ABSTRACT

Title of Document: EXERCISE TRAINING-ASSOCIATED DIFFERENCES IN CIRCULATING MICRORNAS AND SERUM-INDUCED ENDOTHELIAL CELL MIGRATION RATE

Ryan M. Sapp, Master of Arts, 2015

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Cardiovascular Diseases (CVDs) are the primary source of global mortality and morbidity. The initial steps of CVD development occur in the endothelium. MicroRNAs (miRNAs) have recently emerged as novel regulators of cardiovascular physiology and pathology. Interestingly, regular aerobic exercise acts to prevent CVDs and also regulates miRNAs in the circulation (ci-miRNAs). The purpose of this study was to determine the effects of serum from highly active and sedentary, young, healthy individuals on migration rate of endothelial cells *in vitro*. Secondarily, CVD-associated ci-miRNAs in serum were compared between groups. The results of this study represent a novel way by which sedentary behavior may act as an early risk for CVD development before the appearance of other classic risk factors. RT-qPCR array analysis identified nine ci-miRNAs as > 4-fold differentially expressed in serum of trained versus inactive subjects, though more subjects are needed before any conclusions about ci-miRNA differences can be made.

EXERCISE TRAINING-ASSOCIATED DIFFERENCES IN CIRCULATING MICRORNAS AND SERUM-INDUCED ENDOTHELIAL CELL MIGRATION RATE

By

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List of Abbreviations

3'UTR – 3' untranslated region

bFGF – Basic fibroblast growth factor

CAD – Coronary artery disease

cDNA – Complimentary DNA

cel / *C. elegans* – *Caenorhabditis elegans*

CHD – Coronary heart disease

CHF – Chronic heart failure

Ci-miRNA – Circulating microRNA

CO_2 – Carbon dioxide

C_T – Cycle threshold

CVD – Cardiovascular disease

DBP – Diastolic blood pressure

DNA – Deoxyribonucleic acid

EMP – Endothelial microparticle

EPC – Endothelial progenitor cell

FBS – Fetal bovine serum

HDL-C – High Density lipoprotein cholesterol

HR – Hour

HUVEC – Human umbilical vein endothelial cell

IRB – Institutional Review Board

LDL-C – Low density lipoprotein cholesterol

LDLR – Low density lipoprotein receptor

MAP – Mean arterial pressure

miR / miRNA – MicroRNA

MI – Myocardial infarction

miRISC – MiRNA-induced silencing complex

mRNA – Messenger RNA

NIH – National Institutes of Health

NO – Nitric oxide

P-bodies – Processing-bodies

Pri-miRNA – Primary microRNA

RT-qPCR – Quantitative reverse transcription real-time polymerase chain reaction

RNA – Ribonucleic acid

SAS – Statistical Analysis System

SBP – Systolic blood pressure

SD – Standard deviation

SEM – Standard error of the mean

TC – Total cholesterol

VEGF – Vascular endothelial growth factor

VEGFR-2 – Vascular endothelial growth factor receptor-2

VLDL-C – Very low density lipoprotein cholesterol

VO_2max – Maximal volume of oxygen consumption

Introduction

Atherosclerosis is the number one cause of death worldwide, and by 2030 it is projected that over 43% of Americans will be living with some form of cardiovascular disease (CVD) (Mathers and Loncar, 2006; Fuster et al., 2007; Go et al., 2014). Unrepaired injury to the endothelium results in endothelial dysfunction, and this represents the first step in the atherogenic process underlying the majority of CVD cases (Levy, 1981; Lusis, 2000; Vanhoutte, 2010). Early identification and prevention of endothelial dysfunction is, therefore, of great clinical interest. Physical inactivity is a well-recognized independent risk factor for development of CVD (Powell et al., 1987; Fletcher et al., 1996; Beaglehole et al., 2007). Alternatively, regular exercise confers a protective effect against CVD, with research showing a 40% reduction in risk among highly active individuals (Shiroma and Lee, 2010). Beneficial cardiovascular adaptations to exercise training include increased aortic compliance, enhanced ventricular performance (Moore and Korzick, 1995), increased capillarization and arterial enlargement (remodeling) (Brown, 2003), and enhanced endothelial vasodilation and function (Hambrecht et al., 2003). A comprehensive understanding of the molecular mechanisms responsible for these protective adaptations, however, remains to be fully elucidated.

MicroRNAs (miRNAs) are ~22 nucleotide long non-coding RNAs that modulate gene expression by post-transcriptional regulation of specific mRNA targets. There are over 2,500 miRNAs encoded in the human genome that act on the majority of protein-coding mRNAs (Friedman et al., 2008; Ha and Kim, 2014).

MiRNAs exert their control by associating with a protein complex and binding to complementary mRNAs, usually causing either translational repression or mRNA decay by a variety of mechanisms. Owing to the complexity of miRNA gene silencing, a single miRNA may target several mRNAs, while multiple miRNAs can also regulate the same mRNA. Of interest, several miRNAs enriched in various cell types, including endothelial cells, have been shown to regulate physiological and pathological processes such as endothelial function, dysfunction, and every stage of atherosclerosis (Madrigal-Matute et al., 2013; Santoro and Nicoli, 2013; Sun, Belkin and Feinburg, 2013). Thus, clinical treatment of CVDs by miRNA therapy is of great interest, and this concept has already been validated in animal models (Lanford et al., 2010; Loyer et al., 2014).

Recently it was discovered that endothelial cells, along with many other cell types, can release miRNAs into circulating blood both passively and actively (Fichtlscherer, Zeiher, and Dimmeler, 2011; Mcmanus and Ambros, 2011). Some of these circulating miRNAs (ci-miRNAs) are dysregulated in CVD states and have emerged as useful clinical biomarkers (Olson, 2014). Additionally, ci-miRNAs have been shown to be taken up by endothelial cells, where they play important roles in regulating endothelial processes (Finn and Searles, 2012). For example, migration of endothelial cells is vital to angiogenesis and wound healing of the endothelium, with deficiencies in this process potentiating endothelial dysfunction and atherogenesis (Ettenson and Gotlieb, 1993; Gimbrone et al., 2000; Lamalice, Le Boeuf, and Huot, 2007). Jansen et al. showed that in response to injury, miR-126 contained in endothelial microparticles (EMP) is released by endothelial cells and is taken up by

recipient endothelial cells where it promotes endothelial repair by cell migration (2013). This response is decreased in CVD patients, who exhibit reduced ci-miRNA-126 levels (Jansen et al., 2013).

Likewise, human umbilical vein endothelial cells (HUVECs) exposed to physiological levels of laminar shear stress *in vitro* have displayed enhanced wound healing by cell migration (Albuquerque et al., 2000; Mun, Jang, and Boo, 2013). Indeed, laminar shear stress has been shown to influence secretion of miRNAs from HUVECs *in vitro* (Hergenreider et al., 2012), while it also regulates endothelial cell function through altering levels of intracellular miRNAs (Boon, Hergenreider, and Dimmeler, 2012). High shear stress has been shown to up-regulate miRNA-21 in HUVECs (Weber et al., 2010), which was subsequently recognized to modulate cell migration (Sabatel et al., 2011). MiRNA-92a is down-regulated by shear stress, while treatment with its antagonist (antagomir-92a) prevented endothelial dysfunction and decreased atherosclerosis in mice fed a high-fat diet, displaying the promising potential of miRNA therapy (Loyer et al., 2014). Repeated bouts of elevated vascular shear stress is a primary factor mediating the beneficial effects of exercise on the endothelium (Brown, 2003; Hambrecht et al., 2003; Whyte and Laughlin, 2010), suggesting that the *in vivo* effects of exercise on ci-miRNA and endothelial migration may be similar to those of shear stress applied *in vitro.*

Regular exercise has undisputed favorable effects on the endothelium and vasculature as a whole (Brown, 2003). As well as decreasing risk of CVD, regular exercise improves endothelial function and reverses dysfunction through a variety of mechanisms, which cannot be fully attributed to attenuation of traditional risk factors

(Green et al., 2003; Di Francescomarino et al., 2009). Research suggests that circulating factors in the blood may be partially responsible for these observed beneficial effects. For example, a 3-week training program reduced the potency of serum from chronic heart failure (CHF) patients to induce endothelial cell death *in vitro* (Mammi et al., 2011). Furthermore, serum collected from athletes promoted endothelial cell uptake of EMP which was associated with enhanced migration *in vitro* compared to endothelial cells cultured with growth medium alone (Wahl et al., 2014).

Recently, several studies have shown differential expression of intracellular and ci-miRNAs following both acute exercise and exercise training (Baggish et al., 2011; Makarova et al., 2014; Nielson et al., 2014), while Gan et al. showed higher expression of miRNA-499 in muscle of active individuals at rest compared to a healthy, sedentary group (2013). Intriguingly, circulating levels of miRNA-499 are also elevated in acute coronary syndrome patients, and are associated with cardiomyocyte injury (De Rosa et al., 2011; Kuwabara et al., 2011). Similarly, endurance training increased cardiac miRNA-126 expression in rats, as compared to sedentary controls, which was possibly related to exercise-induced cardiac angiogenesis (Da Silva et al., 2012). Examples of ci-miRNAs regulated by exercise and training also include those shown to be involved in vital endothelial cell processes such as angiogenesis (Baggish et al., 2011).

However, the influence of ci-miRNAs on the endothelium is poorly understood. Despite evidence of a favorable ci-miRNA signature associated with regular aerobic exercise, combined with the known beneficial effects of *in vitro* shear

stress on miRNAs involved in endothelial cell migration, no study has examined the potential ci-miRNA induced differences in migration as a result of a highly active versus sedentary lifestyle. Therefore, we proposed to compare the migration rate of endothelial cells cultured with serum from regularly exercising versus sedentary subjects. Furthermore, we aimed to elucidate ci-miRNAs potentially responsible for the hypothesized differences in serum's effect on endothelial cell migration rate as a result of exercise training status. We hypothesized that exercise-training induced secretion of circulating factors, such as ci-miRNAs, contributes to endothelial adaptations and cardiovascular benefits. The results of this research will be of substantial public health and clinical significance, as uncovering ci-miRNAs involved in the development of endothelial dysfunction may provide novel targets for the prevention and/or treatment of CVDs.

Specific Aim 1: To determine cell migration rate differences in HUVECs due to aerobic exercise training-mediated effects on serum.

Hypothesis 1: Serum of subjects who regularly engage in aerobic exercise will cause HUVECs to have a higher migration rate as compared to serum of their inactive counterparts.

