ABSTRACT

Title of Dissertation: SEX- AND RACE-BASED DIFFERENCES IN THE EFFECTS OF ACUTE AND CHRONIC EXERCISE ON VASCULAR FUNCTION AND CIRCULATING MICRORNA

Ryan M. Sapp, Doctor of Philosophy, 2021

Dissertation directed by: Professor James M. Hagberg, Ph.D.
Department of Kinesiology

Cardiovascular disease (CVD) is the leading global cause of death. Disparities in CVD development exist, with greater rates observed in men than women and in African Americans (AA) than Caucasian Americans (CA). It is crucial to determine the molecular mechanisms underlying these disparities in order to formulate preventative strategies. Regular aerobic exercise reduces CVD risk, while acute exercise is a useful stimulus to reveal impairments in cardiovascular function not apparent at rest. This dissertation utilizes approaches to identify sex- and race-based differences in vascular function within young, healthy individuals, indicative of future CVD risk, including the use of acute exercise as a cardiovascular stimulus and the exercise-trained individual as a model of superior cardiovascular health. Aim #1 shows that exercise training is associated with beneficial effects of the circulating factors in serum on

vascular endothelial cells, in a sex-specific manner, suggesting that circulating factors are differently affected by exercise training in men and women. Aim #2 shows that endothelial function and central arterial stiffness respond similarly to acute exercise in AA and CA. Carotid arterial compliance, however, is increased only in CA during exercise recovery. MicroRNAs (miRs) are epigenetic modulators of gene expression implicated in CVD development. Blood-borne circulating miRs (ci-miRs) are paracrine/endocrine molecules and preclinical biomarkers, yet sex- and race-based differences in ci-miRs are understudied. Additionally, ci-miRs are altered with exercise and may mediate training-induced vascular adaptations. Aim #3 of this dissertation reveals that the resting concentrations of select vascular-related ci-miRs differ based on sex and exercise training status, but not race. In response to acute exercise however, several anti-inflammatory ci-miRs increased significantly in CA, but not AA. Additionally, the changes in one anti-inflammatory ci-miR exhibited race-specific correlations with the changes in carotid arterial compliance identified in Aim #2. Aim #4 investigates the hypothesis that exercise elicits endothelial integral damage, and that this may mediate changes in vascular function and endothelial-derived ci-miRs. By measuring different endothelial-derived circulating factors, we show that exercise likely does not cause endothelial cell detachment or apoptosis. Thus, ci-miR are likely released via a selective method of secretion, rather than passively leaking from damaged endothelium.

SEX- AND RACE-BASED DIFFERENCES IN THE EFFECTS OF ACUTE AND CHRONIC EXERCISE ON VASCULAR FUNCTION AND CIRCULATING MICRORNA

by

Ryan M. Sapp

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Advisory Committee:

Professor James M. Hagberg, Ph.D., Chair

Assistant Professor Sarah Kuzmiak-Glancy, Ph.D.

Assistant Professor Steven J. Prior, Ph.D.

Assistant Professor Sushant M. Ranadive, Ph.D.

Associate Professor Steven M. Jay, Ph.D., Dean's Representative

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Chapter 1: Background and Specific Aims

Background

An estimated 122 million Americans live with cardiovascular disease (CVD), which is also the leading global cause of death (517). Chronic physical inactivity is an important modifiable cardiovascular risk factor that promotes CVD development, while regular physical activity is well-established to maintain cardiovascular health and decrease CVD risk by up to 40% (346, 459). Endurance exercise training further lowers the risk for CVD, as increases in cardiorespiratory fitness are associated with even greater protection (542). Exercise training reduces CVD risk partially via beneficial effects on traditional cardiovascular risk factors including lowering blood pressure (BP), circulating glucose/glycosylated hemoglobin (HbA1c), and triglycerides (157, 346). However, the major protective effects of exercise training are thought to be due to the direct arterial adaptations that it elicits, including enhanced endothelial vasodilator function, increased arterial diameter, and decreased arterial stiffness (19, 182, 183, 252, 451). Conversely, sedentary behavior leads to the reverse, detrimental impairments in these measures of vascular health (6, 54, 182, 381).

The mechanisms underlying the vascular adaptations to exercise training are not completely understood. Vascular adaptations are largely mediated by the accumulated effects of the regular increases in laminar shear stress due to increased blood flow to active tissues during each bout of acute exercise (182). Yet, adaptations also occur in vessels that do not experience substantial increases in shear stress during exercise, potentially due to the paracrine/endocrine effects of circulating blood-borne

factors (157, 158, 382). Numerous molecules that are released into circulation by various cell-types throughout the body in response to exercise have been proposed as exercise factors that may mediate responses to exercise and adaptations to training (157, 158).

Recently, microRNAs (miRs) have been implicated as one such novel class of molecules important in maintaining cardiovascular health and mediating adaptations to exercise training (115, 295, 434, 436, 546). MiRs are short, non-coding RNAs that act as post-transcriptional regulators of gene expression primarily by inhibiting translation or causing the degradation of mRNA (34, 152, 360). Circulating miRs (ci-miRs) released from cells into the bloodstream are proposed as novel biomarkers and potential mediators of cardiovascular exercise responses and training adaptations, as well as all types of CVD (249, 369, 434, 436). Several miRs that regulate critical pathways within vascular cells exhibit altered circulating concentrations in response to acute and chronic exercise, suggesting potential roles in arterial adaptations (434, 436). The expression levels of ci-miRs related to vascular function are also altered in the presence of traditional and non-traditional CVD risk factors, such as overweight/obesity, hypertension, hyperglycemia and diabetes, dyslipidemia, aging, smoking, and insufficient sleep (55, 60, 214–216, 242, 487, 511). Identifying associations between specific ci-miRs and vascular function in at-risk individuals before the development of CVD could reveal important biomarkers and targets for prevention.

Rates of CVD within the population vary based on demographics such as sex and race (517). Premenopausal women exhibit lower rates of CVD as compared with age-matched men, which is largely attributed to differences in sex hormones (46, 473,

517). Primarily, estrogen exerts beneficial effects on vascular health in women by increasing nitric oxide bioavailability and decreasing oxidative stress and inflammation in the endothelium (46, 473). Indeed, men exhibit declines in endothelial function at earlier ages than women, which is important considering endothelial dysfunction is a crucial precursor to atherosclerotic CVD (73, 466, 508). However, sex differences in vascular health are not thought to solely result from the direct effects of estrogen and other sex hormones. For example, the concentrations of several circulating proteins and miRs with roles in cardiovascular physiology differ between young men and women (12, 28, 98, 281). Thus, the mechanisms underlying sex disparities in vascular function and CVD development remain to be fully elucidated. Identifying the contributions of specific factors to sex differences in vascular health is of vital importance for developing strategies to combat CVD development in both men and women. As mentioned, exercise training induces beneficial arterial adaptations and can therefore reveal mechanisms important to vascular health. It is unclear if exercise training differently affects circulating factors in men and women, potentially contributing to sex differences in the mechanisms underlying vascular function.

Racial disparities in CVD development also exist. Specifically, African Americans (AA) experience greater rates of CVD as compared with most other ethnic groups, also developing CVD at younger ages and demonstrating higher mortality rates (148, 517). Even young, otherwise healthy AA often have lower endothelial function (66, 135, 209, 257, 379, 392, 408, 449, 474) and higher arterial stiffness (60, 207, 209) as compared with their Caucasian American (CA) counterparts. Recent research suggests that differences in epigenetic mechanisms, such as miR, stemming from the

influence of socio-cultural-environmental factors on past and present generations may underlie racial differences in CVD risk (272, 332, 515). Differences in ci-miR expression are apparent between older AA and CA patients with diseases such as hypertension (129), though it is unclear if there are racial differences in ci-miR among young, healthy individuals that may be related to disparities in vascular function.

Furthermore, AA have been shown to exhibit exaggerated or impaired vascular responses to stressful stimuli, even when impairments are not apparent at rest (65, 68, 357, 441, 475, 522). Identifying these responses and the underlying mechanisms is crucial, as they may contribute to the early development of CVD in AA. In this respect, acute exercise is a stimulus that can reveal mechanisms underlying impairments in cardiovascular function. Acute exercise represents a challenge to the cardiovascular system, disturbing vascular homeostasis in an intensity-dependent manner via increases in blood flow, reactive oxygen species (ROS), inflammatory factors, and sympathetic nervous system activity (10, 22, 178, 248, 328). Therefore, the ability of the vasculature to respond appropriately to the stress of acute exercise is a sensitive measure of cardiovascular health that can reveal impairments not evident at rest.

In the studies discussed herein, acute exercise was used as a tool to elicit cardiovascular stress and exercise trained individuals were used as models of superior cardiovascular health. Young, healthy individuals were studied in order to identify differences preceding CVD initiation, making our findings relevant to the development of early preventative strategies. **Thus, the overall goals of my dissertation work were to 1) determine sex- and race-based differences in the vascular responses to chronic and acute exercise, respectively, 2) identify ci-miR potentially involved in**

these divergent vascular responses, and 3) examine a potential mechanism responsible for exercise-induced changes in endothelial function and ci-miRs. My initial investigation focused on identifying associations between sex and habitual activity level with the effects of circulating blood-borne factors on cultured endothelial cells. My second study sought to determine racial differences in the effects of acute exercise on endothelial function and arterial stiffness by comparing the exercise responses of AA and CA individuals. Subsequently, I determined sex- and race-based differences in the circulating levels of miRs related to vascular function in order to determine their potential associations with the responses to acute and chronic exercise in the above studies. My final investigation examined the potential role of endothelial integral damage in mediating the acute exercise-induced changes in endothelial function and ci-miR release into circulation.

Specific Aim #1

To determine sex- and habitual activity level-based differences in the effects of serum on endothelial cell function.

The purpose of this study was to identify differences in the entire milieu of circulating blood-borne factors due to both sex and habitual activity status. Blood was drawn from habitually inactive (sedentary) and highly active (endurance-trained) men and women. Differences in circulating factors were then determined by observing the effects of serum on cultured endothelial cells. We hypothesized that endothelial cells would respond better to serum from endurance-trained individuals as compared with serum from sedentary individuals, and to serum from women compared to men in both groups.

Our results reveal sex-dependent associations between habitual activity level and the effects of serum on endothelial cells. Specifically, the migration of endothelial cells in an *in vitro* "wound closure assay" was significantly higher over 24 hours in response to serum of endurance-trained versus sedentary women. In men however, there was no difference in serum-induced endothelial migration due to habitual activity level suggesting the observed effects of habitual endurance exercise on circulating factors are specific to women. Endothelial migration is an important component of angiogenesis and endothelial wound repair, processes important to the maintenance of adequate blood flow and vascular function (97, 116, 233, 274, 287). Our results, therefore, suggest that in women, endurance exercise training alters circulating factors in a manner that may improve these endothelial cell functions.

Additionally, endothelial ROS production was higher in response to serum of sedentary men as compared with serum of their trained male or sedentary female counterparts. In excess, ROS production is detrimental to endothelial function as it decreases NO bioavailability (230). Oxidative stress represents a state of ROS production that exceeds the rate of removal, which chronically is a major mechanism underlying endothelial dysfunction and CVD development (79, 230). Again, the effects that we observed were sex-dependent, as no difference was apparent between trained and sedentary women. Thus, specifically in men, sedentary behavior is associated with a signature of circulating factors that increases ROS production, which could contribute to endothelial dysfunction.

Importantly, all participants in this study were young and healthy, in that they were devoid of traditional risk factors. Women were tested during the early follicular

period of the menstrual cycle and there were no differences between groups in circulating estradiol levels. Thus, our results are likely due to differences in other circulating factors such as cardiovascular-related proteins or ci-miRs. The results of this study provide support for the interrogation of specific circulating factors that may underlie sex disparities in the development of vascular dysfunction and CVD. Additionally, this study highlights the importance of regular exercise on cardiovascular health even in young, healthy individuals, as the effects of serum from sedentary individuals in our study could translate to reduced endothelial function, repair, and angiogenesis due to sedentary behavior.

Specific Aim #2

To evaluate the effects of acute exercise on endothelial function and arterial stiffness in young African American individuals in comparison to matched Caucasian American individuals.

In this study young, healthy AA and CA individuals completed 30 minute bouts of moderate intensity continuous (MOD) and high intensity interval (HII) cycling exercise. Before, 10, and 60 minutes after exercise, endothelial function was measured via flow-mediated dilation (FMD) of the brachial artery and arterial stiffness was measured centrally via carotid to femoral pulse wave velocity (cf-PWV) and locally at the carotid artery (carotid β-stiffness and compliance). Additionally, augmentation index was assessed (normalized to a heart rate of 75 bpm [AIx75]), which gives an indication of afterload imposed on the left ventricle due to wave reflection and can change independently of arterial stiffness. Considering AA often exhibit impaired

endothelial function (66, 135, 209, 257, 379, 392, 408, 449, 474) and reduced vasodilation in response to stimuli (68, 475), we hypothesized that AA would display exaggerated and prolonged reductions in FMD following acute exercise as compared with CA, and that this difference would be greater following HII. Additionally, AA have previously exhibited increases in aortic stiffness following acute bouts of exercise, while CA did not (207, 556, 557). Thus, we hypothesized that arterial stiffness and AIx75 would be increased to a greater degree after exercise in AA as compared with CA.

Contrary to our hypotheses, FMD was not different between the racial groups at baseline and was not altered in response to either exercise bout. Central arterial stiffness increased in both AA and CA individuals 10 minutes after HII exercise, while AIx75 increased only in CA after MOD exercise. Thus, our findings do not support aberrant exercise-induced changes in conduit artery endothelial function or central arterial stiffness in AA. Further, CA exhibited greater post-exercise brachial BP and exercise-induced increases in aortic BP that were not seen in AA. These findings were also unexpected, as prior research has shown AA to display exaggerated increases in BP following physiological or psychological stress, potentially due to greater sympathetic activity and/or sensitivity (65, 357, 475, 522). The discrepancies between our results and those of previous studies demonstrate the need for additional, larger scale investigations aimed at not only determining racial differences in responses of the vasculature to stress, but the factors potentially mediating these differences.

Sixty minutes after HII exercise, we observed a beneficial increase in carotid arterial compliance (the inverse measure of stiffness) of the carotid artery within CA

that was not apparent in AA. The increase in compliance specifically in CA during the recovery from exercise does not appear to be related to changes in BP. Thus, our study reveals that AA lack a potentially beneficial recovery response of large, elastic arterial compliance. The mechanisms underlying these responses are of interest for future studies to determine as they could be related to racial disparities in the effects of exercise training and/or the development of CVD.

Specific Aim #3a

To examine the associations between habitual activity level and sex with concentrations of circulating microRNAs related to cardiovascular health.

Our study in Specific Aim #1 demonstrated differences in serum's effects on endothelial cell functions due to sex and habitual activity level. In this study we focused on ci-miRs as factors potentially contributing to those differences by assessing the levels of vascular-related ci-miRs in the serum of the same endurance-trained and sedentary men and women studied in Aim #1. Previous studies have shown that ci-miRs differ between men and women across varying ages (12, 28, 98) and that exercise training of up to six months alters ci-miR concentrations in a manner that may contribute to beneficial cardiovascular adaptations (33, 434, 436). This study is novel in that we compared ci-miRs in young, healthy individuals who differed in their long-term habitual exercise habits, thus informing on the potential effects of chronic endurance exercise training versus sedentary behavior. To our knowledge, this study is also the first to determine sex-based differences in the association between habitual exercise and ci-miRs. Correlational analyses were additionally performed in order to

determine any sex-specific associations between cardiometabolic factors, endothelial cell functions, and ci-miRs.

We first identified five ci-miRs for further analysis from an initial array of miRs relevant to CVD. Quantifying the levels of those five ci-miRs in addition to five other ci-miRs chosen for investigation *a priori* revealed differences based on sex and habitual activity level. Primarily, there were sex effects on all nine of the ten ci-miRs that were abundant enough for quantification, with greater levels of all in men. These results add to the literature supporting sex differences in circulating factors important to cardiovascular physiology and provide support for ci-miRs as potential mediators of sex disparities in the development of vascular dysfunction and CVD risk. Additionally, there were effects of activity level on two of the ci-miRs, namely ci-miRs- 140-5p and 145-5p. The higher expression of these two ci-miRs in sedentary individuals could reasonably be related to the negative effects of sedentary behavior on vascular health. Ci-miR-145-5p was specifically elevated in sedentary men compared to trained men and sedentary women, revealing a sex-specific association with habitual activity level. Thus, we identified potential biomarkers for future CVD risk in sedentary young men, also with potential mechanistic roles in the development of vascular dysfunction, both of which should be validated in future studies.

Sex-specific correlations between ci-miRs and serum-induced endothelial cell functions were also observed. Several of the ci-miRs investigated were associated with serum-induced endothelial proliferation and ROS production in women, but not men. These findings suggest ci-miRs may be more related to endothelial function in young women compared to men. Additionally, different cardiometabolic factors were related

to ci-miR concentrations in men and women. Determining the directionality of these correlations could be informative in elucidating either sex-specific roles of ci-miRs, or factors regulating ci-miR concentrations.

Specific Aim #3b

To examine racial differences in resting and exercise-induced concentrations of circulating microRNAs with regulatory roles in vascular function.

The purpose of this study was to determine racial differences in ci-miRs within those individuals studied in Aim #2. Blood samples were obtained immediately before and after exercise to determine differences in resting and exercise-induced changes in ci-miR concentrations. We hypothesized that ci-miRs related to vascular inflammation, ROS production, and eNOS expression/activity would differ at rest and would also exhibit different responses to exercise in young, healthy AA as compared with CA individuals. Further, we performed correlational analyses to test the hypothesis that exercise-induced changes in ci-miR would be related to the race-specific changes in arterial stiffness observed in Aim #2, suggesting a potential role of ci-miRs in the vascular responses to exercise.

We found no racial differences in the resting concentrations of select ci-miRs related to vascular function. While ci-miR-150-5p increased significantly in both AA and CA following exercise (both MOD and HII), CA also exhibited significant increases in five other ci-miRs following MOD and/or HII exercise. The ci-miRs that were altered with exercise have been shown to exert primarily anti-inflammatory effects within the vasculature. Therefore, changes in anti-inflammatory ci-miR may be

blunted in AA as compared with CA, which could have implications for vascular responses to exercise and adaptations to training. When the fold changes in ci-miRs were compared between AA and CA there were no significant differences, so additional studies are needed to determine whether our findings may simply reflect our relatively small sample sizes.

In addition, changes in the anti-inflammatory ci-miR-150-5p were correlated with the changes in carotid arterial compliance specifically in CA 10 minutes after HII exercise, while this association approached significance specifically in AA 60 minutes after HII exercise. Therefore, we speculate that the failure of carotid arterial compliance to increase in AA during the recovery from HII exercise may be related to inflammation and related ci-miRs. Future studies should determine whether the lack of an increase in anti-inflammatory ci-miRs within AA could represent a mechanism that contributes to elevated CVD risk and/or impaired exercise training-induced adaptations to large arterial compliance

Specific Aim #4

To determine whether acute exercise alters circulating endothelial-derived factors reflecting disrupted endothelial integrity, and whether this may act as a mechanism of endothelial-derived circulating microRNA release with exercise.

The purpose of this study was to investigate endothelial damage as a potential mechanism underlying the changes in both endothelial vasodilatory function and miR release into circulation in response to acute exercise. Changes in several vascular stimuli have been proposed to underlie the transient exercise-induced changes in

endothelial function, however the responsible mechanisms are still unclear. It is important to determine the mechanisms responsible, as they likely contribute to exercise training-induced vascular adaptations via a hormetic response to repeated bouts of acute exercise. One proposed mechanism is that increases in vascular stimuli elicit integral damage to the endothelium, which would disturb NO signaling. For example, FMD of the radial artery is reduced following catheterization (111, 335). Considering such overt endothelial damage results in reduced FMD for weeks to months, while FMD recovers within the hour(s) after exercise, we hypothesized that endothelial damage does not occur with acute exercise (110, 111, 335, 433).

In addition, exercise results in increases in the circulating concentrations of miRs that are found specifically within endothelial cells in high abundance, strongly suggesting endothelial cells release miRs during exercise (436, 444). However, it is currently not clear whether endothelial cells actively release miR into circulation in response to exercise-induced stimuli, or whether miRs passively leak out of damaged/dying cells. *In vitro* experiments suggest endothelial cells respond to stimuli such as laminar shear stress and inflammation by selectively secreting specific miRs (9, 193, 212, 444, 569). Therefore, we hypothesized that the exercise-induced increase in ci-miRs does not result from passive leakage of miRs from the endothelium.

In order to determine whether acute exercise elicits integral damage to the endothelium we quantified the concentrations of circulating markers of endothelial integrity before and after acute exercise. To test our hypotheses, we also quantified changes in FMD and ci-miRs in order to determine their associations with changes in markers of endothelial damage. The same bouts of MOD and HII exercise used in Aims

#2 and #3b were used here for the determination of intensity-dependent effects, considering the vascular stimuli potentially responsible for exercise-induced endothelial damage exhibit intensity-dependent changes.

The changes in circulating factors that we observed suggest that endothelial denudation and apoptosis do not occur, while slight disruption to the endothelial membrane and glycocalyx may occur in response to both MOD and HII exercise. On average, FMD was unchanged following either exercise bout, suggesting endothelial function at least in the upper limb vasculature was not affected. FMD responses to exercise have been reported to vary based on individual characteristics. Individual changes in endothelial function to MOD and/or HII in our study were associated with changes in markers of endothelial activation, glycocalyx integrity, and ci-miR-126-3p. These mechanisms may potentially contribute to individual responses of endothelial function to exercise, which should be followed up in future investigations. Lastly, miRs- 126-3p and 126-5p, which are highly enriched in endothelial cells, were increased in circulation only following HII exercise. Therefore, the increased release of endothelial miRs is likely in response to some intensity-dependent vascular stimuli during exercise as opposed to cell damage/death.

Chapter 2: Literature Review

The purpose of this literature review is to summarize the vascular responses to acute exercise and adaptations to exercise training, with specific focus on endothelial function, arterial stiffness, and cardiovascular disease risk. Sex and race differences in vascular function, both at rest and in response to exercise, and the relevance to cardiovascular disease risk are also discussed. Lastly, circulating microRNAs are introduced and discussed in the context of the topics above. Chapters 3 and 4 provide a review of the current knowledge regarding circulating microRNAs in exercise physiology.

Cardiovascular Disease

Diseases of the heart and blood vessels, cumulatively referred to as cardiovascular disease (CVD), affect approximately half of all individuals in the U.S. (517). Heart disease, stroke, and hypertension are among the primary causes of disability in the U.S., and it's estimated that the yearly costs associated with CVD exceed $350 billion (517). Globally, CVD is the primary cause of death (517). Atherosclerosis is a disease involving the progressive development of lesions within the inner arterial walls that is the underlying cause of several other forms of CVD, such as coronary artery disease (CAD), peripheral arterial disease (PAD), and chronic kidney disease, as well as the majority of clinical cardiovascular events (e.g. stroke and myocardial infarction) (310, 420). Atherogenesis is a silent process, often with no apparent signs or symptoms until it manifests as a potentially life-threatening acute

event (7, 310, 420). Clearly, it is of utmost importance to develop preventative strategies aimed at combatting the development of atherosclerosis and CVD.

Endothelial Function and Dysfunction

The endothelium consists of a single layer of cells which line the luminal side of all blood vessels. Once thought to act solely as a passive barrier for the diffusion of molecules, the endothelium is now recognized as a critical regulator of vascular health and pathology. The location of the endothelium adjacent to both the circulating blood and vascular smooth muscle cells allows it to act as a sensor of mechanical (i.e. pressure and shear stress) and chemical stimuli in order to maintain normal arterial function. Endothelial cells regulate processes including vasomotion, hemostatic and fluid balance, vascular permeability, vessel wall hypertrophy, immune function, inflammation, and the formation of new vessels (138, 139). The regulation of vascular tone is a hallmark function of the endothelium, achieved via the release of myriad vasoactive substances to the underlying smooth muscle cells. Although endothelial cells release numerous molecules that promote either arterial dilation or constriction, nitric oxide (NO) is of central importance (211, 510). Endothelial function is assessed as the degree of endothelial-dependent dilation, primarily mediated by NO release, in response to stimuli such as increased laminar shear stress or acetylcholine (179). Crucially, NO dictates vascular health not only via its ability to induce vasodilation, but by mediating most of the other anti-atherogenic effects of the endothelium listed above, as well as protecting against oxidative stress, leukocyte infiltration, and excessive release of vasoconstrictors (139, 510). Therefore, assessing endothelial

function gives an indication of NO bioavailability and, by extension, overall vascular health.

Under healthy conditions, endothelial cells are in a quiescent state and exert protective effects against atherogenesis (116). Repeated/prolonged exposure to CVD risk factors and pro-atherogenic stimuli alter the endothelial cell phenotype resulting in a state of endothelial activation, whereby the normal homeostatic functions of the endothelium are lost (8, 116, 518). Specifically, CVD risk factors increase reactive oxygen species (ROS) production and inflammation, which decrease NO bioavailability both directly and indirectly (188, 230, 476). The ensuing state of endothelial dysfunction, underlined by a chronic state of intimately linked oxidative stress and inflammation, promotes atherogenesis (188, 230, 476). Endothelial dysfunction is both the key initiating event preceding atherosclerotic CVD development and a contributing process in later lesion progression (508). Impaired endothelial function has been observed in patients with hypertension, CAD, heart failure, renal failure, diabetes (types 1 and 2), and chronic inflammatory conditions (8, 116, 139, 510, 514). Endothelial-dependent dilation of coronary arteries is a strong predictor of future cardiovascular events in subjects with and without CAD (325, 440, 483, 514). Importantly, endothelial function of the peripheral arteries, the common, less invasive site of measurement, also predicts cardiovascular events in patients with vascular diseases (139, 325).

In individuals without overt CVD, endothelial function is inversely correlated with the number of traditional CVD risk factors present (73, 519), and when the risk factors are treated, endothelial function is restored (252). Additionally, endothelial

dysfunction is apparent in the presence of other "non-traditional" CVD risk factors such as overweight/obesity, physical inactivity, and insufficient sleep (26, 122, 134, 510). Thus, it has been proposed that endothelial function may serve as a barometer of CVD risk, since endothelial dysfunction is involved both in the initiating step and later stages of atherosclerotic lesion progression (509, 518). Measurement of endothelial function is increasingly being used to monitor CVD risk, as well as the efficacy of interventions aimed at preventing further development of CVD or acute cardiovascular events (8, 116, 514).

Arterial Stiffness

In addition to endothelial function, arterial stiffness is another indicator of vascular health and future CVD risk. A crucial role of the large arteries is to dampen the pressure waveform resulting from cardiac systole as it travels down the arterial tree and as it is reflected back to the heart (548). The stiffness of an artery refers to its ability to expand and contract in response to such changes in pressure. Under healthy conditions the large central arteries (i.e. the aorta and carotid artery) are elastic, allowing them to distend to accommodate the increased blood flow and pressure resulting from cardiac systole. Stiffer arteries exhibit reduced compliance/distensibility, meaning for any change in pressure there is a lower change in volume (72). A result of reduced arterial distensibility is an increase in the speed of the pressure waveform, which can be measured between two points on the arterial tree as pulse wave velocity (PWV) (72). While increased stiffening of the central arteries occurs naturally with aging, it is exacerbated in the presence of CVD risk factors and

carries several important consequences for cardiovascular health (268, 283, 336, 428). Obesity, smoking, high salt intake, hypercholesteremia, sedentary behavior, and metabolic syndrome are all associated with greater arterial stiffness (283, 428). Elevated arterial stiffness eventually manifests as systolic hypertension and is apparent in patients with essentially all types of CVD (87, 283, 460).

Vascular ventricular coupling refers to the ability of the large elastic arteries to smoothen the pulsatile pattern of blood flow from the heart before it reaches the smaller blood vessels (460). With increased arterial stiffness, vascular ventricular coupling is altered such that large arteries lose their ability to dampen the pulsatile pressure, which can then penetrate and damage the subsequent smaller arterioles, capillaries, and target organs (87, 460). As the artery directly receiving blood from the heart, the aorta plays a substantial role in dampening the pulse pressure and pulsatile nature of blood flow. Importantly, the aorta exhibits the greatest propensity for stiffening, and is therefore a common site of measurement that is also the most clinically relevant (283). PWV from the carotid to the femoral artery (cf-PWV), covering the length of the aorta, is the gold standard measurement of central arterial stiffness (540). A meta-analysis including data from almost 16,000 individuals with and without CVD found that with each 1 m/s increase in aortic PWV there were 14-15% increased relative risks of cardiovascular events and mortality, as well as all-cause mortality (520). Having an aortic PWV that was one standard deviation above the average conferred 42-47% increased relative risks (520). Importantly, the prognostic ability of arterial stiffness remains after adjustment for classic cardiovascular risk factors, illustrating its value as an independent predictor (53, 283).

Another consequence of greater arterial stiffness is an increased speed/intensity of wave reflection back to the heart. As the pulse wave from cardiac systole propagates down the arterial tree, it meets points of physical impedance (i.e. impedance mismatching due to arterial branching points and more muscular resistance arteries) and returns back to the heart as a reflected wave (460). The return of the pressure waveform normally contributes to afterload during systole and coronary perfusion pressure during diastole (548). A decrease in large arterial compliance causes earlier return of the reflected wave which can merge with the systolic wave of the following heartbeat, thereby increasing cardiac work and decreasing oxygen delivery. This results in ventricular pathological remodeling and eventually heart failure (87, 548). The impact of the reflected wave can be determined by measuring augmentation index (AIx), specifically defined as the difference between the first and second systolic peaks of the arterial pressure waveform relative to the pulse pressure (283, 576). While AIx is influenced by PWV, it is also affected by changes in heart rate (HR) and reflection sites, and is therefore not strictly a measure of arterial stiffness (428). AIx increases with age, however unlike PWV which increases nearly linearly, AIx tends to level off in older individuals (336, 428). Additionally, AIx has demonstrated predictive ability that is similar to or greater than that of pulse pressure (291). Especially in younger to middle-aged individuals, AIx is an independent predictor of CVD (262, 535). Furthermore, a 10% increase in AIx is associated with 18-19% increased risks of cardiovascular events and all-cause mortality (291).

Lastly, local arterial stiffness can be measured by observing the relationship between changes in volume and pressure at specific regions (283). Specifically, the

carotid and femoral arteries are common sites of atherosclerosis. Considering arterial stiffness may contribute to atherogenesis and vice versa, assessing these sites could give an indication of atherosclerotic progress and risk (72, 283, 507). Stiffness of the carotid artery is also of interest considering the brain is susceptible to damage from elevated pulsatile pressure, which can contribute to cognitive decline (354, 394, 428).

Given the clear clinical importance of arterial stiffness, therapeutic strategies have been developed to reverse or mitigate changes in the underlying cellular/molecular mechanisms. Arterial stiffness of the large arteries is primarily determined by the relative proportions of load-bearing extracellular matrix proteins in the tunica media. An increased degradation of elastin fibers and production of stiffer collagen fibers contributes to reduced arterial compliance (384, 394). Other processes that interact along with changes to the elastin/collagen ratio to increase arterial stiffness include vascular calcification, inflammation, smooth muscle cell stiffening, and endothelial dysfunction (87, 384, 394). Aerobic exercise training, nutritional, and pharmacological interventions have all proven effective in decreasing or mitigating age-related increases in arterial stiffness (548, 574).

Acute and Chronic Exercise

Role of Regular Exercise in Protection from CVD

Physical inactivity is a major risk factor for CVD development, and sedentary behavior time is positively associated with the risks for CVD and all-cause mortality (353, 517). Conversely, regular physical activity protects against CVD development, conferring a 40% reduction in risk among the most active individuals (346, 459).

Increases in cardiorespiratory fitness are associated with even greater protection, illustrating the health benefits of exercise training (542). In this sense, aerobic, endurance type activities cause superior cardiovascular adaptations as opposed to strength training (225). Aerobic exercise training-induced structural and functional changes to the heart, vasculature, and blood combine to improve functional capacity and endurance. Additionally, exercise training-based rehabilitation is an effective therapy to improve symptoms, enhance quality of life, and reduce mortality rates in patients with all types of CVD (499, 517).

Vascular Adaptations to Exercise Training

Classically, exercise training protects against CVD via its beneficial effects on traditional risk factors, though at least 40% of the reduction in risk is not explained by changes in either established or novel risk factors (180, 182, 382). Current research suggests that the direct conditioning effects of exercise training on the vasculature convey a major portion of this additional protective effect (180, 182, 382). Physiological adaptations of the large arteries to endurance exercise training include improved endothelial function (20, 194), increased compliance (342), arterial enlargement (57, 338, 443), and reduced wall thickness (421, 422, 498). The overall effect of these adaptations is to increase vascular conductance, which accommodates enhanced cardiac output and allows for greater skeletal muscle blood flow.

There is a positive relationship between the intensity of exercise training and improvement in endothelial-dependent dilation as measured by FMD (20). Importantly, exercise training is able to reverse endothelial dysfunction in young and older adults,

including those with CVD risk factors (122, 182). Even larger improvements in FMD are seen in individuals with cardio-metabolic diseases (182). Contrary to the conduit arteries, exercise training does not appear to increase endothelial function of the resistance arteries in young, healthy individuals (182). In those with impaired resistance artery function however, such as those with CVD risk factors and disease, exercise training may improve resistance artery endothelial function via increased NO bioavailability (182). Changes in endothelial-independent dilation are usually not observed after training, suggesting no adaptations in smooth muscle vasodilatory function (364).

Changes in hemodynamic stimuli during acute exercise, specifically laminar shear stress, mediate much of the vascular adaptations to training (182). The increases in shear stress during exercise have been shown to be necessary for both functional and structural adaptations of the vasculature to training (182). This was illustrated in a study by Tinken and colleagues, who had subjects perform bilateral handgrip exercise training with a cuff applied to one arm in order to prevent increases in brachial artery shear stress (501). After two and four weeks of training, the brachial artery of the un-cuffed arm exhibited increased FMD, while the cuffed arm did not. Both arms showed increases in forearm size and grip-strength, suggesting equivalent muscular adaptations. Improved endothelial function with training is largely due to increased endothelial NO production resulting from elevated eNOS expression, phosphorylation, and stabilization (20, 167, 182, 194). Concurrently, a greater expression of antioxidant proteins and reduced expression of pro-oxidant proteins serves to increase NO bioavailability by decreasing ROS (20, 167, 182, 194). Other stimuli proposed to play a role in training-induced vascular adaptations include a reduction in autonomic tone (180, 252) and beneficial alterations in other vasoactive molecules such as prostacyclin, endothelin-1, and angiotensin II (182). These changes likely do not play a

role in adaptations in healthy individuals, but are important in those with CVD risk factors or disease in whom imbalances in such pathways may mediate endothelial dysfunction (182).

The above study by Tinken and colleagues further showed that peak reactive hyperemic blood flow was increased after four to eight weeks of bi-lateral handgrip training only in the un-cuffed arm, revealing structural changes in the resistance arteries that were not observed in the cuffed arm (501). That study also suggested a now generally accepted time-course of conduit artery adaptations. Functional changes in endothelial-dependent dilation occur in the first few weeks of training, after which structural changes (i.e. increased diameter) occur, presumably as a means to "permanently" normalize exercise-induced increases in shear stress. Indeed, exercise training increases conduit artery lumen diameter and endurance-trained individuals exhibit larger arteries (e.g. aorta, carotid, coronary, peripheral) than non-trained individuals (182). As a result, mean and peak resting and exercise-induced shear levels are similar before and after training, as well as between endurance trained and sedentary individuals, despite higher blood flow in the trained state (338, 443). As a result of increased arterial diameter, endothelial-dependent dilation also returns back towards baseline.

Structural adaptations in peripheral arteries are also seen only in the physically active limbs after exercise training. Single leg or arm training results in increased conduit artery size only in the trained limb (339, 575). Experiments in animals have illustrated the necessity of endothelial-derived NO in structural adaptations by showing that enhanced arterial diameter induced by increases in blood flow could be abolished by blocking NO (182). As mentioned previously, blocking shear stress in humans using a cuff also prevents structural adaptions (501). Interventions utilizing different stimuli to increase shear stress

including leg cycling and heating of the limbs have resulted in similar vascular adaptations to exercise training (41, 69, 182, 362, 501). Thus, endothelial shear stress mediates structural remodeling of conduit arteries with exercise. Additionally, a decrease in conduit artery wall thickness occurs with training, although this is not mediated by shear stress (182, 498). Brachial artery wall thickness decreased similarly in both arms after bilateral handgrip training with a cuff applied to one arm (498). Further, various athletes do not exhibit specific adaptations to arterial wall thickness in the limb(s) involved in their sport (421, 422).

As with conduit arteries, resistance arteries exhibit structural adaptations only in the active limbs and this is dependent on increases in shear stress (182, 501). Tennis players have higher peak reactive hyperemic blood flow in the trained arm compared to the non-dominant arm (182, 463). After bilateral handgrip training, an increase in peak blood flow was seen in the un-cuffed arm, but not the cuffed arm (501). However, similar experiments with cycling training or repeated leg heating that caused increased shear stress in the arms did not cause similar adaptations, suggesting structural adaptations to the resistance arteries are also matched to skeletal muscle metabolic work (41, 362).

The age-related increases in aortic and carotid arterial stiffness are attenuated in individuals who regularly exercise (451). It is hypothesized that the maintenance of arterial compliance may be due to the protective effects of aerobic exercise training against the aging-associated increases in oxidative stress and inflammation (451). Additionally, aerobic exercise training interventions result in reductions in both PWV and AIx (19). Hasegawa et al. showed reductions in central/aortic PWV in both rodents and humans following exercise training interventions consisting of either moderate continuous or high intensity interval aerobic exercise (199). The change in aortic stiffness correlated with an

increase in eNOS phosphorylation in rodents, while humans also exhibited increases in plasma nitrite/nitrate levels (199). Those results suggest decreased arterial stiffness may be related to increased NO bioavailability following exercise training. A significantly greater decrease in stiffness is usually observed in peripheral arterial stiffness measured by brachial to ankle PWV, as compared to aortic stiffness measured by cf-PWV (19). This could be due to the increase in NO-mediated endothelial function that occurs in peripheral arteries with training, and suggests the more muscular peripheral arteries may be more susceptible to reductions in stiffness as compared to the more elastic central conduit arteries (19, 182). Greater reductions in stiffness also occur with longer durations of training and in those with stiffer arteries at baseline (19). Additionally, decreases in AIx occur with training and are associated with the intensity of exercise, suggesting higher intensity exercise may elicit greater changes in stimuli that are beneficial to lowering AIx (19). The responsible exercise-induced stimuli are unclear, but the reduced AIx may be related to increased compliance of peripheral arteries and decreased HR (19).

Vascular Responses to Acute Exercise

Vascular adaptations to training result from the accumulation of responses to repeated bouts of acute exercise. Each acute exercise bout elicits stress that disturbs homeostasis of the cardiovascular system, causing responses that protect the system from subsequent challenges. Thus, an understanding of the stimuli and cellular/molecular responses to acute exercise can reveal information underlying the CVD protection conferred by training. Both endothelial vasodilator function and arterial stiffness have been measured to gauge vascular responses to acute exercise.

Endothelial Function

A general biphasic response of endothelial function has been proposed to occur in response to acute exercise in young, healthy individuals (110). On average, exercise causes an immediate decrease in FMD that rebounds back to or above baseline values by ~one hour after exercise (110). Studies have also reported divergent responses, likely because the response is modulated by numerous variables such as exercise intensity, duration, mode and individual fitness status (110). Studies have almost all reported no exercise-induced change in endothelial-independent dilation in response to direct NO-donors, suggesting no effect of exercise on the ability of smooth muscle to relax in response to NO (110, 364).

Generally, high intensity exercise (>80% VO_{2max}) results in an immediate post-exercise decrease in FMD, while moderate intensity exercise (50-80% VO_{2max}) results in an attenuated decrease, no change, or even an increase in FMD (110). However, this is not always the case. For example, Siasos et al. found increased FMD immediately after both moderate and high intensity interval cycling (461). Other studies in adolescents and older individuals have found a greater increase in FMD following high intensity interval exercise compared to moderate intensity continuous exercise (48, 234). It is also possible that high intensity interval exercise exerts a different effect than high intensity continuous exercise. A recent study by Shenouda et al. showed no effect of 3 x 20 second all-out cycling bouts on FMD one or 24 hours post-exercise in sedentary men and women (456), although an immediate post-exercise response may have been missed.

Exercise duration also impacts the immediate response of endothelial function. A comparison of FMD responses to running at 80% VO_{2max} for either short (17-22 minutes) or moderate (30 minutes) duration showed that FMD was lower (below baseline) immediately after moderate duration compared to short duration (247). FMD was also lower after 60 minutes than after 30 minutes of exercise at 50% VO_{2max} (247). Thus, increasing the duration of exercise at any intensity may cause greater reductions in immediate post-exercise FMD. The effect of exercise intensity and duration on FMD one hour after exercise is less clear. It has been proposed that exercise "dose" based on caloric expenditure is more important than either intensity or duration (247), although this has also been refuted (227). Still, the effects of exercise lasting greater than one hour on endothelial and smooth muscle function are not well studied so the impact of exercise duration is not completely understood.

The response of microvascular function to acute exercise is not as well studied as that of macrovascular function. One study in adolescents found that both peak and total reactive hyperemia increased immediately and remained elevated up to two hours following both moderate intensity and high intensity interval cycling exercise (48). The increase was greater following the high intensity bout than the moderate intensity bout at all post-exercise time points (48). This differed from the FMD responses, which showed the classic biphasic response after high intensity interval exercise and no changes after the moderate intensity bout (48). Thus, microvascular responses to acute exercise may be unique from that of the larger arteries.

There are several potential mechanisms that have been proposed to mediate the effects of exercise on endothelial function. Exercise can induce oxidative stress via

increases in the generation of ROS (202, 259). The reduction of bioavailable NO due to the quenching effects of ROS would lead to reduced endothelial-dependent dilation. Additionally, ROS can uncouple eNOS, contributing to further production of superoxide. Higher intensities and longer durations of exercise are associated with greater production of ROS, which could explain the findings of greater reductions in endothelial function as compared to lower intensity exercise (45, 247). This could also explain the finding that exercise-trained individuals often exhibit attenuated reductions in FMD following exercise as compared to untrained subjects. For example, following a maximal treadmill test, FMD was reduced in sedentary individuals but remained unchanged in regular exercisers (227). Exercise training increases antioxidant capacity by upregulating the expression of antioxidants in response to the transient increases in ROS with acute exercise bouts (20, 167, 170, 182, 194). Recent research also suggests that transient increases in blood pressure (BP) may cause resistance-trained individuals to switch from NO to H_2O_2-mediated vasodilation during/after exercise (418). It is not yet clear whether this adaptation occurs in endurance-trained individuals.

Due to practicality issues with the technique, FMD is usually assessed in the brachial artery in studies of exercise. While antegrade shear increases in the vessels supplying active skeletal muscle during exercise, retrograde shear usually increases in the inactive limbs, which may normalize as exercise progresses due to vasodilation of microvasculature in the skin for thermoregulatory purposes (182). Retrograde shear is associated with increased endothelial ROS production, reduced NO, and reduced endothelial function in a dose-dependent manner (307, 448, 497). Considering leg cycling exercise is the modality most often utilized in studies to date, increased

retrograde shear in the brachial artery has been proposed as a potential mechanism explaining post-exercise reductions in FMD (110). In this context, cycling at low intensity and duration with a cuff on the upper arm in order to induce increased retrograde shear prevented a post-exercise increase in FMD, which was seen in the un-cuffed contralateral limb (246, 500). Prior supplementation with an antioxidant (vitamin C) prevented the negative effects of the cuff on FMD, suggesting the increased retrograde shear during exercise may reduce endothelial function via increased ROS production (246).

Other factors that increase dose dependently with exercise and could potentially contribute to acute decreases in endothelial function include inflammatory factors, systolic BP, and sympathetic nervous activity. In a study by Atkinson et al., a 30 min bout of cycling at 75% HR_{max} caused an immediate reduction in FMD (22). When an α-adrenoreceptor blocker was given before exercise to block sympathetic activation, there was actually an increase in post-exercise FMD (22). There were no significant differences in retrograde shear rates during exercise between the placebo and drug condition (22). Thus, sympathetic nervous system activation may impact endothelial-dependent dilation by directly interacting with NO bioavailability as opposed to increasing retrograde shear. It is likely that all of the factors discussed above contribute in part to the transient decrease in endothelial function in response to exercise.

Arterial Stiffness

The effects of acute exercise on arterial stiffness have primarily been investigated within the hour after exercise (359). Arterial stiffness is most affected immediately post-exercise (0-5 min post) after which it gradually returns to or below

baseline levels (359). Many studies that did not include an immediate measure of arterial stiffness reported no changes regardless of the parameter or the arterial segment analyzed (359). Further, differences appear to exist in the responses of the central and upper limb compared to the lower limb arterial segments. In the period immediately after exercise (0-5 min), PWV of central and upper body peripheral arterial segments are increased or not different than baseline values (359). In the >5 min recovery period, there is often a decrease below resting values in central and upper limb PWV, suggesting a potential biphasic response similar to that seen for endothelial-dependent dilation responses (359). Interestingly, the application of a supra-systolic cuff to increase retrograde shear in the brachial artery caused an increase in upper limb PWV coinciding with an associated decrease in FMD (478). Thus, acute changes in arterial stiffness may be at least partially driven by changes in endothelial function and this may explain exercise-induced changes in arterial stiffness within the upper limbs. Conversely, PWV in the lower limbs, which are active during lower body exercise, often decreases immediately after exercise and tends to gradually return to baseline in the first hour of recovery (359).

Changes in arterial stiffness with acute exercise have been shown to be independent of changes in BP and HR, supporting that changes to properties inherent to the vessels mediate changes in stiffness. Indeed, changes in arterial stiffness with exercise are thought to be largely mediated by local changes in vascular tone. This is supported by the fact that exercise exclusively using the upper or lower limbs differentially impacts peripheral arterial stiffness. Ranadive et al. (407) found that after a maximal leg cycling test, PWV was decreased in the legs but not the arms. After a

maximal test on an arm ergometer, PWV was reduced in both the arms and the legs (407). Neither arm nor leg cycling affected central arterial stiffness (407). Additionally, following both single leg resistance and aerobic exercise, PWV is reduced in the exercising but not the non-exercising leg, further suggesting local vasodilation could be related to decreased arterial stiffness (208, 480). It is possible that changes in upper limb arterial stiffness following exercise are due to shifts in load from the more compliant elastin fibers to the stiffer collagen fibers, which could result from the increase in sympathetic-mediated vascular tone during exercise (264, 359). Conversely, in the lower limbs there is increased vasodilation, blood flow, and shear stress which could work in the opposite manner. With a reduction in smooth muscle tone, stress is transferred from collagen to elastin fibers making the vessels less stiff (264). Vasodilation may, therefore, reduce arterial stiffness via a decrease in tension generated by the smooth muscle. Arterial stiffness at rest is regulated by endothelium-derived NO, as infusion of an NO inhibitor or donor into the brachial arterial bloodstream decreased and increased arterial compliance, respectively (265). Conversely, one study reported that the exercise-induced reduction in peripheral arterial stiffness is not dependent on NO (480). Inhibition of NO in the exercising leg did not prevent a reduction in leg PWV with exercise (480). Nonetheless, the decrease in peripheral PWV could still be dependent on vasodilation, since vasodilation would have occurred due to other mechanisms (e.g. prostaglandins).

A reduction in leg PWV has also been observed in response to external compressions via cuff inflation (205). This suggests muscular compression of arteries due to skeletal muscle contractions may act to reduce arterial stiffness by decreasing

transmural pressure (205). Since the vast majority of studies on aerobic exercise have utilized running or cycling, this could contribute to unchanged or increased arterial stiffness in the arms, where there is vasoconstriction and no/very little muscle contraction during such lower body exercises.

The effects of exercise intensity and duration on arterial stiffness are unclear, as studies directly investigating these variables are limited. Exercise of different intensities may similarly affect arterial stiffness considering cf-PWV was unchanged and leg PWV was reduced similarly following both high intensity interval and moderate intensity cycling bouts (461). However, other studies suggest leg PWV may be reduced exclusively or to a greater degree after high intensity interval as compared to moderate intensity continuous cycling (390, 502).

Augmentation index (AIx) has consistently been shown to decrease in response to exercise, though this is likely mediated by an elevated HR (359). Considering this, AIx is generally normalized to a HR of 75 bpm (AIx75) in order to assess changes in the reflected waveform. An increase in AIx75 usually occurs after exercise (359, 393). AIx75 was increased to a greater degree from 5 to 35 minutes after high intensity interval cycling as compared to after moderate intensity continuous cycling exercise, suggesting exercise intensity-dependent differences (196).

Sex Differences in Vascular Function and Risk of CVD

The prevalence of CVD is higher in men than women before the age of ~55-59 years, after which rates are about equal between the sexes (517). The lower risk of CVD among young women is primarily due to the protective effects of the sex hormone

estrogen on vascular health (46). The loss of sex hormones over the menopausal transition largely explains the sudden increased risk of CVD in aging women (46, 473). In support of the protective role of estrogen, younger women who can no longer produce estrogen due to oophorectomy have an increased risk of developing CVD, which is then decreased when estrogen is restored by exogenous administration (416). Estrogen exerts its beneficial effects on the vasculature via both genomic and non-genomic mechanisms (46, 473). Among numerous other actions, estrogen increases NO production/bioavailability and decreases ROS and inflammation through a number of pathways (46, 473). Of course, other sex hormones impact vascular health, such as progesterone and follicle-stimulating hormone in women, and androgens in men (473). However, the roles of these other hormones have not proven to be as important to vascular function in women, and the effects of androgens on the vasculature are still not clear (46, 348, 473).

The protective effects of estrogen are reflected by differences in vascular function between premenopausal women and age-matched men. Endothelial function is lower in young men and starts to decline in the 4th decade of life, whereas in women it starts to decline in the 6th decade of life at a greater rate (73, 466). As mentioned, the enhanced endothelial function in young women is largely thought to result from the pro-vasodilatory effects of estrogen (46, 473). The loss of estrogen during the menopausal transition then causes a decline in endothelial function. Indeed, the endothelial dysfunction observed in postmenopausal women is explained by the resulting reduction in NO bioavailability due to oxidative stress and elevated inflammation (348).

The higher FMD often observed in premenopausal women may also result from a smaller arterial diameter in comparison to men (380). Previous studies have found that when arterial diameter is controlled for, the sex differences in FMD are lost (117, 258). Another recent study controlling for arterial diameter found that young men actually had higher FMDs than women, and suggested that the failure of previous studies to do so may explain the observations of greater endothelial function in women (455). Contradicting evidence exists regarding sex differences in smooth muscle function as measured by endothelial-independent vasodilation. Studies have found higher vasodilation in response to direct NO donors either in men, women, or neither sex (73, 117, 455).

