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The Effects of Exercise Training on Cardiovascular-related Circulating MicroRNAs

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ABSTRACT

PURPOSE: MicroRNAs (miRNAs) are small regulatory RNAs that post transcriptionally modify mRNAs and control gene expression. Circulating miRNAs are significantly altered following a single session of exercise, however the effects of exercise training on the circulating miRNA profile is unclear. Therefore, the purpose of the present study was to determine the effects of endurance exercise training on the abundance of targeted circulating miRNAs and the association of changes in miRNA levels with concomitant changes in cardiometabolic traits, in a subsample of adults from the HERITAGE Family Study.

METHODS: This exploratory analysis examined 20 previously sedentary adults from the HERITAGE Family Study who completed 20 weeks of endurance exercise training. miRNAs were isolated from serum samples taken at baseline and post-training. The expression of 84 miRNAs related to cardiovascular disease and development was measured at both time points by performing RT-qPCR on the Human Cardiovascular Disease miScript miRNA PCR array (Qiagen, Hilden, Germany). Fold change was calculated as $2^{-\Delta\Delta Ct}$ using the global geometric mean signal of all detected microRNAs as the normalizer value. Paired t-tests were used to examine the effects of exercise training on individual miRNA levels.

RESULTS: Exercise training resulted in nominally significant down-regulation of five miRNAs (miR-155-5p, let-7b-5p, let-7e-5p, miR-486-5p, and miR-7-5p) compared to baseline (Fold change: 0.33-0.76, p=0.01-0.04). Change in miR-486-5p expression was

moderately correlated with change in small high-density lipoprotein particle concentration (r = -0.55, p=0.01) and change in low-density lipoprotein particle size (r=0.53, p=0.01). Additionally, change in miR-7-5p was correlated with change in very low-density lipoprotein particle concentrations (r = -0.47, p=0.04).

CONCLUSIONS: Exercise training altered the expression of specific miRNAs associated with cardiovascular disease, which was related to concomitant changes in the plasma lipoprotein profile. MiRNAs therefore represent a potential mechanism that partially mediates the beneficial effects of exercise on cardiometabolic traits. Further research is needed to understand the complete effects of exercise on the circulating miRNA profile.

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CHAPTER 1
INTRODUCTION

MicroRNAs (miRNAs) are small non-coding regulatory RNAs that control gene expression via post-transcriptional modification of messenger RNA (mRNA).[1, 2] MiRNAs silence mRNAs through two distinct mechanisms: mRNA degradation or repression of mRNA translation.[2] Over 2,500 different miRNAs have been identified in humans, and despite accounting for an estimated 1-5% of the human genome, miRNAs are estimated to regulate greater than 30% of protein coding genes.[2-4] MiRNAs are transported into circulation within exosomes, protein complexes, or microvesicles.[2, 5] The transport of miRNAs in these complexes prevents their breakdown and allows miRNAs to circulate to and act on target cells throughout the body.[1, 2] Thus, circulating miRNAs represent a novel biomarker for diseases such as cancer and cardiovascular disease (CVD).[2, 6, 7]

Altered circulating miRNA profiles have been identified in patients with many different diseases, including different forms of cancer, diabetes, and forms of CVD.[8, 9] Additionally, Zampetaki et al.[10] found that baseline miRNA profiles were associated with incident myocardial infarction over ten years in an elderly cohort, suggesting that miRNAs can also be used to predict future disease. MiRNAs have also been associated with many steps during the progression towards atherosclerosis, and associated with acute myocardial infarction, coronary artery disease, and unstable angina.[7, 11]

Additionally, miRNAs play an essential role in many biological processes such as angiogenesis, mitochondrial metabolism, and cardiac/skeletal muscle hypertrophy.[12-14] These pathways have implications beyond disease risk reduction and are associated with adaptations often found following exercise training. Indeed, miRNAs have recently been shown to correlate with cardiovascular adaptation to exercise,[15] demonstrating an active functional role of miRNAs in human physiology. The associations of miRNAs with disease, as well as the physiological effects of miRNAs on multiple biological pathways, demonstrate the potential clinical relevance of circulating miRNAs as biomarkers of disease.

Although circulating miRNAs have been well studied as biomarkers of various disease state, the effects of exercise on the circulating microRNA profile are not completely understood. Current literature shows an effect of acute exercise on the circulating miRNA profile and it has been suggested that miRNAs play a role in physiological adaptations to exercise.[16, 17] However, less is known about the effects of exercise training on the circulating miRNA profile. Baggish et al.[15] found that 90 days of rowing significantly altered resting levels of selected circulating miRNAs. Another study found that 4 weeks of cycling significantly decreased circulating levels of miR-486 and that this miRNA was correlated with changes in maximal oxygen consumption $\dot{V}O_2max$.[18] Thus, limited evidence of the effects of exercise training on circulating miRNAs is available. Another limitation of the current literature is that many studies examine fewer than 10 targeted miRNAs, with little overlap of miRNAs between studies.[17] Taken together, the effects of exercise training on circulating miRNAs are still largely unclear. Therefore, the purpose of the current study was to examine the effects of

exercise training on a panel of 84 miRNAs associated with CVD in 20 individuals from the HERITAGE Family Study. We hypothesized that exercise training would significantly alter the circulating miRNA profile. We will test these hypotheses with the following aims:

Aim 1: Determine the effects of endurance exercise training on the abundance of targeted circulating miRNAs in a subsample of previously sedentary adults from the HERITAGE Family Study.

We hypothesize that endurance exercise training will significantly alter the circulating miRNA profile of targeted miRNAs.

Aim 2: Determine the association between circulating miRNA expression levels with cardiometabolic risk factors at baseline and as an adaptation to exercise training (i.e., change score).

We hypothesize that baseline levels and exercise induced changes in circulating miRNAs will be significantly associated with concomitant levels of lipids and inflammatory markers.

CHAPTER 2

A BRIEF HISTORY OF HDL AND MICRORNAS

Cardiovascular disease (CVD) involves the heart or blood vessels and is associated with multiple physiological and behavioral risk factors including dyslipidemia, smoking, hypertension, diabetes, and obesity.[19] As CVD is the leading cause of death in the United States[20], much of research and resources have focused on the prevention and treatment of CVD. The association between low high-density lipoprotein cholesterol (HDL-C) and negative health outcomes was first reported in 1966, when HDL-C levels were found to be inversely associated with ischemic heart disease risk.[21] In 1989 Gordon et al.[22] showed that low levels of high-density lipoprotein cholesterol (HDL-C) was a risk factor for coronary heart disease. Low HDL-C levels have repeatedly been associated with increased CVD risk in many different populations across the world.[23-26] Similar results are found in animal models. Overexpression of the human apolipoprotein A-I (ApoA-I) gene in mice results in an increase in HDL-C concentration that is accompanied with protection against atherosclerosis.[23] Rubin et al.[27] found that genetically elevating ApoA-I and HDL-C concentrations in mice protected against the formation of atherosclerotic lesions from a high fat diet compared to control mice, demonstrating a protective effect of HDL-C and ApoA-I. In ApoE deficient mice, who are predisposed to atherosclerosis, transgenic elevation of HDL-C diminished atherosclerotic lesion formation and HDL-C levels accounted for 78% of the observed variance in the lesion

size.[28] This combination of human and animal research led to the hypothesis that elevated HDL-C levels protects against atherosclerosis and CVD.

The HDL hypothesis has recently been called in to question due to the ineffectiveness of treatments that raise HDL-C levels to attenuate CVD risk. The 2013 ACC/AHA Guidelines concluded that statins were effective at lowering low density lipoprotein cholesterol (LDL-C) levels and that these reductions were effective in reducing CVD risk.[29] However, targeted drug treatments for raising HDL-C do not yield similar results. A meta-analysis by Keene et al.[30] of 39 drug trials of niacin, fibrates, and cholesterol esterase transfer protein (CETP) inhibitors showed that while many of the trials were successful in raising HDL-C levels, no change in CVD risk was found. In fact, their meta-analysis showed that despite elevated HDL-C levels some of these drugs were associated with elevated risk of CVD when compared to control groups.[30] Additionally, mendelian randomization studies have shown that single nucleotide polymorphisms (SNPs) associated with elevated HDL-C levels are not associated with reduced CVD risk.[31] These studies turned the HDL hypothesis upside down, as HDL-C levels by themselves do not seem to be protective against CVD. The HDL hypothesis therefore has recently shifted toward the HDL "function" hypothesis, where the functionality of HDL particles (HDL-P) is the important factor in determining CVD risk.

