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Circulating MicroRNA Following High Intensity Interval Cycling With and Without Post-Exercise Nutrient Consumption

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Table of Contents

List of Tables

List of Figures

Abstract

Introduction: MicroRNA (miRNA) are small, non-coding RNA that act post-transcriptionally to regulate gene expression. miRNA levels are modulated by acute aerobic exercise, yet little is known about how miRNA levels may change in response to high-intensity interval exercise. Further, almost nothing is known about the impact of post-exercise nutrition (carbohydrate and/or protein) on miRNA levels. Thus, the purpose of this study is to examine the effects of high-intensity interval cycling and different post-exercise nutrients on ci-miRNA levels. **Methods:** Nine recreationally active males (age 21.9 ± 2.0yrs; VO_{2max} 49.6 ± 4.0mL/kg/min) competed three trials, each including identical exercise protocols. Protocol involved two Wingate tests separated by four sets of high-intensity (3 minutes @ 90% W_{max} separated by 1 minute @ 50% W_{max}) intervals, along with warm-up and cooldown. Finger stick and venous blood samples were collected pre- and up to four hours post-exercise. Additionally, a different nutrition treatment (i.e. carbohydrate, carbohydrate + protein, or control) was administered immediately post-exercise. Serum samples were analyzed for content of twelve target miRNA (miR-1, -21, -126, -133a, -146a, -150, -206, -210, -221, -222, -486, and -499). miRNA levels were expressed as fold changes relative to baseline of 1 and paired-samples t-tests and *post hoc* two-way, repeated measures ANOVAs were used to detect changes in miRNA levels across chosen timepoints and treatments. **Results:** miR-210 and miR-486 were unaffected by exercise at any timepoints and all remaining targets were either unchanged or upregulated immediately post-exercise. Most targets (except miR-1) were returned to

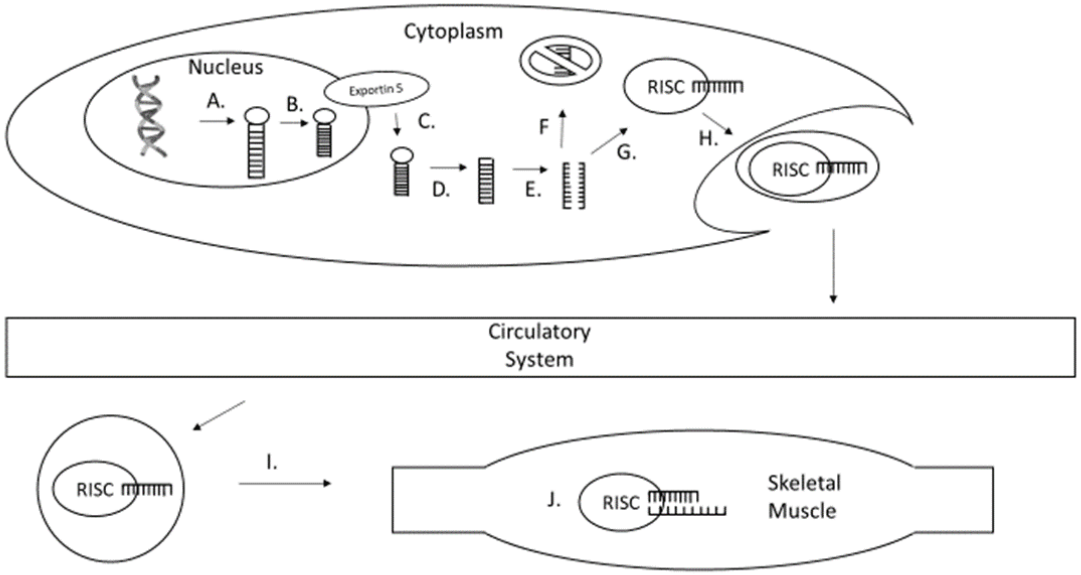
baseline at four-hours post-exercise. Nutrition only affected miR-150, downregulating it at one-hour post-exercise. *Post hoc* analysis revealed a main effect for time for all targets immediately post-exercise. Further, a main effect for time was observed one-hour post-exercise for miR-1 and miR-210 and at four hours pos-exercise for miR-210 and mIR-146a. **Conclusion:** High-intensity cycling impacted miRNA implicated in skeletal and cardiac phenotype, angiogenesis, and inflammation, though post-exercise nutrition was inconsequential except for miR-150. It is currently unknown the extent to which intracellular miRNA activity may be reflected in circulation, thus further work is needed to study how nutrition may influence miRNA response to exercise.

Chapter 1

Introduction

Epigenetics is a field of study focused on how lifestyle and environmental factors can alter gene expression. The role that microRNA (miRNA) has in modulating gene expression is of growing interest. miRNA are small (˜22 nucleotides in length), noncoding RNA that degrade specific mRNA (i.e. post-transcriptional regulation) as a strategy to decrease the probability of protein-specific translation. miRNA are encoded in the genome and transcribed in the nucleus by RNA polymerase II to produce a primary RNA transcript (pri-miRNA) (1). A pri-miRNA typically consists of a stem, approximately 33-35 base pairs in length, and a terminal loop structure (2). Upon cleavage by a complex consisting of the Drosha enzyme (3) and the Di George Syndrome critical region 8 protein (DGCR8) (4), the pri-miRNA is converted into a pre-miRNA, which is exported into the cytoplasm of the cell via the Exportin 5 protein (5). The pre-miRNA is further cleaved by the Dicer enzyme, producing a mi-RNA duplex. The duplex is split, at which point the mature miRNA strand binds with an argonaute protein (6) to form the RNA-induced silencing complex (RISC). This RISC assembly is able to modulate protein translation by binding to target messenger RNA (mRNA) and inducing degradation or silencing (1) (Figure 1).

Figure 1A. miRNA Biogenesis



(A) miRNA is transcribed from coding gene in the nucleus by RNA polymerase II, producing pri-miRNA. (B) pri-miRNA gets cleaved by Drosha/Di George Syndrome critical region gene 8 (DGCR8) complex, producing pre-miRNA. (C) pre-miRNA exits the nucleus into the cytoplasm via Exportin-5 protein. (D) pre-miRNA is cleaved by Dicer protein, producing miRNA/miRNA duplex. (E) Duplex splits, with one half being discarded (F), and the other half being bound to its corresponding argonaute protein (ARG) to form the miRNA-induced silencing complex (RISC) (G). (H) RISC enters into circulation, via passive leak or active release in extracellular vesicles. (I) RISC is brought into neighboring cell, where it can bind to target mRNA and regulate protein translation (J).

Initially discovered in 1993 in *C. elegans* (7), there are now 2,588 documented miRNA found in humans (8). While originating within the cell, miRNA have recently been found in the circulation, as a result of passive leak through the cell membrane or active secretion in extracellular vesicles (9–11), expanding their potential for use as mechanisms of intercellular communication (12, 13), or biomarkers of cellular injury or disease (14, 15). A single miRNA can regulate multiple sets of different mRNA (16), suggesting the potential for miRNA to have widespread influence on mRNA translation. Importantly, miRNA expression is cell-type specific (17) . McCarthy detailed a set of muscle-specific miRNA named ‘myomiRs’ , consisting of miR-1, miR-133a, miR-206, miR-208a, miR-208b, miR-486, and miR-499, which are believed to have a major role in skeletal muscle fiber type (18). Moreover, earlier work by the McCarthy lab demonstrated downregulation of miR-208b and miR-499 activity during skeletal muscle atrophy, which was associated with depression of beta-myosin heavy chain (β-MHC) protein (19). Furthermore, knockout studies in rodent models suggest these miRNA may also impact muscle mass via downregulation of myostatin expression, allowing for increased muscle growth (20). Finally, Yamakuchi described a group of miRNA involved in vascular development and homeostasis, as well as endothelial cell senescence (21). These studies suggest that groups of miRNA interact to regulate or modulate a multitude of physiological processes.

While acute exercise perturbs homeostasis, repeated exposure to acute exercise (i.e. training) can evoke favorable changes in phenotype such as increased muscle mass, aerobic capacity, and capillary growth (22–24). A better understanding of the molecular events that elicit adaptations to exercise has the potential to instruct interventions to improve health, performance, and quality of life. Because of the relatively new discovery of miRNA, and its presence in circulation, very little is understood about the behavior of miRNA following exercise and how miRNA may therefore affect adaptations. The influence of acute exercise on circulating miRNA (ci-miRNA) levels was detailed by Banzet et al. who reported that plasma miRNA were elevated in the early hours following 30 minutes of walking (25). Not only did an acute session of walking alter miRNA levels but also the response was specific to the type of walking. Downhill walking, which accentuates eccentric muscle contractions, elicited an increase in muscle-enriched myomiRs (miR-1, 133a, 133b, and 208b) that have been shown to influence myocyte differentiation (26). Alternatively, uphill walking led to an increase in miR-181b and miR-214. The idea that miRNA levels are mediated in a contraction type-specific or mode-specific manner was extended by Uhlemann et al. miRNA levels following different doses of aerobic exercise (incremental max test vs. 4 hours of cycling vs. marathon footrace) were compared to the response to eccentric resistance exercise. ci-miR-126 was elevated in each dose of aerobic exercise and miR-133 was elevated following the marathon race and the eccentric resistance exercise (27). This study helped demonstrate both mode-specific and dose-related miRNA patterns following exercise. Additional

evidence for the effect of exercise dose on ci-miRNA response was found following different doses of acute aerobic exercise (10-km or marathon). Distinctly different inflammatory ci-miRNA patterns were observed between both distances, suggesting the potential use of ci-miRNA as biomarkers for inflammation (28).

In addition to the transient changes in miRNA following different types of acute exercise, several studies have revealed that basal and post-exercise ci-miRNA is influenced by training status. Specifically, there were higher levels of cardiovascular and inflammatory miRNA in endurance-trained athletes compared to resistance-trained athletes (29). A subsequent investigation demonstrated that training can modulate miRNA, as miR-486 was lower following acute cycling and 4 weeks of cycle training (30). More specifically, 6 weeks of endurance training downregulated miRNA that target mRNA known to code for various transcription factors, which in turn led to the upregulation of these transcription factors (31). Importantly, miRNA may serve as useful indicators of the potential for individuals to respond to training programs. Davidsen et al. observed differential changes in several miRNA following a 12-week resistance training program. miRNA-451 was upregulated in “high responders” to the training while miR-26a, miR-29a, and miR-378 were downregulated in “low responders” and unchanged in the higher responders (32). Taken together these data may provide mechanistic insight into long-term training adaptations.

High intensity interval training (HIIT) is an increasingly popular form of aerobic exercise partly due to its time efficient nature. HIIT is characterized by short intervals (30 seconds to 4 minutes) of high-intensity work coupled with lower intensity recovery intervals. HIIT has been shown to improve aerobic capacity and body composition (33, 34) at magnitudes similar to continuous aerobic training, but with much less training volume (35, 36). Gillen and Gibala have proposed that as little as 3 HIIT sessions per week, with <10 min of high intensity per session, may produce favorable adaptations in aerobic capacity, muscle oxidative capacity, and exercise tolerance (37). HIIT has also been shown to greatly improve skeletal muscle fat and carbohydrate oxidative capability (38), even in as few as seven sessions over two weeks (39). Additionally, mitochondrial content and biogenesis have been demonstrated to increase following HIIT, providing physiological evidence for improved peripheral oxygen extraction (40, 41). Finally, HIIT has been shown to improve insulin sensitivity (34) and promote muscular hypertrophy (42), demonstrating a wide array of health benefits.

Despite the recent attention to HIIT, little is known about the impact of high-intensity exercise on post-exercise miRNA response, as only a few studies have used a high-intensity exercise mode to profile miRNA levels. While low-volume sprint interval cycling (2 x 30 sec at maximum effort, separated by 4 min active recovery) in healthy, adult males has been shown to influence ci-miRNA profiles (43), similar changes in miRNA were observed between high-intensity

interval running (7 x 4 min at 85 - 95% HRmax) and distance-matched, continuous running (44). Additionally, further research is needed to illustrate the time-course of the miRNA response following high-intensity exercise, as neither of the previously mentioned studies collected blood samples past the immediate post-exercise period. This is a major limitation due to the fact that ci-miRNA levels have been shown to fluctuate in the hours following acute exercise (45).

Post-exercise carbohydrate (CHO) and protein (PRO) intake can impact the molecular response to exercise (46). Specifically, acute post-exercise protein consumption has been shown to improve net protein balance following both resistance and endurance exercise (47, 48). Furthermore, the inclusion of protein in a post-exercise carbohydrate supplement has a positive effect on skeletal muscle transcriptome response to endurance exercise (49). While studies have repeatedly demonstrated the benefits of post-exercise nutrient consumption, virtually nothing is known about the effects of different nutritional interventions post-exercise on miRNA levels. Drummond et al. observed an increase in several muscle-enriched miRNA (miR-1, miR-23a, miR-208b, and miR-499, as well as pri-miR-206) following the consumption of 10 g of essential amino acids (EAA), but without any exercise intervention (50). Perhaps more applicable to the proposed study, Camera et al. demonstrated increase of several miRNA implicated in exercise adaptation (increase of miR-23a, miR-23b, miR-133b, miR-181, and miR-378 with a decrease of miR-494) with PRO ingestion following concurrent resistance and endurance exercise. Interestingly, miR-494 decreased

post-exercise in the placebo treatment, but following protein consumption post-exercise, remained unchanged. The target genes for several of these miRNA are involved in translation initiation and muscle protein synthesis, suggesting a regulatory potential for miRNA in the formation of proteins vital to exercise adaptation (51). More importantly, the aforementioned data provide preliminary evidence that nutrient intake can impact miRNA. To our knowledge, nothing is known about how carbohydrate influences the post-exercise miRNA response. The goal of this study is to characterize the effects of HIIT and different post-exercise nutrition composition on circulating miRNA.