Specific Aim 2: To identify ci-miRNAs that are differentially expressed between the highly active and inactive groups that could potentially be responsible for altered cell migration rate associated with exercise training status.

Hypothesis 2: Group differences in endothelial cell migration rate will be associated with differences in specific ci-miRNA levels.

Methods

Subjects: Men and women between the ages of 20-39 were recruited for participation. All subjects gave written informed consent prior to taking part in the study, which was approved in advance by the University of Maryland IRB. Subject characteristics were acquired as part of a previous study (Landers-Ramos et al., 2015). Participants were placed into groups based on self-reported level of habitual endurance exercise over the past 5 years. Endurance-trained/highly active subjects (n=10) reporting >4 days/week or >4 hours/week of moderate intensity aerobic exercise (including competition) were matched by age and BMI with inactive counterparts (n=10), who reported <20 min/day and <2 days/week of exercise. Height and weight were obtained for calculation of BMI. Body fat percentage was determined using the 7-site skinfold procedure (Jackson and Pollock, 1978) and VO_2max was measured via a maximal graded treadmill test. Blood chemistry was examined for a number of CVD risk factors. Exclusion criteria included: systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 90mmHg, fasting glucose ≥ 100 mg/dl, serum total cholesterol ≥ 200 mg/dl, low-density lipoprotein ≥ 130 mg/dl, and high-density lipoprotein ≤ 35 mg/dl. All women were tested during the follicular phase of their menstrual cycle to control for the effects of estrogen.

Serum Collection and Storage: Sample collection was performed as previously described (Landers-Ramos et al., 2015). In brief, blood was collected by cubital vein phlebotomy into non-coated tubes and allowed to sit for at least 30 minutes to allow for coagulation of clotting factors. Samples were then centrifuged at 1,500 x g for 20

minutes at 4°C to separate serum, which was immediately frozen in 200-500 µl aliquots at -80°C. All blood sampling occurred after an overnight fast (~12 hours) and 16-24 hours following each subject's normal exercise routine in the highly active group.

Endothelial Cell Migration Assay: HUVECs were initiated from cryopreserved aliquots and passed once before use. Once harvested from culture, HUVECs (P3) were re-suspended in endothelial growth medium (EGM-2, Lonza) and seeded onto the Radius™ 96-well cell migration plate (Cell Biolabs, San Diego, CA) at a concentration of 30,000 cells per well. Plates were cultured at 37°C, 5% CO2 until HUVECs reached 80% confluency. At this point the media was removed and replaced with a gel removal solution for 30 minutes, followed by multiple washes with EGM-2 without fetal bovine serum (FBS). EGM-2 without FBS plus 10% serum was then added for each subject. The 10% serum concentration was chosen based on the fact that it induced the fastest rate of migration in a dose-response experiment (data not shown). Serum from subjects was studied in triplicate (i.e. 3 wells per each condition). Control wells were cultured with EGM-2 without either FBS or human serum, and exhibited no migration after 24 hours (data not shown). Pictures were taken manually using a microscope at 10x magnification immediately (0 hr) upon addition of samples. Migration was tracked by taking pictures every 4 hours for up to 24 hours. The outer edge of migrating cells was traced and the area was quantified at each time point using ImageJ (NIH). Pictures were coded so that quantification was performed blinded to condition and time point. Cell migration rate was calculated

based on percentage of wound closure over time (100 – (size of wound area at time point / size of initial wound area x 100)).

MiRNA Isolation: Ci-miRNA was purified from serum samples using the miRNeasy Serum/Plasma kit (Qiagen). First, 200 µl of serum was mixed with QIAzol lysis reagent and chloroform. Following centrifugation, the upper aqueous layer containing RNA was extracted, mixed with ethanol, and applied to the RNeasy MinElute spin column for several more centrifugation steps. The resulting purified total RNA containing miRNA was eluted in 14 µl RNase free water. Additionally, prior to addition of chloroform, a synthetic spike-in control (*C. elegans* miR-39 (cel-miR-39), Qiagen) was added as an internal control for extraction efficiency analysis and normalization of real time quantitative polymerase chain reaction (RT-qPCR) data.

MiRNA Array: Three samples were chosen from each group to be investigated in the miRNA array based on their performance in the migration assay. The three fastest migrating samples over all time points were from trained individuals, while the three slowest were from inactive subjects. Thus, RNA from these subjects was pooled for each group and 2 µl was reverse transcribed using the miscript II RT kit (Qiagen). The miScript SYBR Green PCR Kit (Qiagen) was used to detect and quantify the expression of miRNAs by means of RT-qPCR performed on an Applied Biosystems 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Prior to use in the array, cDNA samples were checked for quality using the miRNA QC PCR Array (SABiosciences, Frederick, MD), which includes probes to assess miRNA recovery, reverse transcription performance, and PCR performance. The Human Cardiovascular Disease miRNA PCR Array (SABiosciences) was used to investigate the expression

of 84 miRNAs previously implicated in CVD pathologies. The array included cel-miR-39 primer assays for internal normalization. A web-based analysis tool was used to analyze the array data (Qiagen, GeneGlobe Data Analysis Center). This tool first calibrated the C_T values based on recovery of cel-miR-39 and then normalized to the average of the five most invariant miRNAs between the groups. Fold-difference was calculated to express the difference in expression between groups using the $2^{-\Delta\Delta CT}$ method of relative quantification.

Individual ci-miRNA RT-qPCR Assays: MiRNA-specific primer assays (Qiagen) were used to analyze ci-miRNA expression in all samples. Targets for examination were chosen based on those showing at least a 4-fold difference in the array, in combination with results of previous studies on exercise training and ci-miRNAs. The miRNA exhibiting the least difference between groups in the array (miR-210) was selected as a control. Spike-in control cel-miR-39 was quantified in each sample and used to calibrate expression of each ci-miRNA. Groups were compared using the $2^{-\Delta\Delta CT}$ method of relative quantification (relative to respective cel-miR-39 C_T values).

Statistical Analysis: Statistical Analysis Software (SAS) was used for the cell migration and individual miRNA RT-qPCR data analyses. For the HUVEC migration assay, a two-way repeated measures ANOVA was used with time and activity status as factors. Independent t-tests were used for each ci-miRNA PCR assay. Significance for all tests was set at $p \leq 0.05$.

Results

Subject Characteristics: Subject demographics are detailed in Table 1. The exercise trained group consisted of 6 women and 4 men (n=10) and the inactive group included 5 women and 5 men (n=10). There were no significant differences in age or BMI between groups ($p>0.05$). However, the exercise trained subjects had a 52% higher relative VO_2max ($p<0.001$) and 55% lower body fat ($p<0.01$) compared to the inactive group, confirming the differences in self-reported training status. All subjects exhibited healthy blood profiles (e.g. resting blood pressure, glucose, and cholesterol) and were non-smokers with no known risk factors for CVD (besides sedentary lifestyle in the inactive group).

Table 1. Subject characteristics

	Trained	Inactive
n	10	10
Age, y	27.7 ± 5.5	28 ± 4.9
BMI, kg/m^2	21.5 ± 1.7	23.5 ± 2.8
Body Fat, %	11.4 ± 4.6	20 ± 5.4*
Absolute VO_2max, ml/min	3901 ± 846	2533 ± 710.4*
Relative VO_2max, $ml \cdot kg^{-1} \cdot min$-1	62.4 ± 5.6	36.7 ± 6*
SBP, mmHg	119.7 ± 5.6	121.9 ± 13.8
DBP, mmHg	72.6 ± 6	79.6 ± 6.2*
MAP, mmHg	88.3 ± 4.4	93.7 ± 8.1
Glucose, mg/dl	84.2 ± 6.3	82.9 ± 9.7
Cholesterol, mg/dl	172.7 ± 24.9	170.3 ± 30
HDL-C, mg/dl	65.3 ± 12.9	55.3 ± 16.3
LDL-C, mg/dl	93.2 ± 20.5	99.8 ± 25.4
VLDL-C, mg/dl	14.2 ± 4.7	15.2 ± 6.1
TC/HDL	2.7 ± 0.5	3.3 ± 0.9
LDL/HDL	1.5 ± 0.4	2 ± 0.8
Triglycerides, mg/dl	71.4 ± 23.6	76.4 ± 30.1

Values are reported as mean ± SEM. n, number of subjects; VO_2max, maximal volume of oxygen consumption; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL-C, very low-density lipoprotein cholesterol; TC, total cholesterol. *$p \leq 0.05$, statistically significant difference from endurance trained.

Endothelial Cell Migration: Representative images of the migration assay can be seen in Figure 1. HUVECs exposed to serum from endurance trained individuals migrated 8% more in the first 4 hours ($p<0.05$) and 13% more after 8 hours ($p=0.058$) compared to those exposed to serum from inactive subjects (Figure 2). Following 12, 16, and 24 hours, migration was 20% ($p=0.055$), 19% ($p>0.05$), and 21% ($p=0.08$) greater respectively, with serum of trained subjects (Figure 2).