Differences in arterial stiffness between men and women across the lifespan support beneficial effects of female sex hormones. Before puberty, girls have higher central and peripheral PWV and pulse pressure than boys (5). After puberty however, all of these parameters of arterial stiffness are increased only in boys such that sex differences are abolished or reversed (5). Although central PWV is higher in adult men than women, after adjustment for age and BP there are essentially no differences (495). Yet, studies using other methods to assess central arterial stiffness do support higher arterial stiffness in men than women before the menopausal transition. One study that estimated the mechanical properties of the abdominal aorta *in vivo* in men and women of various ages found that from the ages of 25-70 years, aortic stiffness increased at over twice the rate in men as compared to women, and these differences were related to changes in collagen and elastin (21). Aortic distensibility measured using magnetic resonance was also higher in young women, but lower in older women, as compared to

their age-matched male counterparts (363). Interestingly, AIx is higher in women than men across all ages, even after adjustment for differences in height and/or HR (133, 330, 336). The mechanisms underlying the sex differences in AIx are unclear.

Furthermore, a large-scale cross-sectional study found that local arterial distensibility of the femoral artery is greater in women than men across the age spectrum (51). Supporting a potential role of sex hormones, femoral artery distensibility exhibits a steep decline in women after the age of ~60 years, whereas it decreases more gradually in men after the age of ~50 years (51). Mechanistically, estrogen is thought to protect against age-related extracellular matrix remodeling (373). Estrogen deficiency is associated with arterial stiffening in animal models via increased collagen synthesis and decreased degradation (373). The protective effects of estrogen against endothelial dysfunction also play a major role in preventing the age-related increases in arterial stiffness (136, 373).

The measurement of vascular function in women is complicated by potential fluctuations over the menstrual cycle due to cyclical changes in estrogen and the other circulating hormones. Changes in FMD over the different phases of the menstrual cycle have been observed (4, 541), with multiple studies reporting that FMD is lowest in the menstrual/early follicular phase when circulating estrogen is also at its lowest (197, 200, 260). However, several recent investigations have reported no changes in FMD over the menstrual cycle (403, 439, 455). Central and peripheral PWV generally do not change over the menstrual cycle (4, 136, 201, 378, 541), although carotid arterial compliance may increase and AIx may decrease in association with increased estrogen

over the menstrual cycle (4, 201, 417, 541). These changes may also be a result of associated fluctuations in BP over the menstrual cycle (4, 136).

Differences in other factors besides the direct effects of sex hormones likely contribute to sex-based disparities in cardiovascular health. Using proteomics for example, the concentrations of 61 out of 71 circulating proteins identified as biomarkers for CVD were found to differ between young men and women (281). Specifically, proteins related to inflammation and adipokine signaling were higher in women, while proteins involved in fibrosis and platelet homeostasis were higher in men (281). After menopause, women exhibited fewer differences compared to age-matched men, although differences in a greater number of proteins were maintained in those women undergoing hormone replacement therapy (281). Those results suggest that the presence of sex hormones may explain some, but not all, of the differences in the concentrations of circulating proteins related to CVD development between men and women.

In addition, studies on human umbilical vein endothelial cells (HUVECs) support that sex differences are intrinsic to endothelial cells. Female HUVECs display greater eNOS mRNA, protein expression, and phosphorylation as compared to male HUVECs (3, 71). Female HUVECs also proliferate at a greater rate (3) and form more tubes in an angiogenesis assay (71, 565), functional differences which may be due to greater eNOS activity (71). Furthermore, transcriptional profiling revealed the differential expression of 1,021 (~2.5% of total) mRNAs between male and female HUVECs, with only ~44 being X- or Y-chromosome-specific genes (303). Female HUVECs also exhibited greater overall changes in gene expression in response to shear

stress, in terms of both number of genes and magnitude of changes (303). The expression levels of both estrogen receptors and androgen receptors are similar between male and female HUVECs, showing that these differences exist in the absence of the influence of sex hormones (3, 13).

Vascular Adaptations to Exercise Training

Few studies have directly tested sex differences in the vascular adaptations to exercise training in young individuals. Cross-sectional studies suggest that both young male and female endurance athletes exhibit similar endothelial function, but greater arterial diameters, when compared to sedentary controls (344, 412). A meta-analysis suggested no effects of sex on exercise training-induced improvements in PWV (19). Habitual exercise training also attenuates or prevents the age-related development of endothelial dysfunction and arterial stiffness in both men and women (451, 490, 491). Further, exercise training interventions improve carotid arterial compliance in both older men and postmenopausal women with or without estrogen treatment (324, 349, 451, 489, 491).

While endothelial function improves with exercise training in older men, these effects are usually not seen in older women (122, 395, 451, 545). Improvements in endothelial function with exercise training are apparent only in postmenopausal women with the most severe impairments in endothelial function, potentially due to the presence of other CVD risk factors (451, 484, 545). The loss of estrogen over the menopausal transition in older women appears to explain the lack of an effect of exercise training on endothelial function. Endurance exercise training improves FMD

in postmenopausal women treated with estrogen, but has no effect in the presence of a placebo (351). In support of a mediating effect of estrogen, premenopausal women who are amenorrheic also have reduced FMD, which is restored to levels of eumenorrheic athletes with recovery of a normal menstrual cycle (412, 560). Evidence suggests that estrogen plays a permissive role in the endothelial adaptations to training in women by decreasing oxidative stress and increasing NO bioavailability (350, 351, 451). Additionally, estrogen is required for the beneficial effects of increased laminar shear stress on arterial diameter and eNOS expression/activation in women (350, 492).

Racial Differences in Vascular Function and Risk of CVD

Within the US, the prevalence of CVD is highest among African American (AA) men (60%) and women (57%), with hypertension making up the large majority of cases (517). Furthermore, AA have the highest mortality rates resulting from CVD as compared to all other races/ethnicities (517). AA also experience CVD events at younger ages than their Caucasian American (CA) counterparts (148). Supporting the fact that CVD development may begin earlier in life, even young, otherwise healthy AA individuals often exhibit impairments in vascular health as compared to CA. Endothelial-dependent vasodilation is usually lower in healthy AA than matched CA (66, 209, 257, 379, 392, 408, 449, 474), and this may be apparent as early as adolescence (135). *In vitro* studies comparing endothelial cells derived from AA and CA suggest the reduced endothelial function in AA is likely due to higher levels of endothelial ROS and inflammation that act to reduce NO bioavailability (58, 146, 147, 254). Those results are corroborated by the finding that antioxidant supplementation

increases FMD in AA so that it is no longer lower than that of CA (256, 257). Additionally, elevated levels of circulating inflammatory markers have been observed in AA individuals across all ages (319, 352, 468). The expression of eNOS is not different or greater in AA endothelial cells, suggesting a detriment in NO production is not the cause of endothelial dysfunction (146, 147, 254).

On average, AA also have higher central and peripheral PWV, carotid arterial stiffness, and AIx/AIx75 as compared to CA (60, 207, 209). These racial differences in arterial stiffness exist despite similar central BPs, suggesting other mediating factors (60). The elevated arterial stiffness in AA may therefore reflect differences in vessel wall properties. Multiple studies have reported higher intima-media thickness of the carotid artery in AA than CA, which may be related to increased collagen content and vascular stiffness (209, 408, 419).

In addition, AA often demonstrate exaggerated or impaired vascular responses to physiological and psychological stimuli, and this has been proposed to underlie the heightened CVD risk in AA. The mechanisms mediating these responses likely contribute to the development of CVD. Importantly, these divergent responses are apparent even when baseline differences are not evident. AA children display larger increases in systolic and/or diastolic BP than CA in response to an orthostatic test, cold pressor test, isometric handgrip test, and video game play (357, 522). Those findings may reflect greater adrenergic activation in AA adolescents in response to cardiovascular stressors. In adults, AA did in fact have larger increases in mean arterial pressure and muscle sympathetic nerve activity following a cold pressor test, despite no racial differences at rest (65). There is other evidence that AA have elevated

sensitivity to α-adrenergic-mediated vasoconstriction and β-adrenergic-mediated vasodilation, rather than increased sympathetic activity as measured by catecholamine release and spillover (475).

Forearm blood flow was greater in CA than AA following a mental stress test, and this difference was abolished when NO synthesis was blocked (68). The AA group in that study showed a reduced vasodilatory response to sodium nitroprusside (an NO donor), suggesting impaired smooth muscle reactivity to NO (68). A mental stress test also induced a decrease in circulating NO metabolites in AA, compared to an increase in CA (441). Further, circulating concentrations of the vasoconstrictor endothelin-1 increased to a greater degree in AA and could contribute to impaired vasodilatory responses (441).

Vascular Responses to Acute Exercise

Acute exercise is a well-established stressor for the cardiovascular system that causes intensity-dependent increases in a multitude of vascular stimuli including shear stress, ROS, inflammatory factors, and sympathetic nerve activity. The BP and arterial stiffness responses to exercise have been found to differ in AA when compared to CA. Following a bout of exercise, AA men display increases in brachial, aortic, and carotid diastolic BP that are not seen in CA men (555–557). After exercise, AA also exhibit increased central and upper limb PWV compared to no change observed in CA, while they do not have the decreased lower limb PWV seen in CA (207, 556, 557). Additionally, carotid arterial stiffness decreased in CA, but not AA, after 45 minutes of moderate intensity treadmill exercise (557). On the other hand, exercise responses

in AIx/AIx75 may not differ between AA and CA (556). The effects of exercise on endothelial function in AA are less well studied. While AA had lower resistance artery function than CA at baseline, they exhibited similar increases in vasodilation after a maximal bout of cycling exercise (449).

MicroRNA

MicroRNAs (miRs) are short, noncoding RNA that regulate protein expression at the post-transcriptional level. MiRs are exported from seemingly all cell types into the circulating bloodstream and other biofluids. These circulating (ci-) miRs facilitate communication between cells by acting as paracrine and endocrine molecules, and have emerged as useful biomarkers for a variety of physiological and pathological processes. An introduction to miRs/ci-miRs, their discovery and canonical biogenesis pathway are given in Chapter 3. The evidence supporting the above roles of ci-miRs in the context of human exercise physiology is reviewed in Chapters 3 and 4.

Roles in Vascular Function, Dysfunction, and CVD

MiRs contribute to the normal development and functions of cells throughout the cardiovascular system. Initial, compelling evidence for this was provided by Yang et al. (558), who generated mice lacking functional Dicer, a critical enzyme in miR biogenesis. Absence of functional Dicer was embryonically lethal and led to defective blood vessel formation/maintenance, showing that Dicer and, therefore, mature miRs are required for normal blood vessel development and integrity (558). Important roles for many specific miRs within the vasculature have since been identified. For example, miR-126 is highly and primarily expressed within endothelial cells, where it regulates

angiogenesis (156, 198, 529). Deletion of miR-126 in mice leads to partial embryonic lethality and loss of vascular integrity (529), similar to the effects of Dicer deficiency (558). Several miRs have now been described to regulate key functions in endothelial cells, as well as in other cardiovascular cell types such as vascular smooth muscle cells, cardiomyocytes, leukocytes, fibroblasts, endothelial progenitor cells and other cardiovascular-related stem/progenitor cells (17, 152, 239, 295, 524, 554). MiRs with important roles in cellular functions usually regulate several pathways in one or more cell type, often directly targeting mRNAs for multiple proteins within the same pathway. In this way, miRs act as influential fine-tuners of physiological processes within the cardiovascular system.

MiRs regulate normal endothelial function by influencing the expression of key proteins and pathways governing NO production, redox balance, and inflammation (17, 84, 152, 174, 295, 322, 554). Conversely, the dysregulated expression of miRs contributes to pathophysiological processes. Important roles of several miRs have been identified in the development of endothelial dysfunction, arterial stiffness, and in all steps of atherosclerosis (17, 128, 131, 152, 271, 360). Additionally, the dysregulated levels of specific miRs are associated with apparently all CVDs and have been proposed as useful biomarkers in CVD pathologies (114, 164, 361, 369, 450, 521, 572). Studies have reported that specific ci-miRs can detect the presence and severity of CVDs including HF, CAD, MI, PAD, arrhythmias, and hypertension (164, 361, 450, 521, 571). Importantly, ci-miRs are also aberrantly expressed in the presence of CVD risk factors such as overweight/obesity, hypertension, hyperglycemia and diabetes, dyslipidemia, aging, smoking, and insufficient sleep (55, 59, 214–216, 242, 487, 511).

In addition to acting as potential biomarkers, the differential expression of ci-miRs in patients with CVD or CVD risk factors compared to healthy controls provides information on potential mechanistic roles of miRs in CVD pathologies.

The expression levels of select ci-miRs have been shown to further differentiate between 1) stable CAD vs acute coronary syndrome, 2) unstable angina vs acute myocardial infarction, 3) ST-elevation myocardial infarction (STEMI) vs non-STEMI, 4) HF with preserved vs reduced ejection fraction, and 5) insulin resistance/prediabetes vs Diabetes (361, 450, 512). Ci-miRs are also able to predict future cardiovascular adverse events such as MI and HF in the general population and in patients with CVD, as well as future comorbidities and mortality in CVD patients (361). Certain ci-miRs have been shown to outperform the diagnostic ability of classic markers such as cardiac troponin, brain natriuretic protein (BNP), and C-reactive protein (CRP) (361). The potential utility of ci-miRs as useful biomarkers is strengthened due to several characteristics including their high stability in circulation and collected samples, minimal invasiveness, and small sample requirement (95, 114, 171). Thus, the use of ci-miRs as both diagnostic and prognostic clinical biomarkers for CVD pathologies and associated comorbidities is the most probable, immediate clinical application for miRs.

Animal and cell-based research has revealed mechanistic roles for miRs in the development of CVDs and suggested their potential as therapeutic targets for intervention. By administering synthetic miRs subcutaneously, intravenously, or intraperitoneally to alter target miR expression within the cell type(s) of interest, a number of pre-clinical studies in rodents and pigs have shown the ability of miR-based interventions to prevent cardiac pathological remodeling and to improve recovery,

function, and survival following heart failure or myocardial infarction (38, 47, 308). MiR therapies have also successfully improved neovascularization after hindlimb ischemia and reduced the formation/progression of atherosclerotic plaques (67, 305, 313). Thus, current evidence indicates a strong potential of miR therapies for the treatment of heart and vascular diseases in humans. However, there are a number of roadblocks at the moment preventing clinical trials for CVDs, primarily concerning toxicity, stability, and difficulty in specifically delivering miRs to target cells/tissues (308).

Sex and Race Differences

Epigenetic mechanisms such as the regulation of gene expression by miR may underlie both sex and race-based differences in CVD development (272, 332, 410, 515). The circulating blood-borne levels of certain miRs, including those with roles in cardiovascular function, have been found to differ between men and women of varying ages (12, 28, 98). Additionally, ci-miRs exhibit sex-specific associations with cardiorespiratory fitness level (i.e. VO_{2max}) (62) and the presence of metabolic syndrome risk components (531). The X chromosome encodes over 100 miRs and approximately 15% of genes escape X chromosome inactivation, suggesting some X-linked miRs may be more highly expressed in women (160, 454, 470). Conversely, only 2 miRs are located on the Y chromosome (160, 454). Additionally, estrogen regulates miR expression within vascular cells by controlling the transcription of specific miRs, as well as the expression of several proteins in the miR biogenesis pathway including Dicer and Argonaute-2 (160, 290, 391). For example, treating

HUVECs with estradiol increased the expression of miR-126-3p, which decreased VCAM-1 expression and monocyte adhesion (290). The same investigators showed that injecting miR-126-3p mimics into $ApoE^{-/-}$ mice attenuated atherogenic lesion development, providing a potential miR-mediated mechanism by which estrogen may be atheroprotective in women (290). Also in HUVECs, a physiological dose of estradiol (1 nmol/L) was found to alter the expression of a number of miRs predicted to regulate pathways important to CVD development (516). In response to transverse aortic constriction, the expression of several estrogen-regulated miRs is altered in mouse hearts in a sex-specific manner and may contribute to sex differences in pressure overload-induced cardiac fibrosis (400, 410). Thus, miRs may mediate sex differences in CVD pathogenesis and particularly the cardiovascular protective effects of estrogen.

It is currently not known whether miR expression differs between healthy AA and CA individuals, though data on diseased populations suggest differences in miRs may contribute to racial differences in disease etiologies. The expression of select miRs differ in tumor samples from AA versus CA patients with cancer (175, 477) and in serum of AA and CA patients with Hepatitis C (123). MiR expression within PBMCs were uniquely reduced in AA women with hypertension as compared to both hypertensive CA women and normotensive AA women (129). Within endothelial cells, the differentially expressed miR were experimentally shown to alter the expression of proteins with roles in endothelial inflammation and dysfunction (129).

Mechanistic Insight from Exercise Studies in Animals

Numerous studies have demonstrated that the levels of specific miRs are altered in the heart or vasculature of animals in response to exercise training (64, 149, 150, 185, 243, 293, 387, 399, 549). Corresponding changes in the expression of proteins with roles in training-induced adaptations that are targeted by those miRs suggest potential mechanisms underlying corresponding cardiovascular adaptations (64, 149, 151, 185, 243, 293, 387, 399, 549). A few studies provide compelling evidence for such mediating roles of miRs in cardiovascular adaptations to exercise training in the contexts of health and disease. D'Souza (103) found that miR-423-5p is upregulated specifically in the sinus node of mice following swimming training, where it mediates training-induced bradycardia by downregulating HCN4. Blocking miR-423-5p in trained mice raised the HR to that of sedentary mice (103). MiR-222 is increased in the hearts of mice following two distinct exercise training programs that induced cardiac hypertrophy, as well as in the serum of heart failure patients after a cardiopulmonary exercise test (300). It was determined that miR-222 positively regulates the hypertrophy (increased), proliferation (increased), and apoptosis (decreased) of cardiomyocytes, and that these effects are mediated by the direct targets of miR-222 including p27, Hipk1, Hipk2, and Hmbox1 (300). Inhibiting miR-222 via injection of an antagonist miR in mice undergoing exercise training completely inhibited training-induced cardiac hypertrophy (300). Additionally, the heart size and function of transgenic mice overexpressing miR-222 specifically in cardiomyocytes were not different compared to controls, showing that miR-222 is necessary, but not sufficient for physiological cardiac remodeling (300). However, compared to control mice, the

transgenic mice were protected from adverse cardiac remodeling in response to induced ischemia-reperfusion injury, showing some protective effects of miR-222 (300). More recently, it was demonstrated that the exercise training-induced increase in cardiac miR-222 is also required for the generation of new cardiomyocytes following myocardial infarction (523).

Lastly, exercise training confers protective effects against myocardial ischemia/reperfusion injury, and the intercellular transfer of miR-342-5p was shown to mediate these effects (222). The miR-342-5p levels in circulating exosomes were ~two-fold higher in exercise trained humans and rats as compared to their sedentary counterparts (222). MiR-342-5p was primarily derived from endothelial cells and could be transferred to cardiomyocytes via exosomes, where it was shown to prevent cardiomyocyte apoptosis by targeting Caspase 9 and Jnk2, and enhance Akt phosphorylation by downregulating the phosphatase Ppm1f (222). Injection of exosomes from trained rats into the hearts of rats prior to myocardial ischemia/reperfusion injury reduced infarct area and improved cardiac function as compared to rats treated with a vehicle control or exosomes isolated from sedentary rats; effects which were greatly attenuated by inhibiting miR-342-5p (222). Furthermore, laminar shear stress increased HUVEC expression and release of exosomal miR-342-5p, providing a mechanism by which the circulating levels and intercellular transfer of miR-342-5p may be increased with exercise training (222).

Chapter 3: Circulating microRNAs in Acute and Chronic Exercise: More than Mere Biomarkers

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Circulating microRNAs in acute and chronic exercise: more than mere biomarkers

Ryan M. Sapp, Daniel D. Shill, Stephen M. Roth, James M. Hagberg

Department of Kinesiology, University of Maryland, College Park, MD, USA

Correspondence: James M. Hagberg, 2242 Valley Drive, College Park, Maryland 20742-2611. (301)-405-2487 (TEL), (301)-405-5578 (FAX). hagberg@umd.edu

Running Title: Ci-miRs in exercise and training

Abstract

MicroRNAs (miRs) are short, non-coding RNAs that influence biological processes by regulating gene expression post-transcriptionally. It was recently discovered that miRs are released into the circulation (ci-miRs) where they are highly stable and can act as intercellular messengers to affect physiological processes. This review provides a comprehensive summary of the studies, to date, that have investigated the effects of acute exercise and exercise training on ci-miRs in humans. Findings indicate that specific ci-miRs are altered in response to different protocols of acute and chronic exercise in both healthy and diseased populations. In some cases, altered ci-miRs correlate with fitness and health parameters, suggesting causal mechanisms by which ci-miRs may facilitate adaptations to exercise training. However, strong data supporting such mechanisms are lacking. Thus, a purpose of this review is to guide future studies by discussing current and novel proposed roles for ci-miRs in adaptations to exercise training. In addition, there are substantial, fundamental gaps in the field that need to be addressed. The ultimate goal is that an understanding of ci-miRs roles in physiological adaptations to exercise training will one day translate to therapeutic interventions.

Introduction

Scientists first identified microRNAs (miRs or miRs) circulating in human serum/plasma in approximately 2008 (86, 284, 337). These circulating miRs (ci-miRs) are secreted into the circulation by a variety of cell types, and can be taken up by other cells where they may regulate numerous physiological processes (80). In this way, they mediate communication between cells and tissues throughout the body. Due to their remarkable stability and ease of access, the promise of ci-miRs as useful biomarkers of diseases was recognized immediately (337). Since then, ci-miRs have been investigated as biomarkers and moderators of essentially all human diseases (191, 513, 527). Specifically, the roles of ci-miRs as biomarkers for cardiovascular diseases (CVD) and skeletal muscle pathologies have been detailed (16, 361). Conversely, miRs are crucial to the normal development and health of these systems (218, 297). Considering the well-established beneficial effects of exercise training on the cardiovascular system, skeletal muscle, and body as a whole, it seems logical that ci-miRs would be altered in response to exercise, and may be involved in mediating/moderating some or all of the adaptations to exercise training. Indeed, this has very recently become an area of increased research interest and several studies have shown changes in a number of ci-miRs in response to various modes of acute and chronic exercise.

While recent "-omics" approaches (e.g. genomics, proteomics, transcriptomics, metabolomics) have been used to interrogate the pathways underlying physiological training adaptations, results of these studies to date have been modest. In this respect, ci-miRs may be important missing links, given their potential to modulate pathways upstream in the "-omic" adaptations to exercise training. Ci-miRs hold the promising

potential to unlock a greater understanding of the integrative physiology of exercise and individual exercise responses. Therefore, the goals of this review are threefold: [1] Present a comprehensive review of studies examining the effect of acute exercise and/or training on ci-miR expression in human serum, plasma, or whole blood. [2] Highlight potential mechanisms by which ci-miRs may induce adaptations with training. [3] Identify current gaps limiting knowledge in the field and suggest future directions for the study of exercise and ci-miRs. While we will address all studies on exercise/physical activity and ci-miRs in humans, the primary focus of this review paper will be on aerobic exercise and endurance training, since that has been the major focus of the field thus far.

MicroRNAs

MiRs are ~22 nucleotide long, non-coding RNAs that regulate gene expression at the post-transcriptional level. The first miR was discovered in the nematode Caenorhabditis elegans (C. elegans) in 1993 by Lee, Feinbaum, and Ambros (286), who in simultaneous experiments with Ruvkun's group (537), reported that the small lin-4 RNA could base pair with a segment of the 3' untranslated region of another RNA, lin-14, to decrease abundance of the LIN-14 protein. In 1998, Fire and colleagues (155) demonstrated the experimental potential of the RNA interference mechanism when they injected small, double-stranded RNA into C. elegans and observed potent downregulation of specific endogenous mRNA. Since these initial discoveries, the field of miR research has expanded immensely. The miR pathway is now known to be highly conserved in mammals and vital in biological processes such as cell growth, cell

proliferation, motility, tissue differentiation, and embryological development (163, 306, 426, 543). Displaying their importance, the number of miRs within the genome correlates with the complexity of the organism (40). Currently, 2,588 miRs have been identified in the human genome. They are expressed in seemingly all cell types and it is predicted that over 60% of protein coding-genes are conserved targets (163).

Genes for miRs are encoded in both intergenic and intronic regions typically located in polycistronic transcription units (189). Interestingly, miRs encoded within intronic regions often repress their co-transcribed host gene(s) and, in addition, have their own promoters, enabling independent transcription and distinct expression from their host gene(s) (404). For an in-depth review of canonical miR biosynthesis we direct the readers to other excellent reviews (189, 543). Briefly, Polymerase II transcribes miR genes into a ≥1,000 base pair (bp) initial primary miR (pri-miR) containing the mature miR sequence in a stem-loop structure (543). The pri-miR is cleaved within the nucleus by Drosha into a ~65 bp pre-miR, which then associates with Exportin-5 to be transported into the cytoplasm. Here, Dicer further cleaves the pre-miR near its terminal loop resulting in a 21-25 bp double stranded RNA comprised of a mature miR and its complimentary strand. The miR duplex quickly unwinds and the mature miR is loaded onto an Argonaute protein, which associates with Dicer to form the miR-induced silencing complex (miRISC) (70, 464). The mature miR then acts as a guide for miRISC by complimentarily binding to the 3'-untranslated region of target mRNA transcripts, while the associated 'passenger' strand is usually released and degraded (189, 464). The miRISC-targeted mRNA may be perfectly or imperfectly complementary, having implications on the mechanism of silencing that is carried out.

Target recognition and binding due to complementarity are determined primarily by nucleotides 2 to 8 on the 5' end of the mature miR (141). Imperfect binding is advantageous in that an individual miR can target numerous mRNAs. In fact, individual miRs can regulate up to 200 mRNA transcripts, while conversely, it is possible for multiple miRs to share the same mRNA target (141). After binding, miRISC partially or completely suppresses gene expression via induction of translational repression, mRNA deadenylation, mRNA degradation, or sequestration in processing-bodies (P-bodies) (237). Recent evidence also indicates that miR-mediated up-regulation of transcription can occur in certain cases (377). Lastly, miRs often exhibit tissue specific expression and functions, which are occasionally contradictory. For instance, in liver and intestinal cancers, miR-26 acts as a tumor suppressor, while it is an oncogene in brain cancer (226, 269, 561). Thus, miRs act in an intricate and coordinated manner to fine-tune protein expression, though knowledge about the mechanisms governing their expression and regulation is incomplete.

Circulating MicroRNAs

The fairly recent discovery of miRs in human biofluids, including serum, plasma, saliva, sweat, cerebrospinal fluid, urine, and milk, has revealed new possibilities for miR research (80, 337). Such ci-miRs are actively or passively secreted into the bloodstream where they circulate in association with extracellular vesicles (EVs) (microvesicles, exosomes, or apoptotic bodies), proteins (e.g. Argonaute), or high-density lipoproteins (HDL). Ci-miRs can then prompt downstream effects upon uptake by target recipient cells by regulating translation of complimentary mRNAs (49,

212, 409, 562, 566). Only select miRs within cells are released into the circulation, and while in some cases miRs are released passively due to cell damage or senescence (e.g. miR-1 following myocardial infarction) (55), evidence supports the deliberate packaging and release of miRs in response to stimuli, as a means of intercellular communication (212). The intercellular transport of miRs and subsequent functional regulation of gene expression in recipient cells is now a well-supported mechanism of cell-to-cell communication, involving a variety of cell types and transport methods (49, 212, 409, 562, 566). However, the exact mechanisms underlying miR secretion/packaging and uptake by recipient cells are not known.

MiRs with aberrant expression in the circulation are reportedly associated with at least 70 diseases and ci-miRs have been extensively investigated as useful disease biomarkers given their ease of acquisition, high stability, and convenient amplification (154, 191, 195, 279, 306). The potential use of ci-miR-based therapeutic interventions is also compelling. Long-term suppression of the hepatitis C virus by intravenous infusion of a complimentary antagonist to miR-122 has been demonstrated in chimpanzees (279). This and other miR-based therapies are already in preclinical development and clinical testing for diseases such as coronary heart failure and myocardial infarction.

While the majority of studies have focused on differentially expressed ci-miRs in disease settings, the effects of other physiological alterations have also been investigated, though to a much lesser extent. Exercise is one such stressor that induces a well-characterized response and if repeated (training), results in beneficial adaptations within numerous physiological systems. Yet, the study of ci-miRs in the

context of acute exercise and exercise training has lagged behind, with only a recent upsurge in interest. To date, there have been 30 published studies, written in English, on the effects of acute exercise, training, or fitness level on ci-miR levels (Table 3.1). These papers were found through a comprehensive search of the PubMed database and by reviewing references of included studies. Of the studies performed thus far, most have investigated responses to acute exercise, with the vast majority focused on aerobic exercise. Still, results from these few investigations are promising. Ci-miRs altered with exercise have been proposed as potential biomarkers with clinical applications (172), and are proposed to participate in mechanisms governing the acute response to exercise and adaptations to exercise training. The studies reviewed in the following sections are presented according to both exercise modality and (dis)agreements in results, which facilitates a discussion of proposed roles for ci-miRs in the physiological responses to exercise. Additionally, the results of investigations on the two groups of most studied ci-miRs that are enriched in striated muscle and the endothelium are concisely summarized in Table 3.2 and Table 3.3, respectively.

Ci-miRs in the Response to Acute Exercise

The first investigation of the effect of exercise on ci-miRs came in 2011 from Baggish and colleagues (24). They examined the effect of a cycle ergometer maximal oxygen consumption (VO_{2max}) test on 12 ci-miRs in young, moderately-trained men. Immediately after exercise, ci-miRs -146a, -222, -21, and -221 increased in plasma while the others remained unchanged. These four miRs are highly enriched in the endothelium compared to other cell types and were chosen a priori based on their roles

in angiogenesis and/or inflammation. Along with ci-miRs -126 and -210, this group of endothelial derived ci-miRs is now among the best described in regards to the response to exercise (Table 3.3). Interestingly, absolute ci-miR-146a level immediately after exercise exhibited a significant linear correlation with absolute VO_{2max} (r=0.63), prompting the investigators to suggest its potential as a biomarker for cardiorespiratory fitness and peak exercise capacity, though this has not been verified.

More recently, Backes et al. similarly assessed the effect of an incremental, maximal cycle ergometer test in young men and women (23). They compared the ci-miR changes in whole blood of elite endurance athletes to that of moderately active controls. Using a microarray of 1,205 miRs, they identified three possible targets, but found no significant differences upon validation using qRT-PCR. Discrepancies between these two studies may have resulted from the sample type used (whole blood versus plasma), small sample sizes/gender differences, and/or time of sampling. RNA isolated from whole blood contains intracellular miR from circulating cells and therefore cannot be compared directly to plasma or serum. Backes et al. included six men and six women in each of their two groups, but they did not report any sex-based comparisons (23). Ci-miRs were shown to be associated with age, gender, and BMI in a population-based cohort study and there are known hormonal and genetic differences in miR regulation between sexes (12, 454). Moreover, it was shown in another study that ci-miRs varying at rest between high VO_{2max} and low VO_{2max} groups were different depending on gender (62). Ci-miR-21 was higher in the low VO_{2max} versus the high VO_{2max} group only in men (62). Lastly, Backes et al. (23) sampled blood 30 minutes after the cessation of exercise, while Baggish et al. (24) found significant changes

immediately after exercise, but showed that all ci-miRs were back to baseline after one hour.

Van Craenenbroeck and colleagues (505) compared the response of 12 ci-miRs after a symptom-limited maximal cycling test in patients with ambulatory chronic kidney disease (CKD) to that of healthy controls. Immediately after exercise, ci-miR-150 increased in all subjects, while -146a decreased only in the CKD patients. Additionally, the same research group recently showed a negative correlation of ci-miRs -146a, -150, and -210 with VO_{2peak} in CKD patients, which was lost when corrected for arterial stiffness, as measured by carotid-femoral pulse wave velocity (506). Interestingly, this negative correlation of ci-miR-146a with aerobic capacity is opposite to the positive correlation previously found in young, healthy men (24). Both miR -146a and -150 also correlated with arterial stiffness in CKD patients and have been implicated in numerous mechanisms of CVD development and disease (506). Further work is necessary to determine their use as biomarkers or therapeutic targets in this population. MiR-150 is also involved in physiological left-ventricular hypertrophy (323), raising the possible role for ci-miR-150 in this beneficial adaptation to training.

Conversely, patients with heart failure have exhibited no change in ci-miRs -146a or -150 immediately following a symptom-limited maximal test, though ci-miRs -21, -378, and -940 all increased (552). Changes in these ci-miRs did not correlate with VO_{2max}, markers of inflammation, or muscle damage. The fact that the cardiac muscle-enriched miR-940 increased in circulation, while other cardiac and skeletal muscle-enriched miRs as well as markers of muscle damage and inflammation did not, implies distinct mechanisms of miR release from muscle into the circulation with exercise. This

is supported in a report by Banzet et al. (29) comparing the response to downhill backwards walking with that of uphill walking in young, recreationally active, healthy men. For this study, subjects performed 30 minutes of both types of treadmill walking at a 25% grade, while wearing a weighted backpack, and changes in eight muscle-related ci-miRs were assessed for up to 72 hours after. The only change with uphill exercise was an increase in ci-miRs -181b and -214 immediately after. On the other hand, there was no change immediately following downhill exercise, though ci-miR-1 was increased two hours after, and ci-miRs -1, -133a, -133b, and -208b were increased six hours after. While the four ci-miRs that increased with downhill running were postulated to be released as a result of skeletal muscle damage, miR-181b and -214 are not muscle specific, but are present in many tissues. For example, they are upregulated in muscle in response to hypoxia (270, 437), and miR-181b is upregulated in neutrophils and peripheral blood mononuclear cells following cycling exercise (401, 402). Although the cellular origins are unknown in this study, it seems likely that these ci-miRs are released actively, as opposed to passively which may be the case with those released after the muscle-damaging downhill exercise, for example.

Notably, Guescini et al. have shown that at rest, muscles secrete EVs containing miR into the circulation (186). They then found increased ci-miR-181a-5p in EVs isolated from plasma of healthy men after a 40 minute treadmill run at 80% VO_{2max}. Ci-miRs -1, -133b, -206, and -499 were also present in muscle derived EVs, all of which exhibited a positive correlation between expression and relative VO_{2max}. A failure of these other common muscle-specific ci-miRs to increase, though, indicates a mechanism of selective miR packaging and release in EVs from skeletal muscle in

response to exercise. The mechanisms underlying this process, the ultimate destination and purpose of these EV-encapsulated ci-miRs, and whether they may explain adaptations to exercise, are all relevant questions that are yet to be investigated.

In another investigation of muscle-enriched ci-miRs, Aoi and colleagues (15) had healthy men undergo cycling at 70% VO_{2max} for 60 minutes. Out of seven ci-miRs, only ci-miR-486 showed a response, decreasing immediately after and returning to baseline three hours after exercise. There was a negative correlation between the fold-change in ci-miR-486 with exercise and relative VO_{2max} (r=0.58). To explain this, the authors suggested the possibility of increased uptake of ci-miR-486 by contracting skeletal muscle, which may then affect energy metabolism. They did not investigate ci-miRs -181b or -214, but the lack of a response in ci-miRs -1, -133a, and -133b implies that they are not released by muscle contraction alone, and supports the hypothesis that increased secretion of these ci-miRs may be due to muscle damage. More recently, ci-miR-486 was also shown to decrease in whole blood of young, recreationally inactive men immediately following a maximal treadmill test (120). Further, in a larger sample including both endurance-trained and inactive men, resting levels of ci-miR-486 correlated positively with VO_{2max} (r=0.20) and negatively with resting heart rate (r=-0.31), signifying it may play a positive role in cardiovascular fitness (120). Ci-miRs -1 and -133a additionally decreased following the max test, and resting levels of ci-miR-1 correlated with VO_{2max} (r=0.25) (120).

Nielsen and colleagues had healthy, trained men cycle for 60 minutes at 65% of maximal power output (367). Eight ci-miRs were downregulated immediately after, including -146a. Following one and three hours of rest after the exercise, seven and

four different ci-miRs were upregulated, respectively. The fact that there was no overlap of differentially expressed ci-miRs at any time point suggests distinct time-sensitive mechanisms of release. The investigators performed an initial global screening of 752 ci-miRs and a validation of 188 of these targets, which also raises the possibility of false positives. Since most exercise and ci-miR studies only examine a small number of targets chosen a priori, the results of this study are not necessarily in disagreement with previous results, but should be verified in another cohort. Cui et al. (100) likewise found a reduction in serum ci-miRs immediately after exercise in an investigation of seven a priori chosen targets. Ci-miRs -1, -133a, -133b, -122, and -16 all decreased following two Wingate tests separated by a four minute period of rest. In addition, ci-miR-133b correlated positively with peak power of the first Wingate ($r=0.712$), while ci-miR-122 correlated positively with the ratio of peak power of sprint one to that of sprint two ($r=0.665$). What role these ci-miRs may play in anaerobic capacity deserves attention. The same investigators more recently compared a session of repeated bouts of sprinting (high intensity interval exercise (HIIE)) to a bout of vigorous intensity continuous exercise (VICE) in endurance-trained men, using a global screening of ci-miRs (101). Immediately following both exercise bouts, an upregulation of 12 ci-miRs occurred, including ci-miRs -1, -133a, and -133b. The only difference was a lower ci-miR-1 level following HIIE compared to VICE, suggesting the sprint intervals may induce more/different muscle damage than the sustained endurance exercise. Kilian et al. (261) performed a similar comparison, although it was in adolescent, competitive, male cyclists, and they isolated ci-miRs directly from capillary blood obtained from the ear. Both 20 minutes into and 60 minutes after VICE,

ci-miR-126 was increased. At the 30 minute post-exercise time point, ci-miR-16 was increased due to VICE, while ci-miR-21 was decreased due to HIIE. The decrease of ci-miR-21 with HIIE and increase of ci-miR-126 with VICE may reflect specificity of exercise adaptations, since both of these miRs are positive regulators of the pro-angiogenic protein vascular endothelial growth factor (VEGF) and are upregulated in endothelial cells by sustained laminar shear stress (343, 534).

MiR-126 is a well-characterized endothelial-enriched miR that induces angiogenesis by inhibiting SPRED1 and PIK3R2, resulting in the upregulation of VEGF (156). Da Silva Jr. et al. (107) evaluated the change in whole blood ci-miR-126 and other circulating angiogenic factors after a maximal treadmill walking test in patients with intermittent claudication. Further, the effect of the antioxidant drug N-acetylcysteine (NAC) was assessed. They first found that NAC supplementation improved redox balance one hour after supplementation and following the exercise bout. However, the expression of angiogenic factors ci-miR-126, VEGF, and eNOS increased in circulation 30 minutes after exercise in the placebo session, but did not change in the test following NAC supplementation. Also, PIK3R2 mRNA, the anti-angiogenic target of miR-126, increased in circulation after exercise only in the NAC session. These results were contrary to the authors' hypotheses and led them to conclude that miR-126 signaling is redox sensitive in patients with intermittent claudication. This role of exercise-induced oxidative stress in stimulating angiogenic ci-miR-126 release from the endothelium is intriguing and remains to be elucidated. Whether this process is also true in healthy individuals, or is a result of peripheral arterial disease, should also be determined.

Ci-miR-126 has been shown to increase in healthy, middle-aged individuals immediately after a maximal cycle ergometer test (504). In addition, Uhlemann et al. (504) measured changes in ci-miR-126 and -133 in response to three other modes of exercise. Ci-miR-126 increased 30 minutes into cycling at 70% ventilatory threshold, and remained elevated throughout the entire four hour bout. Following a marathon, both ci-miR-126 and -133 were elevated, while only -133 was upregulated after resistance exercise with added eccentric load. This lends support for ci-miR-133 as a marker of skeletal muscle damage. The authors argued that the increase in ci-miR-126 is evidence of endothelial damage/lysis during aerobic exercise, though they did not provide any evidence to support this claim. In their study, ci-miR-126 was increased after just 30 minutes of cycling at 70% ventilatory threshold, while a previous paper showed no evidence of endothelial damage, determined by number of circulating endothelial cells, until two hours into cycling at the same intensity (340, 504). Together, these data do not point towards endothelial damage as the cause of increased ci-miR, indicating that other mechanisms are responsible for the secretion of endothelial ci-miR-126. In addition to the potential role of redox balance discussed above, laminar shear stress is known to upregulate endothelial cell expression of miRs -126 and -21 (343, 534), as well as secretion of miRs -143, -145, and -150 *in vitro* (212, 238). Moreover, Jaé et al. (238) recently showed that shear stress-responsive transcription factor KLF2 induces selective packaging and export of miRs from endothelial cells that is independent of intracellular levels of up-regulation. Lastly, miR-126 is released from circulating angiogenic $CD34^+$ cells (341), so their contribution to circulating levels cannot be discounted.

Long distance running is a well-characterized exercise stimulus in relation to ci-miR response. In 2014, Baggish et al. (25) examined the changes of seven ci-miRs in healthy men following the Boston Marathon. Immediately after the run, ci-miRs -1, -126, -133a, -134, -146a, -208a, and -499-5p were all increased, and returned to baseline by 24 hours after the run. Meanwhile, creatine phosphokinase, troponin I, NH2-terminal prohormone of brain natriuretic peptide (NT-proBNP), and high-sensitivity C-reactive protein (hsCRP) as markers of skeletal muscle damage, cardiomyocyte stress, cardiomyocyte damage, and inflammation respectively, all remained elevated 24 hours after the marathon. This adds credence to miR-specific mechanisms of secretion and uptake from circulation, even in the case of muscle damaging exercise. That same group of researchers (334) also found increases in the skeletal muscle-derived ci-miRs -1, -133a, -134, and -206 in individuals with hypercholesterolemia after the Boston Marathon. Again, all returned to baseline after 24 hours, though ci-miR-499-5p was increased at this time point only in those runners using statins. These data were supplemented with the ci-miR response of contracting mouse C2C12 myotubes exposed to statins, as an *in vitro* model of exercise and statin exposure. In conjunction with the human data, ci-miRs -1, -133a, -134, and -206 were increased in the culture media following contraction and regardless of statin exposure. An increase in miR-499-5p secretion from the myotubes was seen only in response to a combination of statin exposure and contraction. Statins are known to cause skeletal muscle dysfunction/damage when combined with exercise (331), and this study identifies ci-miR-499-5p as a potential biomarker for this phenomenon. Interestingly, statins have also been shown to attenuate the normal increase in skeletal muscle

mitochondrial content with aerobic exercise training (333), while intracellular miR-499 has been shown to regulate the expression of mitochondrial proteins (564). Thus, future studies should investigate the role that secretion of miR-499-5p plays in statin-induced muscular dysfunction and whether it is taken up by non-active muscle, or other tissues, to cause a systemic response. Contrarily, statins are known to elicit beneficial vascular adaptations (513). In one study of statin use, plasma levels of ci-miR-122 and -370 were higher in patients with hyperlipidemia than healthy controls, but were lower in statin users than non-users (165). It is unclear if ci-miRs -499-5p, -122, -370, or other vascular related miRs are involved in statin-induced vascular adaptations, and also what interaction exercise might have.

In 2015, de Gonzalo-Calvo et al. (113) measured an array of 106 ci-miRs involved in inflammation, as well as four muscle-specific ci-miRs, in nine trained men after both a 10 km and a marathon race. Immediately after the 10 km run, only ci-miR-150 was increased. After the marathon, a total of 12 inflammatory ci-miRs were upregulated, not including ci-miR-150, and all returned to baseline following 24 hours of rest. Inflammatory markers interleukin (IL)-6, IL-8, IL-10, and CRP also increased immediately after the marathon, but not the 10 km run. This study provides evidence for a dose-dependent response of inflammatory ci-miRs to exercise and suggests they play a role in exercise-induced inflammation. Still, it cannot be determined whether these ci-miRs were a cause or merely a response to the inflammation, as mentioned by the authors (113). Ci-miR-150 also correlated positively with the increased leukocyte and neutrophil (as mediators of inflammation) counts immediately after the 10 km run, suggesting them as possible sources. Surprisingly, there was no change in the four

muscle-specific ci-miRs -1, -133a, 133b, or -206 after either race. Increases in skeletal and heart secreted ci-miRs immediately after both a marathon and half marathon have been shown in other studies of endurance-trained men (172, 345). Although the study by Gomes et al. (172) included only five subjects, they found an increase in ci-miRs -1, -133a, and -206 immediately after a half marathon. Uniquely, only the study by Mooren et al. (345) has reported a sustained elevation of ci-miRs -1, -133a, and -206 after 24 hours of rest. The reason(s) for this discrepancy is unknown, given the subjects were healthy, middle-aged men who were exercise trained, the same characteristics as those used in the studies by Baggish et al. (25), de Gonzalo-Calvo et al. (113), and Min et al. (334). In addition, a positive correlation was reported for the increases in ci-miRs -1, 133a, and -206 with relative VO_{2max} and running speed at lactate threshold (345). Ci-miR-133a was also correlated to thickness of the intraventricular septum. Relatedly, Clauss et al. (93) found a negative correlation between ci-miR-1 and -133a levels immediately after a marathon and left atrial diameter. This correlation was true for left atrial diameter both immediately and 24 hours after the marathon only in "elite" marathon runners. This result indicates that training intensity and/or volume may alter the ci-miR response to a single exhaustive bout of exercise. Further, the potential roles of ci-miRs -1 and -133a in endurance training-induced atrial remodeling should be explored.

There are currently only three studies examining the effect of resistance exercise on ci-miRs. As mentioned, Uhlemann et al. (504) found increased ci-miR-133 after three sets, 15 repetitions of lat pulldown, leg press, and butterfly with added eccentric load. Sawada and colleagues (438) found no immediate response to five sets,

10 repetitions of bench press and leg press at 70% of one rep maximum. There was a delayed increase in ci-miR-149* one day after, and a decrease in -146a and -221 three days after the exercise. What role the endothelial-enriched ci-miRs -146a and -221 play in adaptations to resistance training and whether they regulate muscle hypertrophy remains to be seen. Moreover, changes in ci-miR-21 correlated with adrenaline and noradrenaline, while changes in -221 correlated with insulin-like growth factor-1 and testosterone (438). Although Uhlemann et al. (504) examined only two ci-miRs selected a priori, Sawada et al. performed a global screening of miRs in circulation.

While the previous two studies of resistance exercise were performed on relatively young participants, Margolis et al. (320) compared the response to three sets, 10 repetitions of bilateral leg extension and leg press in young (22±1 years) and older men (74±2 years). Out of 90 serum miRs assessed using qRT-PCR, there were no changes immediately after exercise, though nine were significantly increased six hours after exercise only in the young men. By combining this data with changes in skeletal muscle mRNA expression from biopsies (vastus lateralis) of the same participants, presented in a separate article (415), the investigators performed an Ingenuity miR target filter analysis (320). Positive correlations were observed for six of the altered ci-miRs (ci-miRs -19a-3p, miR-19b-3p, miR-20a-5p, miR-26b-5p, miR-143-3p, and miR-195-5p) with p-AktSer473 and p-S6K1Thr389, phosphorylated proteins upstream and downstream of mTORC1 important to the anabolic/hypertrophic response to resistance exercise (320). Interestingly, several of the miRs that increased with exercise are part of the miR-17~92 cluster transcribed from the same primary transcript. These miRs overlap in function and target phosphatase and tension homolog (PTEN), a positive

regulator of Akt-mTOR signaling (550). The lack of responses seen for these ci-miRs that may facilitate hypertrophy are consistent with the blunted response to resistance exercise that is characteristic of older individuals. Thus, failure of these ci-miRs to become upregulated may partially explain the resistance to anabolic stimuli with age, through inadequate regulation of important gene targets. Although causal mechanisms cannot be determined, this paper provides some of the strongest evidence that ci-miRs are involved in training adaptations by corroborating ci-miR and tissue gene expression data using integrative analytic techniques. The authors suggested that these findings support ci-miRs as predictive markers of age-associated changes in body composition and metabolic health, and as potential future biomarkers for adaptive responses to resistance exercise (320). Still, the ci-miR response to acute resistance exercise is a largely unexplored area. Several questions stand unanswered, such as the effects of gender, age, exercise intensity/volume, and resistance training status.

Effects of Exercise Training on ci-miRs

In addition to the investigations of acute exercise responses discussed above, several studies have examined the effects of different exercise training protocols. Aoi et al. (15) found a similar ci-miR response to four weeks of moderate intensity cycling training for three days a week, as that found for a 60 minute acute bout of cycling. Before training, out of seven muscle-enriched ci-miRs, only ci-miR-486 decreased immediately after exercise. Resting levels of ci-miR-486 were then decreased after training, and this change correlated positively with serum insulin level ($r=0.43$) (15). Mir-486 is known to target PTEN, which is a negative regulator of the PI3k/AKT

pathway downstream of insulin signaling. Thus, during acute exercise, ci-miR-486 may be taken up by the muscle, where it stimulates glucose uptake by suppressing PTEN. More muscle uptake of miR-486 during sustained endurance exercise may therefore translate to lower exercise capacity, as those with higher aerobic capacity will rely more heavily on lipid utilization (220). Furthermore, a reduction in ci-miR-486 at rest may reflect higher concentration in skeletal muscle, reduced PTEN, and enhanced insulin signaling as an adaptation to endurance training. Interestingly, in a cohort including inactive, young and older men, fat mass and blood glucose concentration explained 52% of the variance in serum miR-486 level (r=.72) (320), providing evidence that ci-miR-486 may be involved in pathways underlying metabolic health. These are attractive hypotheses that merit more extensive investigations.

Baggish et al. (24) compared the acute exercise response before and after 90 days of rowing training. The training stimuli consisted of daily sessions at low intensities, but long duration (one to three hours). First, they showed that resting levels of ci-miRs -146a, -222, -21, -221, and -20a were increased following training. The ci-miRs -146a and -222 were further increased after acute exercise in the trained state, though there was no increase of -21 and -221 as was seen with acute exercise before training (24). Conversely, following intense cycling training five days a week for 12 weeks, Nielsen et al. (367) found that 11 ci-miRs were downregulated, including -21 and -133a, while -103 and -107 were upregulated. They also observed no change in ci-miRs -146a or -221 (367). Only ci-miR-133a was affected by both training and acute exercise, also increasing three hours after 60 minutes of cycling performed before training. On the other hand, Clauss et al. (93) found no effect of 10 weeks endurance

training on five ci-miRs involved in atrial remodeling. Thus, there is no strong consensus on the ci-miR response to exercise training in healthy individuals, and more studies are needed. All training protocols used in studies thus far have varied substantially and the crossover of ci-miRs studied has been minimal. Further, no study has included healthy women in order to investigate possible sex differences in training-induced ci-miR responses.