Aims and Hypotheses

Aim 1: To profile ci-miRNA implicated in skeletal muscle or cardiovascular function, angiogenesis, or inflammation immediately, 1 hour, and 4 hours following high intensity interval cycling.

Hypothesis 1: High-intensity interval cycling will increase muscle, cardiovascular, and inflammatory miRNA in circulation post-exercise, and levels will remain elevated in the four hours following exercise.

Aim 2: To investigate the potential impact of different post-exercise nutrition treatments on miRNA expression observed in circulation.

Hypothesis 2: Post-exercise carbohydrate + protein will decrease ci-miRNA profiles for muscle-enriched miRNA compared to non-nutritive placebo, but will have no influence on other target miRNA. Additionally, carbohydrate will have minimal impact on ci-miRNA profiles.

Significance

miRNA are now understood to influence a wide array of physiological processes (13, 18, 52). While research has examined how miRNA levels are altered in disease states (14, 53), less research has been focused on the effect of exercise on modulation of miRNA levels. While exercise has shown a dose (27) and mode-dependent (28) influence on miRNA found in circulation, few studies have specifically investigated the effects of HIIT on miRNA.

High-intensity interval training (HIIT) is an increasingly popular form of exercise due to its high efficiency. HIIT has been shown to produce similar improvements in aerobic capacity as continuous aerobic exercise (33), but with significantly less training volume required (35). Furthermore, HIIT has been shown to improve body composition and insulin sensitivity (33, 34), suggesting its efficacy for both healthy and clinical populations. While low-volume HIIT has been shown to alter miRNA levels (43), no miRNA measurements were taken beyond the immediate post-exercise period, leaving the time-course for miRNA return to baseline following HIIT largely unexplored.

While studies have examined the impact of protein consumption, with and without exercise, on miRNA patterns (50, 51), the impact of carbohydrate on miRNA has yet to be explored. This study provides a unique opportunity to examine the effects of carbohydrate, separate from and in conjunction with protein. Since post-exercise nutrition is understood to influence the transcriptome (49), a better understanding of the impact of nutrient composition on miRNA

following exercise may better elucidate the role of nutrition on gene expression following exercise.

Chapter 2

Methodology

Subject Recruitment

Nine recreationally active, healthy males will be recruited via word of mouth, flyers, and email from James Madison University and the Harrisonburg area. All subjects will be nonsmokers and cleared for participation of vigorous exercise, according to ACSM guidelines. Based on ACSM guidelines, people are approved for vigorous exercise if they are without signs, symptoms, or diagnosis of cardiovascular, metabolic, or renal disease (54). All procedures will be approved by the James Madison University Institutional Review Board.

VO_{2max} Testing

Upon obtaining informed consent, height, and weight, subjects will undergo a graded exercise test to determine maximal aerobic capacity (VO_{2max}). Testing will be performed on an electronically braked cycle ergometer (Velotron Inc. Seattle, WA). Subjects will begin the test at a self-selected workload described as “comfortable, but not easy pace for a 1-hour ride”. Workload will increase by 25W every 60 seconds until volitional fatigue or failure to maintain a minimum cadence of 50 rpm. Oxygen uptake will be measured throughout the test via Moxus (Pittsburgh, PA, USA) or Vmax®Encore PFT (Vyaire Medical Inc, Yorba Linda, CA) metabolic cart and the maximum wattage (W_{max}) obtained during the VO_{2max} test will be used to assign work rates for the exercise trial

protocols. Heart rate (HR) and rate of perceived exertion (RPE) will be assessed during the last 15 seconds of each stage.

Exercise Trials

Exercise Protocols

Exercise procedures are detailed below (Figure 1). Subjects will perform three experimental trials consisting of identical exercise protocols with three different post-exercise nutrient conditions. Following a 5-minute warm up at a workload of 50 watts, subjects will complete an interval cycling protocol consisting of a 30 second, maximal effort sprint (Wingate test), 4 sets of 3 minutes cycling at 90% W_{max} (with 1 minute of cycling at 50% W_{max} between intervals), and a second Wingate test. Subjects will recover for 3 minutes between the first Wingate test and the intervals, and 1 minute between the intervals and the second Wingate test. Following post-exercise blood sampling, a 5-minute cooldown at a self-selected workload will be performed. The workload during the cooldown will be recorded and replicated in each subsequent trial. Heart rate will be obtained during the final 30 seconds of each interval (Polar; Lake Success, NY, USA).

Nutrition Treatments

Following exercise, subjects will receive one of the following treatments:

CHO+PRO: 20 oz chocolate milk (Great Value, Dean Foods, Dallas, TX)

CHO: matched for CHO-content in CHO+PRO treatment (approximately 65 g) mixed in 20 oz water (CLIF Bar & Company, Emeryville, CA)

PLA: 20 oz water

The treatments will be consumed within 30 minutes of exercise completion. The amount of time taken to consume the beverage during the initial trial will be replicated across all exercise trials.

Blood Samples

Venous blood samples will be obtained pre-exercise, immediately post-exercise, 1-hour post-exercise, and 4 hours post-exercise. Samples will sit at room temperature for 30-45 minutes to allow clotting. Samples will be centrifuged at 4°C for 15 minutes at 1500 rcf and 500 µL aliquots of serum will be pipetted. The aliquoted serum will be stored at -80°C in the Human Performance Lab at James Madison University for the duration of data collection. Upon completion of data collection, samples will be sent to the University of Maryland for miRNA quantification analysis. The following miRNA will be measured:

Skeletal muscle: 1, 133a, 206, 486, 499

Cardiovascular: 21, 126, 150, 210

Inflammation: 146a, 221, 222

Furthermore, hematocrit will be measured at each sample timepoint via HemoPoint® H2 Hemoglobin Meter (Stanbio Laboratory, Boerne, TX) in order to

mathematically correct for hydration status. In addition to venous blood samples, finger stick blood samples will be obtained pre-exercise and every 30 minutes post-exercise for 4 hours to measure blood glucose levels. Blood glucose levels will be measured via YSI 2900 Series Analyzer (YSI Inc. Yellow Springs, OH).

Standardization Procedures

Subjects will report to the laboratory between 5:30-9am following an overnight fast. They will be instructed to refrain from tobacco or alcohol consumption or exercise for 24 hours pre-trial and refrain from caffeine consumption for 12 hours pre-trial. Subjects will be asked to record dietary intake for 24 hours and physical activity for 72 hours before the trials. They will be given copies of their dietary and physical activity logs and asked to replicate them between trials. Subjects will receive a small, standardized meal 2 hours post-exercise (90 minutes post-treatment beverage).

Statistical Analysis

Changes in ci-miRNA will be reported as fold changes. Magnitude-based inferences about the data will be derived using methods described by Hopkins and colleagues (55, 56). The threshold value for a substantial treatment effect will be defined as 0.2 x within-subject standard deviation, under resting conditions.

Published spreadsheets (57) will be used to determine the likelihood of the true treatment effect (of the population) reaching the substantial change threshold; these percent likelihoods were classified as: <1% almost certainly no

chance, 1-5% = very unlikely, 5-25% = unlikely, 25-75% = possible, 75-95% = likely, 95-99% = very likely, and >99% = almost certain. Clinical inference criteria will be used to classify the effects of condition on performance. Specifically, if the percent chance of the effect reaching the substantial change threshold is <25% and the effect is clear, it will be classified as 'trivial'. If the percent chance of the effect reaching the substantial change threshold for benefit exceeds 25% but the chance for harm is >0.5% the effect will be classified as unclear. An exception to the 0.5% chance of harm criterion will be made if the benefit/harm odds ratio is >66, in which case the effect will be interpreted as clear and an inference was assigned.

Separate analyses will be performed on each nutritional intervention (PLA, CHO, CHO + PRO) using the spreadsheet mentioned above (57). The outcomes from each condition will be compared using the same spreadsheet (57). The classification system detailed above will be applied but mechanistic criteria will be used such that if 90% confidence intervals include values that exceed the substantial change threshold for both a positive and negative effect, effects will be classified as unclear (>5% chance of reaching the substantial threshold for both a positive and negative effect).

Chapter 3

Manuscript

Circulating MicroRNA Following High Intensity Interval Cycling With and Without Post-Exercise Nutrient Consumption

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Abstract

Introduction: MicroRNA (miRNA) are small, non-coding RNA that act post-transcriptionally to regulate gene expression. miRNA levels are modulated by acute aerobic exercise, yet little is known about how miRNA levels may change in response to high-intensity interval type exercise. Further, almost nothing is known about the impact of post-exercise nutrition (carbohydrate and/or protein) on miRNA levels. Thus, the purpose of this study is to examine the effects of high-intensity interval cycling and different post-exercise nutrition treatments on ci-miRNA levels. **Methods:** Nine recreationally active males (age 21.9 ± 2.0yrs; VO_{2max} 49.6 ± 4.0mL/kg/min) competed three trials, each consisting of the same exercise protocol. Protocol consisted of two Wingate tests separated by four sets of high-intensity (3 minutes @ 90% W_{max} separated by 1 minute @ 50% W_{max}) along with warm-up and cooldown. Finger stick and venous blood samples were collected pre- and up to four hours post-exercise. Additionally, a different nutrition treatment (i.e. carbohydrate, carbohydrate + protein, or control) was administered immediately post-exercise. Serum samples were analyzed for content of twelve target miRNA (miR-1, -21, -126, -133a, -146a, -150, -206, -210, -221, -222, -486, and -499). miRNA levels were expressed as fold changes relative to baseline of 1 and paired-samples t-tests and *post hoc* two-way, repeated measures ANOVAs were used to detect changes in miRNA levels across chosen timepoints and treatments. **Results:** Two targets (miR-210 and miR-486) were unaffected by exercise at any timepoints and all remaining targets were either unchanged or upregulated immediately post-exercise. Most targets (except miR-1) were returned

to baseline at four-hours post-exercise. Nutrition only affected miR-150, downregulating it at one-hour post-exercise. *Post hoc* analysis revealed a main effect for time for all targets immediately post-exercise. Further, a main effect for time was observed one-hour post-exercise for miR-1 and miR-210 and at four hours pos-exercise for miR-210 and mIR-146a. **Conclusion:** High-intensity cycling impacted miRNA implicated in skeletal and cardiac phenotype, angiogenesis, and inflammation, though post-exercise nutrition was found to be inconsequential for all targets except miR-150. It is currently unknown the extent to which intracellular miRNA activity may be reflected in circulation, thus further work is needed to study how nutrition may influence miRNA response to exercise.

Introduction

Repeated exposure to acute exercise elicits phenotype changes that lead to improvements in health and performance. Post-exercise alterations in the availability of messenger RNA (mRNA) for translation into protein is one of the key mechanisms that facilitates these adaptations. mRNA availability is regulated by both gene transcriptional activity (i.e. gene to mRNA) and alterations to mRNA following transcription. The latter is believed to be predominantly mediated by small (~20-24 nucleotides) non-coding microRNA (miRNA). Upon maturation, miRNA and argonaute proteins (Ago) form RNA-induced silencing complex (RISC) that targets mRNA and, through silencing or degradation, downregulate mRNA translation to protein (1, 6). A single miRNA can have multiple target mRNA, allowing for miRNA to have widespread influence on protein-specific synthesis (16). Not only have certain miRNA been generally associated with certain types of cancer (58), obesity (59), diabetes (60), and cardiovascular disease (61), there is clear evidence that manipulating miRNA levels can directly impact phenotype. Among many other possible examples, the skeletal muscle-specific suite of miRNA (myomiRs) are now believed to play an integral role in skeletal muscle fiber type, as overexpression of miR-499 in the mouse soleus was shown to induce conversion of all fast myofibers to slow myofibers (18). Further, knockout of miR-126, a proangiogenic miRNA, promoted leukocyte adherence to endothelial cells via decreased inhibition of vascular cell adhesion molecule-1 (VCAM-1) (62).

Though miRNA were first discovered intracellularly, miRNA are also present in circulation (ci-miRNA), thereby introducing the possibility that exogenous

miRNA could facilitate intercellular communication and, more practically, providing easier access for the study of miRNA (63). ci-miRNA originate from host tissues and enter circulation packaged in microvesicle exosomes (10), high density lipoproteins (64), or bound to argonaut transport proteins (65). The extent and control of cell specific targeting and miRNA sorting/packaging for export is currently unknown. However, the parent cell miRNA is ultimately transported and taken up by target cells whereby the parent miRNA exerts an endogenous-like miRNA effect (64). Like intracellular miRNA, ci-miRNA are also associated with a variety of diseases (66) and are sensitive to environmental stressors such as exercise (67), altogether indicating that the miRNA response to exercise probably plays a role in adaptations to exercise training.

It is clear that exercise modulates levels of certain miRNA and that these changes are influenced by contraction type (25), exercise mode (27), and dose (28), Of the little that is known about how exercise influences ci-miRNA, virtually all has been documented following traditional aerobic and resistance type exercise. High intensity interval training (HIIT) is an increasingly popular form of exercise. This is due to the combination of its time efficient nature (30-second to 4-minute intervals of high-intensity work coupled with lower intensity recovery intervals) and favorable adaptations (i.e. improved aerobic capacity (37), mitochondria content (40), abdominal fat loss (34), and improved fat oxidation (38)). Despite the recent attention to HIIT, little is known about the impact of high-intensity exercise on the post-exercise miRNA response. There is some evidence that sprint interval cycling and running alter levels of skeletal muscle specific ci-

miRNA (43, 44). However, the targets in these studies were almost all muscle-specific; thus, further research is warranted to examine the effects of high-intensity exercise on miRNA of other tissues.