Ample evidence exists for a physiological basis of the HDL hypothesis. HDL plays a critical role in reverse cholesterol transport (RCT), a known anti-atherogenic function or pathway, by accepting cholesterol on to lipid poor apoA-I, the main protein constituent of HDL, which is largely mediated by ATP binding cassette transporter A1 (ABCA1).[32] HDL also inhibits the expression of endothelial adhesion molecules *in vitro*[33]

and protects against LDL oxidation and apoptosis.[34] Finally, HDL can stimulate the release of nitric oxide from the endothelium and induce vasodilation.[35, 36] Perhaps the most well-established HDL function is the facilitation of RCT. Briefly, HDL and apoA-I promote cholesterol efflux from peripheral macrophages and other peripheral tissues primarily through the ABCA1 and ABCG1 transporters.[37] Mature, lipid rich, HDL can then transfer cholesterol to the liver via scavenger receptor class B type 1 (SR-B1) or indirectly via CETP mediated transfer for excretion.[37] Several recent population studies have shown cholesterol efflux capacity is associated with prevalent and incident of CVD[38-40] and CVD mortality.[41] In a prospective study of 2,924 adults from the Dallas Heart Study, Rohatgi et al.[39] found a 67% reduction in atherosclerotic cardiovascular risk in the highest quartile of cholesterol efflux capacity compared to the lowest quartile after adjustment for traditional risk factors, HDL-C, and HDL-P concentration. Additionally, cholesterol efflux capacity of HDL has been reported as independent of traditional cardiovascular risk, as well as HDL-C and HDL particle concentration.[39] Another atheroprotective function of HDL is the proposed anti-inflammatory properties through this particle's ability to inhibit the expression of cell surface adhesion molecules in endothelial cells.[42] HDL has also been reported to inhibit a key enzyme in the production of endothelial cell adhesion molecules, however this inhibition has a great deal of variability in humans, likely due to the heterogeneity of HDL molecules themselves.[42] The relationship between the anti-inflammatory properties of HDL and CVD risk are still unclear, but circulating levels of these endothelial cell adhesion molecules (e.g., VCAM-1, ICAM-1) are associated with CVD risk.[43, 44] Therefore, the connection between HDL

function and CVD risk goes beyond the role of HDL in RCT and is likely a result of the combination of all HDL functions.

A recently discovered function or role of HDL is the ability of HDL particles to transport and deliver microRNAs (miRNAs) to target tissues.[1] MiRNAs are small regulatory RNAs that control gene expression by targeting and binding messenger RNA (mRNA) and preventing protein production.[1, 2] One estimate is that miRNAs account for 1-5% of the human genome and regulate greater than 30% of protein coding genes.[2] Over 2,500 different miRNAs have been identified in humans, with more constantly being discovered.[3, 4] As such, circulating miRNA levels may represent a new type of biomarker for diseases like cancer and CVD.[2, 6, 7] The miRNA profile of patients with non-ischemic systolic heart failure was found significantly altered compared to healthy controls and correlated with the severity of the heart failure.[8] MiRNAs may also be able to predict cardiovascular events, as Zampetaki et al.[10] found that baseline miRNA profiles were associated with incident myocardial infarction over ten years in an elderly cohort. These studies illustrate the potential viability of miRNAs as biomarkers of disease and perhaps even future disease development.

MiRNAs are synthesized in the nucleus and undergo modifications in the cytoplasm where they form a double stranded duplex before being exported via exosomes, microvesicles, HDL-P, or in a protein complex.[2, 5] The binding of miRNAs to HDL likely follows a mechanism similar to the binding of small RNAs with liposomes which means that HDL incorporates the miRNAs into a protected space on the complex.[1] Additionally, the HDL-miRNA profile is altered in disease states compared to healthy controls and compared to circulating exosomal miRNA, potentially representing a

distinct novel biomarker for disease from circulating miRNA.[1, 45] Wagner et al.[45] found similar results showing that HDL-miRNA profiles were altered in patients with acute coronary syndrome compared to controls, and that three vascular- and inflammatory-related miRNAs were most prominent on HDL. Choteau et al.[46] examined HDL-miRNAs in patients with acute coronary syndrome and coronary artery disease (CAD) found that HDL miR-223 levels were correlated with CAD score ($r=0.383$, $p=0.016$) and may reflect the progression of CAD. In further support of the importance of the HDL-miRNA profile, Vickers et al.[1] found that HDL was effective at delivering miRNAs to target cells. Hepatocytes were treated with synthesized HDL-miRNA complexes, which led to an increase in intracellular miRNA and a reduction in the specific miRNA targets.[1] This study also showed that the delivery of HDL-miRNA is SR-B1 dependent, consistent with the known interaction between HDL and SR-B1 during cholesterol efflux.[1] The delivery of miRNAs by HDL to target cells at least in part mediates the known function of HDL inhibiting cellular adhesion molecules.[47] The discovery of the HDL mediated transport and functional delivery of miRNAs represents a potential new biomarker for disease and a target for intervention. Additionally, HDL-miRNAs are an exciting new potential mechanism by which HDL mediates its antiatherogenic effects.

As discussed above, drug interventions targeted at raising HDL-C levels have not resulted in improved CVD risk, so alternative therapies for improving HDL function and cardiovascular health are needed. Regular exercise is an alternate therapy that has been suggested for reducing CVD risk.[48, 49] Regular exercise has favorable effects on both lipid and lipoprotein profiles[50] including increasing plasma HDL-C levels.[51] However, the effects of exercise training on HDL function are still largely unclear. A recent study by

Khan et al. 2018 found that weight loss combined with exercise in patients with metabolic syndrome improved HDL composition and cholesterol efflux capacity.[52] Recently Sarzynski et al.[53] examined the effects of exercise training on HDL mediated cholesterol efflux capacity across two training studies, and found that only the highest dose of high intensity exercise resulted in significant changes in global radiolabeled efflux capacity (+6.2%) in one cohort and non-ABCA1 radiolabeled efflux capacity (+5.7%) in another cohort. Furthermore, results from the remaining limited literature on the effects of exercise on HDL mediated cholesterol efflux are mixed. A study in patients with peripheral artery disease found no effect of exercise training on cholesterol efflux capacity,[54] however this may have been due to the inability of these patients to reach the intensities necessary to see a change. Koba et al.[55] studied patients with acute coronary syndrome and found that cardiac rehabilitation, even at relatively low intensities, significantly increased cholesterol efflux capacity compared to baseline but not compared to controls. Exercise may also impact the anti-inflammatory properties of HDL. A short-term diet and exercise intervention was effective at converting pro-inflammatory HDL to anti-inflammatory in overweight and obese men.[56] Some evidence does exist for a beneficial effect of exercise on the anti-oxidative properties of HDL. In patients with type II diabetes, four months of endurance training improved the ability of HDL to inhibit LDL oxidation, but this improvement was not seen in the healthy control group.[57] However, the effects of exercise on the anti-inflammatory and anti-oxidative properties are still largely unclear, as there are limited studies and most of the beneficial effects of exercise on HDL function have been found in studies examining diseased populations.

While the effects of exercise on HDL function are somewhat unclear, ample evidence exists of an effect of exercise on circulating miRNA levels. In 32 healthy trained men, acute endurance exercise up regulated selected miRNAs one to three hours after the last exercise session, and basal levels of nine selected miRNAs were significantly altered following 12 weeks of endurance training.[58] The authors proposed that a mechanism for the alteration of circulating miRNAs following training is the associated/concomitant alteration of lipoprotein levels.[58] Xu T et al.[5] in a recent review speculated that the rise in circulating miRNAs following acute exercise may be due to muscle damage and subsequent release of miRNAs from damaged tissue. The authors also noted that the effects of chronic exercise on circulating miRNAs are still unclear, as some miRNAs seem to be upregulated following chronic exercise while others are down regulated.[5] The fact that miRNA levels themselves change with exercise has significant implications for disease and may mediate some of the beneficial effects of exercise.[59] Uhlemann et al.[60] showed that endurance training acutely increases endothelial specific miRNA and resistance training increased skeletal muscle specific miRNA.