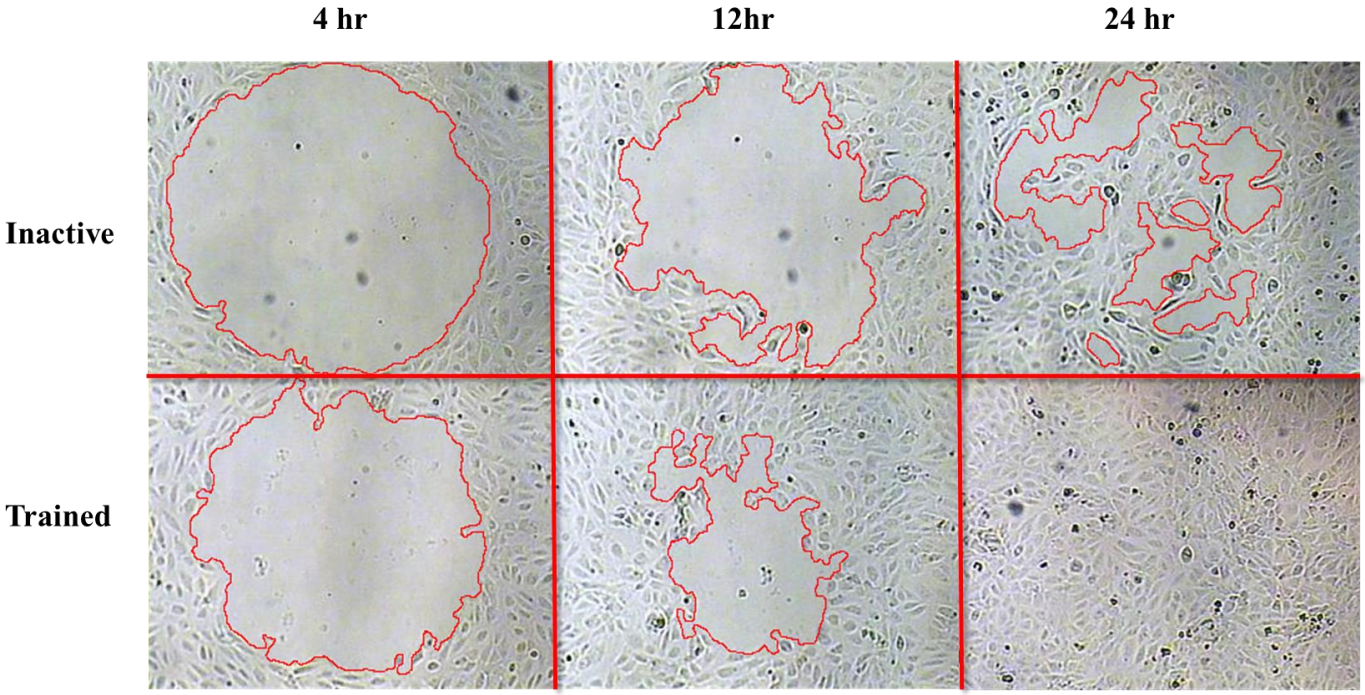


Figure 1. Representative images of HUVEC migration due to serum from one endurance trained and one inactive subject. Images are 4, 12, and 24 hours following addition of serum and initiation of migration. The 24 hour image for the trained condition is equivalent to 100% migration.

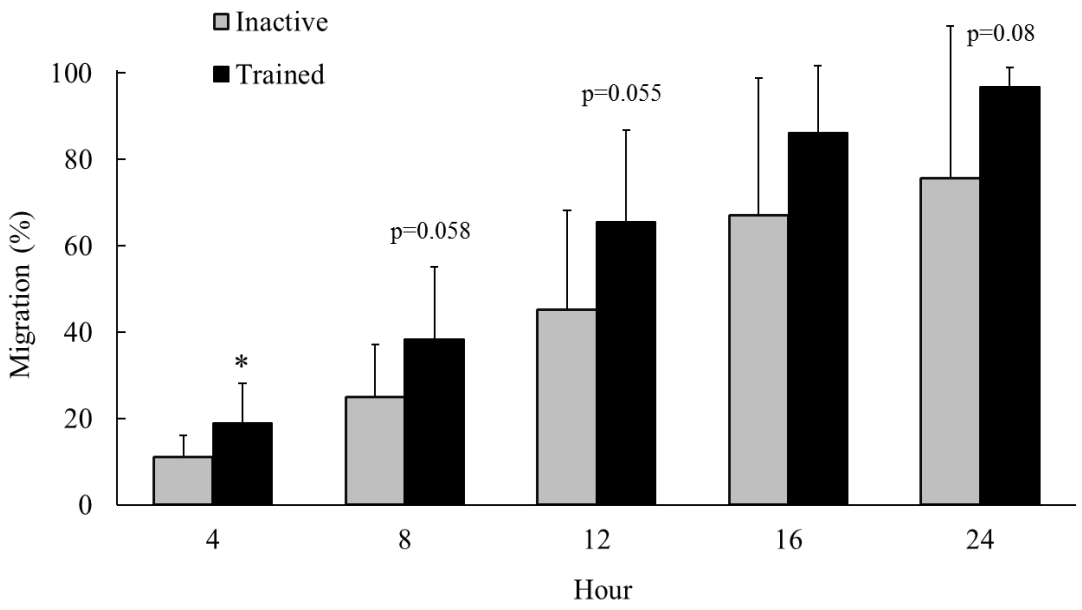


Figure 2. Quantification of HUVEC migration upon application of serum from endurance trained (n=10) or inactive (n=10) individuals over 24 hours. Migration was calculated as (100 – (size of wound area at time point / size of initial wound area x 100)). Images were taken at 10X magnification and area was quantified using imageJ (NIH). Values are reported as mean + SD. *$p \leq 0.05$, statistically significant difference from inactive group at that time point.

MiRNA Array: Of the 84 miRNA targets investigated, 71 showed appropriate abundance for analysis ($C_T \leq 35$). C_T values were first calibrated based on recovery of spiked-in cel-miR-39. Reverse transcription and PCR control primer assays indicated acceptable performance for analysis (data not shown). The data were further normalized using the five most invariant miRNAs between groups in the array (miRs-210, 122, let-7c, 424, and 100). The average of the C_T values for these five miRNAs was used for the $\Delta\Delta C_T$ calculations. The $2^{-\Delta\Delta CT}$ method of relative quantification was used to compare groups. Fold-difference was calculated as the normalized $2^{-\Delta\Delta CT}$

value for the trained sample over that of the inactive sample. Fold-difference values less than 1, indicating lower expression in the trained sample, were transformed to negative values by taking the inverse (i.e. 1 / fold-difference value). MiRNAs showing >4-fold difference between groups were said to be differentially expressed. One miRNA was more highly expressed in the trained sample, while eight where higher in the inactive sample (Figure 3). Those miRNAs and their respective fold-differences (trained group/inactive group) are miR-31-5p (300.7), miR-125a-5p (-4.7), miR-140-5p (-20.3), miR-145-5p (-5.6), miR-199a-5p (-7.4), miR-208b-3p (-4.0), miR-23b-3p (-5.3), miR-30c-5p (-4.4), and miR-93-5p (-6.0).

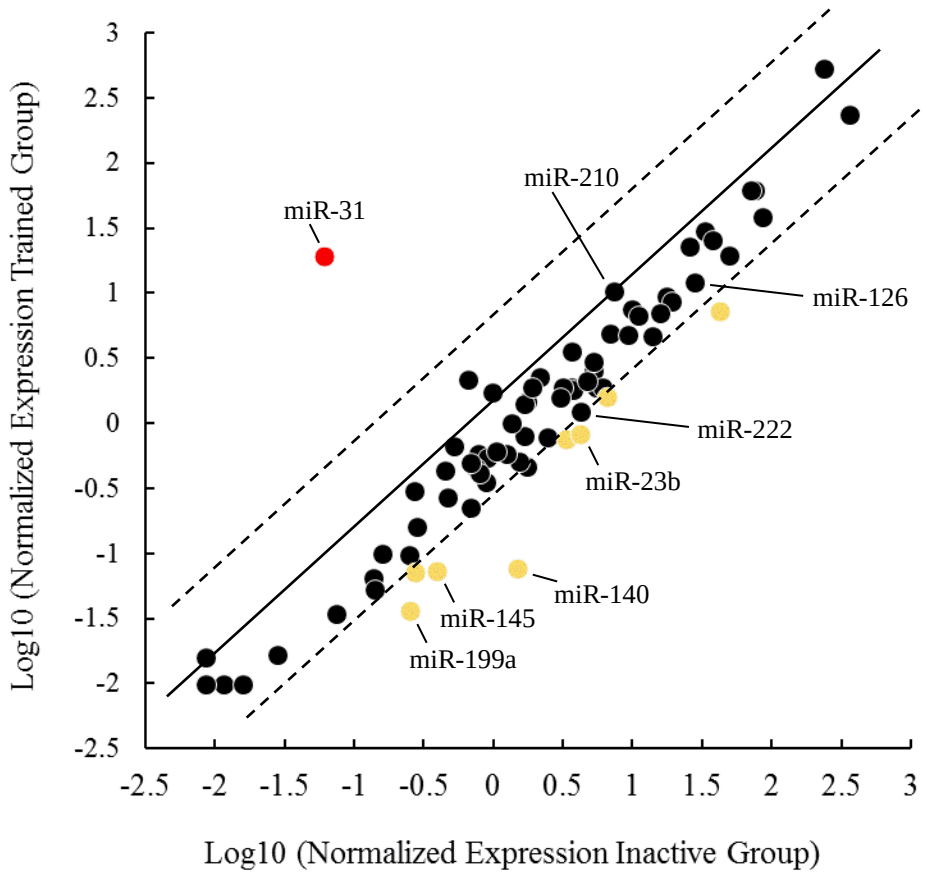


Figure 3. Analysis of CVD-related miRNAs in an RT-qPCR array. Total RNA from serum of three subjects per group was isolated, pooled, and reverse transcribed. Complimentary DNA from groups was then compared using a RT-qPCR array of CVD-related miRNAs. A total of 71 miRNAs showed appropriate abundance for analysis (Ct≤35) in both the pooled, trained and inactive cDNA samples. Expression is represented as the common logarithm of the $2^{-\Delta\Delta CT}$ value for the trained sample / that of the inactive sample. Those miRNAs exhibiting ≥4-fold difference between groups were considered differentially expressed. In addition, miRs-210, 126 and 222 were selected for further evaluation in all samples.

Validation of Differentially Expressed ci-miRNA: The coefficient of variation for the C_T values of the cel-miR-39 spike-in control was 2.6% (Figure 4), which is in line with a previous study (Rosjo et al 2014). None of the ci-miRNA targets chosen for analysis in all samples exhibited different expression between the groups (Figure 5). Variation in expression between sexes is apparent however, highlighting the need for more subjects.

