The Role Of Circulating Mirnas As Clinical Biomarkers Of Insulin Sensitivity And Exercise Adaptation In Young Offspring Of Type 2 Diabetes Patients

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Dedication

I dedicate my thesis work to my family and mentor, whose support never went unnoticed:

To my mother, thank you for exemplifying what it means to be a good person and encouraging my career aspirations. Your selflessness and hard work are values I hope to emulate. While I get to say I am a first-generation graduate, this is not possible without you.

To my father, thank you for encouraging my career and instilling the values of work ethic and humility. I appreciate your involvement in my studies growing up and making sure I exceed my potential.

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THE ROLE OF CIRCULATING MIRNAS AS CLINICAL BIOMARKERS OF INSULIN SENSITIVITY AND EXERCISE ADAPTATION IN YOUNG OFFSPRING OF TYPE 2 DIABETES PATIENTS

by

CESAR ARTURO MEZA, B.S.

THESIS

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Abstract

**Background:** A family history of Type 2 Diabetes (FH) is considered a risk factor for development of type 2 diabetes (T2D). Exercise training is an effective strategy to improve insulin resistance. Recently, micro-RNAs (miRNAs) have emerged as mediators of various intracellular processes, including insulin signaling, and may act as signaling molecules when released into circulation. The miRNAs -29a, -133a and -155 have been implicated in the regulation of insulin sensitivity, however their roles in exercise training-induced improvements in insulin sensitivity are yet to be elucidated. The purpose of this study was to determine whether circulating miRNA-29a, miRNA-29b, miRNA-29c, miRNA-133a, miRNA-133b and miRNA-155 were altered by a FH in healthy, normoglycemic men. In addition, we examined the effects of eight weeks of combined exercise training on circulating expressions of miRNA-29a, miRNA-29b, miRNA-29c, miRNA-133a, miRNA-133b and miRNA-155. **Methods:** 19 sedentary, normoglycemic Mexican American men (mean ± SEM; age: 23.3 ± 0.6; BMI: 26.9 ± 0.9) with/without a family history of T2D (FH-/FH+) underwent eight weeks (3x/wk) of combined exercise training (endurance and resistance exercise). A five-day standardized diet was provided prior to test days. Insulin sensitivity was assessed via the gold-standard hyperinsulinemic euglycemic clamp and serum was collected for miRNA detection. Total RNA was extracted and reverse transcribed to cDNA followed by miRNA analysis via qRT-PCR. Differences in miRNA expression between FH- and FH+ at baseline were analyzed by independent t-tests. Group and time effects of the exercise training intervention were analyzed by two-way ANOVA. Relationships between miRNA expression and clinical outcome measures were determined using Pearson correlation analyses. **Results:** While insulin sensitivity was similar between groups at baseline (p > 0.05), exercise training significantly increased insulin sensitivity in FH- and FH+ (FH-: 3.01 ± 0.38 to 4.16 ± 0.35; FH+: 3.86 ± 0.61 to 4.16 ± 0.35 mg/kg EMBS/min; two-way ANOVA p = 0.0005). Baseline circulating miRNA expression levels were similar between FH- and FH+ (FH- vs. FH+: miRNA-29a: -3.82 ± 4.92 vs. 3.56 ± 1.48, p = 0.17; miRNA-133a: 0.89 ± 3.97 vs. 1.76 ± 3.85, p = 0.88; miRNA-133b: -1.73 ± 4.4 vs. -3.52 ± 5.44, p = 0.80; miRNA-155: 2.01 ± 5.06 vs. 3.31 ± 9.47, p = 0.89). Exercise training did not significantly alter circulating miRNA expression regardless of FH (two-way ANOVA: miRNA-29: p = 0.05; miRNA-133a: p = 0.19; miRNA-133b: p = 0.31; miRNA-155: p=0.37). There were significant negative associations between fasting blood glucose at baseline and miRNA-29a ($r^2 = 0.63$, p =
0.01), miRNA-133a \((r^2 = 0.52; p = 0.001)\), miRNA-133b \((r^2 = 0.30, p = 0.02)\) and miRNA-155 \((r^2 = 0.80, p = 0.0002)\). The exercise training-induced reductions in fasting blood glucose were significantly associated with miRNA-29a \((r^2 = 0.46, p = 0.003)\), miRNA-133a \((r^2 = 0.43; p = 0.004)\), miRNA-133b \((r^2 = 0.39, p = 0.008)\) and miRNA-155 \((r^2 = 0.50, p = 0.01)\). **Conclusions:** Insulin sensitivity was not impaired in young, sedentary men with a FH and eight weeks of combined exercise training improved insulin sensitivity, independent of FH. Circulating miRNAs (miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155) were not altered by a FH or combined exercise training. Our findings suggest circulating miRNAs (miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155) are unaltered between normoglycemic individuals with/without a FH or following eight weeks of combined exercise training. However, fasting glucose levels may be regulated by circulating miRNAs -29a, -133a, -133b and -155, despite normoglycemia.
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Chapter 1: Introduction

BACKGROUND AND SIGNIFICANCE

Obesity and Type 2 Diabetes (T2D) have reached epidemic proportions across the world, with an estimated 2.8 million people dying each year as a result of being overweight or obese (1). Globally, nearly 2 billion adults are overweight and 650 million are considered obese (2). These numbers have nearly tripled in recent decades and contributed to metabolic impairments, including insulin resistance (2). Thus, the number of people with T2D - a major cause of blindness, kidney failure, heart attacks, stroke and lower limb amputation – increased 4-fold from 1980-2014 (3). With rises in the prevalence of overweight/obesity and T2D, economies have experienced a parallel increase in the resulting cost of diabetes management. According to the American Diabetes Association (ADA), the total estimated cost of diagnosed diabetes in 2017 was $327 billion, including $237 billion in direct medical costs (4).