Despite numerous studies examining changes in circulating miRNAs with exercise, to our knowledge no study has examined the effects of exercise on HDL-miRNAs. Additionally, most of the research on the effects of exercise on circulating miRNAs has examined the acute effects of exercise.[16] Thus, a need exists for further research concerning the effects of exercise training on circulating miRNAs in different populations.

CHAPTER 3

METHODOLOGY

HERITAGE Family Study. The HERITAGE Family Study (hereafter referred to as HERITAGE) is one of the largest, most well-controlled, standardized exercise training studies to date. HERITAGE was designed to examine the role of genetic factors on cardiometabolic responses to endurance exercise training. The HERITAGE cohort is composed of 481 whites (232 men and 249 women) from 99 families and 250 blacks (88 men and 162 women) from 105 family units that completed a 20-week endurance exercise program at one of four clinical centers (Indiana, Minnesota, Québec, Texas). The study design and training protocol have been described in detail elsewhere.[61] Briefly, the participants were sedentary at baseline, normotensive or mildly hypertensive (<160/100 mm Hg) without medications for hypertension, diabetes, or dyslipidemia.

Exercise training program. The training program consisted of three weekly sessions of cycling at the heart rate associated with 55% of baseline VO_2max for 30 minutes for the first two weeks. The duration and intensity were then gradually increased every two weeks until the heart rate associated with 75% of baseline VO_2max for 50 minutes was achieved. This level was maintained for the final six weeks of training. All training was performed on Universal Aerobicycles (Cedar Rapids, IA). Power output was controlled directly relative to heart rate by using the Universal Gym Mednet (Cedar Rapids, IA) computerized system. The protocol was standardized across all clinical

centers and supervised to ensure that the equipment was working properly and that the participants were compliant with the protocol.

Exercise tests. Three exercise tests were administered prior to training and three additional tests were given at the end of training. All tests were completed on a stationary cycle ergometer. The first test at each time point was used to establish the participants $\dot{V}O_2max$. During the second test of each battery, participants cycled at 50 watts and 60% of their $\dot{V}O2max$ for 8 minutes each. The third and final test of each battery consisted again of 8 minutes each of 50 watts and 60% $\dot{V}O_2max$, after which the power output was increased to 80% $\dot{V}O_2max$ for three minutes, and continually increased until exhaustion. During the third test blood was collected via a venous catheter. [61]

Subjects for current study. Twenty white subjects from HERITAGE that completed the 20-week exercise program were selected for this study based on discordance for exercise-induced changes in HDL particle size. All subjects selected for this exploratory study were from the Québec center, as the Québec participants were also part of an ancillary study that involved skeletal muscle biopsies and have available gene expression data.

Blood collection. Blood samples were obtained from an antecubital vein into sealed Vacutainer tubes in the morning after a 12-hour fast with participants in a semi-recumbent position. The blood samples were collected at baseline and again at 24 hours after the last training session. Blood samples were allowed to clot at room temperature and serum was separated via centrifugation according to standard methods. For eumenorrheic women, all samples were obtained in the early follicular phase of the menstrual cycle when blood plasma cholesterol alterations are minimal.[61]

HDL isolation. HDL isolation was previously performed on these 20 subjects using the following procedures. HDL plasma was separated from whole serum via fast performance liquid chromatography (FPLC) with size exclusion chromatography. Briefly, 370 µL of whole serum was injected into an Akta Pure FPLC and run through two gel filtration columns (Superdex 200 increase columns, GE Healthcare). HDL fractions were collected and pooled together then aliquoted for storage in a -80°C freezer. HDL plasma was then concentrated by adding 500 µL of plasma HDL to an Amicon Ultra 0.5 mL centrifugal filter with a 3000 dalton cellulose membrane in a 2 mL centrifuge tube and spinning at 14,000 x g for 30 minutes at room temperature. The flow through was discarded and the filter device removed and placed upside down in a clean tube and spun at 1,000 x g for 2 minutes at room temperature, yielding approximately 60 µL of concentrated HDL serum.

Note: The miRNA PCR analysis of serum HDL samples was largely unsuccessful, therefore for the remainder of the document, analysis will focus on circulating miRNAs.

MiRNA analysis. MiRNAs were extracted on the QIAcube workstation from baseline and post-training whole serum samples using miRNeasy Serum/Plasma Advanced Kits (Qiagen, Hilden, Germany). Samples were spiked with a known amount of synthetic C. elegans miR-39-3p. MiRNA quantification was then performed using reverse transcriptase real-time polymerase chain reaction (RT-qPCR). Briefly, cDNA was transcribed from the extracted miRNA and then aliquoted into the Human Cardiovascular Disease miScript miRNA PCR Array (Qiagen, Hilden, Germany). This array contains primers for 84 miRNA sequences identified as exhibiting altered expression during CVD

and development. The selected miRNAs are listed in Table 3.1. All cDNA steps and PCR setup were performed by the QIAgility instrument (Qiagen, Hilden, Germany) using an automated pipetting protocol. Real-time qPCR was performed on the miRNA PCR array in the Rotor-Disc 100 format by the Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany). Rotor-Gene PCR cycling conditions were performed according to manufacturer's suggested protocol and conditions.

Cardiometabolic trait measurement. Total cholesterol and triglyceride levels in plasma and lipoproteins were measured by enzymatic methods using the Technicon RA-1000 analyzer.[61] Lipoprotein subclass and particle size measurements were performed using NMR spectroscopy previously described.[62] Substrate utilization and ventilation during submaximal exercise were measured via standard methods previously described.[61]

Statistical methods. The effect of training on circulating miRNAs (Aim 1) was assessed using paired t-tests of pre- and post-training miRNA levels. Cycle threshold (Ct) represents the cycle number at which there is an exponential increase in miRNA fluorescence. Delta Ct was calculated by subtracting the global geometric mean signal of all detected miRNAs from individual Ct values. Delta delta Ct was then calculated by subtracting delta Ct values of baseline samples from delta Ct values post-training. Fold change was calculated as $2^{-\Delta\Delta Ct}$ using the global geometric mean signal of all detected microRNAs as the normalizer value. Pearson's correlations were used to examine the associations between baseline and fold change in miRNA levels with change in select cardiometabolic phenotypes(Aim 2). All analyses were performed using SAS 9.4 (Cary, NC).

Table 3.1. List of miRNAs included on Qiagen's Human Cardiovascular Disease miScript miRNA PCR Array.

Mature miRNA ID or Gene Symbol					
hsa-let-7a-5p	hsa-miR-130a-3p	hsa-miR-181b-5p	hsa-miR-223-3p	hsa-miR-30c-5p	hsa-miR-92a-3p
hsa-let-7b-5p	hsa-miR-133a-3p	hsa-miR-182-5p	hsa-miR-224-5p	hsa-miR-30d-5p	hsa-miR-93-5p
hsa-let-7c-5p	hsa-miR-133b	hsa-miR-183-5p	hsa-miR-23a-3p	hsa-miR-30e-5p	hsa-miR-98-5p
hsa-let-7d-5p	hsa-miR-140-5p	hsa-miR-185-5p	hsa-miR-23b-3p	hsa-miR-31-5p	hsa-miR-99a-5p
hsa-let-7e-5p	hsa-miR-142-3p	hsa-miR-18b-5p	hsa-miR-24-3p	hsa-miR-320a	cel-miR-39-3p
hsa-let-7f-5p	hsa-miR-143-3p	hsa-miR-195-5p	hsa-miR-25-3p	hsa-miR-328-3p	SNORD61
hsa-miR-1-3p	hsa-miR-144-3p	hsa-miR-199a-5p	hsa-miR-26a-5p	hsa-miR-342-3p	SNORD68
hsa-miR-100-5p	hsa-miR-145-5p	hsa-miR-206	hsa-miR-26b-5p	hsa-miR-365a-3p	SNORD72
hsa-miR-103a-3p	hsa-miR-146a-5p	hsa-miR-208a-3p	hsa-miR-27a-3p	hsa-miR-378a-3p	SNORD95
hsa-miR-107	hsa-miR-149-5p	hsa-miR-208b-3p	hsa-miR-27b-3p	hsa-miR-423-3p	SNORD96A
hsa-miR-10b-5p	hsa-miR-150-5p	hsa-miR-21-5p	hsa-miR-29a-3p	hsa-miR-424-5p	RNU6-6P
hsa-miR-122-5p	hsa-miR-155-5p	hsa-miR-210-3p	hsa-miR-29b-3p	hsa-miR-451a	miRTC
hsa-miR-124-3p	hsa-miR-15b-5p	hsa-miR-214-3p	hsa-miR-29c-3p	hsa-miR-486-5p	PPC
hsa-miR-125a-5p	hsa-miR-16-5p	hsa-miR-22-3p	hsa-miR-302a-3p	hsa-miR-494-3p	
hsa-miR-125b-5p	hsa-miR-17-5p	hsa-miR-221-3p	hsa-miR-302b-3p	hsa-miR-499a-5p	
hsa-miR-126-3p	hsa-miR-181a-5p	hsa-miR-222-3p	hsa-miR-30a-5p	hsa-miR-7-5p	