All other training studies have been conducted on unhealthy and diseased populations. Van Craenenbroeck et al. (505) examined ci-miR concentrations in CKD patients following a 12-week home-based cycling program, both at rest and after a symptom-limited maximal cycling test. There were no changes in resting ci-miR concentrations after training. The patients exhibited a decrease in ci-miR-210 immediately after exercise in the trained state, the degree of which correlated with change in VO_{2peak} with training (rho=-0.236). However, there was no change in ci-miRs -150 and -146, as was seen with the maximal test before training. As discussed above, such differences indicate that alterations in these ci-miRs may facilitate some of the cardiovascular adaptations to training.

In overweight/obese men and women, a 16-week diet and resistance-training program resulted in an increase of ci-miRs -221-3p and -223-3p (385). This response was not different between high and low responders in regards to weight loss, but ci-miR-140 was higher in the low responding group than the high weight loss group at the end of the intervention. FNDC5 is a target of ci-miR-140 that is processed into the circulating myokine IRISIN, which is secreted into the circulation with exercise and is responsible for stimulating thermogenic processes in adipose tissue (52). The authors

proposed a mechanism by which increased ci-miR-140 expression may explain the low weight loss response of some individuals to training/diet, through downregulating FNDC5 and attenuating potential IRISIN-induced increases in energy expenditure (385). If this mechanism were elucidated, the use of an antagonist miR (antagomir) to miR-140, in conjunction with exercise/dieting might then be a useful method to enhance weight loss in these individuals. Beyond that, whether miRs -221 and -223 respond primarily to resistance training or reduced caloric intake in overweight/obese individuals is unclear, since the individuals in this study received both interventions.

At baseline, older, pre-diabetic patients exhibited higher ci-miR-192 and -193 levels than either diabetic or healthy controls (386). After a 16-week aerobic and resistance training program, pre-diabetic individuals had reduced ci-miR-192 and -193b to levels seen in healthy controls, who did not exhibit differences with training. These results were also replicated in glucose-intolerant mice who underwent exercise training and caloric restriction, indicating ci-miRs -192 and -193b play some role in the shift from healthy to unhealthy glucose metabolism, and vice versa with exercise training. The reductions in ci-miRs -192 and -193b cannot be attributed to the effects of exercise training per se, however, since the pre-diabetic subjects in this study were given diet recommendations in combination with the training program. Lastly, in a study of patients with impaired glucose tolerance/fasting glucose or diabetes, both groups had higher ci-miR-126 levels after a six month exercise training and diet treatment (301). Unfortunately, the exercise training protocol in this study was not described. In sum, there is vast evidence that ci-miR alterations facilitate the progression of diseases. Likewise, the ability of exercise training to prevent and

mitigate diseases is well characterized, so it seems likely that ci-miRs may mediate such processes. The few studies detailed here lend early support to this notion, although more mechanistic approaches are obviously needed, as well as time-course and longer term, well controlled training studies.

Correlations of Fitness and Activity Level with ci-miRs

Cross-sectional comparisons of individuals based on fitness characteristics have been performed in four studies. Significant findings have predominantly been on ci-miRs enriched in or specific to the endothelium. As mentioned earlier, Bye et al. (62) compared high and low VO_{2max} groups containing both men and women. Ci-miRs -210 and -222 were higher in the low versus high VO_{2max} group, and -21 was higher only in men with low VO_{2max}. Negative correlations of ci-miR-210 (r=-0.35) and -21 (r=-.20) to relative VO_{2max} were also observed. The investigators recognized that miR-210 is a regulator of the response to hypoxia (223), including moderating hypoxia-induced angiogenesis (144). The extent to which higher levels of ci-miR-210 and -21 may play a role in individuals with low VO_{2max} is yet to be elucidated.

Wardle et al. (533) compared elite, competitive athletes who were endurance-trained, to those who were strength-trained, as well as non-trained controls. Ci-miRs -21, -221, -222, and -146a were all higher in the endurance-trained group than the strength-trained group, with the control group tending to fall in between. These four ci-miRs each correlated with a number of performance related fitness variables. Overall, these data indicate that fitness related ci-miRs may be regulated in opposite directions based on aerobic/endurance versus strength training. This coincides with previous data

showing that all or some of these ci-miRs increase following acute endurance exercise and training (24, 25), and decrease three days after a bout of resistance exercise (438). Nonetheless, the fact that the athletes in the study by Wardle et al. (533) were competitive elite athletes raises the possibility of underlying genetic differences between the groups. Indeed, subjects were chosen from a larger pool of athletes because they had the largest relative VO_{2max} or best performance in strength/power tests. Thus, the observed results attributed to training modes may be confounded by genetic predispositions. On the other hand, Denham and Prestes (120) compared non-elite, endurance trained men and women to inactive, healthy controls, using relatively large sample sizes (n=67 and n=61, respectively). They assessed resting levels of five skeletal or cardiac muscle-enriched ci-miRs in whole blood. Ci-miRs -1, -486, and -494 were higher in endurance athletes than controls, and both ci-miRs -1 (r=0.25) and -486 (r=0.20) correlated positively with VO_{2max}. The finding of higher ci-miR-486 in the endurance-trained individuals is seemingly contradictory to the finding of decreased serum miR-486 expression after four weeks of endurance training that was observed by Aoi and colleagues (15). This may indicate time-dependent changes, since all endurance athletes had been exercising for at least one year, or may simply be a result of sampling type discrepancies.

Finally, Zhou et al. (570) grouped older men and women with metabolic syndrome into quartiles based on habitual physical activity. The group with the lowest metabolic equivalent (MET) hours per week had higher ci-miR-126 and -130a than the most highly active group. Higher levels of ci-miR-126 were associated with an increased risk for metabolic syndrome. The association of higher physical activity with

decreased risk of metabolic syndrome was also abolished when adjusted for ci-miR-126 level. Thus, ci-miR-126 may partially mediate the decreased risk of metabolic syndrome due to regular physical activity, though the mechanism is unknown.

Future Directions of Study

In addition to the roles of ci-miRs in exercise physiology suggested thus far, we have identified some novel areas for future study that may reveal potential mechanisms of action for ci-miRs. Shear stress is well characterized as the primary mechanism responsible for endothelial and vascular adaptations to exercise training (162, 501), as it beneficially modulates endothelial cell phenotypes through mechanotransduction-mediated effects (102). Intriguingly though, adaptations may not be directly dependent on shear stress, since similar adaptations are also observed in vasculature perfusing skeletal muscles and organs that are inactive during exercise (382). Since these vessels are not exposed to a significant degree of increased shear stress, this suggests that other systemic mechanism(s), such as other hemodynamic or circulating factors, may also be in effect (382). The relationship between shear stress and vascular-related ci-miRs *in vivo* and in relation to exercise has not been investigated, though *in vitro*, miR expression and secretion from endothelial cells is determined by the amount of shear stress (212, 534). Further, endothelial cells secrete miR-containing microvesicles, which may be incorporated into vascular smooth muscle cells or other endothelial cells (9, 212). Endothelial cells have been shown to secrete exosomes containing miRs -143 and -145 in response to shear stress, which subsequently confer atheroprotective effects by regulating gene expression in smooth muscle cells (212). Lastly, endothelial cells

transfer miR-126 to other endothelial cells through microparticles *in vivo*, which promotes repair of vascular injury by stimulating migration and proliferation (242). The inflammatory protein TNF-α can further modulate the miR "cargo" of released microparticles in order to induce either pro- or anti-atherogenic effects in recipient endothelial cells (9). The idea that shear stress and/or other factors experienced by endothelium of active skeletal muscle vasculature during exercise may induce the secretion of ci-miRs to then be taken up by endothelial cells of inactive skeletal muscle vasculature, as a means of inducing systemic endothelial adaptations (Figure 3.1), has not been explored.

Besides ci-miRs derived from the endothelium itself, ci-miRs released from circulating cell types may contribute to endothelial adaptations with exercise and training. Platelet-derived microvesicles are increased in response to intense cycling exercise, are related to brachial and femoral artery shear rate, and induce pro-angiogenic effects when applied to cultured endothelial cells (538). Monocytes secrete microvesicles containing miR, including miR-150, which stimulates and enhances migration in recipient endothelial cells *in vitro* (566). Pro-angiogenic exosomes highly enriched in miRs -126 and -130a are secreted by $CD34^+$ stem cells and are also taken up by endothelial cells (427). Shear stress has been shown to alter miR expression in similar $CD34^+$ endothelial progenitor cells (83). MiRs are also transported to endothelial cells by HDL (485). HDL isolated from patients with chronic heart failure and cultured with endothelial cells reduced endothelial expression of pro-angiogenic miRs -126, -21, and -222 (414). After 15 weeks of aerobic exercise training, this effect of HDL was attenuated, suggesting their composition/cargo was altered. Whether

HDL-encapsulated ci-miRs were altered was not assessed. If this were the case, it would raise the attractive prospect of isolating HDL from CVD patients, altering their miR content, and re-introducing them as a means to enhance endothelial function and combat disease. Other circulating EVs such as exosomes are also candidates for such a treatment (275).

While we have focused here on endothelial adaptations, the roles of ci-miRs likely extend to training adaptations seen in other tissues (e.g. the heart, skeletal muscle, adipose tissue, nervous system, etc.), as suggested by some of the studies reviewed above. A key to elucidating these roles will be combining changes in ci-miR with corresponding intracellular miR and mRNA data. Only one study has recently done this (320), combining ci-miR data with previously published gene expression data from skeletal muscle biopsies (415). Currently, these studies remain difficult due to the fact that the origin(s) and destination(s) of ci-miRs are largely unknown, though some miRs are highly enriched in distinct tissues. Additionally, the assessment of predicted downstream target mRNA and protein levels, as well as pre-miR levels and components of the miR biogenesis pathway will be necessary for a thorough understanding of the ci-miR response to exercise. While investigations of most tissues will require *in vitro* and animal models, the use of muscle biopsies can shed light on these pathways within skeletal muscle of humans. As a notable example, Russell et al. (424) took biopsies following both a 60 minute bout of cycling and 10 days of cycling training. Drosha, Dicer, and Exportin-5, as well as miRs -1, -133a, and -133b were upregulated in skeletal muscle three hours after acute exercise, while miRs known to be involved in muscle wasting and disease were downregulated, indicating miRs can be regulated

independently of the primary components of biogenesis. They further observed that some of the predicted targets of affected miRs exhibited altered mRNA or protein expression and miR levels correlated with the levels of their predicted protein targets (424). It would be interesting to supplement this type of study with ci-miR data, though the time course of ci-miR packaging, release, uptake, and translational regulation within target cells make it a more complicated endeavor. These processes, as well as the origin(s) and destination(s) of specific ci-miRs may first need to be characterized.

Methodological Considerations

Currently, there is no standardized protocol for the study of ci-miRs. Methods differ in regard to sample type and timing, RNA extraction, normalization, and quantification. As mentioned, the time course for miR appearance and clearance from circulation has yet to be characterized. In addition, plasma and serum differ in ci-miR content. Either serum or plasma have been shown to exhibit a higher concentration of ci-miR, possibly as a result of the coagulation process or remnant cellular components, respectively (166, 329, 488, 528). Previously, the sample type selected for study has apparently been chosen arbitrarily, and the degree to which this accounts for differences between studies is unknown. Hemolysis is another possible confounder of ci-miR concentration, since many miRs are present in erythrocytes (329). Hemolysis is only occasionally accounted for, though Nielsen et al. (367) have proposed a protocol for hemolyzation quality control. Furthermore, there is currently no known invariant "house-keeping" miR in circulation, and methods for normalization vary. The most popular method is to introduce a known-amount of a synthetic spike-in miR that is not

expressed in humans, during the RNA isolation process. This miR can then be quantified following isolation and used to normalize samples based on efficiency of extraction. Other methods include normalizing based on the mean expression of all miRs in an array, to the least variant ci-miR found between groups in an array, or to a priori chosen ci-miR(s) that usually show low variation between samples (318). A universally accepted, gold standard method of normalization would be useful to increase reproducibility and confidence in the legitimacy of results. Strategies such as using algorithms to pick the most stable ci-miR or combining the use of endogenous and exogenous controls have been proposed (318, 472). Lastly, both the extraction kit and qRT-PCR system used may affect accuracy and reproducibility of results (472, 488). Thus, it is important to consider the methods used in studies of ci-miRs, as they may play a significant role in data interpretation and may provide an explanation for some apparent discrepancies between results.

Conclusions

To date, available review papers summarizing studies of ci-miR responses to acute exercise and exercise training have either not been exclusively focused on ci-miRs or the effects of exercise, or were published before a majority of more recent ci-miR and exercise studies. For instance, the most recent reviews published in 2015 include five (11), six (551), eight (172), and ten (161) primary articles on exercise/training and ci-miRs, whereas we have identified and presented 30 such peer-reviewed papers. A previous review paper (172) has highlighted the potential of ci-miRs as biomarkers in exercise and training. In addition to that, we have discussed

possible mechanisms of action and proposed areas of future study. There is a need for more studies describing ci-miR responses to different protocols of exercise and training in diverse populations. The majority of studies performed to date have used small sample sizes of men. Of those that have used women, only one has reported sex-based comparisons (62). In light of known ci-miR gender differences, more studies on women are needed. Additionally, most studies have examined a small number of ci-miRs chosen a priori. This is understandable due to the large number of miRs and added cost of high-throughput methods, but may be limiting our current knowledge. Of those studies that have examined large numbers of ci-miRs, some use samples from the same subjects for follow-up qRT-PCR validation, while others did not validate their array findings. This step is important due to the increased sensitivity and specificity of qRT-PCR and the fact that results of initial microarray and screening results often fail to be reproduced (81). Findings from these studies should be replicated in other, larger cohorts.

As of now, only one study has investigated the effects of aerobic training in completely sedentary/untrained men (15). More studies are needed comparing young, healthy, sedentary and trained subjects, either cross-sectionally or in longitudinal training studies, in order to more completely understand the effects of exercise training. Meanwhile, no study has explored the effect of a resistance-training program on ci-miRs in healthy individuals. Conversely, studying the effect of training cessation in well-trained individuals may be a useful paradigm to shed light on ci-miRs roles in adaptations to long-term training. The intensity of exercise and the amount of muscle mass that must be recruited to significantly alter miR levels in circulation is also

unclear, as well as whether a systemic response to exercise is necessary to upregulate ci-miRs, or if localized responses are sufficient. A primary gap in knowledge limiting the progress of the ci-miR field as a whole is the unknown cellular origin of ci-miRs. While extensive work has been completed on miRs enriched in, or specific to, certain cell types, the sources of many ci-miRs are speculative or unknown. Likewise, the significance of the distinct modes by which ci-miRs travel in circulation (associated with proteins, vesicles, or HDL) is largely unknown, especially in the context of exercise. The different processes of packaging, secretion, and uptake/clearance may be important determinants of ci-miR effects. In conclusion, miRs appear vital to the acute exercise response and adaptations to training, though the majority of data is still correlative (149, 266). Future research should move beyond simply describing exercise-induced alterations in ci-miR, and should focus on identifying the mechanisms and pathways by which ci-miRs elicit responses to acute exercise and adaptations to exercise training. The biological roles of ci-miRs in these processes remain to be elucidated, but once understood may offer future opportunities for therapeutic interventions (172, 376, 457).

Table 3.1. Studies of ci-miRs in response to acute exercise, exercise training, or fitness in humans

Authors/Year	Subjects	Stimulus	Samples	Ci-miR responses	Ci-miRs investigated
Baggish et al. 2011	Male student athletes (n=10) age 19.1±0.6 yr	1) Exhaustive incremental cycling exercise test; 2) 90 days rowing training 1-3 h/day	Plasma - immediately (immed.) and 1 hr after an exercise test, both before (baseline) and after training	Baseline immed. after exercise: 146a, 222, 21, 221 ↑ At rest after training v. baseline: 146a, 222, 21, 221, 20a ↑ After training immed. after exercise: 146a, 222 ↑	A priori: 12 ci-miRs
Aoi et al. 2013	Healthy, untrained men (n=10) age 21.5±4.5 yr	1) Acute: Cycling at 70% VO_{2max} for 60 min. 2) Training: Cycling at 70% VO_{2max}, 30 min., 3 days/wk for 4 wk	Serum - 1) immed., 3 h, and 24 h after acute exercise 2) at rest after training	Immed. after ex: 486 ↓ 48 h after training: 486 ↓	A priori: 7 muscle-enriched ci-miRs
Banzet et al. 2013	Healthy, recreationally active men (n=9) age 27-36 yr	1) Downhill backwards walking 2) uphill walking 30 min, 1m/s, with loaded backpack (12% body weight)	Plasma - immed., 3 h, 6 h, 24 h, 48 h, 72 h after each exercise session	Immed. after uphill ex: 181b, 214 ↑ 2 h after downhill ex: 1 ↑ 6 h after downhill ex: 1, 133a, 133b, 208b ↑	A priori: 8 muscle specific/related ci-miRs
Bye et al. 2013	Healthy men (n=38) and women (n=38) age 45.5±3.3 yr	None; subjects categorized in high (145.2 ± 20.7 ml/kg$^{0.75}$/min) or low (101.1 ± 18.0 ml/kg$^{0.75}$/min) VO_{2max} categories	Serum - at rest	Low VO_{2max} vs. high VO_{2max}: 210, 222 ↑ Low VO_{2max} vs. high VO_{2max} (males only): 21 ↑	Screening of 720 ci-miRs (50 detected); 8 selected for further validation
Sawada et al. 2013	Healthy, recreationally active men (n=12) age 29.9±1.2 yr	bench press and bilateral leg press, 5 sets of 10 reps at 70% 1 RM	Serum - immed., 1 hr, 1 day, and 3 days after exercise	1 day after: 149* ↑ 3 days after: 149* ↑ 146a, 221 ↓	Global screening of all miRs (3 selected for further validation) plus 8 ci-miRS selected a priori
Tonevitsky et al. 2013	Highly trained, male, national level ski athletes (n=8) age 21.7±2.6 yr	Treadmill running at 80% VO_{2peak} for 30 min	Whole blood - immed., 30 min, and 60 min after exercise	Microarray results not validated	Screening of 200 ci-miRs
Backes et al. 2014	Elite endurence athletes (6 men and 6 women) age 24±5 yr and age/sex matched moderately active controls	Exhaustive stepwise cycling exercise test	Whole blood - 30 min after exercise	No significant differences using qRT-PCR	Screening of 1,205 ci-miRs (154 detected in all samples); 3 selected for further validation
Baggish et al. 2014	Healthy, Caucasion, male Boston Marathon runners (n=21) age 51.8±1.4 yr	Marathon	Plasma - immed. and 24 h after exercise	Immed. after: 134, 126, 1, 133a, 499-5p, 208a, 146a ↑	A priori: 7 ci-miRs
Gomes et al. 2014	Male, recreational runners (n=5) age 31.6±4.4	Half marathon	Plasma - immed. after exercise	1, 133a, 206 ↑	A priori: 3 muscle specific ci-miRs

Liu et al. 2014	Patients with impaired glucose tolerance(IGT)/impaired fasting glucose (IFG) (male=82, female=75) age 47.9±7.8. Patients with diabetes mellitus (DM) (male = 78, female=82) age 50.2±6.7	Six month exercise "training" and diet control treatment	Serum - at rest after exercise "training"	IGT/IFG and DM: 126 ↑	A priori: 1 ci-miR
Mooren et al. 2014	Moderately endurance trained men (n=14) age 42.8±6.0 yr	Marathon	Plasma - immed. and 24 hr after	Immed. after: 1, 133a, 206, 208b, 499 ↑ 24 hr after: 1, 133a, 206 ↑	A priori: 7 skeletal/heart muscle-related ci-miRs
Nielsen et al. 2014	Healthy, trained men (n=13 for acute exercise and n=7 for training) age 28±8 and 28±5 yr	1) Acute exercise: 60 min cycling at 65% Pmax 2) Training: Cycling 5 times/wk for 12 wk	Plama - immed., 1 hr, and 3 hr after acute exercise. 3-5 days after training	Immed. after exercise: 106a, 221, 30b, 151-5p, let-7i, 146a, 652, 151-3p ↓ 1 hr after exercise: 338-3p, 330-3p, 223, 139-5p, 143, 145, 424 ↑ 3 hr after exercise: 1, 424, 133a, 133b ↑ After training: 342-3p, let-7d, 766, 25, 148a, 185, 21, 148b, 133a, 92a, 29 ↓ 103, 107 ↑	Global screening of 752 miRs. Validation of 188 ci-miRs
Uhlemann et al. 2014	Subjects varied for each exercise stimulus, including healthy men or both men and women; average age range 30.4 - 56.8 yr	1) Single-symptom limited maximal exercise test on cycle ergometer 2) 4 hr cycling at 70% ventilatory threshold 3) Marathon 4) Resistance exercise with additional eccentric load	Plasma - sampling times varied across different exercise stimuli	At highest workload and 5 min post maximal cycling test: 126 ↑ 30 min and at each hour during 4 hr cycling: 126 ↑ Immed after marathon: 126, 133 ↑ Immed after resistance exercise: 133 ↑	A priori: 2 ci-miRs
Zhou et al. 2014	Men and women with metabolic syndrome (n=209) age 62.5±7.1	None; subjects grouped into quartiles by habitual physical activity (metabolic equivalent hours per week)	Serum - at rest	Highest activity versus lowest activity quartiles: 126, 130a ↓	A priori: 55 physical activity related miRs
Cui et al. 2015	Healthy, regularly active men (n=18) age 20.2±1	Two Wingate tests separated by 4 min active recovery	Plasma - immed. after exercise	1, 133a, 133b, 122, 16 ↓	A priori: 7 ci-miRs
Gonzalo-Calvo et al. 2015	Healthy, trained, male runners (n=9) age 39.1±2.2	1) 10 km race 2) Marathon	Serum - before, immed. after, and 24 h after each run	Immed. after 10 km run: 150 ↑ Immed. after marathon: let-7d-3p, let-7f-2-3p, 125b-5p, 132-3p, 143-3p, 148a-3p, 223-3p, 223-5p, 29a-3p, 34a-5p, 424-3p, 424-5p ↑	106 ci-miRs involved in inflammatory processes and 4 muscle specific ci-miRs
Guescini et al. 2015	Healthy male students (n=7) age 26±4.8 yr	40 min treadmill running at 80% VO_{2max}	Extracellular vesicles isolated from Plasma - 1 h after exercise	181-5p ↑	A priori: 10 ci-miRs

Parrizas et al. 2015	Prediabetic (n=6) and healthy control (n=12) men and women; age 57.3±3.2 and 53.8±3.4 yr	16 wk monitored aerobic and resistance exercise 2 times/wk and diet recommendations	Plasma - after intervention	Prediabetic group: 192, 193b ↓	A priori: 2 ci-miRs
Silva Jr. et al. 2015	Male patients with history of intermittent claudication (n=10) age 62±2 yr	Maximal exercise test (treadmill walking at 2 mph, 0% grade increased by 2% every 2 min) following antioxidant or placebo ingestion	Whole blood -30 min after exercise	Placebo group only: 126 ↑	A priori: 1 ci-miR
Van Craenenbroeck et al. 2015	Ambulatory chronic kidney disease (CKD) patients (n=32) and healthy controls (n=12) age 49.6±15.3 and 43.4±4.7 yr	1) Maximal symptom limited exercise test on cycle ergometer both before and after training 2) 12 wk of home-based cycling 4 times/day for 10 min each (CKD patients only)	Plasma - 1) 10 min after acute exercise (both at baseline and after training) 2) At rest following 12 wk training	All subjects after exercise at baseline: 150 ↑ CKD patients only after exercise at baseline: 146 ↓ After acute exercise, following training: 210 ↓	A priori: 12 ci-miRs
Wardle et al. 2015	Male strength (n=10) and endurance (n=10) competitive athletes, and non-exercising controls (n=10) average age 22.2 - 24.0 yr	None; subjects compared based on training type	Plasma - at rest	Endurance compared to strength athletes: 222, 21, 146a, 221 ↑	A priori: 14 ci-miRs
Clauss et al. 2016	"Elite" (n=15) and "non-elite" (n=15), healthy, male, Munich marathon runners; age 40.0±1.7 and 40.1±1.4	1) 10 week endurance training program: elite group ran 73.9±3.9 km/wk; non-elite group ran 33.9±2.7 km/wk 2) Marathon run	Plasma - Baseline (V1) After 10 wk training (V2) Immed. after Marathon (V3) 24 h after marathon (V4)	All runners V3 vs. V2: 1, 133a, 30a ↑ Elite runners only V4 vs. V3: 26a ↓	A priori: 5 ci-miRs associated with atrial remodeling
Cui et al. 2016	Healthy, endurance trained men (n=26) age 20.4±0.1	1) High intensity interval exercise (HIIE) 2) Vigorous intensity continuous exercise (VICE)	Plasma - immed. after each exercise session	Immed after both HIIE and VICE: 1, 133a, 133b, 206, 485-5p, 509-5p, 517a, 518f, 520f, 522, 553, 888 ↑	Global screening of ci-miRs (449 identified as candidates); 12 selected for further validation
Denham and Prestes 2016	Healthy, endurance trained (n=67) and inactive (n=61) men and women; age 33.9±10.8 and 28.7±10.6	1) Endurance trained v. Inactive at rest 2) Maximal treadmill test	Whole blood - 1) at rest 2) immed after exercise	Endurance-trained compared to Inactive: 1, 486, 494 ↑ Immed after exercise in Inactive: 1, 133a, 486 ↓	A priori: 5 skeletal/heart muscle-enriched ci-miRs
Hecksteden et al. 2016	Trained cyclists (n=15) and strength athletes (n=14)	6 day fatiguing training program	Plasma and whole blood - after training	Differences suggested for all comparisons (not validated by qRT-PCR)	

Kilian et al. 2016	Healthy, trained, competitive male cyclists (n=12) age 14.4±0.8 yr	1) High intensity interval exercise (HIIT) 2) High volume continuous exercise (HVT)	Capillary blood - during exercise at 20, 30, and 60 min.; immed., 30, 60, and 180 min after	30 min after HVT: 16 ↑ 30 min after HIIT: 21 ↓ 20 min into and 60 min after HVT: 126 ↑	A priori: 3 ci-miRs
Margolis et al. 2016	Healthy, inactive, young (n=9) and old (n=9) age 22±1 and 74±2 yr	Bilateral leg extension and leg press, 3 sets of 10 reps at 80% 1RM	Serum - immed. and 6 hrs after	Six hours after exercise in young: 17-5p, 19a-3p, 19b-3p, 20a-5p, 26b-5p, 93-5p, 106-5p, 143-3p, 195-5p ↑	90 miRNAs (an array of 84 plus 6 skeletal muscle-enriched chosen a priori)
Min et al. 2016	Healthy (except for hypercholesterolemia) male and female boston marathon runners taking (n=28) or not taking (n=28) statins; age 53.0±6.5 and 56.6±8.2 yr	Marathon run	Plasma - immed. and 24 hr after	All immed. after: 134, 133a, 1, 206 ↑ Statin users only 24 hr after: 499-5p ↑	A priori: 4 muscle specific and 1 brain-enriched ci-miR
Parr et al. 2016	Low (n=18) and high (n=22) responding men and women to a weight loss intervention; age 46.3±5.7 and 48.2±6.2 yr	16 wk diet and resistance exercise training (3 times/wk) intervention	Plasma - after intervention	Both groups: 221-3p, 223-3p ↑	A priori: 13 ci-miRs
Xu et al. 2016	Male heart failure patients (n=28) age 59.1±1.8 yr	Maximal symptom limited exercise test on cycle ergometer	Serum - immed. after	21, 378, 940 ↑	A priori: 18 ci-miRs (9 skeletal/heart muscle-related)

Ci-miRNA responses are compared with resting/baseline values unless otherwise stated. CKD, chronic kidney disease; DM, diabetes mellitus; HIIE, high-intensity interval exercise; HVT, high-volume continuous exercise; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; immed, immediately; VICE, vigorous intensity continuous exercise.

Table 3.2. Summary of results on skeletal and cardiac muscle enriched/specific ci-miRNAs

	Protocol	Subjects	Ci-miRNA response							Reference
Acute			**1**	**133(a)**	**133b**	**206**	**208a**	**208b**	**499**	
	Maximal cycling test	Healthy, trained men		↔						Baggish et al. 2011
	Maximal cycling test	Healthy men and women		↔						Uhlemann et al. 2014
	Maximal cycling test	Men with heart failure	↔	↔	↔		↔	↔	↔	Xu et al. 2016
	Maximal running test	Healthy, inactive men	↓	↓						Denham et al. 2016
	Sprint cycling	Healthy, active men	↓	↓	↓	↔			↔	Cui et al. 2015
	High intensity interval	Healthy, trained men	↑	↑	↑	↑				Cui et al. 2016
	Endurance cycling	Healthy, trained men	↑	↑	↑	↑				Cui et al. 2016
	Endurance cycling	Healthy, trained men	↑	↑	↑	n.d.	n.d.			Nielsen et al. 2014
	Endurance cycling	Healthy, trained men		↔						Uhlemann et al. 2014
	Endurance cycling	Healthy men	↔	↔	↔	↔		n.d.	n.d.	Aoi et al. 2013
	Uphill walking	Healthy, active men	↔	↔	↔	n.d.	n.d.	↔	↔	Banzet et al. 2013
	Downhill backwards	Healthy, active men	↑	↑	↑	n.d.	n.d.	↑	↔	Banzet et al. 2013
	Endurance running	Healthy men	↔	↔	↔	↔			↔	Guescini et al. 2015
	Half marathon	Healthy, active men	↑	↑		↑				Gomes et al. 2014
	Marathon and 10 km run	Healthy, trained men	↔	↔	↔	↔				de Gonzalo-Calvo et al.
	Marathon	Healthy, trained men	↑	↑			↑		↑	Baggish et al. 2014
	Marathon	Healthy, trained men	↑	↑		↑		↑	↑	Mooren et al. 2014
	Marathon	Healthy, trained men		↑						Clauss et al. 2016
	Marathon	Trained men and women with hypercholesterolemia	↑	↑		↑			↔	Min et al. 2016
	Marathon	Trained men and women with hypercholesterolemia using statins	↑	↑		↑			↑	Min et al. 2016
	Marathon	Healthy, trained men		↑						Uhlemann et al. 2014
	Resistance exercise	Healthy, active men		↔						Sawada et al. 2013
	Resistance exercise	Trained men and women		↑						Uhlemann et al. 2014
	Resistance exercise	Healthy, inactive men	↔	↔		↔		↔		Margolis et al. 2016
Chronic										
	Cycling training	Healthy men	↔	↔	↔	↔		n.d.	n.d.	Aoi et al. 2013
	Cycling training	Healthy, trained men		↓						Nielsen et al. 2014
	Rowing training	Healthy, trained men		↔						Baggish et al. 2011

n.d., not detected

Table 3.3. Summary of results on endothelium enriched/specific ci-miRNAs

	Protocol	Subjects	Ci-miRNA response						Reference
Acute			**21**	**126**	**146a**	**210**	**221**	**222**	
	Maximal cycling test	Healthy, trained men	↑		↑	↔	↑	↑	Baggish et al. 2011
	Maximal cycling test	Healthy men and women		↑					Uhlemann et al. 2014
	Maximal cycling test	Men with heart failure	↑	↔	↔	↔	↔		Xu et al. 2016
	Maximal cycling test	Men and women with CKD	↔	↔	↓	↔			Van Craenenbroeck et al. 2015
	Maximal cycling test	Trained men and women with CKD	↔	↔	↔	↓			Van Craenenbroeck et al. 2015
	Maximal walking test	Men with intermittent claudication		↑					da Silva Jr. et al. 2015
	High intensity interval cycling	Healthy, trained boys	↓	↔					Kilian et al. 2016
	Endurance cycling	Healthy, trained boys	↔	↑					Kilian et al. 2016
	Endurance cycling	Healthy, trained men	↔		↓		↓		Nielsen et al. 2014
	Endurance cycling	Healthy, trained men		↑					Uhlemann et al. 2014
	Marathon and 10 km run	Healthy, trained men	↔	↔	↔		↔		Gonzalo-Calvo et al. 2015
	Marathon	Healthy, trained men		↑	↑				Baggish et al. 2014
	Marathon	Healthy, trained men	↔						Mooren et al. 2014
	Marathon	Healthy, trained men		↑					Uhlemann et al. 2014
	Resistance exercise	Healthy, active men	↔		↓	↔	↓	↔	Sawada et al. 2013
	Resistance exercise	Trained men and women		↔					Uhlemann et al. 2014
	Resistance exercise	Healthy, inactive men	↔	↔	↔	↔	↔	↔	Margolis et al. 2016
Chronic									
	Cycling training	Healthy, trained men	↓		↔		↔		Nielsen et al. 2014
	Cycling training	CKD patients	↔	↔	↔	↔			Van Craenenbroeck et al. 2015
	Rowing training	Healthy, trained men	↑		↑	↔	↑	↑	Baggish et al. 2011
	Exercise training and diet control	Patients with impaired fasting glucose/glucose tolerance or		↑					Liu et al. 2014
	Resistance training and diet intervention	Overweight and obese men and women	↔	↔			↑		Parr et al. 2016

Figure 3.1

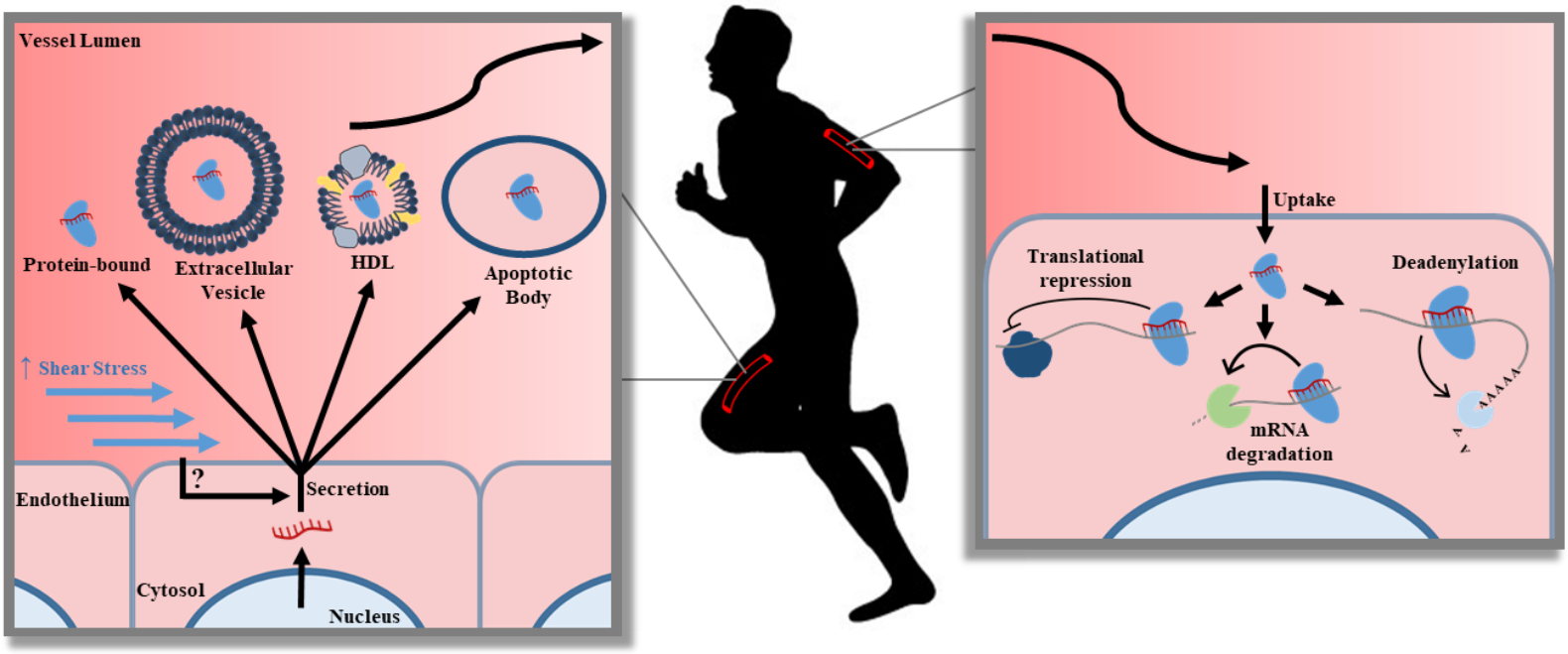

Figure 3.1. Circulating microRNAs (ci-miRs) are secreted from various tissues, including the endothelium, in response to exercise. MiRs are transported from the nucleus (details of biogenesis are omitted) into the cytoplasm, where they associate with proteins (Argonaute, miRISC) and are secreted into the bloodstream. Ci-miRs may circulate in association with proteins or may additionally be packaged into extracellular vesicles (exosomes or microvesicles), high-density lipoproteins (HDLs) or released in apoptotic bodies. Increased shear stress along the endothelium is one mechanism potentially responsible for the altered ci-miR profile observed with exercise, because it may stimulate the secretion of specific miRs from endothelial cells. Ci-miRs may then be absorbed from the circulation by distant tissues, where they exert their effects by regulating gene expression. MiRs most often cause downregulation or inhibition of translation in target cells by inducing translational repression, mRNA degradation, or deadenylation of complementary mRNAs. Ci-miRs altered with acute and chronic exercise are proposed to mediate/moderate beneficial adaptations to training through translational downregulation of their target mRNA transcripts. We propose that ci-miRs released from the endothelium in response to exercise-induced increases in shear stress may be taken up by endothelial cells in vessels of inactive tissues to facilitate adaptations. For specific details of biogenesis, see (189, 404).

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Chapter 4: Circulating microRNAs: Advances in Exercise Physiology

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Circulating microRNAs: Advances in exercise physiology

Ryan M. Sapp and James M. Hagberg

Department of Kinesiology, School of Public Health, University of Maryland,

College Park, MD, USA

Correspondence: Ryan M. Sapp, 2242 Valley Drive, College Park, Maryland 20742-

2611. (301)-405-2487 (TEL), (301)-405-5578 (FAX). rsapp@umd.edu

Abstract

Circulating microRNAs (ci-miRs) are post-transcriptional regulators of gene expression released by cells into blood or other biofluids. Acute and chronic exercise have been shown to alter the profile of ci-miRs. The past few years have seen an upsurge in research detailing the exercise responses of ci-miRs and investigating their utility as biomarkers for various conditions. The functions of ci-miRs as paracrine/endocrine mediators of systemic adaptations to exercise training are also strongly suggested, but direct evidence is still lacking. The purpose of this review is to provide an update on recent advancements concerning ci-miRs in the field of exercise physiology.

Introduction

MicroRNAs (miRs) are small non-coding RNAs that act as fine-tuning regulators of the transcriptome by repressing the translation of target mRNAs (34). Cells secrete miRs into circulating blood and other biofluids either actively or passively in response to a range of stimuli. Circulating miRs (ci-miRs) are promising biomarkers of both diseases and exercise responses that may also be missing links in understanding the mediators of systemic adaptations to exercise training. Recently there has been an upsurge in research on the effects of acute and chronic exercise on ci-miRs. Current focal points and advances in the field are discussed in this review.

Concurrent Analyses in Skeletal Muscle and Different Circulating Fractions

One current hypothesis is that cells release miRs in response to exercise as a means of offloading them in order to allow increased intracellular translation of proteins necessary for adaptation (406). This hypothesis is supported by results showing that increased ci-miR expression six hours after a resistance exercise bout correlated positively with intramuscular expression of phosphorylated proteins in the MTORC1 pathway, favoring hypertrophy (320). Several of the altered ci-miRs are part of the miR-17~92 cluster that regulates PTEN, an inhibitor of Akt-mTOR signaling. Additionally, following four weeks of endurance training, mice exhibited increased ci-miR-133a coinciding with decreased skeletal muscle expression of miR-133a and upregulated mRNA expression of its target, serum response factor (SRF) (406). Those results also raise the potential of ci-miRs as biomarkers reflecting exercise responses of their source tissue. For example, ci-miRs found to increase in young men following resistance exercise and correlating with hypertrophic responses in muscle were not altered in older men, potentially reflecting resistance to anabolic stimuli with aging (320).

In contrast, D'Souza et al. (106) found that only one (miR-23b-3p) of 38 miRs showed a correlation between plasma and muscle expression at rest, while changes in the two sources with resistance exercise were not related (104). Of note, miR-133a-3p increased in both muscle and plasma following exercise, though at different time-courses (104). Further, ci-miRs were found to be poor predictors of muscle size and strength (106). In another investigation, D'Souza et al. (105) compared exercise-induced changes in miR expression within the circulating exosome fraction, as well as

in plasma and muscle. There were differences between fractions relative to which miRs were altered with exercise, as well as time-courses and magnitudes of changes. The results of that study do not support the hypothesis that ci-miRs reflect the exercise-induced offloading of miRs from muscle, as no miRs were decreased in muscle and increased in either circulating fraction. However, it does suggest that miRs are selectively altered and exported in the exosomal or non-encapsulated forms, probably reflecting unique purposes. This supports another hypothesis, that miRs are released by cells to serve as paracrine/endocrine molecules taken up by distant recipient cells, resulting in adaptations. These hypotheses represent two paradigms regarding ci-miRs in the field of exercise physiology, as they are currently regarded as potential biomarkers of exercise responses and paracrine/endocrine mediators of systemic physiological processes (Figure 4.1).

Ci-miRs are Sensitive to Different Protocols of Acute Exercise and Training

A number of recent studies have assessed the differential responses of ci-miRs to specific exercise protocols. Cycling protocols differing greatly in volume and intensity had contrasting effects on the vascular-related ci-miRs-21 and -126 (525). By having men run at varying speeds for a set duration, and varying durations at a set speed, Ramos et al (406) were able to identify ci-miRs exhibiting dose-dependence to either exercise intensity or duration, as well as those affected at a certain threshold but with no dose-response. Similarly, out of a panel of 74 ci-miRs related to cardiac (patho)physiology, five and 19 ci-miRs were altered in response to a 10km and marathon race, respectively (112). Only ci-miR-103a-3p was affected by both exercise

bouts, suggesting specific ci-miRs respond to distinct doses of exhaustive exercise (112). Those results are similar to a previous study by the same authors comparing the responses of inflammation-related ci-miRs, where only ci-miR-150-5p increased immediately after a 10km race, compared to 12 ci-miRs that increased after a marathon, not including ci-miR-150-5p (113). Resistance exercise protocols matched for absolute workload but differing in volume, intensity, and rest interval length caused contrasting responses of ci-miRs related to muscle and the vasculature (99). Effects were seen immediately, one hour, and up to 24 hours after exercise (99).

Schmitz et al. (446, 447) investigated the effects of different exercise training protocols on resting concentrations of select ci-miRs, as well as the effects of acute bouts of exercise before and after training. Four weeks of high intensity interval (HII) running training protocols differing in intensity, volume, or work/rest interval durations had distinct effects on resting levels of specific ci-miRs. There were also contrasting effects of the different acute protocols, as well as differences in the acute effects of each protocol before compared to after training (446, 447). Multiple studies have found that ci-miRs altered by HII cycling training were unchanged with an acute bout (118, 119). Additionally, there were both similar and unique changes in resting levels of select ci-miRs following 5-8 weeks of explosive as compared to hypertrophic-based resistance training or HII running (219). Thus, ci-miRs appear to be sensitive to exercise training status, type, dose, intensity, and work/rest interval durations.

The functional relevance of training intensity-dependent changes in ci-miRs has been suggested. In mice, the amount of miR-126 in circulating exosomes originating from circulating angiogenic cells showed dose-dependent increases with training

intensity (312). Further, the exosomes exerted protective effects on cultured endothelial cells that were greatest from the most highly trained mice and were dependent on transferred miR-126.

Acute and chronic exercise reveal the potential utility of ci-miRs in various conditions

Aging

As mentioned, ci-miRs may reflect changes in skeletal muscle function with aging. Ci-miRs-21 and -146 increased over a ten year period and, combined with age, predicted declines in sprint time and measures of strength in masters athletes (255). Baseline levels of ci-miR-181a-5p and changes in ci-miR-92a-3p, but not other health/performance measures, predicted changes in walking gait speed following a five month training intervention in older obese individuals (563). Elevated levels of a set of four muscle-specific ci-miRs were strongly associated with lower rates of whole body protein synthesis in older men (321). In older, postmenopausal women, specific ci-miRs did not differentiate sarcopenia or osteoporosis status, but were found to correlate with muscular power and bone mineral content (82). Relatedly, similar ci-miRs associated with bone fracture risk were decreased in young men following HII training and were more sensitive than other biomarkers of bone turnover (432).

Muscle Damage and Repair

Separate from the idea of selective secretion discussed above, damaged muscle cells passively leak miRs into circulation. Therefore, ci-miRs are promising biomarkers of muscle damage. While classical biomarkers cannot discriminate fiber-type specific

damage, Siracusa et al. (465) identified a highly accurate set of three ci-miRs (133b-3p, 206-3p, and 434-3p) able to differentiate slow versus fast skeletal muscle damage. Additionally, these ci-miRs were elevated 12 hours after the muscle-damaging event, which would allow earlier detection of damage compared to some conventional biomarkers (465). Such distinct time courses may explain why changes in ci-miRs-133a and -206 failed to correlate with changes in circulating creatine kinase (CK) or cardiac troponin immediately following a half marathon, despite all markers increasing (108). These results also do not rule out the possibility that these miRs were secreted actively in response to the exercise bout and suggest that, at least immediately after exercise, they should not be used as markers of exercise-induced muscle damage.

Skeletal muscle-specific miRs were unaltered in extracellular vesicles (EVs) two and 24 hours after muscle damaging exercise, though miR-31 content was decreased at 24 hours post-exercise (304). Interestingly, miR-31 targets the satellite cell activator Myf5 to promote quiescence. Thus, reduced transport of miR-31 to satellite cells following muscle damage may allow for greater activation and muscle repair, although this is highly speculative and such a mechanism remains to be demonstrated. Ci-miRs-29a-3p and-495-3p were proposed as potential contributors to muscle repair/recovery, as resting levels in patients with critical limb ischemia due to severe peripheral arterial disease were different from those in elite cyclists specifically in the exercise recovery period, but not at rest (192).

Training Load and Adaptations in Athletes

Ci-miRs are altered in athletes in response to soccer- or basketball-specific training (130, 292). After three months of basketball training, changes in ci-miR-221 correlated with exercise capacity and serum CK (292), while following two months of soccer training, the exosomal content of ci-miR-29a correlated with estimated VO_{2max} (130). Further, the ci-miR response following a tennis match differed between two athletes, while other physiological markers responded similarly, suggesting changes in ci-miR may vary in well-matched athletes exposed to a similar stimulus (217). Ci-miRs could be used as biomarkers of training load or adaptation in athletes. For example, changes in ci-miRs following resistance exercise correlated with changes in circulating inflammatory markers and hormones, including IL-10, cortisol, testosterone/cortisol ratio, and IGF-1 (99). Thus, exercise-induced ci-miR responses in athletes could reflect the balance between states of adaptation and overtraining, though this requires further study.

Hicks and colleagues (213) recently used saliva as a source to determine exercise-induced changes to the entire profile of ci-miR and mRNA. Salivary ci-miRs were altered in distance runners following a long training run, as were several mRNAs predicted as targets of the altered ci-miRs. The predicted pathways targeted by the exercise-regulated ci-miRs were involved in metabolism, fluid regulation, and cardiac conduction. A number of ci-miRs additionally correlated with post-exercise heart rate changes, while there were also sex-specific differences. This study provides evidence that salivary ci-miRs are potentially attractive non-invasive biomarkers in exercise physiology.

Obesity

Substantial recent work has assessed the effects of acute and chronic exercise on vascular and inflammation-related ci-miRs in obese individuals. Multiple studies have found higher resting levels of pro-inflammatory ci-miRs in obese individuals compared to healthy weight controls (30, 425). A 30 minute bout of moderate intensity running also increased endothelial and inflammation-related ci-miRs to a greater degree in obese individuals (30), while a three month physical activity intervention lowered pro-inflammatory ci-miR-146a-5p to levels comparable to their lean counterparts (425). Combined use of the baseline level and change in ci-miR-146a-5p was suggested as a predictive biomarker able to identify responders and non-responders to an exercise training intervention (425).

After six weeks of an exercise training and caloric restriction intervention, obese adolescents displayed increased ci-miR-126 to levels comparable to normal weight controls, which were strongly correlated with changes in BMI, microvascular endothelial function, and circulating NO/ET-1 ratio (132). Eight weeks of HII training increased microparticle number and content of miR-146a, but not -126, in both obese women and normal weight controls (124). Other miRs related to inflammation and vascular health were increased in one or both groups. While no correlations were found between the microparticle abundance of any miRs and microvascular function, miR-150 was found to correlate with circulating nitrite and advanced oxidation protein products (oxidative stress) (124). Following gastric bypass surgery, an exercise training intervention altered the expression of ci-miRs related to metabolism and vascular function (370). Changes in ci-miRs correlated with changes in insulin resistance, body

composition, lipids, and measures of β-cell and liver function (370). Thus, ci-miRs are altered with exercise training in obese individuals and have the potential to act as biomarkers of metabolic, inflammatory, and vascular adaptations to exercise training. The above results also suggest the potential for mechanistic roles of ci-miRs in these processes.

Cardiometabolic Diseases

Ci-miRs are proposed as prognostic and diagnostic biomarkers of cardiometabolic diseases. An acute bout of resistance, but not aerobic, exercise increased ci-miR-146a in older diabetic patients, while nondiabetic patients did not respond to either bout (347). Combining the results of a VO_{2max} test with pre- or post-exercise levels of select ci-miRs allowed the accurate discrimination between patients with coronary artery disease (CAD) and healthy controls (327). In contrast to that study which investigated 187 ci-miRs, Hortman et al. (221) focused specifically on the exercise-induced changes of three ci-miRs but did not find any clinical value related to the diagnosis of CAD. In pulmonary hypertension patients, 6 min walk distance at baseline correlated with the change in a set of ci-miRs with reported roles in muscle function to three weeks of exercise training or one week of nightly supplemental oxygen interventions (184).

Neuromuscular Disease

Individuals with spinal cord injury are at elevated risk for cardiovascular disease (CVD). Athletically active spinal cord-injured individuals displayed a different ci-miR profile than their sedentary counterparts, which was also more similar to able-bodied participants (383). Several of these ci-miRs correlated with circulating oxidized

LDL and carotid intima-media thickness. Thus, ci-miRs could act as biomarkers for CVD risk in spinal cord-injured individuals and may additionally act as underlying regulators of CVD development with chronic inactivity. Amyotrophic lateral sclerosis (ALS) patients display higher levels of skeletal muscle-derived ci-miRs than healthy controls (493). Six weeks of concurrent aerobic and resistance exercise training effectively lowered muscle-derived ci-miRs in patients with ALS (389).