Despite the fact that post-exercise nutrition can influence recovery and adaptation to exercise, the influence that post-exercise nutrient uptake may have on mi-RNA levels has been largely overlooked. Post-exercise carbohydrate (CHO) and protein (PRO) intake can impact the molecular responses to exercise (46). Acute post-exercise PRO ingestion can improve net protein balance following both resistance (47) and aerobic exercise (48). Further, the addition of PRO to a post-exercise CHO supplement has a positive effect on the skeletal muscle transcriptome response to endurance exercise (49). However, few studies have investigated the impact of different post-exercise nutritional interventions on ci-miRNA levels. The consumption of 10 g of essential amino acids, absent any exercise, increases several muscle-enriched miRNA in muscle biopsy samples (50). Likewise, PRO ingestion following concurrent resistance and exercise enhances several miRNA implicated in exercise adaptations (51). While PRO consumption can alter miRNA activity, to our knowledge, nothing is known about how PRO influences miRNA in combination with high-intensity exercise, particularly ci-miRNA. Additionally, nothing is known about how CHO impacts post-exercise ci-miRNA. Therefore, the purpose of this study is to explore the effects of high-intensity interval cycling and different post-exercise nutrition composition on circulating profiles for miRNA implicated in skeletal and cardiac muscle plasticity, cardiovascular function, angiogenesis, and inflammation.

Methods

Subject Information

Fifteen recreationally active, healthy adult males were recruited from James Madison University and the surrounding area. Six subjects withdrew from the study due to scheduling difficulties and other factors not related to the project. Therefore, complete data were obtained on nine subjects. Subject demographics are reported in Table 1. All procedures were approved by the James Madison University Institutional Review Board.

Physical Activity and Diet Standardization Procedures

Subjects reported to the laboratory between 5:30-9:00 am following an overnight fast. To standardize pre-fast dietary intake, all subjects were provided nutrition shakes (Ensure, Abbott Laboratories, Shawnee, KS) equivalent to 20% of daily resting energy expenditure, as estimated via the Harris-Benedict equation (68). They were instructed to refrain from tobacco, alcohol consumption, or exercise for 24 hours pre-trial and refrain from caffeine consumption for 12 hours pre-trial. Subjects recorded dietary intake for 24 hours and physical activity for 72 hours before the trials. They were provided with copies of their dietary and physical activity logs and asked to replicate them between trials. Subjects also consumed a small, standardized meal (440 calories, 3 g fat, 93 g carbohydrate, 10 g protein) 2 hours post-exercise (90 minutes post-treatment beverage).

Exercise Protocol

Subjects performed a graded exercise test to determine maximal aerobic capacity (VO_{2max}). Testing was performed on an electronically-braked cycle ergometer (Velotron Inc. Seattle, WA). Subjects began the test at a self-selected workload described as a “comfortable, but not easy pace for a 1-hour ride”. Workload was increased by 25 W every 60 seconds until volitional fatigue or cycling cadence dropped below 50 rpm. Oxygen uptake was measured throughout the test via Moxus (Pittsburgh, PA, USA) or Vmax®Encore PFT (Vyaire Medical Inc, Yorba Linda, CA) metabolic cart and the maximum wattage (W_{max}) obtained during the VO_{2max} test was used to assign work rates for the exercise protocols. Heart rate (HR) and ratings of perceived exertion (RPE) were assessed during the last 15 seconds of each stage.

Subjects performed three experimental trials consisting of identical exercise protocols with three different post-exercise nutrient conditions (Figure 1). Following a 5-minute warm up at a workload of 50 watts, subjects completed an interval cycling protocol consisting of a 30 second, maximal effort sprint (Wingate test), 4 sets of 3 minutes cycling at 90% W_{max} (with 1 minute of cycling at 50% W_{max} between intervals), and a second Wingate test. Subjects recovered for 3 minutes between the first Wingate test and the intervals, and 1 minute between the intervals and the second Wingate test. Following post-exercise blood sampling, a 5-minute cooldown at a self-selected workload was completed. The workload during the cooldown was recorded following the initial trial and replicated in each subsequent

trial. Heart rate was obtained during the final 30 seconds of each interval (Polar; Lake Success, NY, USA).

Nutrition Treatment

Following exercise, subjects consumed one of the following treatments within 30 minutes following exercise (consumption finish time was repeated across trials) in a randomized, counterbalanced order:

- Carbohydrate + Protein; ~600 ml of Chocolate Milk (Great Value, Dean Foods, Dallas, TX) containing 65g carbohydrate and 20g protein
- Carbohydrate; ~600 ml of water with added chocolate flavored CLIF Bar gel to match the carbohydrate content of the Carbohydrate + Protein treatment (CLIF Bar & Company, Emeryville, CA)
- Control; 600 ml of water

Blood Sample Collection

Venous blood samples were obtained pre-exercise, immediately post-exercise, 1-hour post-exercise, and 4 hours post-exercise. Samples sat at room temperature for 30-45 minutes to allow clotting. They were then centrifuged at 4°C for 15 minutes at 1500 rcf. The aliquoted serum was stored at -80°C until analysis. Furthermore, hematocrit was measured at each sample timepoint via HemoPoint® H2 Hemoglobin Meter (Stanbio Laboratory, Boerne, TX) to mathematically correct for changes in plasma volume. In addition to venous blood samples, finger stick blood samples were obtained pre-exercise and every 30 minutes post-exercise for 4 hours to measure blood glucose levels. Blood glucose levels were measured via

automated glucose/lactate (YSI 2900 Series Analyzer, YSI Inc. Yellow Springs, OH). Plasma volume shifts are reported in Table 4.

miRNA Isolation and Analysis

Serum samples were thawed at room temperature and spun at 16,000 g for 10 minutes at 4ºC. Total RNA was isolated from 50µL serum using the miRNeasy serum/plasma kit (Qiagen, Germantown, MD), with slight changes to the manufacturer's protocol. The change involved using 50 µL serum samples with volumes of Qiazol lysis reagent and chloroform recommended for 200µL serum samples in attempt to improve RNA yield, as was previously reported (69). Specifically, serum was mixed with 20 volumes of Qiazol and 4 volumes of chloroform. A standard amount of synthetic spike-in control miR (C. elegans miR-39) was added to all serum samples during isolation for assessment of recovery and calibration of PCR results. RNA was eluted in 14µL RNAse-free water. Reverse transcription was performed to generate cDNA from 10µL RNA using the miScript II RT Kit (Qiagen, Germantown, MD), and the product was diluted in 200µL water. Real-time quantitative PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) using the miScript SYBR Green PCR Kit (Qiagen, Germantown, MD). Samples were assessed in duplicate using 2.5µL diluted cDNA. The levels of twelve *a priori* chosen miRNA (1, 21, 126, 133a, 146a, 150, 206, 210, 221, 222, 486, 499a) were determined using specific primer assays (Qiagen). A disassociation curve analysis was performed to check for non-specific amplification. Further, those miRNA with CT values greater than 35 were considered undetected and unreliable for analysis. Levels of ci-miRNA are

presented as fold-changes relative to baseline with baseline set equal to 1. Expression values were compared using the $2^{-\Delta\Delta CT}$ method of relative quantification as previously described (70). For each miRNA, ΔCT was calculated as the CT of the miRNA of interest – CT of spike-in control miRNA. The ΔΔCT was determined as the ΔCT for an individual subject/time point –baseline ΔCT from the subject for the respective treatment.

Statistical Analysis

Raw ci-miRNA data were tested for normality via Shapiro-Wilk test. Since no ci-miRNA were normally distributed, raw data were log transformed and re-assessed for normality. Any remaining non-normal data were adjusted for normality as described by Templeton (71). Once normality was established, Two-way, repeated measures ANOVAs were conducted to determine a main effect for time. Comparisons were made between pre- and each post-exercise timepoint (i.e. post, post1hr, post4hr). Further, *post hoc* comparisons were made for changes in ci-miRNA across timepoints and between nutrition treatments using paired-samples t-tests. Glucose values at the post1hr (1-hour post-exercise) timepoint were compared using paired-samples t-test. All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) Version 24 (IBM Corporation, Armonk, New York).

Results

Exercise Stimulus

Heart rate responses and workloads during the exercise trials are reported in Table 2. Wingate power outputs are reported in Table 3.

Circulating miRNA

miRNA levels are expressed as fold changes relative to a baseline of 1 (Figures 3-6). Three target miRNA (miR-133a, miR-206, and miR-499) were undetected in serum, and were excluded for analysis. Further, miR-1 was undetectable at baseline in one subject during the Pro trial, so this subject was excluded from analysis of miR-1. Additionally, miR-150 data for one subject were found to be an outlier (>2 SD) and were excluded from analysis.

Skeletal Muscle miRNA; miR-1 & miR-486:

miR-1 was elevated above baseline at Post and Post-1 hr (main effect for time, $p<0.05$). *Post hoc* analysis revealed that miR-1 was elevated at post- and post-1 ($p<0.05$) with PLA. No nutritional effects were detected.

miR-486 was increased above baseline at Post (main effect for time, $p<0.01$). *Post hoc* analysis revealed that miR-486 was not elevated at any timepoints post-exercise. Additionally, no nutritional effects were observed.

Cardiovascular miRNA; miR-21, miR-126, miR-150, & miR-210:

miR-21 was upregulated at Post (main effect for time, $p<0.01$). *Post hoc* analysis showed that miR-21 was increased at post with PLA ($p<0.05$). No nutritional effects were observed.

miR-126 was elevated at Post (main effect for time, $p<0.01$). *Post hoc* analysis demonstrated that miR-126 was increased at post with PLA and CHO ($p<0.05$). No nutritional effects were observed.

miR-150 was upregulated at Post (main effect for time, $p<0.01$). *Post hoc* analysis showed that miR-150 was increased with CHO ($p<0.05$). Additionally, miR-150 demonstrated a nutritional effect at Post-1hr (main effect for treatment, $p<0.01$). Specifically, *post hoc* analysis revealed that PRO and CHO were decreased compared to PLA ($p<0.05$).

miR-210 was enhanced at Post, Post-1 hr, and Post-4 hr (main effect for time, $p<0.05$). However, *post hoc* analysis revealed no changes from baseline post-exercise. No nutritional effects were observed.

Inflammatory miRNA; miR-146a, miR-221, & miR-222:

miR-146a was elevated at Post and Post-4 hr (main effect for time, $p<0.01$ and $p<0.05$, respectively). *Post hoc* analysis demonstrated that mR-146a was increased with PLA and CHO ($p<0.05$) and was trending toward significance ($p=0.05$) with PRO at post1. No nutritional effects were observed.

miR-221 was upregulated at Post (main effect for time, $p<0.01$). *Post hoc* analysis revealed that miR-221 was elevated at post by PLA ($p<0.05$) and CHO ($p<0.01$). No nutritional effects were observed.

miR-222 was increased at Post (main effect for time, $p<0.01$). *Post hoc* analysis showed that miR-222 was increased at post by all treatments ($p<0.01$). No nutritional effects were observed.

Blood Glucose Measurement

Blood glucose was affected by CHO treatment at one-hour post-exercise, as glucose in the CHO trial was different than in the PLA and PRO trials (Table 4).

Tables & Figures

Table 1. Subject demographics

Age (yrs)	Height (cm)	Weight (kg)	VO_{2max} (mL/kg/min)	Peak Watts (W)
21.9 ± 2.0	177.8 ± 6.8	78.5 ± 10.7	49.6 ± 4.0	300 ± 35.4

All data are expressed as means ± SD

Table 2. Interval workloads and heart rate responses

	PLA		PRO		CHO	
Interval	HR (bpm)	Workload (W)	HR (bpm)	Workload (W)	HR (bpm)	Workload (W)
1	177 ± 11	268 ± 33	177 ± 6	269 ± 31	175 ± 8	268 ± 26
2	180 ± 9	247 ± 33	176 ± 12	251 ± 27	176 ± 8	241 ± 19
3	179 ± 10	217 ± 29	176 ± 9	224 ± 27	173 ± 10	213 ± 29
4	181 ± 10	212 ± 31	176 ± 10	210 ± 27	176 ± 9	212 ± 25

All data are expressed as means ± SD

Table 3. Wingate power outputs

PLA		PRO		CHO	
Wingate 1 (W)	Wingate 2 (W)	Wingate 1 (W)	Wingate 2 (W)	Wingate 1 (W)	Wingate 2 (W)
513 ± 93	458 ± 99	504 ± 82	458 ± 68	499 ± 94	463 ± 91

All data are expressed as means ± SD

Table 4. Plasma Shift post-exercise (% change from baseline). Negative values represent plasma loss relative to baseline