CHAPTER 4

RESULTS

Baseline characteristics including mean values for the standard lipid panel and other cardiovascular risk factors are shown in Table 4.1. No significant differences were found between sexes. The expression levels of five miRNAs were nominally ($p<0.05$) down regulated with exercise. Exercise resulted in a fold change of 0.82 ($p=0.04$) for mir-155-5p, 0.49 ($p=0.01$) for let-7b-5p, 0.70 ($p=0.04$) for let-7e-5p, 0.56 ($p=0.02$) for miR-486-5p, and 0.42 ($p=0.01$) for miR-7-5p, all compared to baseline expression levels. In addition to fold change, average Ct values increased in all five miRNAs (Figure 4.1), indicating reduced expression following exercise. Change in miR-486-5p expression was moderately correlated with change in small high-density lipoprotein (HDL) particle concentration ($r=-0.55$, $p=0.01$) and change in low-density lipoprotein (LDL) particle size ($r=0.53$, $p=0.01$). Additionally, change in miR-7-5p was correlated with change in very low-density lipoprotein (VLDL) particle concentrations ($r=-0.47$, $p=0.04$). Fold change of miR-155-5p and let-73-5p were correlated with change in plasma free fatty acids at 50W and 60% of max workload, as well as change in circulating glucose at 60% max workload (Table 4.2).

Baseline levels of numerous miRNAs were nominally ($p<0.05$) correlated with changes in cardiometabolic traits following exercise training. Baseline expression levels of miR-124-3p, miR-143-3p, and miR-199a-5p were moderately positively correlated

with changes in $\dot{V}O_2$max (r=0.48-0.54, p=0.02-0.04). Expression levels of 24 circulating miRNAs at baseline were positively correlated with change in plasma apoA-1 levels (range: r=0.47-0.64, p=0.003-0.04) (Table 4.3). Baseline levels of 12 different miRNAs were correlated with exercise induced changes in HDL cholesterol levels (Table 4.4), and baseline expression levels of 7 different miRNAs were correlated with change in mean arterial pressure with exercise (Table 4.5). The baseline miRNA expression level of 51 miRNAs were negatively correlated with change in end tidal carbon dioxide pressure ($PETCO_2$) during submaximal exercise (range: r=-0.49 to -0.81, p<0.0001-0.01). These 51 miRNAs are listed in Table 4.6.

Table 4.1. Participant baseline characteristics given as means (standard deviation).

	(n=20)
Age (years)	43.4 (13.1)
BMI (kg/m^2)	26.4 (3.7)
VO_2 max (L/min)	2.1 (0.5)
HDL-C (mg/dL)	43.4 (10.7)
LDL-C (mg/dL)	134.4 (34.3)
TC (mg/dL)	203.7 (37.5)
TG (mg/dL)	159.1 (84.3)
CRP (mg/dL)	0.29 (0.3)
SBP (mmHg)	116.7 (12.8)
DBP (mmHg)	65.9 (9.9)
Waist (cm)	90.5 (11.6)
Percent fat	28.9 (6.9)

BMI: body mass index, HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol, TC: total cholesterol, TG: triglycerides, CRP: C-reactive protein, SBP: systolic blood pressure, DBP: diastolic blood pressure,

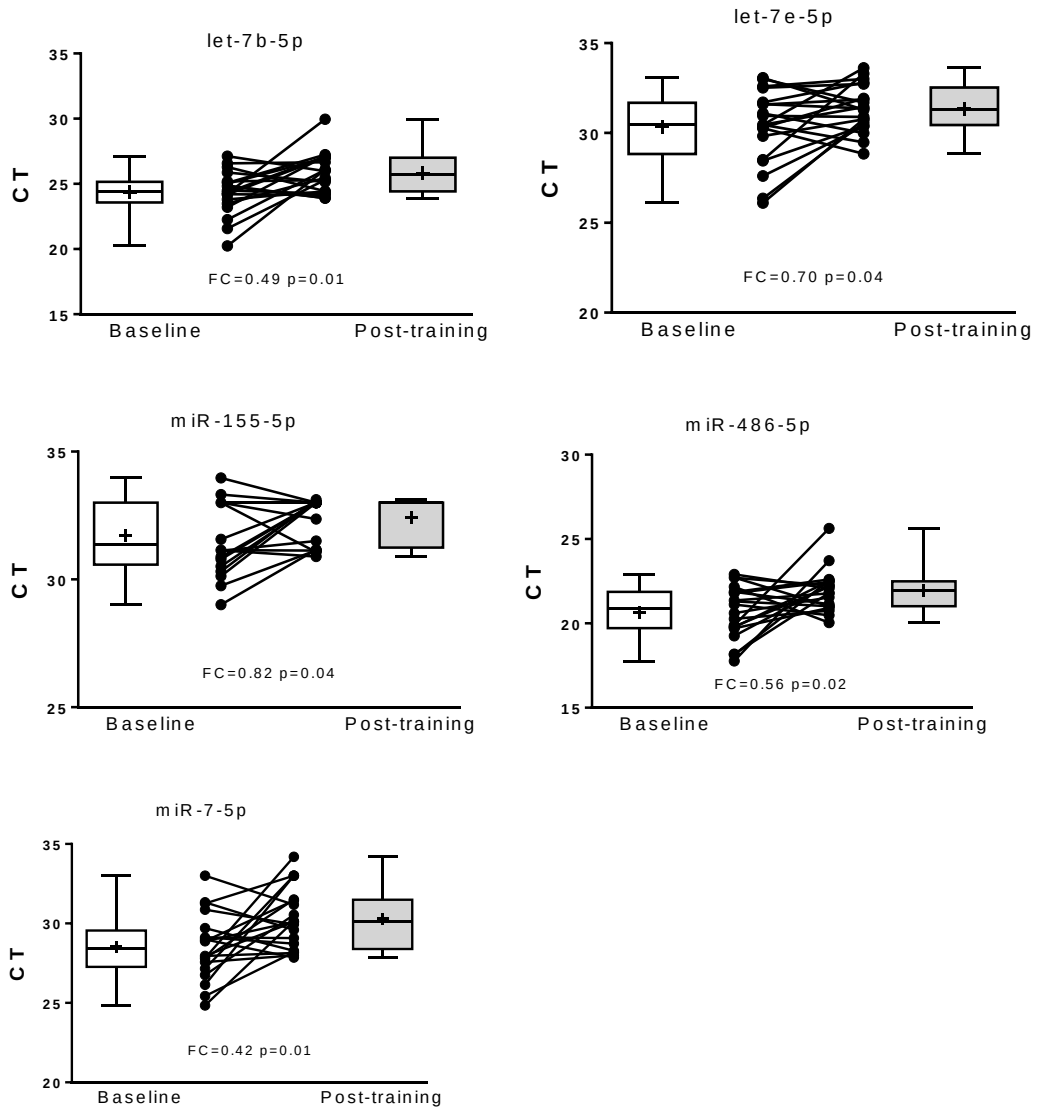

Figure 4.1. Baseline and post-training cycle threshold (CT) values along with individual exercise response for miRs: let-7b-5p let-7e-5p, miR-155-5p, miR-486-5p, and miR-7-5p.

