these genes between subjects.

For CFU-Hill derived cells, all genes related to NO tended to decreased after rPA, however these decreases were not significant (Figure 16 A, B). The expression of eNOS and iNOS decreased by a little over ~ 1 fold ($p=0.07$, $p=0.12$ respectively) while the expression of Sirt1 and Cav1 decreased by less than 1 fold after rPA ($p>0.05$ for both). No significant differences were found for genes in the oxidative stress group Nox2, Nox4, p47phox and SOD1 (Figure 15A) in either CD34⁺ enriched cells or CFU-Hill cells (Figure 17 A) ($p>0.05$). Figure 15 B and Figure 17 B illustrate the individual variability in the response to rPA.

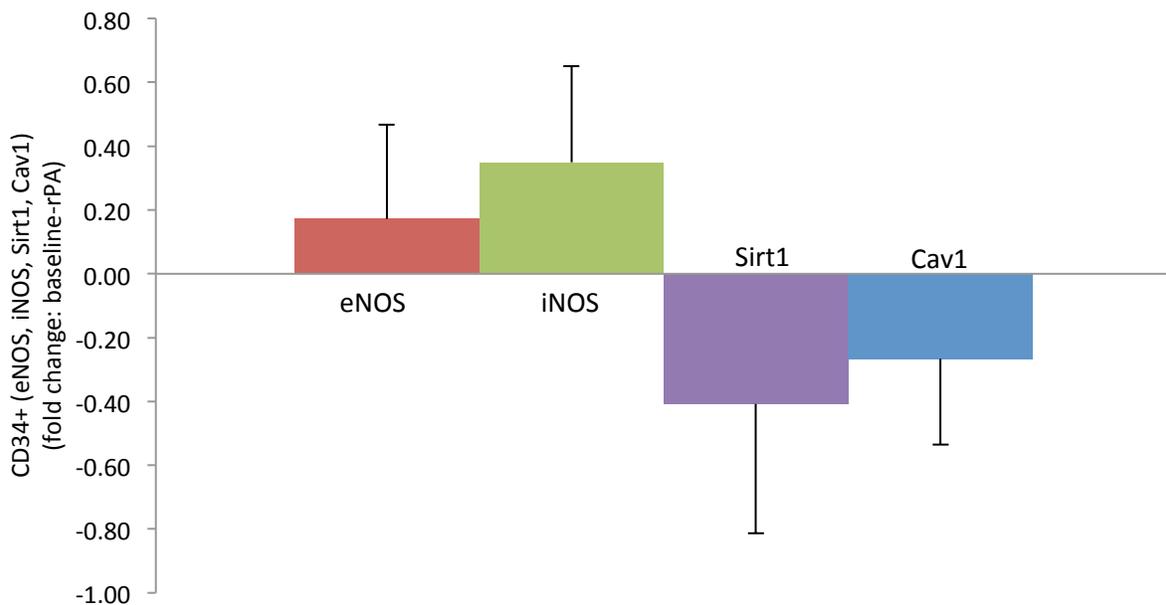


Figure 14 A. CD34⁺ cells eNOS, iNOS, Sirt1, Cav1 fold change at rPA, relative to baseline ($p>0.05$, $n=8$)

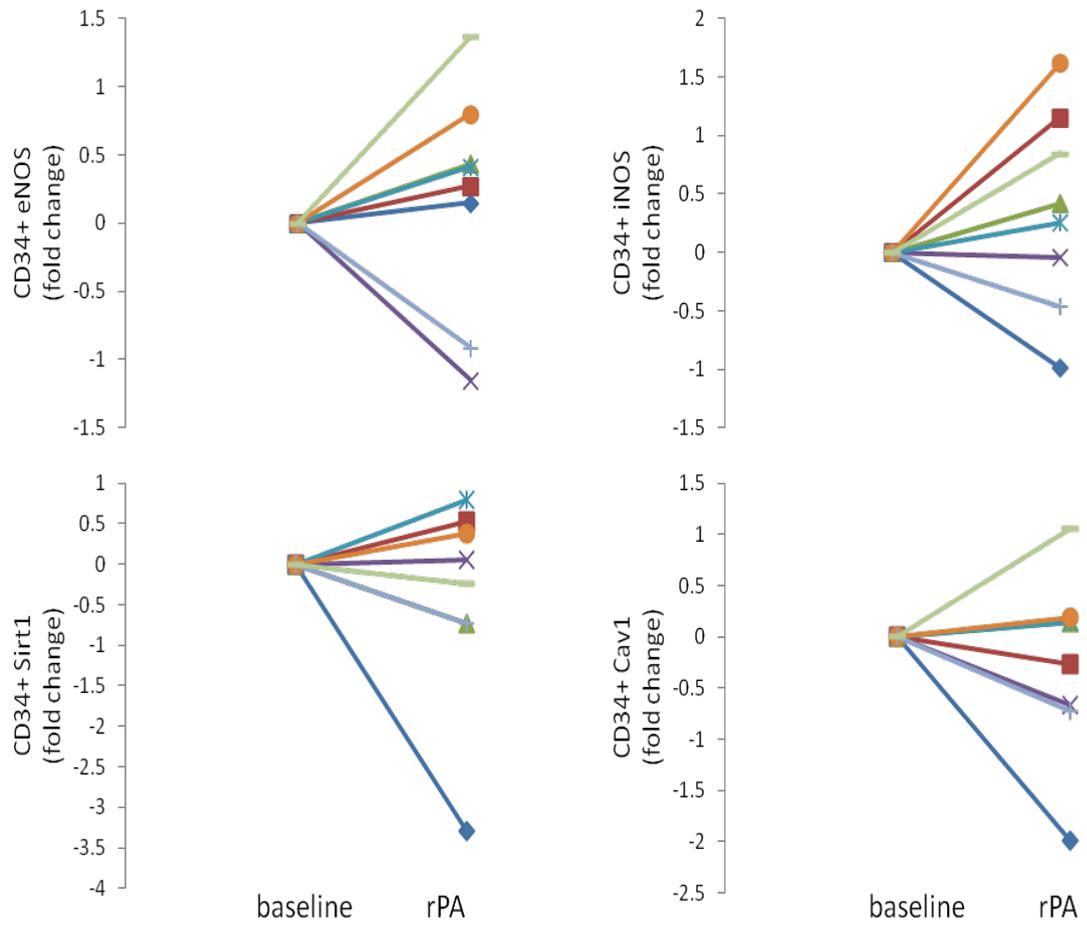


Figure 14 B. CD34⁺ cells eNOS, iNOS, Sirt1, Cav1 fold change at rPA, relative to baseline, individual data (p>0.5, n=8)

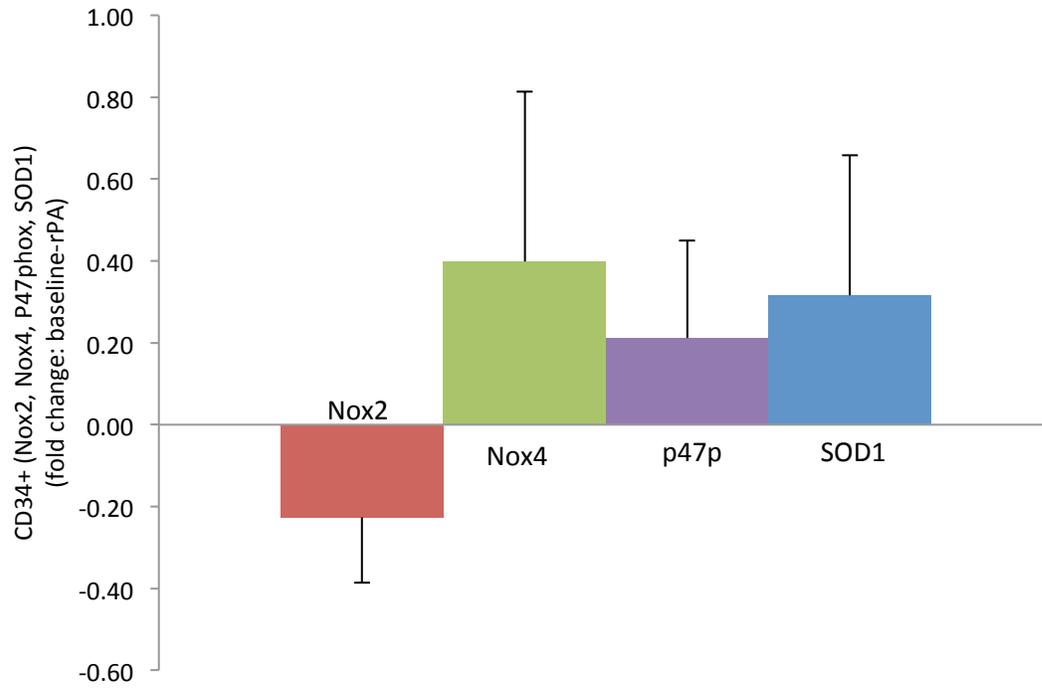


Figure 15 A. CD34⁺ cells Nox2, Nox4, p47phox and SOD1 fold change at rPA, relative to baseline (p>0.5, n=8)

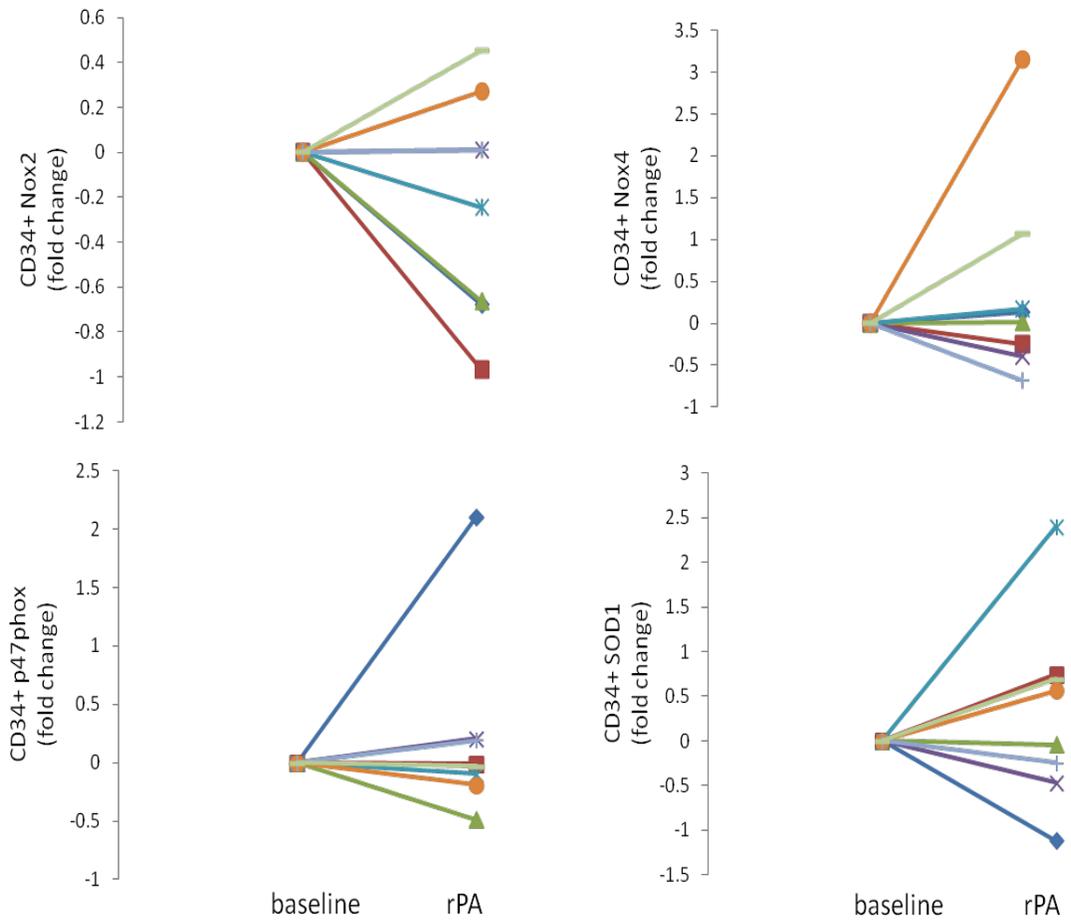


Figure 15 B. CD34⁺ cells Nox2, Nox4, p47phox and SOD1 fold change at rPA, relative to baseline individual data (p>0.5, n=8)

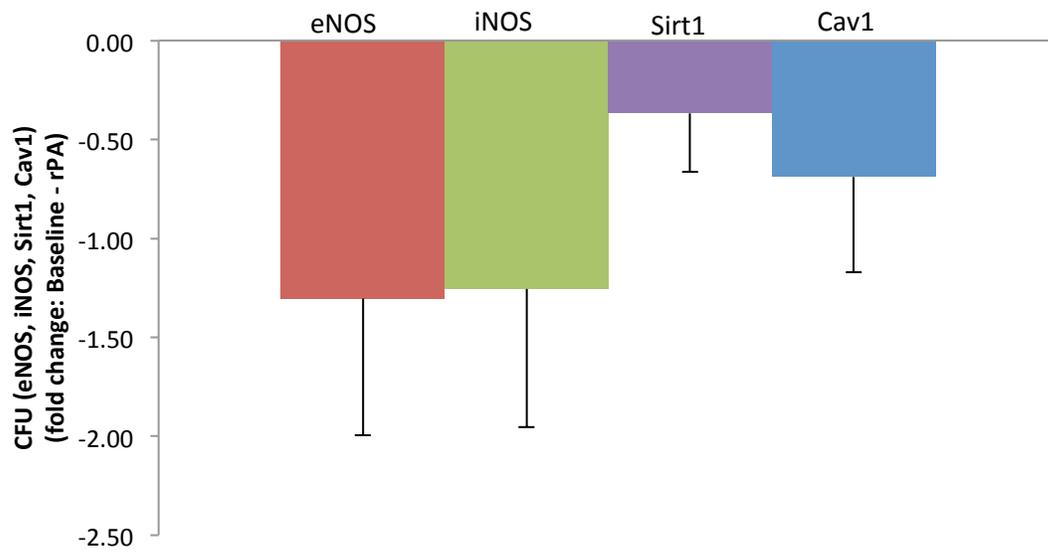


Figure 16 A. CFU-Hill cells eNOS, iNOS, Sirt1, Cav1 fold change at rPA, relative to baseline
($p > 0.5$, $n = 8$)