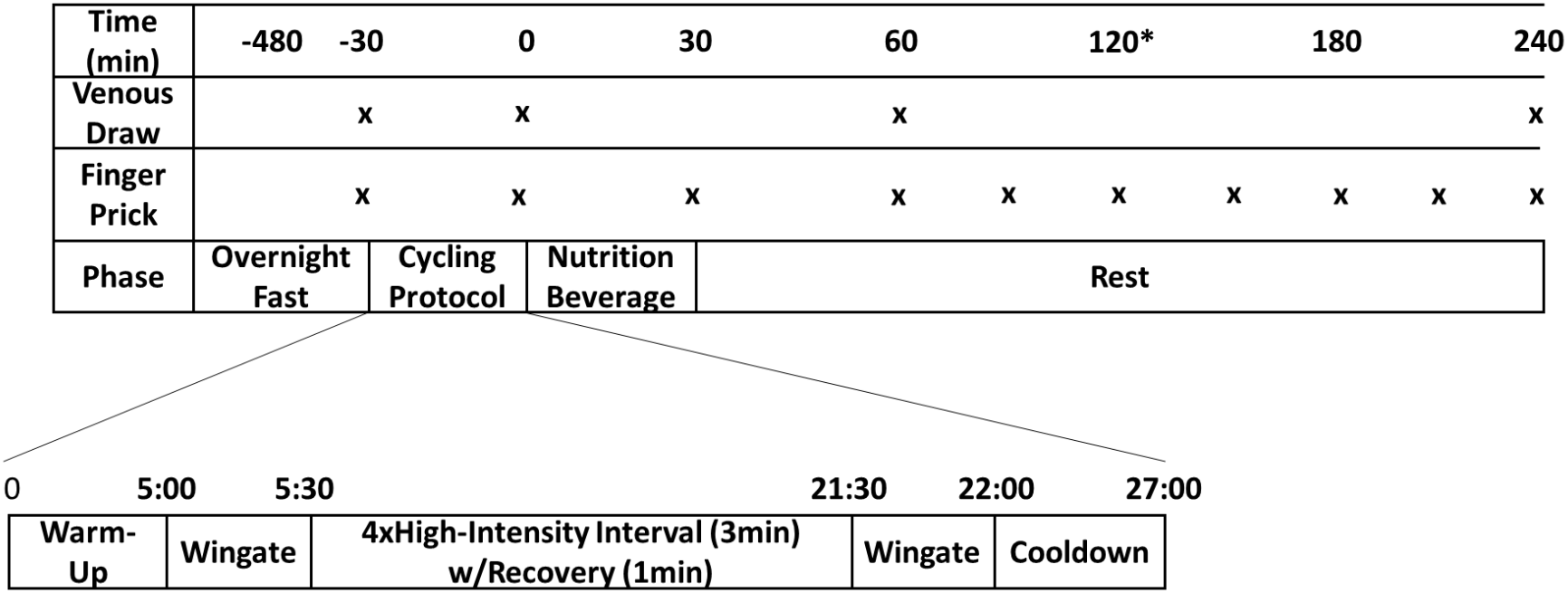
PLA			PRO			CHO		
Hct Post	Hct Post1	HctPost4	Hct Post	Hct Post1	HctPost4	Hct Post	Hct Post1	HctPost4
-13.5 ± 11.0	6.3 ± 8.1	7.2 ± 10.6	-16.1 ± 6.3	2.6 ± 8.3	2.8 ± 5.2	-13.1 ± 8.1	1.9 ± 4.3	-0.4 ± 4.8

Table 5. Blood glucose levels (mg/dL)

	Pre	Post	Post-1 hr	Post-4 hr
PLA	76.6 ± 6.9	101.4 ± 16.0	69.0 ± 3.1*	76.1 ± 5.4
PRO	87.1 ± 37.2	97.6 ± 19.5	69.4 ± 6.6*	76.0 ± 7.5
CHO	83.2 ± 14.7	96.0 ± 16.8	91.6 ± 12.1	75.1 ± 7.2

All data are expressed as means ± SD. *$p<0.01$ vs. CHO

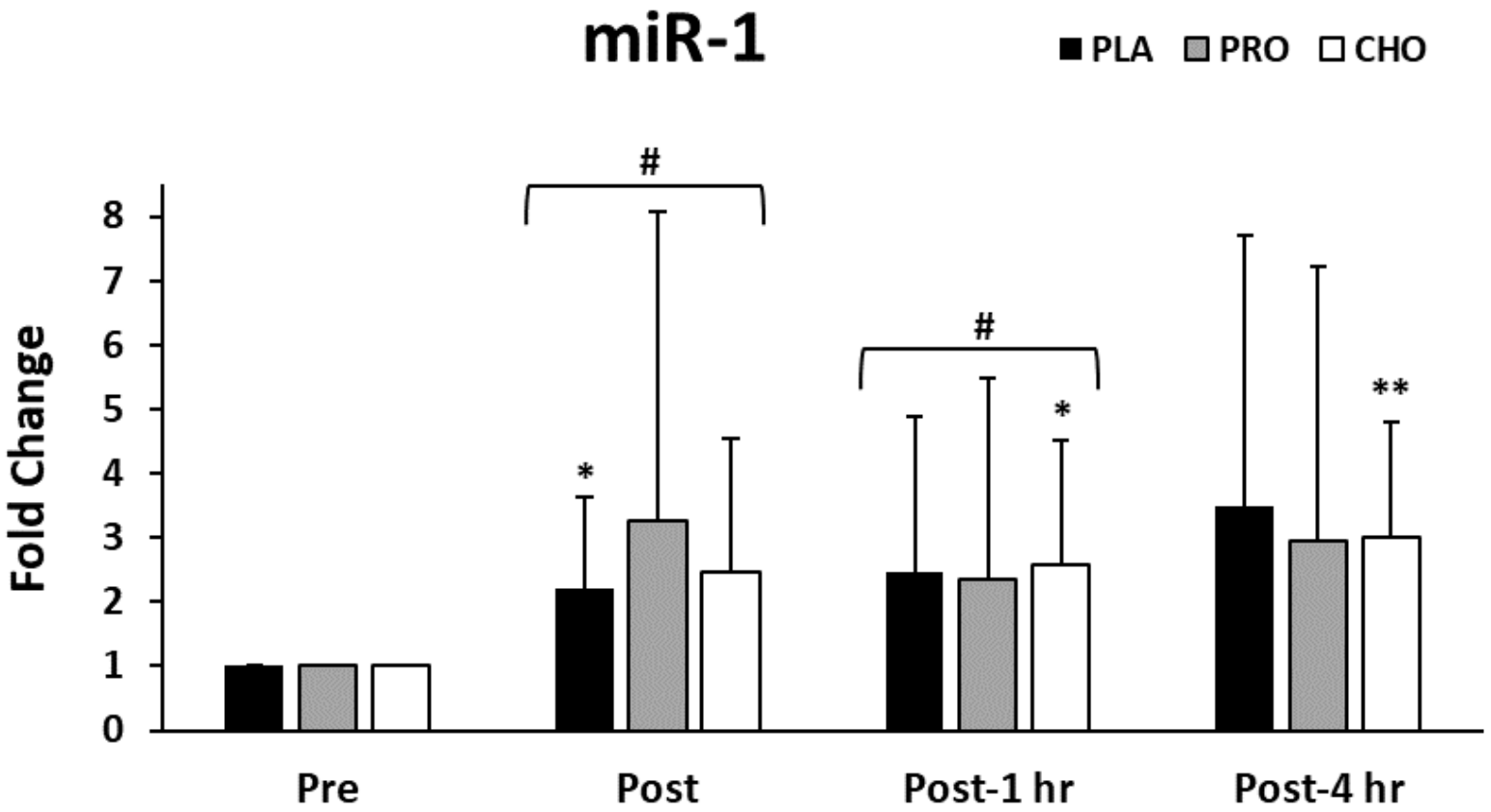
Figure 1. Experimental Trial Design



*** Denotes administration of standardized meal**

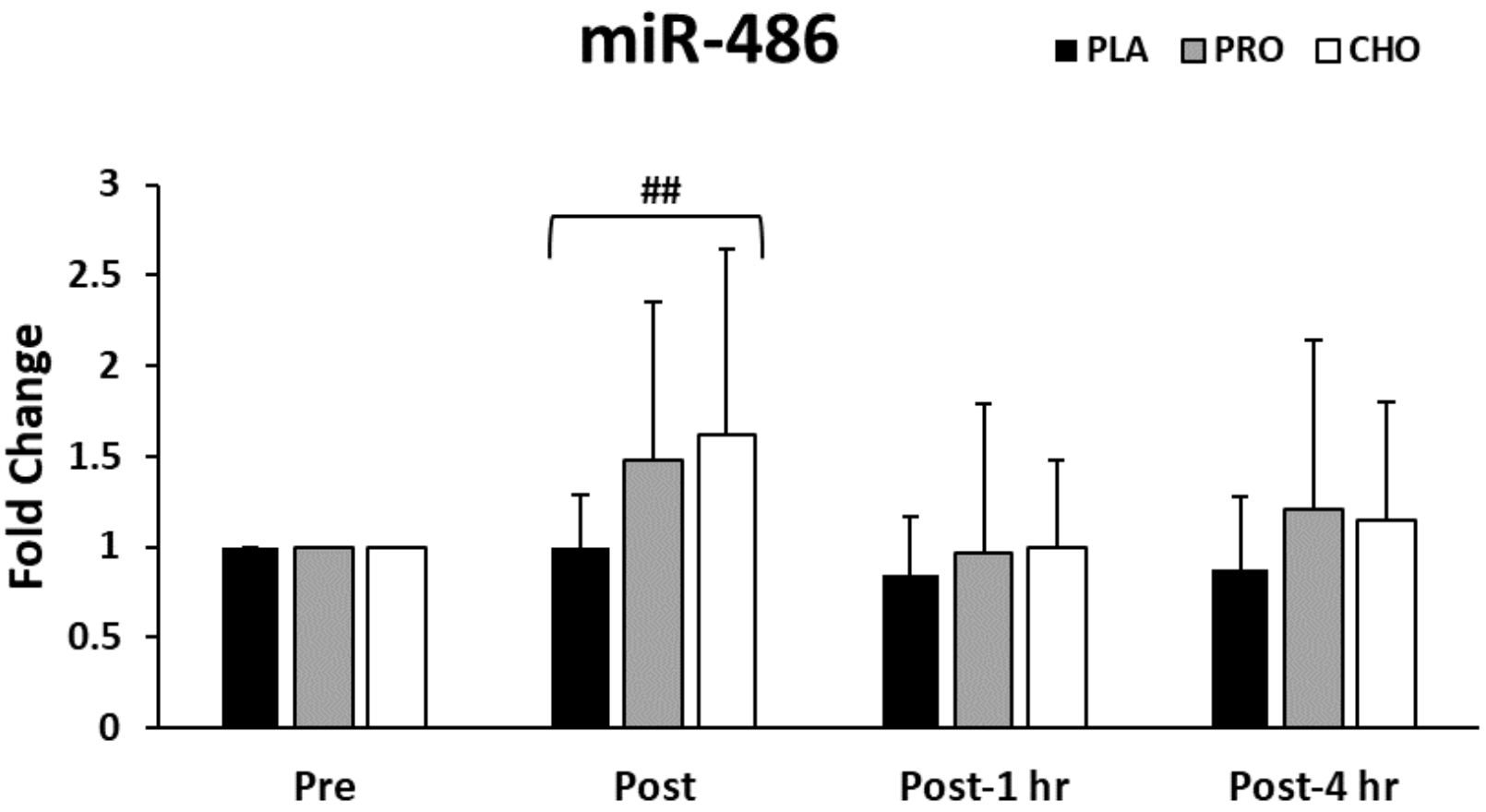
Visual schematic of exercise trial design (upper). Negative time-values denote time preceding the completion of the exercise protocol (lower).

Figure 2A. Muscle-specific miRNA (miR-1)



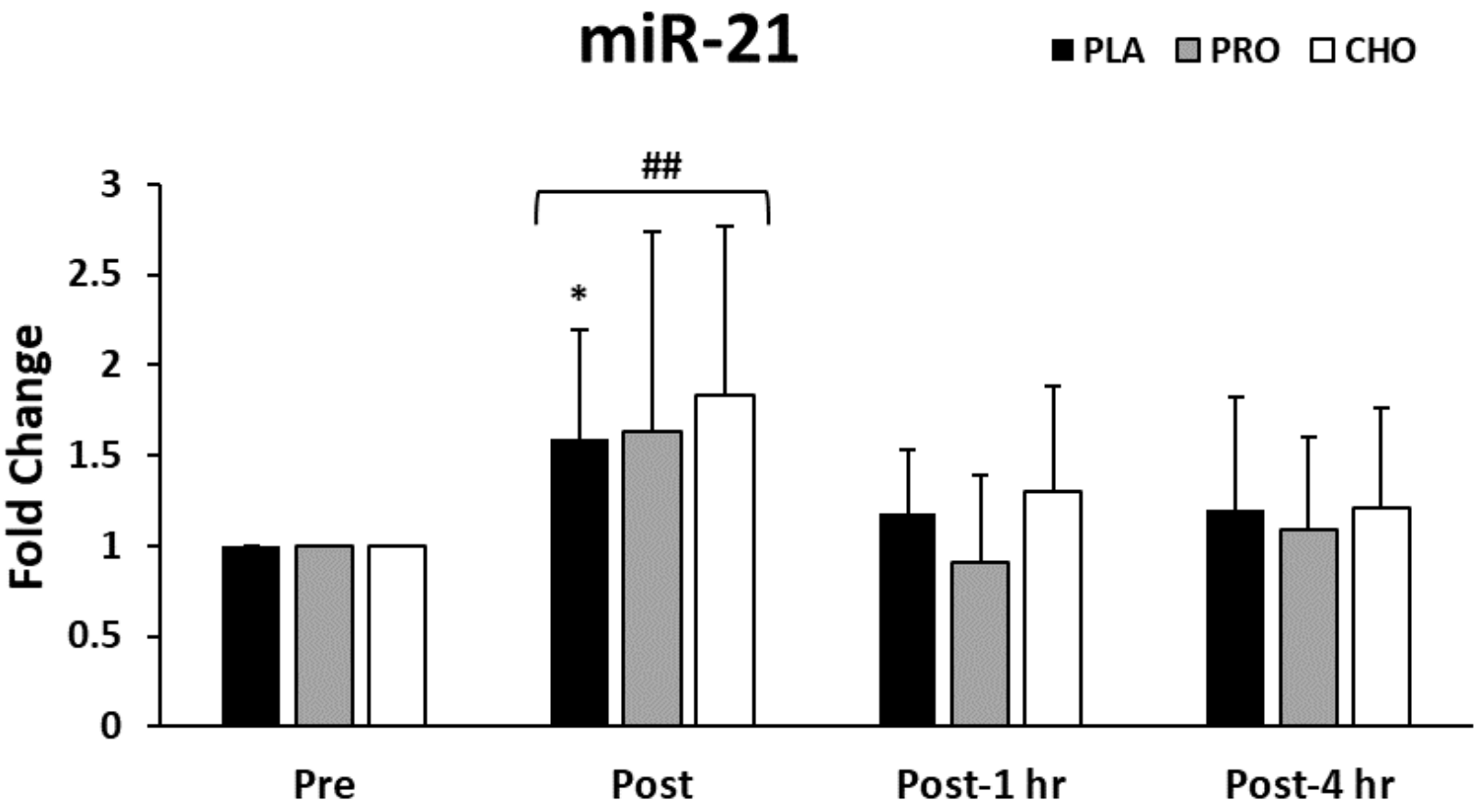
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * p<0.05; ** p<0.01; # p<0.05 vs. baseline