Table 4.2. Correlation between fold-change in select miRNAs and concomitant change in substrate utilization

	FFA 50W	FFA 60%	Glucose 60%	Aldosterone
miR-155-5p	0.73	0.59	0.49	NS
Let-7b-5p	NS	NS	NS	0.61
Let-7e-5p	0.72	0.60	0.53	NS
miR-486-5p	NS	NS	NS	NS
miR-7-5p	NS	NS	NS	0.58

All correlations listed were $p<0.05$. NS, not significant ($p>0.05$). FFA 50W: free fatty acids at 50 watts workload, FFA 60%: free fatty acids at 60% workload, Glucose 60%: glucose at 60% workload

Table 4.3. Correlation between baseline miRNA expression and change in apoA-1.

	r	p-value
Let-7c-5p	0.64	0.003
Let-7d-5p	0.47	0.04
miR-10b-5p	0.53	0.02
miR-130a-3p	0.48	0.04
miR-140-5p	0.47	0.04
miR-142-3p	0.47	0.04
miR-150-5p	0.47	0.04
miR-16-5p	0.50	0.03
miR-195-5p	0.53	0.02
miR-221-3p	0.55	0.01
miR-222-3p	0.57	0.01
miR-24-3p	0.48	0.04
miR-25-3p	0.53	0.02
miR-26a-5p	0.49	0.03
miR-29a-3p	0.49	0.03
miR-29b-3p	0.53	0.02
miR-30a-5p	0.55	0.01
miR-30c-5p	0.52	0.02
miR-30e-5p	0.59	0.01
miR-320a	0.55	0.01
miR-342-3p	0.54	0.02
miR-451a	0.52	0.02
miR-93-5p	0.59	0.01
miR-99a-5p	0.49	0.03

Table 4.4. Correlation between baseline miRNA expression levels and change in HDL-C levels

	r	p-value
Let-7a-5p	0.51	0.03
Let-7c-5p	0.50	0.03
miR-130a-3p	0.52	0.02
miR-17-5p	0.48	0.04
mir-185-5p	0.50	0.03
miR-195-5p	0.54	0.02
miR-223-3p	0.49	0.03
miR-23b-5p	0.53	0.02
miR-31-5p	-0.46	0.04
miR-320a	0.49	0.03
miR-98-5p	0.49	0.03
miR-99a-5p	0.47	0.04

Table 4.5. Correlation between baseline miRNA expression levels and change in mean arterial pressure.

	r	p-value
Let-7a-5p	-0.59	0.01
Let-7c	-0.54	0.02
Let-7f-5p	-0.49	0.03
miR-210	-0.49	0.03
miR-26b-5p	-0.57	0.01
miR-98-5p	-0.50	0.03
miR-99a-5p	-0.61	0.01

Table 4.6. List of baseline miRNAs significantly correlated with change in $PETCO_2$ during submaximal exercise

Let-7c	Let-7d-5p	miR-103a-3p	miR-124-3p	miR-125a-5p	miR-126-3p
miR-130a-3p	miR-140-5p	miR-143-3p	miR-144-3p	miR-145-5p	miR-146a-5p
miR-15b-5p	miR-16-5p	miR-17-5p	miR-181b-5p	miR-185-5p	miR-18b-5p
miR-195-5p	miR-199a-5p	miR-21-5p	miR-210	miR-214-3p	miR-221-3p
miR-223-3p	miR-224-3p	miR-23a-3p	miR-24-3p	miR-25-3p	miR-26a-5p
miR-26b-5p	miR-27a-3p	miR-27b-3p	miR-29a-3p	miR-29b-3p	miR-29c-3p
miR-30a-5p	miR-30c-5p	miR-30d-5p	miR-30e-5p	miR-320a	miR-342-3p
miR-365a-3p	miR-423-3p	miR-424-5p	miR-451a	miR-494	miR-92a-3p
miR-93-5p	miR-98-5p	miR-9a-5p			

CHAPTER 5
DISCUSSION

Exercise training nominally down-regulated 5 miRNAs related to cardiovascular disease compared to baseline in 20 healthy, previously sedentary adults from the HERITAGE Family Study. Exercise induced changes in miRNA expression levels were correlated with concomitant changes in select cardiometabolic factors such as HDL-C and plasma free fatty acids during submaximal exercise.

The down regulation of the five miRNAs in the current study could be explained by a few different mechanisms. One would be a degradation of the miRNAs in circulation with exercise, however miRNAs are transported within vesicles or within protein complexes that prevent degradation,[1, 2] so this seems unlikely. Exercise could also influence the expression of the miRNAs themselves, however little is known about potential mechanism(s) by which exercise could accomplish this. MiRNA expression can certainly be regulated and altered, but the regulation of miRNA is complex and not fully understood.[63] Another potential mechanism would be the promotion of selective uptake of these miRNAs by skeletal muscle with exercise. Several studies have demonstrated the ability of target cells, such as skeletal muscle, to take up circulating miRNAs.[1, 2, 64] One plausible explanation is that the reduction of these five miRNAs in circulation with exercise training may in part be due to increased uptake of these miRNAs by skeletal muscle. Exercise training however does not likely increase the uptake of all miRNAs by

skeletal muscle and may in fact increase the release of certain miRNAs into circulation. Baggish et al.[15] found that exercise training resulted in an up-regulation of miR-222, miR-21, and miR-221. Interestingly these same three miRNAs were also up-regulated following acute exercise, indicating a potential additive effect of acute bouts with training that serves to upregulate certain miRNAs.[15]

The current study found that exercise training down-regulated miR-486-5p. Similarly, in a study of 11 healthy young men both a single cycling exercise session and four weeks of exercise training significantly decreased circulating levels of miR-486-5p.[18] Additionally, the directionality of the change in circulating miR-486-5p with exercise was the same following an acute bout and following exercise training,[18] giving further evidence for an additive effect of acute bouts that may lead to a training effect. MiR-486-5p targets phosphatase and tensin homolog (PTEN), a negative regulator of Akt signaling, and therefore increases insulin-dependent glucose uptake.[65] It is well established that exercise training improves insulin dependent glucose uptake in skeletal muscle.[66, 67] Therefore, the reduction of circulating miR-486-5p may be the result of an increased uptake of the miRNA into the skeletal muscle. This uptake of miR-486-5p may in turn be responsible for improvements in insulin dependent glucose uptake by the skeletal muscle following exercise training.

MiR-155-5p is down regulated in mice following 12 weeks of endurance exercise training.[68] Data from this study supports the current study's finding that exercise training down regulates circulating miR-155-5p. MiR-155-5p is drastically upregulated in atherosclerosis, and miR-155-5p targets eNOS and inhibits nitric oxide production.[69, 70] MiR-155-5p expression also promotes cardiac inflammation and failure in mice.[71] Bone

marrow transplant into miR-155-5p knockout mice was effective at rescuing the cardiac hypertrophy, demonstrating the importance of reducing miR-155-5p expression even in a diseased state.[71] The physiological role of miR-155-5p with atherosclerosis and heart failure highlights the potential clinical importance of strategies that reduce circulating levels of this miRNA. Further research is needed to determine if the reduction in circulating miR-155-5p is associated with a similar reduction in vascular miR-155-5p.

Little is known about the effects of exercise on the let-7 family of miRNAs, and this study may be the first to show a down regulation of let-7b and let-7e following exercise training. This reduction may be beneficial as plasma levels of let-7b are elevated during an ischemic stroke and has been identified as a potential biomarker of stroke.[72] However, the physiological basis for this increase is unclear. The let-7 family of miRNAs has been implicated in both the immune and inflammatory response.[73, 74] Let-7e has been identified as a pro-inflammatory miRNA and may play a role in atherosclerosis.[73] Therefore, the reduction of circulating levels of let-7b and let-7e with exercise may have clinical relevance for a reduction in inflammation.

Baggish et al.[15] reported that exercise training significantly altered the expression of select miRNAs and that this alteration was associated with changes in $\dot{V}O_2$max, indicating a potential role of miRNAs as mediators of exercise induced cardiovascular and metabolic adaptations. Similarly, we found that baseline expression levels of three miRNAs were moderately positively correlated with exercise-associated changes in $\dot{V}O_2$max, further implicating miRNAs in response of $\dot{V}O_2$max to training. The current study found that change in circulating miR-486-5p was correlated with changes in size of both HDL particles and LDL particles. MiR-486 has been reported to associated with

HDL_2 (large HDL) but not HDL_3 (small HDL),[75] suggesting a relationship between miR-486 and lipoprotein size. Additionally, changes in miR-155-5p and let-7e-5p were correlated with change in circulating free fatty acids and plasma glucose during submaximal exercise. Both circulating free fatty acids and plasma glucose are measures of substrate utilization during exercise. Exercise training improves the utilization of lipids during moderate intensity exercise.[76] An increase in circulating free fatty acids during exercise may indicate greater mobilization of fats for use and may represent a shift toward lipid metabolism. This increase in circulating free fatty acids is expected near the end of an acute bout of moderate exercise as substrate utilization shifts toward lipids,[77] therefore an increase in circulating free fatty acids during submaximal exercise following training may indicate improved lipid metabolism following training. Thus, the change in select circulating miRNAs may mediate metabolic changes observed during submaximal exercise following exercise training.