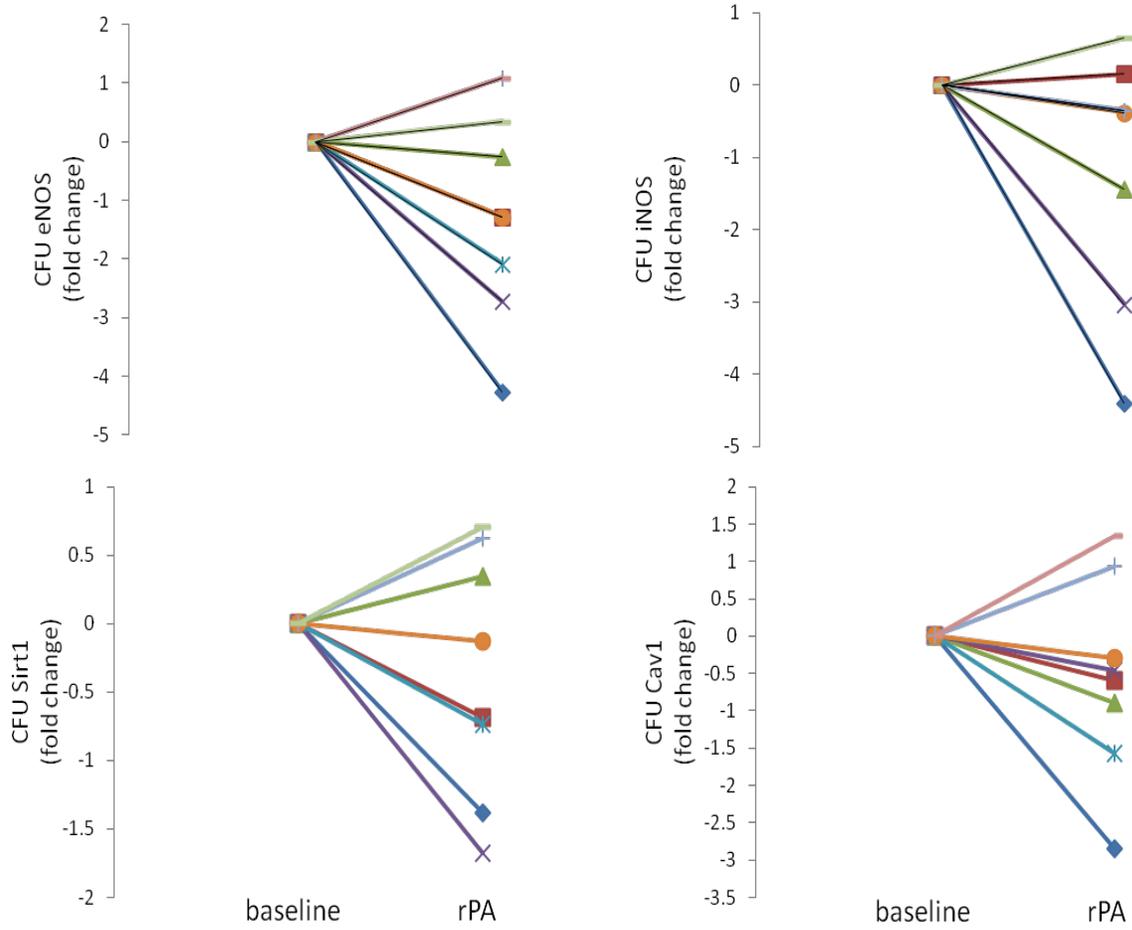


Figure 16 B. CFU-Hill cells eNOS, iNOS, Sirt1, Cav1 fold change at rPA, relative to baseline, individual data (p>0.5, n=8)

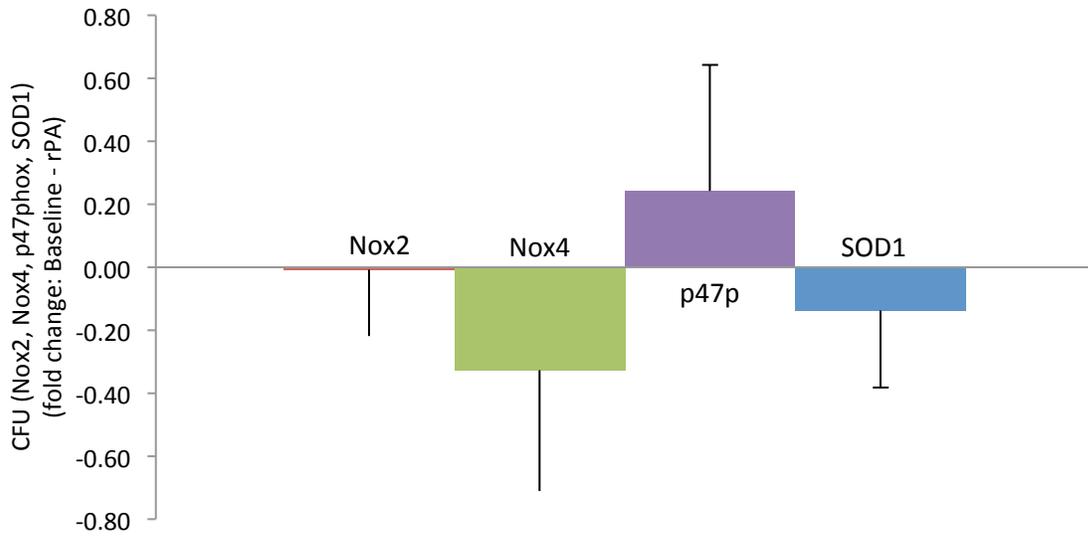


Figure 17 A. CFU-Hill cells Nox2, Nox4, p47phox and SOD1 fold change at rPA, relative to baseline ($p > 0.5$, $n = 8$)

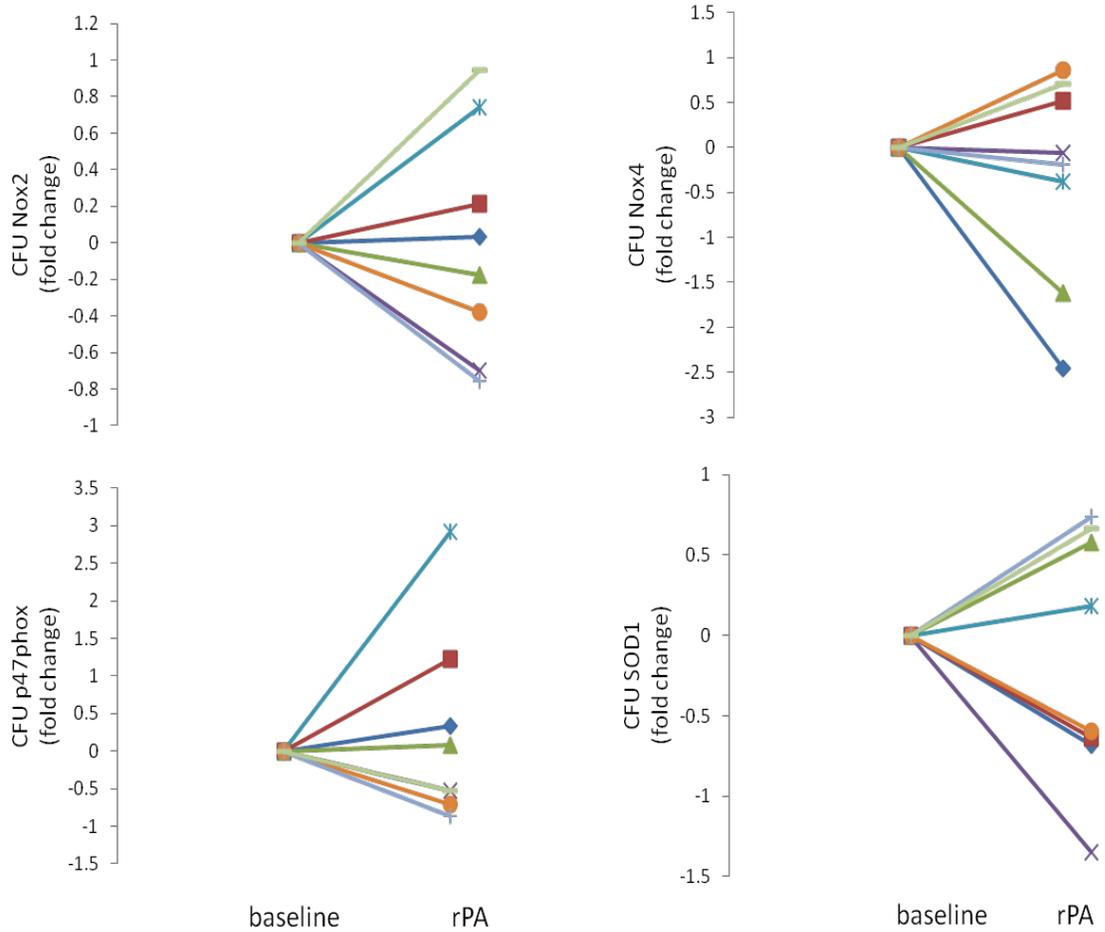


Figure 17 B. CFU-Hill cells Nox2, Nox4, p47phox and SOD1 fold change at rPA, relative to baseline, individual data (p>0.5, n=8)

4.6 Correlations and Regressions

Correlations were calculated based on the change in rPA values from baseline, for all outcomes. In the CD34⁺ enriched cells, correlations that initially appeared to be significant were found to be driven entirely by one subject when graphed. Therefore, only the outcomes from CFU-Hill derived cells have been presented below.

CFU colony numbers had a strong positive correlation with CFU NOi (p=0.096, R²=0.68), CFU eNOS (p=0.014, R²=0.86) and CFU-Sirt1 (p=0.001, R²=0.96), as illustrated in Figure 18. A-C.

We also found that CFU NOi was positively correlated with 3 out of the 4 genes that contribute towards NO production- CFU eNOS ($p=0.021$, $R^2=0.83$), CFU-Sirt1 ($p=0.043$, $R^2=0.77$) and CFU Cav1 ($p= 0.049$, $R^2=0.76$), as seen in Figure 19 A-C.

We found positive correlations between CFU eNOS with CFU iNOS ($p=0.015$, $R^2= 0.811$), CFU Sirt1 ($p= 0.001$, $R^2= 0.92$) and CFU Cav1 ($p=0.006$, $R^2= 0.86$), which were also genes that contribute towards NO production, as seen in Figure 20 A-C.

Multiple linear regression analyses were run on each response outcome that was found to have strong correlations. Therefore, our response outcomes and their respective predictor variables were:

Response outcome 1: CFU colonies, predictor variables: CFU NOi, CFU eNOS, CFU Sirt1

Response outcome 2: CFU NOi, predictor variables: CFU eNOS, CFU Sirt1, CFU Cav1

Response outcome 3: CFU eNOS, predictor variables: CFU eNOS, CFU iNOS, CFU Sirt1

We did not find any set or sub-set of predictor variables that could predict their respective response outcomes. However, we did confirm that they were statistically significant as independent predictors ($p < 0.05$).

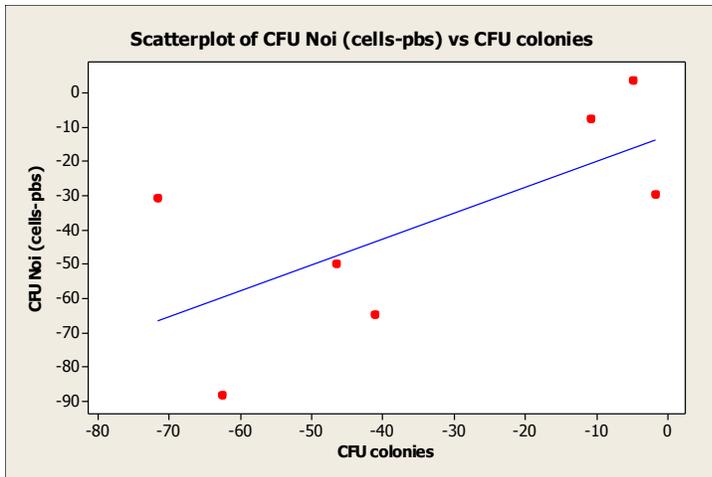


Figure 18A: Correlation of CFU-Hill colonies with CFU NOI ($p=0.096$, $R^2=0.68$)

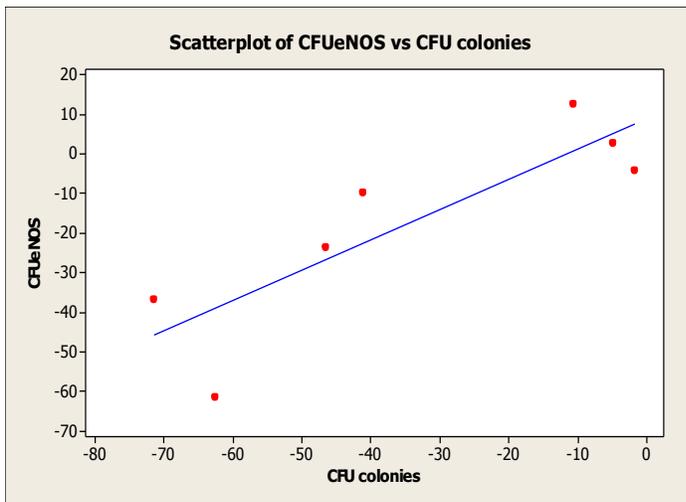


Figure 18B: Correlation of CFU-Hill colonies with CFU eNOS ($p=0.014$, $R^2=0.86$)

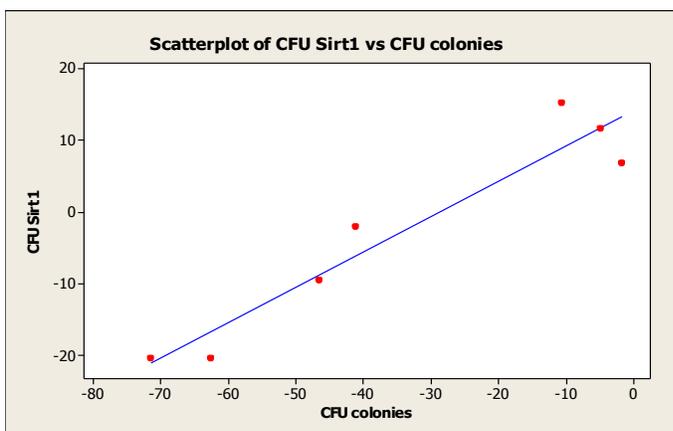


Figure 18C: Correlation of CFU-Hill colonies with CFU Sirt1 ($p=0.001$, $R^2=0.96$)

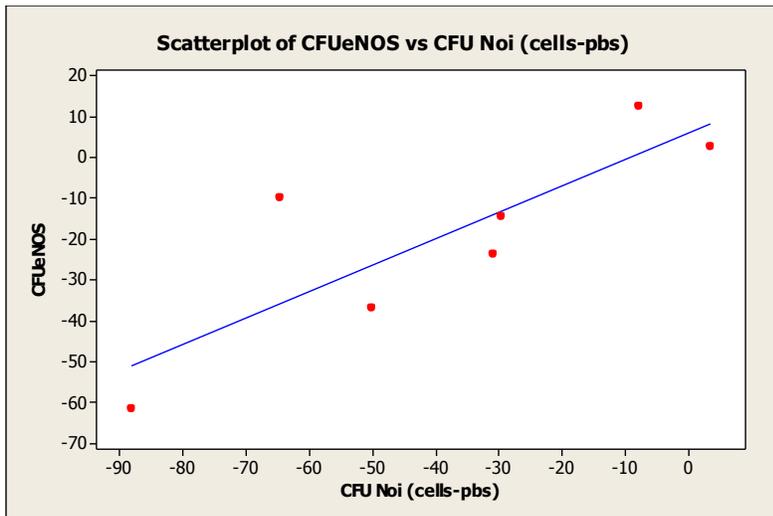


Figure 19A: Correlation of CFU NOi with CFU eNOS ($p=0.021$, $R^2=0.83$)

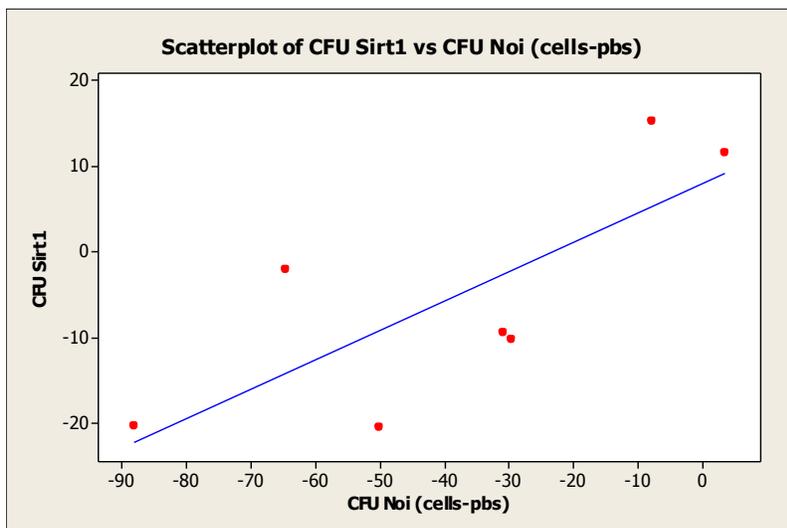


Figure 19B: Correlation of CFU NOi with CFU Sirt1 ($p=0.043$, $R^2=0.77$)