Figure 2B. Muscle-specific miRNA (miR-486)



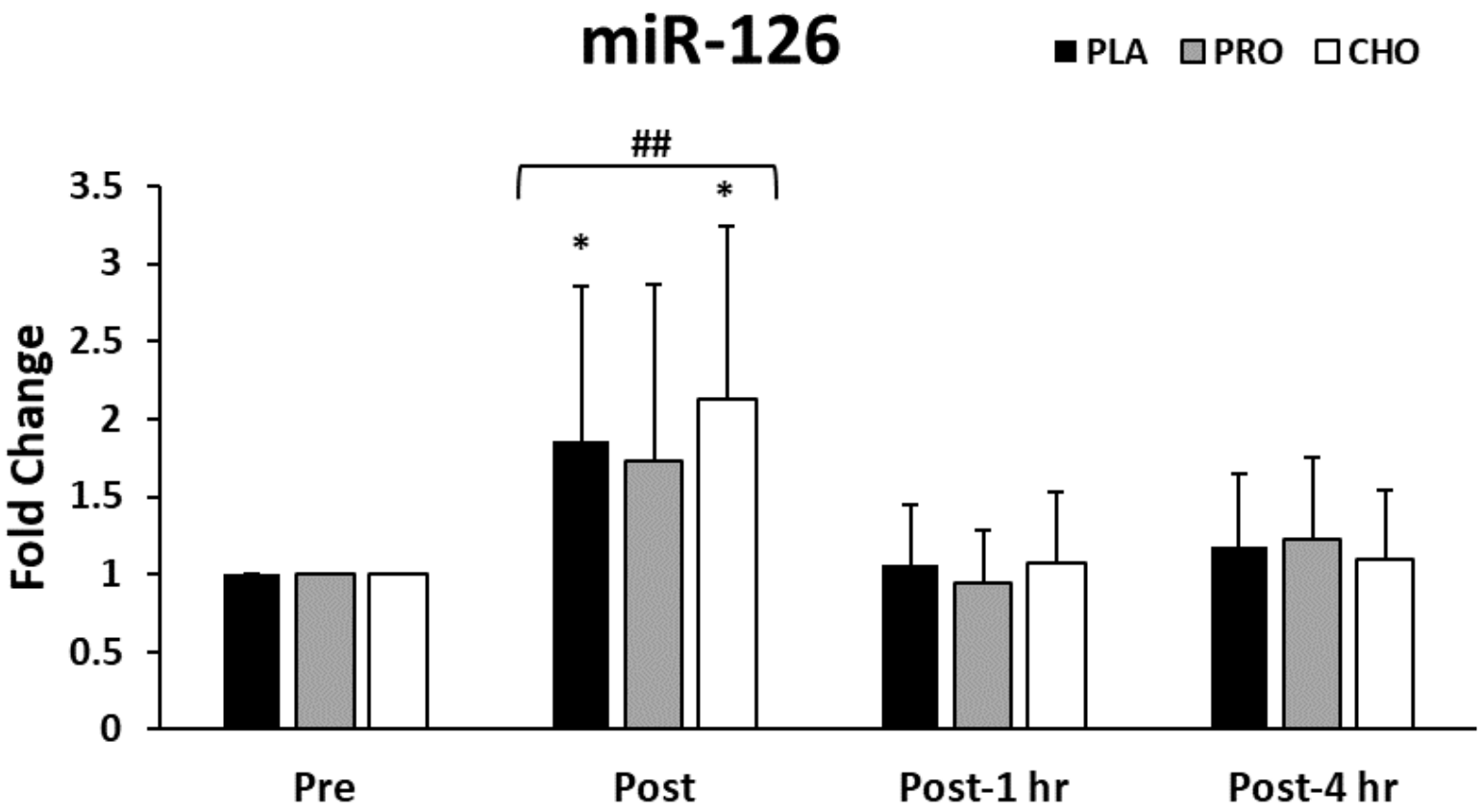
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). ## $p<0.01$ vs. baseline

Figure 3A. Cardiovascular miRNA (miR-21)



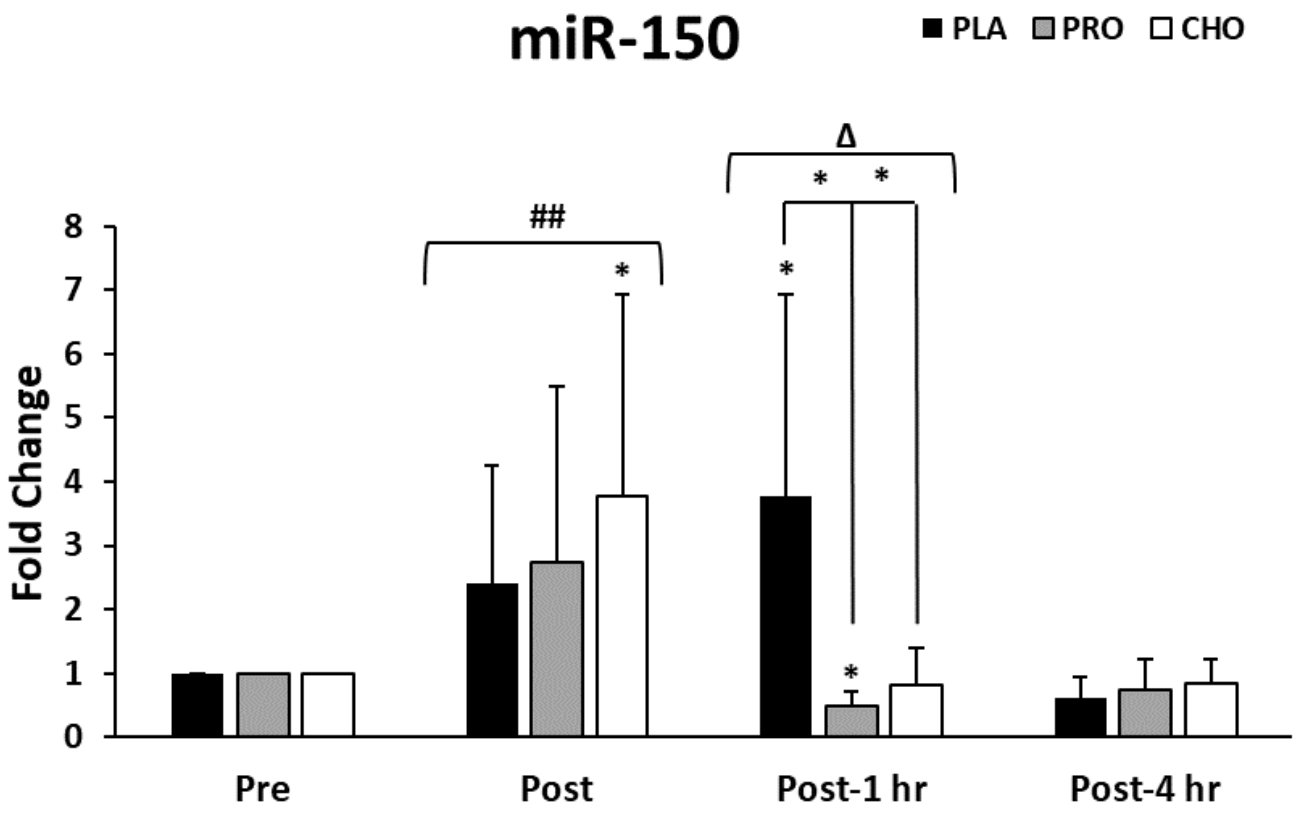
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * p<0.05; ## p<0.01 vs. baseline

Figure 3B. Cardiovascular miRNA (miR-126)



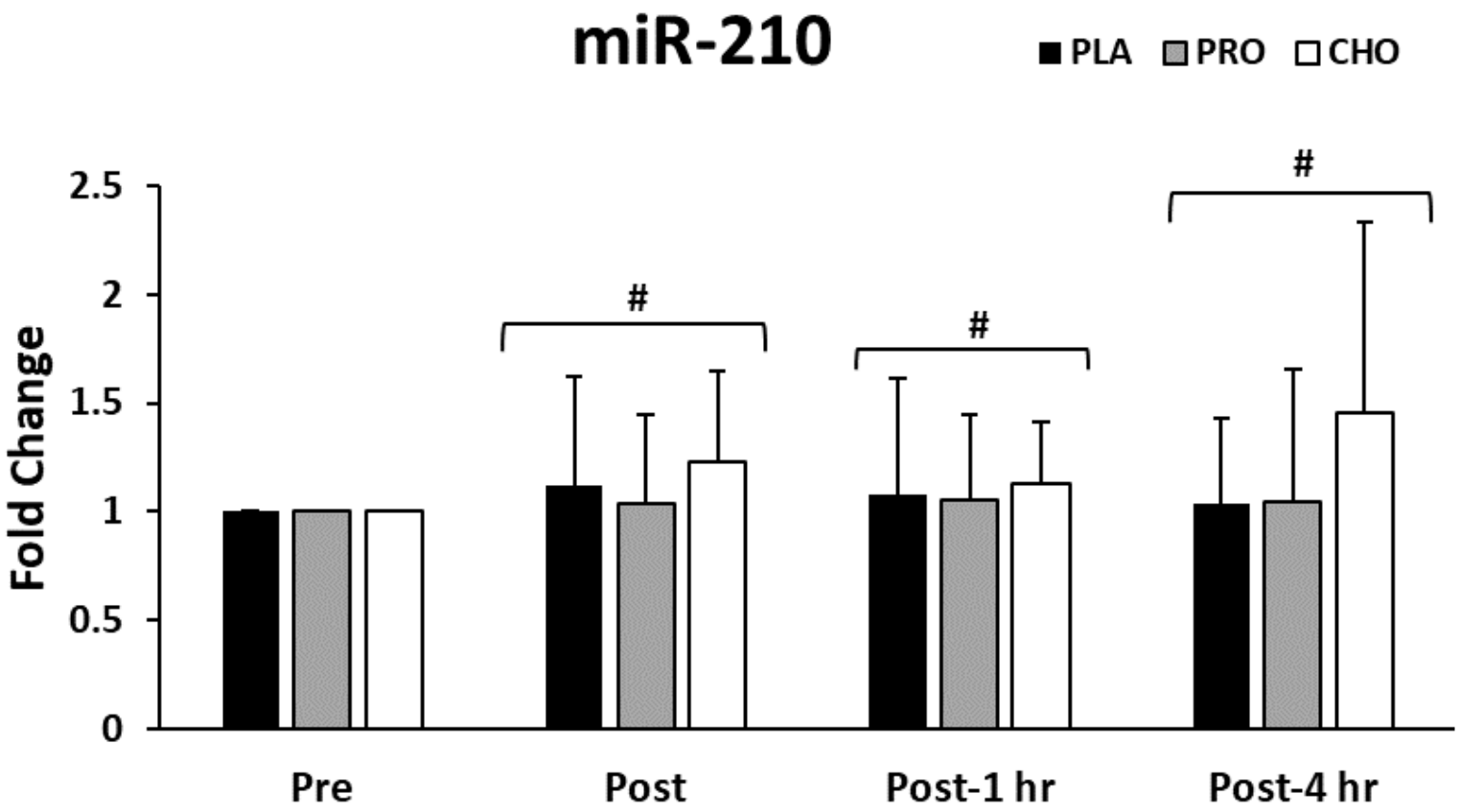
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * p<0.05; ## p<0.01 vs. baseline

Figure 3C. Cardiovascular miRNA (miR-150)