This study also examined associations between the baseline miRNA profile and changes in cardiometabolic risk factors. Baseline levels of several miRNAs were positively correlated with change in HDL-C levels and change in apoA-1 levels. As discussed previously, HDL-C has long been used as a biomarker for cardiovascular health,[21] however focus has recently shifted to the functionality of HDL instead of HDL-C levels. ApoA-1 is the primary functional protein of HDL and has been inversely associated with a reduction in risk of atherosclerosis.[27] The potential ability of miRNAs to predict response of both HDL-C and apoA-1 levels to exercise training would allow for the tailoring of exercise prescriptions for individuals with low HDL-C or apoA-1 levels. Strikingly, over 50 baseline miRNAs were negatively correlated with change in

$PETCO_2$ during submaximal exercise at 60% VO_2max. $PETCO_2$ decreases as a result of decreased pulmonary blood flow and decreased cardiac output and is a surrogate measure for tolerance to exercise.[78, 79] $PETCO_2$ is decreased in patients with chronic obstructive pulmonary disease, indicative of diminished exercise tolerance.[79] Therefore an increase in $PETCO_2$ during submaximal exercise following training is indicative of an improved ventilatory tolerance to exercise. The current results suggest that the circulating miRNA profile at baseline may be able to predict the response of $PETCO_2$ during acute exercise to exercise training.

Our study benefitted from the use of a miRNA PCR array that included 84 miRNAs associated with cardiovascular disease and development. However, the relatively small and heterogeneous sample may not be representative of other populations. Additionally, this was the first study to examine the effects of exercise for many of the included miRNAs, thus further research is needed to confirm our findings. Further research is also needed to determine the mechanisms by which exercise alters circulating miRNA expression and the association of changes in the circulating miRNA profile with changes in clinical outcomes.

Exercise training may alter the circulating miRNA profile of select cardiovascular disease related miRNAs and these alterations may have physiologically significant roles. The reduction in select miRNAs with exercise may be clinically significant given the diverse role of these miRNAs in disease development. The current study provides further support for the effects of exercise training on circulating miRNAs such as miR-486 and miR-155 and gives new evidence miR-7 and miRNAs in the let-7 family. Additionally, the current study provides preliminary evidence for the use of circulating miRNAs as

biomarkers for exercise training efficacy and predicting exercise training response, which may be useful in clinical exercise prescription. Future research is needed on the effects of exercise on the untargeted circulating miRNA profile to identify novel miRNAs associated with exercise and confirm the existing findings.

REFERENCES

1. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD and Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13:423-33.
2. Macfarlane LA and Murphy PR. MicroRNA: Biogenesis, Function and Role in Cancer. *Curr Genomics*. 2010;11:537-61.
3. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006;34:D140-4.
4. Hammond SM. An overview of microRNAs. *Adv Drug Deliv Rev*. 2015;87:3-14.
5. Xu T, Liu Q, Yao J, Dai Y, Wang H and Xiao J. Circulating microRNAs in response to exercise. *Scand J Med Sci Sports*. 2015;25:e149-54.
6. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB and Tewari M. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105:10513-8.
7. Feinberg MW and Moore KJ. MicroRNA Regulation of Atherosclerosis. *Circ Res*. 2016;118:703-20.
8. Vogel B, Keller A, Frese KS, Leidinger P, Sedaghat-Hamedani F, Kayvanpour E, Kloos W, Backe C, Thanaraj A, Brefort T, Beier M, Hardt S, Meese E, Katus HA and Meder B. Multivariate miRNA signatures as biomarkers for non-ischaemic systolic heart failure. *Eur Heart J*. 2013;34:2812-22.
9. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, Li Q, Li X, Wang W, Zhang Y, Wang J, Jiang X, Xiang Y, Xu C, Zheng P, Zhang J, Li R, Zhang H, Shang X, Gong T, Ning G, Wang J, Zen K, Zhang J and Zhang CY. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*. 2008;18:997-1006.
10. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S and Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol*. 2012;60:290-9.
11. Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW and Jing Q. Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans. *Eur Heart J*. 2010;31:659-66.
12. Zhang C. MicroRNAs in vascular biology and vascular disease. *J Cardiovasc Transl Res*. 2010;3:235-40.

13. Chan SY, Zhang YY, Hemann C, Mahoney CE, Zweier JL and Loscalzo J. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. *Cell Metab*. 2009;10:273-84.
14. Williams AH, Liu N, van Rooij E and Olson EN. MicroRNA control of muscle development and disease. *Curr Opin Cell Biol*. 2009;21:461-9.
15. Baggish AL, Hale A, Weiner RB, Lewis GD, Systrom D, Wang F, Wang TJ and Chan SY. Dynamic regulation of circulating microRNA during acute exhaustive exercise and sustained aerobic exercise training. *J Physiol*. 2011;589:3983-94.
16. Flowers E, Won GY and Fukuoka Y. MicroRNAs associated with exercise and diet: a systematic review. *Physiol Genomics*. 2015;47:1-11.
17. Sapp RM, Shill DD, Roth SM and Hagberg JM. Circulating microRNAs in acute and chronic exercise: more than mere biomarkers. *J Appl Physiol (1985)*. 2017;122:702-717.
18. Aoi W, Ichikawa H, Mune K, Tanimura Y, Mizushima K, Naito Y and Yoshikawa T. Muscle-enriched microRNA miR-486 decreases in circulation in response to exercise in young men. *Front Physiol*. 2013;4:80.
19. Yusuf S, Hawken S, Ounpuu S, Dans T, Avezum A, Lanas F, McQueen M, Budaj A, Pais P, Varigos J, Lisheng L and Investigators IS. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet*. 2004;364:937-52.
20. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, de Ferranti SD, Floyd J, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Mackey RH, Matsushita K, Mozaffarian D, Mussolino ME, Nasir K, Neumar RW, Palaniappan L, Pandey DK, Thiagarajan RR, Reeves MJ, Ritchey M, Rodriguez CJ, Roth GA, Rosamond WD, Sasson C, Towfighi A, Tsao CW, Turner MB, Virani SS, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P, American Heart Association Statistics C and Stroke Statistics S. Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation*. 2017;135:e146-e603.
21. Gofman JW, Young W and Tandy R. Ischemic heart disease, atherosclerosis, and longevity. *Circulation*. 1966;34:679-97.
22. Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR, Jr., Bangdiwala S and Tyroler HA. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*. 1989;79:8-15.
23. Toth PP, Barter PJ, Rosenson RS, Boden WE, Chapman MJ, Cuchel M, D'Agostino RB, Sr., Davidson MH, Davidson WS, Heinecke JW, Karas RH, Kontush A, Krauss RM, Miller M and Rader DJ. High-density lipoproteins: a consensus statement from the National Lipid Association. *J Clin Lipidol*. 2013;7:484-525.
24. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, Thompson A, Wood AM, Lewington S, Sattar N, Packard CJ, Collins R, Thompson SG and Danesh J. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA*. 2009;302:1993-2000.
25. Assmann G, Schulte H, von Eckardstein A and Huang Y. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. *Atherosclerosis*. 1996;124 Suppl:S11-20.