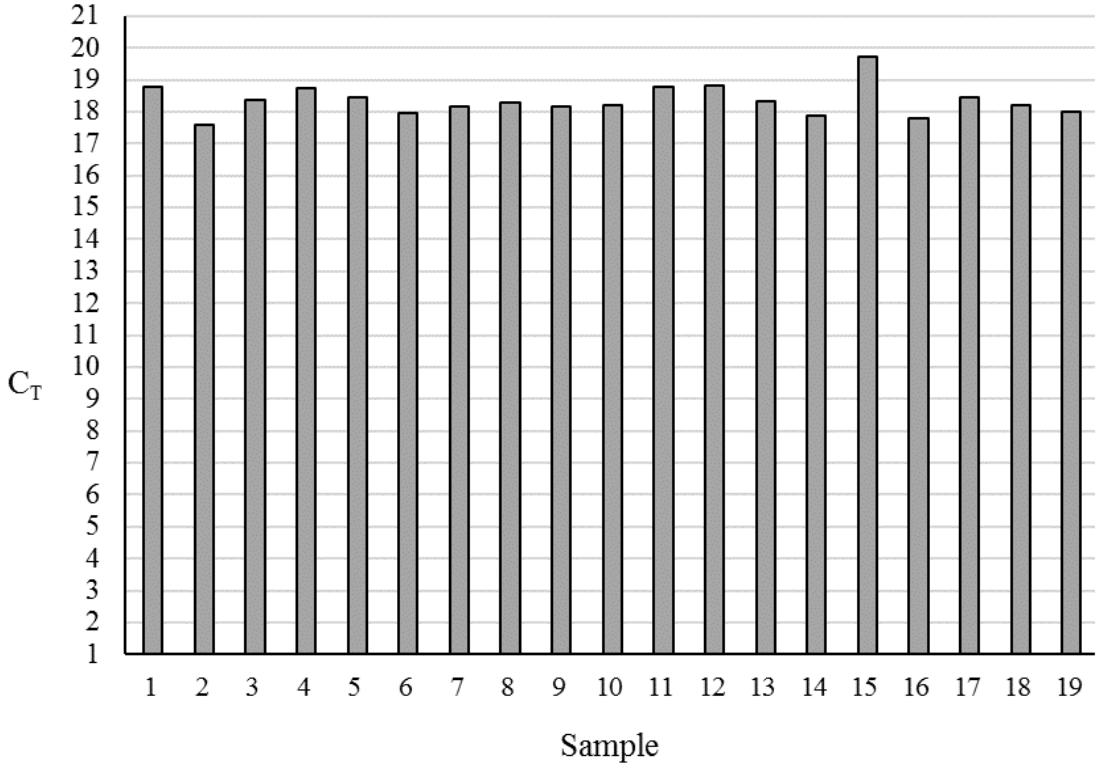
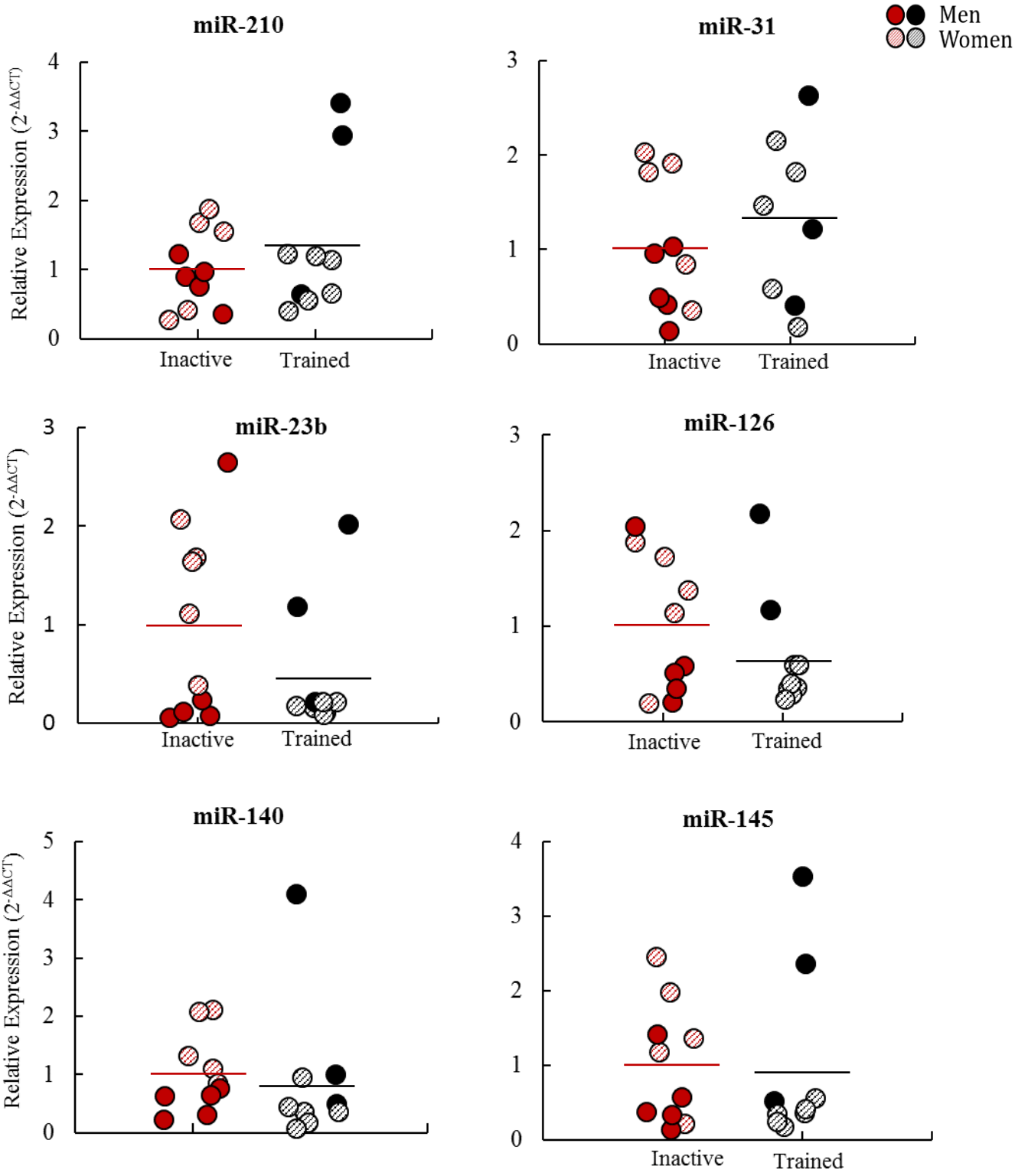


Figure 4. Recovery of cel-miR-39 spiked in during the isolation step was determined in all samples by RT-qPCR. A low variation in RNA extraction from all samples was verified (CV=2.6%). C_T, cycle threshold.


miR-210
miR-31
miR-23b
miR-126
miR-140
miR-145
Men
Women
Relative Expression (2-ΔΔCT)
Inactive
Trained

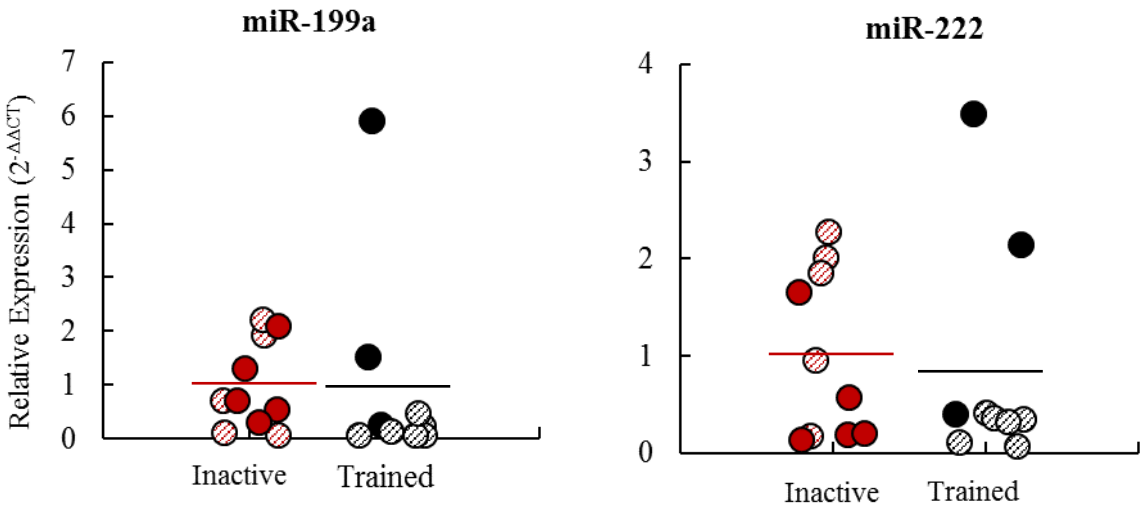


Figure 5. Individual miRNA RT-qPCR analyses in all samples using specific primer assays. Each point represents an individual subject (solid dots, men; striped dots, women) and lines represent group means. No significant differences were observed for any ci-miRNA ($p>0.05$).

Discussion

To our knowledge, this is the first study to compare the effects of serum from trained and inactive individuals on endothelial cells in a migration assay. In 2013, Beijer and colleagues determined human serum's ability to stimulate HUVEC proliferation *in vitro* both before and after a six week resistance training program and found no difference due to training. Wahl et al. previously examined the effects of serum collected from trained triathletes and cyclists on human coronary artery endothelial cells in a scratch migration assay (2014). Serum accelerated migration as compared to control growth medium, however there were no differences seen with serum collected at rest or following a single 180 minute acute bout of cycling exercise. In our study, EGM-2 without serum was used as a control condition and did not induce a migration response (i.e. 0% migration). In line with our hypothesis, serum of inactive subjects induced a slower migration rate than that of trained individuals. HUVECs cultured with 'trained serum' migrated significantly faster at the 4 hour time point, and trended toward significance in the same direction at almost all other time points (Figure 2). This suggests circulating factor(s) in serum of endurance trained individuals may beneficially stimulate especially the initial stages involved in endothelial cell migration, though later mechanisms may be affected as well (Gotlieb and Lee, 1999).

Considering both groups consist of young, healthy individuals devoid of other classic CVD risk factors, our results suggest a potential early effect of exercise training to preserve endothelial integrity. It is likely that the difference in migration rate would have been more dramatic in older individuals and further in those with

CVD risk factors or overt CVD. For example, HUVECs cultured in the presence of serum from CHF patients released more lactate dehydrogenase and displayed greater DNA fragmentation, signs of cell death and apoptosis respectively, compared to age-matched controls (Mammi et al., 2011). Following three weeks of fairly intensive physical rehabilitation, both markers were reduced, though not to levels seen in healthy controls. It is also important to note that the controls in that study were labeled 'healthy' despite the presence of dyslipidemia, hypertension, and/or diabetes.