Although the mechanisms and pathogenesis of T2D are not completely understood, impairments in insulin sensitivity and β-cell function are considered two key precursors in development of the disease (5). To prevent the onset of these two conditions, glucose and lipid homeostasis must be maintained by a reciprocal balance between β-cell feedback and insulin action in peripheral tissues (i.e., skeletal muscle, liver, adipose tissue and cardiac muscle) (6). As a result, insufficiencies to either part of the relationship can present challenges to preserving optimal metabolic health. Evidence has suggested that an inability to store excess fat in subcutaneous adipose tissue compartments results in an influx of lipid in ectopic sites such the liver and skeletal muscle, that subsequently contributes to insulin resistance (7). In sedentary, obese populations, there is a reduced capacity to match lipid oxidation with lipid availability in oxidative tissues, thus a lower adipose turnover results in lipotoxicity (8,9). Opposed to lean, active counterparts, obese individuals display elevated lipid metabolites, including ceramides and diacylglycerols (DAGs) that hinder insulin action via increased serine phosphorylation of the insulin receptor and insulin receptor substrate-1 (IRS-1) and/or reduced serine phosphorylation
of protein kinase B (PKB/Akt) (10,11). In rats infused with lipids, insulin resistance occurs with accumulation of DAGs and inhibition of IRS-1 mediated by activated protein kinase C (PKC) (12). Notably, insulin resistance was independent of changes in muscle triacylglycerol (TAG), suggesting the link between insulin resistance and lipid accumulation lies in the lipid intermediates, not the level of intramuscular triglycerides. To support this link, exercise training results in increased muscle TAG, but decreased DAGs and ceramides (13). The adjustment of lipid oxidation to match lipid availability is therefore a critical component in maintaining glucose and lipid homeostasis (14) and an important characteristic of metabolic flexibility - the capacity to adapt fuel oxidation to fuel availability (15).

In conditions of obesity without T2D, animal models have shown that the pancreas compensates for hyperglycemia with increased insulin secretion and β-cell mass (16). However, during the progression of obesity to T2D, the mechanisms which replicate β-cells become deficient, resulting in greater β-cell apoptosis, providing a mechanistic explanation for β-cell dysfunction observed in T2D patients (17). Moreover, due to the clinical state with elevated triglycerides, lipotoxicity in β-cells has been shown to significantly reduce expression of the insulin gene (18), a prerequisite to compromised insulin secretion and a common manifestation associated with T2D. Together, these models suggest that the presence of hyperglycemia and elevated fatty acid concentrations contribute to defective β-cell function and a T2D phenotype. The evidence provides a prominent hypothesis that it is important to understand the mechanisms mediating healthy β-cell function and insulin sensitivity to prevent the onset of insulin resistance and T2D.

In addition, the liver is considered a key regulator of energy homeostasis and subject to be negatively impacted by T2D. The causal relationships between hepatic insulin resistance, skeletal muscle insulin resistance and β-cell dysfunction are not completely understood, however it has been shown that a fatty liver increases the risk for T2D independent of poor metabolic profiles in humans (i.e., elevated fasting insulin, glucose, triglycerides) (19). During starvation, the liver is responsible for maintaining blood glucose levels via generation of glucose from local
glycogen stores or non-carbohydrate sources, gluconeogenesis, and in response to increased glucagon secretion from the pancreas. Conversely, these mechanisms are reversed during the postprandial state, where hepatic glucose production is suppressed and glucose uptake is increased (14). However, in a state of overnutrition, the liver’s transition from a starved to a fed state becomes compromised (20). Patients with diagnosed non-alcoholic fatty liver disease (NAFLD), demonstrate lower basal hepatic glucose production (HGP) and insulin-stimulated suppression of HGP during the hyperinsulinemic euglycemic clamp, two classical indicators of liver insulin resistance (21). Kelley and colleagues (22) showed that individuals with the greatest degree of fatty liver demonstrated the most pronounced whole-body insulin resistance. In addition, the authors found an association between the severity of fatty liver and free fatty acid (FFA) concentrations (22). Under normal conditions, insulin is responsible for suppressing FFA concentrations, however with insulin resistance, a clinical state with hyperinsulinemia and elevated FFA’s is often observed. These data, together with evidence showing upregulated hepatic lipid esterification (23), suggest insulin resistance is influenced by dysfunctional regulation of insulin to suppress lipolysis combined with excess lipid influx in the liver.

Furthermore, the consequences of being overweight/obese are not consistent among ethnic groups. Mexican Americans have been considered 50% more likely to die from T2D compared to non-Hispanic Whites (24). This disparity constitutes a grave concern, as two-thirds of the 56 million Hispanics in the U.S. are overweight and among Hispanics, Mexican Americans suffer from the greatest rate of T2D (24). Notably, a considerable number of adults live with undiagnosed diabetes, particularly in minority populations (25). In Mexican-Americans, the proportion of adults with undiagnosed diabetes rose by 60% from 2005-2010, while the number of whites with undiagnosed diabetes declined (25). T2D is the seventh leading cause of death and with 1.5 million new cases in the U.S. each year, largely comprised of Hispanics, it is imperative to identify therapeutics, or more importantly develop interventions to prevent insulin resistance.
Although it is likely that lifestyle factors such as diet and physical activity have a significant influence in the development of T2D, genetics may also explain a portion of the risk (26,27). Individuals with a family history of T2D (FH), with one or more first-degree relatives, have a greater risk for developing the disease and thus more likely to suffer from metabolic dysfunction (28). The interactions between a FH and insulin sensitivity require further research, however insulin resistance is observed in the healthy offspring of patients with T2D (29–32) including decreased skeletal muscle insulin-stimulated IRS-1 phosphorylation (31) and mitochondrial activity (33). Notably the aberrations in insulin sensitivity with a FH can be detected by the gold-standard clamp method as well as less intensive oral glucose tolerance tests (OGTT) (34–36). Gulli et al. (37) characterized the metabolic abnormalities associated with a FH, demonstrating significantly elevated fasting insulin levels in adults with normal glucose tolerance. Although it may be expected that compensatory hyperinsulinemia in response to elevated blood glucose may confer greater glucose uptake, it was found that insulin-stimulated glucose disposal was up to 43% lower than counterparts without any FH. Importantly, Gulli et al. (37) reported that a FH resulted in diminished insulin-stimulated suppression of lipid oxidation.