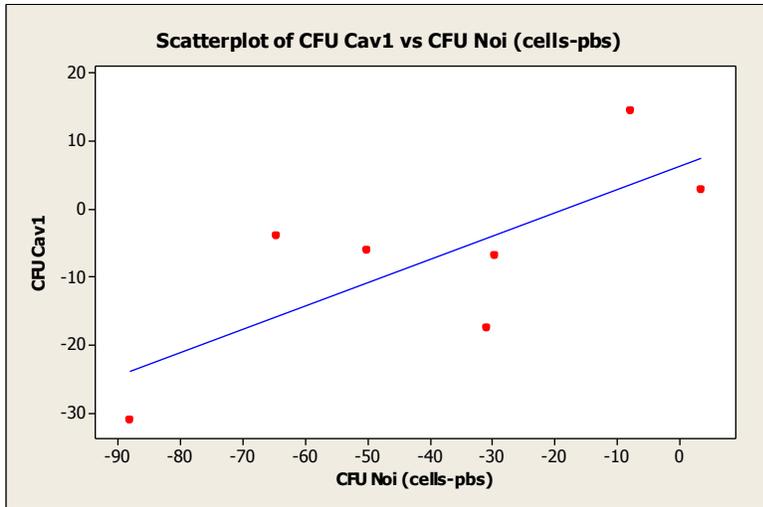


Figure 19C: Correlation of CFU NOi with CFU Cav1 ($p= 0.049$, $R^2=0.76$)

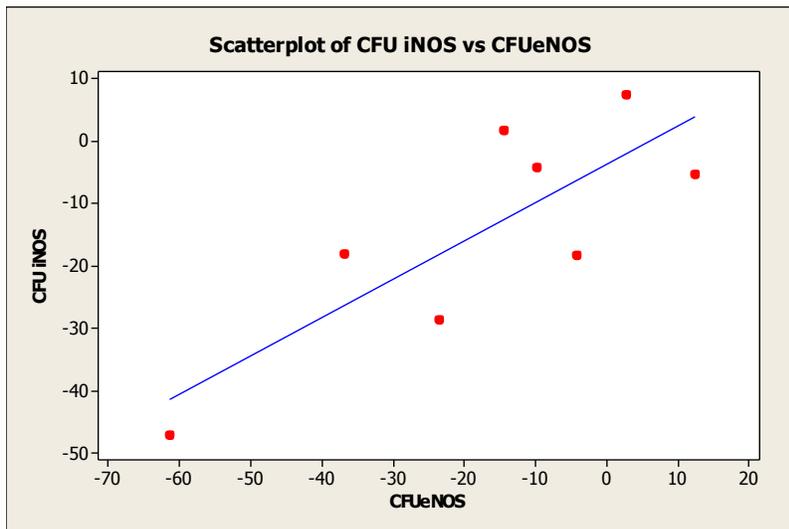


Figure 20A: Correlation of CFU eNOS with CFU iNOS ($p=0.015$, $R^2= 0.811$)

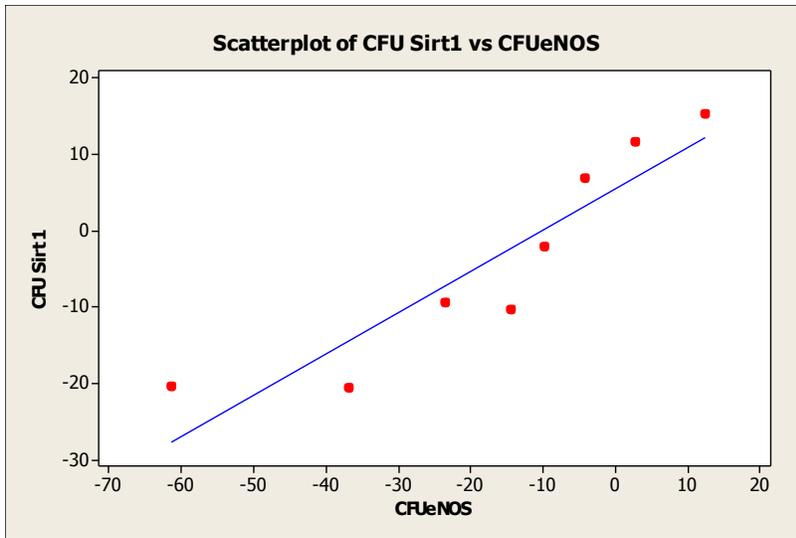


Figure 20B: Correlation of CFU eNOS with CFU Sirt1 ($p=0.001$, $R^2=0.92$)

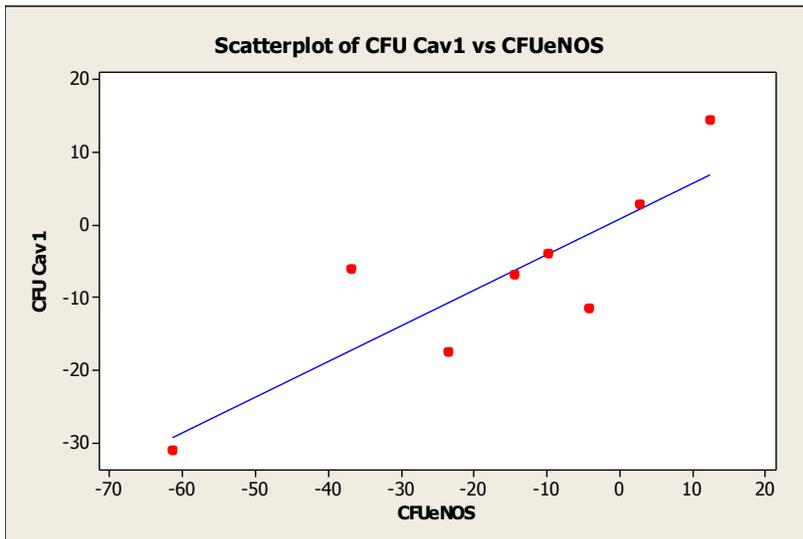


Figure 20C: Correlation of CFU eNOS with CFU Cav1 ($p=0.006$, $R^2=0.86$)

CHAPTER 5

DISCUSSION

Exercise has been shown to have positive effects on the number and function of CACs, a putative, novel and early biomarker of vascular function and cardiovascular health. In this study, we were interested in the effect of reduced physical activity in healthy, active men on two populations of CACs. We hypothesized that the reduction in physical activity would negatively impact CAC number and intracellular nitric oxide-related factors. Some of the key findings of our study were: 1) the two types of CACs we evaluated- CD34⁺ enriched cells and CFU-Hill cells, responded very differently to rPA, with decreased CFU-Hill number and NOi after rPA, and no significant changes in CD34⁺ cells and 2) changes in nitric oxide-related gene expression were correlated to changes in CFU-Hill number and NOi. Importantly, we were able to objectively measure the physical activity of our subjects to confirm that it was reduced during rPA and use energy expenditure data to ensure that participants remained in energy balance and did not increase weight during rPA.

5.1 Reduced physical activity model

The purpose of this study was to understand the effect rPA had on CACs, in healthy individuals. We accomplished this by recruiting healthy men who were primarily runners, and asking them to reduce their physical activity (rPA), which we defined as discontinuing any structured physical activity (i.e. regular work-outs) for a period of ten days. Holloszy et al.⁸⁶ and Witkowski et al.⁷⁵ have used a similar model where male athletes were asked to stop exercising for a period of 10 days; they found significant declines in glucose tolerance⁸⁶ and CAC number⁷⁵

respectively. However, both studies noted a large degree of individual variability. By using physical activity monitoring as an objective measure in our model, we were able to ascertain whether or not all subjects adhered to the protocol. This strategy was effectively used to eliminate one of our subjects from all analyses since he increased his MVPA. Neither of the studies mentioned above measured physical activity, objectively.

The significant reduction in vigorous and very vigorous activities (>6METs) from Actigraph data (Figure 8) of the 8 subjects that were compliant, helped us confirm their adherence to the protocol. Further, the change in sedentary time for our subjects during rPA was not significant (Figure 12). Existing models of inactivity such as the ones used in bed-rest studies³² and spinal cord injury studies³⁹ do not take into consideration the added or independent impact of increasing sedentary behavior on the cardiovascular system.³⁷ We were also able to avoid potential confounding factors such as weight gain (table 1) and increased energy intake during rPA by taking into account their baseline energy intake (via dietary recalls) and energy expenditure (via Actigraph monitoring), and recommending individual kcal amounts to each subject, so that they would remain in energy balance during the ten days. We infer that energy balance was maintained during rPA given that their weight did not change after rPA and based on our kcal recommendations. Therefore, we can conclude that the changes in cellular factors that we observed were likely due to the reductions in structured physical activity (vigorous and very vigorous activity) over the 10 days.

5.2 rPA response of CFU-Hill CAC

We found that in response to rPA, CFU-Hill number and NOi significantly decreased, which was correlated to changes in NO-related gene expression, whereas CD34⁺ cells were not

affected by rPA. CFU-Hill derived cells are predictive of major cardiovascular events.⁶⁶ The Framingham risk score for CVD is inversely related with the number of CFUs.²¹ At the level of the vascular endothelium, there is a strong correlation between the CFU-Hill colony count and flow-mediated brachial reactivity.²¹ With 10 days of rPA, we were able to observe a significant decrease in the number of CFU-Hill colonies (Figure 14). These findings are similar with results from a recent study by Witkowski et al.⁷⁵ however, the reduction in CFU-Hill colonies with 10 days of detraining was not statistically significant in their population of older endurance trained men. Two main differences between the studies were the objective activity monitoring and the age and training history of the men. Objective monitoring of rPA allowed us to assess changes in compliant individuals, whereas compliance was not verified with monitoring in the Witkowski et al⁷⁵ study. Second, Witkowski et al⁷⁵ recruited older men with a long-term training history (over 20 years), therefore the age of the men may have added variability due to unknown factors related to age and CFU-Hill colonies.

CFU-Hill colonies are a mixed population of cells that consist of monocytes and lymphocytes and few endothelial progenitors.^{83,84,85} Their primary function appears to be support of angiogenesis and endothelial repair via a paracrine mechanism, or the release of vaso-supportive factors.^{13,56} The generation of a colony in this assay requires appropriate cell communication and motility. With fewer colonies, rPA appeared to disrupt that signaling and communication.

Nitric Oxide has been shown to be essential for proper function in angiogenic cell populations.^{1,34,60} NO is important for CAC motility and may be an important component of

paracrine signaling to promote vascular repair and growth.¹ We observed significant decreases in CFU-Hill NOi with rPA and a strong correlation between the change in CFU-Hill colony number and CFU-Hill NOi ($p=0.046$, $R^2=0.82$). We also saw correlations between changes in CFU-NOi with changes in CFU gene expression for eNOS, Sirt1 and Cav1. While all 3 genes tended to decrease, eNOS tended to decrease the most after rPA. eNOS is one of the primary contributors of NO, shown to greatly affect CAC mobility and health. eNOS expression is altered in health and disease, with and without exercise training.⁶⁷

Oxidative stress, primarily contributed by NADPH oxidase in endothelial cells and CACs, has been known to decrease NO production and thought to do so via eNOS uncoupling.²⁷ In a study by Jenkins et al²⁷ they compared NOi in CFU-Hill cells between active and sedentary men. They also observed a significant difference in the NOi of the CFU-Hill cells between the two groups. Their group of sedentary control subjects were matched for age, weight and BMI to the active group, and the differences in the NOi between groups was partially explained by NADPH oxidase-related oxidative stress in the sedentary group whereas NADPH oxidase inhibition did not increase NOi any further in the active group. Interestingly, our gene expression results also do not indicate any increased oxidative stress for our active participants since we did not see any changes in Nox2, Nox4 and p47phox or SOD1. Interestingly, our gene expression results do not indicate any increased oxidative stress since we did not see any changes in Nox2, Nox4 and p47phox or SOD1. Further, iNOS is only activated when inflammation is present (such as IL-6 or TNF- α) and in response to oxidative stress.³¹ While not significant, the CFU iNOS from our study tended to decrease. Therefore, the reduction in NOi (intracellular NO) observed in CFU-Hill cells

does not appear to be due to increased oxidative stress with rPA. Another mechanism is likely involved in the decreased CFU-Hill NOi observed after rPA.

Our gene expression data show some evidence that eNOS downregulation, potentially through SIRT1, may be the mechanism involved in reduced CFU-Hill NOi. Cav1 (a component of eNOS) and Sirt1 (an indirect regulator of eNOS) were similarly decreased and were both independently correlated with the changes in CFU-NOi. Sirt1, an NADP⁺ dependent molecule, works indirectly via deacetylation of eNOS.⁴⁹ It has most commonly been studied in reference to insulin resistance and its upregulation has shown to positively improve outcomes via activation (deacetylation) of eNOS.⁷⁶ Blocking of Sirt1 has shown to decrease NO-bioavailability and endothelial-dependent vasorelaxation.⁴⁹ Interestingly, caloric restriction has also been shown to cause the deacetylation of eNOS via a Sirt1 pathway,³⁸ which is one of the many reasons why we attempted to ensure that our subjects remained in energy balance during the 10 days of rPA and followed a very specific low-nitrate diet 3 days prior to coming in for both testing visits. Therefore the correlations seen between Sirt 1, eNOS and CFU-NOi were not likely due to changes in caloric intake.

It is unclear what stimulus evoked by rPA caused changes in CFU-Hill NOi. NO availability is essential for the migration and homing of CACs from the bone marrow³⁴ but it is not known what stimulus related to exercise alters cellular factors within CACs. Shear stress due to increased blood flow (e.g. during running) is one of two basic pathways for increased NO production in mature endothelial cells.³¹ The second pathway for NO production is receptor-stimulated NO formation (ex. Ach, bradykinin). During rPA, we can assume that the high

amounts of shear-stress on the cardiovascular system was greatly reduced since their vigorous and very vigorous activity was significantly decreased (Figure 8). Additionally, our subjects were asked to follow a low-nitrate diet for 3 days prior to each testing visit and they were asked to avoid alcohol, caffeine and any medication for 24 hours prior to their visit, factors that could influence NO. Further, we believe that there was no external influence of the second (receptor-stimulated) pathway by which NO formation could be influenced since we were able to control for possible stimulants from food and medication. Therefore the significantly decreased NOi in the CFU-Hill-derived cells (Figure 16) would indicate that this effect was most likely mediated by the decreased shear stress during rPA. With a protocol similar to our current protocol, Witkowski. et al⁷⁵ showed that changes in CAC were strongly correlated with flow mediated dilation. Since we know that changes in FMD are primarily nitric-oxide mediated,^{10,57} it supports the possibility that the significantly decreased CFU-Hill numbers and NOi we observed were due to a reduction in shear stress during rPA.

5.3 CD34⁺ cell response

CD34⁺ cells are hematopoietic and endothelial progenitors. When CACs were first discovered by Asahara et al. in 1997, they were identified as CD34⁺ cells.³ The number of CD34⁺ cells are negatively correlated with CVD risk and they are an independent predictor of future cardiac events.¹³ The CD34⁺ surface marker is still one of the most widely used and accepted markers to define CACs and they have shown great therapeutic potential through their application in a number of clinical trials.^{13,56} Clinical trials for individuals with acute myocardial infarction, peripheral vascular disease and severe coronary artery disease used enriching CD34⁺

cells for their intervention.⁵⁶ Trials have reported generally positive outcomes such as improvements in symptoms and LVEF (left ventricular ejection fraction).