There are both symptom overlap and problems with currently used questionnaires to diagnose disorders of fatigue/depression, so ci-miRs offer an attractive objective diagnostic alternative. Baraniuk et al. (32) performed the first study on the effects of exercise on ci-miRs in cerebrospinal fluid, from patients with Gulf War Illness or Chronic Fatigue Syndrome, as well as healthy controls. There were no differences in baseline levels of ci-miRs between groups, though there were distinct effects of exercise that distinguished each group. These results suggest that exercise-induced cerebrospinal fluid ci-miRs are potential biomarkers able to help diagnose and discriminate between these similar diseases and even separate phenotypes of Gulf War Illness.

Breast Cancer

There were no changes in skeletal muscle or cancer-associated ci-miRs following 16 weeks resistance training in breast cancer survivors (190). However, when separated as responders and non-responders based on strength changes, responders had increased levels of ci-miR-133a-3p and -370-3p with training. In another study of breast cancer survivors who underwent a 6 month exercise and diet intervention, ci-

miR-106b-5p, a prognostic marker of breast cancer risk and recurrence (568), was decreased (1).

Ci-miRs as physiological mediators of systemic responses to exercise

The roles of ci-miRs as paracrine/endocrine molecules mediating mechanistic responses to exercise are suggested mostly by correlational relationships with various phenotypes or other molecular markers in previous studies (436) and those discussed above. However, direct experimental evidence is lacking. The function of ci-miRs as cell-to-cell messengers is supported by the facts that ci-miRs contained either in EVs or freely bound to a protein carrier (Argonaute 2) are selectively secreted, highly stable, and can be selectively taken up by recipient cells where they are biologically active (193, 241, 242, 312, 569). Still, major hurdles in the field are the unknown cellular origin and destination of ci-miRs. Only a handful of ci-miRs are highly enriched in specific cell types (e.g. endothelium or striated muscle).

Another question yet to be answered is what stimuli trigger the release of ci-miRs with exercise. Suggested stimuli include laminar shear stress of blood along endothelial cells (436, 446, 525), hypoxia (446, 525), lactate (447), inflammation (30, 425), biomechanical stimulation (432), muscle contraction (186) and cell damage (304, 465). Shear stress, hypoxia, and inflammation have been shown to modulate the cellular release of miRs *in vitro* (9, 193, 212, 569). It is also currently unknown whether changes in ci-miR are primarily a result of de novo production or export of stored endogenous miRs. The balance between production, export, uptake and degradation of

ci-miRs must be taken into consideration when attempting to explain their kinetics, and little is understood regarding these aspects.

Conclusions

The literature on ci-miRs in exercise physiology has more than doubled over the past few years. The majority of studies have sought to determine the utility of blood-borne ci-miR responses to acute and chronic exercise as biomarkers. In this context, novel sample types including cerebrospinal fluid and saliva have recently been investigated as ci-miR sources (32, 213). Additionally, innovative methods of ci-miR quantification such as single droplet digital PCR (221) and multiplex via flow cytometry (Firefly) (1) or NanoString nCounter (563) have been employed and carry advantages over real-time PCR (429). Small RNA sequencing has also been used to study the effects of acute exercise and training on the entire profile of circulating small RNA species (119, 453). Despite promising advances, hesitation has been expressed regarding the utility of ci-miRs as biomarkers, given the current lack of similarities in study designs/methods and reproducibility of findings (153). These limitations are to be expected in such a nascent field, and we believe that studies over the coming years will clarify issues and begin to reveal utility of exercise-regulated ci-miRs in clinical and sports medicine settings. Specifically, larger-scale, validation studies with longer participant follow-ups will be needed. Sample processing and analysis/normalization will also need to become more standardized before ci-miRs can become reliable biomarkers (285). Lastly, investigations aimed at elucidating mechanisms underlying

ci-miR responses to exercise and the mechanistic roles of these ci-miRs will be crucial in revealing their true purpose and potential.

Table 4.1. Associations with circulating microRNAs in studies of exercise and fitness

Relevant condition	Ref.	Subjects	Stimulus	Sample type	Circulating microRNAs	Associations
Aging & muscular function	Margolis et al. 2017a	Young & older, healthy men	Acute resistance exercise	Serum	(A, B) 19b-3p, 206, 486 (C) 19a-3p, 19b-3p, 20a-5p, 26b-5p, 143-3p, miR-195-5p	(A) age (B) fat-free mass (C) skeletal muscle p-Akt^{Ser473} & p-S6K1^{Thr389}
	D'Souza et al. 2019	Middle-aged, healthy men	–	EDTA plasma	(A, C) 146a-5p (B, C) 451a (D) 222-3p, 361-5p	(A) age (B) total body lean mass (C) leg lean mass (D) thigh muscle cross-sectional area
	Kangas et al. 2017	Masters athletes	10 year follow-up	Serum	(A, B) 21-5p, 146a-5p (C) 146a-5p	(A) knee flexion strength (B) bench press strength (C) 60m sprint time
	Zhang et al. 2017	Sedentary, obese, older men & women	5 month aerobic training	EDTA plasma	BL 181-5p, Δ 92a-3p	Δ gait speed
	Margolis et al. 2017b	Older, overweight men	4 weeks 30% energy restriction diet	Serum	1 + 133a-3p + 133b + 206	Whole body protein synthesis
	Chen et al. 2019	Older, postmenopausal women	–	Serum	(A, B) 125b-5p (C) 21-5p (D) 133a-3p (E) 23a-3p	(A) age (B) jump velocity & power (C) trochanter bone mineral content (D) total body bone mineral content (E) serum TRAP5B
	Sansoni et al. 2018	Young, healthy men	8 weeks sprint interval training or control	EDTA plasma	(A) 93-5p, 122-5p (B) 100-5p, 122-5p (C) 93-5p, 100-5p, 122-5p, 148-3p	(A) serum sclerostin (B) osteoprotegerin (C) osteocalcin (control group only)
Muscular damage	Siracusa et al. 2018	Male & female Wister rats	Muscle crushing injury	EDTA plasma	133b-3p + 206-3p + 434-3p	Damage specific to EDL (fast) or soleus (slow) muscles
Adaptations in athletes	Cui et al. 2017	Young, healthy men	Acute resistance exercise	EDTA plasma	(A) 532 (B) Δ 133a	(A) IGF-1 & IL-10 (B) Δ cortisol & cortisol/testosterone ratio
	Schmitz et al. 2018	Young, healthy men & women	Acute bout HII exercise & 4 weeks HII training	Whole blood	(A) post-training 222-3p & 29c-3p (B) Δ 222-3p with acute exercise	(A) post-training circulating TGF-β1 (B) Δ running speed at individual "anaerobic threshold" with training
	Domanska Senderowska et al. 2017	Young, male soccer athletes	2 months soccer training	Serum exosomes	Post-training 29a	Post-training VO_{2max}
	Li et al. 2018	Young, male basketball athletes	3 months basketball training	Serum	(A) Δ 208b (B) post-training 221	(A) Δ "anaerobic threshold" VO_2 (B) post-training "anaerobic threshold", peak workload, & creatine kinase
	Hicks et al. 2018	Young, male & female distance runners	~18km run	Saliva	(A) Δ twenty-six ci-miRs (B) twenty-four ci-miRs (C) twenty-three ci-miRs (D) two ci-miRs	(A) Δ target mRNA(s) (B) Δ heart rate (C) sex (D) age

Obesity	Russo et al. 2018	Middle-aged, obese men & women	3 months exercise training	EDTA plasma	(A) BL 146a-5p (B) post-training 146a-5p	(A) BL total cholesterol & waist circumference (B) post-training age & body weight
	Donghui et al. 2018	Obese adolescent boys	6 weeks exercise training & caloric restriction	Serum	Δ 126	Δ BMI, Δ reactive hyperemia index, Δ NO/ET-1
	Dimassi et al. 2018	Normal weight or obese, young women	8 weeks HIl training	Plasma microparticles	(A) 124a (B) 150	(A) HDL, triglycerides, TNF-α (B) nitrites, TBARS, AOPP, adiponectin
	Lopez et al. 2018	Obese men & women	6 months exercise training or control following gastric bypass surgery	Heparin plasma	BL: 7, 15a, 106b, 135b, Δ: 7, 15a, 106b, 135b, 122, 206, 149, 221, 34a, 149	Measures of β-cell function & insulin resistance, bone mass, BMI, waist circumference, fat mass, cholesterol, LDL, measures of liver function
Coronary artery disease	Mayr et al. 2018	Middle-aged to older men & women with or without CAD	VO_{2max} test	EDTA plasma	BL 150-5p + post-exercise 101-3p + 141-3p + 200b-3p	Presence of CAD
Pulmonary hypertension	Grunig et al. 2018	Middle-aged to older men & women with pulmonary hypertension	3 weeks exercise training or 1 week nightly oxygen supplementation	Heparin plasma or serum	Δ 22-3p + 21-5p	6 minute walk distance
Spinal-cord injury	Paim et al. 2018	Young, active or sedentary spinal cord-injured patients	–	Serum	(A, B) 146a-5p, 328-3p, 191-5p, 103a-3p, 125b-5p, 30b-5p (A) 125a-5p (B) 301a-3p, 766-3p, 28-5p, 146b-5p, 126-5p, 145-5p, 26b-5p, 26a-5p	(A) oxidized LDL (B) carotid intima-media thickness
Disorders of fatigue/depression	Baraniuk et al. 2017	Men & women with Gulf War Illness	Acute submaximal cycling exercise	Cerebrospinal fluid	Post-exercise 22-3p, 9-3p	START vs. STOPP Gulf War Illness phenotypes
Breast cancer	Hagstrom and Denham 2018	Breast cancer surviving women	16 weeks resistance training or control	Serum	(A) Δ 133a-3p, 133b-3p (B) Δ 370-3p	(A) Δ Leg press strength (B) Δ non-surgical arm strength
	Adams et al. 2018	Postmenopausal, breast cancer surviving women	–	Serum	191-5p, 17-5p. 103a-3p, 93-5p, 22-3p, 122-5p, 126-3p, 150-5p, 27a-3p, 195-5p, 10a-5p, 30d-5p	Measures of body composition, bone mineral content & density, CRP, IL-6, glucose, insulin, leptin

BL, baseline; Δ, change in; EDL, extensor digitorum longus; IGF-1, insulin-like growth factor 1; IL, interleukin; HII, high intensity interval exercise; TGF-β1, transforming growth factor beta 1; VO_{2max}, maximal oxygen consumption; BMI, body mass index; NO/ET-1, nitric oxide/endothelin-1; HDL, high density lipoprotein; LDL, low density lipoptrotein; TNF-α, tumor necrosis factor alpha; TBARS, thiobarbituric acid reactive substances; AOPP, advanced oxidation protein products; CAD, coronary artery disease; START, stress test activated reversible tachycardia; STOPP, stress test originated phantom perception; CRP, c-reactive protein

Figure 4.1

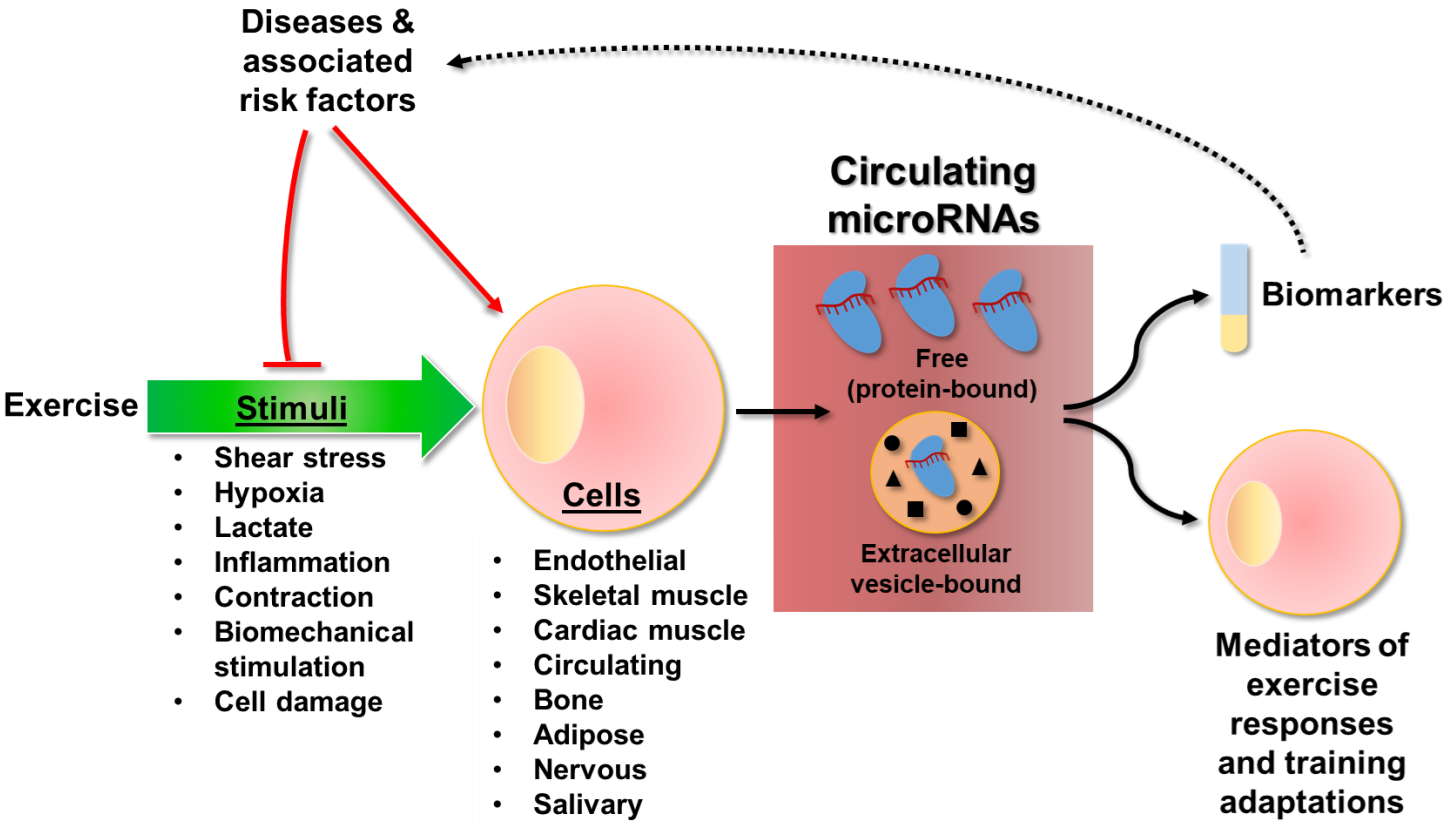

Figure 4.1. Circulating microRNAs (ci-miRs) are secreted by various cell types in response to acute and chronic exercise. Exercise stimuli influencing the release of ci-miRs are suggested, but require experimental validation. Diseases and associated risk factors (e.g. aging, obesity) influence the ci-miR profile and responses to exercise. Therefore, exercise-induced ci-miRs are potential biomarkers for athletes, as well as prognostic and diagnostic biomarkers of diseases. Further, they are potential paracrine/endocrine mediators of systemic responses to exercise and adaptations to exercise training.

Conflict of interest statement: Nothing declared.

Chapter 5: Sex-specific alterations in blood-borne factors in physically inactive individuals are detrimental to endothelial cell functions

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Sex-specific alterations in blood-borne factors in physically inactive individuals are detrimental to endothelial cell functions

Ryan M. Sapp[1], Rian Q. Landers-Ramos[2], Daniel D. Shill[1], Catherine B. Springer[1], James M. Hagberg[1]

[1]Department of Kinesiology, School of Public Health, University of Maryland, College Park, MD, USA

[2]Department of Kinesiology, Towson University, Towson, MD, USA.

Running title: Circulating factors associated with sex & habitual exercise

Correspondence: Ryan M. Sapp, 2242 Valley Drive, College Park, Maryland 20742-2611. (301)-405-2487 (TEL), (301)-405-5578 (FAX). rsapp@umd.edu

Abstract

Mechanisms underlying the protective effects of both habitual endurance exercise and the female sex on vascular function are incompletely understood. Blood-borne circulating factors, such as circulating microRNAs (ci-miRs), may partially explain these effects. Blood samples were obtained from young, healthy men and women who either habitually performed endurance exercise (endurance trained) or were relatively inactive (sedentary). Women were tested during the early follicular phase of the menstrual cycle or placebo pill of oral contraceptive to control for estrogen. Cultured human umbilical vein endothelial cells (HUVEC) were exposed to participants' serum in migration, proliferation, and reactive oxygen species (ROS) assays. RT-qPCR was used to quantify an initial array of 84 CVD-related ci-miRs, followed by validation of 10 ci-miRs. All participants were devoid of traditional CVD risk factors, and circulating estradiol concentration was not different between groups. Serum of endurance trained women induced greater HUVEC migration compared with serum of sedentary women. HUVEC ROS production was greater in response to serum of sedentary men compared to endurance trained men and sedentary women. There were sex-effects on the levels of 9 ci-miRs, with greater levels in men, while ci-miRs-140-5p and 145-5p were also higher in sedentary compared to endurance trained men and/or women. In a sex-specific manner, habitual endurance exercise was associated with enhanced endothelial migration and reduced ROS production in response to serum. Thus, alterations in circulating factors may contribute to the protective effects of habitual endurance exercise on vascular health. Additionally, sex had a greater impact than habitual activity level on the levels of vascular-related ci-miRs.

Introduction

Physical inactivity is a major independent risk factor for cardiovascular disease (CVD), which remains the primary global cause of death (39, 397). The endothelium is a monolayer of cells lining the lumen of all blood vessels that is critical in maintaining proper vascular function and preventing atherogenesis (63). The effects of chronic inactivity manifest as impaired endothelial-dependent dilation and adverse structural remodeling of the arteries (54, 451, 498). Conversely, regular physical activity improves endothelial function and protects against CVD, with the most active individuals showing up to a 40% reduction in CVD risk (122, 182, 451, 459). The primary roles of both reductions in traditional risk factors and changes in hemodynamics in mediating the positive effects of regular exercise, and negative effects of inactivity, on vascular health have been well described (157, 182, 381, 501). However, the training-induced decrease in CVD risk and improvements in vascular function cannot be fully attributed to the attenuation of traditional risk factors (158, 162, 183), and are not likely fully explained by changes in blood flow (382). Research suggests that circulating factors in the blood may be partially responsible for the vascular protection conferred by regular exercise, though the association between habitual exercise and the effects of the circulating milieu on endothelial cells has not been determined (157, 158, 316, 382).

Additionally, premenopausal women exhibit lower rates of CVD in comparison to age-matched men (39). These sex-based disparities are hypothesized to be largely dependent on sex hormones, primarily estrogen which exerts numerous protective effects on the endothelium in women (46, 181, 350, 473). However, a recent study

including thousands of participants found sex differences in 61 of 71 investigated circulating proteins involved in CVD development, with the greatest differences apparent between premenopausal women and age-matched men (281). Thus, other circulating factors likely contribute to disparities in CVD risk between men and women, and it may be useful to determine sex-based differences in the effects of all blood-borne factors on the endothelium, excluding differences in estrogen.

MicroRNAs (miRs) are a relatively novel class of blood-borne molecules proposed to mediate the effects of exercise on endothelial health via post-transcriptional regulation (34, 436). Exercise training alters cardiovascular-related circulating miRs (ci-miRs) (350, 366) but given the difficulty in performing long-term exercise training studies, long-term effects (>6 months) of habitual exercise on the ci-miR profile are not known. Additionally, sex-based differences in the expression of select miRs have been identified and may underlie disparities in CVD development (28, 62, 98, 400, 410), but it is unclear whether habitual exercise impacts ci-miRs differently in men and women. Therefore, we employed a cross-sectional study comparing young, healthy men and women who either habitually performed endurance exercise (endurance trained) or were relatively inactive (sedentary).

We sought to determine the associations of habitual activity level and sex with 1) the effects of the circulating milieu (in serum) on endothelial cell function and 2) the levels of cardiovascular-related ci-miRs. By comparing the effects of serum derived from young individuals devoid of traditional CVD risk factors and differing only in chronic exercise habits, the aim was to identify alterations in the circulating milieu that likely play a role in the early development of CVD in physically inactive individuals.

In order to exclude acute effects of circulating estrogen, women were tested during the menstrual/early follicular phase (first three days) of the menstrual cycle or the placebo phase of oral contraceptive, when estradiol is at its lowest concentration and is similar to that in men (455, 462). It was hypothesized that endothelial cells would respond better (enhanced endothelial migration and proliferation, reduced ROS production) to serum from endurance-trained individuals as compared with serum from sedentary individuals, and to serum from women compared to men in both groups. Likewise, we hypothesized habitual endurance exercise- and sex-based differences in the levels of cardiovascular-related ci-miRs, such that ci-miRs which promote inflammation and oxidative stress would be greater in sedentary compared to trained individuals, and in men compared to women.

Methods

Ethical Approval

All procedures and documents conformed to the Declaration of Helsinki and were approved by the University of Maryland Institutional Review Board (IRB) prior to participant recruitment. Before beginning any testing, all participants were provided both a verbal and written explanation of the study, and gave their written informed consent.

Participants

Healthy men and women of any race/ethnicity between the ages of 18-39 years who reported being either endurance trained or sedentary were recruited for this study. Physical activity status was determined first by a questionnaire regarding habitual

physical activity level over at least the past five years. Self-reported habitual activity level was then corroborated by a VO_{2max} test. Endurance trained participants reported structured aerobic exercise (including running, cycling, swimming, and/or rowing) >30 minutes per day on 4 or more days/week and/or >4 hours/week. On average, the endurance trained participants had been training continuously for the past 11.5±6.4 years. The majority of the endurance trained individuals also reported that they regularly trained for and competed in endurance races. While some participants reported that they occasionally (i.e. not on a regular weekly basis) participated in other types of exercise (e.g. resistance training, yoga), endurance exercise was by far the major exercise type for each individual. Sedentary individuals did not engage in regular scheduled exercise and reported ≤20 minutes/day on ≤2 days/week of structured physical activity.

In order to confirm health status, participants completed a health history questionnaire, as well as further testing. Measurements included height and weight, seated blood pressure (BP) and heart rate (HR), and body fat measurement via the 7-site skinfold method (236). A resting, fasted blood sample was also used to assess blood chemistry for CVD risk factors. Exclusion criteria included any one of the following: prior CVD or metabolic diseases, current or past smoker, use of any potential study-confounding medications such as non-steroidal anti-inflammatories, body weight <110 pounds, body mass index (BMI) >29 kg/m2, resting HR >100 bpm, systolic BP (SBP) >140 mmHg or diastolic BP (DBP) >90 mmHg, fasted total cholesterol >200 mg/dL, low density lipoprotein cholesterol >130 mg/dL, high density lipoprotein cholesterol <40 mg/dL, or blood glucose >100 mg/dL.

Blood Sampling

Participants arrived to the laboratory in the morning after an overnight fast of ≥8 hours. Participants were also asked to refrain from caffeine overnight, alcohol, NSAIDS, or other medications for 24 hours, and from exercise for at least 16 hours prior to the visit. All women were tested during the first 3 days of their menstrual cycle or during the placebo pill phase of oral contraceptive, which were self-reported by the participants. After seated rest of ≥5 minutes, ten milliliters of blood was drawn from an antecubital vein into a serum separator tube and was allowed to clot at room temperature for 45 minutes, after which it was centrifuged at 1,500 xg for 15 minutes at 4ºC. Serum was aliquoted and stored at -80ºC until use. An additional 8-10 ml of blood was also obtained and sent for analysis of blood chemistry for screening (Quest Diagnostics, Baltimore, MD). Serum samples from some participants were used in other studies and were therefore limited. Serum from all participants was used in the endothelial migration and proliferation assays, while the number of samples used in the estradiol enzyme-linked immunoassay (ELISA), endothelial reactive oxygen species (ROS) assay, and real-time qPCR for each group were: endurance trained men (n=12), endurance trained women (n=12), sedentary men (n=11), sedentary women (n=9).

Maximal Oxygen Consumption (VO_{2max}) Test

Participants had the option to perform the VO_{2max} test on the same visit as the fasted blood draw, or on a separate, non-fasted visit. Considering this, the menstrual cycle phase/oral contraceptive phase in which VO_{2max} was assessed in women varied. Previous research suggest no effects of either menstrual cycle phase or oral contraceptive use on VO_{2max} (232, 250, 326). Following ~5 minutes of warmup,

participants completed either a treadmill or cycle ergometer exercise test during which they had a mask covering their nose and mouth to measure their oxygen consumption via indirect calorimetry. HR was monitored during the exercise with a chest strap monitor (POLAR T31). Participants ran at a self-selected speed or cycled at ≥80 rpm for the entire test, during which the treadmill grade increased by 2-3% or cycling intensity increased by 25-50 Watts every 2 minutes until volitional exhaustion. A true maximum was confirmed by achievement of at least three of the following criteria: a plateau in VO_2 (increase in VO_2 of <250 ml/min despite increased work load), HR_{max} ±10 bpm within age-predicted max, respiratory exchange ratio ≥1.15, rating of perceived exertion ≥17, or post-exercise blood lactate ≥8 mmol/L.

Estradiol ELISA

The concentration of estradiol in serum was quantified using an ELISA according to the manufacturer's protocol (Eagle Biosciences, Nashua, NH). Samples were assayed in duplicate according to the manufacturer's instructions. A spectrophotometer (Synergy H1 Hybrid Reader; BioTek, Winooski, VT) was used to measure absorbance at 450 nm immediately after stopping the reaction.

Endothelial Migration Assay

Pooled donor HUVECs were purchased (Lonza, Basel, Switzerland), grown out to passage 2 (P2), and cryopreserved until further use. Experiments were performed on HUVECs (P3-P5) grown in endothelial growth medium (EGM-2, Lonza) supplemented with 2% fetal bovine serum (FBS) at 37ºC and 5% CO2. HUVECs were harvested using trypsin plus EDTA and seeded onto the RadiusTM 96-well cell migration plate (Cell Biolabs Inc., San Diego, CA) at 25,000 cells/well overnight. Once

HUVECs reached confluency around the central circular gel the media was removed and replaced with a gel removal solution in EGM-2 without FBS for 30 minutes, followed by multiple washes using EGM-2 without FBS. EGM-2 without FBS, plus 10% human serum was then added using serum from each participant. In a dose-response experiment (data not shown), 10% human serum was chosen as the optimal concentration to induce endothelial migration and to detect differences between samples. Serum at higher concentrations resulted in impaired and incomplete migration for the majority of samples tested. Participants' serum was assessed in triplicate. Control wells in each 96-well plate were cultured with media containing neither FBS nor human serum, which induced minimal migration over 24 hours (negative control) (435). Pictures were taken manually using a microscope at 10x magnification immediately upon addition of samples (0 hour time-point). Migration was then tracked by taking pictures every 4 hours for 24 hours. The outer edge of migrating cells was traced using ImageJ (NIH) and the area was quantified at each time point. Quantification was performed separately by two blinded investigators. Migration rate at each time point was calculated as percentage of area closure over time (100 – (size of area at time point / size of initial area x 100)). Area under the curve (AUC) was then calculated using migration at time-points 0, 4, 8, 12, 16, and 24 hours.

Endothelial Proliferation Assay

HUVECs were seeded into a white-walled, clear bottom 96-well plate at a concentration of 4,000 cells/well and allowed to attach overnight. The media was then removed, cells were washed, and EGM-2 without FBS containing 20% serum from each participant was added based on previous literature and dose-response experiments

(316). Serum from each participant was added to wells in triplicate. Control wells were cultured with the same conditions used in the migration assay. After 36 hours, proliferation was assessed using a fluorometric cell proliferation assay kit (BioVision, Inc., Milpitas, CA). Wells were washed with endothelial basal media to remove any dead cells or debris, after which a cell lysis buffer and nuclear cell dye were added and the 96-well plate was gently shaken for 15 minutes. Fluorescence was read on a Fluorescence microplate reader (BioTek FLx800TBIE) at Ex/Em=485/528 nm.

Reactive Oxygen Species Assay

The effects of serum on ROS production in HUVECs was determined using the 2', 7'–dichlorofluorescin diacetate (DCFDA) Cellular ROS Detection Assay Kit (abcam, Cambridge, MA). DCFDA diffuses into cells and is converted to the fluorescent molecule 2', 7'–dichlorofluorescein (DCF) by ROS. Cells were seeded into a black, clear bottom 96-well plate at 15,000 cells/well in EGM-2 + 2% FBS without phenol red and allowed to attach overnight. HUVECs were then washed with PBS and stained with 25 μM DCFDA for 45 minutes. After removing the DCFDA and washing again, duplicate wells of cells were exposed to 20% human serum from participants in EGM-2 without FBS or phenol red for 6 hours. Following the incubation, fluorescence was read at Ex/Em=485/535 nm.

Circulating microRNA Quantification

Ci-miR concentrations were assessed in serum using quantitative real-time PCR (RT-qPCR). Although some studies have found differences in ci-miR concentrations between sample types (serum vs. plasma) potentially due to the coagulation process, ci-miR concentrations between serum and plasma are generally well correlated (171,

337, 528). We chose to use serum as a sample type since it was used in the culture-based endothelial cell assays. Total RNA was first isolated from 200 µl serum using the miRNeasy serum/plasma kit (Qiagen, Germantown, MD). Prior to addition of chloroform during the isolation process, a synthetic spike-in control (Caenorhabditis elegans miR-39 (cel-miR-39)) was added for normalization of RT-qPCR data. Two µl RNA from each sample was reverse transcribed using the miscript II RT kit (Qiagen). RT-qPCR was performed using 2.5µL input cDNA with the miScript SYBR Green PCR Kit (Qiagen, Germantown, MD). The PCR arrays were run on an ABI 7300 Real-Time PCR System (Applied Biosystems), while an Agilent Mx3005P qPCR system was used for individual ci-miR quantification.

PCR array

The Human CVD miR PCR Array (SABiosciences) was first used to investigate the expression of 84 miRs previously implicated in CVD pathologies. Samples from three participants of each activity level were chosen to be pooled and investigated in the miR array based on their performance in the migration assay. Three participants from the endurance trained group whose serum induced expedited and complete (100%) HUVEC migration in the migration assay, and three participants from the sedentary group whose serum induced impaired migration were included. This consisted of two women and one man of each activity level. RNA from these participants was pooled for each group and 2 µl was reverse transcribed into cDNA using the miscript II RT kit (Qiagen). Analysis of the array data was performed using a web-based tool (Qiagen, GeneGlobe Data Analysis Center). This tool first calibrated the cycle threshold (CT) values based on recovery of spiked-in cel-miR-39 and then

normalized to the average CT values of the five most invariant miRs between the groups for the ΔΔCT 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 sedentary 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).

Individual miR

For quantification of individual miR targets chosen both based on the results of the PCR array and selected a priori, specific miScript primer assays (Qiagen) were used and each reaction was performed in duplicate. Ci-miRs chosen for analysis based on results of the array included miRs- 23b-3p, 31-5p, 140-5p, 145-5p, and 199a-5p, while those chosen for analysis a priori included miRs- 126-3p, 146a-5p, 181b-5p, 221-3p, and 222-3p . MiR expression levels were determined using the $2^{-\Delta\Delta CT}$ method of relative quantification. For each miR within each sample, ΔCT = CT of miR – CT of spike-in control miR; ΔΔCT = ΔCT for individual sample – average ΔCT of all endurance trained individuals. Ci-miRs quantified in all samples in less than 35 cycles (CT<35) were determined as sufficiently expressed.

Statistics

All statistical analyses were performed using GraphPad Prism 8. Migration (AUC values), proliferation, ROS, and ci-miR data were analyzed using two-way ANOVAs (activity level x sex). The effects of oral contraceptive use on serum-induced endothelial responses were also determined in separate comparisons of women by two-way ANOVAs (oral contraceptive use x activity level). When a significant or

borderline significant interaction was identified, planned Fisher's LSD post-hoc comparisons were performed to compare activity levels within the sexes and sexes within the activity levels. Effect sizes were calculated for differences between groups using Hedges' g_s (273). Pearson's correlation coefficients were calculated to determine associations between ci-miR concentrations and HUVEC responses to serum in the assays. All tests were two-sided with $P<0.05$ considered statistically significant and $0.05<P<0.09$ considered borderline (i.e. trending towards) significant.

Results

Participant Characteristics (Table 5.1)

Participants were all healthy and groups were generally well-matched. There were no differences between groups in age or BMI. On average, the relative VO_{2max} of the endurance trained men ($P<0.0001$, $g_s=3.23$) and women ($P<0.0001$, $g_s=3.40$) was greater than those of their sedentary counterparts by ~50-57% (~20 $ml \cdot kg^{-1} \cdot min^{-1}$), while men in both groups also had significantly greater VO_{2max} than women of the same activity level (trained $P=0.0002$, $g_s=1.42$; sedentary $P<0.0001$, $g_s=1.86$). Similar statistical differences were seen between groups in body fat percentage, with the sedentary groups and women having greater body fat levels than the trained groups and men, respectively (all $P\leq0.0003$, $g_s=1.51$-2.47). Men had greater SBP than women of the same activity level (trained $P=0.0001$, $g_s=1.55$; sedentary $P=0.004$, $g_s=1.18$), though there was only a significant difference in mean arterial pressure between endurance trained men and women ($P=0.01$, $g_s=0.92$). Sedentary men ($P=0.002$, $g_s=1.23$) and trained women ($P=0.02$, $g_s=0.88$) both had significantly higher glucose

than sedentary women, while trained women also had significantly higher HDL cholesterol than trained men (P=0.003, g_s=1.10). Seven women in the endurance trained group (39%) and seven women in the sedentary group (50%) reported being on oral contraceptives. There were no significant differences between groups in circulating estradiol concentration (P=0.40 – 0.99, g_s=0.01-0.32).

Endothelial Cell Assays

Two-way ANOVA revealed a borderline significant interaction effect (P=0.053) of serum on endothelial cell migration AUC over 24 hours (Figure 5.1). There was a significant effect of habitual activity level (P=0.03) with no effect of sex (P=0.67). Post-hoc tests indicated that serum from endurance trained women induced significantly greater migration compared to serum from sedentary women (P=0.004, g_s=1.12), and also approached statistical significance compared to trained men (p=0.08, g_s=0.63). There were no significant interaction (P=0.15), activity level (P=0.12), or sex (P=0.87) effects on endothelial cell proliferation (Figure 5.2). There was a borderline significant interaction effect (P=0.08) on ROS production of HUVECs in response to serum (Figure 5.3). There was a significant sex effect (P=0.004) on ROS levels, with no effect of activity level (P=0.29). Specifically, serum from sedentary men caused significantly greater ROS production compared to both their endurance trained male (P=0.04, g_s=0.66) and sedentary female (P=0.002, g_s=1.09) counterparts. There was no significant difference in endothelial ROS production in response to serum of trained compared to sedentary women (P=0.61, g_s=0.40).

Comparisons of serum from women based on the use of oral contraceptives revealed no significant interaction effects or effects of oral contraceptive use on

endothelial migration (P=0.13, P=0.66), proliferation (P=0.30, P=0.40), or ROS production (P=0.90, P=0.25) (Figure 5.4). There remained a significant effect of activity level on serum-induced endothelial migration (P=0.002). Additionally, in these comparisons that only included women there was a borderline significant effect of activity level on serum-induced endothelial proliferation (P=0.066), though there was still no effect on ROS production (P=0.40).

Human Cardiovascular Disease miR PCR Array

Of the 84 miRs investigated, 70 showed appropriate abundance for analysis in samples from both the endurance trained and sedentary groups (CT ≤ 35) (Table 5.2). Controls for reverse transcription and PCR indicated acceptable performance for analysis. CT values were first calibrated based on recovery of spiked-in cel-miR-39 and data were further normalized using the five most invariant ci-miRs between groups in the array (miRs- 210-3p, 122-5p, let-7c-5p, 424-5p, and 100-5p). Ci-miRs showing >4-fold difference between groups were said to be differentially expressed. One ci-miR was more highly expressed in the trained sample, while seven were higher in the sedentary sample. Those ci-miRs and their respective fold-differences (endurance trained group/sedentary group) were miR-31-5p (317), miR-23b-3p (-5.0), miR-30c-5p (-4.2), miR-93-5p (-5.7), miR-125a-5p (-4.5), miR-140-5p (-19), miR-145-5p (-5.3), and miR-199a-5p (-7.0).

Specific ci-miR assays

Individual ci-miR quantification was performed on a set of five chosen from the array and on a set of five selected a priori based on previous literature (Figure 5.5 and 5.6). The coefficient of variation (CV) for the spike-in cel-miR-39 in each sample

was 2.2% (CT=24.7±0.54), suggesting the methods of miR isolation, reverse transcription, and PCR were reliable and did not contribute substantially to variability between samples. Ci-miR-31-5p was not reliably detected (CT>35) in all samples and was therefore excluded from further analysis. There were no significant interaction effects on any ci-miR, though there was a borderline significant interaction effect (P=0.07) on ci-miR-145-5p (Figure 5.5A). All ci-miRs exhibited significant sex effects, while there were also significant effects of activity level on ci-miRs- 140-5p (P=0.02) (Figure 5.5B) and 145-5p (P=0.02), with greater levels in men. Specifically, endurance trained (P=0.07, gs=1.08) and sedentary men (P=0.0002, g_s=1.38) had greater levels of ci-miR-145-5p compared to their female counterparts. Compared to trained men, ci-miR-145-5p was also higher in sedentary men (P=0.004, gs=0.94).

Correlations

The correlations between serum-induced endothelial cell functions and ci-miRs with cardiovascular and metabolic factors for both men and women separately are shown in Table 5.3. In men, serum-induced endothelial migration correlated positively with age and LDL concentration, while in women it correlated positively with relative VO_{2max} and negatively with resting HR. Serum-induced endothelial proliferation was related to endothelial ROS production and body fat percentage in men and endothelial migration, DBP, and circulating glucose concentration in women. Circulating glucose concentration was also associated with endothelial ROS production in women only. In men, ci-miR-181b-5p correlated with endothelial migration and ci-miRs- 126-3p, 146a-5p, and 199a-5p correlated with SBP. In women, there were select associations between ci-miRs and endothelial proliferation and ROS production, circulating

triglycerides, and glucose. Circulating estradiol correlated only with serum-induced endothelial ROS production in men.

Discussion

As evidenced by the effects of serum on cultured endothelial cells, we found that the circulating milieu differs in association with habitual activity level in a sex-specific manner. There was a significant effect of activity level on serum-induced endothelial cell migration and a significant effect of sex on serum-induced endothelial ROS production. Compared to serum from sedentary individuals of the same sex, serum from endurance trained women induced greater HUVEC migration, while serum of trained men caused lower ROS production. There were also sex-based and some activity level-based differences in the concentrations of ci-miRs, suggesting they may contribute to known disparities in endothelial function and CVD development. Importantly, all participants were matched by age and BMI, as well as being devoid of any CVD risk factors. Due to our study design, circulating estradiol concentration was also not different between men and women, thus ruling out a major contributor to sex-based differences in endothelial function.

We sought to determine endothelial cell migration and proliferation in response to serum as a method to identify differences in the cumulative effects of blood-borne factors on endothelial cell functions. The migration and proliferation of endothelial cells are important cellular functions in endothelial repair and angiogenesis (97, 116, 233, 274, 287). These processes may be modulated by a variety of factors, with faster migration and proliferation indicating superior function (125, 274, 287). In a previous

report, six weeks of lower-body resistance exercise training in young men did not alter serum-induced HUVEC proliferation (36). Similarly, we did not observe a difference in endothelial migration or proliferation in response to serum of endurance trained compared to sedentary men. However, we did observe a significant effect of activity level, with serum of endurance trained women acting to increase endothelial migration in comparison to their sedentary counterparts, indicating potentially favorable alterations in circulating factors due to habitual exercise.

To our knowledge, we are the first to examine the effects of serum from young, healthy women on endothelial cell functions. In a study of postmenopausal women, 13 weeks of walking-based training improved serum's capacity to induce chemoattractive migration and capillary-like tube formation of HUVECs (235). This is interesting in light of the common phenomenon that endothelial function of postmenopausal women measured at the vascular level commonly does not respond to exercise training, purportedly due to the loss of estrogen's permissive effect on training adaptations (350, 351). Indeed, the beneficial effects of estrogen on endothelial function are well-recognized (46, 473). Estrogen exerts both genomic and non-genomic effects that are anti-inflammatory and antioxidant, also promoting vasodilation, endothelial repair, and angiogenesis (46, 56, 75, 231). In order to exclude the acute effects of estrogen in our study, women were sampled during the early follicular phase of the menstrual cycle or the placebo phase of oral contraceptive use, and circulating estradiol concentrations were similar to those found in men. Our results show that in young women, sedentary behavior is associated with a profile of circulating factors that reduces endothelial migration. This may represent a physiological adaptation to chronic endurance

exercise/inactivity that is specific to women. Identification of the responsible circulating factors could benefit vascular therapies aimed at enhancing endothelial repair and/or angiogenesis in sedentary, young women or in postmenopausal women.

The effects of oral contraceptives on the endothelium are currently unresolved (14, 169, 210, 294, 374, 455, 459). Although we controlled for circulating estradiol, additional hormones and other circulating factors may be affected by the use of oral contraceptives (127, 142, 168). Thus, we performed exploratory comparisons including the endurance trained and sedentary groups of women in our study and found no significant effects of oral contraceptive use on serum-induced endothelial cell migration, proliferation, or ROS production. Future targeted studies should determine the effects of the diverse types of oral contraceptives, length of oral contraceptive use, and the different phases of oral contraceptives and the menstrual cycle on circulating factors and their impacts on the endothelium.

Traditional and non-traditional CVD risk factors are detrimental to endothelial cell health/function largely via the induction of chronic oxidative stress, a state of elevated ROS production that exceeds the rate of removal by antioxidants and nitric oxide (NO). Excessive ROS reduces NO bioavailability and leads to increased vascular inflammation and cellular damage (230). Thus, a chronic state of oxidative stress is a major mechanism underlying the development of endothelial dysfunction and CVD (79, 230). In young, healthy men and women, longitudinal exercise training and cross-sectional studies comparing athletes and sedentary controls suggest that the antioxidant capacity of the blood may increase with training (158). In our study, we observed a significant effect of sex on serum-induced endothelial ROS production. The interaction

effect additionally approached statistical significance and endothelial ROS production was specifically found to be higher in response to serum of sedentary men compared to endurance trained men, as well their sedentary female counterparts. Habitual activity level was not associated with a difference in serum-induced endothelial ROS production in women, suggesting the observed differences in endothelial cell migration were due to other mechanisms. Thus, alterations in blood-borne factors that increase endothelial ROS production may represent an early mechanism underlying CVD development due to inactivity in otherwise healthy men.

A previous study found that compared to serum of anaerobic male athletes (soccer players and sprinters), serum of male triathletes had the highest NO bioavailability, induced the lowest ROS production (measured by thiobarbituric acid reactive substances (TBARS)), and caused the greatest survival and proliferation of cultured endothelial cells (96). Those results suggest aerobic-type exercise training may be the most beneficial in terms of endothelial cell health due to alterations in the circulating milieu. While that study included only men, our results suggest that exercise training does not influence the effects of serum from young women on endothelial ROS production. It is possible that sedentary, yet otherwise healthy, young women are protected from the endothelial ROS production that was apparent in men in our study, due to the residual effects of estrogen on other antioxidant molecules in circulation (37).

Classically, endurance exercise training improves the circulating cardiometabolic risk factor profile, including circulating glucose, triglycerides, and cholesterol levels (157, 346). In an effort to exclude these factors as the cause of

differences in HUVEC functions, all participants included in our study had healthy levels of circulating glucose, cholesterol, and triglycerides. Indeed, our study groups were very well matched in regard to their metabolic profile, despite large differences in VO_{2max}. Within sexes, the only significant difference in circulating metabolic profile was a higher glucose concentration in endurance trained compared to sedentary women. Surprisingly, circulating glucose concentration exhibited a positive correlation with serum-induced endothelial proliferation and ROS production in women, but not men. Both of these endothelial measures were also associated with the levels of several ci-miRs exclusively in women. Conversely, only a negative correlation between endothelial proliferation and body fat percentage was observed in men. Adipose tissue is known to secrete several circulating factors (i.e. adipokines) which regulate cardiovascular health (137, 158). These findings support the conclusion that different circulating factors may contribute to endothelial health in men and women. Interestingly, cardiorespiratory fitness, as measured by VO_{2max} and resting HR, correlated with endothelial migration only in women.

Exercise training is also proposed to protect against atherogenesis at least partially via alterations in the circulating concentrations of cytokines (157, 158). Previously however, our laboratory found no differences in the concentrations of a number of growth factors or inflammatory proteins, including VEGF, IL-6, and TNF-a, between young, healthy endurance trained and sedentary men (276). To our knowledge, similar studies in women have not been performed. A recent large-scale study found differences in many circulating CVD biomarkers between healthy, premenopausal women and age-matched men; unfortunately they did not focus on

fitness or activity levels (281). Thus, studies determining sex- and habitual activity-based differences in blood-borne circulating factors important in early CVD development are needed. Here, we determined differences in the levels of ci-miRs, as they are novel proposed mediators of both exercise training adaptations and CVD development.

Endurance exercise training has been shown to influence the circulating levels of miRs important to endothelial health, though the effects of chronic endurance exercise are not well known, and women are understudied (434, 436). Recently, using serum samples from the HERITAGE Family Study, the effects of a 20-week endurance exercise training program on ci-miRs related to cardiovascular health were determined in previously sedentary, but healthy, men and women (aged 43.7±12.8 years) (33). By using the same PCR array of ci-miRs related to cardiovascular health used in our study, the investigators identified 14 ci-miRs with either up- or down-regulated expression after training, including ci-miRs- 126-3p, 146a-5p, and 221-3p (33). In our study, after validation only ci-miRs-140-5p and 145-5p were different in association with habitual activity level. Both exhibited higher levels in sedentary individuals, with ci-miR-145-5p higher specifically in sedentary men. MiR-140-5p increases ROS production by directly targeting NRF2 and SIRT2, while miR-145-5p decreases NO production by targeting SLC7A1, an L-arginine transporter protein (298, 530). Additionally, both inhibit angiogenesis, with miRs- 140-5p and 145-5p directly targeting VEGF and integrin β8, respectively (94, 481). Thus, a reduction in these ci-miRs with training could contribute to increased capillarization and protection against oxidative stress.

Due to major differences in the limited studies performed to date on the association between habitual physical activity and ci-miR expression in healthy individuals, it is difficult to make conclusions on the associations between fitness level and habitual exercise with ci-miRs (62, 120, 533). In our study, sex was a more important factor than habitual activity level in determining ci-miR concentrations. We found significant sex effects on all nine vascular-related ci-miRs that were detected during validation, with higher levels in men compared to women. While we cannot infer the directionality of the ci-miR correlations identified, it appears that ci-miRs may also be related to different cardiometabolic factors in young men and women. These results add to the literature showing sex differences in ci-miR concentrations in a variety of age groups and health conditions (12, 28, 98, 120, 454). In this context, estrogen regulates proteins in the miR biogenesis pathway within endothelial cells, as well as the expression of specific miRs within vascular cells and the circulation (160, 187, 391). The levels of ci-miRs have been found to stay consistent over the course of the menstrual cycle and, therefore, do not appear to be altered due to acute changes in circulating hormones in women (411). Thus, sex-differences in ci-miRs likely persisted in our study due to these long-term genomic mechanisms of regulation in women.

Specifically, the anti-inflammatory and antioxidant effects of estrogen are likely partially accomplished via its influence on the expression of several miRs within endothelial cells (160, 391). The ci-miRs assessed in our study regulate targets to induce primarily anti-inflammatory effects, although they have varying effects on NO and ROS. For example, miRs-23b-3p, 145-5p, 146a-5p, and 181b-5p all either directly or indirectly downregulate NFkB expression or activity (84, 224, 322, 482). MiR-126-

3p may upregulate eNOS activity at least partially via the PI3k/Akt pathway, while miR-199-5p and 221-3p may act to downregulate this pathway (89, 251, 366). Endothelial ROS production may be increased by miRs-181b-5p, 199a-5p, and 221-3p/222-3p downregulating SIRT1, SOD1, and PGC-1α, respectively, and decreased by miR-146a-5p-induced NOX4 suppression (203, 251, 553). The cumulative effects of the higher levels of these ci-miRs in men compared to women is unclear, but they may contribute to the elevated serum-induced endothelial ROS production that we observed.

There are some limitations to our study that should be acknowledged. Our study was cross-sectional in nature, so follow-up longitudinal exercise training studies should be performed to investigate changes in circulating factors in sedentary individuals. While we focused on functional assays of endothelial cell function, future studies should also investigate the effects of exercise training-induced changes in circulating factors on intracellular pathways related to endothelial function. Although the sample sizes used in our study were comparable to those used in similar previous studies performed by us and others (96, 277), they may have limited our ability to detect statistically significant differences considering some of our comparisons approached significance. While estradiol is the major sex hormone promoting vascular protection in women, there are other sex hormones with roles in endothelial function that we did not measure or control for, such as progesterone and other estrogens (473). Still, circulating progesterone is low in the early follicular/menstrual phase and was likely similar to concentrations in men considering the estradiol concentrations were not different (455, 462). We also did not control for the effects of androgens in men, such as testosterone, which may exert either positive or negative cardiovascular effects

depending on concentration (46, 473). Due to financial restraints, we pooled samples including men and women for the initial PCR arrays. The results of the array likely would have been different if we had performed arrays separately for samples from men and women, considering we found sex differences in ci-miR levels. Lastly, while we found differences in the concentrations of specific ci-miRs and determined correlations, we did not experimentally determine their mechanistic roles in the endothelial cell assays in our study.

In conclusion, we have found that the blood-borne circulating milieu in young, sedentary, but otherwise healthy individuals exerts different effects on cultured endothelial cells as compared to that of their endurance trained counterparts. These effects are sex-specific, as are the cardiometabolic factors that they correlate with. Identification of the major responsible circulating factors may be useful for the development of early cardiovascular interventions in individuals unable to exercise, as well as therapies for postmenopausal women who commonly do not respond to exercise training. Additionally, in our population, ci-miRs related to vascular health are influenced by sex more so than habitual activity level. Mechanisms underlying the regulation of these miRs, such as estrogen, as well as the long-term effects of these alterations on cardiovascular health are of interest, as they could contribute to sex disparities in CVD risk. Lastly, ci-miRs- 140-5p and 145-5p were upregulated in inactive individuals. Their roles in vascular training adaptations and the development of vascular diseases due to sedentary behavior require further investigation.