In a study to assess genetic predisposition for diabetes due to a FH, non-obese adults demonstrated dysfunctional and enlarged adipocytes in subcutaneous adipose tissue compared to individuals without a FH, which were associated with reduced insulin sensitivity (38). Similarly, circulating levels of adiponectin, an adipokine shown to activate adenosine 5′-monophosphate-activated protein kinase (AMPK) (39), have been shown to be significantly lower in individuals with a FH compared to those with no family history of the disease (40). In the study of healthy Mexican Americans, Civitarese and colleagues (40) showed that mRNA levels of both adiponectin receptors (AdipoR1 and AdipoR2) in skeletal muscle were lower with a FH. Interestingly, expression of adiponectin receptors was positively correlated with insulin sensitivity measured by the gold-standard hyperinsulinemic euglycemic clamp (40). In addition to impairments in glucose metabolism observed in adult offspring with a FH, studies have reported markers of insulin resistance as early as pre-pubescent years. The children (boys and
girls, ~12 years of age) of patients diagnosed with T2D have demonstrated impaired insulin sensitivity coupled with poor insulin action and β-cell feedback – a trio of hallmark prerequisites that are fundamental to the development of insulin resistance and T2D (29). The collective clinical and physiological data have provided a convincing argument that a FH plays an important regulatory role in the risk for offspring to develop the disease.

The diminished capacity to switch from a predominant lipid oxidation state to carbohydrate oxidation upon insulin-stimulated conditions refers to metabolic inflexibility (41). Whereas individuals with metabolic flexibility may rely primarily on fat as a fuel source, healthy individuals can also switch to carbohydrate oxidation promptly with glucose ingestion. Research has shown that a FH may or may not affect metabolic flexibility (34,42,43), however it well understood that the ability to oxidize lipids while fasting and glucose after a meal, has important implications in metabolic health (44).

Evidence points to a FH as a significant contributor of insulin resistance, however there is considerable data showing exercise can prevent T2D. Despite the abundance of evidence demonstrating many benefits to physical activity and exercise (45), physical inactivity constitutes a major risk factor for the development of metabolic diseases (46). Only one third of the global population is estimated to meet physical activity guidelines (47), while 9% of premature mortalities are directly attributable to physical inactivity (48). The American College of Sports Medicine (ACSM) recommends 150 min/week of moderate to vigorous endurance exercise, and two or three days each week of resistance exercises that train each major muscle groups (49). According to recent reports by the Centers for Disease Control and Prevention (CDC) and American Heart Association (AHA), 80% of U.S. adults fail to meet these exercise recommendations (50). In an effort to control non-communicable diseases, the World Health Organization (WHO) implemented an initiative to combat physical inactivity as one of its four primary objectives (1).

In addition to promoting physical activity, strategies to manage and/or protect against T2D include pharmaceutical and dietary approaches. Significant progress has been made in
recent years to understand the effects of drugs (51) and diet (52) on energy metabolism, however the complexity of these environmental factors warrants further investigation to understand the underlying mechanisms. The HERITAGE Family Study reported that the trainability of cardiorespiratory fitness varies up to 50% due to familial and genetic components (53). In addition, there is large inter-individual variability in exercise training-induced weight loss (54), response to various diets (55) and the effects of metformin (56), a commonly prescribed T2D drug. Therefore, a current trend in obesity and diabetes research, including among exercise physiologists, is to identify early detection markers and molecular mechanisms related to insulin sensitivity that influence adaptations (or lack of adaptations) to exercise, diet and pharmacological agents. Within the last decade, there has been a surge of interest in identifying the role of microRNAs (miRNAs) in mediating processes of exercise adaptation including endothelial function (57), mitochondrial function (58) and muscle hypertrophy (59,60).

As demonstrated by Arslanian et al. (29), the manifestations of metabolic dysfunction may arise in the early years of adolescence. However the gold-standard clamp method to assess insulin sensitivity presents several challenges due to its cost and labor-intensive nature. Therefore it is important to identify a valid, early detection marker of insulin resistance that is responsive to changes in lifestyle modifications such as exercise training. In 2010, Nielson et al. (61) identified changes in the expression of a small genetic marker in skeletal muscle following an acute bout of exercise and after chronic training. The novelty, herein, lied in the class of short RNA molecules, miRNAs, that have since been found to be differentially expressed in several disease states including cancer, cardiovascular disease and T2D (62). The work of Nielson and colleagues was recently supplemented by Denham and Prestes (63) who showed that the same RNA molecule, miRNA-133a, was significantly lower in the blood of endurance trained athletes following an acute bout of exercise, suggesting that an endurance trained phenotype alters circulating levels of miRNAs.