For our study, we attempted to isolate the effects of exercise training by recruiting young, healthy individuals who differed only in their aerobic physical activity habits (Table 1). Thus, it is likely that the trained group differed from the inactive group only through endurance exercise training-induced mechanisms (i.e. exposure to regular increases in vascular shear stress). Aerobic exercise training has been shown to improve endothelial function and reverse dysfunction in young and old, healthy and diseased populations (Higashi et al., 1990; Desouza et al., 2000; Hambrecht et al., 1998). Improvements in endothelial function following exercise training are in large part attributed to increased bioavailability and/or signaling of NO (Di Francescomarino et al., 2009). In a study of CHF patients, the correction of endothelial dysfunction due to training corresponded to improvements in basal NO formation in the resistance arteries (Hambrecht et al., 1998). The authors of this study further postulated that because the vascular smooth muscle cells did not show any alterations in response to exogenous NO, the improvement in endothelial function was due to shear-stress-induced release of endothelial-relaxing factors (Hambrecht et al., 1998). Indeed, the concomitant episodes of increased vascular, laminar shear

stress due to exercise training beneficially increase endothelial NO production and decrease oxidative stress through mechanotransduction-induced pathways (Di Francescomarino et al., 2009). Furthermore, both shear stress and NO have been shown to play important roles in the regulation of endothelial processes such as angiogenesis, endothelial cell migration, and wound repair (Ziche et al., 1994; Murohara et al., 1998; Lee et al., 1999; Li, Huang, and Hsu, 2005). As shown by Wahl et al. though, a prolonged bout of acute exercise did not alter serum's effect on endothelial cell migration, independent of shear stress (2014). There are, in fact, myriad other factors that may influence endothelial cell migration in one way or another. Further, the relatively ephemeral shear stress-mediated mechanotransduction effects would not likely explain longer-term differences in serum of trained versus inactive populations at rest. For instance, in 2012, Conti et al. observed differences in serum oxidative markers and NO levels in different types of athletes 15 days after the last bout of exercise. When they cultured endothelial cells with the serum, they found higher survival and proliferation rates as a result of the aerobically trained athletes' serum compared to the two more anaerobically trained athlete groups (Conti et al., 2012). This suggests an exercise training effect on circulating factors in serum, independent of the acute effects of elevated laminar shear-stress. A 15 day period without exercise is likely to induce effects of de-training, however (Coyle et al., 1984), so these results may have depicted de-training effects on/of serum, as opposed to an accurate depiction of the exercise trained state. For our study, blood was collected 16-24 hours following the last exercise bout. This avoided any possible

immediate, short-term effects of elevated shear stress, while still maintaining the effects of exercise training.

As mentioned, endothelial cell migration is a highly regulated process. In addition to hemodynamic factors, it is modulated by numerous soluble molecules and intracellular pathways (Gotlieb and Lee, 1999; Dimmeler, Dernbach, and Zeiher, 2000; Lamalice et al., 2007). Most notably, vascular endothelial growth factor (VEGF) potently regulates endothelial cell migration, primarily through binding to the cell receptor VEGFR-2 and stimulating multiple signaling pathways (Lamalice et al., 2007). Interestingly, at rest, circulating levels of VEGF and similar angiogenic factors involved in endothelial cell migration appear to be unaffected by endurance exercise training status (Kraus et al., 2004; Landers-Ramos et al., 2014; Lamalice et al., 2007). Conversely, miRNAs are strikingly mutable in response to aerobic exercise training (Nielson et al., 2014; Wardle et al., 2015). Many intracellular and ci-miRNAs are regulated by shear stress and can also modulate endothelial cell migration (Boon et al., 2012; Jae et al., 2015; Mondadori et al., 2015; Sabatel et al., 2011). Ci-miRNAs from several origins, such as monocytes, EPCs, and endothelial cells are incorporated by the endothelium and subsequently stimulate migration or play other atheroprotective roles (Zhang et al., 2010; Sahoo et al., 2011; Zernecke et al., 2009). In a recent study, HDL isolated from CHF patients was co-cultured with human aortic endothelial cells (Riedel et al., 2015). Compared to HDL from healthy controls, CHF HDL induced significantly less endothelial expression of pro-angiogenic miRs-126, 21, and 222 (Riedel et al., 2015). This difference was attenuated following a moderate exercise training program (Riedel et al., 2015). Although this study isolated the

effects of HDL from other serum factors, it is interesting nonetheless since HDL are also known to transport miRNAs to endothelial cells, where they influence gene expression (Tabet et al., 2014). We thus sought to investigate differences in serum ci-miRNAs between endurance trained and inactive subjects.

In our initial screening, we chose to use an array of miRNAs previously shown to regulate and/or respond to a variety of CVDs in humans. Several of these miRNAs have additionally been observed as markers of exercise and/or training. Analysis of 84 miRNAs in the array produced 71 targets observable within the detectable range for both groups ($C_T \leq 35$) (Figure 3). From this total, nine miRNAs showed $\geq$ 4-fold difference between groups, of which eight displayed lower expression in the trained group. Baggish et al. previously examined ci-miRNA levels in men before and after a 90 day aerobic exercise training program (2011). They assessed eight ci-miRNAs, five of which were previously shown to be involved in angiogenesis (Baggish et al., 2011). Of these, miRs-20a, 221, and 222 were elevated at rest following training. In a more robust examination, Nielson and colleagues screened for all miRNAs present in the circulation of men and compared ci-miRNA profiles before and after 12 weeks of cycling training (2014). They found a total of 13 differentially expressed ci-miRNAs, 11 of which were downregulated following the training protocol (Nielson et al., 2014).

MiRNAs are postulated to play a role in exercise training-induced adaptations. There is ample evidence that unique miRNAs are involved in the left ventricular hypertrophy response to aerobic exercise training, as opposed to those active in the pathological hypertrophy response (Soci et al., 2011; Martinelli et al., 2014;

Ramasamy et al., 2015). Similarly, in rats, cardiac miR-126 and ventricular capillary-to-fiber ratio increased as a function of exercise training intensity (Da Silva et al., 2012). The authors of this study further provided evidence for, and proposed a mechanism by which miR-126 downregulates inhibitors of the VEGFR-2 signaling pathway, leading to increased cardiac angiogenesis (Da Silva et al., 2012). In humans, ci-miRNAs that correlate with VO_2max, running speed at lactate threshold, and thickness of intraventricular septum have been identified (Mooren et al., 2014). Moreover, plasma miRNAs have been shown to differ between elite strength and endurance athletes (Wardle et al., 2015). Both elite athlete groups were further compared to a third inactive control group, although CVD risk factors were not examined (Wardle et al., 2015). Of the 14 ci-miRNAs examined, four were higher in endurance athletes than strength athletes, and only miR-222 was different between all groups (Wardle et al., 2015).

Ours is the first study, to our knowledge, to cross-sectionally compare the ci-miRNA signatures of endurance trained and inactive individuals, with an emphasis on all subjects being healthy with no CVD risk factors. We chose to study a subset of ci-miRNAs identified as ≥ 4-fold differentially expressed in the array, plus two others commonly seen in the CVD and exercise literature (miRs-126 and 222; 2.5- and 3.75-fold lower in our trained group, respectively (Figure 3)). The miRNA exhibiting the least difference between groups in the array, miR-210, was used as a control. Ci-mir-210 is a marker of atherosclerosis and has previously been shown as unresponsive to aerobic exercise training (Raitoharju, Oksala, and Lehtimaki, 2013; Baggish et al., 2011). Upon further RT-qPCR validation of ci-miRNAs chosen from the array, using

all samples, no significant differences were seen (Figure 5). However, this is likely due to normal variability of ci-miRNA expression and the fact that we chose to study both men and women. Even in studies of diseases, where ci-miRNA expression is dysregulated, large inter-individual differences in both the disease and control groups are common (Vasu et al., 2014; Han et al., 2015). Indeed, in a recent review, wide variation in ci-miRNA expression was noted as one of the major challenges of defining them as useful biomarkers of CVDs (Min and Chan, 2015).

Our data also suggest possible sex-based differences and thus render our sample sizes too small to draw any conclusions. Virtually all studies reporting altered ci-miRNA expression in association with exercise training in healthy populations thus far have studied only men. Conversely, in a study using whole blood from men and women, Backes et al. observed no significant differences in ci-miRNAs between elite endurance athletes and moderately active controls (2014). Interpreting the results of this study raises several considerations, however. For one, the whole blood miRNA profile includes the intracellular miRNAs from all cells in circulation and, therefore, does not reflect the ci-miRNA profile of serum or plasma. Further, the control group consisted of healthy, age- and sex-matched "moderately active controls", although the exact activity/training habits were not reported and only maximal workload achieved on a cycling test was measured as an indicator of training status. Though subjects were described as healthy, no blood chemistry analyses or other CVD risk factors were reported. In addition, the elite endurance trained group competed at an international/Olympic level, so most likely exhibited performance-related genetic differences in addition to a presumed greater training load. For instance, well-

matched individuals with the same physical activity habits separated into high and low VO_2max groups, exhibit differential ci-miRNA expression (Bye et al., 2013). Further differences were also seen by separating out men and women (Bye et al., 2013). Though more subjects are needed for any conclusive statements, our data similarly suggest possible sex-based differences. Since our groups also display significantly different VO_2max values due to the effects of exercise training, it would be interesting to compare our results to those of Bye et al. following the recruitment of more subjects.

In conclusion, we have identified a novel phenomenon by which aerobic exercise training enhances the ability of serum to stimulate endothelial cell migration. This highlights a potential early risk factor for CVD development in sedentary, otherwise healthy, young people. We have also identified a potential role for differential regulation of ci-miRNAs in the cardiovascular response to exercise training. Though we have postulated ci-miRNAs as mediators of this response, other circulating factors should not be ruled out. Future studies are needed in order to identify the factor(s) involved so that potential early biomarkers and/or interventions can be developed. Furthermore, more subjects will be recruited to supplement the data presented here in the hopes of elucidating training- and sex-dependent effects on the ci-miRNAs identified in our array.

Literature Review

The cardiovascular system

The mammalian cardiovascular system consists of the heart, blood vessels, and blood. The heart is a muscular pump which creates the pressure necessary to move blood through the closed circuit of blood vessels. Blood travels from the heart through the arterial system, into capillaries associated with all tissues of the body, and back to the heart through the veins. Vessels on the arterial side are considered the resistance vessels because they largely control blood pressure by vasomotion, while those on the venous side are considered capacitance vessels due to their distensibility. Capillaries consist almost exclusively of a single layer of endothelial cells and are, therefore, ideal for the vital exchange of small molecules between the blood and tissues. Indeed, all blood vessels are lined on the luminal side by this single layer of endothelial cells known as the endothelium. The endothelium was once thought to act only as a passive barrier between the blood and tissues, but is now known to play a variety of essential physiological roles in maintaining homeostasis.

Blood is made up of plasma (water), cells (red blood cells, leukocytes, platelets, etc.), and other circulating factors (hormones, proteins, cholesterol, etc.). Thus, the circulatory system is responsible for maintaining life by transporting oxygenated blood from the lungs to muscle and other cells for the production of energy and, conversely, transporting carbon dioxide (CO_2) and other waste products from the cells for excretion. It acts to integrate all tissues of the body by providing a means of transporting nutrients, hormones, and other factors between distant tissues.

Additionally, the cardiovascular system regulates pH balance, body temperature, and immune function. Therefore, preserving proper cardiovascular function is of great importance to maintenance of healthy life.