26. Sharrett AR, Ballantyne CM, Coady SA, Heiss G, Sorlie PD, Catellier D, Patsch W and Atherosclerosis Risk in Communities Study G. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: The Atherosclerosis Risk in Communities (ARIC) Study. *Circulation*. 2001;104:1108-13.
27. Rubin EM, Krauss RM, Spangler EA, Verstuyft JG and Clift SM. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature*. 1991;353:265-7.
28. Plump AS, Scott CJ and Breslow JL. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A*. 1994;91:9607-11.
29. Stone NJ, Robinson JG, Lichtenstein AH, Bairey Merz CN, Blum CB, Eckel RH, Goldberg AC, Gordon D, Levy D, Lloyd-Jones DM, McBride P, Schwartz JS, Shero ST, Smith SC, Jr., Watson K, Wilson PW and American College of Cardiology/American Heart Association Task Force on Practice G. 2013 ACC/AHA guideline on the treatment of blood cholesterol to reduce atherosclerotic cardiovascular risk in adults: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2014;63:2889-934.
30. Keene D, Price C, Shun-Shin MJ and Francis DP. Effect on cardiovascular risk of high density lipoprotein targeted drug treatments niacin, fibrates, and CETP inhibitors: meta-analysis of randomised controlled trials including 117,411 patients. *BMJ*. 2014;349:g4379.
31. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Holm H, Ding EL, Johnson T, Schunkert H, Samani NJ, Clarke R, Hopewell JC, Thompson JF, Li M, Thorleifsson G, Newton-Cheh C, Musunuru K, Pirruccello JP, Saleheen D, Chen L, Stewart A, Schillert A, Thorsteinsdottir U, Thorgeirsson G, Anand S, Engert JC, Morgan T, Spertus J, Stoll M, Berger K, Martinelli N, Girelli D, McKeown PP, Patterson CC, Epstein SE, Devaney J, Burnett MS, Mooser V, Ripatti S, Surakka I, Nieminen MS, Sinisalo J, Lokki ML, Perola M, Havulinna A, de Faire U, Gigante B, Ingelsson E, Zeller T, Wild P, de Bakker PI, Klungel OH, Maitland-van der Zee AH, Peters BJ, de Boer A, Grobbee DE, Kamphuisen PW, Deneer VH, Elbers CC, Onland-Moret NC, Hofker MH, Wijmenga C, Verschuren WM, Boer JM, van der Schouw YT, Rasheed A, Frossard P, Demissie S, Willer C, Do R, Ordovas JM, Abecasis GR, Boehnke M, Mohlke KL, Daly MJ, Guiducci C, Burtt NP, Surti A, Gonzalez E, Purcell S, Gabriel S, Marrugat J, Peden J, Erdmann J, Diemert P, Willenborg C, Konig IR, Fischer M, Hengstenberg C, Ziegler A, Buysschaert I, Lambrechts D, Van de Werf F, Fox KA, El Mokhtari NE, Rubin D, Schrezenmeir J, Schreiber S, Schafer A, Danesh J, Blankenberg S, Roberts R, McPherson R, Watkins H, Hall AS, Overvad K, Rimm E, Boerwinkle E, Tybjaerg-Hansen A, Cupples LA, Reilly MP, Melander O, Mannucci PM, Ardissino D, Siscovick D, Elosua R, Stefansson K, O'Donnell CJ, Salomaa V, Rader DJ, Peltonen L, Schwartz SM, Altshuler D and Kathiresan S. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet*. 2012;380:572-80.
32. Vergeer M, Holleboom AG, Kastelein JJ and Kuivenhoven JA. The HDL hypothesis: does high-density lipoprotein protect from atherosclerosis? *J Lipid Res*. 2010;51:2058-73.

33. Cockerill GW, Rye K-A, Gamble JR, Vadas MA and Barter PJ. High-Density Lipoproteins Inhibit Cytokine-Induced Expression of Endothelial Cell Adhesion Molecules. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 1995;15:1987-1994.
34. Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST and Fogelman AM. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. *J Lipid Res*. 2001;42:1308-17.
35. Yuhanna IS, Zhu Y, Cox BE, Hahner LD, Osborne-Lawrence S, Lu P, Marcel YL, Anderson RG, Mendelsohn ME, Hobbs HH and Shaul PW. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat Med*. 2001;7:853-7.
36. Nofer JR, van der Giet M, Tolle M, Wolinska I, von Wnuck Lipinski K, Baba HA, Tietge UJ, Godecke A, Ishii I, Kleuser B, Schafers M, Fobker M, Zidek W, Assmann G, Chun J and Levkau B. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor S1P3. *J Clin Invest*. 2004;113:569-81.
37. Rader DJ, Alexander ET, Weibel GL, Billheimer J and Rothblat GH. The role of reverse cholesterol transport in animals and humans and relationship to atherosclerosis. *J Lipid Res*. 2009;50 Suppl:S189-94.
38. Khera AV, Demler OV, Adelman SJ, Collins HL, Glynn RJ, Ridker PM, Rader DJ and Mora S. Cholesterol Efflux Capacity, High-Density Lipoprotein Particle Number, and Incident Cardiovascular Events: An Analysis From the JUPITER Trial (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin). *Circulation*. 2017;135:2494-2504.
39. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, Neeland IJ, Yuhanna IS, Rader DR, de Lemos JA and Shaul PW. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med*. 2014;371:2383-93.
40. Saleheen D, Scott R, Javad S, Zhao W, Rodrigues A, Picataggi A, Lukmanova D, Mucksavage ML, Luben R, Billheimer J, Kastelein JJ, Boekholdt SM, Khaw KT, Wareham N and Rader DJ. Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study. *Lancet Diabetes Endocrinol*. 2015;3:507-13.
41. Ritsch A, Scharnagl H and Marz W. HDL cholesterol efflux capacity and cardiovascular events. *N Engl J Med*. 2015;372:1870-1.
42. Barter PJ, Nicholls S, Rye K-A, Anantharamaiah GM, Navab M and Fogelman AM. Antiinflammatory Properties of HDL. *Circulation Research*. 2004;95:764-772.
43. Luc G, Arveiler D, Evans A, Amouyel P, Ferrieres J, Bard JM, Elkhalil L, Fruchart JC, Ducimetiere P and Group PS. Circulating soluble adhesion molecules ICAM-1 and VCAM-1 and incident coronary heart disease: the PRIME Study. *Atherosclerosis*. 2003;170:169-76.
44. Jude EB, Douglas JT, Anderson SG, Young MJ and Boulton AJ. Circulating cellular adhesion molecules ICAM-1, VCAM-1, P- and E-selectin in the prediction of cardiovascular disease in diabetes mellitus. *Eur J Intern Med*. 2002;13:185-189.
45. Wagner J, Riwanto M, Besler C, Knau A, Fichtlscherer S, Roxe T, Zeiher AM, Landmesser U and Dimmeler S. Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs. *Arterioscler Thromb Vasc Biol*. 2013;33:1392-400.