Table 5.1. Participant characteristics

	Men		Women	
	Trained (n=16)	**Sedentary (n=16)**	**Trained (n=18)**	**Sedentary (n=14)**
Age, y	23 ± 5	24 ± 5	25 ± 6	25 ± 4
VO_{2max}, $L \cdot min^{-1}$	4.35 ± 0.62*†	3.05 ± 0.36†	3.06 ± 0.49*	1.94 ± 0.36
VO_{2max}, $mL \cdot kg^{-1} \cdot min^{-1}$	62.6 ± 7.9*†	41.6 ± 4.2†	52.4 ± 6.0*	33.4 ± 4.4
BMI, kg/m^2	22 ± 2	23 ± 2	22 ± 2	22 ± 2
Body fat, %	8.5 ± 2.7*†	16.1 ± 5.7†	17.6 ± 4.2*	23.6 ± 3.3
SBP, mmHg	123 ± 5†	123 ± 10†	113 ± 7	111 ± 9
DBP, mmHg	73 ± 9	76 ± 9	69 ± 7	74 ± 9
MAP, mmHg	89 ± 6†	92 ± 9	81 ± 10	86 ± 8
Glucose, mg/dl	89 ± 7	90 ± 6†	88 ± 6*	81 ± 9
Total cholesterol, mg/dl	151 ± 25	152 ± 27	164 ± 23	166 ± 31
HDL-C, mg/dl	56 ± 12†	55 ± 9	71 ± 15	63 ± 17
LDL-C, mg/dl	81 ± 20	83 ± 15	79 ± 19	88 ± 21
VLDL-C, mg/dl	14 ± 4	14 ± 6	14 ± 5	14 ± 5
Triglycerides, mg/dl	72 ± 22	70 ± 28	70 ± 21	70 ± 23
Estradiol, pg/ml	101 ± 70	100 ± 59	156 ± 219	144 ± 218

Means ± SD. Participant demographics were compared between groups using independent t tests. BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure; SBP, systolic blood pressure; VLDL, very low-density lipoprotein. *$P < 0.05$ vs. sedentary of the same sex; and †$P < 0.05$ vs. women with same activity status.

Table 5.2. Human Cardiovascular Disease miR PCR Array

	Average Ct		2^(-Δ(Ct))		Up-Down Regulation (comparing to control group)
microRNA	Control Group	Group 1	Control Group	Group 1	
hsa-let-7a-5p	26.84	28.71	2.478599	0.76493	-3.2403
hsa-let-7b-5p	26.25	26.52	3.725735	3.497927	-1.0651
hsa-let-7c-5p	29.08	28.92	0.525195	0.662504	1.2614
hsa-let-7d-5p	26.27	27.43	3.690266	1.858966	-1.9851
hsa-let-7e-5p	29.23	30.24	0.473168	0.265779	-1.7803
hsa-let-7f-5p	27.34	29.43	1.753971	0.464098	-3.7793
hsa-miR-1-3p	35	34.31	x	x	x
hsa-miR-100-5p	29.3	29.55	0.45179	0.429521	-1.0518
hsa-miR-103a-3p	27.4	28.68	1.682172	0.781545	-2.1524
hsa-miR-107	35	35	x	x	x
hsa-miR-10b-5p	33.29	34.25	0.028268	0.016512	-1.7119
hsa-miR-122-5p	27.02	27.16	2.185442	2.240002	1.025
hsa-miR-124-3p	28.16	27.55	0.992834	1.710123	1.7225
hsa-miR-125a-5p	26.4	28.74	3.355959	0.751477	-4.4658
hsa-miR-125b-5p	28.5	29.14	0.781721	0.570382	-1.3705
hsa-miR-126-3p	23.32	24.76	28.451346	11.846591	-2.4016
hsa-miR-130a-3p	27.52	29.33	1.550491	0.497201	-3.1184
hsa-miR-133a-3p	31.87	33.2	0.075768	0.03405	-2.2252
hsa-miR-133b	35	35	x	x	x
hsa-miR-140-5p	27.58	32.03	1.480131	0.076931	-19.2398
hsa-miR-142-3p	25.67	27.44	5.560925	1.849198	-3.0072
hsa-miR-143-3p	29.95	30.98	0.287997	0.158857	-1.8129
hsa-miR-144-3p	27.32	27.77	1.776977	1.472125	-1.2071
hsa-miR-145-5p	29.49	32.08	0.39574	0.074094	-5.3411
hsa-miR-146a-5p	28.3	29.85	0.900454	0.347937	-2.588
hsa-miR-149-5p	15	35	x	x	x
hsa-miR-150-5p	24.84	25.44	9.941923	7.413218	-1.3411
hsa-miR-155-5p	24.01	25.12	17.649128	9.227245	-1.9127
hsa-miR-15b-5p	25.73	27.01	5.350319	2.496661	-2.143
hsa-miR-16-5p	21.88	22.4	76.88472	60.724214	-1.2661
hsa-miR-17-5p	25.25	24.99	7.459221	10.100116	1.354
hsa-miR-181a-5p	28.27	29.22	0.922119	0.538157	-1.7135
hsa-miR-181b-5p	28.68	30.49	0.69372	0.223121	-3.1092
hsa-miR-182-5p	30.79	31.66	0.160921	0.098926	-1.6267
hsa-miR-183-5p	35	35	x	x	x
hsa-miR-185-5p	27.7	28.35	1.369952	0.984866	-1.391
hsa-miR-18b-5p	34.57	35	x	x	x
hsa-miR-195-5p	23.07	23.43	33.813443	29.7971	-1.1348
hsa-miR-199a-5p	30.13	33.11	0.252652	0.036292	-6.9616
hsa-miR-206	27.2	35	x	x	x
hsa-miR-208a-3p	35	35	x	x	x
hsa-miR-208b-3p	30.03	32.13	0.27216	0.071496	-3.8067
hsa-miR-21-5p	23.88	25.23	19.270539	8.543931	-2.2555
hsa-miR-210-3p	30.03	30.08	0.271839	0.297137	1.0931
hsa-miR-214-3p	33.88	35	x	x	x

hsa-miR-22-3p	20.25	19.29	239.208526	523.193207	2.1872
hsa-miR-221-3p	26.22	27.48	3.80613	1.79303	-2.1227
hsa-miR-222-3p	26.05	28.06	4.274705	1.204555	-3.5488
hsa-miR-223-3p	21.7	23.08	87.306974	37.836323	-2.3075
hsa-miR-224-5p	34.1	35	x	x	x
hsa-miR-23a-3p	24.15	25.54	16.039141	6.88139	-2.3308
hsa-miR-23b-3p	26.09	28.59	4.170803	0.832833	-5.008
hsa-miR-24-3p	28.54	29.48	0.763884	0.448071	-1.7048
hsa-miR-25-3p	24.66	25.61	11.22719	6.589642	-1.7038
hsa-miR-26a-5p	25.52	27.41	6.190367	1.886223	-3.2819
hsa-miR-26b-5p	25.88	27.27	4.805961	2.079591	-2.311
hsa-miR-27a-3p	25.34	26.06	6.99937	4.797557	-1.4589
hsa-miR-27b-3p	26.48	27.41	3.186836	1.887138	-1.6887
hsa-miR-29a-3p	27.39	27.84	1.694342	1.396679	-1.2131
hsa-miR-29b-3p	23.45	23.83	26.071921	22.581979	-1.1545
hsa-miR-29c-3p	28.46	29.61	0.807607	0.411282	-1.9636
hsa-miR-302a-3p	23.85	35	x	x	x
hsa-miR-302b-3p	35	35	x	x	x
hsa-miR-30a-5p	24.33	26.13	14.070727	4.596705	-3.061
hsa-miR-30c-5p	25.43	27.66	6.581083	1.583043	-4.1572
hsa-miR-30d-5p	27.82	29.14	1.252599	0.569631	-2.199
hsa-miR-30e-5p	24.9	26.08	9.492743	4.740371	-2.0025
hsa-miR-31-5p	32.18	24.05	0.061134	19.405585	317.4257
hsa-miR-320a	26.55	27.67	3.031065	1.571563	-1.9287
hsa-miR-328-3p	21.99	22.38	71.378862	61.682956	-1.1572
hsa-miR-342-3p	25.76	26.78	5.252211	2.9142	-1.8023
hsa-miR-365b-3p	28.73	27.24	0.667817	2.116822	3.1698
hsa-miR-378a-3p	31	32.3	0.138487	0.063809	-2.1703
hsa-miR-423-3p	28.06	29.07	1.061221	0.598283	-1.7738
hsa-miR-424-5p	27.21	27.41	1.91524	1.881261	-1.0181
hsa-miR-451a	19.63	20.48	366.641174	229.635201	-1.5966
hsa-miR-486-5p	22.52	24.06	49.546972	19.251514	-2.5737
hsa-miR-494-3p	35	35	x	x	x
hsa-miR-499a-5p	35	34.61	x	x	x
hsa-miR-7-5p	30.96	32.58	0.142618	0.052236	-2.7302
hsa-miR-92a-3p	22.89	23.66	38.248334	25.379645	-1.507
hsa-miR-93-5p	22.76	25.45	41.869419	7.346719	-5.6991
hsa-miR-98-5p	28.67	29.35	0.698932	0.491853	-1.421
hsa-miR-99a-5p	30.14	31.7	0.251342	0.096395	-2.6074
cel-miR-39-3p	18.66	18.32	716.848779	1027.626298	1.4335
cel-miR-39-3p	17.74	18.08	1363.335576	1214.544807	-1.1225
SNORD61	32.57	33.86	0.046802	0.021526	-2.1743
SNORD68	34	33.12	0.017275	0.036011	2.0846
SNORD72	35	18.9	0.008666	690.166195	79641.0709
SNORD95	28.1	29.04	1.036144	0.610939	-1.696
SNORD96A	28.52	30.81	0.773689	0.178142	-4.3431
RNU6-6P	32.74	32.32	0.041413	0.062721	1.5145
miRTC	20.33	19.87	225.381455	352.138705	1.5624
miRTC	20.1	19.77	264.042252	376.002906	1.424
PPC	16.72	16.26	2765.117362	4297.255971	1.5541
PPC	17.33	17.13	1812.449396	2344.395081	1.2935

Fold-regulation values greater than 2 are indicated in red; fold-regulation values less than -2 are indicated in blue. Ct, cycle threshold. MicroRNAs with a CT > 35 were defaulted to 35.

Table 5.3. Pearson's correlations (r values) between serum-induced endothelial cell functions and circulating microRNAs (miR) with demographic and cardiometabolic factors in men and women.

	Migration	Prolif.	ROS	Age	BMI	VO_{2max}	SBP	DBP	HR	%Body Fat	HDL	LDL	Triglyc.	Glucose	Estradiol
Men															
Migration	x	0.33	0.35	**0.35***	-0.02	0.26	0.11	-0.19	-0.17	-0.04	-0.12	**0.38***	0.10	0.23	-0.16
Prolif.	0.33	x	**0.58***	0.02	0.30	-0.10	0.17	0.15	-0.02	**0.39***	-0.11	0.10	-0.03	0.30	0.33
ROS	0.35	**0.58***	x	0.03	0.17	-0.17	0.27	0.40	0.01	0.28	0.02	-0.14	0.01	0.20	**0.43***
miR-23b	0.21	-0.08	0.07	0.25	-0.01	0.09	0.37	-0.12	0.22	-0.08	-0.20	-0.33	-0.02	-0.31	-0.23
miR-126	0.21	-0.18	0.18	-0.05	-0.26	0.01	**0.46***	0.19	0.16	-0.16	-0.06	-0.26	-0.18	-0.06	-0.04
miR-140	0.33	0.19	0.20	0.099	0.07	-0.23	0.16	-0.04	0.16	0.05	-0.00	-0.13	-0.12	-0.06	-0.14
miR-145	0.19	-0.11	0.22	0.058	-0.18	-0.19	0.25	0.05	0.34	-0.08	-0.01	-0.36	-0.19	-0.12	-0.11
miR-146a	0.24	-0.18	0.21	-0.04	-0.31	-0.01	**0.42***	0.22	0.19	-0.15	-0.06	-0.27	-0.20	-0.00	-0.04
miR-181b	**0.44***	0.25	0.33	0.15	0.08	0.05	0.30	-0.01	0.09	-0.05	-0.15	-0.25	-0.07	-0.12	-0.04
miR-199a	0.22	-0.21	0.17	-0.10	-0.34	0.00	**0.45***	0.30	0.16	-0.19	0.02	-0.24	-0.21	0.06	-0.04
miR-221	0.23	-0.17	0.13	-0.06	-0.33	-0.00	0.34	0.15	0.14	-0.16	-0.08	-0.30	-0.15	0.02	-0.08
miR-222	0.30	-0.08	0.18	0.03	-0.23	-0.03	0.32	0.03	0.10	-0.11	-0.15	-0.34	-0.11	-0.04	-0.12
Women															
Migration	x	**0.42***	0.15	0.00	0.07	**0.45***	0.20	-0.29	**-0.55***	-0.21	-0.07	-0.31	0.02	0.34	-0.01
Prolif.	**0.42***	x	0.06	-0.15	-0.04	0.31	-0.18	**-0.43***	-0.18	0.02	0.04	-0.23	0.07	**0.53***	-0.03
ROS	0.15	0.06	x	0.33	-0.03	0.20	0.38	0.17	-0.30	-0.14	0.30	0.17	0.25	**0.57***	0.25
miR-23b	-0.04	-0.14	**0.45***	-0.15	0.07	-0.04	0.13	-0.07	0.05	0.11	-0.22	0.29	**0.55***	0.18	-0.24
miR-126	-0.30	-0.41	**0.48***	-0.16	-0.04	-0.10	-0.04	0.07	0.37	0.07	-0.12	-0.07	**0.74***	0.41	-0.07
miR-140	0.01	-0.43	0.07	-0.15	-0.12	-0.19	0.11	0.20	0.20	0.14	-0.08	-0.04	0.36	0.02	-0.26
miR-145	-0.03	**-0.50***	0.14	-0.34	-0.06	0.00	-0.13	0.32	0.31	0.15	-0.14	0.02	0.29	0.34	-0.13
miR-146a	-0.22	-0.42	**0.44***	-0.26	-0.14	-0.11	-0.04	-0.00	0.29	0.02	-0.21	-0.09	**0.78***	0.26	-0.12
miR-181b	-0.09	-0.42	0.31	-0.39	-0.09	0.04	0.05	-0.01	0.16	0.01	-0.36	0.05	**0.63***	0.15	-0.26
miR-199a	-0.17	**-0.45***	**0.44***	-0.21	-0.08	0.07	-0.06	-0.03	0.22	-0.08	-0.15	-0.04	**0.70***	**0.46***	-0.16
miR-221	-0.19	**-0.44***	**0.44***	-0.25	-0.04	0.02	-0.03	0.03	0.20	-0.03	-0.25	0.03	**0.69***	0.36	-0.18
miR-222	-0.19	**-0.46***	0.42	-0.26	0.02	-0.01	0.00	0.05	0.22	0.01	-0.27	-0.01	**0.68***	0.32	-0.11

BMI, body mass index; DBP, diastolic blood pressure (n = 59); Glucose (n = 63); HDL, high-density lipoprotein (n = 63); HR, resting heart rate (n = 43); LDL, low-density lipoprotein (n = 63); Prolif., proliferation; ROS, reactive oxygen species; SBP, systolic blood pressure (n = 59); Triglyc., triglycerides (n = 63); VO_{2max}, maximal oxygen consumption (n = 61); % Body fat (n = 63). Sample sizes are specified for those variables missing data for some participants not already specified in the text. *P < 0.05. Significant correlations are in bold.

Figure 5.1

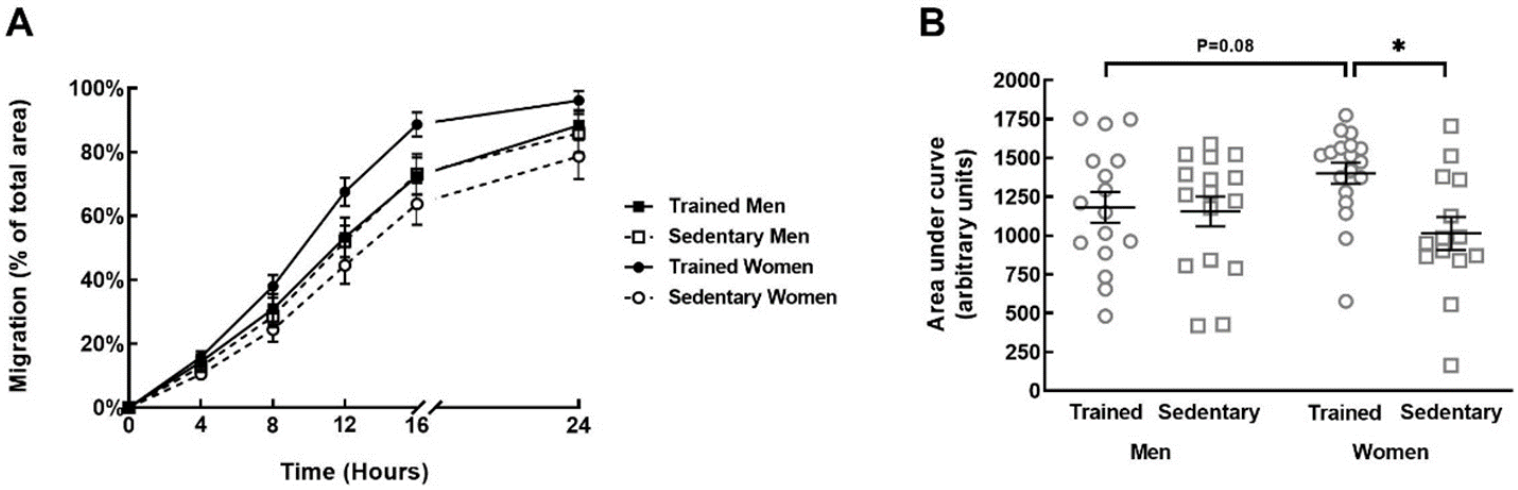

Figure 5.1. [A] Endothelial cell migration over 24 h. [B] Area under the curve in response to 10% serum from endurance-trained men (n = 16) and women (n = 18) and sedentary men (n = 16) and women (n = 14). Means ± SEM; *P < 0.05.

Figure 5.2

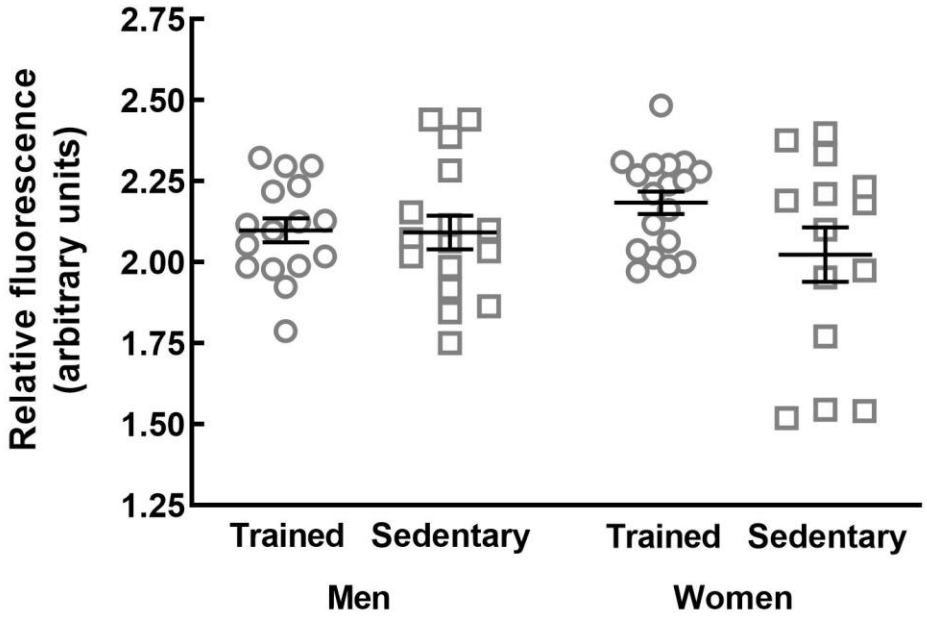

Figure 5.2. Endothelial cell proliferation in response to 20% serum from endurance-trained men (n = 16) and women (n = 18) and sedentary men (n = 16) and women (n = 14). Fluorescence is reported relative to the endothelial growth medium no serum negative control (set as 1). Means ± SEM.

Figure 5.3

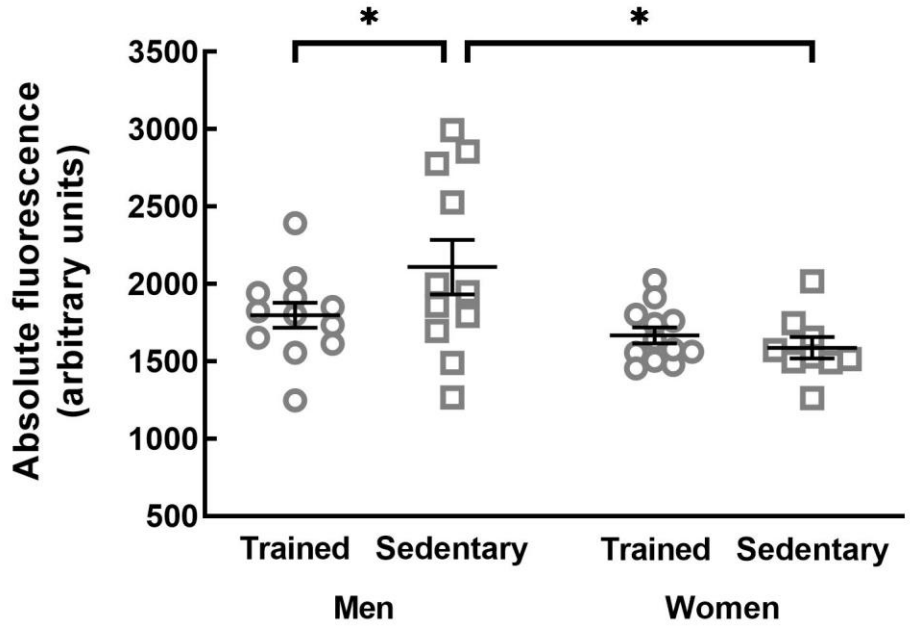

Figure 5.3. Endothelial cell reactive oxygen species production in response to 20% serum from endurance-trained men (n = 12) and women (n = 12) and sedentary men (n = 11) and women (n = 9). Means ± SEM; *P < 0.05.

Figure 5.4

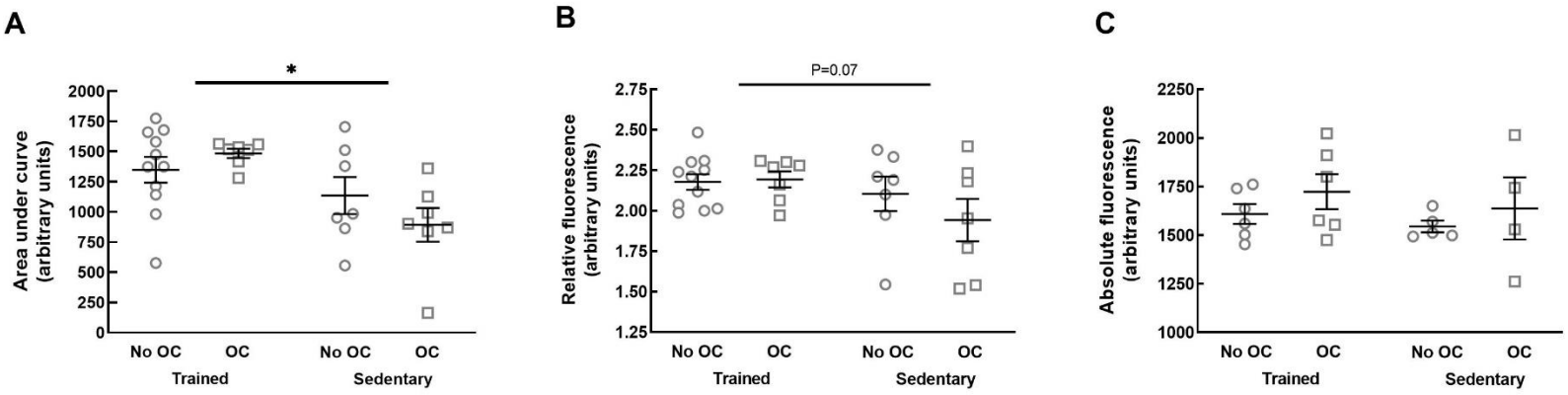

Figure 5.4. The effects of serum from trained and sedentary women using oral contraceptives (OC) compared with those not using OC (no OC) on endothelial migration [A], proliferation [B], and reactive oxygen species production [C]. For migration and proliferation: no OC trained (n = 11), OC trained (n = 7), no OC sedentary (n = 7), and OC sedentary (n = 7). For ROS production: no OC trained (n = 6), OC trained (n = 6), no OC sedentary (n = 5), and OC sedentary (n = 4). Means ± SEM; *P < 0.05.

Figure 5.5

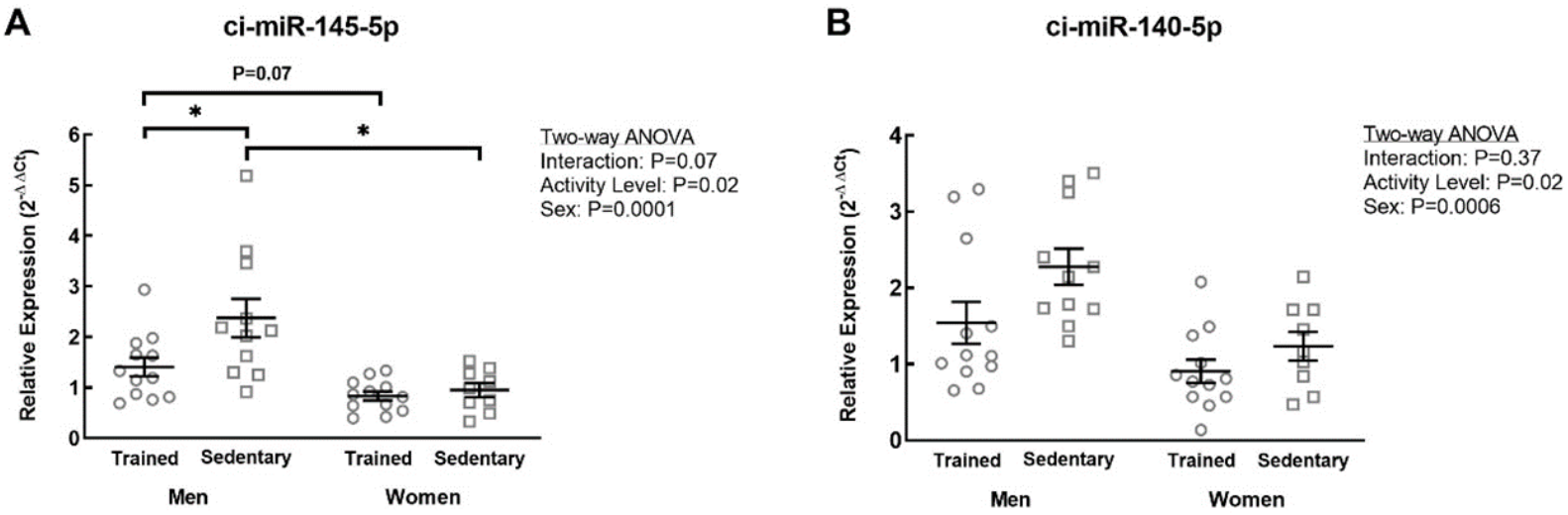

Figure 5.5. Circulating microRNA- (ci-miR-) 145-5p [A] and 140-5p [B] expression in serum from endurance-trained men (n = 12) and women (n = 12) and sedentary men (n = 11) and women (n = 9). Means ± SEM; *P < 0.05.

Figure 5.6

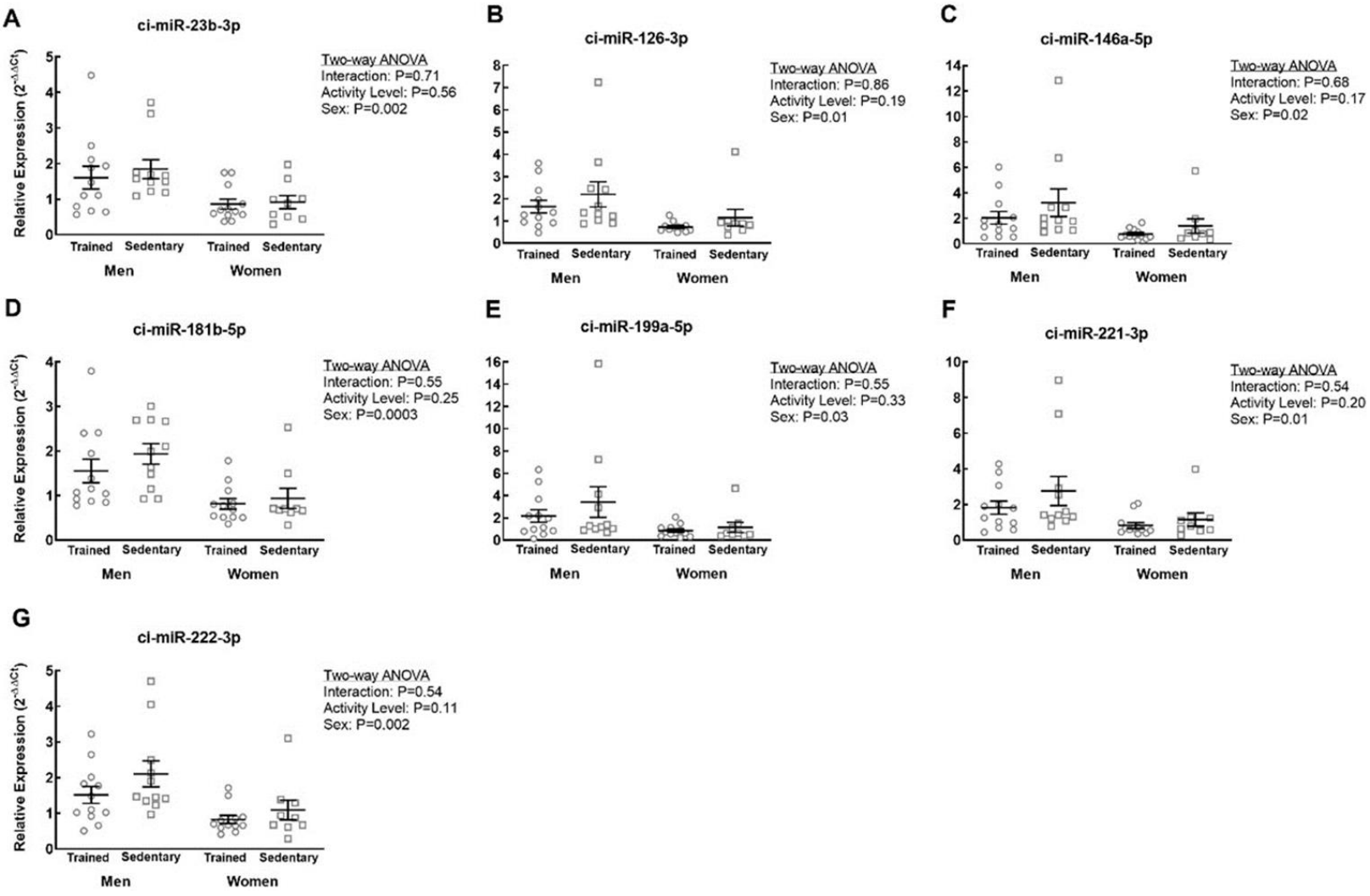

Figure 5.6. Circulating microRNA- (ci-miR-) 23b-3p [A], 126-3p [B], 146a-5p [C], 181b-5p [D], 199a-5p [E], 221-3p [F], 222-3p [G] expression in serum from endurance-trained men (n = 12) and women (n = 12) and sedentary men (n = 11) and women (n = 9). Means ± SEM.

Author contributions: RMS, RQLR, and JMH conceived and designed the study. RMS, RQLR and DDS collected the data. RMS and CBS analyzed the data. RMS, RQLR, DDS, CBS, and JMH interpreted the data. RMS drafted the manuscript and prepared the figures. RMS, RQLR, DDS, CBS, and JMH revised and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing interests: The authors report no potential conflicts of interest.

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Chapter 6: Racial differences in the responses of vascular function and related circulating microRNAs to acute exercise

Introduction

Compared with most other ethnic groups, African Americans (AA) carry a greater risk of developing cardiovascular disease (CVD) (148, 517). In addition, they develop CVD earlier in life and exhibit the highest associated mortality rates (148, 517). Decrements in vascular health including impaired endothelial function (measured as flow-mediated dilation (FMD) of the brachial artery) and elevated arterial stiffness (measured as the pulse wave velocity from the carotid to the femoral artery (cf-PWV)) are often apparent even in young, otherwise healthy AA individuals (60, 66, 135, 207, 209, 257, 379, 392, 449, 474). Importantly, FMD and cf-PWV are both non-invasive gold-standard methods to assess vascular health that are early predictors of future cardiovascular risk (53, 229, 283, 498, 520).

An aberrant vascular response to stressful stimuli has also been proposed to further contribute to the heightened risk for CVD in AA (65, 68, 357, 441). Compared with their CA counterparts, young AA display adverse vascular responses such as elevated blood pressure (BP), reduced forearm blood flow, and an exaggerated increase in sympathetic nervous system activity in response to either physiological or psychological stimuli including orthostatic, cold pressor, isometric handgrip, and mental stress tests (65, 68, 357, 522). Such responses can precede impairments in resting vascular function and may augment the development of vascular pathologies. Acute changes in arterial function due to stressful stimuli may therefore be a more sensitive indicator of future CVD risk as compared to measures taken at rest. The

impaired vascular responses to cardiovascular stress in AA, as well as the precise underlying mechanisms, remain to be fully described

Acute exercise is a well-established stressor for the cardiovascular system that can reveal detriments in cardiorespiratory function. Multiple studies have shown that arterial responses to exercise differ in AA when compared with CA. Following a bout of exercise, AA men display increases in brachial, aortic, and carotid diastolic BP (DBP), as well as central and upper limb arterial stiffness, that are not seen in CA men (204, 555–557). Acute exercise also caused stiffness of the carotid artery and lower limbs to transiently decrease in CA, but not AA (204, 557). Still, racial differences in the effects of exercise on macrovascular endothelial function are unknown. Additionally, a majority of the studies on exercise-induced racial differences have utilized a maximal exercise test (204, 449, 556). Considering vascular stimuli such as hemodynamics, reactive oxygen species (ROS), and inflammatory factors increase in an exercise intensity-dependent manner, it may be useful to determine the effects of both moderate and high intensity exercise of longer duration. Thus, this study sought to determine racial differences in the effects of moderate intensity continuous (MOD) and high intensity interval (HII) exercise bouts on macrovascular endothelial function and arterial stiffness. Altered vascular responses in AA would provide insight for future studies aiming to determine specific mechanisms underlying CVD risk in AA.

Recent research suggests that differences in epigenetic mechanisms stemming from the influence of socio-cultural-environmental factors may underlie racial differences in CVD risk (272, 332, 515). MicroRNAs (miRs) are epigenetic modulators of gene expression that act to regulate the translation of target mRNA. Critical

mechanistic roles for several miRs have been identified in vascular function and pathologies (76, 131, 295, 554). Racial differences in miRs exist among patients with diseases such as cancer and hypertension (129, 174), but are understudied in young, healthy individuals. Thus, miRs are novel molecules that could potentially contribute to vascular dysfunction and the increased risk of CVD in AA.

Furthermore, acute exercise induces the release of biologically active miRs into the circulating bloodstream (434, 436). These circulating miRs (ci-miRs) are released in response to changes in vascular stimuli and can be subsequently taken up by the vasculature, where they can then exert their effects to alter endothelial or vascular smooth muscle cell function (9, 31, 369, 444, 567, 569). Thus, ci-miRs reflect the state of the vasculature and may potentially exert autocrine, paracrine, and endocrine effects, partially mediating the systemic vascular effects of exercise (369, 436). Differential responses of vascular-related ci-miRs between AA and CA may provide mechanistic insight into racial differences in the vascular responses to exercise.

Therefore, the purpose of this study was to compare the changes in measures of vascular function/stiffness and ci-miRs between young, healthy AA and CA following the acute cardiovascular stress induced by both MOD and HII exercise. We hypothesized that within AA, arterial endothelial function would exhibit an exaggerated and prolonged impairment, while arterial stiffness would increase to a greater degree following acute exercise as compared with CA. Likewise, we hypothesized that changes in ci-miRs would differ in AA as compared with CA both at rest and in response to exercise. Further, we thought that changes in ci-miRs would be related to changes in vascular function/stiffness, revealing novel biomarkers and

potential mechanisms underlying early development of vascular dysfunction. We hypothesized that racial differences would be more apparent/greater following HII exercise as compared with MOD exercise.

Methods

Participants

All study procedures and documents were approved by the University of Maryland Institutional Review Board (IRB) and conformed to the Declaration of Helsinki. Individuals between the ages of 18-39 yr. who self-reported as being either AA or CA were recruited. In order to control for potential effects of either exercise training or sedentary lifestyle, we recruited moderately active individuals reporting physical activity where heart rate (HR) was continuously elevated for at least 30 minutes on 1-4 days per week. The study consisted of three morning visits to our laboratory. Prior to each visit, participants were required to refrain from food and drink other than water for at least 10 hours, as well as non-steroidal anti-inflammatory drugs (NSAIDS), alcohol, and exercise for at least 24 hours. During the initial visit, participants were first informed of the study procedures and gave their written informed consent. Following completion of the informed consent, participants completed health history and physical activity questionnaires. Participants were excluded from participation based on the use of antihypertensive medication, the presence of CVD, or if they did not meet the moderately active criteria. Seated resting BP followed by height and weight were assessed, and participants were excluded if they had a body mass index (BMI) > 30 kg/m^2, brachial systolic blood pressure (SBP) > 140 mmHg, or DBP > 90

mmHg. Participants were then moved to a phlebotomy bed and a resting blood sample was drawn from an antecubital vein for blood chemistry analysis.

Exercise bouts

Following completion of all paperwork and baseline measurements during the first visit, participants completed a peak power output (PPO) test on a cycle ergometer (Monark Ergomedic 894E). The test began with a 1-3 minute warmup followed by 2 minute stages of increasing load until volitional exhaustion. After warming up with a load of 1 kg, men began the test at a standardized load of 1.6 kg. Women warmed up with the cycle ergometer unloaded and started the first stage at a load of 1 kg. For both sexes, the load increased by 0.3 or 0.6 kg with each subsequent stage. During the test, participants were asked to maintain a cadence between 70-80 revolutions per minute (rpm). HR was recorded at the end of each stage using a chest strap HR monitor (POLAR T31). When participants were unable to maintain at least 70 rpm, the test was terminated and the final load (kg) was used to calculate the participant's PPO (kg·m/s).

The second and third visits consisted of a moderate intensity continuous (MOD) cycling bout (30 min, 60% PPO) and a high intensity interval (HII) cycling bout (30 min, 6 min at 40% PPO followed by 3 min intervals at 85% PPO interspersed with 4 min intervals at 40% PPO), which were matched for total time and workload (Figure 6.1A). HR was recorded each minute during exercise. The exercise bouts were completed in a randomized, counterbalanced order and visits were separated by at least one week to control for potential carryover effects of exercise. Upon arrival to the laboratory for visits 2 and 3, participants lay supine on a phlebotomy bed for at least 10 minutes before vascular testing (arterial function and stiffness) and a resting blood

draw. Participants then moved to the cycle ergometer, completed the exercise bout, and moved back to the bed as quickly as possible for a post-exercise blood draw. Post-exercise vascular testing was then performed 10 and 60 minutes after exercise cessation.

Flow-mediated dilation

Endothelium-dependent dilation was measured via flow-mediated dilation (FMD) of the brachial artery according to current guidelines (496). A high-resolution ultrasound (Hitachi-Aloka Arietta 70, Tokyo, Japan) with a 5–18 MHz linear transducer was aligned parallel above the brachial artery and stabilized with a probe holder. The location of the probe on the arm was marked and kept consistent at each time point per visit. A rapid inflator BP cuff (Hokanson, Bellevue, WA) was placed around the thickest section of the forearm, immediately distal to the antecubital fossa. Image capture and analysis were conducted using Quipu Cardiovascular Suite FMD Studio (Pisa, Italy). Baseline arterial diameter was initially measured during 1 minute of rest, after which the cuff was inflated to a suprasystolic pressure (220 mmHg) for 5 minutes. The cuff was then deflated and arterial diameter was recorded for 2.5 minutes to measure endothelial-dependent dilation in response to the cuff-induced reactive hyperemia. FMD was calculated as the percentage change in arterial diameter from the mean of the baseline to the peak of the 2.5 minute post-cuff deflation. To control for differences in brachial arterial diameter, allometrically-scaled FMD was calculated by dividing the %FMD by the resting brachial arterial diameter.

Pulse wave velocity

Central pulse wave velocity from the carotid to femoral artery (cf-PWV) was determined via pressure waveforms obtained at the carotid artery using a handheld tonometer and at the femoral artery using cuff-based applanation tonometry (SphygmoCor, AtCor Medical, Sydney, Australia). The cf-PWV path length was determined by measuring the distances from the sternal notch to the carotid and femoral artery sites, and from the femoral artery pulse site to the top of the thigh BP. The path length was then determined by subtracting the distance between the carotid artery and sternal notch from the carotid to femoral artery distance. Cf-PWV was calculated as the path length distance (m) divided by the change in time (s), where the change in time is the time delay between pulse arrivals at the carotid and femoral arteries. All assessments were made on the right side of the body.

Carotid arterial compliance and β-stiffness

Ultrasound was used to image the right common carotid artery via a 7.5 MHz linear-array probe (Aloka, Hitachi) while HR was simultaneously measured using a three-lead electrocardiogram (ECG) and brachial arterial BP was measured using an automatic sphygmomanometer. An automated wall detection echo-tracking software system carried out image analysis and calculated carotid arterial compliance and β-stiffness calibrated to brachial arterial BP.

Augmentation index

Cuff-based applanation tonometry (SphygmoCor, AtCor Medical, Sydney, Australia) was used to obtain a brachial artery pressure waveform, which was used to reconstruct an aortic pressure waveform using a generalized validated transfer function

(78, 356). Augmentation index (AIx) was determined as the difference between the first and second systolic peaks of the aortic waveform divided by pulse pressure, expressed as a percentage. To control for the effects of changes in HR on AIx, values were normalized to a HR of 75 beats per minute (AIx75).

Blood collection

Immediately before and after exercise, approximately 10 mL of blood was drawn from an antecubital vein into a serum separator tube. The blood samples sat at room temperature for 45 minutes to allow for clotting, after which they were spun at 1,500 xg for 15 minutes at 4ºC. The serum was then aliquoted on ice and stored at -80ºC.

Circulating microRNA

Serum samples were thawed at room temperature and centrifuged at 16,000 xg for 5 minutes to pellet any cell debris. Total RNA was then isolated from 50 µL of serum using the miRNeasy Serum/Plasma kit with slight alterations to the manufacturer's protocol (Qiagen, Germantown, MD). The serum was mixed with 20 volumes of Qiazol Lysis Reagent and 5 volumes of chloroform. Additionally, 1 µg of yeast RNA (ThermoFisher, Waltham, MA) was added to each sample during the isolation process in order to increase yield of RNA recovered (405). A standard amount of spike-in control miR (Caenorhabditis elegans (cel) miR-39-3p) was also added to each sample for use in calibration of PCR results. The RNA was eluted in 14 µL water. Reverse transcription was then performed using 5 µL of the resulting RNA samples and the miScript II RT kit (Qiagen, Germantown, MD). The final reaction mix including the cDNA was diluted in 200 µL of RNase-free water. The miScript SYBR®

Green PCR Kit (Qiagen, Germantown, MD) was used for quantitative real-time polymerase chain reaction (RT-qPCR) quantification of ci-miR in conjunction with 2.5 µL cDNA from each sample and specific miScript primer assays (Qiagen, Germantown, MD) for each ci-miR of interest (cel-miR-39-3p: MS00019789, miR-21-5p: MS00009079, miR-34a-5p: MS00003318, miR-92a-3p: MS00006594, miR-126-3p: MS00003430, miR-146a-5p: MS00003535, miR-150-5p: MS00003577, miR-155-5p: MS00031486, miR-181b-5p: MS00006699, miR-221-3p: MS00003857, miR-222-3p: MS00007609). Reactions were run on an Agilent Mx3005P qPCR system and all samples were assayed in duplicate for each ci-miR. MiR expression levels were determined using the $2^{-\Delta\Delta CT}$ method of relative quantification. For each miR within each sample, ΔCT = CT of miR – CT of spike-in control miR; $\Delta\Delta CT$ = CT for individual sample – average CT of all baseline samples. Ci-miRs quantified in all samples in < 35 cycles (CT < 35) were determined as sufficiently expressed. Ci-miRs-34a-5p and 155-5p were not reliably detected in all samples (CT > 35) and were excluded from analysis.

Statistics

Statistical analyses were performed using GraphPad Prism 8. Subject characteristics were compared between the racial groups using unpaired t-tests. Baseline (pre) and post-exercise HR, BP and vascular data were assessed using three-way repeated measures mixed-effects model analyses with exercise/time (baseline, 10 minutes post-exercise, 60 minutes post-exercise), intensity of exercise (MOD vs. HII), and race (AA vs. CA) as factors. Ci-miR data were also analyzed using three-way mixed-effects model analyses with only two time points (baseline vs. immediately post-

exercise). For each exercise intensity, an individual's data for a particular variable was excluded if data was missing from one or more time points. Statistical tests were two-sided with $P<0.05$ considered significant and $0.05<P<0.09$ considered approaching (i.e., borderline) significance. When a significant or borderline significant interaction or main effect was identified, independent and paired Fisher's LSD tests were performed for individual comparisons between and/or within races for each exercise intensity, respectively. Effect sizes were calculated for effects within groups (hedges g_{av}) and differences between groups (hedges' g_s) at specific time points (273). Pearson's correlation coefficients were calculated to determine the associations between the changes in ci-miRs and vascular measures that were significantly affected by exercise.

Results

Participant characteristics

Of the 27 participants recruited, 25 completed all testing and were included for analyses. Participant characteristics are presented in Table 6.1. The AA and CA groups were well matched, as there were no statistically significant differences in age, BMI, any blood chemistry parameters, BP, or PPO. Baseline resting values for vascular measures, averaged between the two exercise visits, are presented in Table 6.2. Carotid arterial compliance was greater in AA than CA ($g_s=0.94$) and was the only measure significantly different between groups at baseline. Additionally, differences in cf-PWV and brachial arterial diameter both approached statistical significance, with AA having numerically lower cf-PWV ($g_s=1.03$) and greater brachial arterial diameter ($g_s=1.17$).

To determine the day-to-day variability of vascular measures, average CVs were calculated using each participants' two resting, baseline values (from visits 2 and 3) for FMD (CV=39.9%), brachial arterial diameter (CV=4.6%), allometrically-scaled FMD (CV=41.4%), cf-PWV (CV=5.2%), carotid β-stiffness (CV=15.8%), carotid arterial compliance (CV=10.8%), and AIx (CV=60.5%). For reference, the CVs for baseline HR, brachial MAP, and aortic MAP were 5.8%, 2.8%, and 3.5%, respectively. Correlations were further calculated to determine the associations between the participants' baseline measures acquired on the separate visits. Correlations were significant for FMD ($r=0.45$, $P=0.03$), brachial arterial diameter ($r=0.83$, $P<0.0001$), allometrically-scaled FMD ($r=0.54$, $P=0.007$), cf-PWV ($r=0.68$, $P=0.0002$), carotid arterial compliance ($r=0.54$, $P=0.007$), and AIx ($r=0.45$, $P=0.02$). Carotid β-stiffness was the only vascular measure for which the baseline measures from visits 2 and 3 did not exhibit a significant correlation ($r=0.21$, $P=0.32$).

Heart rate and blood pressure

The HR responses to HII and MOD exercise are shown in Figure 6.1B-C. Baseline and post-exercise (10 and 60 minutes) HR values were analyzed by three-way repeated measures mixed-effects model analysis. There was no significant three-way interaction ($P=0.57$) nor two-way interactions for race x intensity ($P=0.42$) or race x exercise ($P=0.30$). There was a significant interaction effect of exercise x intensity ($P=0.0003$). Compared to baseline, HR remained significantly elevated 10 minutes after (both $P<0.0001$) and 60 minutes after (both $P<0.01$) HII in both AA and CA. HR was also elevated 10 minutes after MOD in both AA and CA (both $P<0.0001$), and remained elevated after 60 minutes in CA ($P=0.02$), but not AA ($P=0.12$).

The effects of MOD and HII exercise on brachial artery and aortic BP are shown in Figures 6.2 and 6.3, respectively. Brachial systolic BP (SBP) was not different between races at rest, but was significantly greater in CA than AA 10 minutes after both MOD (P=0.02, g_s=0.77) and HII (P=0.03, g_s=0.86) exercise (Figure 6.2A). Compared to baseline, brachial diastolic BP (DBP) was significantly increased in CA 10 minutes after (P=0.01, g_{av}=0.57), while it was decreased in AA 60 minutes after (P=0.045, g_{av}=0.49) HII exercise (Figure 6.2B). Further, brachial mean arterial pressure (MAP) was significantly lower in AA as compared with CA 60 minutes after HII exercise (P=0.04, g_s=0.89) (Figure 6.2C). Both aortic DBP (P=0.0002, g_s=0.87) and MAP (P=0.001, g_s=0.67) were significantly increased only in CA 10 minutes after HII exercise (P=0.07, g_s=0.64 and P=0.12, g_s=0.49 in AA) (Figure 6.3B-C). Aortic MAP was also increased only in CA 10 minutes after MOD exercise (P=0.03, g_s=0.55 vs. P=0.28, g_s=0.32 in AA).

Flow-mediated dilation

There were no significant interaction or main effects on FMD (Figure 6.4A). The three-way interaction for brachial arterial diameter approached statistical significance (Figure 6.4B). Post-hoc comparisons revealed that baseline differences for the HII exercise bout approached significance, with AA trending towards having larger baseline diameters than CA (4.08±0.19 vs. 3.87±0.16 mm, P=0.06, g_s=0.76). Brachial arterial diameter also increased in CA over time following the HII bout (10 min. g_{av}=0.30, 60 min. g_{av}=0.38). Thus, to control for differences in brachial arterial diameter, allometrically-scaled FMD (relative to brachial arterial diameter) was

analyzed (Figure 6.4C). There remained no significant interaction or main effects on allometrically-scaled FMD.