From a molecular biology perspective, the investigation of gene expression provides a link with the adaptations that occur at the protein level (64,65). The classical central dogma of
biology identifies the progression of genetics as the foundation for physiological responses to an environmental stimulus and the subsequent changes at the protein level (Figure 1) (66,67). According to the National Human Genome Research Institute (NHGRI), the genetic code of an organism is embedded in DNA that is transcribed by mobile mRNA that serves as the template for synthesis of a particular protein (Figure 1) (66). Studying the modulations of gene expression imposed by exercise, allow for the understanding of early-stage cellular responses that have consequences related to the subsequent biochemical pathways and proteins that are activated (68). The human genome is comprised of approximately 24,000 protein-coding genes, while only a fraction of the genes in a cell are expressed at a given time (69). Gene expression profiles are therefore a reflection of the current energetic state and demands imposed by human health and the external environment. This flexibility in modifying gene expression affords an advantage for adjusting the cellular environment to meet the requirements of events such as exercise (68). Conversely, chronic disease states, including T2D, alter gene expression profiles in a manner that is less favorable for optimal health (70). Regardless of the health or clinical status of an individual, studying the patterns in gene expression can be translated to understanding the response to an environmental stimulus as well as understanding the potential for future adaptations.

With the discovery of circulating miRNA’s (i.e., stable, minimally invasive makers), there is an immense potential for developing therapeutic approaches to improve obesity/T2D management in response to exercise training. There are likely cross-talk mechanisms that are
exhibited by circulating miRNAs, whereby the molecules are released into the bloodstream to regulate the function of tissues elsewhere (70,71). Given that the regulation of β-cell function and insulin sensitivity are considered fundamental to preventing T2D, investigating the role of circulating miRNA profiles in mediating exercise-induced improvements can progress our strategies to identify insulin resistance in early stages and modify exercise prescription to promote optimal responses and adaptations.

**miRNA Synthesis and Function**

miRNAs are classified as small, non-coding RNAs, approximately 22 nucleotides (nt) in length, that regulate gene expression by binding to the 3’ untranslated regions (UTR) of messenger RNA (mRNA), hence inhibiting translation or degrading mRNA. Each miRNA is capable of targeting several mRNAs and likewise, each mRNA is subject to be targeted by several miRNA. It is estimated that miRNAs regulate approximately 60% of the human protein-coding genome (72) and have been implicated in the regulation of several biological processes including cell proliferation, apoptosis and metastasis (73). While the functions of miRNAs in tissues continue to be explored, a wide regulatory network and multi-tissue coordination has been identified in the context of insulin resistance (74) and other disease-states such as cancer (75). The discovery of miRNAs two decades ago changed the traditional philosophy that protein-coding genes should be the focus of studying disease, and more recent techniques have continued to progress approaches to studying gene expression as clinical biomarkers in plasma/serum rather than subjecting patients to tissue biopsies.

The first miRNA, lin-4, was discovered in 1993 by the works of Ambros and Ruvkun in the roundworm, *Caenorhabditis elegans* (*C. elegans*), who identified that lin-4 had the ability to repress the expression of the mRNA, lin-14, by function of its 3’ UTR (76). At this point, lin-4 was referred to as a non-coding RNA (ncRNA), until the subsequent discovery of the second ncRNA seven years later. Let-7 was identified by a member of Ruvkin’s laboratory as a 21nt RNA sequence in *C. elegans* that also had the ability to repress lin-14 (77). Notably, let-7 was
found to be conserved in humans and its expression was later confirmed in several tissues (77). These discoveries ignited a trend in molecular biology research to understand the roles of these small, non-coding genes that counterintuitively have considerable downstream effects on mRNA and protein expression.

The biogenesis of miRNA has been heavily studied and is adeptly understood, considering its relatively recent discovery. miRNA biosynthesis involves processing of precursor miRNA transcripts by a cluster of RNases before the mature, functional miRNA is acquired (Figure 2) (78). Beginning with a primary miRNA (pri-miRNA) transcript, the strand is cleaved inside the nucleus by the Drosha enzyme, a RNaseII endonucleaseIII, into a 60-110nt sequence. The resulting structure is a precursor miRNA (pre-miRNA) with a hairpin loop, which is transported out of the nucleus by a nuclear transport protein (Exportin-5 [XPO5]) for further processing. In the cytoplasm, a RNaseII, Dicer-1, cleaves the pre-miRNA into a short double-stranded miRNA. Lastly, the intrinsic helicase activity of Dicer unwinds the double-strand to allow incorporation of the mature miRNA with a member of the Argonaute family of RNA-induced silencing (RISC) proteins. Once the miRNA-loaded RISC complex is formed, the structure probes the cytoplasm for mRNAs with potential complementarity. miRNAs target the 3’ UTR of mRNA, regions with specialized subunits to allow RNA interference (RNAi), and bind with imperfect base pair complementarity to effectively block ribosomes from initiating translation (73). The incomplete base paring between miRNA and mRNA has since been understood as a central function of miRNA’s ability target several mRNAs and thus have profound impacts on different biological processes (Figure 2) (78).
CIRCULATING miRNAs as Biomarkers

The conventional paradigm of identifying molecular markers in vivo involves biopsy techniques that are highly invasive and often distressing for patients. Nonetheless, access to tissue samples (skeletal muscle, liver, adipose, etc.) provides the standard for extensive assessment of normal and pathological events of an organism (79–81). Recently, however, there has been a focus to identify biomarkers in human fluids (serum/plasma, urine, saliva) that are readily available, minimally invasive and provide biological information that is comparable to biopsy samples. In 2008, Chim et al. (82) first reported the use of miRNAs as diagnostic tools with the detection of placental miRNA in the plasma of maternal patients. The discovery of miRNA in the circulation sparked the understanding that these small genetic biomarkers have the potential to identify diseases during pre-clinical stages, assess disease predisposition and observe responses to interventions. Thus, identification of miRNA profiles or changes in certain miRNAs with exercise and/or with conditions of insulin resistance, provide a promising prospective for improving the health of our society.