46. Choteau SA, Cuesta Torres LF, Barraclough JY, Elder AMM, Martinez GJ, Chen Fan WY, Shrestha S, Ong KL, Barter PJ, Celermajer DS, Rye KA, Patel S and Tabet F. Transcoronary gradients of HDL-associated MicroRNAs in unstable coronary artery disease. *Int J Cardiol*. 2018;253:138-144.
47. Tabet F, Vickers KC, Cuesta Torres LF, Wiese CB, Shoucri BM, Lambert G, Catherinet C, Prado-Lourenco L, Levin MG, Thacker S, Sethupathy P, Barter PJ, Remaley AT and Rye KA. HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. *Nat Commun*. 2014;5:3292.
48. Eckel RH, Jakicic JM, Ard JD, de Jesus JM, Houston Miller N, Hubbard VS, Lee IM, Lichtenstein AH, Loria CM, Millen BE, Nonas CA, Sacks FM, Smith SC, Jr., Svetkey LP, Wadden TA, Yanovski SZ and American College of Cardiology/American Heart Association Task Force on Practice G. 2013 AHA/ACC guideline on lifestyle management to reduce cardiovascular risk: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *J Am Coll Cardiol*. 2014;63:2960-84.
49. Stone NJ, Robinson JG, Lichtenstein AH, Goff DC, Jr., Lloyd-Jones DM, Smith SC, Jr., Blum C, Schwartz JS and Panel AACG. Treatment of blood cholesterol to reduce atherosclerotic cardiovascular disease risk in adults: synopsis of the 2013 American College of Cardiology/American Heart Association cholesterol guideline. *Ann Intern Med*. 2014;160:339-43.
50. Durstine JL, Grandjean PW, Davis PG, Ferguson MA, Alderson NL and DuBose KD. Blood Lipid and Lipoprotein Adaptations to Exercise: A Quantitative Analysis. *Sports Medicine*. 2001;31:1033-1062.
51. Couillard C, Despres JP, Lamarche B, Bergeron J, Gagnon J, Leon AS, Rao DC, Skinner JS, Wilmore JH and Bouchard C. Effects of endurance exercise training on plasma HDL cholesterol levels depend on levels of triglycerides: evidence from men of the Health, Risk Factors, Exercise Training and Genetics (HERITAGE) Family Study. *Arterioscler Thromb Vasc Biol*. 2001;21:1226-32.
52. Khan AA, Mundra PA, Straznicky NE, Nestel PJ, Wong G, Tan R, Huynh K, Ng TW, Mellett NA, Weir JM, Barlow CK, Alshehry ZH, Lambert GW, Kingwell BA and Meikle PJ. Weight Loss and Exercise Alter the High-Density Lipoprotein Lipidome and Improve High-Density Lipoprotein Functionality in Metabolic Syndrome. *Arterioscler Thromb Vasc Biol*. 2018;38:438-447.
53. Sarzynski MA, Ruiz-Ramie JJ, Barber JL, Slentz CA, Apolzan JW, McGarrah RW, Harris MN, Church TS, Borja MS, He Y, Oda MN, Martin CK, Kraus WE and Rohatgi A. Effects of Increasing Exercise Intensity and Dose on Multiple Measures of HDL (High-Density Lipoprotein) Function. *Arterioscler Thromb Vasc Biol*. 2018;38:943-952.
54. Albaghdadi MS, Wang Z, Gao Y, Mutharasan RK and Wilkins J. High-Density Lipoprotein Subfractions and Cholesterol Efflux Capacity Are Not Affected by Supervised Exercise but Are Associated with Baseline Interleukin-6 in Patients with Peripheral Artery Disease. *Front Cardiovasc Med*. 2017;4:9.
55. Koba S, Ayaori M, Uto-Kondo H, Furuyama F, Yokota Y, Tsunoda F, Shoji M, Ikewaki K and Kobayashi Y. Beneficial Effects of Exercise-Based Cardiac Rehabilitation on High-Density Lipoprotein-Mediated Cholesterol Efflux Capacity in Patients with Acute Coronary Syndrome. *J Atheroscler Thromb*. 2016;23:865-77.

56. Roberts CK, Ng C, Hama S, Eliseo AJ and Barnard RJ. Effect of a short-term diet and exercise intervention on inflammatory/anti-inflammatory properties of HDL in overweight/obese men with cardiovascular risk factors. *J Appl Physiol (1985).* 2006;101:1727-32.
57. Ribeiro IC, Iborra RT, Neves MQ, Lottenberg SA, Charf AM, Nunes VS, Negrao CE, Nakandakare ER, Quintao EC and Passarelli M. HDL atheroprotection by aerobic exercise training in type 2 diabetes mellitus. *Med Sci Sports Exerc*. 2008;40:779-86.
58. Nielsen S, Akerstrom T, Rinnov A, Yfanti C, Scheele C, Pedersen BK and Laye MJ. The miRNA plasma signature in response to acute aerobic exercise and endurance training. *PLoS One*. 2014;9:e87308.
59. Silva GJJ, Bye A, El Azzouzi H and Wisloff U. MicroRNAs as Important Regulators of Exercise Adaptation. *Prog Cardiovasc Dis*. 2017;60:130-151.
60. Uhlemann M, Mobius-Winkler S, Fikenzer S, Adam J, Redlich M, Mohlenkamp S, Hilberg T, Schuler GC and Adams V. Circulating microRNA-126 increases after different forms of endurance exercise in healthy adults. *Eur J Prev Cardiol*. 2014;21:484-91.
61. Bouchard C, Leon AS, Rao DC, Skinner JS, Wilmore JH and Gagnon J. The HERITAGE family study. Aims, design, and measurement protocol. *Med Sci Sports Exerc*. 1995;27:721-9.
62. Jeyarajah EJ, Cromwell WC and Otvos JD. Lipoprotein particle analysis by nuclear magnetic resonance spectroscopy. *Clin Lab Med*. 2006;26:847-+.
63. Gulyaeva LF and Kushlinskiy NE. Regulatory mechanisms of microRNA expression. *J Transl Med*. 2016;14:143.
64. Mittelbrunn M, Gutierrez-Vazquez C, Villarroya-Beltri C, Gonzalez S, Sanchez-Cabo F, Gonzalez MA, Bernad A and Sanchez-Madrid F. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun.* 2011;2:282.
65. Small EM, O'Rourke JR, Moresi V, Sutherland LB, McAnally J, Gerard RD, Richardson JA and Olson EN. Regulation of PI3-kinase/Akt signaling by muscle-enriched microRNA-486. *Proc Natl Acad Sci U S A.* 2010;107:4218-23.
66. Ross R, Dagnone D, Jones PJ, Smith H, Paddags A, Hudson R and Janssen I. Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men. A randomized, controlled trial. *Ann Intern Med*. 2000;133:92-103.
67. Jakicic JM, Marcus BH, Gallagher KI, Napolitano M and Lang W. Effect of exercise duration and intensity on weight loss in overweight, sedentary women: a randomized trial. *JAMA*. 2003;290:1323-30.
68. Wu XD, Zeng K, Liu WL, Gao YG, Gong CS, Zhang CX and Chen YQ. Effect of aerobic exercise on miRNA-TLR4 signaling in atherosclerosis. *Int J Sports Med*. 2014;35:344-50.
69. Shi L and Fleming I. One miR level of control: microRNA-155 directly regulates endothelial nitric oxide synthase mRNA and protein levels. *Hypertension*. 2012;60:1381-2.
70. Sun HX, Zeng DY, Li RT, Pang RP, Yang H, Hu YL, Zhang Q, Jiang Y, Huang LY, Tang YB, Yan GJ and Zhou JG. Essential role of microRNA-155 in regulating

endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. *Hypertension*. 2012;60:1407-14.
71. Heymans S, Corsten MF, Verhesen W, Carai P, van Leeuwen RE, Custers K, Peters T, Hazebroek M, Stoger L, Wijnands E, Janssen BJ, Creemers EE, Pinto YM, Grimm D, Schurmann N, Vigorito E, Thum T, Stassen F, Yin X, Mayr M, de Windt LJ, Lutgens E, Wouters K, de Winther MP, Zacchigna S, Giacca M, van Bilsen M, Papageorgiou AP and Schroen B. Macrophage microRNA-155 promotes cardiac hypertrophy and failure. *Circulation*. 2013;128:1420-32.
72. Long G, Wang F, Li H, Yin Z, Sandip C, Lou Y, Wang Y, Chen C and Wang DW. Circulating miR-30a, miR-126 and let-7b as biomarker for ischemic stroke in humans. *BMC Neurol*. 2013;13:178.
73. Lin Z, Ge J, Wang Z, Ren J, Wang X, Xiong H, Gao J, Zhang Y and Zhang Q. Let-7e modulates the inflammatory response in vascular endothelial cells through ceRNA crosstalk. *Sci Rep*. 2017;7:42498.
74. Teng GG, Wang WH, Dai Y, Wang SJ, Chu YX and Li J. Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. *PLoS One*. 2013;8:e56709.
75. Niculescu LS, Simionescu N, Sanda GM, Carnuta MG, Stancu CS, Popescu AC, Popescu MR, Vlad A, Dimulescu DR, Simionescu M and Sima AV. MiR-486 and miR-92a Identified in Circulating HDL Discriminate between Stable and Vulnerable Coronary Artery Disease Patients. *PLoS One*. 2015;10:e0140958.
76. Coggan AR, Swanson SC, Mendenhall LA, Habash DL and Kien CL. Effect of endurance training on hepatic glycogenolysis and gluconeogenesis during prolonged exercise in men. *Am J Physiol*. 1995;268:E375-83.
77. Friedberg SJ, Sher PB, Bogdonoff MD and Estes EH, Jr. The Dynamics of Plasma Free Fatty Acid Metabolism during Exercise. *J Lipid Res*. 1963;4:34-8.
78. Stickland MK, Butcher SJ, Marciniuk DD and Bhutani M. Assessing exercise limitation using cardiopulmonary exercise testing. *Pulm Med*. 2012;2012:824091.
79. Thirapatarapong W, Armstrong HF, Thomashow BM and Bartels MN. Differences in gas exchange between severities of chronic obstructive pulmonary disease. *Respir Physiol Neurobiol*. 2013;186:81-6.