Arterial stiffness and augmentation index

Ten minutes after HII exercise, cf-PWV was significantly elevated in both AA (P=0.047, g_{av}=0.54) and CA (P=0.0003, g_{av}=1.03) (Figure 6.5A). At this time point, the difference in cf-PWV between AA and CA approached significance (P=0.06, g_s=0.83). To control for changes in central MAP, which can influence cf-PWV and were seen only in CA, cf-PWV was normalized to aortic MAP (cf-PWV/aortic MAP) and further analyzed (Figure 6.5B). There were no significant interaction or main effects on cf-PWV / MAP, although the effect of race did approach significance.

For the MOD exercise bout, carotid arterial compliance was greater in AA than CA at all time points (baseline P=0.005, g_s=0.93 and 10 min. P=0.005, g_s=1.24) although the difference only approached statistical significance 60 minutes after exercise (P=0.06, g_s=0.70) (Figure 6.6A). Carotid arterial compliance was also lower in CA than AA 10 minutes after HII exercise (P=0.03, g_s=1.12). Sixty minutes after HII exercise, carotid arterial compliance was significantly increased as compared to baseline only in CA (P=0.01, g_{av}=0.66). There were no significant interaction or main effects on carotid arterial β-stiffness index (Figure 6.6B).

AIx was increased 10 minutes after MOD exercise only in CA (P=0.02, g_{av}=1.05 vs. P=0.78, g_{av}=0.09 in AA), while it was decreased 60 minutes after both MOD (P=0.04, g_{av}=0.69) and HII exercise (P=0.03, g_{av}=0.82) only in AA (P=0.48, g_{av}=0.22 and P=0.55, g_{av}=0.21 in CA, respectively) (Figure 6.7A). When normalized to a HR of 75 bpm to control for changes in HR, AIx75 increased only in CA 10 minutes

after MOD exercise (P<0.0001, g_{av}=1.82 vs. P=0.12, g_{av}=0.51 in AA) (Figure 6.7B). Ten minutes after the HII exercise bout, AIx75 was increased in both CA (P=0.0003, g_{av}=1.18) and AA (P=0.03, g_{av}=0.65) individuals.

Circulating microRNA

To determine the day-to-day variability of ci-miR levels, average CVs were calculated using each participant's two resting, baseline values for ci-miRs- 21-5p (CV=23.4%), 92a-3p (CV=42.2%), 126-3p (CV=26.3%), 146a-5p (CV=31.2%), 150-5p (CV=17.7%), 181b-5p (CV=16.5%), 221-3p (CV=21.4%), and 222-3p (CV=23.1%). Correlations were further calculated to determine the associations between ci-miR levels in the participants' baseline samples acquired on the separate visits. Correlations were significant for ci-miRs- 21-5p (r=0.71, P=0.0005), 150-5p (r=0.58, P=0.007), 181b-5p (r=0.68, P=0.0009), and 222-3p (r=0.58, P=0.007), but were not significant for ci-miRs- 92a-3p (r=0.043, P=0.86), 126-3p (r=0.36, P=0.12), 146a-5p (r=0.023, P=0.92), 221-3p (r=0.17, P=0.47).

Ci-miR-150-5p was the only ci-miR to significantly increase in AA in response to exercise, while it also exhibited the largest exercise-induced increases in CA (Figure 6.8). Ci-miR-150-5p was significantly increased by ~150% in AA and ~230% in CA following both MOD (AA P=0.006, g_{av}=1.00 and CA P=0.002, g_{av}=1.55) and HII (AA P=0.002, g_{av}=1.05 and CA P=0.0008, g_{av}=1.37) (all P<0.01). There were increases in both ci-miR-21-5p and ci-miR-126-3p after MOD exercise that were significant in CA (P=0.03, g_{av}=0.73 and P=0.04, g_{av}=0.99) and approached significance in AA (P=0.05, g_{av}=0.69 and P=0.07, g_{av}=0.47) (Figure 6.9A-B). Both ci-miR-21-5p and ci-miR-126-3p also increased in CA (P=0.003, g_{av}=0.89 and P=0.002, g_{av}=0.84), but not AA

(P=0.16, g_{av}=0.41 and P=0.09, g_{av}=0.86), after HII exercise. Ci-miR-222-3p was significantly increased in CA following both MOD (P=0.01, g_{av}=0.80) and HII (P=0.005, g_{av}=1.03) exercise, but was not significantly altered in AA (P=0.26, g_{av}=0.58 and P=0.20, g_{av}=0.68) (Figure 6.10A). CA also displayed significant increases in ci-miRs- 146a-5p (g_{av}=0.73; Figure 6.10B) and 221-3p (g_{av}=0.85; Figure 6.10C) specifically following HII exercise (both P=0.007), which were not seen in AA (P=0.14, g_{av}=0.84 and P=0.55, g_{av}=0.27, respectively). Ci-miRs- 92a-3p and 181b-5p were the only ci-miRs not to exhibit significant effects of exercise (Figure 6.11A-B). Comparing the exercise-induced changes (%) in ci-miR between AA and CA yielded no significant differences for any ci-miR in response to either exercise bout (data not shown).

Exploratory correlations were determined to assess the associations between the exercise-induced changes in ci-miRs and vascular measures at 10 and/or 60 minutes after exercise in each race separately. For each exercise intensity, only those ci-miRs and vascular measures that exhibited significant effects of exercise were analyzed. The only significant correlation was between ci-miR-150-5p and carotid arterial compliance 10 minutes after HII exercise, which was significant in CA (r=0.82, P=0.001), but not AA (r=0.06, P=0.88) (Figure 6.12A). There were a few other correlations that approached statistical significance in only one race after HII exercise. Specifically, carotid arterial compliance with ci-miR-21-5p in CA 10 minutes post (r=0.54, P=0.07 vs. r=0.16, P=0.68 in AA) and with ci-miR-150-5p in AA 60 minutes post (r=0.61, P=0.083 vs. r=0.35, P=0.27 in CA) (Figure 6.12B). Additionally, ci-miR-

21-5p was negatively correlated with AIx75 in AA 10 minutes after HII exercise (r=0.67, P=0.050 vs. r=-0.078, P=0.81 in CA).

Discussion

The major findings of this study are that (a) brachial artery endothelial function did not differ between young, healthy, moderately active AA and CA individuals at baseline and was unaffected up to 60 minutes after either MOD or HII exercise (b) both AA and CA exhibited increases in central arterial stiffness 10 minutes after HII exercise, (c) CA had higher brachial MAP than AA 60 minutes after HII exercise and exhibited increases in aortic MAP after both exercise intensities, while AA did not (d) on average, carotid arterial compliance was lower in CA than AA at baseline as well as 10 minutes after both MOD and HII exercise, however it increased 60 minutes into recovery from HII exercise only in CA (e) AIx75 was elevated 10 minutes after MOD exercise only in CA, though it increased in both races 10 minutes after HII exercise (f) there were no resting racial differences in the concentrations of select ci-miRs related to vascular function, (g) several ci-miRs with known anti-inflammatory effects displayed significant exercise-induced increases only in CA, and (h) changes in anti-inflammatory ci-miR-150-5p correlated with the changes in carotid arterial compliance 10 minutes after HII exercise specifically in CA.

Endothelial function

Several previous investigations have reported lower conduit (66, 257, 319, 392) or resistance artery (135, 209, 379, 449, 474) endothelial function in AA as compared with CA. Reduced nitric oxide (NO) bioavailability along with elevated reactive

oxygen species (ROS) and inflammation contribute to the relative endothelial dysfunction in AA (58, 147, 254, 319, 468). Similar to a recent report by Kappus et al. (256) however, we observed no racial differences in resting macrovascular endothelial function as measured by FMD of the brachial artery. The reasons for the discrepancy between our results and those of prior studies demonstrating lower endothelial function in AA are unclear, but may be related to the fact that all participants in our study were moderately active. Although we did not measure cardiorespiratory fitness via VO_{2max}, the AA and CA groups had similar PPO on the cycle ergometer test, suggesting similar cardiorespiratory fitness. Therefore, the AA participants in our study may have had superior cardiovascular function as compared to average values, considering it has previously been shown that cardiorespiratory fitness is lower in AA than CA on average (449, 467, 555–557). In agreement with at least one other study (392), brachial arterial diameter trended towards (i.e. approached statistical significance) being larger in AA as compared with CA. Still, there remained no racial differences in endothelial function after FMD was allometrically-scaled to control for differences in brachial arterial diameter.

AA have also been reported to display altered vascular reactivity to stressful stimuli. As compared with CA, previous studies have shown that AA had lower endothelial-dependent vasodilation following a mental stress test (68) and greater vasoconstriction after a cold pressor test (475), despite no racial differences at rest. Thus, we hypothesized that FMD would be reduced to a greater degree and/or for a longer duration in AA following acute bouts of exercise. However, we did not observe any changes in FMD in either racial group following either MOD or HII exercise.

Classically, there is thought to be a biphasic response in brachial artery FMD following acute exercise, with an immediate decrease that rebounds back to/above baseline levels within the first hour of recovery (110). The decrease in FMD may be greater following higher intensity exercise (42, 48, 110, 248), yet the effects of exercise of different intensities on endothelial function in distinct populations, including those with CVD risk factors, are unclear.

To our knowledge, only one other study has determined the effects of acute exercise on endothelial function in AA and CA. Recently, Schroeder et al. (449) determined racial differences in endothelial-dependent dilation of the forearm resistance arteries 30 minutes after a maximal exercise test, in young, sedentary AA and CA individuals. Despite baseline microvascular endothelial function being greater in CA, they found similar exercise-induced increases in endothelial function among young AA and CA (449). Therefore, the limited data available to date do not suggest that endothelial function is differently affected by exercise in young, healthy AA as compared with their CA counterparts. Unfortunately, limited comparisons can be made between our results and those of Schroeder et al. (449) due to contrasts in participant habitual activity levels, exercise intensity/duration, type of artery assessed, the presence/absence of baseline racial differences, and post-exercise time-points of data collection. Considering impairments in resistance artery function may precede those in conduit arteries within AA (256), future investigations should seek to determine whether macro- and microvascular function are similarly affected by exercise of different intensities in AA and CA individuals.

Arterial stiffness

Central arterial stiffness is typically increased in the early minutes initially following exercise, after which it progressively returns to or below baseline levels (359). We observed this biphasic response in cf-PWV for both racial groups following HII exercise, while there were no effects of MOD exercise. The effects of exercise intensity on central arterial stiffness are not well studied, but our results suggest higher intensity exercise may be necessary to elicit increases. Our findings were contrary to our hypothesis that AA would have greater increases in arterial stiffness than CA, as cf-PWV actually approached statistical significance to be greater in CA than AA 10 minutes after HII exercise. Three previous investigations have compared exercise-induced changes in cf-PWV between AA and CA, all employing a graded maximal exercise test and taking measurements 15 and/or 30 minutes into recovery (207, 449, 556). Of those, only Yan et al. (556) observed racial differences in the response to exercise. Although their analyses precluded comparisons of post-exercise and baseline values, the change in cf-PWV differed between AA and CA 30 minutes after exercise, with AA exhibiting a numerical increase and CA a numerical decrease (556).

Transient exercise-induced increases in BP may increase arterial stiffness by causing a transfer in load bearing from the compliant elastin fibers to the stiffer collagen fibers making up the extracellular matrix (209, 264, 365). Indeed, the increases in cf-PWV disappeared when changes in aortic MAP were controlled for, suggesting the increase in central arterial stiffness after HII exercise was primarily due to elevated aortic MAP. In our study however, aortic MAP was increased after both exercise bouts, specifically in CA. The facts that cf-PWV was not increased with MOD

exercise, and that AA had increased cf-PWV without concomitant changes in MAP, suggest that increased central MAP may be neither sufficient nor necessary to cause increases in central arterial stiffness. The increased aortic MAP specifically in CA was unexpected, considering AA have been shown to exhibit heightened sympathetic nervous system activity and BP responses following psychological and physiological stress tests (65, 357, 522). Interestingly, Yan et al. (556) also reported that aortic DBP was elevated in CA men 15 minutes after maximal exercise, compared to no change in AA. However, in their study aortic MAP and cf-PWV were similarly unchanged in men of both races at the same time point, suggesting the change in aortic DBP was not sufficient to increase central arterial stiffness (556).

In our study, central arterial stiffness at rest trended ($P = 0.08$) towards being greater in CA and resting carotid arterial compliance was significantly lower in CA than AA. These results showing that CA had lower resting arterial compliance than AA are in contrast to prior reports. A recent meta-analysis found that, on average, cf-PWV was elevated by >0.28 m/s in young AA as compared with CA (60). Prior investigations also suggest that carotid arterial stiffness may be higher in AA as compared with CA (126, 206, 209, 449, 557). As with endothelial function, the reasons for the discrepancies between our findings and those of previous studies are unclear. Additional research comparing arterial stiffness measured at different sites and employing larger samples sizes are needed to clarify possible differences among young, healthy AA and CA individuals.

Compliance of the carotid artery also displayed a different recovery response to HII exercise between AA and CA. AA did not exhibit the same beneficial increase in

compliance one hour after HII exercise that was seen in CA. The race-specific response in carotid arterial compliance to exercise may have been related to the fact that CA had lower average baseline carotid arterial compliance. However, there were no significant racial differences in baseline carotid arterial compliance before the HII exercise bout, suggesting resting differences may not entirely account for the different recovery response of the carotid artery to HII exercise. Previously, carotid β-stiffness was shown to be higher in AA than CA 90 minutes after a moderately intense bout of running (557). Although we did not see any effects of exercise on carotid arterial β-stiffness in our study, those findings support a potential beneficial exercise recovery response in carotid arterial compliance within CA as compared with AA.

While cf-PWV is a measurement of stiffness over a segment of the arterial tree including elastic (i.e. carotid and aorta) and more muscular arteries (i.e. femoral), local assessment of carotid arterial stiffness specifically reflects the stiffness of a large elastic artery (283). Thus, mechanisms governing changes in cf-PWV and carotid arterial stiffness after exercise likely differ (359). Previously, AA have been shown to have higher carotid arterial BP and wall thickness as compared with CA, both of which are proposed to mediate elevations in arterial stiffness (209, 557). Although we did not specifically measure carotid BP, AA in our study did not experience elevated peripheral (brachial) or central BP 60 minutes after exercise, suggesting BP changes likely did not contribute to the lack of a decrease in carotid arterial compliance as was seen in CA. Thus, it is unclear what mediated the differential effect of exercise on carotid arterial stiffness. Nevertheless, the divergent exercise-induced changes in carotid arterial compliance may carry potentially important implications. Exercise can reveal

impairments in cardiovascular health not apparent at rest. For example, a greater acute exercise-induced increase in BP is related to elevated CVD risk (494) and is a predictor of future cardiovascular events (288) and mortality (355). Resting arterial stiffness is predictive of future CVD and related mortality (53, 283, 520), thus it is plausible that a relationship may also exist with exercise-induced changes in arterial stiffness and future CVD risk. Additional studies are needed to determine a potential relationship between carotid arterial compliance during the recovery from exercise and the risk of future CVD in AA who may or may not have elevated resting arterial stiffness.

The reduced carotid arterial compliance in CA 60 minutes after HII exercise reflects a beneficial transient effect of exercise that may also have implications for adaptations to exercise training. For instance, the change in FMD following an acute bout of exercise is positively correlated with the degree of adaptation following exercise training (109). Thus, a similar relationship could exist for acute and chronic exercise-induced changes in arterial stiffness, though this remains to be determined. The fact that AA did not display the same beneficial increase in carotid arterial compliance as CA suggests the possibility that exercise training may not induce the same beneficial effects as those in CA. Central arterial stiffness is generally reduced with aerobic exercise training, with greater reductions observed in those with stiffer arteries (>8 m/s) and in response to longer durations of exercise training (>10 weeks) (19). Ranadive et al. (408) found that both AA and CA men had reduced carotid arterial pulse pressure after eight weeks of aerobic exercise training, though cf-PWV and carotid β-stiffness were unaffected by training in both races. Additionally, carotid arterial diameter decreased only in CA and carotid intra-media thickness decreased

specifically in AA after training (408). Therefore, there may be race-specific adaptations to exercise training and these could be related to different responses to acute exercise. A longer exercise training intervention, especially in individuals with elevated arterial stiffness, could potentially reveal differential effects of exercise training on carotid arterial stiffness in AA.

Augmentation index

AIx is indicative of the afterload imposed on the left ventricle due to wave reflection and can change independently of arterial stiffness (398). Importantly, AIx is a potentially better predictor of future CVD in young to middle-aged individuals as compared to cf-PWV (262, 330, 535). With acute exercise there is generally a transient decrease in AIx that is largely driven by increased HR (359, 393). When AIx is normalized to a HR of 75 bpm (AIx75) in order to control for this, AIx75 typically increases immediately after exercise (359, 393). Indeed, AIx75 was increased similarly in both AA and CA 10 minutes after HII exercise, although only CA exhibited a significant increase 10 minutes after MOD exercise. To our knowledge, ours is the first study to determine the effects of non-maximal exercise bouts on AIx75 in AA and CA individuals. Previous studies have found that a maximal graded exercise test similarly affected AIx75 in AA and CA (449, 556). Thus, the differential response may be specific to moderate intensity exercise and suggests AIx75 could be more sensitive to changing in CA as compared with AA.

Exercise-induced increases in AIx75 may be mediated by elevated central arterial stiffness or mismatching between central and peripheral vascular tone (impedance mismatching) due to vasoconstriction of resistance arteries to areas of non-

active skeletal muscle (393). Central arterial stiffness does not appear to have played a role in the increased AIx75 that was seen in CA after MOD exercise, since cf-PWV was not significantly altered with MOD exercise and the exercise response did not differ between the races. The elevated AIx75 response in CA could alternatively be due to greater sympathetically driven peripheral vasoconstriction in response to the MOD exercise bout, although prior research suggests that AA have greater sympathetic activity and/or vascular reactivity in response to stress (65, 475). There were also no significant racial differences in brachial artery FMD or diameter after MOD exercise, suggesting peripheral resistance may have been similar. Therefore, the mechanisms underlying the elevation in AIx75 specifically in CA remain to be clarified.

Circulating microRNA

To our knowledge, this is the first study comparing ci-miR expression between young, healthy AA and CA individuals. Differences in epigenetic mechanisms, such as miR expression, stemming from sociocultural and environmental factors experienced by past and present generations are proposed to mediate the elevated CVD risk in AA (129, 272, 332, 515). In addition, AA have been shown to have higher circulating markers of inflammation and ROS (145, 319, 468), as well as greater endothelial inflammation, ROS, and lower NO production as compared with CA (147, 254). Thus, we hypothesized that ci-miRs related to vascular inflammation, ROS production, and eNOS expression/activity may differ between young, healthy AA and CA individuals. However, we found no differences in the resting levels of any of the ci-miRs related to vascular function that we measured. Still, this does not preclude any racial differences in resting ci-miR expression since we only examined a small *a priori* selected set of ci-

miRs. Future studies should use an unbiased approach to identify possible differentially expressed miRs between AA and CA individuals.

In our study, several ci-miRs related to vascular function increased in response to HII and/or MOD exercise and all but one of these were significantly increased only in CA. These results suggest ci-miRs may be more sensitive to changing in response to exercise in CA, or the response may be more heterogeneous among AA. When the fold changes in ci-miRs were compared between AA and CA there were no significant differences, while effect sizes for changes in some ci-miRs were also similar between the races. Thus, additional studies are needed to determine whether our findings are simply a result of our relatively small sample sizes or if ci-miRs actually exhibit a blunted response in AA following exercise as compared to in CA. Sex differences also exist in ci-miR expression, so separate comparisons of men and women are also needed.

Ci-miRs can be selectively taken up by recipient vascular cells where they exert their effects by regulating gene expression (31, 369, 567). Chronically, ci-miRs that are released during exercise may, therefore, contribute to the adaptations in endothelial function and arterial stiffness with exercise training. The ci-miRs that we assessed are generally anti-inflammatory and increase NO and/or decrease ROS production (74, 76, 84, 89, 91, 198, 375, 554, 569). Thus, their transiently increased expression with exercise would be expected to induce beneficial adaptive effects on the vasculature.

Interestingly, many of the ci-miRs that increased in response to exercise in CA exhibit anti-inflammatory effects. For example, miRs- 146a-5p, 221-3p, and 222-3p were increased after exercise specifically in CA and have been shown to curb vascular inflammation. MiR-146a-5p is upregulated in response to inflammatory stimuli and

acts to counteract inflammation-induced endothelial activation by preventing the increased expression of NF-kB and decreased expression of eNOS, via the downregulation of separate upstream targets (85). Additionally, miR-146a-5p negatively regulates toll-like receptor (TLR) signaling (486). MiRs- 221-3p and 222-3p are highly related paralogues that are abundantly expressed in both endothelial cells and vascular smooth muscle cells (74, 91, 299, 479, 573). Within endothelial cells, both miRs-221/222 attenuate TNFα-induced increases in NF-kB expression, as well as the expression of the pro-inflammatory transcription factor Ets-1 (296, 573). MiRs-221/222 further regulate processes in inflammation-induced vascular remodeling associated with atherosclerosis, including the promotion of vascular smooth muscle cell proliferation (91, 121, 299). Therefore, we hypothesize that anti-inflammatory ci-miRs may increase in response to exercise-induced inflammation and may act to curb future inflammation and changes in vascular function/stiffness.

MiRs- 92a-3p and 181b-5p were the only ci-miRs detected that did not increase in response to either exercise bout. Both miRs -92a-3p and 181b-5p are upregulated in response to low/oscillatory vascular shear stress (305, 554), likely explaining why they did not increase in response to exercise, which is associated with increases in laminar shear stress. MiR-181b-5p is also downregulated in endothelial cells in response to inflammatory stimuli (i.e. TNFα) and is reduced in the circulation of patients with inflammatory diseases (84). Thus, exercise-induced inflammation may have also acted to prevent increases in ci-miR-181b-5p. Interestingly, both of these miRs may exert detrimental effects on vascular function. MiR-92a-3p exerts pro-inflammatory effects by suppressing KLF2 expression and indirectly increasing NF-kB expression (84, 305).

On the other hand, miR-181b-5p indirectly suppresses NF-kB activity, but may act to increase ROS production and decrease NO bioavailability by directly targeting SIRT1 (84, 532).

We further determined correlations between the exercise-induced changes in ci-miRs and vascular measures in order to determine associations that might suggest potential roles of ci-miRs in contributing to vascular responses. While the effects of ci-miRs likely are not apparent within the first hour after exercise due to the time required for cellular transport and for alterations in translation to manifest as altered protein expression, they may be 1) related to vascular responses because they are released in response to vascular stimuli that also alter endothelial function or arterial stiffness, and/or 2) released as "compensatory signals" that are meant to induce adaptations that prevent future disturbances in vascular function/stiffness. The only significant correlation and most of the borderline significant correlations were between ci-miRs and carotid arterial compliance, which showed racial differences in the response to HII exercise. Specifically, the change in ci-miR-150-5p was positively associated with the change in arterial compliance 10 minutes after HII only in CA. Interestingly, the correlation between ci-miR-150-5p and arterial compliance 60 minutes after HII approached significance in AA, but was not significant in CA. MiR-150-5p inhibits the NF-KB pathway, maintaining endothelial cell function and reducing the production/secretion of inflammatory cytokines from macrophages (173, 309, 431).

Speculatively then, the fact that carotid arterial compliance did not increase in AA during the recovery from HII exercise may be related to inflammation since the majority of anti-inflammatory ci-miRs failed to significantly increase, and the

association between the changes in anti-inflammatory ci-miR-150 and carotid arterial compliance differed between the races. Whether this may stem from greater resting and/or exercise-induced inflammation is unclear. Further, the increase in these ci-miRs with exercise in CA may constitute a hormetic response meant to curb future exercise-induced inflammation. The lack of a similar response in AA suggests a possible mechanism by which exercise training adaptations may be blunted, and this requires future study.

Conclusions

Contrary to our hypotheses, AA did not display impaired endothelial function or exaggerated increases in central arterial stiffness in response to exercise as compared with CA. In disagreement with prior research, CA actually exhibited lower resting arterial compliance and greater responses in central BP and AIx75 following MOD exercise, as compared with AA. Apparent discrepancies between our results and those of previous studies could be related to the fact that prior studies have largely employed maximal graded exercise tests as the exercise stimulus (207, 449, 556). Therefore, additional studies are needed to determine the vascular responses of AA following more diverse intensities and durations of exercise. There was, however, an increase in carotid arterial compliance in CA 60 minutes after HII exercise that did not occur in AA. This apparently adverse recovery response in AA could be related to mechanisms underlying a greater risk for CVD development. Therefore, future studies should seek to elaborate on this differential response and determine potential mediating factors. In our study, a number of ci-miRs with beneficial actions within the vasculature were significantly increased following exercise in CA, but not AA. Additionally, the

association between the anti-inflammatory ci-miR-150-5p and the change in arterial compliance differed between CA and AA. These findings suggest potential mechanisms that may underlie the differential effects of exercise, as well as possible differences in the adaptations to exercise training, within AA.

Table 6.1. Subject characteristics

	African American (n=11)	Caucasian American (n=14)	P
male/female (% female)	8/3 (27%)	10/4 (29%)	
Age, y	21 ± 2	22 ± 3	0.40
BMI, kg/m^2	24 ± 2	23 ± 3	0.52
Peak power output, kg·m/s	25 ± 5	26 ± 6	0.89
Resting HR, bpm	66 ± 6	65 ± 7	0.63
SBP, mmHg	119 ± 12	121 ± 10	0.53
DBP, mmHg	70 ± 10	67 ± 7	0.44
MAP, mmHg	86 ± 10	85 ± 7	0.80
Glucose, mg/dl	87 ± 6	89 ± 7	0.60
Hemoglobin A1c, %	5.2 ± 0.3	5.0 ± 0.3	0.09
Total cholesterol, mg/dl	153 ± 24	164 ± 33	0.38
HDL-C, mg/dl	55 ± 11	58 ± 11	0.51
LDL-C, mg/dl	84 ± 19	90 ± 29	0.59
VLDL-C, mg/dl	13 ± 3	15 ± 6	0.38
Triglycerides, mg/dl	55 ± 17	71 ± 30	0.13

BMI, body mass index; DBP, diastolic blood pressue, HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; MAP, mean arterial pressure; SBP, systolic blood pressure; VLDL-C, very-low-density lipoprotein cholesterol. Means ± SD.

Table 6.2. Average baseline vascular measures

	African American	Caucasian American	P
FMD, %	5.84 ± 2.27	7.59 ± 3.74	0.19
Brachial arterial diameter, mm	4.22 ± 0.58	3.78 ± 0.59	0.08
Allometrically scaled FMD, %·mm^{-1}	1.46 ± 0.69	2.17 ± 1.26	0.11
cf-PWV, m/s	5.39 ± 0.41	5.79 ± 0.62	0.08
Carotid β-stiffness, a.u.	4.81 ± 1.14	4.94 ± 0.66	0.74
Carotid arterial compliance, mm^2/kPa	1.12 ± 0.24	0.94 ± 0.13	**0.02**
AIx, %	6.77 ± 8.09	5.36 ± 5.55	0.61
AIx75, %	1.05 ± 11.27	3.64 ± 6.12	0.47

AIx, augmentation index; AIx75, augmentation index relative to heart rate of 75 bpm; cf-PWV, carotid-to-femoral pulse wave velocity; FMD, flow-mediated dilation. Means ± SD.

Figure 6.1

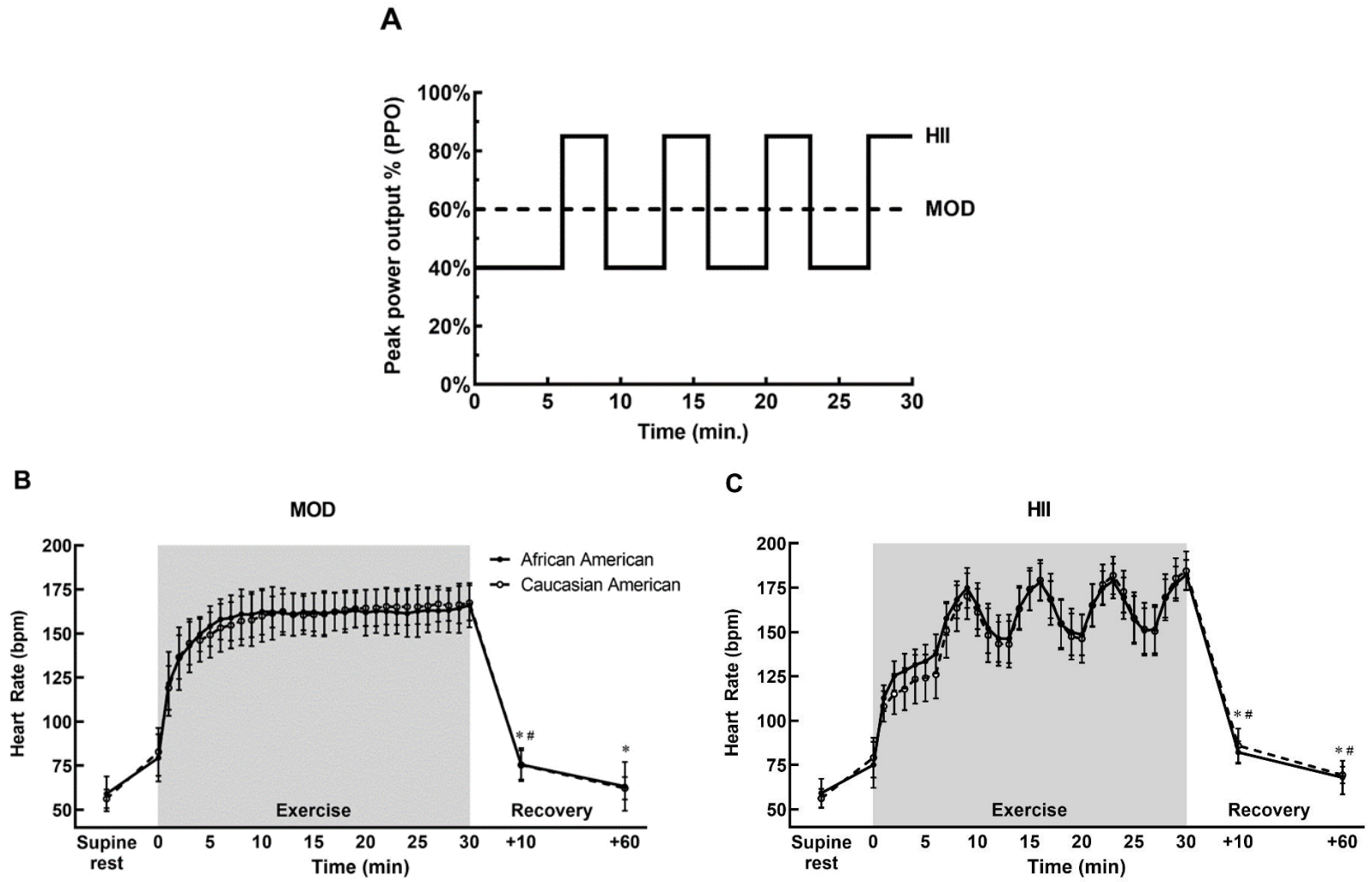

Figure 6.1. The moderate intensity continuous (MOD) and high intensity interval (HII) cycling protocols [A] were matched for time and average peak power output (PPO). Heart rate responses to the MOD [B] and HII [C] exercise bouts in the African American (AA) and Caucasian American (CA) groups. Data are Means ± SD. For MOD, AA n = 10, CA n = 13. For HII, AA n = 11, CA n = 13. * $P < 0.05$ vs. baseline for CA. # $P < 0.05$ vs. baseline for AA.

Figure 6.2

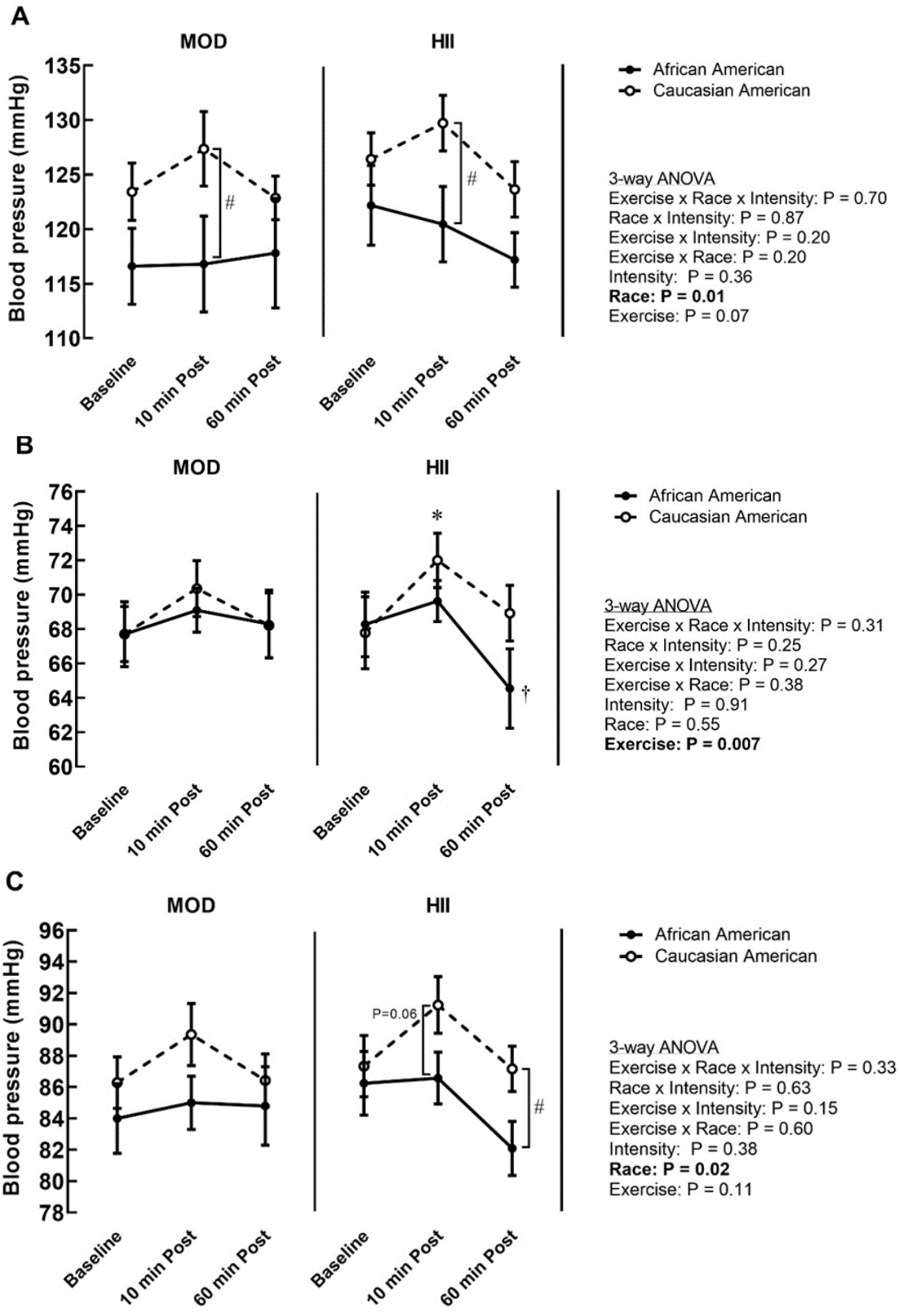

Figure 6.2. Brachial [A] systolic, [B] diastolic, and [C] mean arterial pressure in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 14) and high intensity interval (HII) (AA n = 11, CA n = 14) exercise. Data are means ± SEM. † $P < 0.05$ vs. baseline for AA. * $P < 0.05$ vs. baseline for CA. # $P < 0.05$ between races.

Figure 6.3

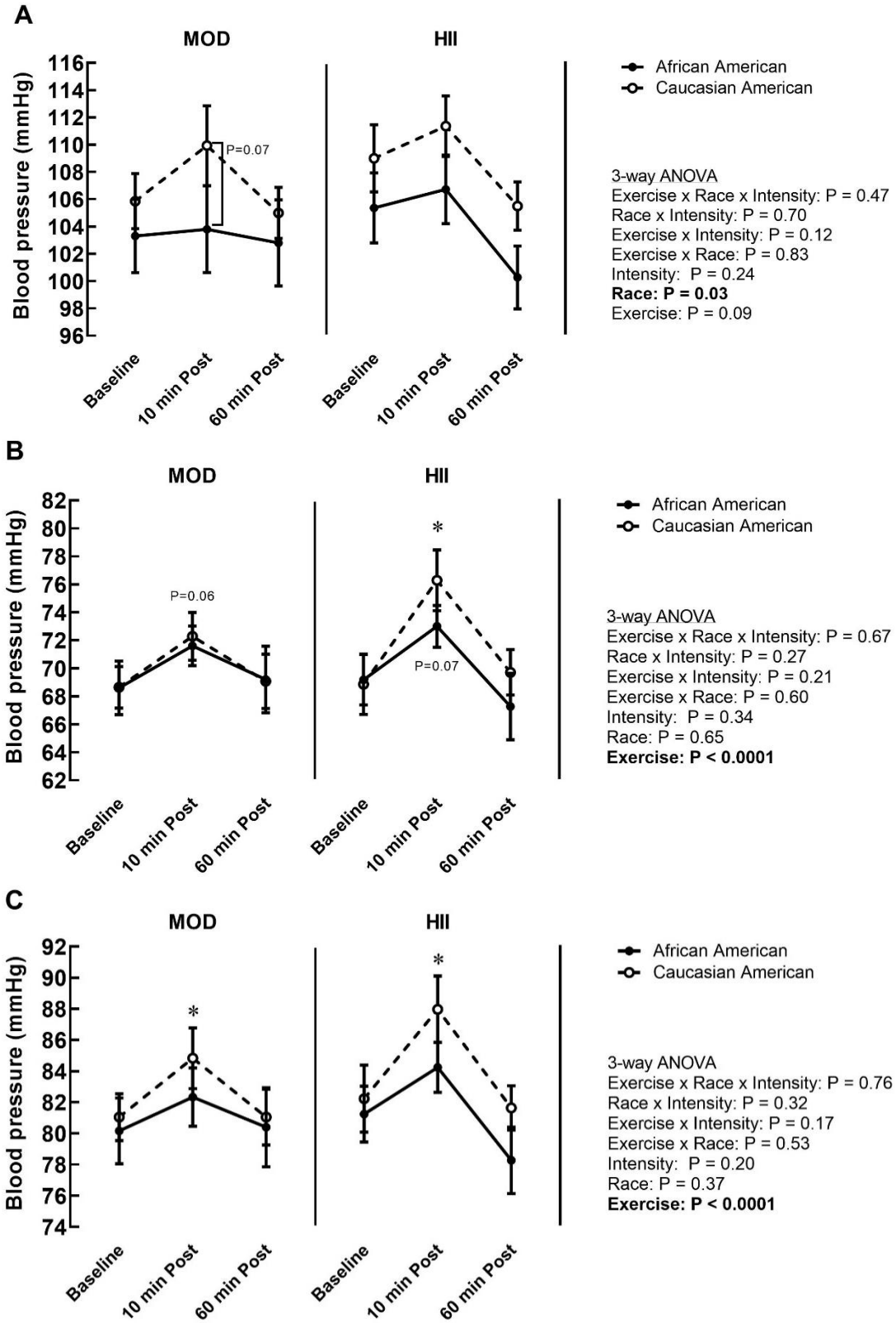

Figure 6.3. Aortic [A] systolic, [B] diastolic, and [C] mean arterial pressure in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 14) and high intensity interval (HII) (AA n = 11, CA n = 14) exercise. Data are means ± SEM. * $P < 0.05$ vs. baseline for CA.

Figure 6.4

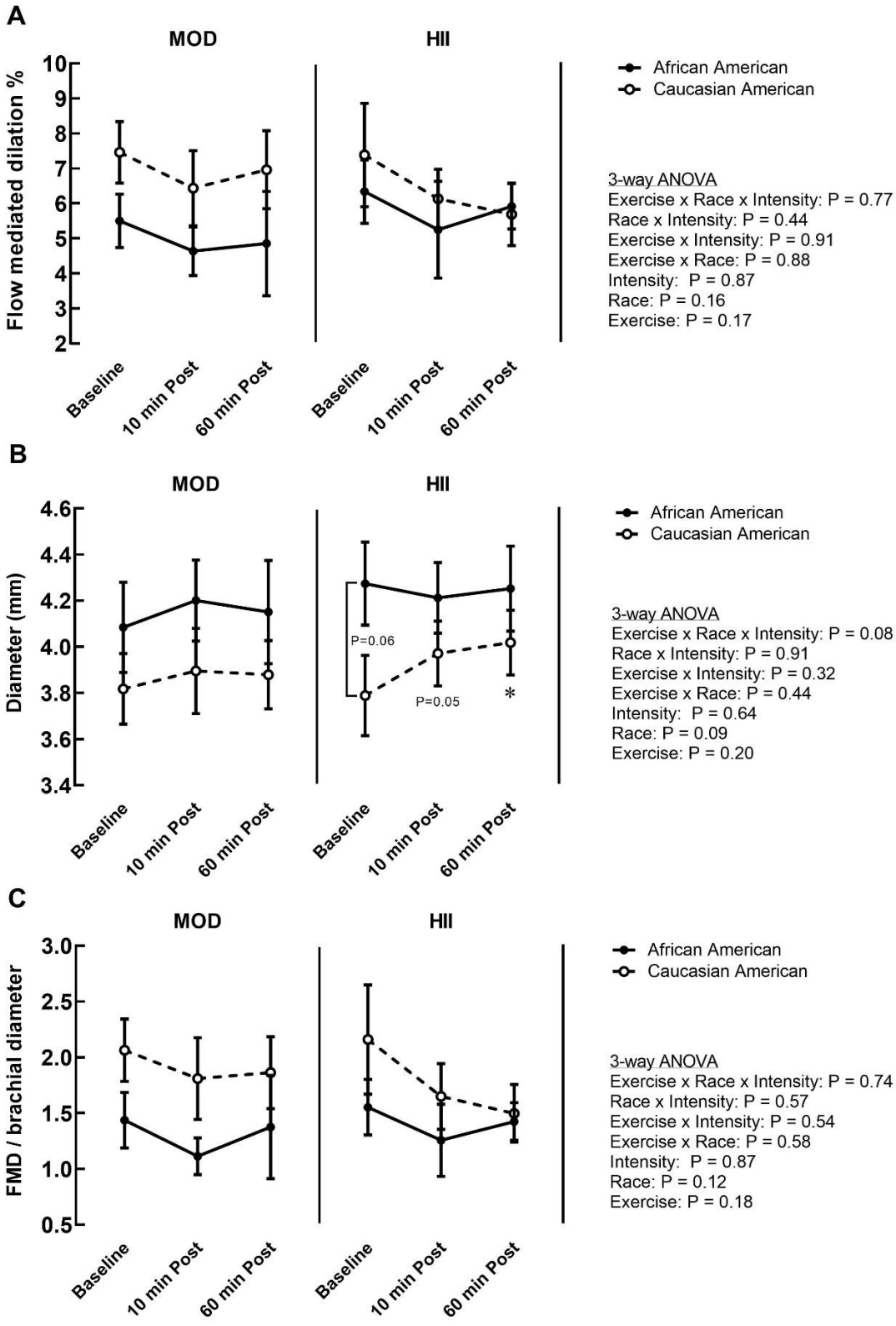

Figure 6.4. [A] Flow mediated dilation (FMD), [B] brachial arterial diameter, and [C] FMD allometrically-scaled to brachial arterial diameter in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 14) and high intensity interval (HII) (AA n = 11, CA n = 13) exercise. Data are means ± SEM. * $P < 0.05$ vs. baseline for CA.

Figure 6.5

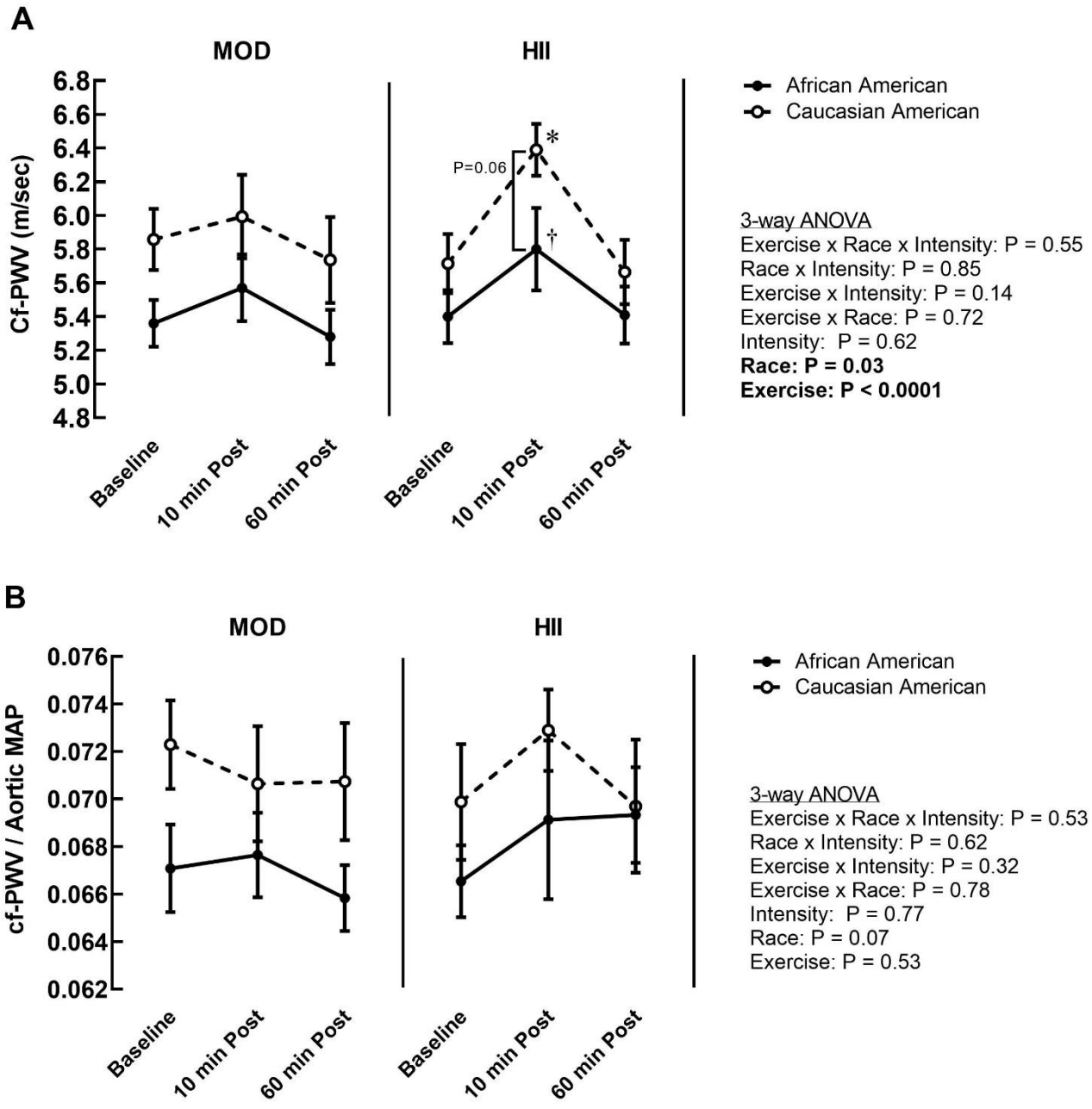

Figure 6.5. [A]Carotid to femoral pulse wave velocity (cf-PWV), and [B] cf-PWV relative to aortic mean arterial pressure (MAP) in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 14) and high intensity interval (HII) (AA n = 11, CA n = 14) exercise. Data are means ± SEM. † $P < 0.05$ vs. baseline for AA.
* $P < 0.05$ vs. baseline for CA.

Figure 6.6

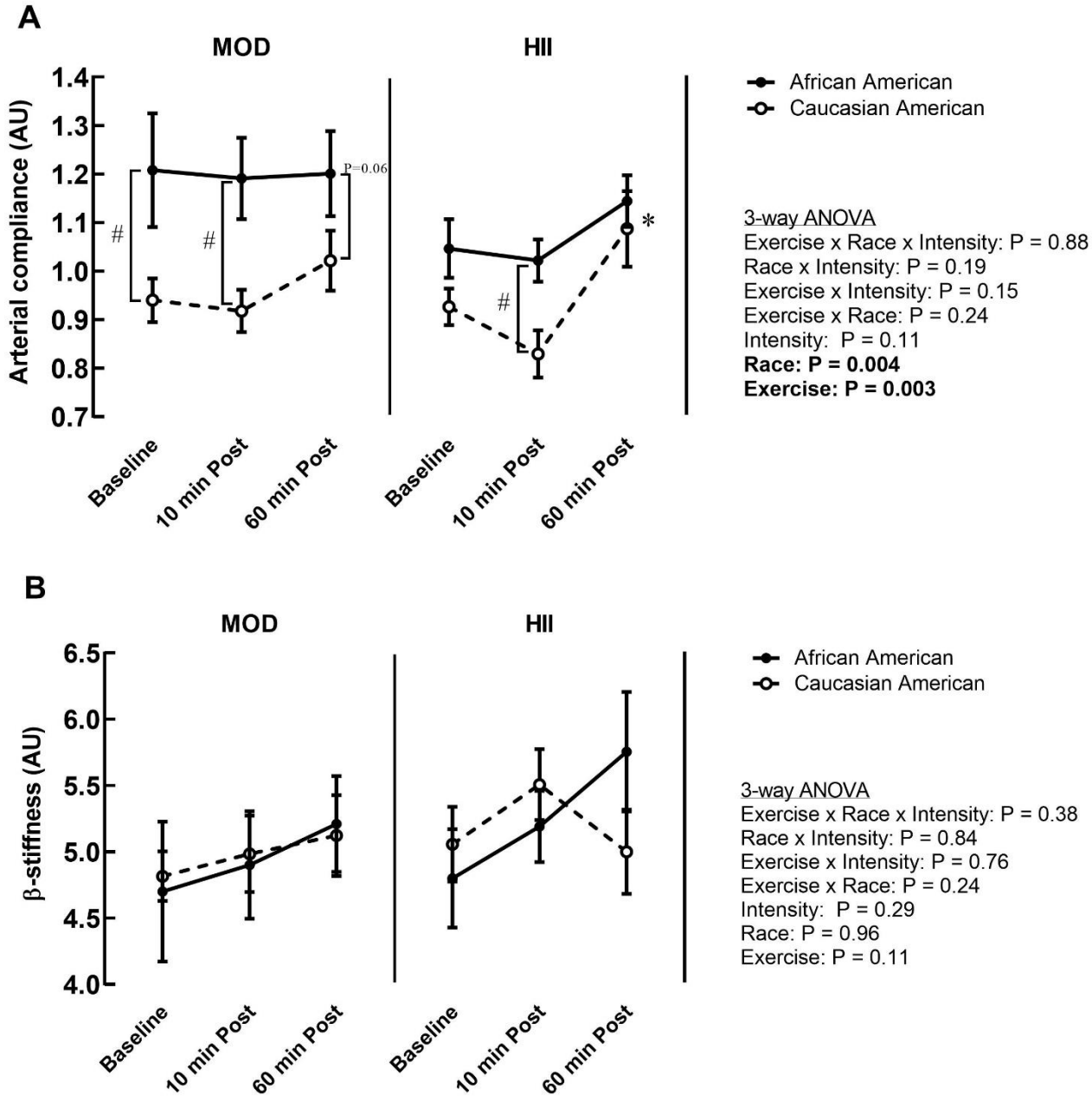

Figure 6.6. [A] Carotid arterial compliance and [B] β-stiffness in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 13) and high intensity interval (HII) (AA n = 11, CA n = 14) exercise. Data are means ± SEM. * $P < 0.05$ vs. baseline for CA. # $P < 0.05$ between races.