Cardiovascular disease

A long term disturbance in normal homeostasis of the cardiovascular system leads to the development of disease. Cardiovascular disease (CVD) is the world's leading cause of mortality, accounting for 17.3 million deaths per year (Mozaffarian et al., 2015). Likewise, in the United States, CVD is the underlying cause of about 1 of every 3 deaths and >1 of every 3 adults lives with at least one CVD, presenting a major economic burden (Mozaffarian et al., 2015). By 2030, yearly global deaths from CVD are projected to rise to over 23 million (Mathers and Loncar, 2006) and total costs of the diseases are predicted to reach $918 billion (Mozaffarian et al., 2015). It is clear that improving our understanding of CVD pathologies for the purposes of developing prevention and treatment methods is of paramount importance. To this end, much is known about the risk factors that potentiate CVD development. Traditional risk factors include aging, family history of CVD, cigarrete smoking, high blood pressure, impaired fasting glucose, dyslipidemia, obesity, and sedentary lifestyle (Mozaffarian et al., 2015; Yusuf et al., 2001). All of these factors act to increase risk of CVD via direct and indirect atherogenic effects on the endothelium. (Hadi, Carr, and Suwaidi, 2005; Bonetti, Lerman, and Lerman, 2002; Vanhoutte, 2010).

Atherosclerosis

Atherosclerosis is a disease of the arteries and is the underlying cause of several other CVDs, such as coronary heart disease (CHD) and chronic kidney disease, as well as the majority of clinical cardiovascular events (Mozaffarian et al., 2015). Atherogenesis, the development of atherosclerosis, is a silent process with no overt signs or symptoms, often until the occurrence of a potentially life-threatening acute event. CHD is the leading cause of mortality in the U.S., alone accounting for about half of all CVD deaths (Mozaffarian et al., 2015). The study of atherosclerosis pathology stems to the 1800's and was most notably advanced in 1856 by Rudolf Virchow, who postulated that atherosclerotic lesions were a response to inflammatory injury to the vessel wall (Ventura, 2000; Ross, Glomset, and Harker, 1977). However, owing to the concealed nature of the atherogenic process, CHD was only first reported in 1912 (Levy, 1981), despite evidence of atherosclerotic plaques in Egyptian mummies that lived almost 4000 years earlier (Allam et al., 2011). This fairly recent discovery of CHD is in large part due to the prolonged lifespan of humans since the threat of infectious diseases was effectively nullified as the leading cause of mortality (Fuster et al., 2007). As a result, CVD has only relatively recently become an epidemic, spreading from predominantly wealthy classes and developed countries in the 1920s (Levy, 1981), to developing nations and the less affluent (Global Status Report on Noncommunicable Diseases, 2010). Still, the complex molecular and cellular details of the pathologic process of atherogenesis continue to be discovered (Lu and Daugherty, 2015).

Impressively, Virchow was correct in that atherosclerosis is a progressive inflammatory disease of the arteries developed in response to injury, though his hypothesis has since been modified (Ross et al., 1977; Lusis, 2000). The initiating injury of atherosclerosis refers to denudation of endothelial cells from the endothelium, which, if unabated and combined with continuous exposure to injurious agents (risk factors) leads to endothelial dysfunction (Ross, 1999; Francis and Pierce, 2011). Impaired ability of the endothelium to function normally, results in unfavorably increased permeability, adhesiveness, and altered release of vasoactive molecules. Impaired vasodilation is a hallmark sign of endothelial dysfunction and atherosclerosis (Francis and Pierce, 2011). Continued inflammatory response to endothelial dysfunction results in the development of lesions, primarily in the large and medium sized arteries. The earliest 'fatty streak' lesions are commonly seen in the aorta of infants and children (Ross, 1999; Lusis, 2000). Lesions advance as the atherogenic process continues over decades until a lesion grows large enough to impede blood flow in an artery, or an acute rupture/erosion leads to thrombosis and complete occlusion of a vessel. Such events are often debilitating and/or life threatening (e.g. angina, myocardial infarction (MI), stroke, etc.).

Endothelial function

Maintaining a healthy, properly functioning endothelium is of utmost importance due to the central role of endothelial dysfunction in the atherogenic process. As mentioned, the endothelium consists of a single intraluminal layer of endothelial cells that lines all blood vessels. In capillaries, endothelial cells provide a

barrier between blood and the underlying body tissues. Far from existing solely as a passive barrier, the endothelium is now considered the largest organ in the body (Gimbrone et al., 2000; Endemann and Schiffrin, 2004; Eckers and Haendeler, 2015). In a healthy state, the endothelium is a dynamic network that regulates vascular tone, hemostatic and fluid balance, vascular permeability, vessel wall hypertrophy, immune function, and formation of new vessels (Smith and Fernhall, 2011; Endemann and Schiffrin, 2004; Eckers and Haendeler, 2015). Endothelial cells accomplish these actions by responding to both chemical and physical stimuli (blood flow [i.e. shear stress] and pressure), producing myriad substances and releasing them into the bloodstream or underlying vascular smooth muscle cells (Endemann and Schiffrin, 2004). Further, endothelial cells are adaptable to their environment; there is evidence of vast endothelial heterogeneity between and within different vascular beds throughout the body (Aird, 2012). Endothelial cells are highly mutable under conditions such as exercise training (Padilla et al., 2011) or exposure to CVD risk factors (Deanfield, Halcox, and Rabelink, 2007).

Endothelial damage and dysfunction

Damage to the endothelium and excessive exposure to CVD risk factors alters the endothelial cell phenotype and hampers its ability to appropriately respond to stimuli (Versari et al., 2009). A state of endothelial dysfunction results, in which the endothelium loses its protective abilities and adopts pro-atherogenic characteristics. This switch reflects the activation of a defense response, whereby endothelial cells promote vasoconstriction, inflammation, and atherogenesis (Vita and Keaney, 2002).

The most notable functional characteristic is impaired endothelium-dependent vasodilation due to reduced bioavailability of vasodilators, primarily nitric oxide (NO), combined with increased expression of vasoconstricting agents (Bonetti et al., 2002).

Endothelial dysfunction was first described in humans by Ludmer et al., who showed impaired endothelium-dependent vasodilation of coronary arteries with atherosclerosis (1986). Panza and colleagues later observed reduced dilation in the forearms of hypertensive patients (1990). Furthermore, endothelial dysfunction precedes CVD development (Taddei et al., 1996), and endothelial function is inversely correlated with the number of CVD risk factors present (Vita et al., 1990). In a prospective study, Halcox et al. showed that endothelial dependent function of coronary arteries was a strong predictor of future cardiovascular events in subjects with and without coronary artery disease (CAD) (2002). Thus, it has been proposed that endothelial function could serve as a barometer of CVD risk, since endothelial dysfunction represents the initial step towards atherogenesis (Vanhoutte, 2010). Importantly, endothelial dysfunction appears also to be involved in the later stages of atherosclerotic lesion progression (Vita and Keaney, 2002).

Maintenance of an intact endothelial cell monolayer is vital to normal functioning of blood vessels, so injury to the endothelial cell layer is an important initiating event in endothelial dysfunction (Ross et al., 1977; Vanhoutte, 2009). Injury may stem from prolonged and/or repeated exposure to a variety of different stimuli, such as CVD risk factors (e.g dyslipidemia, smoking, hyperglycemia, aging, sedentary lifestyle), inflammatory disorders, chemical factors, metabolites, infections,

and/or mechanical factors (Ross et al., 1977; Deanfield et al., 2007; Eckers and Haendeler, 2015; Vanhoutte, 2010; Woywodt et al., 2002). Under normal, healthy conditions, endothelial cells are in a quiescent state and endothelial turnover occurs at a slow rate. With aging, endothelial cell apoptosis and turnover increase, a process which is accelerated by the presence of CVD risk factors (Vanhoutte, 2010; Vanhoutte, 2009). Thus, injurious agents can lead not only to endothelial cell dysfunction, but also compromised integrity of endothelial cells, apoptosis or senescence, and eventual detachment from the endothelium (Deanfield et al., 2007; Ross et al., 1977).

The mechanism(s) of endothelial denudation are not well understood, though alterations in blood flow, loss of contact with neighboring cells, integrins, neutrophils, cytokines, and proteases may all play a role (Ballieux et al., 1994; Woywodt et al., 2002; Ross et al., 1977). Sites of denudation are then susceptible to platelet adhesion to the sub-endothelial layer which cause vasoconstriction and subsequent smooth muscle cell migration and proliferation (Ross et al., 1977; Vanhoutte, 2009). Restoration of the endothelial monolayer is possible if the injury and its response are limited. However, repeated cycles of this process over a period of years leads to the accumulation and invasion of proliferated smooth muscle cells into the lumen (Ross et al., 1977). At this point, endothelial dysfunction results and an atherosclerotic lesion is initiated, as the mechanisms of repair and lesion regression are no longer effective (Ross et al., 1977).

Endothelial repair by migration

Damage to the endothelium is dependent on the balance between injury and repair. Regeneration of the endothelial monolayer is achieved by neighboring endothelial cells adjacent to the damaged area, though more recently circulating endothelial progenitor cells (EPCs) have been shown as an alternative mechanism (Ettenson and Gotlieb, 1993; Asahara et al., 1997). It has been postulated that under healthy conditions in the absence of risk factors, the endogenous repair mechanisms of adjacent endothelial cells would be sufficient to maintain endothelial integrity throughout life (Deanfield et al., 2007). Endogenous repair is achieved primarily by migration of adjacent endothelial cells into the site of injury, followed by proliferation. Proliferation is both preceded by and dependent on prior migration (Coomber and Gotlieb, 1990; Gotlieb and Lee, 1999). Further, migration, as opposed to proliferation, appears to be the major mechanism responsible for wound healing *in vitro*, at least in the initial 1-2 days following injury (Li et al., 2005). In response to denudation of an area of the endothelium, adjacent endothelial cells transition from a quiescent state to one which promotes migration. This process relies heavily on microfilament/cytoskeleton reorganization and involves the reduction of cell-cell adhesion with neighboring cells, elongation, cell spreading, and translocation (Gotlieb and Lee, 1999). Once the wounded area is covered and the endothelium monolayer is restored, endothelial cells may return to their normal, resting state.