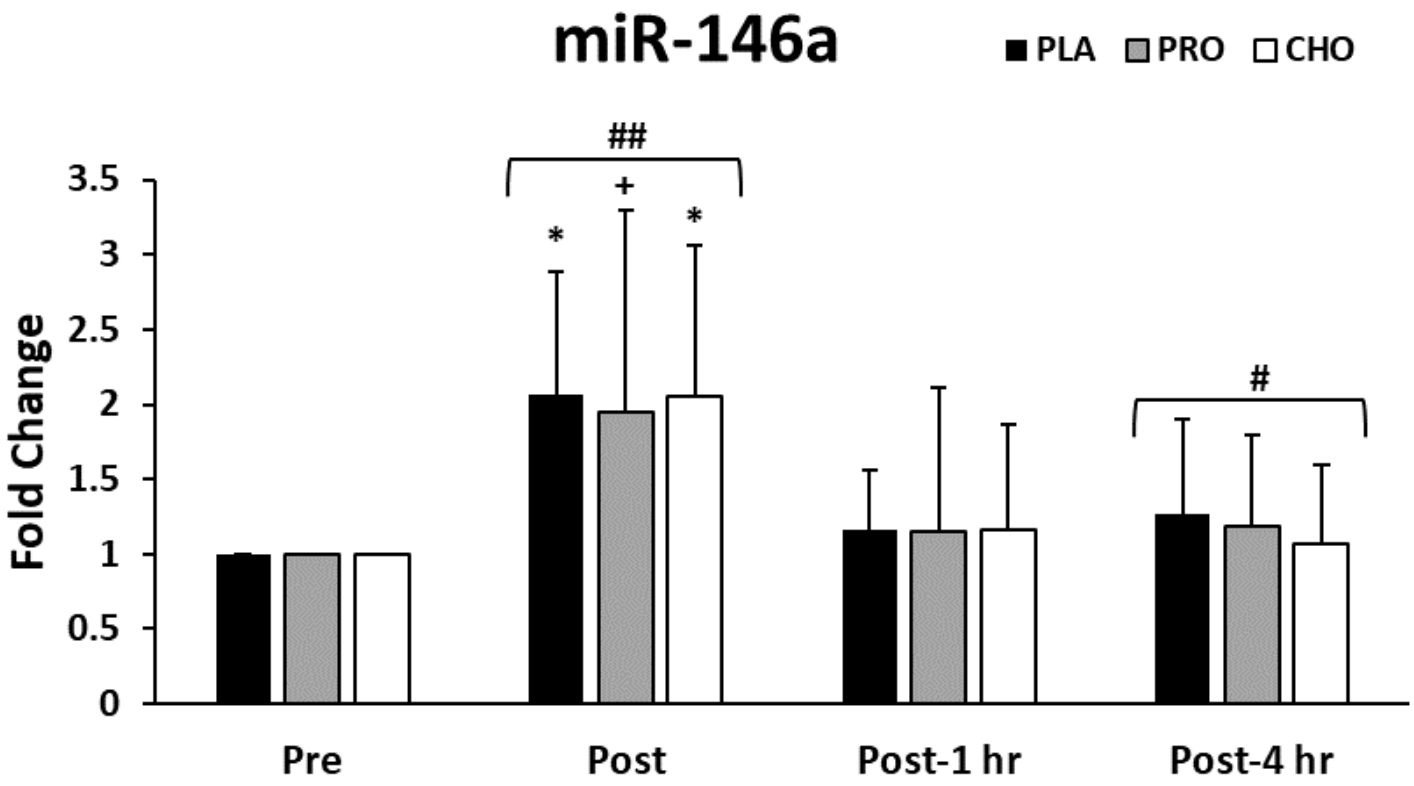
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * $p<0.05$; ## $p<0.01$ vs. baseline; Δ $p<0.01$ PLA vs. PRO/CHO

Figure 3D. Levels of circulating angiogenesis-associated miRNA (miR-210)



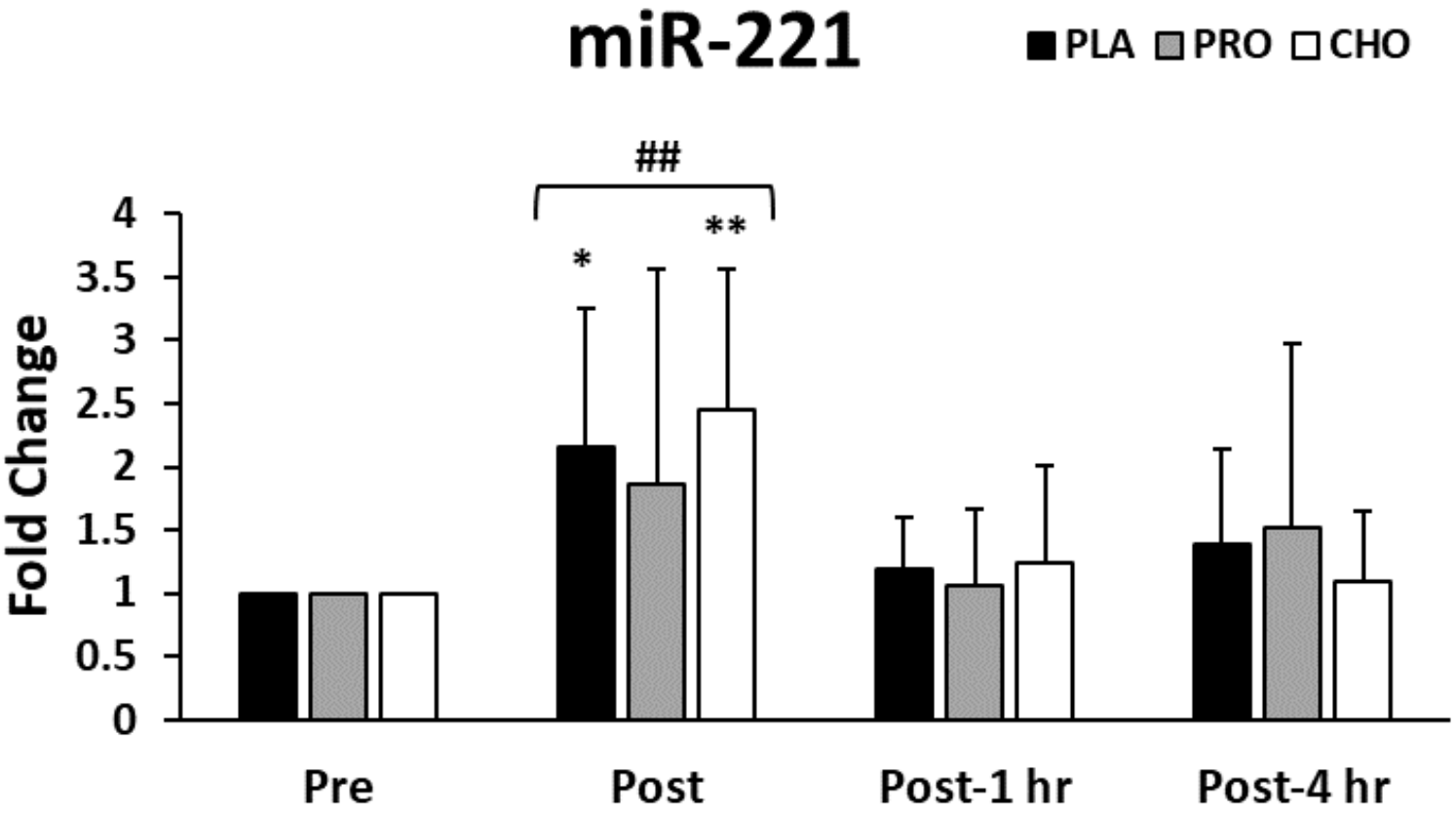
Circulating miRNA levels expressed as fold changes relative to baseline (Pre); # $p<0.05$ vs. baseline

Figure 4A. Inflammatory miRNA (miR-146a)



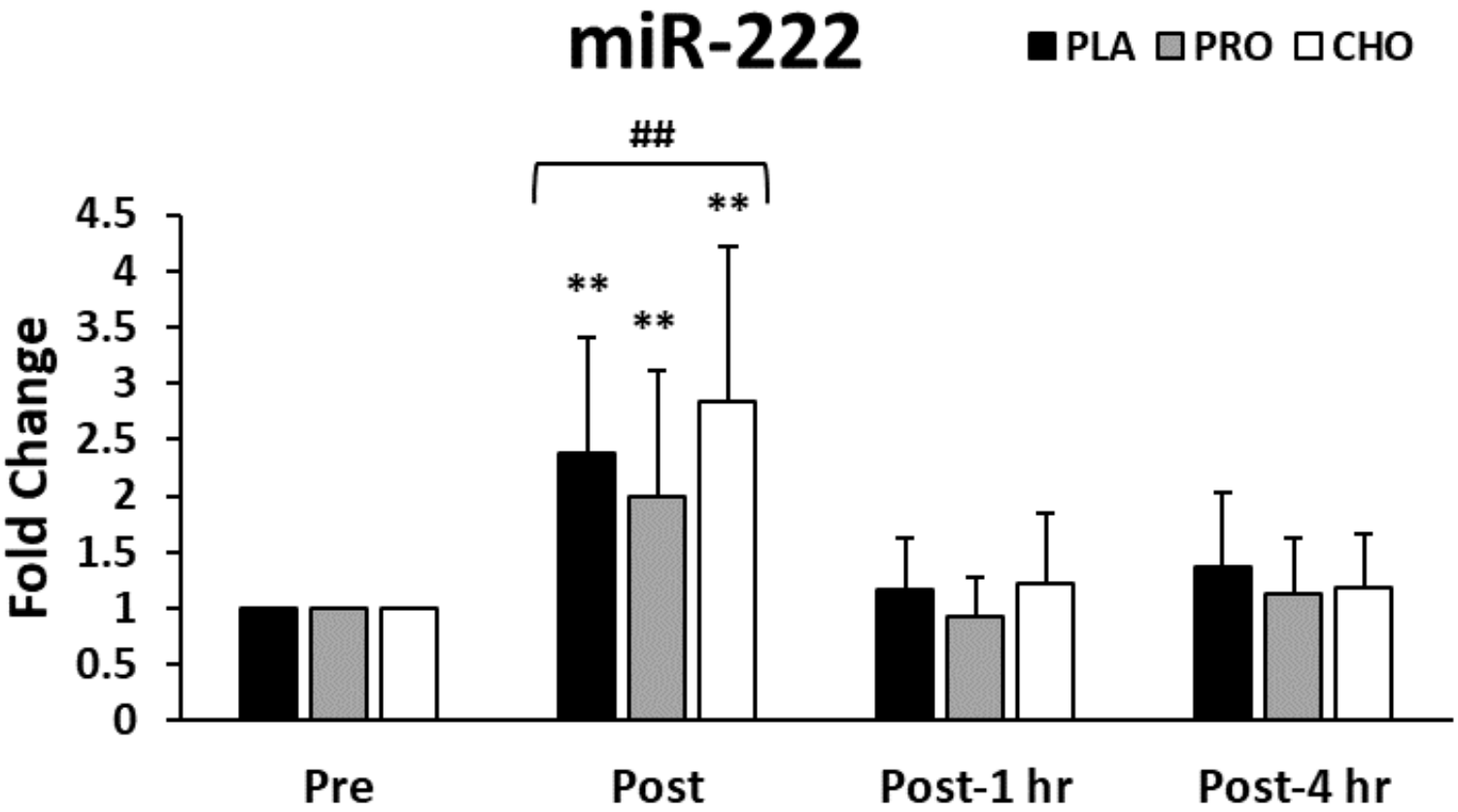
Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * $p<0.05$; + $p=0.05$; # $p<0.05$ vs. baseline; ## $p<0.01$ vs. baseline

Figure 4B. Inflammatory miRNA (miR-221)



Circulating miRNA levels expressed as fold changes relative to baseline (Pre). * p<0.05; ** p<0.01; ## p<0.01 vs. baseline

Figure 4C. Inflammatory miRNA (miR-222)



Circulating miRNA levels expressed as fold changes relative to baseline (Pre). ** p<0.001; ## p<0.01 vs. baseline

Discussion

The primary objectives of this study were to examine the effects of high-intensity cycling, along with different post-exercise nutritional treatments, on circulating levels of select miRNA. The primary finding was that high-intensity cycling altered all of the measured miRNA targets. This is in line with recent evidence that high-intensity exercise can affect ci-miRNA implicated in muscle and cardiovascular function (43, 72). This can now be extended to include miR-146a, miR-150, miR-210, miR-221, and miR-222. Surprisingly, post-exercise nutrition had no effect on ci-miRNA levels, with the exception of miR-150. This seems to suggest that phenoytype adaptations to high intensity training mediated by these target miRNA may not be accentuated by early post-exercise nutritional strategies. A note of caution should be made when interpreting any of the increases in immediate post-exercise levels, as the observed decrement in plasma volume shift (13-16%) (Table 4) at this timepoint almost certainly contributed to the increase in miRNA concentration.

The current findings are in general agreement with the scarce research on high-intensity exercise and ci-miRNA levels; however, the only targets that overlap between the current study and earlier reports are miR-1, miR-21, and miR-126. Specific to miR-1, the post-exercise increase in miR-1 reported by Cui et al. was not only confirmed here but we also found that miR-1 remains elevated for four hours post-exercise, particularly in the CHO condition (44). Interestingly, an earlier study by the Cui group demonstrated a downregulation of miR-1 post-exercise (43). It is difficult to reconcile the differences in findings, as the Cui protocol was

similar to the protocol used in the present study. When combined, it appears that miR-1 levels are largely upregulated following high-intensity exercise, though more work is needed to confirm this to determine whether there are specific exercise conditions that may have the opposite effect. Regarding targets -21 and -126, we report an increase in the endothelial miR-21 and miR-126, which is consistent with the findings of Wahl et al. using a nearly identical high-intensity exercise protocol (72). Interestingly, they observed higher magnitude changes in miR-21 and miR-126 with other exercise protocols, such as their prolonged exercise (130 minutes at 55% W_{max}) or sprint-interval exercise (4 x 30s all-outcompared to the protocol similar to our own (72), This may be due to greater sheer stress endured by the endothelial cells during severe exercise bouts, as previous research demonstrated that miR-21 is stimulated by shear stress (74).

Muscle-specific miRNA: It has been documented that several myomiRs are found at very low levels in plasma, and may be difficult to detect in circulation (30). This was confirmed here, as , the only other myomiR detected at any timpepoint, besides miR-1, was miR-486. As mentioned above, our findings for miR-1 are in general agreement with previous reports. However, we are unaware of any previous studies examining the effects of high-intensity cycling on miR-486, and only one exercise study in general. In contrast to increase in immediate post-exercise levels observed here, miR-486 has been shown to decrease in circulation following one hour of cycling at 70% VO_{2max} (30). The reason for this discrepency is unclear, though it may be related to exercise intensity or duration, as the exercise in the present study totalled less than half the exercise duration in the above study.

miR-486 has been shown to target phosphatase and tensin homolog (PTEN), an established negative regulator of phosphoinositide-3-kinase (PI3K)/Akt signalling (76), implicating its potential to influence insulin signalling. Though purely speculative, if miR-486 does influence insulin signalling, then it may be beneficial to maintian or upregulate miR-486 during exercise in order to ensure adequate glucose uptake by exercising muscle. It may be that the exercise in the aformentioned study was of long enough duration and low enough intensity to allow for increased lipid utlization for energy, sparing glucose more and making maintenance of miR-486 during exercise less crucial for performance.