Circulating miRNAs have been shown to have several attributes that result in excellent stability and allow for sensitive detection. For example, Mitchell et al. (71) demonstrated that incubation of human plasma at room temperature for up to 24h and subjecting samples to up to eight cycles of freeze-thawing, had minimal effects on the expression of three miRNAs. In addition, it was shown that the miRNAs were unaffected by endogenous plasma RNase activity and that there were strong associations in the measurements of miRNAs in plasma compared with serum (71). Others have shown that the stability of miRNAs in serum/plasma is owed to packaging by lipid or lipoprotein complexes (83) and secretory particles, microvesicles (up to 1μm) or smaller exosomes (50-100nm) (84,85). The mechanisms by which miRNA are secreted and incorporated with circulating protein complexes remain unclear, however given the ability of miRNA to regulate gene expression, it is evident that these small, non-coding RNA exhibit a significant capacity to coordinate metabolism between different tissues. In addition, the resistance to degradation in biofluids presents favorable opportunities for detection with genetic
techniques/applications and ultimately for using circulating miRNAs as biomarkers for metabolic disease.

**The Role of miRNA in Insulin Resistance**

Insulin resistance is a multi-factorial metabolic condition that is characterized by impairment of glucose and insulin responsiveness in peripheral tissues, including adipose, liver and skeletal muscle. It is hypothesized that overnutrition and inflammation alter the function of these various cell types and contribute to metabolic abnormalities. In conditions of overweight/obesity and insulin resistance, insulin signaling and glucose uptake become compromised in addition to exhibiting dysfunctional β-cells. Collectively, this myriad of metabolic aberrations characterizes the complexity of understanding the causal events that underlie T2D. Given the need to examine the coordination of multiple tissues, there is an emerging role of circulating miRNAs as biomarkers in obesity and insulin resistance research.

**miRNA-143**

While human studies involving miRNA profiling and individual detection are emerging, the contributions of miRNAs in animal and cell culture models of obesity and insulin resistance have been well studied. In the liver of obese mice, overexpression of miRNA-143 resulted in impaired glucose homeostasis due to insulin resistance, but not β-cell dysfunction (86). Compared with control littermates, induction of miRNA-143 overexpression did not alter fasting blood glucose concentrations, however significantly lowered glucose tolerance, insulin tolerance and increased homeostatic model of insulin resistance (HOMA-IR). To study loss-of-function, the authors induced gene knockout (KO) of miRNA-143 after high fat feeding and observed miRNA-143 KO mice were protected against insulin resistance (86). Similarly, Zu et al. (87) tested the hypothesis that obesity progression is comprised by efflux of FFA and adipokines such as tumor necrosis factor-α (TNF-α), leptin and interleukin-6 (IL-6). Indeed, it was shown that exogenous incubation of preadipocytes with FFAs, leptin or resistin significantly downregulate miRNA-143 expression (87). When β-cell function was analyzed in miRNA-143 transgenic
(overexpressing) mice via assessment of glucose-stimulated insulin secretion, β-cell mass and morphological changes in β-cell islets, no abnormalities were observed (86). However, miRNA-143 overexpression induced impairments in insulin-stimulated PI3K-Akt signaling in the liver that were not evident in skeletal muscle. Specifically, insulin-stimulated phosphorylation of pAkt\textsuperscript{Ser473} and pAkt\textsuperscript{Thr308} were significantly impaired compared to controls, suggesting a role for miRNA-143 in regulating liver insulin action and whole-body glucose control while sparing β-cells (86).

In vitro studies assessing miRNA-143 have shown dysfunctional adipose tissue that may contribute to insulin resistance. Opposed to the initial paradigm of adipose tissue as solely a reservoir for excess nutrients, adipose tissue is now recognized as a functional tissue involved in regulating energy metabolism (66). In adipose-tissue-derived stromal cells, precursors to adipocytes, Chen et al. (88) demonstrated that overexpression of miRNA-143 resulted in impaired adipocyte formation via a mitogen-activated protein-dependent kinase (MAPK) signaling pathway, an elaborate signaling complex involved in PGC-1α (89) and UCP-1 (90) activation. By overexpressing miRNA-143, it was shown that miRNA-143 targets the MAP2K5 mRNA and promotes terminal differentiation of adipose tissue precursors into mature adipocytes (88). The implications for dysfunctional adipocyte formation are connected to several biological regulations of mature adipocytes including lipolytic activity, glucose uptake and cytokine secretion (91).

Similarly, Zhang et al. (92) showed that overexpression of miRNA-143 in bovine skeletal muscle satellite cells negatively regulated myocyte proliferation and differentiation. The function of miRNA-143 was found to be regulated by targeting insulin-like growth factor binding protein-5 (IGFBP5), an important component in IGF signaling, and thus repressing MAPK signaling (92). In skeletal muscle, p38 MAPK is known to increase fatty acid oxidation in concert with AMPK via stimulation by the adipokine, adiponectin (93).