Selective activation of beneficial genes (e.g. eNOS) and selective blockade of non-beneficial genes (e.g. NADPH oxidase) in CD34⁺ cells has shown to correct the vasoreparative dysfunction in diabetic individuals.^{73,74} Jenkins et al. observed that NO and NADPH in CD34⁺ cells were significantly different in sedentary versus active individuals.⁷⁷ Using a detraining model that was similar to our study, Witkowski et al. found that the number of CD34⁺ cells significantly decreased after 10 days of detraining in highly active individuals.⁷⁵ Based on these data, we hypothesized that rPA would lead to a decrease in CD34⁺ cell number, mediated by decreases in NO and increased NADPH. However, we observed that our CD34⁺ cells were resistant to the effects of rPA.

Studies investigating the acute effect of exercise on men (mean age ~33yrs), have shown CD34⁺ cells increase after acute exercise.⁸¹ Further, while the study by Witkowski et al. found a decrease in CD34⁺ cells after detraining, they are the only study to date that has used a detraining model to show the effect of exercise cessation on CD34⁺ cells.⁷⁵ Differences in changes in CD34⁺ cell number with rPA between the two studies might also be attributable to the difference in methods used to isolate and quantify the CD34⁺ cells. We used a magnetic separation technique to isolate our CD34⁺ cells, and Witkowski et al. used flow cytometry to quantify the number of CD34⁺ cells.⁷⁵ Flow cytometry was used by them to count the number of CD34⁺ cells within the population of mononuclear cells that they collected, by using specific antibodies to detect them. Magnetic separation on the other hand positively selects for the

CD34⁺ cells using a CD34⁺ specific antibody. However, the cells attained through magnetic separation are an enriched population of CD34⁺ cells that have been positively selected. Therefore, when we make inferences regarding any of our outcomes, they must be made keeping in mind that ours is an enriched, not pure population of cells. Using the flow cytometry analyses for our study would have been useful. However, it would have been at the cost of not being able to measure gene expression or NOI.

One of the major differences between the current study and that by Witkowski, et al, as mentioned above, is the mean age of the participants; the mean age of their participants was ~63yrs compared to 24yrs in our population.⁷⁵ It has been found that older subjects have lower CD34⁺ cells compared to young individuals.¹³ Therefore, it is possible that since our population was young and active, their CD34⁺ cells were less susceptible to the effect of rPA than older, active men.

Stem and progenitor cells are known to have a robust antioxidant capacity and are therefore believed to be more resilient in an oxidative-stress environment.⁷⁸ Upon exposure to increased ROS concentrations, CAC expression of antioxidant enzymes such as catalase, glutathione peroxidase and manganese superoxide dismutase (MnSOD), was found to be significantly higher. This makes them equipped to be protected against an oxidative stress environment and is characteristic of their progenitor cell character.⁷⁸ As CD34⁺ cells are hematopoietic and endothelial progenitors, it is possible that the CD34⁺ cells from our subject population were unaffected by rPA because they were more fortified compared with CFU-Hill CAC to deal with systemic changes associated with reduced physical activity. Also, if changes in

systemic shear stress with rPA is responsible for the changes in CFU-Hill CACs as noted above, CD34⁺ CACs may not be as responsive to changes in shear as CFU-Hill CACs. However, the exact mechanism by which CD34⁺ cells are resistant to rPA compared to the CFU-Hill cells is not known and warrants further investigation.

5.4 Limitations

One limitation of our work is the challenge of isolating a pure population of CD34⁺ cells. In order to have 100% of our isolated cells be CD34⁺ we would have to draw approximately 2L of blood. While we certainly had an enriched population of CD34⁺ cells, the other mononuclear cells present in the samples might explain some of the variability of the results observed in our outcomes. Further, in order to obtain as many cells as possible for analysis, we identified our CD34⁺ CAC population on only 1 cell surface marker. CD34⁺ cell marker is ubiquitous to hematopoietic and endothelial progenitor cells and was the original marker that identified endothelial progenitor cells.³ While it is still currently used as the identifying marker for isolation and use of cells in cardiovascular regenerative therapies,⁵⁶ identification of specific CAC populations would require multiple markers and improve understanding of the diverse CAC family.

Several studies have found a large degree of individual variability in CAC response to changes in physical activity, especially in healthy individuals. Although we attempted to obtain a profile of individuals who were primarily runners with similar characteristics, no CVD risk factors, and carefully monitor physical activity and energy expenditure, we still observed some

individual variability, indicating that there are still unknown factors that contribute to the number and function of CACs.

A second limitation of our study was the measurement of sedentary behavior using the activity monitor Actigraph GT3X. While the Actigraph is well suited to measure moderate, vigorous and very vigorous activities, it is not the most accurate at measuring sedentary behavior.⁸² The primary objective of this study was to ensure that our subjects decreased their structured physical activity (vigorous and very vigorous activity) during rPA, which is a goal we were able to achieve using the Actigraph monitor. However, we must be cautious when drawing conclusions about sedentary behavior using the Actigraph outputs since this particular monitor is not sensitive enough to measure it accurately.

5.5 Conclusion

Our hypotheses for this study were that rPA would cause a decrease in CAC number, intracellular NO and genes related to NO. We further hypothesized that the number of CAC and nitric oxide related factors would be related to one another. Based on our results, we found that the CFU-Hill cells did follow the direction of our hypothesis while the CD34⁺ remained unaffected.

We observed decreases in CFU-Hill colony numbers, NO_i and eNOS and found strong correlations between all these outcomes. Further NO_i was strongly correlated with Sirt1 and Cav1 (NO regulators). Since we did not see any increases in oxidative stress markers that can cause to decrease NO, further research is needed in order to understand the cellular mechanisms behind the decreases observed. Just as shear stress is hypothesized as the cause for increased CACs during exercise, we suspect that the reduction of high-intensity shear stress

during the 10 days of rPA was one of the mechanisms through which the CFU-Hill cells were decreased in number and function.

Our data is novel in that we were able to show how the cells derived from this assay are sensitive to reductions in physical activity, in healthy individuals. Given the inverse relationship between CFU-Hill colony numbers and the Framingham risk score for CVD, there is a larger implication with these findings. Fewer than 5% of Americans adhere to the minimum physical activity guidelines and could be at risk for CVD early on. It is important for us to take advantage of this novel biomarker and further investigate ways it can be used for early detection and prevention of CVD.

APPENDIX

QUESTIONNAIRES, INFORMED CONSENT AND PROTOCOLS

6.1: Informed consent

Consent Form for Participation in a Research Study

University of Massachusetts Amherst

Principal Investigator: Sarah Witkowski, Ph.D.
Study Title: Inactivity and Cardiovascular Disease
Sponsor: UMASS Amherst Faculty Research Grant

1. WHAT IS THIS FORM?

This form is called a Consent Form. It will give you information about the study so you can make an informed decision about participation in this research study.

This consent form will give you the information you will need to understand why this study is being done and why you are being invited to participate. It will also describe what you will need to do to participate and any known risks, inconveniences or discomforts that you may have while participating. We encourage you to take some time to think this over and ask questions. If you decide to participate, you will be asked to sign this form and you will be given a copy for your records.

2. WHO IS ELIGIBLE TO PARTICIPATE?

Healthy men and women age 18-50 years old who have been participating in regular endurance running exercise for over 10 years are eligible to participate in this study. Participants must weigh over 110lbs. Regular running exercise history should include at least 3 days/week of moderate to intense activity. All participants must be healthy, non-smoking and free of cardiovascular disease, diabetes, lung disease, and cancer. Participants cannot be taking medications for high cholesterol, high blood pressure, erectile dysfunction, or diabetes and cannot be taking vitamins or supplements. Women must not be pregnant.

3. WHAT IS THE PURPOSE OF THIS STUDY?

The purpose of this research study is to evaluate the effect of short-term inactivity (detraining) on age-related factors of cells found in your blood that may be related to the development of cardiovascular diseases. Our objective is to determine whether stopping regular exercise will result in changes in factors that protect your blood cells from aging and disease.

4. WHERE WILL THE STUDY TAKE PLACE AND HOW LONG WILL IT LAST?

The research for this study will be performed in the Kinesiology Department at the University of Massachusetts in Amherst, MA. Each participant will be asked to come to our laboratory 3 times, a screening visit, and 2 testing visits. There will be 1 week between the first and second testing visits and 10 days between the second and third testing visit. The 1st visit will last about 1 hr, the 2nd and 3rd visit last for 2 hrs each, for a total of about 4 hours. Your total participation will last a span of 3 weeks.

5. WHAT WILL I BE ASKED TO DO?

If you agree to take part in this study you will be given a health history and physical activity questionnaires to fill out. Following this you will be asked to come to the lab for a screening visit.

Visit #1: Screening/Familiarization: During this visit you will: 1) Review the informed consent, 2) clarify any questions we might have about your health history, training/competition history, 3) provide us with documentation from your doctor with results from your most recent physical (within the past 9 months), 4) have your blood pressure taken, 5) have a blood sample taken for a general blood chemistry screen, 6) get fitted with two activity monitors and 6) be familiarized you with the protocols we will be performing for visits 2 and 3.

Blood pressure: During this visit, to evaluate your blood pressure, we will ask you to sit quietly for several minutes. A cuff will be placed around your upper arm. We will inflate this cuff then slowly release the pressure while we listen for the blood pressure in the vessel with a stethoscope. We will repeat this procedure a few times to get an accurate reading.

Blood chemistry screen: To evaluate your blood chemistry profile, we will use a needle to withdraw about 1 tablespoon of blood from the vein in your arm (about 15ml)

Activity monitors: We will also fit you with an activity monitor (ActiGraph) as well as a pedometer (Omron) that you will wear at your hip to evaluate the amount of physical activity and step count over the course of 1 week. During this week, we will ask that you wear these monitors and perform the tasks (exercise and otherwise) that you perform in a typical week. You will be asked to take the pedometer off during your exercise. After 1 week, you will return to the lab to have the monitors removed and your second testing visit.

The ActiGraph is a wearable activity monitor worn at the hip. It weighs 19 gms and its size is 46mm x 33mm x 15mm and can monitor an individual's activity for up to 7 days. The ActiGraph is secured to the hip using an elastic belt. It can be worn over your clothes or under it. You will be instructed to remove the ActiGraph during bathing, swimming and sleeping. These are the only times the ActiGraph should be removed.

The Omron is a pedometer that weighs 28 gms, its size is 47 mm x 66 mm x 16 mm and you will wear this on the elastic belt with the ActiGraph. The Omron will monitor your step count for 7 days. In addition to keeping the Omron off when the ActiGraph is off, you will also be asked to remove it when you are exercising.

Monitor and Diet logs: You will be given an activity log where you will be asked to record times that the monitors are removed and worn again. Reasons for monitor removal will also be recorded. At the end of the 7-day period, you will return the logs along with the ActiGraph and the Omron.

You will also be given a diet log in order to keep a detailed account of your food intake throughout the day during the 7 days that you will also be wearing the activity monitor. We will explain to you how you need to log these details and you will also be counseled on stopping any dietary supplements and vitamins that you might be taking for the duration of the study.

If you remain qualified for the study, or you meet the inclusion criteria and do not meet the exclusion criteria mentioned above, we will schedule you for 2 testing visits. We will also familiarize you with the measurements we will perform during visits 2 and 3.

Testing Visit #2: Testing Prior to your second testing visit, we will ask that you perform an exercise session between 36- 24 hours prior to your visit and that you do not perform any other exercise in the 24 hours directly before your visit. You will also be asked to refrain from caffeine or alcohol during those 24 hours. In addition, we ask that you refrain from taking any medications (including vitamins) 48 hours prior to coming in. Because eating affects substances in the blood, we will also ask that you refrain from eating or drinking anything other than water for 12 hours prior to your visit. When you arrive in the lab, we will ask you to give us your ActiGraph, Omron, diet and monitor logs.

Following this we will have you lay quietly for a few minutes and then draw blood from the vein in your arm. We will draw about 4-5 tablespoons of blood (about 50ml).

Next, we will have you lay quietly in a room for 30 minutes then perform a flow-mediated dilation (FMD). This will involve the use of an ultrasound machine to visualize an artery in your non-dominant arm. We will then inflate a blood pressure cuff right above your elbow and keep it inflated for 5 minutes. After 5 minutes the cuff will be rapidly deflated so that we can get another image of your artery.

We will provide you with a snack and beverage at the end of the testing visit.

During the next 10 days, you will be asked to be inactive. This means that you cannot perform your regular physical activity workouts. We will also give you the pedometer and activity monitor again that measures your activity. The pedometer, activity monitor, diet and activity logs will be given back to you for these 10 days so you can keep a record like you did for the previous week. You will use the pedometer to help ensure that you are staying as inactive as possible, at less than 5000 steps/day. During these 10 days you will also be counseled on how you will need to decrease your food intake to match your decrease in activity.

Testing Visit # 3: Testing: After 10 days, you will be asked to return to the lab with the monitors and the logs. You will again be asked to refrain from eating or drinking anything besides water for 12 hours prior to this visit and no caffeine or alcohol for 24 hours prior to the visit. In addition, we will ask you to refrain from taking any medications (i.e. aspirin, ibuprofen, decongestants) for the 48 hours prior to this visit.

After having you rest quietly for a few minutes we will draw about 4-5 tablespoons of blood (about 50ml). Next, we will have you lie quietly for 30 minutes and perform a FMD again like we would have during visit # 2. We will provide you with a snack and a beverage following before you leave.

6. What are my benefits of being in this study?

You will earn \$100 for completion of the entire protocol, this means all visits and testing.

We hope that your participation in the study may help investigators to determine whether short-term inactivity has an effect on the biological aging of cells in your blood. This information will help to determine if exercise and inactivity can cause relatively rapid changes in cellular aging in humans.

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

7. WHAT ARE my RISKS OF being in THIS STUDY?

The following risks are associated with your participation in this study:

1) There is risk of bruising and infection associated with blood drawing. These risks will be minimized by the use of sterile techniques and by having experienced personnel draw all blood samples. There may also be a chance of fainting during the blood draw. We will ask about any history of fainting during your screening visit. To minimize the risk of fainting or effects of fainting, blood draws will be performed with you in a reclined position.

2) The risk of stopping your exercise for 10 days is that your risk factors for cardiovascular disease and aging may deteriorate. It is unlikely that substantial changes will take place over the space of the 10 days without exercise. Furthermore, you will be able to start exercising again immediately after these 10 days without exercise.

3) Physical Activity Monitoring: There are no known risks associated with monitoring your physical activity with either the ActiGraph activity monitor or Omron pedometer.

4) You will be asked to fast for 12 hours on two occasions (prior to visits 2 and 3). Fasting for this time period may result in some discomfort. You will be allowed to take in water during this period. In addition, you will be given a snack and beverage following your testing during visits 2 and 3.

5) Your involvement may involve some inconveniences, such as time spent in travel, performing the screening and testing procedures, and stopping your physical activity. Your parking costs will be covered.

8. how will my personal information be protected?

We will do our best to keep your personal information confidential. To help protect your confidentiality, all data are kept in a secure locked office with access available only to study personnel. Furthermore, all electronic files (e.g., database, spreadsheet, etc.) containing identifiable information will be password protected. Computers with data from this study will be protected by a password and only available to be accessed by authorized personnel involved with the study. Only the members of the research staff will have access to the passwords.