Cardiovascular miRNA: The increase in both miR-21 and miR-126 (main effect) immediately post-exercise is in line with previous reports that miR-21 is elevated following a maximal exercise test (70) and miR-126 is higher following a maximal exercise test and throughout 4 hrs of cycling (27). Mechanistically, as previously mentioned, the increase in miR-21 may be due to the shear stress experienced by the endothelium during heavy exercise (74) and may ultimately faciitate angiogenesis (77) whereas the upregulation of miR-126 was likely triggered by the hypoxic stress induced by the prolonged cycling and can enhance pro-angiogenic factors, such as VEGF (78). Both miR-21 and miR-126 are critical for vascular function, as miR-21 was shown to decrease endothelial apoptosis and stimulate the nitric oxide (NO) production pathway in order to produce favorable vascular responses to shear stress (74). Alternatively,, miR-126 was vital for proper vascular health and development as targeted knockout of miR-126 in mice

has resulted in reduced vascular integrity, leading to vascular leakage and hemmorhage (78).

Additionally, miR-150 was upregulated following a 10-km race (28) and after training in mice(79). There is limited data concerning the effects of exercise on miR-150, as it is more commonly examined in disease states (80, 81). However, miR-150 may produce positive cardiovascular effects, as it was shown to protect the heart from injury in mouse MI models (82) and inibit foam cell formation *in vitro* (83).

The angiogenesis-related miRNA assessed in this project, miR-210, was increased throughout the post-exercise period in the current study. This finding contradicts a similar finding by Baggish et al. who reported no change in miR-210 following a maximal exercise test (70). Additionally, miR-210 was not affected by resistance exercise (84). Interestingly, overexpression of miR-21 has been shown to induce HIF-1α in human prostate cancer cells (77) and miR-210 is both induced by, and critical in response to, by hypoxia (85). It has been demonstrated that HIF signalling can induce miR-210 (82), illustrating a possible integrative miRNA signalling network in response to hypoxic conditions. It is possible that the previously mentioned exercise stimuli failed to induce hypoxia to the extent that the current exercise protocol did, and, as a result, did not increase miR-210 as presently observed.

Inflammation-implicated miRNA: miR-146a, -221, and -222, targets associated with inflammation (86–88), were increased immediately post-exercise in this study. Interestingly, while there was no main effect for time observed at one-

hour post-exercise, a main effect for time was detected at four-hours post-exercise for miR-146a. To our knowledge, miR-146a levels have not been examined following high-intensity exercise. Though it has been shown to increase in circulation following a maximal exercise test, it was decreased following one hour of cycling at 65% of maximal power output (89). While Baggish et al. reported a similar, if not greater, fold-increase in ci-miR-146a following a marathon than as we reported (67), de Gonzalo-Calvo et al. reported no change in miR-146a levels from baseline following a marathon (28). Interestingly, neither of the aforementioned studies followed the same blood sampling timecourse as the current study. Baggish et al. collected post-exercise blood samples immediately and 24 hours post-exercise (67), so if miR-146a has any fluctuation in its post-exercise timecourse, the collectoin timepoints may have been too far apart to capture it. Similarly to previous work (70), miR-221 and -222 were elevated immediately post-exercise in the present study. miR-221 and miR-222 appear to regulate vascular function, as miR-221 overexpression was shown to inhibit adiponectin-derived nitric oxide (NO) production (87) and both miRNA work together to regulate cell apoptosis (58).

Perhaps the most surprising finding of this study was that nutrition had no influence on ci-miR levels following high-intensity cycling except for miR-150, though it is not yet known what functional significance this finding may have. Few studies have examined the role of nutrition on ci-miRNA levels, and even fewer have explored the effects of combining nutrition with exercise. Importantly, to our knowledge, all previous studies examining the combined impact of exercise and

nutrition on miRNA activity chose to investigate intramuscular mi-RNA, not circulating levels. It is not clear to what extent ci-miRNA profiles reflect intramuscular miRNA activity, so the results of previous biopsy studies may be difficult to translate to ci-miRNA research. Regardless, while the present study observed no effects of nutrition or exercise on ci-miR-486, Camera et al. demonstrated that protein intake following concurrent exercise lead to greater levels of miR-486 four hours post-exercise (51). Drummond et al. have also shown that nutrition, without an exercise stimulus, can increase miRNA in skeletal muscle (50). One of the unique features of this study was the inclusion of CHO as a nutrition treatment, independent of PRO. To our knowledge, only one study has used CHO as a nutrition treatment to examine its effects on miRNA activity, and it was combined with PRO instead of being administered alone. Margolis et al. provided subjects with a PRO/CHO beverage during 90 min of cycling and weighted load carriage sessions. They reported that nutrient provision downregulated miR-1 compared to placebo, and the decrease was more apparent in the weighted exercise mode (73). The authors speculated that their findings may be explained in part by modulations in muscle protein synthesis by the combined mechanical strain of the exercise (especially the weighted carriage) and nutrition consumption (73). Altogether, more research is needed to establish nutrition strategies that may be used, with or without exercise, to manipulate miRNA activity to obtain favorable adaptations for health or performance.

Altogether, high-intensity cycling was found to alter several ci-miRNA profiles while post-exercise nutrition had very little impact. Given the relatively new research interest in miRNA response to exercise, more research is needed to further our understanding of how exercise impacts miRNA and how these impacts may be used to illucidate the molecular underpinning of adaptations to acute and chronic exercise. Considering the widespread influence of miRNA on exercise response and physiological function, more research is needed to examine the potential use of miRNA for pharmocological modification in order to modify disease risk or augment exercise adaptation and improve performance.

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Appendices

James Madison University

Department of Kinesiology

Consent for Investigative Procedure

I, _____, hereby agree on _____ (date) to participate in the research project conducted by Nicholas D. Luden, Ph.D. and Jeremy Via from James Madison University titled *Circulating MicroRNA Following High Intensity Interval Cycling With and Without Post-Exercise Nutrient Consumption.*

The purpose of this study is to examine the effects of high intensity interval cycling and post-exercise nutrient composition on circulating miRNA profile. Furthermore, this study aims to examine correlations between blood glucose levels and circulating miRNA.

Subject Responsibility

I understand that I will undergo the following testing:

This study consists of 4 separate visits performed, all of which will involve exercise on a stationary bike (cardiovascular fitness test and three exercise trials). All testing will occur in Godwin Hall, room 209, on the campus of James Madison University. You will also be asked about lifestyle behaviors such as smoking and physical activity and complete dietary and physical activity records. The total time commitment is estimated to be less than 8 hours over the course of 4 weeks.

Preliminary Trial (1 visit; 60 min):

After completing this consent form and the health status screening, if you meet the inclusion criteria for the study, researchers will measure your height and body weight.

You will then be asked to perform a maximal cardiovascular fitness test to determine your maximum oxygen consumption (VO2max). You will be asked to ride a stationary bike at an initial workload that is 'fairly easy'. The workload will then be increased every two minutes until fatigue is reached, determined by either: 1) your request to stop due to fatigue, or 2) inability to maintain a cadence of ≥50 revolutions per minute. You will be verbally encouraged to continue to obtain an accurate measurement of VO2max. To access oxygen consumption, you will need to breathe through a mouthpiece/breathing apparatus which collects expired air throughout the test (10-15 minutes). Heart rate and rate of perceived exertion (RPE) will be measured and recorded at conclusion of each stage.

Experimental Phase

Exercise Trial (3, 60 min each)

You will be asked to arrive at the human performance lab between 7-9am, following an overnight fast and not having consumed alcohol or tobacco 24 hrs or caffeine 12 hrs prior to testing.

Blood Sampling – Before beginning the exercise protocols, a resting blood sample will be acquired via venous blood draw. ≤4mL will be obtained for each sample. Furthermore, venous blood samples will be obtained immediately post-exercise, 1-hour post-exercise, and 4 hours post-exercise. Finger stick blood samples will also be collected pre-exercise, immediately post-exercise, and every 30 minutes following exercise for 4 hours. Samples will be between 20-60µL.

Exercise Protocol – Following a 5-minute warm-up at 50W, you will be asked to perform a cycling exercise protocol consisting of: a 30-second, maximum effort sprint (Wingate test), followed by 4x3 minutes at 90% Wmax, and concluding with a second Wingate test. Heart rate will be measured and recorded during each stage via heart rate monitor. A 5-minute cooldown will be performed upon completion of the exercise following the post-exercise blood sample.

Nutrition Treatment:

Upon completion of the exercise protocols in each exercise trial, you will be given a nutrition beverage containing either: placebo, carbohydrate (CHO), or a carbohydrate-protein mix (CHO+PRO). The order of the beverages will be random

and double-blinded. You must consume the beverage within 30 minutes of completing the exercise.

Dietary and Exercise Controls:

You will be asked to record all food and beverage intake for 24 hrs preceding the exercise trials. After the initial exercise trial, you will be provided with copies of your dietary records and instructed to replicate their dietary habits for the following exercise trials. You will be asked to report to all testing after an overnight fast. You will be asked to consume the nutrition beverage within 30 minutes of completing the exercise trial, and the time taken to consume the beverage should be consistent across all trials. Additionally, you will be instructed to refrain from consuming anything other than water during the 4 hours following the trial. You will be given a small, standardized meal 2 hours following completion of the exercise (90 minutes following the consumption of the nutrition beverage). You will also be instructed to record all physical activity 72 hrs prior to the first exercise trial. Additionally, you will be instructed to avoid physical activity between the post-exercise blood samples, and to keep physical activity habits consistent between all exercise trials.

Risks/Benefits:

Cardiovascular Exercise (Wingate Test, Intervals, and VO2max test)

According to the American College of Sports Medicine's Guidelines for Exercise Testing and Prescription, people without signs, symptoms, or diagnosis of cardiovascular, metabolic, or renal disease are cleared for vigorous exercise. The conditions that the exercise sessions are to take place are likely safer than the typical exercise environments of the subjects. If you do not meet ACSM criteria of clearance for vigorous exercise, you will not be allowed to participate in the study. In the unlikely event of cardiac or other complications during exercise, an emergency plan is in place. This includes immediate access to a phone to call emergency personnel. In addition, at least one of the listed investigators will be present during the exercise sessions, and all are CPR certified.

Blood Sampling

The risks of blood sampling using venous blood sampling or finger stick technique include possible mild bruising, and the risk of transfer of blood-borne pathogens,

as well as possible risks of infection or skin irritation. These risks are considered to be minimal, and all safety precautions for handing blood samples will be followed according to OSHA protocols, including: investigators will wear latex gloves at all times during blood sampling and testing. A sharps container lined with a biohazard bag will be used for all sharp objects involved in the blood sampling; all other materials (i.e. gloves, gauze pads, etc.) used during the sampling will be put in a separate waste disposal unit lined with a biohazard bag. All investigators who will be involved in blood draws (and handling of blood) have been trained in these phlebotomy techniques, and completed JMU blood-borne pathogen training. Additional risks include injury to blood vessels, as well as dizziness, vasovagal syncope (fainting), and nausea. A total of <100 milliliters of blood will be obtained throughout the course of the study, which is roughly 21% of the amount of blood typically obtained during blood donation (1 pint or 473 milliliters).

Benefits:

Upon request, you will receive your cardiovascular fitness testing results. If you compete the project, you will be given a free Human Performance Laboratory t-shirt and entered into a lottery to win $150.

Confidentiality:

The results of this research will be presented at conferences and published in exercise science journals. The results of this project will be coded in such a way that your identity will not be attached to the final form of this study. The researcher retains the right to use and publish non-identifiable data. However, you can ask that your data be removed from the study at any point prior to presentation and publication. While individual responses are confidential, aggregate data will be presented representing averages or generalizations about the responses as a whole. All data will be stored in a secure location accessible only to the researcher. Final aggregate results will be made available to you upon request.

Participation & Withdrawal:

Your participation is entirely voluntary. You are free to choose not to participate. Should you choose to participate, you can withdraw at any time without consequences of any kind. Your right to withdraw includes the right to request that your blood samples be discarded at any time. To dispose of your samples, your samples will be rinsed down a chemical drain in our laboratory or will be disposed of in a biohazard container. Again, your sample will not be identifiable without the coding document that will be locked away in a filing cabinet.

Questions:

You may have questions or concerns during the time of your participation in this study, or after its completion. If you have any questions about the study, contact Nicholas D. Luden, Ph.D. at ludennd@jmu.edu or by phone at 540-568-4068.

Giving of Consent:

I have read this consent form and I understand what is being requested of me as a participant in this study. I freely consent to participate. I have been given satisfactory answers to my questions. The investigator provided me with a copy of this form. I certify that I am at least 18 years of age.

Name of Participant (Printed)	Name of Researcher(s) (Printed)
Name of Participant (Signed)	Name of Researcher(s) (Signed)
Date	Date

For questions about your rights as a research subject, you may contact the chair of JMU's Institutional Review Board (IRB). Dr. David Cockley, (540) 568-2834, cocklede@jmu.edu.