Figure 6.7

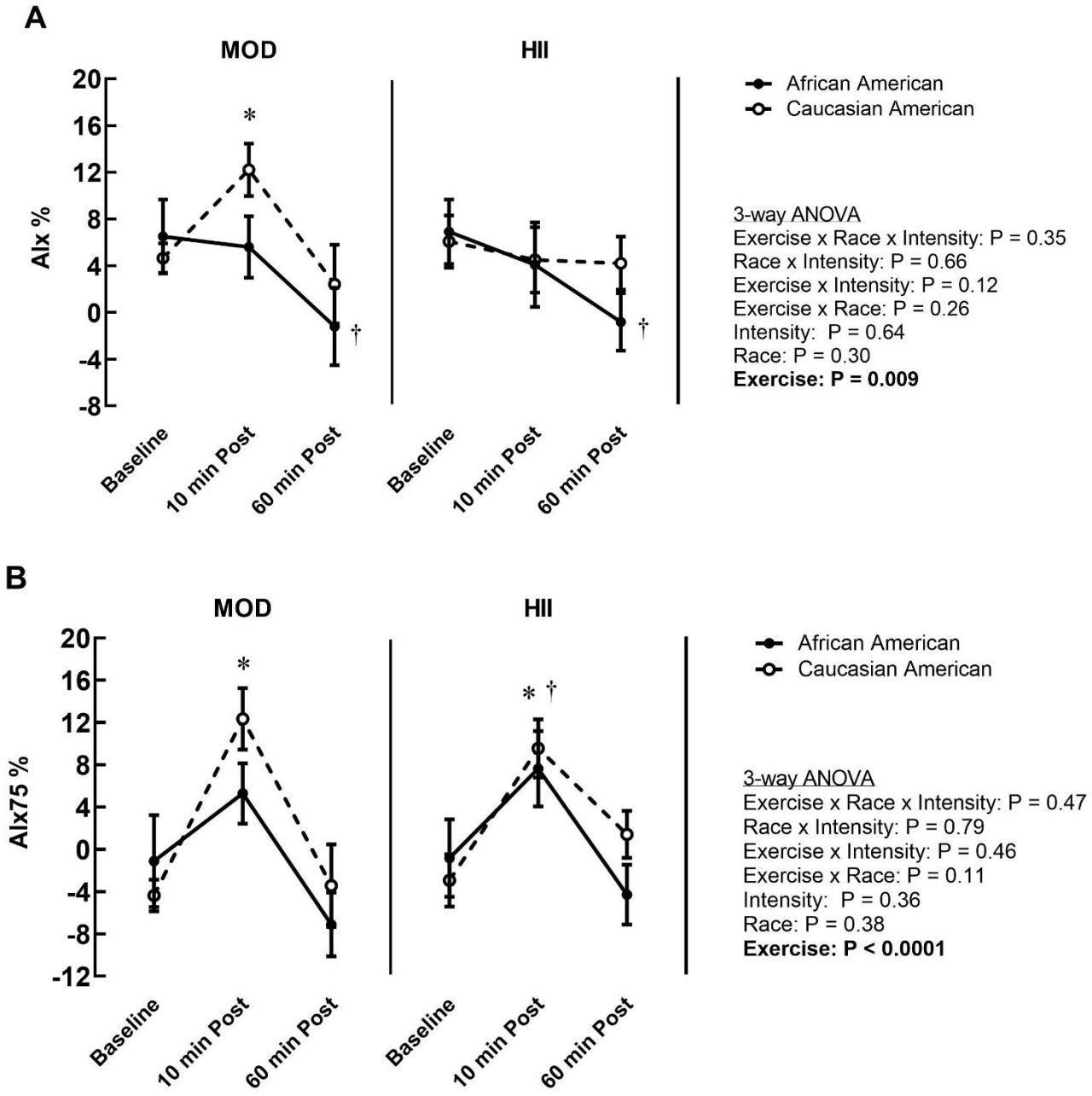

Figure 6.7. [A] Augmentation index (AIx) and [B] AIx normalized to a heart rate of 75 bpm (AIx75) in African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 14) and high intensity interval (HII) (AA n = 11, CA n = 14) exercise. Data are means ± SEM. † $P < 0.05$ vs. baseline for AA. * $P < 0.05$ vs. baseline for CA.

Figure 6.8

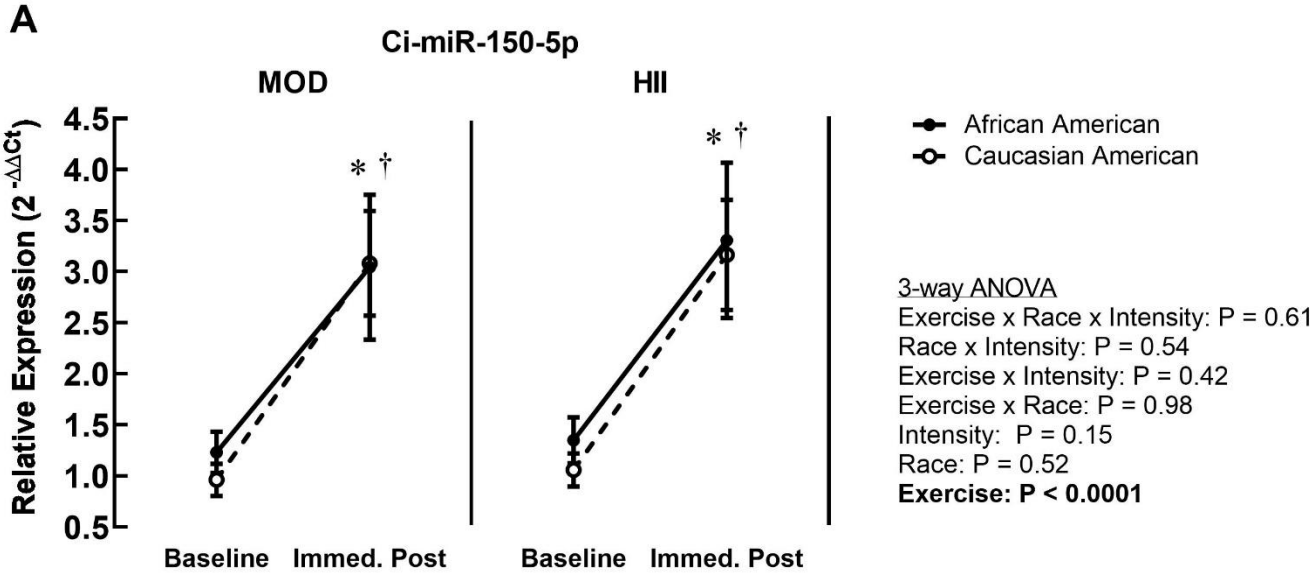

Figure 6.8. Circulating microRNA- (ci-miR-) 150-5p increased in both African American (AA) and Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (AA n = 10, CA n = 11) and/or high intensity interval (HII) (AA n = 9, CA n = 13) exercise. Data are means ± SEM. * CA $P < 0.05$ vs. baseline. † AA $P < 0.05$ vs. baseline.

Figure 6.9

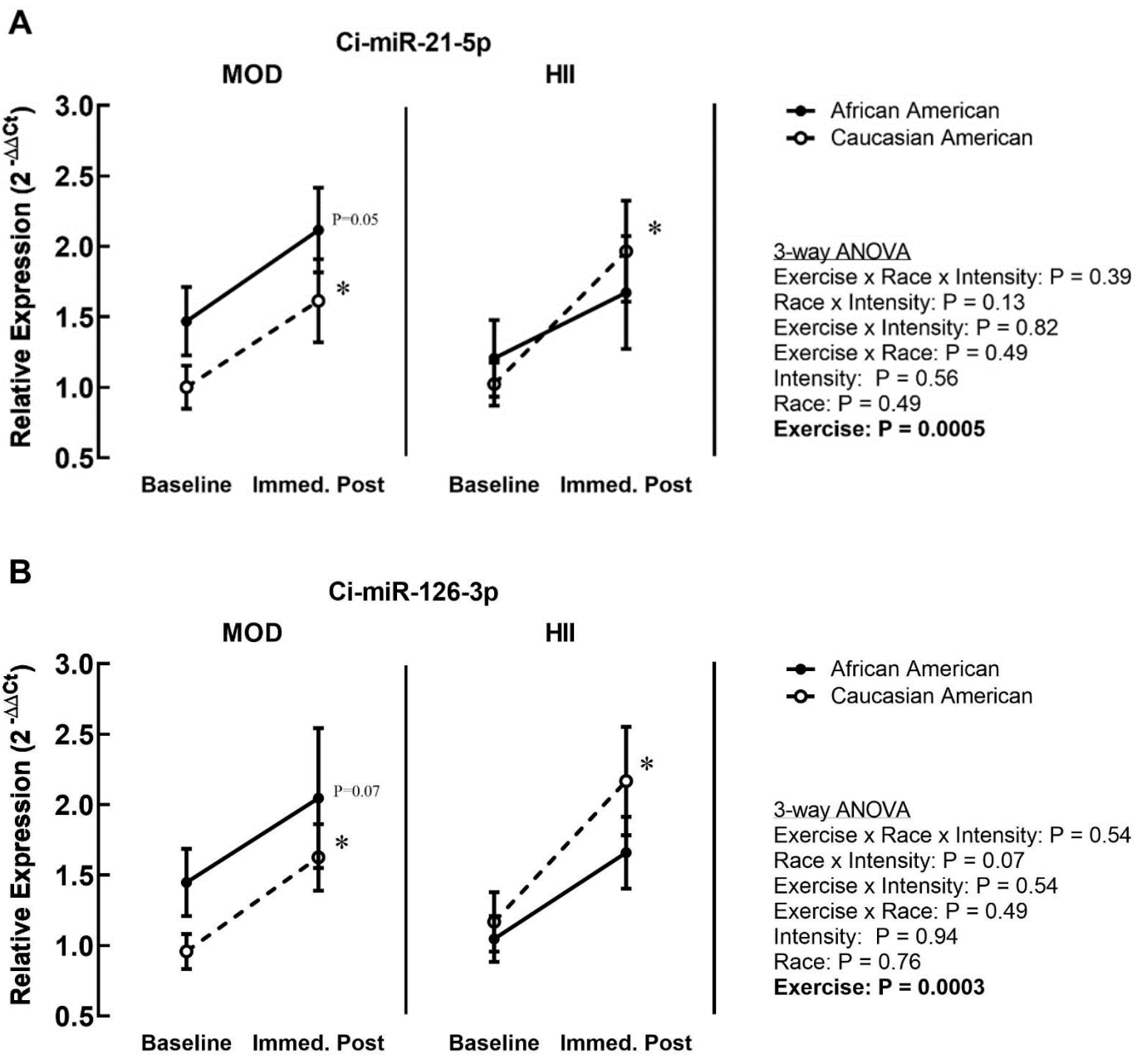

Figure 6.9. Circulating microRNAs- (ci-miRs-) [A] 21-3p and [B] 126-3p increased significantly in Caucasian American (CA) individuals in response to both moderate intensity continuous (MOD) (African Americans n = 10, CA n = 11) and high intensity interval (HII) (AA n = 9, CA n = 13) exercise. Increases only approached statistical significance in AA following MOD exercise. Data are means ± SEM. * P < 0.05 vs. baseline in CA.

Figure 6.10

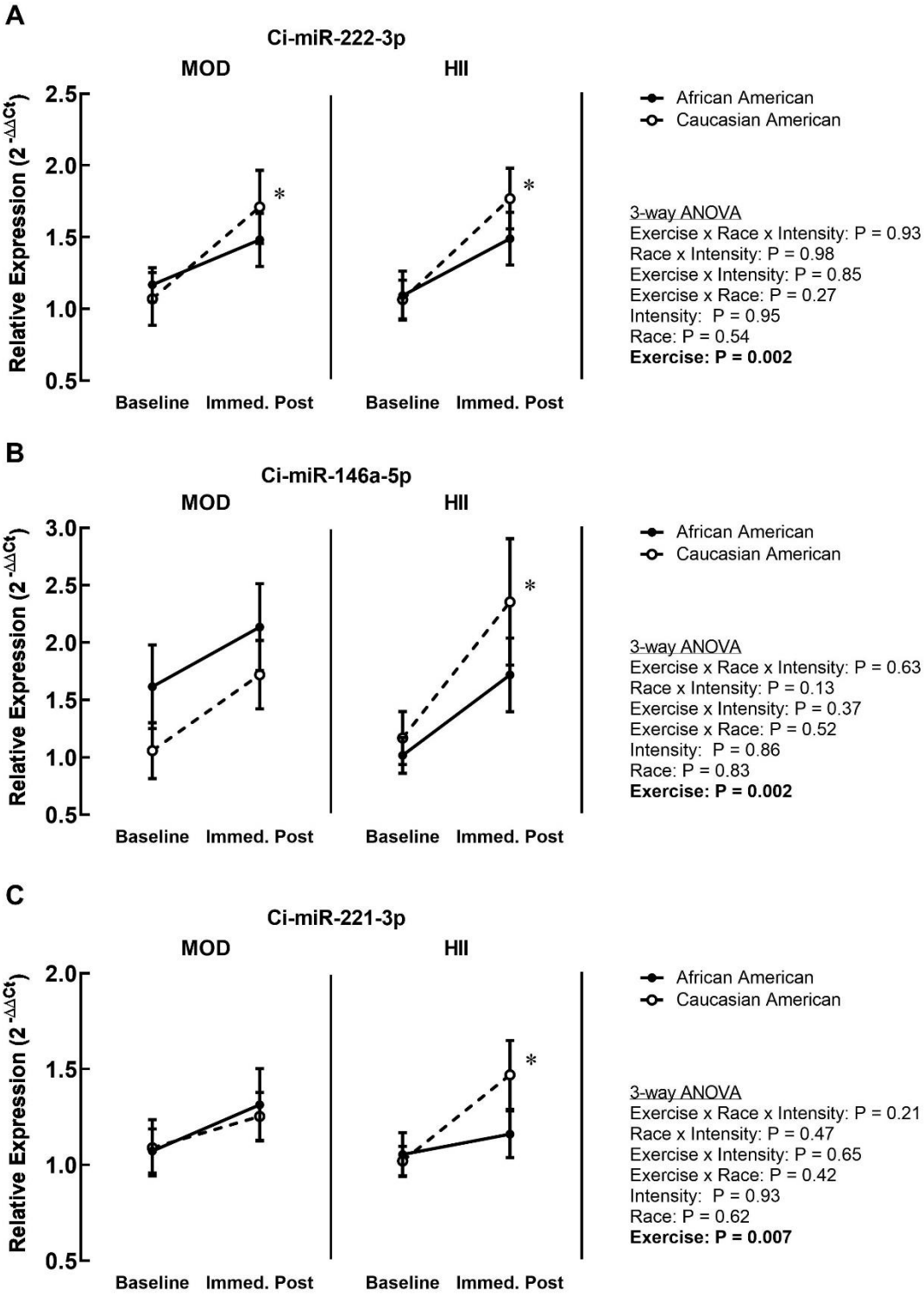

Figure 6.10. Circulating microRNAs- (ci-miRs-) [A] 222-3p, [B] 146a-5p, and [C] 221-3p increased in Caucasian American (CA) individuals in response to moderate intensity continuous (MOD) (African Americans (AA) n = 10, CA n = 11) and/or high intensity interval (HII) (African Americans (AA) n = 9, CA n = 13) exercise, but were unaltered in AA individuals. Data are means ± SEM. * $P < 0.05$ vs. baseline in CA.

Figure 6.11

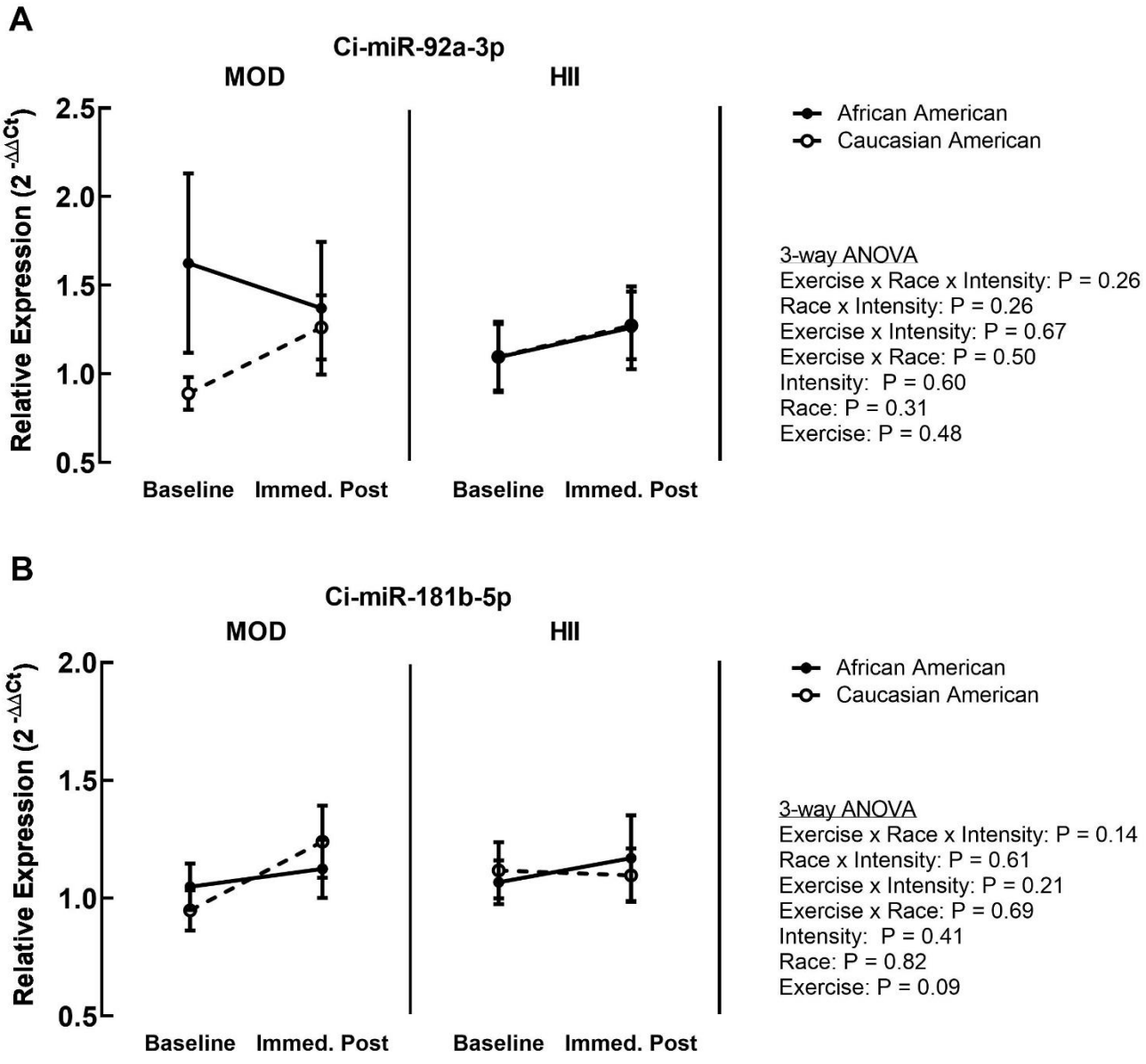

Figure 6.11. Circulating microRNA- (ci-miR-) [A] 92a-3p and [B] 181b-5p were not significantly affected by either moderate intensity continuous (MOD) (African Americans (AA) n = 10, Caucasian American (CA) n = 11) or high intensity interval (HII) (AA n = 9, CA n = 13) exercise. Data are means ± SEM.

Figure 6.12

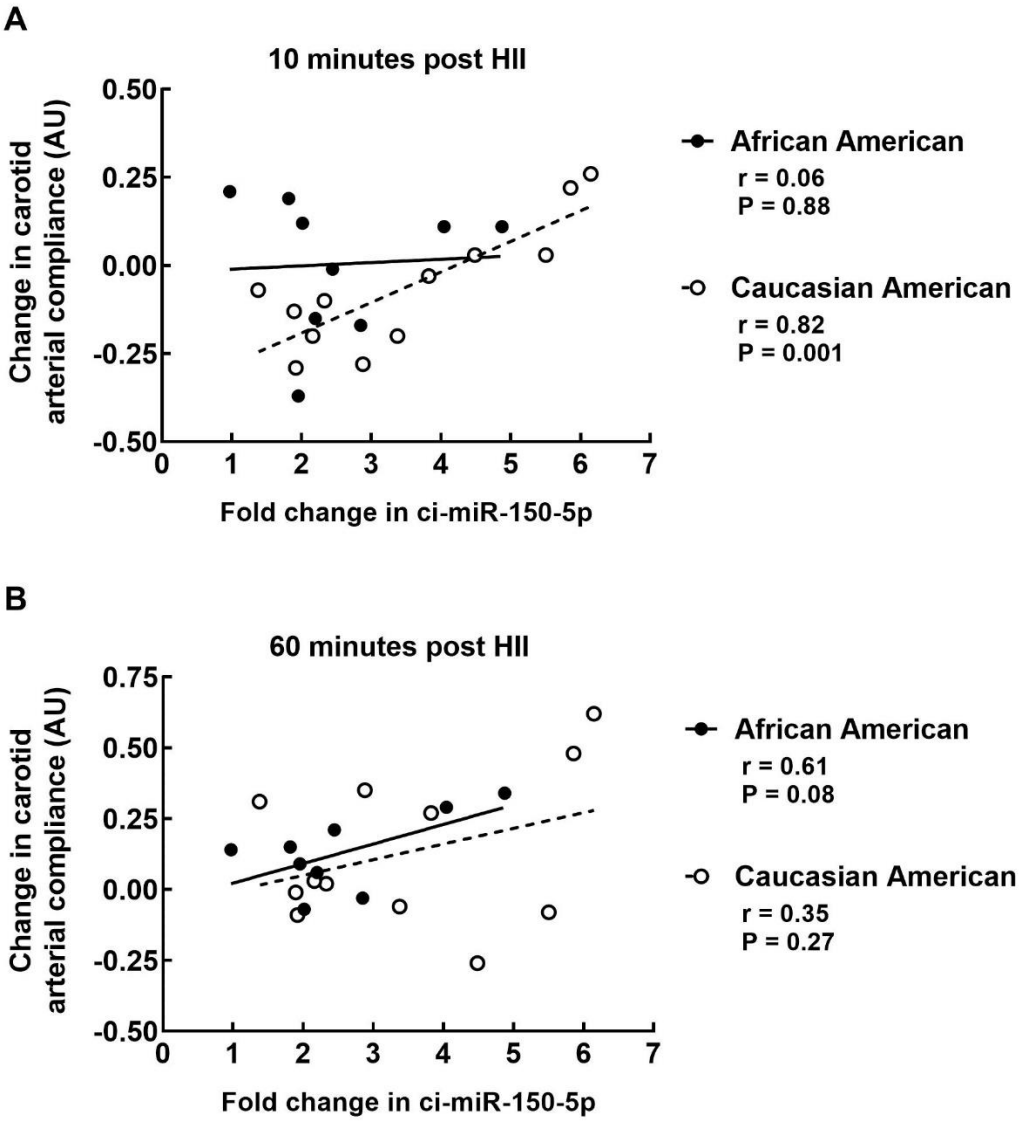

Figure 6.12. Correlations between the changes in circulating microRNA- (c-miR-) 150-5p immediately after and carotid arterial compliance [A] 10 minutes after and [B] 60 minutes after high intensity (HII) exercise.

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Chapter 7: The effects of moderate and high-intensity exercise on circulating markers of endothelial integrity and activation in young, healthy men

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The effects of moderate and high intensity exercise on circulating markers of endothelial integrity and activation in young, healthy men

Ryan M. Sapp[1], William S. Evans[1], Lauren E. Eagan[1], Catalina A. Chesney[1], Evelyn M. Zietowski[1], 2, Steven J. Prior[1], 3, Sushant M. Ranadive[1], James M. Hagberg[1]

[1]Department of Kinesiology, School of Public Health, University of Maryland, College Park, MD, USA

[2]Department of Biology, University of Maryland, College Park, MD, USA

[3]Baltimore Veterans Affairs Geriatric Research, Education and Clinical Center, Baltimore, MD, USA

Running title: Acute exercise and circulating markers of endothelial damage

Correspondence: James M. Hagberg, Address: 2242 Valley Drive, College Park, Maryland 20742-2611. (301)-405-2487 (TEL), (301)-405-5578 (FAX).

hagberg@umd.edu

Abstract

Endothelial function typically exhibits a hormetic response to exercise. It is unknown whether endothelial damage occurs in response to acute exercise and could be a contributing mechanism. We sought to determine the effects of acute exercise on endothelial-derived circulating factors proposed to reflect endothelial integrity and activation. Ten young, healthy men underwent 30-minute moderate continuous (MOD) and high intensity interval (HII) cycling exercise bouts. Venous blood samples were taken immediately before and after exercise for quantification of circulating endothelial cells (CEC), circulating angiogenic cells (CAC), apoptotic and activated endothelial microvesicles (EMV), thrombomodulin (TM), von Willebrand Factor (vWF), syndecan-1, and circulating microRNAs (ci-miRs) 126-3p and 126-5p. Endothelial function was assessed by flow-mediated dilation (FMD) of the brachial artery before, 10min after, and 60min after exercise. Numbers of CEC and EMV were unchanged by either exercise bout ($P>0.05$). Numbers of all measured CAC subtypes decreased in response to MOD (21-34%, $P<0.05$), while only $CD31^{+}/34^{+}/45^{dim/-}$ CAC decreased following HII (21%, $P<0.05$). TM and syndecan-1 increased with both exercise intensities (both ~20%, $P<0.05$). HII, but not MOD, increased vWF (88%, $P<0.001$), ci-miR-126-3p (92%, $P=0.009$) and ci-miR-126-5p (110%, $P=0.01$). The changes in several circulating factors correlated with changes in FMD following either one or both intensities. Changes in circulating factors do not support the concept of exercise-induced endothelial cell denudation, apoptosis, or activation, though slight disruption of endothelial glycocalyx and membrane integrity may occur. A related loss of

mechanotransduction along with mechanisms underlying endothelial activation and ci-miR-126 secretion may relate to changes in endothelial function.

Introduction

Exercise training is beneficial for the cardiovascular system, largely through direct effects of blood flow on the endothelium (182). Primarily, repeated increases in laminar shear stress during acute exercise bouts culminate in long term adaptations in arterial function and structure (182, 501). However, the mechanisms underlying vascular responses to acute exercise and adaptations to training are not completely understood (110, 382). In response to acute exercise, vascular endothelial function commonly exhibits an immediate decrease that rebounds back to or above baseline following ~one hour of recovery, although individual responses may vary considerably (109, 110). The immediate decrease in endothelial function following acute exercise can potentially be explained by the concept of hormesis, which posits that the effects of repeated short-term challenges to the system accumulate to produce beneficial adaptations (109, 110). The molecular mechanisms underpinning the potential hormetic response of endothelial function to exercise training are necessary to understand for the development of optimal exercise-based interventions and molecular therapeutics. Furthermore, the mechanisms by which acute exercise transiently increases the risk for acute cardiovascular events are not fully understood (177).

One recently debated mechanism that could contribute to the acute effects of exercise on endothelial function is that of endothelial cell damage (2, 433). Damage to the membrane of endothelial cells and/or the integrity of the endothelial monolayer in

response to exercise stress would be expected to contribute to an immediate decline in vascular function. This hypothesis is controversial due to a lack of evidence that exercise-induced damage to endothelial cells occurs in healthy individuals (433). Studies that have investigated the effects of exercise on circulating markers reflecting endothelial integrity and activation are sparse and usually do not include a wide range of markers.

A number of circulating factors have been proposed as biomarkers of endothelium damage (61). The most direct circulating markers of disrupted endothelium integrity are circulating mature endothelial cells (CEC), which have denuded from the endothelial monolayer (140, 143, 253). Additionally, endothelial cells release microvesicles (EMV) in response to either apoptosis (identified as $CD31^{+}/42b^{-}$) or activation ($CD62E^{+}$) (244, 442). Vascular diseases are marked by elevations in CEC and EMV, while healthy individuals possess low numbers of both (88, 140, 143, 253, 413). On the other hand, circulating angiogenic cells (CAC) contribute to repair of the endothelium in pathogenic settings and endothelial adaptive responses to exercise training, primarily via paracrine mechanisms (253, 278). The number of several subtypes of CAC increase immediately following exercise (278), but it is unclear whether these changes correspond to the simultaneous occurrence of arterial endothelial damage or activation.

Elevations in other circulating factors such as endothelial-derived proteins have been proposed to reflect endothelial cell injury. Thrombomodulin (TM) and von Willebrand Factor (vWF) are glycoproteins expressed primarily in endothelial cells that increase in circulation after damage or stimulation of the endothelium (43, 44, 92). In

addition, the endothelial glycocalyx is a glycoprotein layer lining the luminal side of the endothelium that acts as a mechanotransducer in shear-induced endothelial-dependent dilation (396). Loss of glycocalyx integrity during exercise could, therefore, mediate a decrease in endothelial function.

Circulating microRNAs (ci-miRs) are molecules proposed as novel biomarkers and mediators of exercise responses (130, 434, 436). MiR-126 is the most highly expressed miR within endothelial cells, where it is almost exclusively expressed (156, 529). This has led researchers to propose increased ci-miR-126-3p, the dominant mature miR-126 strand, as a marker of endothelial damage (504). MiR-126 secretion is constitutive and increases in response to stimuli (9, 193, 212), but there is a lack of evidence that miR-126 is passively leaked into circulation during exercise due to endothelial cell damage.

Thus, the purpose of this study was to assess the effects of acute submaximal exercise on circulating factors derived from the endothelium, in order to test the hypothesis that disrupted endothelial integrity may play a role in the effects of exercise on arterial function. As exercise intensity is an important determinant of the degree of change in vascular stimuli affecting endothelial function (42, 328, 388), we sought to determine the effects of both moderate intensity continuous and high intensity interval exercise bouts on the above circulating endothelial-derived markers, and whether changes in these factors are associated with individual responses in endothelial function following exercise.

Methods

Ethical Approval

All procedures were approved by the University of Maryland Institutional Review Board (IRB) and conformed with the Declaration of Helsinki.

Participants

Healthy men between the ages of 18-39 were recruited to the laboratory for three separate visits. Participants reported being African American (n=5) or Caucasian (n=5). In order to exclude any effects of exercise training or sedentary lifestyle, all participants were moderately active, reporting physical activity where heart rate (HR) was elevated continuously for at least 30 minutes on one to four occasions per week, excluding walking for transportation. Exclusion criteria included BMI >30 kg/m2, systolic blood pressure (SBP) >140 mmHg and/or diastolic blood pressure (DBP) >90 mmHg, use of antihypertensive medication, or existing heart/cardiovascular disease.

For all visits, participants came to the laboratory after an overnight (≥10 hour) fast and were asked to refrain from medications, NSAIDs, alcohol, and exercise for 24 hours prior to the visit. In the first visit, participants gave their written informed consent, completed health and physical activity questionnaires, and demographics testing. A resting blood sample was then taken from an antecubital vein and used for blood chemistry analysis.

Exercise bouts

During the first visit, participants performed a peak power output (PPO) test on a cycle ergometer (Monark Ergomedic 894E), which consisted of two minute stages of increasing load to volitional exhaustion. After a warmup at 1 kg, the load for the first

stage was standardized at 1.6 kg, followed by a 0.3 or 0.6 kg increase each subsequent stage. Participants maintained a cadence of 70-80 rpm throughout the test. Inability to maintain at least 70 rpm resulted in termination of the test, with the final load (kg) used to calculate the participant's PPO (kgm/s). These results were used to calculate the loads for the subsequent exercise bouts to take place during the second and third visits, which entailed a moderate intensity continuous (MOD) bout (30 min, 60% PPO) and a high-intensity interval (HII) bout (30 min, 6 min at 40% PPO followed by 3 min intervals at 85% PPO interspersed with 4 min intervals at 40% PPO) matched for total time and workload (Figure 7.1A). During the exercise bouts, participants maintained 80 rpm and HR was recorded each minute using a chest strap HR monitor (POLAR T31). The exercise bouts were completed in a randomized, counterbalanced order and were separated by at least one week in order to avoid any potential carryover effects of exercise.

Upon arrival to the laboratory for the second and third visits, participants laid supine on a phlebotomy bed for ≥10 minutes, after which vascular assessments and a resting blood draw were taken. Immediately after completion of exercise, participants moved to the phlebotomy bed and laid supine for the post-exercise blood draw and the subsequent hour for vascular testing. Before, 10 minutes, and 60 minutes after exercise, brachial arterial BP was measured using an automated oscillometric sphygmomanometer (HEM-907 XL; Omron Corporation, Kyoto, Japan) and aortic BP was measured using applanation tonometry (Sphygmocor, AtCor Medical, Sydney, Australia).

Blood collection

Immediately before and immediately after exercise, blood was drawn from an antecubital vein into tubes containing either ethylenediaminetetraacetic acid (EDTA) or acid citrate dextrose (ACD) as anticoagulants for plasma, as well as serum separator tubes for serum. ACD tubes were spun immediately at 2,000xg for 20 minutes at room temperature. ACD plasma was then pipetted into 500µL aliquots and stored at -80ºC. After sitting at room temperature for 45 minutes, serum separator tubes were spun at 1,500xg for 15 minutes at 4ºC. Serum was then pipetted into 500ul aliquots on ice and stored at -80ºC.

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from 20 mL of EDTA blood using SepMateTM-50 (Stemcell, Vancouver, BC Canada) as specified by the manufacturer. Isolated PBMCs were suspended at a density of 1.0 x 10^7 cells/mL in Recovery™ Cell Culture Freezing Medium (Gibco, Gaithersburg MD) and placed in a -80°C freezer in a 1°C/min freezing container (Mr. Frosty; Nalgene, Rochester, NY) for 24 hours and then placed in liquid nitrogen until further processing and analysis. Cell phenotyping was determined using direct immunofluorescence labeling of cell surface markers with mouse anti-human monoclonal antibodies. Cells were stained with Live-Dead Zombie Dye (Biolegend, San Diego) and incubated at 4°C for 15 minutes in the dark. Cells were then incubated with FcR blocker (Miltenyl Biotec, Auburn, CA) for 15 minutes followed by addition of pre-titrated antibodies [APC mouse anti-human CD45 (BD Biosciences Cat#555485, Lot#7075571 RRID:AB_398600); PE mouse anti-human CD146 (BD Biosciences Cat#550315,

Lot#7335648 RRID:AB_393604); FITC mouse anti-human CD34 (BD Biosciences Cat# 555821, Lot# 6245812 RRID:AB_396150); V450 mouse anti-human CD31 (BD Biosciences Cat#561653, Lot#8240742 RRID:AB_10896326); (BD Pharmingen, San Jose CA)] in a total volume of 100 µL of phosphate buffer solution. Cells were washed to remove excess antibody, fixed in 2% paraformaldehyde and immediately analyzed by flow cytometry. Appropriate single stains and fluorescence minus one (FMO) controls were run. Cells were run on a FACS Canto II flow cytometer (BD Biosciences, CA USA) and analyzed with FCS Express 6 (De Novo Software, Glendale, CA). For analysis, debris, doublets and dead cells were excluded. CECs were defined as $CD146^{+}/CD31^{+}/CD45^{dim/-}$, while CACs were defined as $CD34^{+}$ with the addition of $CD45^{dim/-}$ and $CD45^{dim/-}/CD31^{+}$ for hematopoietic fate exclusion and endothelial interacting subsets, respectively. Because lymphocytic cell counts increase with exercise (526), events are normalized to 500,000 total events as opposed to non-lymphocytic cell counts.

Endothelial microvesicles

Microvesicle concentrations were determined in batch assays similar to a previous report (452). Briefly, cell-free plasma was obtained from subsequent centrifugations of ACD plasma and labelled with either 20 µl of PE mouse anti-human CD62E (BD Biosciences Cat# 551145, Lot#8135710, RRID:AB_394072) and V450 mouse anti-human Annexin V (BD Biosciences Cat# 560506, Lot#8086800), or PE mouse anti-human CD31 (BD Biosciences Cat# 555446, Lot#7335512 RRID:AB_395839), FITC mouse anti-human CD42b (BD Biosciences Cat# 555472, Lot#8183987, RRID:AB_395864), and V450 mouse anti-human Annexin V (Becton,

Dickinson and Company) antibodies to determine EMV fractions. EMV events were captured on a FACS Canto II (BD Biosciences, CA USA) for 180 seconds from events smaller than 1.0 μm. Microvesicles were classified using 900 nm NIST calibration beads (Polysciences, Inc., Warrington, PA, USA), and plasma concentrations were calculated using CountBright™ Absolute Counting Beads (ThermoFisher Scientific, Waltham, MA, USA). Flow cytometry data were analyzed with FCS Express 6 (De Novo Software, Glendale, CA).

Proteins

Enzyme linked immunosorbent assays (ELISA) were used to quantify concentrations of circulating vWF and syndecan-1 from serum (RayBiotech, Norcorss, GA) and TM from ACD plasma (Boster Biological Technology, Pleasanton, CA) according to manufacturer's instructions. Sample dilutions were performed according to manufacturer's instructions for syndecan-1 and vWF (8- and 8,000-fold, respectively). For TM, plasma was diluted 5-fold. Samples were assayed in duplicate and absorbance measured using a spectrophotometer (Synergy H1 Hybrid Reader; BioTek, Winooski, VT) at 450 nm absorbance.

Circulating microRNA

Serum was thawed at room temperature and spun at 16,000xg for 10 minutes at 4ºC in order to remove cell debris. Total RNA was isolated from 50 μL serum using the miRNeasy serum/plasma kit with slight changes to the manufacturer's protocol (Qiagen, Germantown, MD). Serum was mixed with 20 volumes of Qiazol lysis reagent and four volumes of chloroform. A spike-in control miR (C. elegans miR-39) was added to all samples during isolation for use in calibration of PCR results. Reverse

transcription was performed (miScript II RT kit, Qiagen) using 5 µL RNA and the final reaction mix was diluted in 200 µL RNase-free water. Real-time quantitative PCR was run on an ABI 7300 Real-Time PCR System (Applied Biosystems) using 2.5 µL input cDNA with the miScript SYBR Green PCR Kit (Qiagen, Germantown, MD). The expression levels of miR-126-3p and miR-126-5p were quantified using specific primer assays (Qiagen; MS00003430, MS00006636). MiR expression levels were determined using the $2^{-\Delta\Delta CT}$ method of relative quantification. For each miR within each sample, ΔCT = CT of miR – CT of spike-in control miR; $\Delta\Delta CT$ = ΔCT for individual's post-exercise sample – ΔCT of individual's respective baseline sample (105, 261, 302). Both miRs were sufficiently detected at CT values <35.

Flow-mediated dilation

Endothelium-dependent dilation was assessed via flow-mediated dilation (FMD) of the brachial artery before, 10 minutes after, and 60 minutes after HII and MOD exercise bouts. Brachial artery diameter was determined using a high-resolution ultrasound (Hitachi-Aloka Arietta 70, Tokyo, Japan) with a 5-18 MHz linear transducer aligned parallel atop the brachial artery and stabilized with a probe holder. To minimize intra-subject variability, the location of the probe along the arm was kept consistent at each time point per visit. A rapid inflator cuff (Hokanson, Bellevue, WA) was placed around the thickest section of the forearm, immediately distal to the antecubital fossa. Image capture and analysis were conducted using Quipu Cardiovascular Suite FMD Studio (Pisa, Italy). Following a 60 second period of baseline arterial diameter and blood velocity measurements, the cuff was inflated to a suprasystolic pressure (220 mmHg) for 300 seconds. Arterial diameter and blood velocity were recorded for 150

seconds post-cuff deflation to measure endothelial response to reactive hyperemia. FMD was calculated as the percentage change in arterial diameter from the mean of the baseline to the peak of the 150 seconds post-cuff deflation, as per current FMD guidelines (496).

Statistics

Analyses were performed using Graphpad Prism 8. Circulating markers were analyzed using two-way ANOVAs with exercise intensity and time as factors. Pre-planned contrasts were used to compare baseline with post-exercise values for each exercise intensity separately. FMD was analyzed by a two-way repeated measures ANOVA (intensity x time) followed by pre-planned contrasts comparing each time point within each exercise intensity. Effect sizes were calculated to determine the effects of each exercise intensity on each variable using hedges' g_{av} (273). Coefficients of variation (CV) were calculated using the baseline samples from visits 2 and 3 to determine the day-to-day variability of each circulating marker. Correlations between changes in circulating factors and FMD were determined by calculating Pearson's correlation coefficients. All tests were two-sided with statistical significance set at $P<0.05$.

Results

Participants

Participant characteristics are shown in Table 7.1. The HR responses for the MOD and HII bouts are shown in Figure 7.1B. HR data for one participant was not reliable for the MOD bout and was thus excluded. The exercise bouts were well-

matched, with average HRs of 155±10 bpm and 152±7 bpm over the entire MOD and HII bouts, respectively. Baseline and post-exercise changes in HR and mean arterial pressure of the brachial artery and aorta are shown in Table 7.2.

Cells and EMV

Cell count data was excluded for one participant due to error in sample handling and storage leading to remarkably low numbers (close to zero) of all cell-types at one time-point. Data for all other circulating factors and FMD are presented for all 10 participants. There were no differences (P>0.05) in baseline values between exercise bouts for any measures. The repeatability of circulating cell and EMV levels at baseline were determined using the resting samples from visits 2 and 3 for $CD146^{+}/CD31^{+}/CD45^{dim/-}$ CEC (CV=17.7%; r=0.91, P=0.0007), $CD34^{+}$ CAC (CV=10.4%; r=0.89, P=0.002), $CD34^{+}/45^{dim/-}$ CAC (CV=13.3%; r=0.81, P=0.008), $CD31^{+}/34^{+}/45^{dim/-}$ CAC (CV=11.6%; r=0.75, P=0.02), $CD62E^{+}$ EMV (CV=23%; r=0.25, P=0.49), and $CD31^{+}/42b^{-}$ EMV (CV=28.3%; r=0.55, P=0.10). There was no effect of either the MOD (P=0.50, g_{av}=0.18) or HII (P=0.25, g_{av}=0.39) exercise bout on the number of $CD146^{+}/CD31^{+}/CD45^{dim/-}$ CEC (both P>0.05) (Figure 7.2A). Two-way ANOVA revealed a significant effect of time on $CD34^{+}$ CAC number (P=0.004) with a significant 21% decrease following MOD (P=0.007, g_{av}=0.63) and no significant change following HII (P=0.14, g_{av}=0.24) (Figure 7.2B). There was a significant time effect (P=0.02) on $CD34^{+}/45^{dim/-}$ CAC number, with contrasts revealing a significant decrease of 28% following MOD (P=0.007, g_{av}=0.82), but not HII (P=0.53, g_{av}=0.13) (Figure 7.2C). There was also a significant time effect (P<0.001) on $CD31^{+}/34^{+}/45^{dim/-}$ CAC number, with significant decreases of 34% and 21% following MOD (P<0.001,

g_{av}=0.87) and HII (P=0.02, g_{av}=0.58) exercise, respectively (Figure 7.2D). There were no effects of either exercise bout on $CD62E^{+}$ EMV (Figure 7.3A) or $CD31^{+}/42b^{-}$ EMV numbers (Figure 7.3B).

Proteins

A significant time x intensity interaction (P=0.02) revealed a different effect of the exercise bouts on vWF concentration, with a significant increase of 88% following HII (P<0.001, g_{av}=1.09) and no change following MOD (P=0.49, g_{av}=0.36) (Figure 7.4A). There were significant time effects on both TM (P<0.001; Figure 7.4B) and syndecan-1 (P<0.001; Figure 7.4C) concentrations. Circulating TM increased 20% and 23% in response to the MOD (P=0.02, g_{av}=0.41) and HII (P=0.001, g_{av}=0.65) bouts, respectively. Syndecan-1 concentration similarly increased 18% and 22% following MOD (P=0.006, g_{av}=0.49) and HII (P<0.001, g_{av}=0.76), respectively. The repeatability of circulating vWF (CV=12.6%; r=0.71, P=0.02), TM (CV=15.1%; r=0.66, P=0.04), and syndecan-1 (CV=5.6%; r=0.91, P=0.0002) levels at baseline were also determined.

Circulating microRNA

There was a significant interaction effect (P=0.01) on ci-miR-126-3p levels, with post-hoc contrasts revealing a 1.9-fold increase following HII (P=0.009, g_{av}=0.63) and no significant change following MOD (P=0.35, g_{av}=0.27) (Figure 7.5A). A significant time effect (P=0.01) was observed on ci-miR-126-5p levels, with a 2.1-fold increase in response to HII (P=0.01, g_{av}=0.44), but no change in response to MOD exercise (P=0.36, gav=0.26) (Figure 7.5B). For repeatability of ci-miR levels at baseline, see Chapter 6 results.

FMD and correlations

There were no significant time, intensity, or interaction effects (P>0.05) on FMD or brachial arterial diameter (Figure 7.6). An analysis of FMD normalized to brachial diameter likewise revealed no significant effects (data not shown). There were similar significant correlations for immediate changes in both circulating syndecan-1 (r=0.46, P=0.04) and vWF (r=0.47, P=0.04) with the change in FMD one hour after exercise (including both intensities). There was a borderline significant correlation between the immediate change in ci-miR-126-5p and FMD one hour after exercise (r=0.41, P=0.07). The change in FMD 10 minutes after the MOD bout was significantly, inversely correlated with the immediate change in ci-miR-126-3p (r=-0.77, P=0.009) and was borderline significantly correlated with the change in CEC (r=0.64, P=0.07). The immediate change in vWF concentration was highly correlated with the change in FMD one hour after the HII bout (r=0.86, P=0.001), while the change in $CD62E^+$ EMV correlated with changes in FMD one hour after the MOD bout (r=0.65, P=0.04).

Discussion

The major findings of our study are that immediately after acute MOD and HII exercise there are no apparent changes in endothelial cell denudation, apoptosis, or activation, as indicated by the lack of changes in $CD146^+/CD31^+/CD45^{dim/-}$ CEC, $CD31^+/42b^-$ EMV, or $CD62E^+$ EMV numbers. Conversely, both exercise bouts resulted in slight elevations of circulating TM and syndecan-1, reflecting disruption of the endothelial membrane and glycocalyx, respectively. HII exercise alone caused

substantial increases in vWF, ci-miR-126-3p, and ci-miR-126-5p, indicating that the mechanisms underlying their secretion are related to exercise intensity and not a result of cell damage. Circulating levels of syndecan-1, vWF, $CD62E^+$ EMV, and ci-miR-126-3p correlated with FMD responses following HII and/or MOD exercise, suggesting these mechanisms, rather than endothelial cell damage per se, may be important to the acute response of endothelial function to exercise.

Integrity of the endothelial monolayer can be indirectly determined by quantifying the number of CEC in blood, as turnover rate of mature endothelial cells is normally relatively slow (140, 547). To our knowledge, only a few studies have determined the effects of acute exercise on CEC in healthy individuals and results are complicated by different methods of identification and quantification. Möbius-Winkler et al. reported a significant increase in $CD146^+$ CEC numbers two hours into a four-hour cycling bout and lasting up to two hours after completion of the exercise (340). However, their results may reflect changes in other cell types, since CD146 is also expressed on activated T-lymphocytes, trophoblasts and mesenchymal stem cells (140). Ours is the first exercise study to use the current conventionally recommended combination of cell markers to more specifically identify CEC (143, 317). Our finding of no change in CEC count following exercise is in agreement with others using various cell markers and methods of identification (371, 372, 536) suggesting that denudation of mature endothelial cells does not occur during acute exercise, or may require exercise of prolonged duration (≥2 hours) (340).

Based on previous studies (278) we expected an increase in all measured subsets of CAC in response to exercise. While a decrease in CAC number was

unexpected, it is not without precedent. A decrease in $CD31^+/CD14^+$ cells was observed following cycling at 60–70% VO_{2peak} for an energy expenditure of 598 kcal (280). Several other studies have observed no changes in both $CD34^+$ and $CD31^+$ CAC subtypes in response to submaximal exercise, suggesting cycling exercise may require longer duration or higher intensity to stimulate increases in CAC (27, 278, 282, 311, 368). Mobilization of CAC is followed by homing and attachment to the vessel wall, whereupon they exert their paracrine actions to support endothelial cell functions (289, 544). Thus, one possible explanation for the observed decrease in CAC concentration is their recruitment to the vessel wall, although not necessarily in response to endothelial cell damage.

The current consensus is that in response to acute exercise, both apoptotic and activated EMV concentrations remain unchanged or decrease (539). Those released due to activation may increase in the recovery period, potentially dependent on individual training status and sex (280, 471, 539). Our study adds to the literature showing that both MOD and HII bouts do not induce immediate changes in EMV reflecting endothelial injury or activation in young, healthy, normally active men (458, 539). Laminar shear stress *in vitro* promotes endothelial quiescence and suppresses EMV shedding (90, 263). Unchanged or decreased EMV release with exercise may be a result of the suppressive effects of exercise-induced laminar shear stress in counteraction to stimulatory effects of other factors such as cytokines (245, 539), although these mechanisms require further interrogation. Still, it must be acknowledged that endothelial cells take up EMV, so it cannot be completely ruled out that EMV

release and clearance are simultaneously stimulated during exercise, masking any increased production (539).