Blood samples will be labeled with a unique code, not your name or any other identifying information. By agreeing to participate in this study, you release ownership of blood samples to the University of Massachusetts Molecular Cardiovascular Physiology Laboratory. The study investigators may store some samples for future analysis, for a maximum of 3 years, after which point the samples will be safely destroyed as per the University's Environmental Health and Safety guidelines. The samples will be stored in a -80°C freezer in our lab. They will be identified by a unique code. The key to the codes will be kept in a locked and secure cabinet. Only the study personnel will have access to the codes

If we write a report or article about this research project, your identity will be protected to the maximum extent possible. Information will be presented in summary format and you will not be identified in any publications or presentations. Your information may only be shared with representatives of the University of Massachusetts or government authorities if you or someone else is in danger or if we are required to do so by law. Our records of the research will be maintained for **3 years** following study completion.

Please indicate if you agree or disagree to having us store your samples for a maximum of three years, for further analysis, under the conditions described above (check only one of the two boxes below):

- I agree to have my samples stored for future analysis under the conditions mentioned above
- I do not agree to have my samples stored for future analysis under the conditions mentioned above

9. WHAT IF I HAVE QUESTIONS?

Take as long as you like before you make a decision. We will be happy to answer any question you have about this study. If you have further questions about this project or if you have a research-related problem, you may contact the principal investigator, Sarah Witkowski at switkows@kin.umass.edu or 413-545-6102 or graduate student, Gayatri Guhanarayan at gguhanar@kin.umass.edu or 973-572-8941. If you have any questions concerning your rights as a research subject, you may contact the University of Massachusetts Amherst Human Research Protection Office (HRPO) at (413) 545-3428 or humansubjects@ora.umass.edu.

11. CAN I STOP BEING IN THE STUDY?

You do not have to be in this study if you do not want to. If you agree to be in the study, but later change your mind, you may drop out at any time. There are no penalties or consequences of any kind if you decide that you do not want to participate. You will be notified of all significant new findings during the course of the study that may affect your willingness to continue.

12. WHAT IF I AM INJURED?

The University of Massachusetts does not have a program for compensating subjects for injury or complications related to human subjects research, but the study personnel will assist you in getting treatment. Medical treatment will be available at the University Health Services for a fee.

13. SUBJECT STATEMENT OF VOLUNTARY CONSENT

I have read this form and decided that I will participate in the project described above. The general purposes and particulars of the study as well as possible hazards and inconveniences have been explained to my satisfaction. I understand that I can withdraw at any time.

Participant Signature: Print Name: Date:

By signing below I indicate that the participant has read and, to the best of my knowledge, understands the details contained in this document and has been given a copy.

Signature of Person Print Name: Date:

Obtaining Consent

6.2: Health history questionnaire

Participant ID:

Molecular and Cardiovascular Physiology Laboratory University of Massachusetts
DEPARTMENT OF KINESIOLOGY,
TOTMAN BUILDING, 30 EASTMAN LA, AMHERST MA 01003
(413) 545-6012 (413) 545-2906 FAX

Health History Questionnaire

GENERAL HEALTH

Height _____ Weight _____

How would you describe your overall physical health? ____ excellent ____ good ____ fair
____ poor Have you undergone a physical examination in the last 5 years? Yes No Are
you on a special diet? Yes No If yes, what type: _____ Have
you gained or lost more than 10 lbs in the last 6 months? Yes No Have you had any
illness in the last 2 weeks? Yes No If yes, specify: _____ Do
you have documented heart disease? Yes No If yes, how long ago was it documented?
_____ years Has a doctor ever told you that you have an ulcer? Yes No Has a
doctor ever told you that you have any type of bleeding disorder? Yes No

Have you ever experienced dizziness or fainting during a blood draw? Yes No

Have you ever been advised by your doctor or any medical professional that you should not
exercise or engage in any sort of strenuous physical activity? Yes No

If yes, please explain: _____ Participant ID:

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

Year of

for yes

Onset

_____ High blood pressure

_____ Heart attack /coronary problem

_____ Heart murmur

_____ Heart disease

_____ Pain or tightness in the chest

_____ Palpitations/rapid heart beat

_____ Phlebitis

_____ Stroke

_____ Lung/respiratory problem s

_____ Diabetes

_____ Varicose veins

_____ High cholesterol

_____ Anemia

_____ Thyroid problems

_____ Cancer

6.3: Physical activity questionnaire

Participant ID:

Molecular and Cardiovascular Physiology Laboratory University of Massachusetts

DEPARTMENT OF KINESIOLOGY,

TOTMAN BUILDING, 30 EASTMAN LA, AMHERST MA 01003

(413) 545-6012 (413) 545-2906 FAX

Physical Activity and Performance Questionnaire

Physical Activity Information

How many times a week do you exercise?

How many minutes do you spend exercising per session? _____
minutes

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

Mode(s) of exercise (check all that apply):

Running Cycling Swimming Other _____

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

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

Running _____ Cycling _____ Swimming _____ Other _____

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

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

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

_____ Participant ID:

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

EXERCISE MODE MILEAGE COVERED DURATION

(run/cycle/etc)

SUNDAY _____

MONDAY _____

TUESDAY _____

WEDNESDAY _____

THURSDAY _____

FRIDAY _____

SATURDAY _____

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

6.4: Baseline- activity and diet log

Information Packet

Baseline Week

DIET

During the baseline week you will be asked to continue your regular dietary habits. A researcher will email you twice during the week and once during the weekend, in the morning, to ask you to recall what you ate the day before. Please be as accurate as possible and complete the dietary recall before midnight of the day that you receive the email.

ACTIGRAPH

During the baseline week you will be asked to wear an Actigraph monitor and continue your regular exercise routines. It is important that you record when you put the Actigraph on and when you take it off in the Actigraph Record Chart (next page). Indicate the reason for taking the Actigraph off (sleep, shower, exercise, other). Please follow these instructions:

- The Actigraph is worn on the right hip, in line with the right armpit and knee
- Be sure the monitor firmly placed and snug around the waist and the monitor is oriented correctly with the arrow pointing up
- This monitor will be worn during you waking ours (from the time you wake up until the time you go to bed) for the seven-day baseline period
 - You will record the times you put the monitor on in the morning and the times you took it off at night on your monitor log for each day
 - You will also record other times during the day when you took the monitor off (for things like swimming and showering) for each day
- Do not wear monitor in water
 - If you do bathe or participate in water-based activities, please remove monitors
 - Record the times the monitor is removed and put back on in you monitor log
- Please be sure to record times with AM or PM indicated

PEDOMETER

During the baseline week you will be asked to wear a pedometer throughout the day. Please take the pedometer off only during exercise, sleep, and anytime it would get wet (swimming or bathing). Please record the times you take it off and put it back on on the Pedometer Record Chart (last page).

Actigraph Record Chart

- Record time on and off for each day, Record reason (sleep, shower, exercise, other)
- Indicate AM or PM

	ON	OFF	ON	OFF	ON	OFF	ON	Off
Day 1 Date:								
Day 2 Date:								
Day 3 Date:								
Day 4 Date:								
Day 5 Date:								
Day 6 Date:								
Day 7 Date:								

Pedometer Record Chart

- Record time on and off for each day, Record reason (sleep, shower, exercise, other)

- Indicate AM or PM

	ON	OFF	ON	OFF	ON	OFF	Start of day count	End of day count
Day 1 Date:								
Day 2 Date:								
Day 3 Date:								
Day 4 Date:								
Day 5 Date:								
Day 6 Date:								
Day 7 Date:								

6.5: Baseline- checklist for exercise and diet (for visit#2)

Participant:

Checklist for Cardiovascular Study:

Thank you for being part of our study! Here is a checklist of things we will need you to do before coming in for your testing visit on _____.

- Please follow the low-nitrate diet (based on the recommendations attached) for 3 days prior to your testing visit. Starting on _____
- You must exercise* 24 hours prior to your testing visit. This can be anytime between the hours of _____(time) on _____(date)
*This should consist of your normal exercise routine (which can include running, cycling, swimming) but CANNOT include weight-lifting. Do not over-exert yourself since this can affect the study outcomes.
 - Please do not exercise after this time.
- You CANNOT consume the following substances 24 hours prior to you visit: starting at _____ (time) on _____ (date)
 1. Medications (including vitamins and allergy medications)
 2. Supplements (such as protein shakes etc.)
 3. Caffeine
 4. Alcohol
- In addition to this, you will need to be in a fasted state for 8 hours prior to your visit. Starting at _____ (time) on _____ (date). During those 8 hours you can only have water.
 - We strongly recommend that you have plenty of water before coming in for the visit.
- On the morning of the testing visit on _____ we will need you to take 2 measurements of your pulse over the course of 1 minute BEFORE YOU GET OUT OF BED (carotid or brachial will do). Please note the readings down below and bring them in with you. We will require these readings for the exercise portion of the testing.
 - HR1:_____beats/minute
 - HR2:_____beats/minute

If you have any questions or concerns please don't hesitate to contact me Gayatri (Gaya):Email: gguhanar@kin.umass.edu Phone: 973-572-8941.

6.6: Low-nitrate diet instructions (for 3 days prior to visits# 2 and 3)

1. Dietary Restriction List

Since diet influences your blood pressure and sodium excretion rate, certain dietary restrictions (low nitrate diet) apply to this part of the study. We ask that for 3 days you exclude from your diet the foods and drinks that are listed on this page. Therefore you will be on this diet 2 days prior to your blood pressure measurement and urine collection, and remain on the diet during your blood pressure measurement and urine collection. This will help ensure the accuracy of the results of your urine collection test.

Upon completion of your 6 months of exercise training, this booklet will be given back to you, and you will be asked to mimic this diet prior to your final urine collection and blood pressure measurement.

LOW NITRATE DIET

(<http://www.nhlbi.nih.gov/labs/7east/lowNdiet.htm>)

FOOD GROUP	FOODS ALLOWED	FOODS NOT ALLOWED
<i>Meat or Substitutes</i>	<ul style="list-style-type: none"> • All plain fresh or frozen beef, poultry, lamb, veal or pork 	<ul style="list-style-type: none"> • All seafood (shellfish, salt water or fresh water fish) • All cured, smoked, preserved, canned or processed meats, e.g., cold cuts, bacon, sausage, ham, hotdogs, pepperoni, corned beef, pastrami • All canned meats and fish, including tuna • Tofu, soybeans, legumes • Nuts, peanut butter • Vegetarian burgers made with lentils, soybeans or other legumes
<i>Vegetables</i>	<ul style="list-style-type: none"> • NONE 	<ul style="list-style-type: none"> • All vegetables (raw or cooked) • Pickles, olives, relish • Raw or cooked garlic and onions • All vegetable based products such as tomato sauces, ketchup, and tomato based pizza sauce

<i>Potato or Substitutes</i>	<ul style="list-style-type: none"> • Rice, pasta, noodles 	<ul style="list-style-type: none"> • All white or sweet potatoes including potato chips, french fries • Any prepared with cheeses or vegetables, including tomatoes, tomato sauces
<i>Eggs</i>	<ul style="list-style-type: none"> • Any except as listed 	<ul style="list-style-type: none"> • Any to which "foods not allowed" have been added, e.g., cheese omelet
<i>Dairy</i>	<ul style="list-style-type: none"> • Milk--whole, 2%, skim, chocolate • Yogurt, plain or with allowed fruits • Cottage cheese, cream cheese • Ice cream or ice milk (vanilla, chocolate) • Cream 	<ul style="list-style-type: none"> • Yogurt with non-allowed fruits • All other cheese--e.g., cheddar, swiss, blue, american, mozzarella • Ice cream with non-allowed fruit or nuts • Sour cream
<i>Fruit or Fruit Juice</i>	<ul style="list-style-type: none"> • Any except as listed 	<ul style="list-style-type: none"> • Strawberries • Bananas • Melons
<i>Soup</i>	<ul style="list-style-type: none"> • No commercial soup • (Homemade, made with allowed ingredients is OK) 	<ul style="list-style-type: none"> • All soups, broths, and brines should be avoided
<i>Fats</i>	<ul style="list-style-type: none"> • Butter, margarine, mayonnaise, vegetable oils • Vinegar & oil salad dressing 	<ul style="list-style-type: none"> • Salt pork, salad dressings containing tomato sauce • Worcestershire sauce or other ingredients not allowed
<i>Breads and Cereal</i>	<ul style="list-style-type: none"> • Pretzels • Graham crackers • Any cereal, crackers, doughnuts or sweet rolls, breads/rolls, muffins, snack crackers, except as listed 	<ul style="list-style-type: none"> • Any prepared with "foods not allowed, e.g., cheese bread, popcorn, doughnut with strawberry filling, • Cereals containing nuts (such as some granolas) • Banana bread
<i>Desserts</i>	<ul style="list-style-type: none"> • Cookies, cakes, pies, jello, 	<ul style="list-style-type: none"> • Desserts containing nuts, sour

	popsicles, frozen desserts, except as listed	cream, non-allowed fruits
\Beverages	<ul style="list-style-type: none"> • Allowed bottled water • Montgomery County tap water (limit to 2 cups daily) • Beverages prepared with allowed water • Soda-limit to 1 can/day 	<ul style="list-style-type: none"> • Non-allowed bottled water • Private well water • Beer, wine, liquor, non-alcoholic beer
Miscellaneous	<ul style="list-style-type: none"> • Sugar, syrup, spices, seasonings, herbs, salt, pepper, honey, jelly (not strawberry), mustard, chewing gum, vinegar, vanilla, onion and garlic salt or powder 	<ul style="list-style-type: none"> • Candy with nuts, • Soy sauce, catsup, steak sauce, chili, cocktail sauce, worcestershire sauce, tomato containing sauces, tabasco sauce • Tobacco

SAMPLE MEALS:

Breakfast Menus

- | #1 | #2 | #3 |
|---|--|---|
| <ul style="list-style-type: none"> • Orange Juice • Rice Krispies • Low-fat Milk • Wheat Toast • Grape jelly • Margarine • Tea or coffee • Cream or Sugar | <ul style="list-style-type: none"> • Apple Juice • Pancakes/syrup • Scrambled eggs • English Muffin • Orange Slices • Margarine • Low-fat Milk • Tea or Coffee | <ul style="list-style-type: none"> • Cranberry Juice • Oatmeal • Blueberry Muffin • Peaches (warmed if desired) • Apple Butter • Bagel • Cream Cheese • Tea or Coffee |

Do not use strawberry jelly, strawberry jam, strawberry preserves, strawberry syrup or strawberry cream cheese. Avoid all doughnuts and bagels made with strawberries. Do not add bananas to your dry cereal.

Lunch Menus

- | #1 | #2 | #3 |
|--|---|--|
| <ul style="list-style-type: none"> • Plain hamburger on bun • Pretzels • Fresh Pear • Oreo Cookies • Soda | <ul style="list-style-type: none"> • Sliced turkey Sandwich • Mayonnaise • Fresh Grapes • Blueberry Yogurt • Low Nitrate Bottled Water | <ul style="list-style-type: none"> • Grilled Chicken Sandwich • Plain Pita Chips • Mandarin Oranges • Mayonnaise/Mustard • Brownie (without nuts) • Low-fat Milk |

Please remember that the **only condiments** allowed for burgers and sandwiches are **mustard and mayonnaise**.

Do not add lettuce, tomato, or pickles to sandwiches.

Remember that **strawberry fruited yogurt is not allowed**.

Remember to **drink only 1 can of soda daily**

Fruit salads are great - as long as they **do not contain bananas, strawberries, or melons**.

Dinner Menus

- | #1 | #2 | #3 |
|--|---|---|
| <ul style="list-style-type: none"> • Grilled Pork Chops • Steamed Pasta • Applesauce • Plain Dinner Roll • Grape jelly • Margarine • Tea or coffee • Angel Food Cake | <ul style="list-style-type: none"> • Honey Roasted Chicken • Fried Rice (don't add onions) • Poached Pears • Plain Popovers • Orange Slices • Margarine • Apple Pie • Tea or Coffee | <ul style="list-style-type: none"> • Broiled Steak • Steamed Rice • Microwaved Peach Slices • Peaches (warmed if desired) • French Bread • Margarine • Orange Sherbet • Tea or Coffee |

Rice can be fried in oil with herbs and spices, **but do not add vegetables**.