Shear stress modulates endothelial repair

Cytoskeletal reorganization, and by extension migration, is moderated by hypercholesteremia, altered shear stress, growth factors (e.g. VEGF, bFGF, angiopoietins), and other soluble factors (Gotlieb and Lee, 1999; Dimmeler et al., 2000; Lamalice et al., 2007). Endothelial cells that regenerate a wounded area in the presence of risk factors may exhibit altered phenotypes, resulting in local endothelial dysfunction and predisposing the area to atherogenesis (Vanhoutte, 2010). Conversely, elevated levels of shear stress induce enhanced wound repair and atheroprotective effects (Cunningham and Gotlieb, 2005). Shear stress refers to the frictional force exerted by the parallel flow of blood along the vascular wall (Cunningham and Gotlieb, 2005). Vascular shear stress exerts direct forces on the endothelium and is important in modulating endothelial cell structure and function. Endothelial cells respond to shear-induced mechanotransduction by altering gene expression, cytoskeletal organization, and secreted factors (Cunningham and Gotlieb, 2005; Li et al., 2005; Boon et al., 2012). Notably, each step in the process of endothelial cell migration is influenced by shear stress (Li et al., 2005). Both *in vivo* and *in vitro* studies have shown that shear stress can beneficially enhance endothelial cell migration and wound healing (Wu et al., 1995; Albuquerque et al., 2000). The importance of shear stress is emphasized by the fact that vascular sites of altered and/or decreased shear, such as arterial branching and curving points, are usually the sites of endothelial denudation and are significantly more likely to develop atherosclerotic lesions (Cunningham and Gotlieb, 2005). Conversely, periodical

increases in shear stress, as is seen with repeated bouts of exercise, favorably augments vascular function and remodeling (Tinken et al., 2010).

Beneficial cardiovascular effects of exercise training

Exercise training induces beneficial cardiovascular system adaptations in young and old, healthy and diseased populations. Cardiovascular adaptations are greatest with aerobic, endurance type activities as opposed to strength training (Hurley et al., 1984). Structural and functional changes to the heart, vasculature, and blood due to repeated bouts of aerobic exercise, combine to improve functional capacity and endurance. Further, physical inactivity is a major risk factor for CVD (Morris et al., 1953). Regular physical activity protects against CVD, with the most active individuals showing up to a 40% reduction in risk (Shiroma and Lee, 2010). Still, even greater reductions in risk are seen with increases in cardiorespiratory fitness (Williams, 2001). Regular physical activity is effective in prevention and treatment of the modifiable CVD risk factors and, by extension, atherosclerosis (Thompson et al., 2003). Moreover, exercise training and rehabilitation are effective therapies for CVD patients. Exercise training has been shown to reduce symptoms, enhance quality of life, and/or reduce mortality rates in patients with MI, CAD, CHF, angina, peripheral arterial disease, and claudication (Thompson et al., 2003).

Specific physiological adaptations to endurance training include increased aortic compliance (Mohiaddin et al., 1989), improved ventricular performance (Moore and Korzick, 1995), augmented myocardial (Brown, 2003) and skeletal muscle capillarization (Brodal, Ingjer, and Hermansen, 1977), arterial enlargement

and compliance (Brown, 2003; Vaitkevicius et al., 1993), enhanced number and size of mitochondria (Hoppeler et al., 1973), increased oxidative enzyme content (Holloszy, 1967), expanded plasma volume (Fellman, 1992), and improved endothelial function (Hambrecht et al., 2003). Additionally, exercise training is able to reverse endothelial dysfunction in young and old adults (DeSouza et al., 2000). Such changes in vascular function are not explained solely by exercise training-mediated effects on classic CVD risk factors (Green et al., 2003).

There is ample evidence to suggest shear stress as the major mechanism responsible for training-induced endothelial and vascular adaptations (Tinken et al., 2010; Di Francescomarino et al., 2009). Shear stress alters endothelial cell phenotypes via direct mechanotransduction-mediated effects (Cunningham and Gotlieb, 2005). Intriguingly, similar adaptations have been observed in vasculature perfusing skeletal muscles and organs that are inactive during exercise and, therefore, do not experience a significant degree of increased shear stress (Padilla et al., 2011). This suggests that other systemic mechanism(s) besides shear stress, such as other hemodynamic or circulating factors, may also be in effect. In addition, studies show that the amount of shear stress experienced by endothelial cells affects microRNA (miRNA) expression and secretion into the circulation, which may be taken up by other endothelial cells (Weber et al., 2010; Hergenreider et al., 2012).

MiRNAs

MiRNAs are ~22 nucleotide long, non-coding RNAs that control gene expression at the post-transcriptional level. The first miRNA was discovered in the

nematode *Caenorhabditis elegans (C. elegans)* by Lee, Feinbaum, and Ambros in 1993. They, along with Ruvkun's group, reported that the small *lin-4* RNA could base pair with a section of the 3' untranslated region (3'UTR) of another RNA, *lin-14*, and downregulate abundance of the LIN-14 protein (Lee et al. 1993; Wightman, Ha, and Ruvkun, 1993). In 1998, Fire and colleagues demonstrated the potential of experimental RNA interference when they introduced a small, double-stranded RNA into *C. elegans* and observed potent downregulation of endogenous target mRNA. The field of miRNA research has since grown immensely and the miRNA pathway is now known to be highly conserved in mammals (Saetrom et al., 2006; Friedman et al., 2008). There are currently 2588 identified miRNAs in the human genome and it is predicted that over 60% of protein coding-genes are conserved targets (Kozomara and Griffiths-Jones, 2014; Friedman et al., 2008). Furthering their complexity, individual miRNAs usually regulate several, in some cases up to 200, mRNA transcripts, while multiple miRNAs can share the same mRNA target (Esquela-Kerscher and Slack, 2006). Another characteristic of miRNAs is tissue specific expression and function, which are sometimes contradictory. For instance, miR-26 acts as a tumor suppressor in liver and intestinal cancers, and as an oncogene in brain cancer (Kota et al., 2009; Zeitels et al., 2014; Huse et al., 2009). Thus, miRNAs act in a highly coordinated and intricate fashion to fine-tune protein expression.

Canonical miRNA biosynthesis

MiRNA genes are transcribed by polymerase II into an initial primary miRNA (pri-miRNA), which is usually over 1,000 bp long and contains the mature miRNA

sequence in a stem-loop structure. Genes for miRNAs are encoded in both intergenic and intronic regions, and they are often found in polycistronic transcription units (Ha and Kim, 2014). Interestingly, many miRNAs encoded within intronic regions repress their co-transcribed host gene(s) (Ramalingam et al., 2013). Further, intronic miRNAs also have their own promoters, which allows independent transcription and distinct expression from their host gene(s) (Ramalingam et al., 2013).

The nuclear enzyme Drosha cleaves the pri-miRNA into a ~65 bp pre-miRNA. This double stranded hairpin structure associates with Exportin-5 and is transported through a nuclear pore into the cytoplasm. Here, the pre-miRNA is further cleaved by Dicer near its terminal loop, resulting in a 21-25 bp double stranded RNA consisting of the mature miRNA and its complimentary strand. The miRNA duplex is quickly unwound and is loaded onto an Argonaute protein, associated with Dicer, to form a miRNA-induced silencing complex (miRISC) (Siomi and Siomi, 2009; Carthew and Sontheimer, 2009). The strand that associates with RISC is the mature miRNA, which acts as a guide by complimentarily binding to the 3'-untranslated region of the target mRNA transcript (Ha and Kim, 2014). The associated 'passenger' strand is most often released into the cytoplasm and degraded (Siomi and Siomi, 2009). Nucleotides 2 to 8 on the 5' end of the mature miRNA are most important in target recognition and binding (Esquela-Kerscher and Slack, 2006). The mRNA targeted by miRISC may be perfectly or imperfectly complementary, having implications on the mechanism of silencing that affects the target mRNA transcript. This capability for imperfect binding is also important in that it allows an individual miRNA to target numerous mRNAs. Following binding, miRISC partially or

completely downregulates gene expression by inducing either translational repression, mRNA deadenylation, mRNA degredation, or sequestration in processing-bodies (P-bodies) (Jackson and Standart, 2007). Further complicating matters, more recent studies have provided evidence that miRNA-mediated up-regulation of transcription may also occur under certain conditions (Orang, Safaralizadeh, and Kazemzadeh-Bavili, 2014).

Circulating-miRNAs as biomarkers of disease

The fine-tuning of gene expression by miRNAs is crucially important in biological processes such as cell growth, cell proliferation, tissue differentiation, embryological development, apoptosis, and motility (Wilson and Doudna, 2013; Lu et al., 2008). Owing to their importance, the number of miRNAs in the genome correlates with complexity of the organism (Berezikov, 2011). Accordingly, miRNAs are found in seemingly all cell types and compartments of the body. Specifically, the fairly recent discovery of miRNAs in biofluids, including serum, plasma, saliva, urine, and milk, has opened up new possibilities for miRNA research (Chen et al., 2011). Such circulating miRNAs (ci-miRNAs) are secreted into the circulation either passively or actively. They circulate in association with microvesicles (exosomes or apoptotic bodies), proteins (Argonaute), or HDL. Ci-miRNAs can then be actively taken up by target recipient cells where they prompt downstream effects (Zhang et al., 2010; Hergenreider et al., 2012).

It is unsurprising that dysregulation of miRNAs underlies numerous pathologies. Dysregulated miRNAs are reportedly associated with at least 70

diseases, including cancers, neurological disorders, metabolic diseases, neuromuscular diseases, viral pathologies, liver diseases, pulmonary diseases, CVDs, and more (Lu et al., 2008; Hammond, 2015; Fiorillo et al., 2015; Lanford et al., 2010; Haider et al., 2014). Consequently, ci-miRNAs have been proposed as useful biomarkers of disease since they are easily acquired, highly stable, and conveniently amplifiable. Ci-miRNA disease biomarkers have been extensively investigated, while the potential therapeutic value of ci-miRNA based interventions is also clear. In 2010, Lanford et al. demonstrated long-term suppression of the hepatitis C virus in chimpanzees by intravenous infusion of a complimentary antagonist to miR-122. This treatment is now in phase 2 clinical trials and other miRNA therapies are in preclinical development for diseases such as CHF and MI (Bader and Lammers, 2011).