While one study determined that miRNA-143 may not have a functional role in insulin secretion (94), the aforementioned cell and animal models indicate a role of miRNA-143 in
regulating peripheral insulin signaling and adipose cell differentiation. Considering the reductions in whole-body glucose tolerance and actions in different tissue-sites, miRNA-143 may exhibit a cross-talk mechanism that elicits impairments in various organs. These studies provide evidence for insulin resistance in the absence of T2D, thus serving as a model for diabetes progression and possibly a marker to address T2D in its early stages. There is evidence from one study assessing miRNA-143 expression in whole blood of severely obese vs. lean subjects, which did not identify differential expression (95). Nonetheless, further assessment of circulating miRNA-143 levels in humans is required to determine the potential for miRNA-143 as a biomarker for whole-body glucose homeostasis in different metabolic states.

miRNA-29

In 2007, He and colleagues identified a cluster of miRNAs that were upregulated in oxidative tissues of a diabetic rat model (96). Microarray analysis and Northern blotting of skeletal muscle, liver, and adipose tissues revealed increased expression of three members of the miRNA-29 family, miRNA-29a, miRNA-29b and miRNA-29c with T2D. To investigate the function of miRNA-29 in insulin signaling and glucose homeostasis, the authors induced overexpression of the three miRNA-29 members in 3T3-L1 adipose cells. Elevated total miRNA-29 did not affect basal glucose uptake, however dramatically reduced insulin-stimulated glucose uptake (50% decrease) assessed by [3H] deoxyglucose, suggesting a negative regulation of insulin signaling. This speculation was confirmed by measuring phosphorylation of Akt at the Serine 473 site, a key prerequisite for glucose transporter-4 (GLUT4) activation. Indeed p-Akt Ser 473 protein content was significantly reduced with miRNA-29 overexpression and insulin treatment of 3T3-L1 cells. In contrast, silencing of miRNA-29 reversed the negative effects on Akt activation in the presence of insulin and similar to the effects of overexpression, basal Akt activation was unaffected. In addition, glucose uptake with miRNA-29 inhibition was unchanged (96). These data indicate compensation by other molecules, while miRNA-29 may only have an effect on certain components of the insulin signaling cascade.
Using a combination of *in vitro* and *in vivo* diabetic rat/hepatic insulin resistant models, Kurtz et al. (97) reported a role of miRNA-29 as a transcriptional activator of hepatic lipid metabolism. The authors utilized bioinformatic tools to identify binding sites on the miRNA-29 sequences and detected more than 14 binding sites for forkhead box protein A2 (FOXA2), a transcription factor negatively associated with insulin, thereby promoting lipid catabolism and oxidation. In two different rat models of T2D, high fat diet-fed and Zucker diabetic fatty rats, upregulation of miRNA-29 and FOXA2 in the liver was observed (97). To examine a relationship between FOXA2 and regulation of liver-specific miRNA-29, the FOXA2 mRNA was inhibited or overexpressed in separate isolated human hepatocytes. The authors reported robust reductions and increases of the three miRNA-29 family members with gene knockdown and transfection, respectively. Further, a series of cell culture experiments including inhibition and transfection combined with fluorescent-labeling (luciferase reporter gene assay) of the 3’ UTR of FOXA2 targets, determined a direct negative regulation of miRNA-29 with three FOXA2 targets. Three genes involved in lipid catabolism or mitochondrial fatty acid oxidation, 3-hydroxy-3-methylglutaryl-CoA synthase 2 (HMGCS2), 1-acylglycerol-3-phosphate O-acyltransferase (ABHD5) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A), were significantly decreased with FOXA2 knockdown, while HMGCS2 was identified as a direct target for miRNA-29 (97). The latter determines a direct role for miRNA-29 to regulate mitochondrial function and hepatic lipid homeostasis.

Related to miRNA function in skeletal muscle, Massart et al. (98) reported the role of miRNA-29 and its three family members using a collection of human, animal and cell culture approaches. It was observed that miRNA-29a and miRNA-29c were significantly upregulated in muscle of T2D patients and obese mice (98). When miRNA-29a and miRNA-29c were independently overexpressed in primary human myotubes, several measures of glucose metabolism were impaired including basal and insulin-stimulated glucose uptake, insulin-stimulated glycogen synthesis and insulin-stimulated glucose oxidation (only with miRNA-29c overexpression) (98).
The mechanisms underlying the defects in carbohydrate oxidation were examined via changes in predicted miRNA-29 targets at the mRNA and protein level. The authors reported that with miRNA-29 overexpression in primary human myotubes, insulin receptor substrate-1 (IRS1), phosphatidylinositol 3-kinase (PI3K) regulatory subunit 3 (PIK3R3) and AKT2 mRNA levels were significantly reduced (98). In addition, expression of GLUT1 and hexokinase 2 (HK2), though not direct targets of miRNA-29, were found to be reduced in human skeletal muscle cells. At the protein level, several upstream as well as downstream molecules involved in insulin signal transduction were observed to be modified with overexpression of miRNA-29. IRS1 and insulin-stimulated phosphorylation of Akt\(^{\text{Ser473}}\) were reduced and additionally, glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) protein and insulin-stimulated GSK3\(\alpha/\beta\)\(^{\text{Ser21/Ser9}}\) phosphorylation were reduced (98). Furthermore, the authors confirmed the defects in glucose metabolism by inhibition of miRNA-29a and miRNA-29c as well as in vivo via electroporation of mouse tibialis anterior muscle (98).

Lastly, the authors investigated the role of miRNA-29 on lipid metabolism in primary human myotubes and isolated tibialis anterior muscle. Despite unaltered mitochondrial content by miRNA-29a or miRNA-29c overexpression in vivo, PGC1A, PDK4 and cluster of differentiation 36 (CD36) mRNA levels were significantly reduced (98). The data indicate miRNA-29 may impair fatty acid oxidation and mitochondrial biogenesis before alterations in mitochondrial complexes are observed. Namely, lower CD36, a translocase involved in import of fatty acids to the cytosol, is associated with reduced palmitate oxidation in lean and obese humans (99). One mechanisms that facilitates CD36-mediated fatty acid transport involves AMPK, where AMPK activation induces the localization of CD36 to the cell surface (100). This mechanism may explain the reduction in palmitate oxidation displayed by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)-stimulated myotubes overexpressed with miRNA-29a and miRNA-29c (98). While obese humans do not express low levels of CD36 (101), the mitochondrial fatty acid transporter carnitine palmitoyl transferase-1 (CPT-1) may require interaction with CD36 to increase beta-oxidation (102). These data do not provide a direct
connection between miRNA-29 and CD36 or other mechanisms that may explain abnormal lipid metabolism induced by miRNA-29 overexpression, however it possible that elevated skeletal muscle miRNA-29 in T2D (98) impairs lipid oxidation due to lower mitochondrial fatty acid uptake.