Make sure your tea and coffee are made with low nitrate water, or are part of your tap water allowance.

6.7: rPA- activity and diet log

Information Packet

10 day reduced activity period

DIET

Please Login to www.myfitnesspal.com every other day to enter your dietary information. Start by filling it out on the first and second day that you start your reduced activity so that you are able to modulate your eating habits to meet the daily calorie “goal” that is set for you on the website.

Goal: _____

The login information that you need is to access your account on myfitnesspal is-

Username: _____

Password: _____

Please check your weight whenever possible to make sure you don't gain/lose weight

Weight day2: _____, day4: _____, day6: _____, day 8: _____

ACTIGRAPH

During the ten-day reduced activity period you will be asked to wear an Actigraph and stop all structured physical activity. See note on “How to reduce physical activity.” It is important that you record when you put the Actigraph on and when you take it off in the Actigraph Record Chart. Indicate the reason for taking the Actigraph off (sleep, shower, exercise, other).

Please follow these instructions:

- The Actigraph is worn on the right hip, in line with the right armpit and knee
- Be sure the monitor firmly placed and snug around the waist and the monitor is oriented correctly with the arrow pointing up
- This monitor will be worn during your waking hours (from the time you wake up until the time you go to bed) for the ten-day reduced activity period
 - You will record the times you put the monitor on in the morning and the times you took it off at night on your monitor log for each day
 - You will also record other times during the day when you took the monitor off (for things like swimming and showering) for each day
- Do not wear monitor in water
 - If you do bathe or participate in water-based activities, please remove monitors
 - Record the times the monitor is removed and put back on in you monitor log
- Please be sure to record times with AM or PM indicated

PEDOMETER

During the ten day reduced activity period you will be asked to wear a pedometer throughout the day. Be sure to stay at or below the maximum number of steps per day as advised by the researchers. Please take the pedometer off only during sleep, and anytime it would get wet (swimming or bathing). Please record the times you take it off and put it back on on the Pedometer Record Chart.

Limit for steps/day : _____

HOW TO BE INACTIVE:

1. No running, weight lifting, elliptical, or any form of exercise
2. Avoid walking long distances and sprinting
3. Take the bus, car and use elevator whenever you can
- 4. Sit as much as you can**
5. Do not participate in recreation sports
6. Limit steps per day according to advice of researchers (above).



Actigraph Record Chart

- Record time on and off for each day, Record reason (sleep, shower, other)
- Indicate AM or PM

	ON	OFF	ON	OFF	ON	OFF	ON	OFF
Day 1 Date:								
Day 2 Date:								
Day 3 Date:								
Day 4 Date:								
Day 5 Date:								
Day 6 Date:								
Day 7 Date:								
Day 8 Date:								
Day 9 Date:								
Day 10 Date:								

--	--	--	--	--	--	--	--	--

Pedometer Record Chart

- Record time on and off for each day, Record reason (sleep, shower, other)
- Indicate AM or PM

	ON	OFF	ON	OFF	ON	OFF	Start of day count	End of day count
Day 1 Date:								
Day 2 Date:								
Day 3 Date:								
Day 4 Date:								
Day 5 Date:								
Day 6 Date:								
Day 7 Date:								
Day 8 Date:								
Day 9 Date:								

Day 10 Date:								

6.8: rPA- checklist for diet (for visit#3)

Participant:

Checklist for Cardiovascular Study:

Thank you for being part of our study! Here is a checklist of things we will need you to do before coming in for your testing visit on _____.

- Please follow the low-nitrate diet (based on the recommendations attached) for 3 days prior to your testing visit. Starting on _____
- You CANNOT consume the following substances 24 hours prior to you visit:
starting at _____ (time) on _____ (date)
 5. Medications (including vitamins and allergy medications)
 6. Supplements (such as protein shakes etc.)
 7. Caffeine
 8. Alcohol
- In addition to this, you will need to be in a fasted state for 8 hours prior to your visit. Starting at _____ (time) on _____ (date). During those 8 hours you can only have water.
 - We strongly recommend that you have plenty of water before coming in for the visit.
- On the morning of the testing visit on _____ we will need you to take 2 measurements of your pulse over the course of 1 minute BEFORE YOU GET OUT OF BED (carotid or brachial will do). Please note the readings down below and bring them in with you. We will require these readings for the exercise portion of the testing.
 - HR1: _____beats/minute
 - HR2: _____beats/minute

If you have any questions or concerns please don't hesitate to contact me Gayatri (Gaya):

Email: gguhanar@kin.umass.edu

Phone: 973-572-8941.

6.9: CFU-Hill assay protocol

EPC Colony Forming Unit Protocol

PREPARATION

Supplies/sample

(5) K ₃ EDTA 7 ml blood drawing tubes	Sterile pipettes
(6) 50 ml polypropylene conical tubes	Pipetter
(3) 15 ml polypropylene conical tube	Micropipettes (p20, p200)
(1) 6-well fibronectin coated plate	Pipette tips
Transfer pipette	Hemocytometer

Solutions & Reagents

3% Acetic Acid with Methylene Blue (220 ul/sample)
PBS (~14 ml/sample)
Ficoll Paque PLUS (15 ml/sample)
EndoCult Medium (7ml/sample)
PBS + 2% FBS (up to 52 ml/sample)

Preparation of EndoCult Medium

- 1) Thaw EndoCult Supplement under refrigeration (2-8°C) overnight or at RT
- 2) Add 2 ml Supplement for every 8 ml of Basal Medium
 - a. Alternatively, add entire bottle of Supplement (20 ml) to entire bottle of basal medium (80 ml).
 - b. Mixed EndoCult Medium is stable for ~ 1 month at 2-8°C
- 3) Add 1 ml of Penicillin + Streptomycin Antibiotic Solution per 100 ml of EndoCult Medium
 - a. Thaw antibiotic solution in 37° C water bath until just thawed.
 - b. 1.5 ml aliquots are in -20° freezer. DO NOT REFREEZE ALIQUOTS!
 - c. **Once antibiotic is added, EndoCult medium must be used within 1WEEK.**

Date of media prepared (with Pen Strep): _____

PROTOCOL

Day 0: Density centrifugation and plating

- 1) Set the big centrifuge at 25 deg C
- 2) Collect blood in 5 purple top (EDTA) 10 ml vacutainer tubes and 1 red top tube

- 3) Prepare mononuclear cell suspension
 - a) Add Blood to 50 ml conical tube, then add an equal volume of PBS to the same tube
 - b) Add 15ml of Ficoll-Paque PLUS to a **NEW** 50 ml conical tube
 - i. Carefully layer the diluted blood on top of the 15 ml of Ficoll **DO NOT MIX BLOOD AND FICOLL**
 - ii. **Use the manual pipette or seriological pipette if confident enough**
 - c) Centrifuge at **300 x g (1250 rpm - CCL)** for **25 min** at **RT** with **BRAKE OFF**
 - d) At this point bring the pbs/fbs out to thaw in a 50ml tube, in incubator
 - e) Collect mononuclear layer into a **NEW** 50 ml conical tube using transfer pipette
 - f) Add PBS + 2% FBS to bring final volume up to 40 ml
 - g) Centrifuge at **300 x g (1250 rpm - CCL)** for **7 min** at **RT** with **BRAKE ON** to pellet cells

FOLLOW CD34⁺ MAGNET PROTOCOL USING 2 OF THE 50ML TUBES AFTER THIS STEP (2f)

- 4) Remove supernatant and re-suspend pellet
 - a) Re-suspend with 6 ml of PBS +2% FBS
 - b) Transfer cell solution to 15 ml conical tube
 - c) Rinse the 50 ml conical tube with 6 ml of PBS + 2% FBS and transfer this cell solution to the same 15 ml conical tube
 - d) Centrifuge at **300 x g (1250 rpm - CCL)** for **7 min** at **RT** with **BRAKE ON** to pellet cells

- 5) Remove supernatant and re-suspend cells in 3 ml of EndoCult Medium

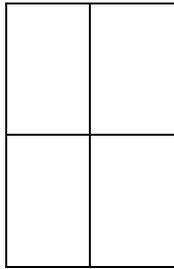
- 6) Count nucleated cells
 - a) Perform 1:20 dilution of cells using 3% Acetic Acid with Methylene Blue

- b) Use 10 ul cells and 190 ul Acetic Acid with Methylene Blue
 - i. This is a dilution factor of 20
- c) Count cells using hemacytometer and 4 outside squares
Carefully clean hemacytometer and coverslip with kimwipes and 70% Ethanol

Place the coverslip over the hemacytometer. Thoroughly mix the cell suspension. Carefully load the 2 V shaped wells of the hemacytometer. Count (under 10x objective) the number of viable (live) cells in the 4 corner boxes of each side.

Cell count (for the cells that are touching the border- only include cells that are in the top and bottom borders of every quadrant)

Do a second cell count if you suspect the numbers are too off from one another.



Average: _____

- d) Cell count per ml = Avg # of cells per square * dilution factor * 10^4

Cell count per ml = _____ * 20 * 10^4

= _____ cells/ml

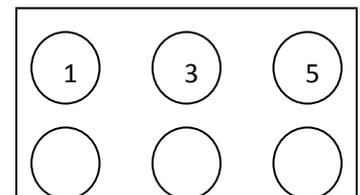
- e) Calculate the volume for $5 \times 10^6 = 5 \times 10^6$ /number from d (above) * 1000uL

- f) Volume of cell solution needed per well = 5×10^6 / _____ * 1000uL

= _____ uL

- 7) Add 2 ml/well of EndoCult medium to a 6-well fibronectin plate

- a) Plate **ALL 6 WELLS!**



b) **Label Plate As follows**

Subject ID: _____

2

4

6

Date: _____

Time: _____

Initials: _____

8) Incubate for **2 days** at **37° C, 5% CO₂, ≥ 95% humidity**

Notes:

Day 2 – Collection and re-plating of non-adherent cells

1. **Setup:** Clean hood and all equipment that goes in the hood with 10% bleach. Bring out the: Methylene blue, 4 of the 15ml conical tubes, 4 microfuge tubes, 1000uL pipettes, 200uL pipettes, 10uL pipettes and tips. Also bring out the media and keep it in the hood to get to get it to be at room temperature when you are going to plate the cells. Take out a 24-well fibronectin plate from the refrigerator and allow it to come to room temperature in a sterile environment.

Pre-label all the microfuge tubes and 15ml tubes.

2. **Collecting non-adherent cells:** Using a 1000ul pipette, pipette the medium in the well up and down 3-4 times. This will remove any non-adherent cells that may be stuck to other cells. The cells that are adherent will be firmly attached to the fibronectin medium so washing can be vigorous.

3. Transfer these non-adherent cells from each well to a separate 15ml tube.

4. Cell count:

a) Perform 1:10 dilution of cells using 3% Acetic Acid with Methylene Blue

b) Use 10 ul cells and 90 ul Acetic Acid with Methylene Blue

i. This is a dilution factor of 10

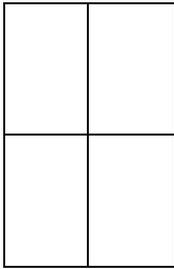
- c) Count cells using hemacytometer and 4 outside squares

Carefully clean hemacytometer and coverslip with kimwipes and 70% Ethanol

Place the coverslip over the hemacytometer. Thoroughly mix the cell suspension. Carefully load the 2 V shaped wells of the hemacytometer. Count (under 10x objective) the number of viable (live) cells in the 4 corner boxes of each side.

Cell count (for the cells that are touching the border- only include cells that are in the top and bottom borders of every quadrant)

Do a second cell count if you suspect the numbers are too off from one another.



Average: _____

- d) Cell count per ml = Avg # of cells per square * dilution factor * 10^4

Cell count per ml = _____ * 10^4

= _____ cells/ml

- e) Calculate the volume for $1 \times 10^6 = 1 \times 10^6$ /number from "d" (above) * 1000uL

Volume of cell solution needed per well = 1×10^6 / _____ * 1000uL

= _____ uL

- f) Volume of CFU-Hill Media that will be needed in each well (the total volume in each well needs to be 1ml or 1000uL) = 1000uL – number from “e” (above)

Volume of media needed = 1000uL – _____

= _____ uL

6. Map out where you are going to plate the cells (see next page). First add 1×10^6 cells in duplicate to a 24-well fibronectin-coated plate. Then add the corresponding type of media (Normal or Glucose) to a final volume of 1.0mL per well. Repeat this process for the other 3 groups.

7. Label Plate As follows (don't write on top of the wells with cells and media)-

Subject ID: _____

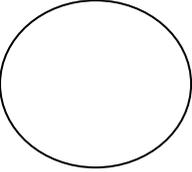
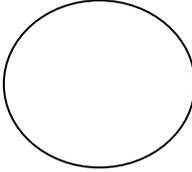
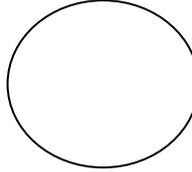
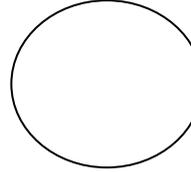
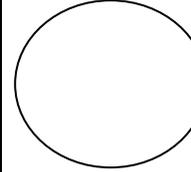
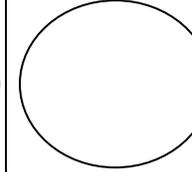
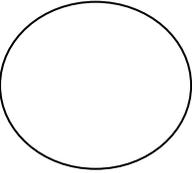
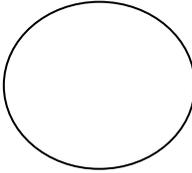
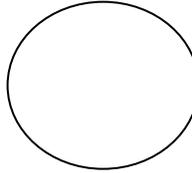
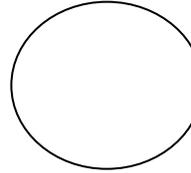
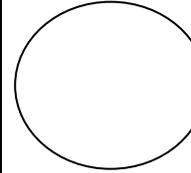
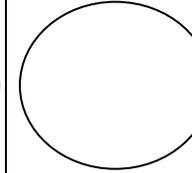
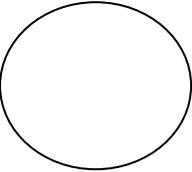
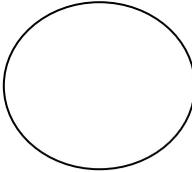
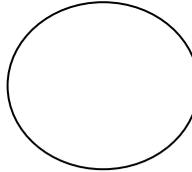
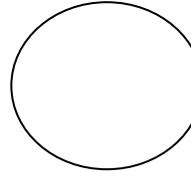
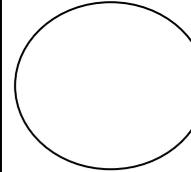
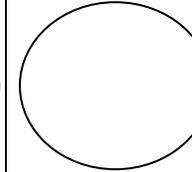
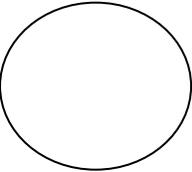
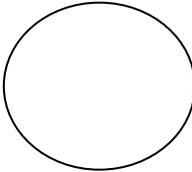
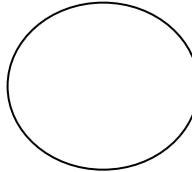
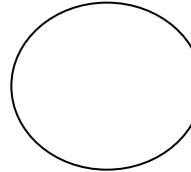
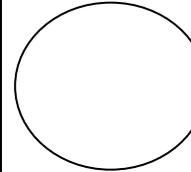
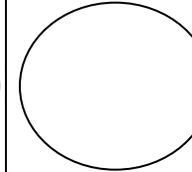
Date: _____

Time: _____

Initials: _____

8. Incubate at 37°C 5% CO₂ with ≥ 95% humidity for 3 days.

Plate set-up (assign 6-8 wells aside for daf-fm, fill up as many as possible with- 18 wells max):

1 	1 	2 	3 	3 	4 
1 	1 	2 	3 	3 	4 
1 	2 	2 	3 	4 	4 
1 	2 	2 	3 	4 	4 

Day 5– Colony Counting

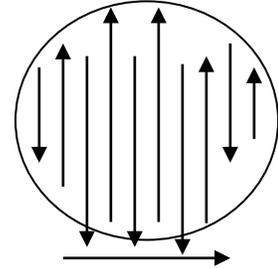
1. Take out fibronectin plate from incubator.