2017 PAR-Q+

The Physical Activity Readiness Questionnaire for Everyone

The health benefits of regular physical activity are clear; more people should engage in physical activity every day of the week. Participating in physical activity is very safe for MOST people. This questionnaire will tell you whether it is necessary for you to seek further advice from your doctor OR a qualified exercise professional before becoming more physically active.

GENERAL HEALTH QUESTIONS

Please read the 7 questions below carefully and answer each one honestly: check YES or NO.	YES	NO
1) Has your doctor ever said that you have a heart condition ☐ OR high blood pressure ☐?	☐	☐
2) Do you feel pain in your chest at rest, during your daily activities of living, OR when you do physical activity?	☐	☐
3) Do you lose balance because of dizziness OR have you lost consciousness in the last 12 months? Please answer NO if your dizziness was associated with over-breathing (including during vigorous exercise).	☐	☐
4) Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)? PLEASE LIST CONDITION(S) HERE: _____	☐	☐
5) Are you currently taking prescribed medications for a chronic medical condition? PLEASE LIST CONDITION(S) AND MEDICATIONS HERE: _____	☐	☐
6) Do you currently have (or have had within the past 12 months) a bone, joint, or soft tissue (muscle, ligament, or tendon) problem that could be made worse by becoming more physically active? Please answer NO if you had a problem in the past, but it *does not limit your current ability* to be physically active. PLEASE LIST CONDITION(S) HERE: _____	☐	☐
7) Has your doctor ever said that you should only do medically supervised physical activity?	☐	☐

If you answered NO to all of the questions above, you are cleared for physical activity.
Go to Page 4 to sign the PARTICIPANT DECLARATION. You do not need to complete Pages 2 and 3.

- Start becoming much more physically active – start slowly and build up gradually.
- Follow International Physical Activity Guidelines for your age (www.who.int/dietphysicalactivity/en/).
- You may take part in a health and fitness appraisal.
- If you are over the age of 45 yr and **NOT** accustomed to regular vigorous to maximal effort exercise, consult a qualified exercise professional before engaging in this intensity of exercise.
- If you have any further questions, contact a qualified exercise professional.

If you answered YES to one or more of the questions above, COMPLETE PAGES 2 AND 3.

Delay becoming more active if:

- You have a temporary illness such as a cold or fever; it is best to wait until you feel better.
- You are pregnant - talk to your health care practitioner, your physician, a qualified exercise professional, and/or complete the ePARmed-X+ at www.eparmedx.com before becoming more physically active.
- Your health changes - answer the questions on Pages 2 and 3 of this document and/or talk to your doctor or a qualified exercise professional before continuing with any physical activity program.

01-01-2017

2017 PAR-Q+

FOLLOW-UP QUESTIONS ABOUT YOUR MEDICAL CONDITION(S)

1. Do you have Arthritis, Osteoporosis, or Back Problems?

If the above condition(s) is/are present, answer questions 1a-1c — If **NO** ☐ go to question 2

1a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments)	YES ☐ NO ☐
1b.	Do you have joint problems causing pain, a recent fracture or fracture caused by osteoporosis or cancer, displaced vertebra (e.g., spondylolisthesis), and/or spondylolysis/pars defect (a crack in the bony ring on the back of the spinal column)?	YES ☐ NO ☐
1c.	Have you had steroid injections or taken steroid tablets regularly for more than 3 months?	YES ☐ NO ☐

2. Do you currently have Cancer of any kind?

If the above condition(s) is/are present, answer questions 2a-2b — If **NO** ☐ go to question 3

2a.	Does your cancer diagnosis include any of the following types: lung/bronchogenic, multiple myeloma (cancer of plasma cells), head, and/or neck?	YES ☐ NO ☐
2b.	Are you currently receiving cancer therapy (such as chemotherapy or radiotherapy)?	YES ☐ NO ☐

3. Do you have a Heart or Cardiovascular Condition? *This includes Coronary Artery Disease, Heart Failure, Diagnosed Abnormality of Heart Rhythm*

If the above condition(s) is/are present, answer questions 3a-3d — If **NO** ☐ go to question 4

3a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments)	YES ☐ NO ☐
3b.	Do you have an irregular heart beat that requires medical management? (e.g., atrial fibrillation, premature ventricular contraction)	YES ☐ NO ☐
3c.	Do you have chronic heart failure?	YES ☐ NO ☐
3d.	Do you have diagnosed coronary artery (cardiovascular) disease and have not participated in regular physical activity in the last 2 months?	YES ☐ NO ☐

4. Do you have High Blood Pressure?

If the above condition(s) is/are present, answer questions 4a-4b — If **NO** ☐ go to question 5

4a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments)	YES ☐ NO ☐
4b.	Do you have a resting blood pressure equal to or greater than 160/90 mmHg with or without medication? (Answer **YES** if you do not know your resting blood pressure)	YES ☐ NO ☐

5. Do you have any Metabolic Conditions? *This includes Type 1 Diabetes, Type 2 Diabetes, Pre-Diabetes*

If the above condition(s) is/are present, answer questions 5a-5e — If **NO** ☐ go to question 6

5a.	Do you often have difficulty controlling your blood sugar levels with foods, medications, or other physician-prescribed therapies?	YES ☐ NO ☐
5b.	Do you often suffer from signs and symptoms of low blood sugar (hypoglycemia) following exercise and/or during activities of daily living? Signs of hypoglycemia may include shakiness, nervousness, unusual irritability, abnormal sweating, dizziness or light-headedness, mental confusion, difficulty speaking, weakness, or sleepiness.	YES ☐ NO ☐
5c.	Do you have any signs or symptoms of diabetes complications such as heart or vascular disease and/or complications affecting your eyes, kidneys, **OR** the sensation in your toes and feet?	YES ☐ NO ☐
5d.	Do you have other metabolic conditions (such as current pregnancy-related diabetes, chronic kidney disease, or liver problems)?	YES ☐ NO ☐
5e.	Are you planning to engage in what for you is unusually high (or vigorous) intensity exercise in the near future?	YES ☐ NO ☐

2017 PAR-Q+

6. **Do you have any Mental Health Problems or Learning Difficulties?** *This includes Alzheimer's, Dementia, Depression, Anxiety Disorder, Eating Disorder, Psychotic Disorder, Intellectual Disability, Down Syndrome*

If the above condition(s) is/are present, answer questions 6a-6b — If **NO** ☐ go to question 7

6a. Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments) YES ☐ NO ☐

6b. Do you have Down Syndrome **AND** back problems affecting nerves or muscles? YES ☐ NO ☐

7. **Do you have a Respiratory Disease?** *This includes Chronic Obstructive Pulmonary Disease, Asthma, Pulmonary High Blood Pressure*

If the above condition(s) is/are present, answer questions 7a-7d — If **NO** ☐ go to question 8

7a. Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments) YES ☐ NO ☐

7b. Has your doctor ever said your blood oxygen level is low at rest or during exercise and/or that you require supplemental oxygen therapy? YES ☐ NO ☐

7c. If asthmatic, do you currently have symptoms of chest tightness, wheezing, laboured breathing, consistent cough (more than 2 days/week), or have you used your rescue medication more than twice in the last week? YES ☐ NO ☐

7d. Has your doctor ever said you have high blood pressure in the blood vessels of your lungs? YES ☐ NO ☐

8. **Do you have a Spinal Cord Injury?** *This includes Tetraplegia and Paraplegia*

If the above condition(s) is/are present, answer questions 8a-8c — If **NO** ☐ go to question 9

8a. Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments) YES ☐ NO ☐

8b. Do you commonly exhibit low resting blood pressure significant enough to cause dizziness, light-headedness, and/or fainting? YES ☐ NO ☐

8c. Has your physician indicated that you exhibit sudden bouts of high blood pressure (known as Autonomic Dysreflexia)? YES ☐ NO ☐

9. **Have you had a Stroke?** *This includes Transient Ischemic Attack (TIA) or Cerebrovascular Event*

If the above condition(s) is/are present, answer questions 9a-9c — If **NO** ☐ go to question 10

9a. Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer **NO** if you are not currently taking medications or other treatments) YES ☐ NO ☐

9b. Do you have any impairment in walking or mobility? YES ☐ NO ☐

9c. Have you experienced a stroke or impairment in nerves or muscles in the past 6 months? YES ☐ NO ☐

10. **Do you have any other medical condition not listed above or do you have two or more medical conditions?**

If you have other medical conditions, answer questions 10a-10c — If **NO** ☐ read the Page 4 recommendations

10a. Have you experienced a blackout, fainted, or lost consciousness as a result of a head injury within the last 12 months **OR** have you had a diagnosed concussion within the last 12 months? YES ☐ NO ☐

10b. Do you have a medical condition that is not listed (such as epilepsy, neurological conditions, kidney problems)? YES ☐ NO ☐

10c. Do you currently live with two or more medical conditions? YES ☐ NO ☐

PLEASE LIST YOUR MEDICAL CONDITION(S) AND ANY RELATED MEDICATIONS HERE: _____

_____

GO to Page 4 for recommendations about your current medical condition(s) and sign the PARTICIPANT DECLARATION.

01-01-2017

2017 PAR-Q+

If you answered NO to all of the follow-up questions about your medical condition, you are ready to become more physically active - sign the PARTICIPANT DECLARATION below:

- It is advised that you consult a qualified exercise professional to help you develop a safe and effective physical activity plan to meet your health needs.
- You are encouraged to start slowly and build up gradually - 20 to 60 minutes of low to moderate intensity exercise, 3-5 days per week including aerobic and muscle strengthening exercises.
- As you progress, you should aim to accumulate 150 minutes or more of moderate intensity physical activity per week.
- If you are over the age of 45 yr and NOT accustomed to regular vigorous to maximal effort exercise, consult a qualified exercise professional before engaging in this intensity of exercise.

If you answered **YES** to **one or more of the follow-up questions** about your medical condition:

You should seek further information before becoming more physically active or engaging in a fitness appraisal. You should complete the specially designed online screening and exercise recommendations program - the **ePARmed-X+** at **www.eparmedx.com** and/or visit a qualified exercise professional to work through the ePARmed-X+ and for further information.

Delay becoming more active if:

- You have a temporary illness such as a cold or fever; it is best to wait until you feel better.
- You are pregnant - talk to your health care practitioner, your physician, a qualified exercise professional, and/or complete the ePARmed-X+ at www.eparmedx.com before becoming more physically active.
- Your health changes - talk to your doctor or qualified exercise professional before continuing with any physical activity program.

- You are encouraged to photocopy the PAR-Q+. You must use the entire questionnaire and NO changes are permitted.
- The authors, the PAR-Q+ Collaboration, partner organizations, and their agents assume no liability for persons who undertake physical activity and/or make use of the PAR-Q+ or ePARmed-X+. If in doubt after completing the questionnaire, consult your doctor prior to physical activity.

PARTICIPANT DECLARATION

- All persons who have completed the PAR-Q+ please read and sign the declaration below.
- If you are less than the legal age required for consent or require the assent of a care provider, your parent, guardian or care provider must also sign this form.

I, the undersigned, have read, understood to my full satisfaction and completed this questionnaire. I acknowledge that this physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if my condition changes. I also acknowledge that a Trustee (such as my employer, community/fitness centre, health care provider, or other designate) may retain a copy of this form for their records. In these instances the Trustee will be required to adhere to local, national, and international guidelines regarding the storage of personal health information ensuring that the Trustee maintains the privacy of the information and does not misuse or wrongfully disclose such information.

NAME _____ DATE _____

SIGNATURE _____ WITNESS _____

SIGNATURE OF PARENT/GUARDIAN/CARE PROVIDER _____

For more information, please contact
www.eparmedx.com
Email: eparmedx@gmail.com

citation for PAR-Q+
Warburton DER, Jamnik VK, Bredin SSD, and Gledhill N on behalf of the PAR-Q+ Collaboration. The Physical Activity Readiness Questionnaire for Everyone (PAR-Q+) and Electronic Physical Activity Readiness Medical Examination (ePARmed-X+). Health & Fitness Journal of Canada 4(2):3-23, 2011.

The PAR-Q+ was created using the evidence-based AGREE process [1] by the PAR-Q+ Collaboration chaired by Dr. Darren E. R. Warburton with Dr. Norman Gledhill, Dr. Veronica Jamnik, and Dr. Donald C. McKenzie [2]. Production of this document has been made possible through financial contributions from the Public Health Agency of Canada and the BC Ministry of Health Services. The views expressed herein do not necessarily represent the views of the Public Health Agency of Canada or the BC Ministry of Health Services.

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01-01-2017

24-HOUR DIET RECORD

*Subject number*_____ *Date*_____ *Day of Week*_____

Time	Food and/or Drink	Method of Preparation	Quantity Consumed	Brand Name

Adapted From: Lee RD, Nieman DC. *Nutritional Assessment.* 2nd ed. United States of America: Mosby; 1996

Physical Activity Records

Subject #_____ Trial #

_____ Date:

_____

Date	Type of Exercise Performed	Duration of Exercise (minutes)	Intensity of Exercise (use scale below)

Intensity Scale

6
7 Very, very light
8
9 Very light
10
11 Fairly light
12
13 Somewhat hard
14
15 Hard
16
17 Very hard
18
19 Very, very hard
20

Subject Prescreening Information

Age:__years

Height_____ Weight_____

Typical Exercise Habits over the Past 3-6 Months:

Average number of days of aerobic exercise per week_____

Average number of hours of aerobic exercise per week_____

Briefly describe your aerobic exercise habits over the past 3-6 months:

Average number of days of resistance exercise/weight lifting per week _____

Average number of hours of resistance exercise/weight lifting per week _____

Briefly describe your resistance training habits over the past 3-6 months:

Do you have a muscle or joint injury/condition that precludes the completion of the exercise protocol? If yes, please explain.

Are you allergic to wheat?

Do you have gluten intolerance?

Are you allergic to latex?

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