The evidence provided by Massart and colleagues (98) aligned with a report by Dooley (103) who conducted a collection of experiments using mice with genetically knocked out (KO) miRNA-29a or miRNA-29c. The defects in glucose tolerance, elevated fasting blood glucose and impaired insulin release, however, were only observed with miRNA-29a KO mice and not with miRNA-29c KO. To identify the mechanisms related to regulation of insulin release by miRNA-29a, islet cells of wild-type mice were transplanted in miRNA-29a⁻/⁻ mice and isolated β-cells were incubated in high glucose concentrations to observe glucose-stimulated insulin secretion (GSIS). The results showed that insulin release was rescued under these conditions, suggesting an important role for miRNA-29a in regulating insulin secretion. Conversely, when exposed to an exogenous insulin challenge, miRNA-29a KO and miRNA-29c KO mice were in fact more insulin sensitive compared to controls. Here, islet transplantation did not correct insulin response to match that of wild-type mice, indicating that there may be defects related to β-cell’s sensitivity to secrete insulin in response to hyperglycemia as well as a compensation in peripheral insulin signaling (103). By injecting a PI3K inhibitor, wartmannin, in miRNA-29a⁻/⁻ mice at the point which the insulin challenge induced the greatest degree of hypoglycemia (60 min), it was observed that glycemic response was corrected to that of wild-types. Thus miRNA-29a deficient mice demonstrated prolonged insulin signaling at the PI3K level, allowing normal insulin sensitivity during an insulin tolerance test (ITT) (103). In contrast, with T2D, where miRNA29a expression has been shown to be chronically elevated, transduction of insulin signaling may be attenuated by reduced PI3K activity.

Clearly, there is strong evidence indicating a role for miRNA-29a, miRNA-29b and miRNA-29c in regulation of multi-organ function during insulin resistance. The collection of experiments conducted by the aforementioned studies demonstrated that miRNA-29 has the
ability to regulate glucose and lipid homeostasis by function of insulin signaling in adipose, skeletal and liver cells. The next important step to determine whether miRNA-29 can be used as a biomarker for insulin resistance, is to validate its detection in circulation and to determine whether its expression in serum/plasma is differentially expressed in humans.

Currently, only a few studies have examined miRNA-29 expression in humans. Dahlmans et al. (104) recently conducted a comprehensive analysis of 25 miRNAs in skeletal muscle of four distinct human metabolic groups including 1) overweight/obese patients with T2D, 2) non-diabetic overweight/obese individuals, 3) young/lean sedentary individuals and 4) endurance athletes. Notably, all participants underwent a hyperinsulinemic euglycemic clamp to assess peripheral insulin sensitivity as well as assessment of in vivo mitochondrial capacity via phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS). The authors found increased expression of all three members of the miRNA-29 family in skeletal muscle of T2D patients compared with non-diabetic overweight/obese individuals. However, there was no difference miRNA-29 isoforms between lean, sedentary individuals and athletes. In addition, expression of each miRNA-29 isoform was negatively associated with glucose infusion rate (GIR) across all metabolic groups (104), suggesting the effects of miRNA-29 are not limited to insulin signaling but may affect whole-body insulin sensitivity. Dahlmans et al. (104) were the first to provide strong evidence in human tissue, confirming the results from Karolina et al. (105) and Kong et al. (106) who also independently reported increased expression of miRNA-29 family members in blood of T2D patients. In these two studies, however, clamps were not conducted. Although, Kong et al. (106) recruited individuals who were “susceptible to T2D,” indicated by an overweight BMI and/or FH (the number of participants with a FH was not reported), miRNA-29a expression in these middle-aged men and women susceptible to T2D was similar compared with individuals with pre-diabetes (impaired glucose tolerance and/or impaired fasting glucose), while significantly lower expression was found compared with T2D. Moreover, Karolina et al. (105) identified upregulated miRNA-29a in four different tissues (pancreas, skeletal muscle, liver, adipose) as well as whole blood of diabetic rats and additionally in blood of T2D patients.
There appears to be a strong relationship between miRNA-29 and insulin sensitivity, but there remain gaps in understanding whether miRNA-29 may identify T2D risk. For instance, only one human study included the gold-standard method to assess insulin sensitivity (104). While Kong et al. (106) recruited individuals who were susceptible to T2D, the authors did not distinguish the number of subjects who’s susceptibility was due to being overweight or a FH (106). Thus, it is not known whether miRNA-29 family members are upregulated young, sedentary individuals with a FH compared with counterparts without a FH.

**miRNA-155**

miRNA-155 was introduced in the context of insulin sensitivity and energy metabolism in 2013 (107) by showing that inhibition of this miRNA in preadipocytes mediated brown adipocyte differentiation and ‘browning’ of white adipocytes. Although brown adipose tissue (BAT) was initially only considered important during fetal development, more recent studies have indicated a role for BAT in maintaining energy homeostasis and controlling adiposity in humans due to its large reservoir of mitochondria (108,109). In addition, three miRNAs typically considered muscle-specific miRNAs – “myomiRs” – were found to be expressed in BAT (110).