2. Prepare microscope with plate stage and 20x objective

3. Identify the wells to be counted.

4. Count colonies by scanning well in an up – to – down pattern as you move from left to right. Be certain to look to the edges for colonies as they like to grow there.

5. Have a second person count to verify the number. Fill it in the box in previous page.



6.10: CD34⁺ cell isolation protocol

EasySep Magnet: CD34⁺ selection

PREPARATION

Supplies/sample

EasySep Magnet (3) K ₃ EDTA 10 ml blood drawing tubes (10) 50 ml polypropylene conical tubes (3) 15 ml polypropylene conical tube Transfer pipette	Sterile pipettes Pipetter Micropipettes (p10, p20, p200) Pipette tips
---	--

Solutions & Reagents (based on 30mls of blood)

~80 ml FBS/PBS 30ml Ficoll Paque PLUS ~10 ml FBS/PBS +1mM EDTA (recommended media for EasySep) EasySep Human CD34 ⁺ selection cocktail EasySep Magnetic nanoparticles
--

PROTOCOL

Preparing a mononuclear cell suspension:

- 9) Prepare 50ml of recommended media- PBS/2%FBS + 1mM EDTA-
 - a) Add 18.612mg EDTA in 50ml of PBS/2%FBS for a 1mM solution- Mol wt of EDTA is 372.24
 - b) Add 500uL of this recommended media to each pellet; to have a total of 1mL of PBS/2%FBS+EDTA. Measure total actual volume by setting pipette to 1000uL and let volume down until liquid comes out. That is the actual volume of the cell solution. Combine the two 500uL tubes to make a cell solution in a 5mL polystyrene tube. Count from here.

CD34⁺ Selection with the Magnet (see diagram):

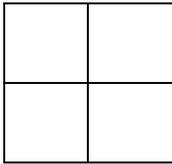
1. Prepare a mononuclear cell suspension in the recommended medium after calculating TOTAL CELLS (see table)-

TOTAL CELLS (starting)*	MEDIUM (PBS/2%FBS+EDTA)
-------------------------	-------------------------

2×10^7	100 uL
$2 \times 10^7 - 2 \times 10^8$	Suspend cells at 2×10^8 cells/ml
$2 - 5 \times 10^8$	1ml

For *TOTAL CELLS (starting) count-

- a) Use 1:20 dilution with acetic acid and methylene blue (AA+MB). Use 10uL of cell solution in 190uL of AA+MB. Load 10uL of this dilution in the hemocytometer after cleaning it with 70% ethanol.



Average: _____

- c) Cell count per ml = Avg # of cells per square * dilution factor (usually 10 or 20) * 10^4

$$\begin{aligned} \text{Cell count per ml} &= \text{_____} * \text{_____} * 10^4 \\ &= \text{_____} \text{ cells/ml} \end{aligned}$$

- d) Total Cells = Cell count per ml from "c" (above) * *Actual measured volume from EDTA step*

$$\begin{aligned} \text{Total Cells} &= \text{_____} * \text{_____} * \text{_____} \\ &= \text{_____} \text{ Total cells} \end{aligned}$$

- b) Adjust volume of the media based on the total cell count using the table above.

Calculations:

If the cells are over-diluted then spin the tube down at 300xg for 5 minutes. Take out the media in there that is in excess. So if the calculations say that it should be in 750uL then spin it down and remove 250uL for the 1ml (or whatever actual volume) that the cells were suspended in.

- c) Cells must be placed in a 5ml (12 x 75mm) polystyrene tube to properly fit into the EasySep magnet.
2. Add EasySep positive selection **cocktail** at 100uL/mL cells (eg. For 1ml of cells, add 100 uL of cocktail). Mix well and incubate at RT for **15 mins**

Calculations:

Amount of cocktail mix added (in uL)= _____

3. Mix Easy Sep Magnetic **nanoparticles** to ensure that they are in a uniform suspension by vigorously pipetting up and down **more than 5 X**. DO NOT VORTEX. Add the particles at 100uL/mL cells (eg. for 1ml of cells, add 100uL of nanoparticles). Mix well and incubate at RT for **10 mins**

Calculations:

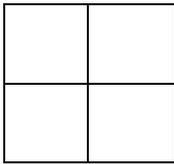
Amount of nanoparticles particle added (in uL)= _____

4. Bring the cell suspension to a **total volume** of 2.5 mL by adding recommended medium. Aliquot about 5ml of medium in a separate tube for this step so that pipette tip doesn't have to be changed. Mix the cells in the tube by gently pipetting up and down 2-3 X. Place the tube **without the cap** into the magnet. Set aside for **10 mins**.
5. Pick up the magnet, and in one **continuous motion** invert the magnet and tube, pouring off the supernatant fraction into another 5ml polysterene tube (labeled "pour 1"). The magnetically labeled cells will remain inside the tube, held by the magnetic field of the EasySep Magnet. Leave the magnet and tube in inverted position for **2-3 seconds**, then return to upright position. DO NOT SHAKE OR BLOT OFF ANY DROPS THAT MAY REMAIN HANGING FROM THE MOUTH OF THE TUBE.

6. Remove the tube from the magnet and add 2.5mL recommended medium. Again, aliquot about 5ml of medium in a separate tube for this step so that pipette tip doesn't have to be changed. Mix the cell suspension by gently pipetting up and down 2-3 X. Place the tube back in the magnet and set aside for **10 mins**. Place the tube from the 1st pour "pour 1" into the 2nd magnet for **10 mins**.
7. After the 10 minutes with your original tube going through its second time and your pour 1 tube going through the first time, pour off the "pour 1" labeled tube in a 15mL conical tube labeled CD34⁻. Pour your original tube into a 5mL polystyrene tube labeled "pour 2." Place "Pour 2" tube into the magnet for **10 minutes**. Pour this into the CD34⁻ labeled conical tube.
8. At this point you should have 3 of the 5ml tubes- the original tube, tube from "pour 1" and tube from "pour 2" with CD34⁺ cells. Aliquot about 1ml of media again. Suspend tubes in 100uL of **1xPBS**, transfer contents of the pour tubes to the original tube using the same pipette tip. Measure total volume by setting the 1000uL pipet and letting out air until just before liquid comes out. This is the final actual volume. The positively selected cells are now ready for use.
9. Spin the CD34⁻ labeled 15mL conical tube at 300xg for 7min. Pour off the supernatant and re-suspend in 500uL of trizol in 1.5mL tubes. Label and put in -80°.

((The following is best done in Morrill. Recount if done in Totman then going to Morrill for NOi.))

10. Count the # of CD34⁺ cells using 1:10 dilution with acetic acid and methylene blue. Use 10uL of cell solution in 90uL of AA+MB



Average: _____

e) Cell count per ml = Avg # of cells per square * dilution factor (10) * 10⁴

Cell count per ml = _____ * _____ * 10⁴

= _____ cells/ml

- f) Total Cells = Cell count per ml from “c” (above) * 1ml (since we diluted cells in 1ml)

$$\text{Total Cells} = \underline{\hspace{2cm}} * \underline{\hspace{2cm}} * \underline{\hspace{2cm}}$$
$$= \underline{\hspace{2cm}} \text{ Total cells}$$

We need $1.5 * 10^5$ cells for the DAF-FM protocol so budget accordingly

Put the rest of the amount of CD 34⁺ and CD34⁻ cells in a total of 500uL trizol.

6.11: CFU-Hill NOi assay protocol

PROTOCOL FOR INTRACELLULAR NITRIC OXIDE in CFU-Hill

A. Preparing DAF-FM diacetate

- 1) Add 20uL of DMSO to the 50ug vial of DAF-FM diacetate (invitrogen)
 - a) This gives us a ~5mM stock solution
 - b) Aliquot in smaller quantities for later use to reduce freeze/thaw

B. Preparing SIN-Dilutions

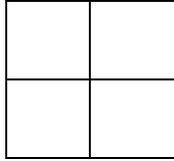
- 1) SIN-1 is 206.63 g/mol (the sin-1 that we have had 4mls initially added to it)
The SIN-1 dilution that has been made with 4ml is 12100uM
- 2) Make a **working stock of 150uM** by adding 796.66uL dH2O + 10uL of the original SIN dilution (which is at 12100uM)

C. Removing cells from Plate

- 1) Remove top media, save and label. Place in -80°
- 2) Add 300uL 1x clear trypsin per well. Rainbow wash x3
- 3) Incubate 10 minutes in 37°
- 4) Combine in 15mL conical tube, check plate on scope for no “left over” colonies/cells. Add more trypsin if cells left on plate
- 5) Spin 15mL conical tube for 2-3 minutes for pellet. Toss supernatant
- 6) Resuspend in 500uL 1x PBS.
- 7) Count cells

D. Combining the CFU-Hill cells and DAF-FM:

- 10) Count the # of CFU-Hill cells using 1:10 dilution with acetic acid and methylene blue. Use 10uL of cell solution in 90uL of AA+MB



Average: _____

a) Cell count per ml = Avg # of cells per square * dilution factor (10) * 10^4

Cell count per ml = _____ * _____ * 10^4 = _____ cells/ml

To determine mL need per well to achieve 1.5×10^5 cells/well:

1.5×10^5 / _____ cells/mL = _____ mL of cell solution per well

b) Total cells = Cell count per ml from "c" (above) * 1ml (since we diluted cells in 1ml)

Total Cells = _____ * _____ * _____

= _____ Total cells

2) Create a DAF-FM dilution of 75uM, unless volume of cells to be added is greater than 130uL; this is atypical see #3.

a) Take out an aliquot of 5mM from -20. Thaw, vortex, and centrifuge. If no aliquot, make one according to part B. Remember DAF-FM is light sensitive.

b) To make 75uM add 197uL dH₂O and 3 uL 5mM DAF-FM to 1.5 mL tube.

c) Vortex and centrifuge the new DAF-FM dilution.

3) Combine cells, PBS, and DAF-FM in the plate

a) Use 96 well plate, black sides, clear, flat bottom, with lid on. Save box!

b) The volume of 75uM DAF-FM to add will be 20uL throughout

The volume of 1xPBS will be 130uL-volume of cell solution to be added

Ex. if cell volume = 30uL then PBS to be added will be 130uL-30uL=100uL

c) Plate in order from largest to smallest volume.

d) Incubate cells in 37degC for 60mins.

4) If cell solution to be added is >130uL but <150uL a 75uM DAF-FM stock will not work

a) Troubleshoot using $M_1V_1=M_2V_2$ To create a working stock that will result in a final DAF-FM concentration of 10uM in the total 150uL/well solution

5) Plate-reader (set this up during the incubation time)

c) The optics used have 3 wires, 2 silver ones (one with blue and one with yellow edges) and a gray wire. Set according to picture



- d) No spacers are needed for these plates
- e) Select “Gaya NOi CFU protocol” for preset settings
- f) Insert plate with lid on and well A1 in upper left hand corner.
- g) Click measure and click the gain adjustment (plate) setting (top left corner)
- h) Click OK
- i) Store readout as an excel file on desktop and upload on thumb-drive

	1	2	3	4	5	6	7
A	CELLS ONLY	CELLS ONLY	DAF-FM ONLY 10uM	DAF-FM ONLY 10uM	CELLS+DAF-FM +PBS	CELLS+DAF-FM +PBS	SIN 50+dH2O 80 + DAFFM 20
B	1X PBS ONLY	1X PBS ONLY			CELLS+DAF-FM +PBS	CELLS+DAF+PBS	SIN 80+dH2O 50 + DAFFM 20

6.12: CD34⁺ cells NOi protocol

PROTOCOL FOR INTRACELLULAR NITRIC OXIDE in CD 34⁺

A. Preparing DAF-FM diacetate

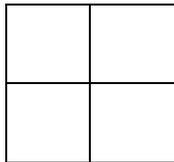
- 1) Add 20uL of DMSO to the 50ug vial of DAF-FM diacetate (invitrogen)
 - j) This gives us a ~5mM stock solution
 - k) Aliquot in smaller quantities for later use to reduce freeze/thaw

B. Preparing SIN-Dilutions

- 6) SIN-1 is 206.63 g/mol (the sin-1 that we have had 4mls initially added to it)
The SIN-1 dilution that has been made with 4ml is 12100uM
- 7) Make a **working stock of 150uM** by adding 796.66uL dH2O + 10uL of the original SIN dilution (which is at 12100uM)

C. Combining the 34⁺ cells and DAF-FM:

- 11) Count the # of CD34⁺ cells using 1:10 dilution with acetic acid and methylene blue. Use 10uL of cell solution in 90uL of AA+MB

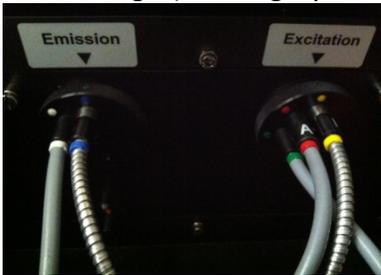


Average: _____

- a) Cell count per ml = Avg # of cells per square * dilution factor (10) * 10⁴
Cell count per ml = _____ * _____ * 10⁴ = _____ cells/ml
To determine mL need per well to achieve 1.5 x 10⁵ cells/well:
1.5 x 10⁵ / _____ cells/mL = _____ mL of cell solution per well
- b) Total cells = Cell count per ml from "c" (above) * 1ml (since we diluted cells in 1ml)
Total Cells = _____ * _____ * _____
= _____ Total cells

- 2) Create a DAF-FM dilution of 75uM, unless volume of cells to be added is greater than 130uL; this is atypical see #3.