Circulating TM increases upon shedding from the endothelial membrane during cellular membrane injury (43, 92). Conversely, vWF is not a marker specific to cellular damage, as it is partially stored in Weibel–Palade bodies and is released in response to various vascular stimuli (43, 44, 92, 244, 469). Both endothelial glycoproteins TM and vWF have previously been shown to increase with exercise (50, 176, 244, 371). In our study, there were equivalent increases in TM following both exercise bouts, suggesting slight disruption of endothelial cell membranes independent of exercise intensity. Increased circulating concentrations of TM may also reflect increased endothelial membrane expression and this cannot be ruled out as contributing to our results (35, 430).

Previously, older patients undergoing an acute stress test for detection of CAD exhibited significant increases in vWF that correlated with increases in CEC number (50). Yet, evidence suggests that vWF release during exercise in healthy individuals is stimulated by β-adrenergic stimulation and shear stress, at least partially through nitric oxide (NO)- and calcium-dependent signaling (176, 244, 358, 469). Our findings that vWF increased in response to HII exercise only and did not correlate with changes in TM or other circulating markers of cellular damage suggest vWF was released due to mechanisms related to exercise intensity rather than endothelial cell injury. There was a positive association between exercise-induced changes in vWF and endothelial function one hour after HII exercise. Therefore, mechanisms governing the endothelial

release of vWF with exercise may likewise be important to the post-exercise recovery response of endothelial function.

Syndecan-1 is an integral component of the glycocalyx that increases in circulation upon damage to the glycocalyx (396). To our knowledge, only one prior study has investigated the response of glycocalyx integrity to acute exercise, finding no effect of a maximal exercise test on levels of circulating syndecan-1 or other markers of glycocalyx damage (314). Our study shows that submaximal exercise bouts lasting 30 minutes are sufficient to disrupt the glycocalyx as evidenced by increased circulating syndecan-1 concentrations, with similar effects of MOD and HII exercise. Importantly, glycocalyx integrity could play a role in the acute exercise response of endothelial function. The glycocalyx is an important mechanosensor and transducer in endothelial cells and its integrity is crucial to shear-mediated NO production (159, 559). In our study, the change in syndecan-1 correlated positively with the change in FMD one hour after exercise. This relationship is opposite to what was expected and suggests a potential compensatory mechanism relating the degree of glycocalyx disruption to a "rebound response" in endothelial function. Glycocalyx shedding during exercise may be related to elevations in reactive oxygen species (ROS) and/or cytokines (77, 423). Moreover, aerobic exercise training may cause increased glycocalyx integrity, as shown by lower resting and post-exercise syndecan-1 after training (315). Those results combined with the present findings suggest a potential mechanism of adaptation, whereby exercise acutely disrupts the glycocalyx, but chronically promotes stability. It would be of interest to explore relationships between the training-induced improvements in glycocalyx integrity and endothelial function.

Increases in ci-miR-126 have frequently been demonstrated following acute exercise bouts ranging from a short maximal test to a marathon, although studies have also found no changes (105, 261, 436, 447, 504, 525). Since miR-126-3p is the most highly enriched miR within endothelial cells, it has been suggested that ci-miR-126-3p may be a biomarker of exercise-induced endothelial damage (504). It has been reported that the vast majority of ci-miR (up to 90%) are found non-encapsulated in association with a protein carrier (AGO2) (18, 503, 569). Leakage of miR-126 from damaged endothelial cells into circulation would result in an increase in this protein-bound fraction of miR. However, ci-miR-126-3p and 126-5p did not appear to increase as a result of passive release due to disruption of the endothelial membrane. Both ci-miRs increased only in response to HII, while TM, a marker of disrupted endothelial membrane integrity, increased in circulation similarly following both exercise intensities. Indeed, endothelial cells *in vitro* are known to selectively secrete miRs non-encapsulated in association with a protein carrier, within exosomes, or within EMV, in response to stimuli including shear stress, inflammatory cytokines, and hypoxia (9, 193, 212, 569), while mechanisms underlying secretion *in vivo* remain to be experimentally determined. Additionally, mir-126 can be selectively packaged and released in apoptotic EMV (156, 525). Although we did not specifically determine miR expression within EMV, both strands of ci-miR-126 increased in response to HII without a concomitant change in apoptotic EMV, and there were no significant correlations between the two factors.

Ci-miRs may play regulatory roles in the vascular responses to acute and chronic exercise. They can act as paracrine and endocrine messengers, since

endothelial-derived ci-miRs are taken up and are biologically active in various cardiovascular cell types including other endothelial cells and vascular smooth muscle cells (31, 193, 369, 569). Supporting their intercellular activity, ci-miRs are selectively secreted and taken up, and are highly stable in circulation (31, 369). Though there is accumulating correlational evidence for their roles in cardiovascular responses and adaptations, direct experimental evidence *in vivo* is lacking (31, 434, 436). Another, more rapid mechanism of action for ci-miRs may be the effect of offloading into the circulation in order to allow increased intracellular translation of miR-regulated targets (320, 406).

Both miR-126 strands regulate targets important in NO, ROS, and inflammation pathways (89, 271). A recent study found that improvements in microvascular endothelial function were associated with increases in ci-miR-126 following six weeks of exercise training and caloric restriction in obese adolescents (132). We found the opposite relationship following an acute bout of MOD exercise, ten minutes after which a reduction in FMD was associated with increased ci-miR-126-3p levels. MiR-126-3p may increase NO and decrease ROS levels by positively regulating several targets including the PI3K/AKT pathway, SIRT1, and SOD2 (89). Additionally it exerts anti-inflammatory effects by suppressing VCAM-1 and ICAM-1 expression (89). Thus, the acute release of miR-126 from the endothelium in response to exercise stimuli could result in an acute decrease in endothelial function, though this is speculative and it is unclear whether changes in miR levels could have such a rapid effect. While less well studied, the complementary miR-126-5p strand is also highly expressed and biologically active within endothelial cells (156, 271), and is altered in circulation with

exercise (447). We chose to assess ci-miR-126-5p in addition to the complimentary -3p strand because any differences in their responses to exercise would further suggest selective secretion from endothelial cells and would not support passive release of miR into circulation. Additionally, several studies reporting the effects of exercise on ci-miR-126 do not specify which strand they have quantified. We found similar increases in both strands of ci-miR-126 in response to HII, with no significant changes following MOD.

We did not observe significant changes in FMD in response to exercise of either intensity. On average, the literature supports an immediate decrease in FMD that rebounds back to or above baseline values by ~one hour post-exercise (110). However, numerous studies have reported different responses including no changes in FMD following exercise, likely because the response is modulated in young, healthy individuals by several variables such as exercise intensity, duration, mode, and subject's sex or fitness status (42, 110, 228, 247, 328). When endothelial function is decreased after exercise, it recovers or improves usually by ~one hour, and ultimately normalizes within 24-48 hours post-exercise (110). This rapid time-course of recovery provides evidence against any substantial loss of endothelial structural integrity during exercise accounting for decreases in endothelial function. In cases where considerable endothelial damage does occur, such as with transradial catheterizations, endothelial function is substantially reduced ($\geq$ 50%) and takes months to recover, implying long-lasting structural effects (111, 335).

There are some limitations of our study that should be acknowledged. We investigated changes in circulating markers reflecting structural endothelial disruption

and activation. We did not investigate other factors that change during exercise and likely contribute to acute changes in endothelial function, such as ROS, cytokines, hemodynamics, and sympathetic stimulation; nor did we determine the post-exercise time-course of changes in circulating factors as we were focused specifically on the immediate effects of exercise stressors on the endothelium. There are select reports supporting the notion that peak changes in some of the factors that we studied (e.g. activated EMV and ci-miR-126) occur during the post-exercise recovery period (105, 261, 267, 471). Nevertheless, it is not likely that these changes reflect continuing endothelial damage since, to our knowledge, there are no reports of elevations in biomarkers of endothelial damage (e.g. CEC, CD31+/42b- EMV, or TM) in healthy individuals during the recovery period following cessation of exercise. Furthermore, endothelial function tends to revert back towards or above baseline levels in the hours following exercise, suggesting endothelial damage does not occur during this period. While we chose to focus on aerobic exercise, resistance exercise is known to acutely reduce endothelial function in untrained individuals, largely due to substantial increases in BP (418). It is plausible that resistance exercise may disrupt endothelial integrity and this requires future study. Lastly, only young, healthy men were included in this study and our results cannot be extrapolated to other populations.

Determining the mechanisms underlying vascular responses to acute exercise and, by extension, adaptations to training are critical for the determination of optimal exercise prescriptions and the development of preventive and therapeutic drugs aimed at improving vascular health. Using circulating markers we have shown that acute exercise does not appear to cause endothelial denudation, apoptosis, or activation, but

may cause disruption of the endothelial cell membrane and glycocalyx integrity. These findings shed light on the potential for endothelial cell damage to contribute to a potential hormetic response of endothelial function to exercise. It is unlikely that endothelial cell death and replacement occurs in response to acute exercise bouts, though damage and repair to the endothelial membrane and glycocalyx may occur. Intuitively, disruption to these structures important to mechanotransduction could contribute to an acute exercise-induced decrease in endothelial-dependent dilation, while with training, increased stability of these structures may contribute to improved endothelial function and protection from future challenges. Potential dose-response effects of exercise volume, as well as the effects of training, should be explored in order to determine the effects of prolonged aerobic exercise on endothelial integrity and function. Mechanisms related to exercise intensity underlying the active endothelial secretion of factors such as vWF and ci-miRs may additionally contribute to changes in endothelial function.

Table 7.1 Subject characteristics

Age, y	22 ± 2
BMI, kg/m^2	24 ± 3
Peak Power Output, kgm/s	29 ± 5
Resting HR, bpm	66 ± 8
SBP, mmHg	127 ± 7
DBP, mmHg	69 ± 10
MAP, mmHg	88 ± 7
Glucose, mg/dl	87 ± 6
Hemoglobin A1c, %	5.0 ± 0.4
Total cholesterol, mg/dl	154 ± 26
HDL-C, mg/dl	55 ± 12
LDL-C, mg/dl	83 ± 15
VLDL-C, mg/dl	14 ± 5
Triglycerides, mg/dl	68 ± 22

Values are means ± SD; n = 10 subjects. BMI, body mass index; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; MAP, mean arterial pressure; SBP, systolic blood pressure; VLDL-C, very-low-density lipoprotein cholesterol.

Table 7.2. Heart rate, brachial MAP, and aortic MAP before, 10 min after, and 60 min after MOD or HII exercise

					Two-way ANOVA		
		Pre	10 min. post	60 min. post	Time	Intensity	Interaction
Heart Rate	MOD	57 ± 10	75 ± 6*	62 ± 14$^{\#}$	**<0.001**	0.13	0.29
	HII	59 ± 9	84 ± 10*†	68 ± 9*$^{\#}$			
Brachial MAP	MOD	88 ± 5	89 ± 8	88 ± 8	**0.04**	0.89	0.20
	HII	89 ± 6	91 ± 7	85 ± 8*$^{\#}$			
Aortic MAP	MOD	84 ± 5	85 ± 8	83 ± 8	**0.007**	0.91	0.27
	HII	83 ± 7	88 ± 7*	80 ± 9$^{\#}$			

Means ± SD. HII, high-intensity interval exercise; MAP, mean arterial pressure; MOD, continuous moderate intensity exercise. *P < 0.05 compared with baseline, #P < 0.05 compared with 10 min post-exercise, †P < 0.05 compared with other exercise intensity.

Figure 7.1

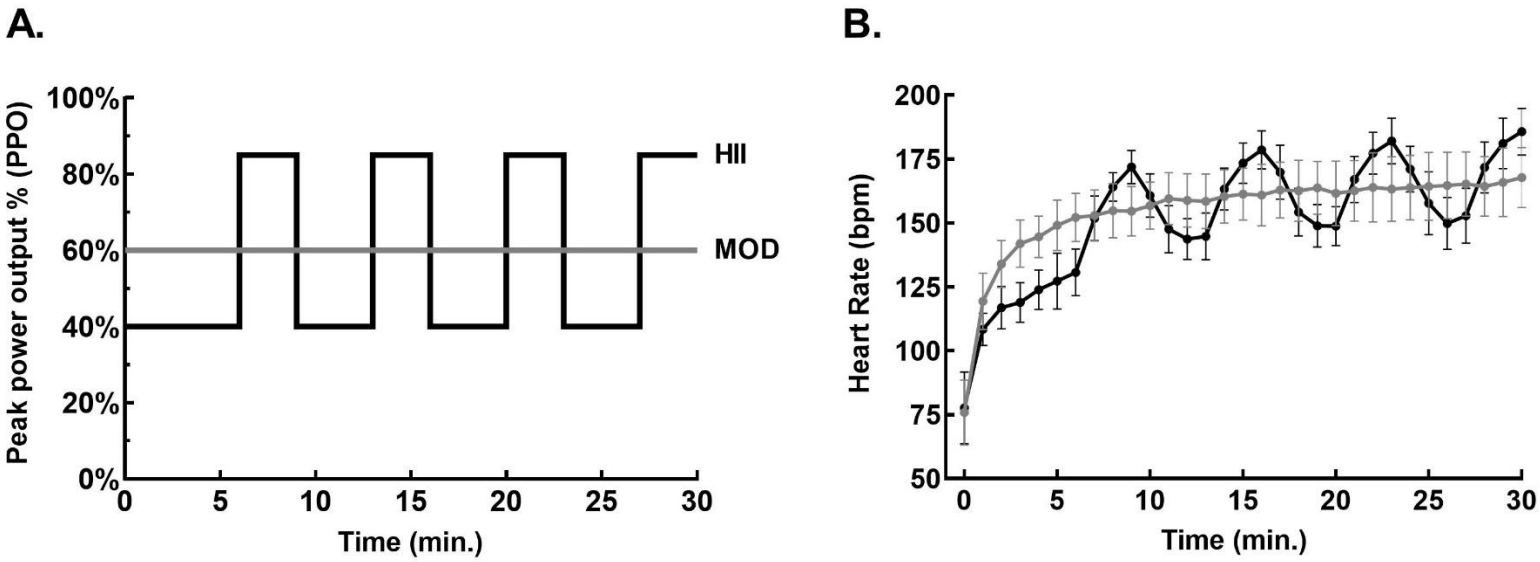

Figure 7.1. [A] High-intensity interval (HII; black line) and moderate intensity continuous (MOD; light gray line) exercise protocols were matched for overall time and average exercise intensity. [B] Heart rate responses during the HII (black line, n = 10) and MOD (light gray line, n = 9) exercise bouts. Means ± SD. PPO, peak power output.

Figure 7.2

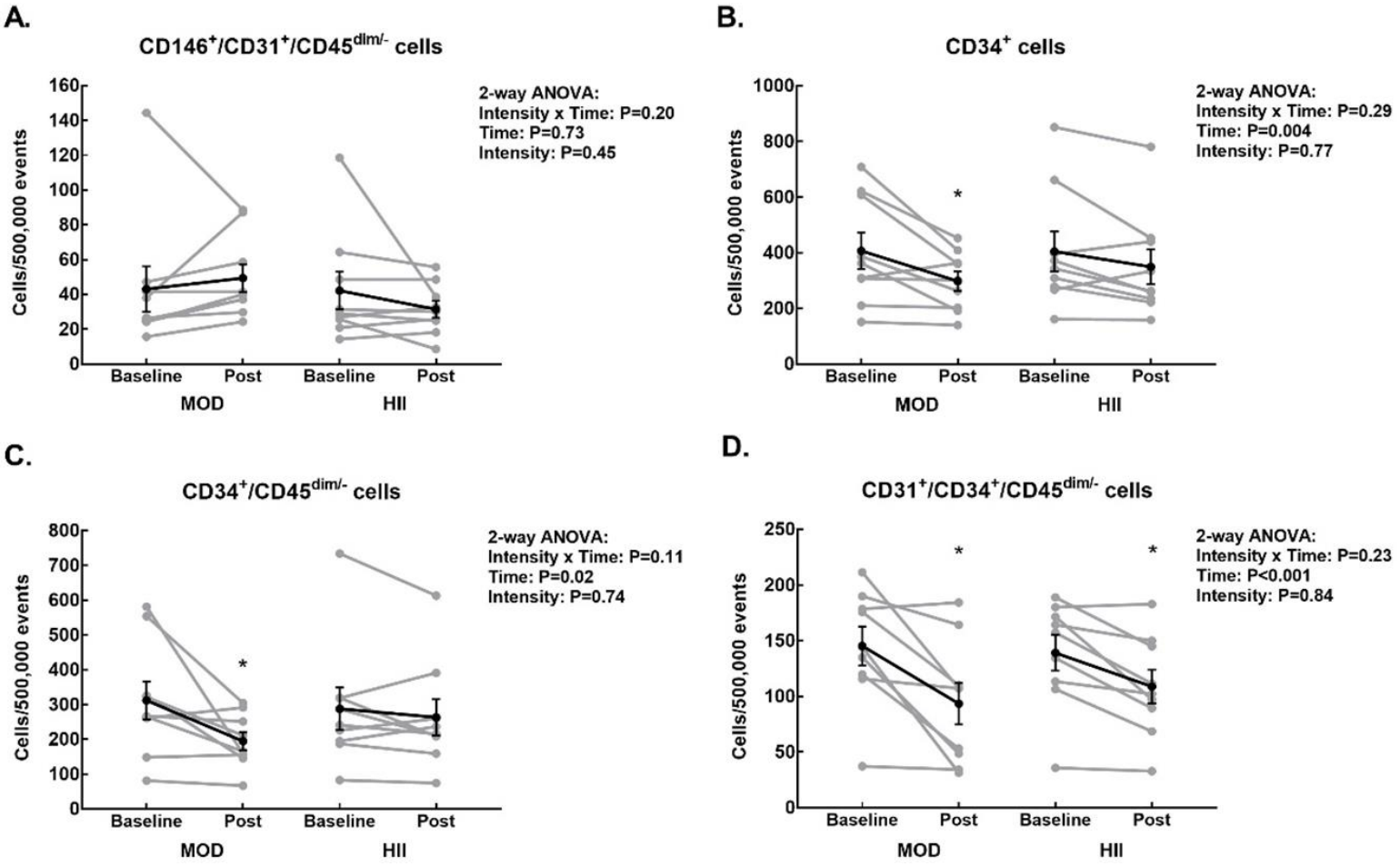

Figure 7.2. Effects of moderate intensity continuous (MOD) and high-intensity interval (HII) exercise on [A] $CD146^{+}/31^{+}/45^{dim/-}$ circulating endothelial cells, [B] $CD34^{+}$ circulating angiogenic cells (CACs), [C] $CD34^{+}/45^{dim/-}$ CACs, and [D] $CD31^{+}/34^{+}/45^{dim/-}$ CACs per 500,000 events. Light gray lines represent individual values; black lines represent means ± SEM. *P < 0.05 vs. respective baseline. Post, immediately post-exercise.

Figure 7.3

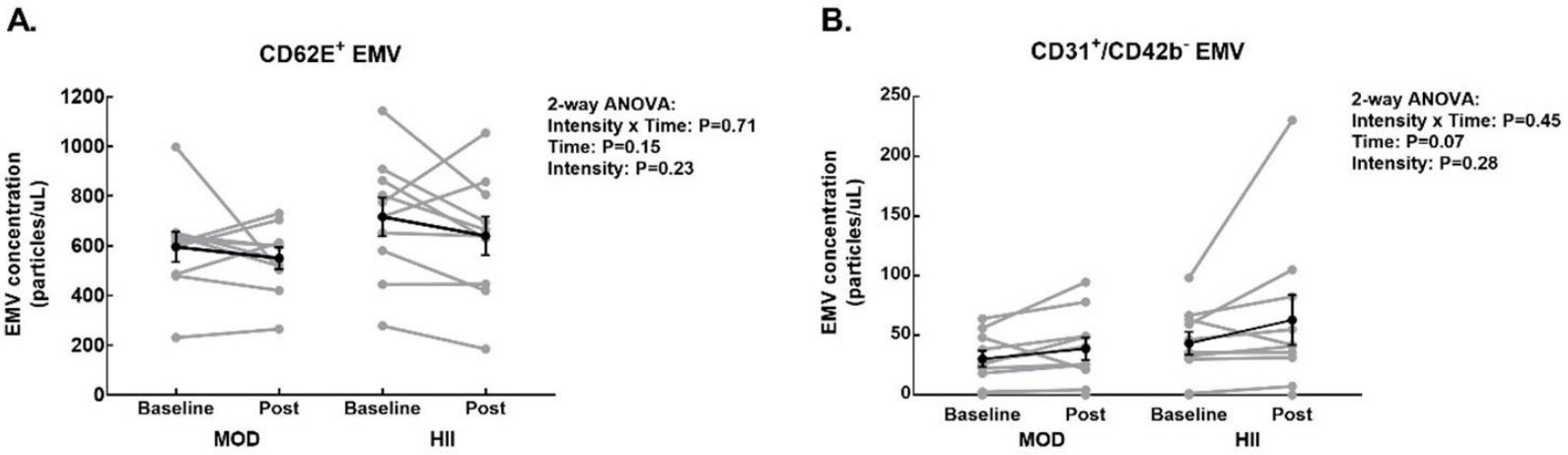

Figure 7.3. Effects of moderate intensity continuous (MOD) and high-intensity interval (HII) exercise on concentrations of [A] $CD62E^{+}$ endothelial microvesicles (EMVs) and [B] $CD31^{+}/42b^{-}$ EMVs. Light gray lines represent individual values; black lines represent means ± SEM. Post, immediately post-exercise.

Figure 7.4

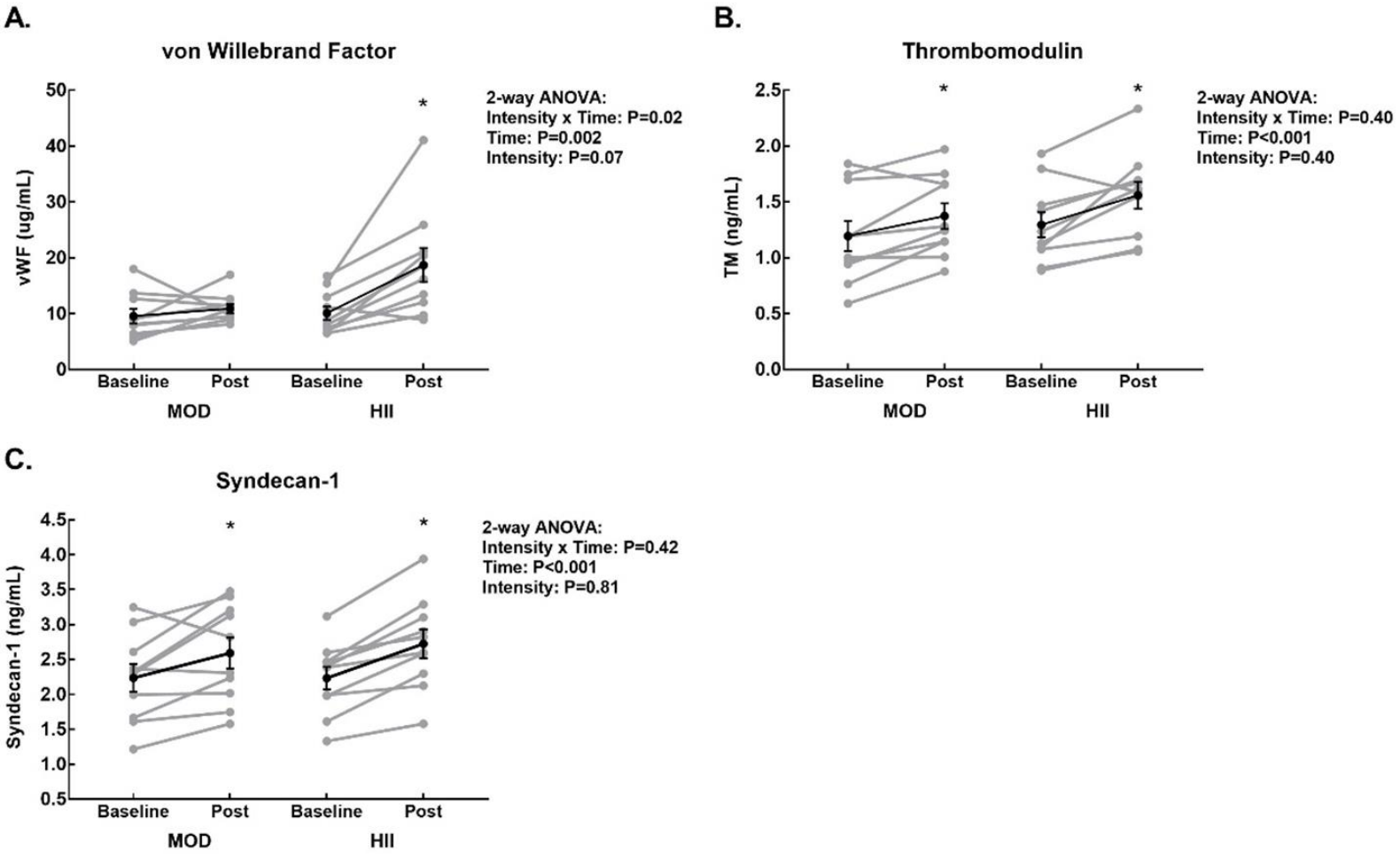

Figure 7.4. Effects of moderate intensity continuous (MOD) and high-intensity interval (HII) exercise on concentrations of [A] von Willebrand factor (vWF), [B] thrombomodulin (TM), [C] and syndecan-1. Light gray lines represent individual values; black lines represent means ± SEM. *P < 0.05 vs. respective baseline. Post, immediately post-exercise.

Figure 7.5

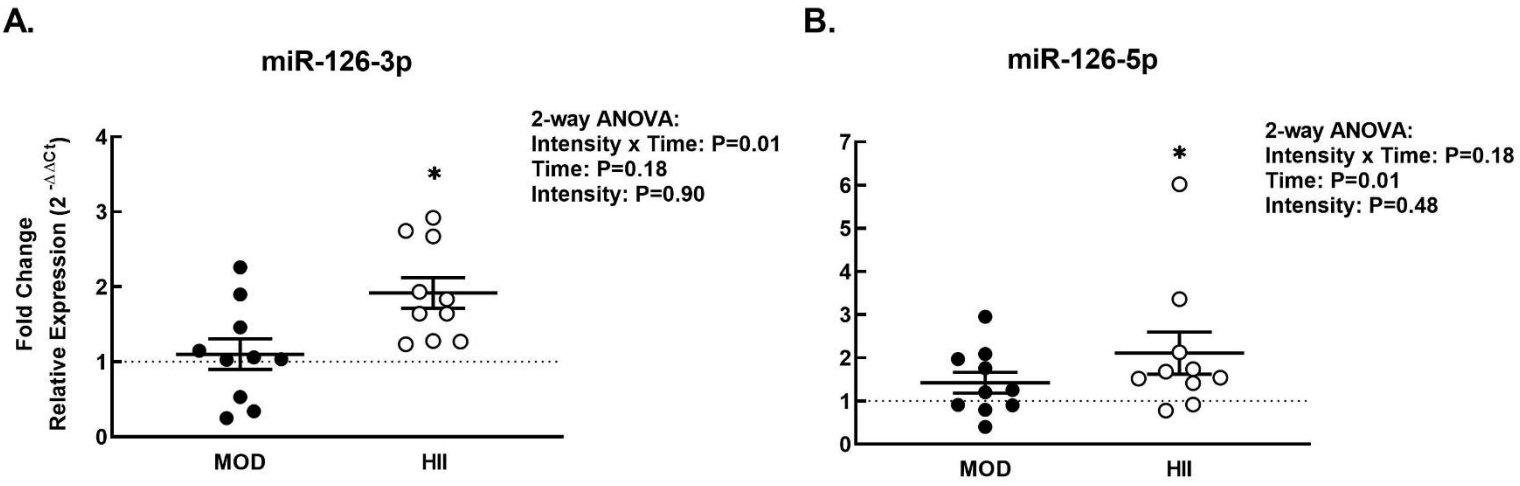

Figure 7.5. Effects of moderate intensity continuous (MOD) and high-intensity interval (HII) exercise on circulating microRNA- (ci-miR-) [A] 126–3p and [B] 126–5p. Two-way ANOVAs were run using the baseline and post-exercise data for each exercise intensity. Data are shown as fold changes with each individual compared with their own baseline values (set as 1). Dots represent individual subject data; lines represent means ± SEM. *P < 0.05 vs. respective baseline.

Figure 7.6

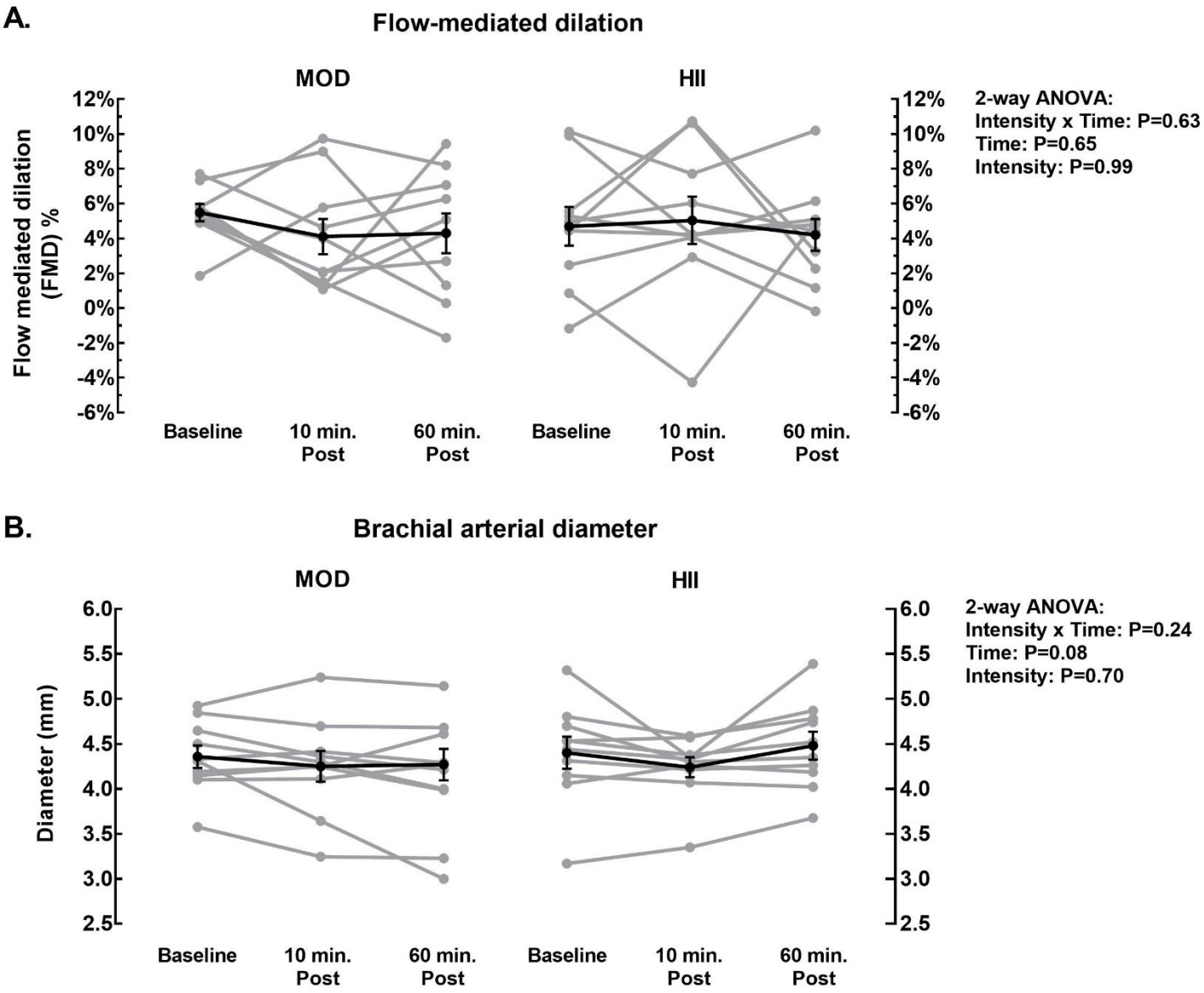

Figure 7.6. [A] Percent flow-mediated dilation (FMD) and [B] brachial arterial diameter before (Baseline), 10 min after, and 60 min after moderate intensity continuous (MOD) and high-intensity interval (HII) exercise. Light gray lines represent individual subject responses; black lines represent means ± SEM.

Figure 7.7

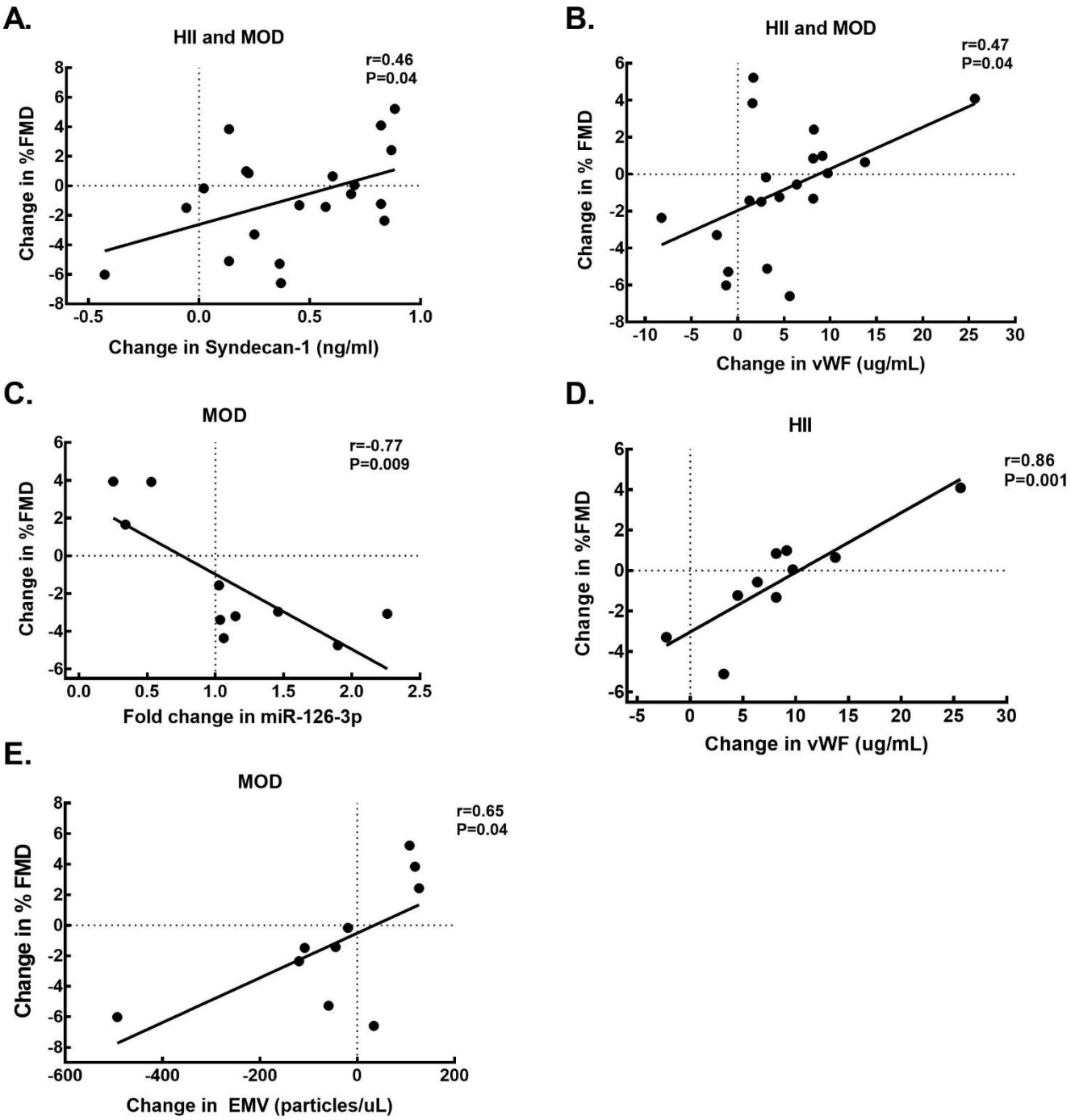

Figure 7.7. Correlations between the change in flow-mediated dilation (FMD) 1 h after both exercise intensities and the immediate change in [A] circulating syndecan-1 and [B] von Willebrand factor (vWF), [C] the change in FMD 10 min after and circulating microRNA 126–3p immediately after moderate intensity continuous (MOD) exercise, [D] change in FMD 1 h after and vWF immediately after high-intensity interval (HII) exercise, and [E] the change in FMD 1 h after and $CD62E^{+}$ endothelial microvesicles (EMV) immediately after MOD exercise.

Author contributions: RMS, SMR, and JMH conceived and designed experiments; RMS, WSE, LEE, CAC, EMZ, and SMR collected data; RMS, WSE, LEE, CAC, EMZ, SJP, SMR, and JMH analyzed data and interpreted results; RMS drafted the manuscript and prepared figures; RMS, WSE, LEE, CAC, EMZ, SJP, SMR, and JMH edited, revised, and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Competing interests: The authors report no potential conflicts of interest.

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Chapter 8: Summary and Future Directions

Summary

The studies comprising this dissertation provide new insight into factors potentially underlying both sex- and race-based disparities in vascular health. We have specifically clarified effects of sex, race, and exercise on the concentrations of ci-miRs related to vascular function. The first two studies have identified sex- and race-based differences in the effects of acute and chronic exercise on cardiovascular measures with significance to future CVD risk. Importantly, these studies were conducted on young, healthy individuals devoid of classic CVD risk factors and, therefore, observed differences likely precede CVD development. Our results suggest that simply measuring classic CVD risk factors in young, apparently healthy individuals is not sufficient. We have identified other mechanisms potentially predisposing such individuals to the future development of vascular dysfunction and CVD based on sex, race, and habitual activity levels. Our results are, therefore, applicable to early strategies aimed at preventing CVD.

The first study presented shows that cumulatively, the circulating blood-borne factors in serum from sedentary individuals induce different effects on cultured endothelial cells as compared with serum of endurance exercise-trained individuals. We also show that the effects of the circulating milieu is associated with exercise training status in a sex-dependent manner. Serum specifically from exercise-trained women increased endothelial cell migration, while serum from sedentary men caused increased ROS production compared to serum from exercise-trained men and sedentary women. Thus, factors within the bloodstream that impact endothelial cell function

appear to be differently altered in response to exercise training/sedentary behavior based on sex. Women were tested during the early follicular period of the menstrual cycle and circulating estradiol levels did not differ between men and women, thereby excluding the female sex hormone commonly proposed to mediate sex differences in vascular health.

The second study shows that compared with CA, AA individuals do not display impaired endothelial function or increased central arterial stiffness either at rest or in response to acute exercise. Conversely, CA actually had lower arterial compliance at rest and greater increases in BP and AIx75 after exercise. Our results are in contrast to those of previous investigations showing that relative to CA, AA have endothelial dysfunction and central arterial stiffness at rest, as well as impaired vasodilation and exaggerated blood pressure responses to stress. However, one hour into recovery from HII exercise CA in our study did exhibit increased carotid arterial compliance, while AA did not. Our results reveal a potentially beneficial recovery response to stress in CA, whereby the compliance of a large, elastic artery that is especially susceptible to atherosclerotic plaque development is increased. The fact that AA did not exhibit the same recovery response suggests that the mechanism(s) underlying this response could be related to racial disparities in future CVD development.

The third aim of this dissertation sought to determine differences in ci-miR that may be related to the sex and race differences identified in studies #1 and #2. In the first study we show that several vascular-related ci-miRs are affected by sex, being greater in men than women, and correlate with different cardiometabolic factors in men and women. Additionally, using an array of ci-miRs implicated in CVD development,

we identify two ci-miRs, ci-miR-140-5p and 145-5p, that are also affected by habitual activity level. Considering these two ci-miRs act to increase ROS, decrease NO production, and inhibit angiogenesis, their greater levels in sedentary individuals may contribute to endothelial dysfunction and reduced capillarization. In the second study we find that the concentrations of several vascular-related ci-miR do not differ between AA and CA at rest. In response to acute exercise, several of these ci-miRs with anti-inflammatory roles were significantly increased in CA, but not AA. Those findings suggest that ci-miRs may be more sensitive to changing in response to exercise in CA, or the response may be more heterogeneous among AA. Further, there were racial differences in the correlations between the change in anti-inflammatory ci-miR-150-5p and the change in carotid arterial compliance from Aim #2. Thus, the increased carotid arterial compliance in CA, and lack thereof in AA, after exercise may be related to inflammation and related ci-miRs. These findings suggest potential mechanisms that may contribute to differential effects of exercise, adaptations to exercise training, and/or development of CVD within AA.

The fourth aim and third study of this dissertation shows that integral damage to the endothelial monolayer likely is not responsible for the transient exercise-induced impairment in endothelial function or endothelial release of miR into circulation. Further, it appears that endothelial cell denudation and apoptosis do not occur with MOD or HII exercise. We do, however, provide evidence that slight disruption of the endothelial membrane and glycocalyx integrity occur with exercise, which may be related to transient changes in endothelial function due to a loss of mechanotransduction.

Cumulatively, the results of these studies provide novel insights into sex- and race-based differences in vascular health within young, healthy individuals. We have used both acute exercise as a stimulus and the exercise-trained individual as a model to elucidate decrements in vascular health that likely precede the initiation of CVD development. Further, we have identified ci-miRs that are differentially expressed due to one or a combination of sex, race, acute exercise, and habitual exercise. These ci-miRs have established mechanistic effects suggesting they may have roles in mediating differences in vascular health due to sex, race, and habitual exercise, as well as the vascular responses to acute exercise. Lastly, we have provided evidence supporting the fact that endothelial-derived miRs are released actively with exercise, as opposed to being passively dumped into circulation as a byproduct of cell damage.

Limitations and Future Directions

While this dissertation provides many novel contributions, there are several limitations of our studies that should be addressed, as well as factors to be considered for future investigations. A weakness of our first study is that we did not measure endothelial function *in vivo* to corroborate our *in vitro* findings on the effects of serum on cultured endothelial cells. Due to conflicting results of previous studies, it remains unclear whether endothelial function is greater in young, healthy men or women (117, 258, 380, 455). However, the effect of long term habitual exercise training on endothelial function likely would not have been apparent because the structural arterial adaptations that occur act to normalize endothelial-dependent vasodilation (182, 501). Additionally, a longitudinal study design may have been preferred to the cross-

sectional design that we employed, so that changes in sedentary individuals could have been tracked in response to an exercise training program. Our goal was to determine differences due to long-term habitual exercise habits and the exercise-trained participants in our study had been training for 11.5 ± 6.4 years on average, making a similar longitudinal exercise training study impractical. Still, future studies focused on circulating blood-borne factors and their effects on endothelial cell function should determine the effects of exercise training on sedentary men and women, as well as the effects of a period of sedentary behavior on endurance-trained individuals. It is also necessary to further characterize the profile of blood-borne circulating factors within men and women, how they cumulatively affect the function of vascular cells, and how they are altered with exercise training/sedentary behavior. Specific factors that are differentially expressed and mediate the effects of serum on vascular cells should next be identified and their roles in sex-specific mechanisms contributing to CVD development determined. Corresponding preventative therapies can then be developed aimed at combatting the early development of vascular dysfunction and CVD in men, as well as the increased risk in postmenopausal women.

A limitation of our second study is that we did not separately study men and women, and instead there were a limited number of women included in each racial group. This was due to a shortened timespan allowed for participant recruitment and data collection due to unforeseen circumstances. As mentioned, sex differences in endothelial function, arterial stiffness, and responses to exercise may differ. Thus, additional studies are needed separately comparing the effects of exercise on vascular function in AA and CA men and women. It is hypothesized that racial differences in

vascular health and CVD risk are due to epigenetic changes resulting from racial discrimination, psychological and physiological stress, and socioeconomic disadvantage imposed on past and present generations (272, 332, 515). Epigenetic markers are understudied in young AA individuals, although they may provide the key to understanding elevated CVD risk. Additional studies should assess differences in miRs and other epigenetic mechanisms, along with vascular function, both at rest and in response to acute and chronic exercise in order to determine specific epigenetic mechanisms potentially underlying racial disparities in CVD development.

A limitation of both our first and second studies is that we did not determine the mechanistic roles of the ci-miRs that we identified as potentially important in mediating sex- or race- differences in the vascular responses to acute or chronic exercise. We used previous research establishing validated mechanistic actions for these miRs to infer their potential roles in the context of our studies. Follow-up studies involving cell culture and animal models are needed to fully elucidate the potential roles of these miRs that our studies in humans suggest. In all of our studies we chose to investigate young, healthy individuals without any CVD risk factors in order to determine differences preceding the onset of CVD development. It is possible that greater differences between groups (sexes and races) than those that we identified may be apparent in older individuals or in those with the presence of CVD risk factors. Further investigations similar to ours are needed to determine differences in these other groups in whom CVD risk is elevated.

In our third study, we assessed the integrity of the endothelium indirectly by measuring the levels of endothelial-derived circulating factors. It would have been

preferred to directly assess integrity by imaging the endothelium. While this may not be possible in humans, it could potentially be performed in animals. To our knowledge, no such studies have been performed to date. Direct imaging of sublingual microvascular endothelial glycocalyx integrity is possible in humans and could potentially inform on the transient effects of exercise on glycocalyx-mediated mechanotransduction (445). In addition, we assessed FMD at the brachial artery similarly to the majority of studies that have determined the effects of acute exercise on endothelial function. Considering our participants underwent cycling exercise, the vasculature in the lower limbs experienced the greatest changes in hemodynamic stimuli. Therefore it is likely that the endothelial-derived circulating factors that we measured may have primarily come from the arteries in the lower limbs and it may have been more representative to measure FMD there as well. Which stimuli are primarily responsible for stimulating miR release from vascular cells during exercise still remains to be elucidated. Future studies should utilize a combination of human, cell, and/or animal models to interrogate the effects of specific stimuli on endothelial function and miR expression/release.

There are still major gaps in our understanding of ci-miRs as they relate to exercise physiology. Importantly, a causal link between ci-miRs and acute exercise responses or exercise training adaptations in has not yet been shown. The studies presented in this dissertation add to the current correlational evidence supporting potential roles of ci-miRs in vascular responses/adaptations. The sources, destinations, and activity of ci-miRs also remain largely unknown, especially in the context of exercise. A recent study has provided the most concrete evidence to date that miRs are

transported between distant tissues (from bone marrow to blood vessels in the lungs) and induce adaptations in recipient endothelial cells (567). Evidence largely points to the fact that miRs are selectively secreted and taken up by cells, but it is unclear what mechanisms govern these processes. The roles of ci-miRs carried in circulation by carrier proteins versus those also encapsulated by vesicles still needs to be clarified as well. There is evidence that both are taken up by cells where they then regulate the translation of their mRNA targets (31, 193, 240–242, 569). Thus, there is ample room for future exploration into ci-miRs in the field of cardiovascular exercise physiology.

Conclusions

The findings presented in this dissertation demonstrate differences in markers of vascular health and related ci-miR due to sex and race that are revealed in the context of habitual exercise or in response to acute exercise. We have identified sex differences in the effects of serum on endothelial cells, with serum of sedentary men inducing greater ROS production compared to sedentary women. However, these sex differences were not apparent between endurance trained individuals, suggesting alterations in circulating factors which promote ROS production may represent a sex-specific mechanism by which sedentary behavior contributes to endothelial dysfunction specifically in men. Importantly, the circulating levels of estradiol were well controlled for and did not differ between the sexes, suggesting this circulating sex hormone is not the only factor mediating sex differences in endothelial function. Determining the specific circulating factors mediating this effect could lead to the development of preventative strategies meant to curb ROS production and the genesis of vascular

dysfunction in men, who develop endothelial dysfunction and CVD at younger ages as compared with women.

In this context, we found that ci-miRs related to vascular function were significantly altered due to sex, and may thereby contribute to sex differences in vascular function. Indeed, the circulating levels of all nine ci-miRs analyzed were greater in men as compared with women. We have added to the literature by showing that sex differences are apparent in vascular-related ci-miRs even among young, healthy individuals who are either sedentary or endurance exercise-trained, when women are in the menstrual/early follicular phase of the menstrual cycle or placebo phase of oral contraceptive use. Thus, in this population sex appears to have a greater effect on ci-miR levels as compared to habitual exercise level. The overall effects of this differential expression of ci-miR on vascular health is not known and will need to be interrogated by subsequent studies.

We have also determined racial differences in both resting and exercise-induced changes in vascular function. The current paradigm is that AA individuals have reduced endothelial function and greater arterial stiffness as compared with CA. In disagreement with previous studies, we found no racial differences in endothelial function and lower carotid arterial compliance in young CA individuals compared with AA individuals. It is unclear exactly why our results were in contrast to prior studies, but it may suggest the AA individuals in our study were healthier than those in previous investigations. This could be due to the fact that we recruited primarily individuals in college who were also moderately active and had healthy BMI, blood pressure, and generally healthy blood chemistry. Our results indicate a need for larger scale

investigations aimed at examining racial differences in vascular function across a wide range of health statuses, socioeconomic statuses, and activity/cardiorespiratory fitness levels in order to determine the factors underlying the often observed impairments in vascular health within AA.

In response to acute exercise (either MOD and/or HII), we observed racial differences in the responses of several parameters including HR, blood pressure, brachial arterial diameter, carotid arterial compliance, and AIx(75). Thus, racial differences in vascular responses to the cardiovascular stress of exercise were apparent. Specifically, CA displayed an increase in carotid arterial compliance following HII exercise, while AA did not. This is a novel finding showing that AA may lack a potential positive response to acute exercise that is seen in CA. This differential response to acute exercise may have implications for the exercise training-induced adaptations to large, elastic arterial compliance, in that AA may not exhibit the same adaptations as CA. Further, the mechanisms underlying this response could be related to the development of future CVD in AA individuals.

To our knowledge, we are the first to show that the resting circulating levels of a set of vascular-related miRs did not differ between young AA and CA individuals. However, several ci-miRs, largely with anti-inflammatory effects, increased only in CA in response to exercise. These findings add to the literature showing that AA exhibit altered responses to physiological stressors as compared with CA, even when differences are not apparent at rest. The different responses of these ci-miR may also contribute to racial differences in the vascular responses to acute exercise. Specifically, the anti-inflammatory ci-miR-150-5p, which was the only ci-miR significantly altered

with exercise in AA, showed race-specific correlations with the changes in carotid arterial compliance after HII exercise. The mechanistic role of this miR should be further determined, but our results provide initial evidence that ci-miR and inflammation may be related to the different changes in arterial compliance between AA and CA.

In sum, further interrogation of the mechanisms identified in the studies presented in this dissertation may inform on 1) the factors underlying sex- and race-based disparities in CVD and 2) the development of strategies to prevent vascular dysfunction and CVD in those at increased risk.

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