MiRNAs in cardiovascular physiology and pathophysiology

MiRNAs appear to play roles in regulating essentially all cardiovascular cell types, processes, and diseases. Studies have revealed roles for miRNAs in the development and function of endothelial cells, vascular smooth muscle cells, cardiomyocytes, early EPCs, and other cardiovascular related stem/progenitor cells (Jakob and Landmesser, 2011). In the first study signifying the importance of miRNAs in cardiovascular development, Yang et al. developed mice lacking Dicer, a critical enzyme in the miRNA biosynthesis pathway (2005). Dicer deficiency was embryonically fatal and embryos displayed defective vessel formation/maintenance (Yang et al., 2005). Numerous miRNAs are now known to be involved in

physiological and pathological angiogenesis (Finn and Searles, 2012). For example systemic injection of a miR-26a antagonist in mice with experimentally induced MI significantly increased myocardial angiogenesis and decreased myocardial infarct size (Icli et al., 2015). Conversely, overexpression of miR-26a reduced skeletal muscle angiogenesis in mice undergoing exercise training (Icli et al., 2015). Feng et al. provided evidence that miR-487b enhances HUVEC migration and angiogenesis (2015). They further showed that ci-miR-487b is up-regulated in plasma of ischemic stroke patients, representing a possible option for therapeutic intervention.

The involvement of ci-miRNAs in CVD pathologies has been extensively detailed (Bronze-da-Rocha, 2014). Further, altered ci-miRNA profiles are associated with CVD risk factors, such as hypertension, diabetes, hyperglycemia, dyslipidemia, aging and smoking (Mcmanus and Ambros, 2011; Jansen et al., 2013; Varga et al., 2013; Bronze-da-Rocha, 2014; Takahashi et al., 2013). As opposed to passive release due to cell damage or senescence, which may happen in some cases (e.g. miR-1 following MI) (Bronze-da-Rocha, 2014), the majority of evidence suggests deliberate packaging and release of miRNAs as a form of intercellular communication (Hergenreider et al., 2012). In 2010, Zhang et al. showed that monocyte secreted microvesicles containing miRNA are taken up by endothelial cells *in vitro*, where transferred miR-150 is then responsible for stimulating and enhancing migration. Exosomes secreted by CD34+ stem cells are also taken up by endothelial cells and account for their proangiogenic paracrine activity (Sahoo et al., 2011). These exosomes are highly enriched in pro-angiogenic miRs-126 and 130a (Sahoo et al., 2011).

Endothelial cells secrete microvesicles of their own, which may be incorporated into vascular smooth muscle cells or other endothelial cells. For instance, in response to shear stress *in vitro*, endothelial cells secrete exosomes containing miRs-143 and 145 which are transferred to the underlying smooth muscle where they regulate gene expression to confer atheroprotective effects (Hergenreider et al., 2012). Endothelial cells also release miR-126 in microparticles, which is taken up by recipient endothelial cells *in vivo* and promotes repair of injured sites by stimulating migration and proliferation (Jansen et al., 2013). Microparticles from serum of diabetic patients exhibited reduced miR-126 content, which is likely associated with decreased capacity for endothelial repair (Jansen et al., 2013). Further, in a setting of atherosclerosis, damaged endothelial cells secrete apoptotic bodies containing miR-126 (Zernecke et al., 2009). Secreted apoptotic bodies act to recruit EPCs, protect neighboring endothelial cells, and stabilize plaques, largely through miR-126-mediated gene regulation (Zernecke et al., 2009). These studies help to emphasize the potential of ci-miRNA based interventions to combat atherosclerosis.

MiRNAs are, in fact, involved in every stage of atherosclerosis, from endothelial dysfunction to cholesterol homeostasis and transport, inflammation, and finally plaque development and rupture (Madrigal-Matute et al., 2013). In 2014, Loyer et al. showed that anti-miR-92a treatment in ldlr knockout mice fed a high-fat diet successfully reduced atherosclerotic lesions, led to more stable plaque composition, and decreased endothelial inflammation. MiR-92a is active in early stages of endothelial damage as well. Treatment with anti-miR-92a improved

HUVEC migration and proliferation *in vitro*, and increased NO production (Iaconetti et al., 2012). More importantly, inhibition of miR-92a significantly improved re-endothelialization and reduced neointimal lesion formation following both carotid artery balloon injury and stenting in rats (Iaconetti et al., 2012). In addition to those previously mentioned, other moderators of wound closure by altered endothelial cell migration include miRs-21, 23, 27, 20a, and 196a (Sabatel et al., 2011; Zhou et al., 2011; Pin et al., 2012; Pin et al., 2012b) Thus, the processes of endothelial cell migration and wound healing are highly regulated by a variety of miRNAs. Interestingly, shear stress modulates miRNAs involved in migration and other endothelial cell processes.

Shear stress modulates endothelial miRNAs

Shear stress has long been known to beneficially improve wound healing by enhanced endothelial cell migration *in vitro* and *in vivo* (Albuquerque et al., 2000; Vyalov, Langille, and Gotlieb, 1996). This finding stems from the fact that atherosclerotic lesions and areas of endothelial damage most often occur in arterial regions of altered/reduced shear (Cunningham and Gotlieb, 2005). Interestingly, Loyer at al. observed increased miR-92a expression in the atheroprone aortic arch as compared to the descending thoracic aorta in normocholesterolemic mice, and in the downstream sections of human carotid plaques, which experience low shear stress as compared to upstream sections (2014). Qin et al. were the first to describe the phenomenon of altered miRNA expression in endothelial cells due to shear stress *in vitro* (2010). High shear stress tends to increase expression of atheroprotective

miRNAs and downregulate atheroprone miRNAs (Boon et al., 2012). Specifically, miR-126 is upregulated in HUVECs exposed to laminar shear stress and subsequently regulates target mRNA expression in favor of an atheroprotective phenotype (Mondadori et al., 2015). Shear stress increases miR-21 expression in HUVECs, decreasing apoptosis and increasing NO production (Weber et al., 2010). Paradoxically, under conditions with no flow or shear stress, miR-21 impairs HUVEC migration and angiogenesis (Sabatel et al., 2011). These results may be reconciled by the fact that miR-21 has different effects depending on the degree and mode of shear stress (Neth et al., 2013). In addition to altering intracellular miRNA expression, shear stress regulates the secretion of ci-miRNAs. Jae et al. recently showed that shear stress-responsive transcription factor KLF2 induces selective packaging and export of miRNAs that is independent of intracellular levels of up-regulation (2015). They further detailed the KLF2 induced exosomal pathway responsible for the secretion of miRs-143 and 150 (Jae et al., 2015).

Exercise training alters miRNAs and circulating factors

Several studies have described the altered characteristics of serum and circulating factors, including ci-miRNAs, in response to either acute exercise or training. Following an aerobic exercise training program, CHF and CAD patients displayed increases in beneficial serum proteins important to cardiac vascular remodeling (Gatta et al., 2012). Furthermore, when serum obtained after training was applied to healthy PBMCs, it beneficially increased colony forming units-endothelial cells as compared to serum from before training (Gatta et al., 2012). Likewise,

exercise training in CHF patients significantly reduced serum capacity to induce release of lactate dehydrogenase and DNA fragmentation in HUVECs, signs of cell death and apoptosis respectively (Mammi et al., 2011). More recently, HDL isolated from CHF patients was co-cultured with human aortic endothelial cells. Compared to HDL from healthy controls, CHF HDL induced significantly less endothelial expression of pro-angiogenic miRs-126, 21, and 222 (Riedel et al., 2015). This difference was attenuated following a moderate exercise training program (Riedel et al., 2015). In pre-diabetic individuals, serum ci-miRs-192 and 193 are elevated as compared to diabetic and healthy subjects (Parrizas et al., 2015). Following an exercise training intervention, prediabetic subjects exhibited healthy fasting glucose levels and ci-miRs-192 and 193 returned to baseline levels (Parrizas et al., 2015).

Surprisingly, at rest, the circulating levels of several angiogenic factors, such as VEGF, appear to be unaffected by endurance exercise training status (Kraus et al., 2004; Landers-Ramos et al., 2014; Lamalice et al., 2007). On the other hand, differential expression of plasma miRNAs occurs following an acute bout of cycling, as well as after aerobic exercise training (Baggish et al., 2011; Nielson et al., 2014). Baggish et al. further characterized the ci-miRNA response to an exhaustive bout of exercise with the finding that muscle, cardiac, and endothelial specific miRNAs were upregulated immediately after a marathon run (2014). Further, they were cleared from circulation faster than their respective, traditional tissue-specific biomarkers (e.g. troponin I and creatine phosphokinase) (Baggish et al., 2014). Similarly, Sawada and colleagues demonstrated that ci-miRNAs exhibit unique time-courses of upregulation following an acute bout of resistance exercise in untrained males (2013).

In 2012, Da Silva et al. demonstrated that cardiac miR-126 and ventricular capillary-to-fiber ratio increased in rats as a function of intensity of exercise training. This suggested the possibility that miRNAs may play a role in exercise training-induced cardiovascular adaptations. It has since been shown that unique miRNAs are involved in the left ventricular hypertrophy response to aerobic exercise training, compared to those active in the pathological hypertrophy response (Soci et al., 2011; Martinelli et al., 2014; Ramasamy et al., 2015). Alternatively, systemic injections of miR-181c markedly reduced exercise capacity and induced signs of CHF in rats by altering mitochondrial function (Das et al., 2014).

In humans, miR-499 is elevated in active versus sedentary thigh muscle, and is associated with increased type 1 fiber percentage (Gan et al., 2013). Furthermore, ci-miRNAs that correlate with VO_2max, running speed at lactate threshold, and thickness of intraventricular septum have been identified (Mooren et al., 2014). As might be expected then, plasma ci-miRNAs do differ between endurance and strength-trained athletes (Wardle et al., 2015). On the other hand, Backes et al. concluded that ci-miRNA expression in whole blood was not significantly different in elite athletes v. moderately active controls, although the fact that both groups were young, healthy, and aerobically active may have meant these subject groups were too similar to see significant differences (2014). Bye and colleagues determined three ci-miRNAs to be differentially expressed between high and low VO_2max groups with identical physical activity habits and CVD risk factors (2013). This suggests a possible genetic basis for ci-miRNA expression divergence, as they also found differences in some miRNAs for men versus women (Bye et al., 2013).

Conclusions

MiRNAs represent important regulators of cardiovascular health and disease. Further, ci-miRNAs are extensively reported as biomarkers of disease and systemic miRNA therapies are becoming more common. Based on the literature, the potential use of miRNA interventions in prevention and treatment of CVDs is vast and compelling. Specifically, numerous miRNAs are involved in wound-healing of the endothelium and *in vivo* studies offer glimpses of their practical importance. Furthermore, miRNAs at least partially mediate many long-established cardiovascular benefits of exercise training. Thus, exercise training represents a powerful model for elucidating novel miRNA pathways relevant to CVD interventions. Evidence of altered circulating and intracellular miRNAs and their targets also provides further mechanistic insight into the ubiquitous health benefits of regular physical activity.

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