- a) Take out an aliquot of 5mM from -20. Thaw, vortex, and centrifuge. If no aliquot, make one according to part B. Remember DAF-FM is light sensitive.
- b) To make 75uM add 197uL dH₂O and 3 uL 5mM DAF-FM to 1.5 mL tube.
- c) Vortex and centrifuge the new DAF-FM dilution.
- 8) Combine cells, PBS, and DAF-FM in the plate
 - a) Use 96 well plate, black sides, clear, flat bottom, with lid on. Save box!
 - b) The volume of 75uM DAF-FM to add will be 20uL throughout
The volume of 1xPBS will be 130uL-volume of cell solution to be added
Ex. if cell volume = 30uL then PBS to be added will be 130uL-30uL=100uL
 - c) Plate in order from largest to smallest volume.
 - d) Incubate cells in 37degC for 60mins.
- 9) If cell solution to be added is >130uL but <150uL a 75uM DAF-FM stock will not work
 - a) Troubleshoot using $M_1V_1=M_2V_2$ To create a working stock that will result in a final DAF-FM concentration of 10uM in the total 150uL/well solution
- 10) Plate-reader (set this up during the incubation time)
 - l) The optics used have 3 wires, 2 silver ones (one with blue and one with yellow edges) and a gray wire. Set according to picture



- m) No spacers are needed for these plates
- n) Select "Gaya NOi 34⁺ protocol" for preset settings
- o) Insert plate with lid on and well A1 in upper left hand corner.
- p) Click measure and click the gain adjustment (plate) setting (top left corner)
- q) Click OK
- r) Store readout as an excel file on desktop and upload on thumb-drive

	1	2	3	4	5	6	7
A	CELLS ONLY	CELLS ONLY	DAF-FM ONLY 10uM	DAF-FM ONLY 10uM	CELLS+DAF-FM +PBS	CELLS+DAF-FM +PBS	SIN 50+dH ₂ O 80 + DAFFM 20
B	1X PBS ONLY	1X PBS ONLY			CELLS+DAF-FM +PBS	CELLS+DAF+PBS	SIN 80+dH ₂ O 50 + DAFFM 20

6.13: RNA protocol

PROTOCOL: RNA Isolation of Endothelial Progenitor Cells

1x PBS
Sterile 1.5ml microcentrifuge tubes
Tri-reagent (trizol) (Sigma T9424) [Totman]
Chloroform (Sigma C2432) (light sensitive) [Totman]
Isopropyl alcohol [Totman]
Glycogen [Totman]
Ethanol (75%)
TE Buffer [Totman]

Samples: EPCs are collected ~24 hours after replating on 24-well fibronectin coated culture plates (Day 5 of Hill CFU Assay) or after isolating CD34⁺ and CD34⁻ cells

RNA ingredients (in uL)- based on starting quantity of trizol (except glycogen and TE)

Trizol (starting qty)	250	500	750
Chloroform	50	100	150
Isopropanol	125	250	375
glycogen	5	5	5
75%Etoh	375	750	1125
1xte buffer	25	25	25

1) **Setup:**

- clean the work space and fume hood with bleach and ethanol
- set the RNA centrifuge at 4^o C
- ice-bucket for keeping the samples
- aliquot reagents (this is per 500uL trizol): 100uLchloroform, 250uLisopropanol, 750uL of 75% ethanol, 25uL TE buffer

2) **Chloroform (light sensitive):**

- Add 20µl Chloroform for every 100µl trizol (1 chloroform : 5 trizol) (100µl per 500uL of Trizol)
- Invert samples several times (turns pink)
- Place in rotator for 10 min

3) Centrifuge 12 min @ 4^o @12,000 x g

4) Carefully remove 50 – 75% of upper clear layer (be conservative, top layer = desired part)

- Be certain not to remove middle DNA layer or lower pink layer
- Draw only from top of clear layer to avoid DNA contamination

- c. Place in second 1.5ml tube
 - d. Discard unwanted trizol solution still in tubes (there is a medicine looking white bottle in the room in Morrill with the centrifuge in which this goes)
- 5) **Isopropyl alcohol & Glycogen:**
- a. Add 50µl isopropyl alcohol for every 100µl trizol (1 isopropyl : 2 trizol) (250µl isopropyl per 500uL trizol)
 - b. Add 5µl glycogen per sample (per 500uL)
 - c. Invert and place in rotator for 10 min.
- 6) Centrifuge for 8 min @ 4⁰ C @ 12,000 x g
- 7) Carefully remove supernatant – do not disturb pellet
- 8) **Ethanol:**
- a. Add 150µl ethanol for every 100 µl of trizol (1.5 ethanol : 1 trizol) (750 µl per 500uL trizol)
 - b. Vortex briefly – pellet should not go into solution, only knocked loose
 - c. Incubate @ RT for 5 min (keep **closed**)
- 9) Centrifuge for 5 min @ 4⁰ C @ 7500 x g
- 10) Remove as much ethanol as possible without disturbing pellet
- 11) Allow pellet to air-dry for ~10 – 15 min (keep **open** to let it dry outside)
- a. Pellet will become clear
 - b. DO NOT let the pellet become completely dry
- 12) Add **25µl** of TE buffer
- 13) Incubate at 55 – 60⁰ C for 10 min (keep **closed**)
- 14) Freeze -80⁰ C (lower right side of freezer)

NUMBER OF SAMPLES: _____

SUBJECT ID/IDs: _____

LOCATION OF SAMPLES: _____

LABELS ON THE TUBES:

SUB ID: _____

DATE: _____

INITIALS: _____

6.14: RNA quantification for cDNA synthesis

Nanodrop- RNA for cDNA synthesis

RNA samples
0.1-2 uL pipettes + tips
Kimwipes
Nucelase free water
1x TE Buffer
Microfuge tubes

Prep:

The original RNA stock should be taken out of the -80 freezer and be placed in a bucket of ice

Dilution:

1. Dilution for RNA from cells- 1:25
 - a. In a new microtube, take 48uL of 1xTE Buffer and add 2uL of your original RNA stock to it
 - b. Pipette mix first and then add 2uL of your original RNA stock to the buffer
 - c. Remember to pipette mix the original stock well after it has been added to the buffer to get a homogenous mixture
2. Dilution for RNA from tissue- 1:50
 - a. In a new microtube, take 98uL of 1xTE Buffer
 - b. Pipette mix first and then add 2uL of your original RNA stock to the buffer
 - c. Remember to pipette mix the original stock well after it has been added to the buffer to get a homogenous mixture

Setup:

1. Clean all equipment with Ethanol, wipe workspace with 10% bleach
2. Clean the pedestal and arm with Kimwipes (if looks contaminated, can you 5.25% bleach solution)
3. Click on ND-1000 icon
4. Choose Nucleic acids option for RNA

Initialize:

5. When the software starts, you should see this message: "click OK to initialize the spectrometer. Load a water sample onto the pedestal first for best calibration"
6. Load a 2uL nuclease free water sample (small bottle with green top) onto the pedestal and close the arm (no need to press it down)
7. Click OK

Blanking:

8. If using sample directly then your 1x TE Buffer is the "blank"
9. Click on BLANK
10. Repeat the first step of blanking
11. This time click MEASURE

12. This result should have a spectrum that varies no more than 0.05 A from the zero baseline

Re-blanking*

- 13. Repeat first step of blanking
- 14. Click Re-BLANK
- 15. The following message should appear “blank applied to displayed spectrum”

Sample ID:

- 16. Enter the sample ID to identify the measurement being made
- 17. Make sure “RNA-40” is selected under sample-type

Load sample:

- 18. Pipette mix again
- 19. Load 2uL of sample
- 20. Place on pedestal and close the arm, but not all the way down
- 21. Click MEASURE

Measures:

Measure 5 readings in TOTAL. Discard the first 2 readings-
NEED A MINIMUM OF 3 READINGS (THAT ARE CONSISTENT) FROM EACH SAMPLE DILUTION

	1	2	3	4	5
A260					
A280					
260/280					
ng/uL					
[RNA] calculated					

If you feel like the A260 readings seem to be off, you can re-blank with the buffer again. However, remember that the sample that was most recently measured will be re-calculated based on the re-blank. And all the measures thereafter will also be calculated based on this new re-blank value.

Can print report for these readings as well but if you are working in pairs, one person can note these values.

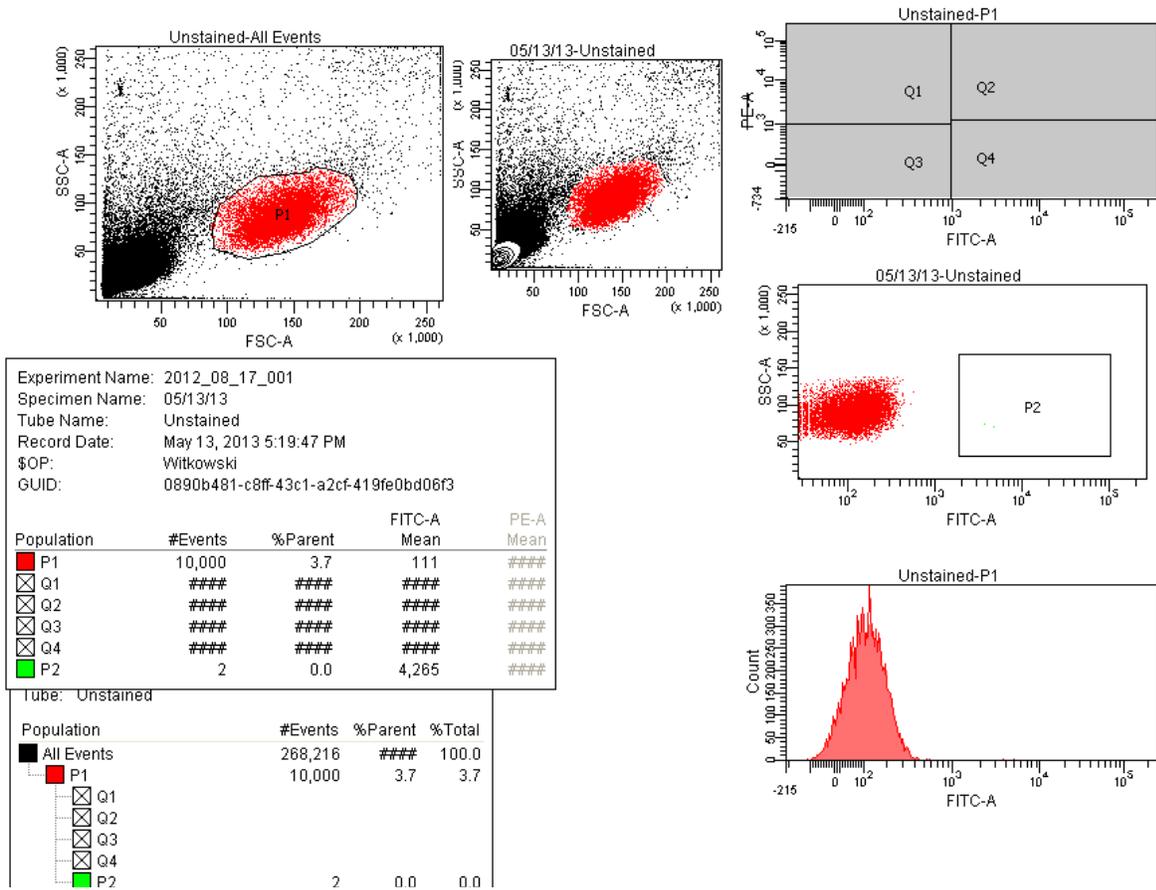
Calculations:

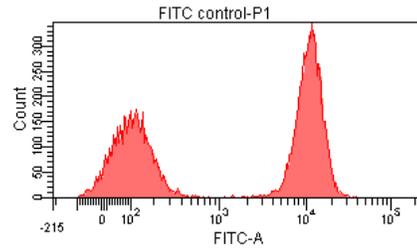
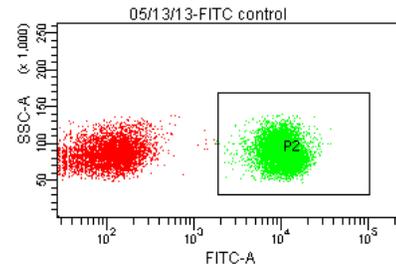
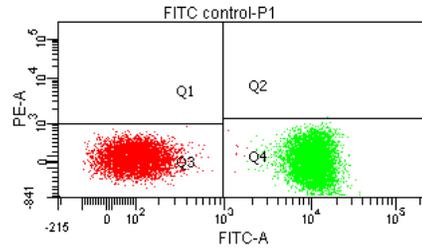
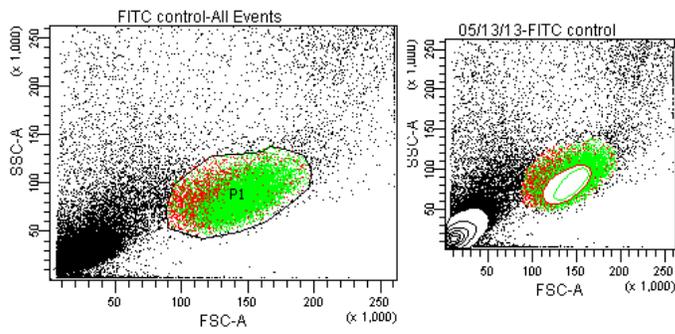
Concentration of RNA-
[RNA]= [A260*40ug/ml*50]/1000

NOTE: 50 = dilution factor of sample used for spec analysis
40= the conversion factor for RNA

Main measurements to look for and what they mean -260/280- ~1.8 is pure for DNA, 2.0 is pure for RNA
260/230- normally 1.8-2.2 range. Lower than this range indicates contamination

6.15: FACs analyses results



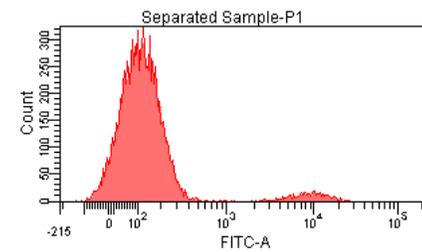
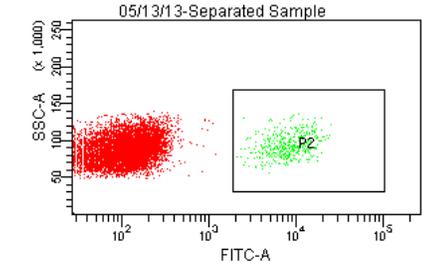
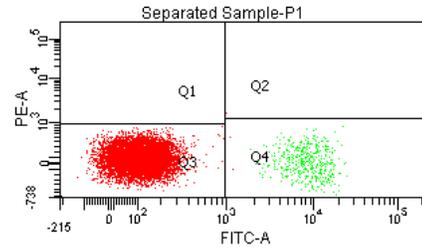
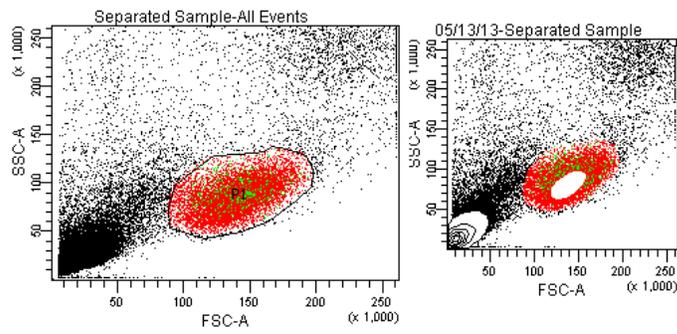


Experiment Name: 2012_08_17_001
 Specimen Name: 05/13/13
 Tube Name: FITC control
 Record Date: May 13, 2013 5:06:20 PM
 \$OP: Witkowski
 GUID: a8a3d996-a766-4aa9-bf82-a74ad09fc3d4

Population	#Events	%Parent	FITC-A Mean	PE-A Mean
■ P1	11,757	12.0	6,228	2
☒ Q1	0	0.0	####	####
☒ Q2	0	0.0	####	####
☒ Q3	5,175	44.0	106	41
☒ Q4	6,582	56.0	11,041	-28
■ P2	6,574	55.9	11,053	-28

Tube: FITC control

Population	#Events	%Parent	%Total
■ All Events	98,059	####	100.0
■ P1	11,757	12.0	12.0
☒ Q1	0	0.0	0.0
☒ Q2	0	0.0	0.0
☒ Q3	5,175	44.0	5.3
☒ Q4	6,582	56.0	6.7
■ P2	6,574	55.9	6.7



Experiment Name: 2012_08_17_001
 Specimen Name: 05/13/13
 Tube Name: Separated Sample
 Record Date: May 13, 2013 5:24:09 PM
 \$OP: Witkowski
 GUID: db90e37d-a006-4d3b-ad09-13a2264ef5a9

Population	#Events	%Parent	FITC-A Mean	PE-A Mean
■ P1	10,000	9.7	530	44
 Q1	0	0.0	####	####
 Q2	1	0.0	998	1,442
 Q3	9,547	95.5	112	46
 Q4	452	4.5	9,370	-13
■ P2	450	4.5	9,406	-14

Tube: Separated Sample

Population	#Events	%Parent	%Total
■ All Events	103,455	####	100.0
■ P1	10,000	9.7	9.7
 Q1	0	0.0	0.0
 Q2	1	0.0	0.0
 Q3	9,547	95.5	9.2
 Q4	452	4.5	0.4
■ P2	450	4.5	0.4

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