Previously reported to target key transcription factors involved in adipogenesis and BAT metabolism, miRNA-155 has been considered an important candidate for understanding miRNA-related mechanisms in glucose and lipid homeostasis. Chen et al. (107) induced miRNA-155 transgenic mice and overexpressed miRNA-155 in preadipocytes, demonstrating reduced BAT mass, altered BAT morphology and reduced abundance of uncoupling protein-1 (UCP1). During the progression from preadipocytes to mature adipose cells, CCAAT/enhancer-binding protein β (C/EBPβ) is a major transcriptional regulator of BAT development and UCP1 expression – a critical component of energy expenditure and fatty acid oxidation (111). The authors showed that miRNA-155 is a negative regulator of BAT function via C/EBPβ-dependent mechanisms (107). Mice overexpressing miRNA-155 showed only slight reductions in C/EBPβ expression, however there were robust reductions in BAT mass and development. When C/EBPβ was overexpressed
in preadipocytes, there was a substantial reduction in miRNA-155 expression, adipocyte differentiation and an increase in triglyceride formation. These results indicated a negative feedback loop between miRNA-155 and adipogenesis as well as BAT function (107). Therefore, miRNA-155 may limit the capacity for adipose cells to act as regulators of energy homeostasis, by inhibiting the formation of mature adipocytes. The mechanisms indicate a relationship between miRNA-155 and C/EBPβ, however clinical studies are needed to investigate whether these molecular changes manifest to whole-body impairments.

These results in vitro are in accordance with Gaudet et al. (112) who showed that deletion of miRNA-155 in adipocytes upregulated UCP1, peroxisome proliferator-activated receptor gamma (PPARγ) and genes involved glucose metabolism, GLUT4 and IRS1. In addition, the authors knocked out miRNA-155 in female mice following a 12-week high fat diet (HFD) or standard chow diet. While chow-fed mice, with intact miRNA-155, increased body weight nearly 3-fold, miRNA-155 KO mice gained no more body weight than KO mice fed a chow diet. This protection against HFD-induced obesity was not due to reduced dietary intake or increased physical activity, as miRNA-155 KO mice did not eat any less than controls and in fact, were 50% less active (112). However, energy expenditure, measured by heat production, was significantly greater in miRNA-155 KO mice, suggesting an important role for thermogenesis in preventing weight gain which was observed by upregulation of UCP1 in vitro. Furthermore, immunohistochemistry (IHC) experiments determined that a HFD induced an enlarged lipid droplet phenotype in white adipose tissue (WAT), which was protected in miRNA-155 KO mice (112).

Related to insulin sensitivity, miRNA-155 KO mice were protected against HFD-induced impairments in glucose tolerance and in miRNA-155 KO preadipocytes, GLUT4 and IRS1 were significantly increased (112). The evidence regarding miRNA-155 in adipose tissue metabolism indicates that low expression of this marker would confer a favorable metabolic phenotype. However there is a strong contradiction reported by Lin et al. (74) who found significantly low
levels of circulating miRNA-155 in serum of T2D patients, which was negatively correlated with HOMA-IR and not with the homeostatic model assessment of β-cell function (HOMA-β).

To understand the mechanisms behind miRNA-155 related impairments in insulin sensitivity and not in β-cell function, the authors generated mice globally overexpressing or knocked out miRNA-155. Interestingly, miRNA-155 had no apparent effect on the pancreas, as β-cell morphology, mass and function were unaltered in either of the experimental mouse conditions (74). However, results from glucose tolerance tests (GTT) and insulin tolerance tests (ITT) identified a positive role for miRNA-155 in controlling glucose handling. Specifically, transgenic mice were more sensitive to an insulin challenge and more effectively lowered blood glucose compared to controls, while GSIS tests determined these improvements were not due to greater insulin secretion. Hence, the improvements in glucose metabolism are likely to due to miRNA-155’s action on peripheral insulin signaling. In addition, global overexpression of miRNA-155 protected against impairments in glucose tolerance induced by a HFD, observed by lower fasting glucose and lower area under the curve (AUC) compared to controls fed a HFD (74).

In addition, assessment of glucose uptake in two different hepatic cells lines (human 7402 and murine hepa 1-6) and in myoblasts (mouse C2C12) was shown to be significantly greater with miRNA-155 overexpression (74). To determine whether glucose utilization mirrored the improvement in glucose uptake, several positive and negative regulators of glucose metabolism in WAT, BAT, the liver and skeletal muscle were assessed in miRNA-155 transgenic mice. Of the positive regulators assessed, glucokinase (key regulator of hepatic glycogen storage), pyruvate kinase M2 (PKM2), GLUT1 and GLUT4 mRNA levels were increased while pyruvate dehydrogenase kinase 4 (PDK4) was downregulated (74). Among the negative regulators of insulin sensitivity, C/EBPβ, histone deacetylase 4 (HDAC4), phosphatase and tensin homolog (PTEN), suppressor of cytokine signaling 1 (SOCS1) and SOCS3 were all reduced in oxidative tissues. At the protein level, insulin-stimulated phosphorylation of IRS-1 and Akt was enhanced in all four tissues observed, confirming the hypothesis that peripheral insulin signaling was
mediating the improvements in whole-body insulin sensitivity with upregulated miRNA-155 (74).

The studies described here portray opposing functions of miRNA-155 in animal and cell culture models of obesity/insulin resistance. Although the primary outcome measures for the investigations conducted by Chen et al. (107) and Gaudet et al. (112) did not include insulin sensitivity, the experiments in both studies included assessments of lipid and glucose metabolism. Gaudet and colleagues (112) showed that miRNA-155 deletion in mice, protected against glucose intolerance induced by a HFD, whereas Lin et al. (74) found that miRNA-155 overexpression rather than deletion, protected against HFD-induced glucose intolerance. Notably, in the study conducted by Gaudet et al. (112), most of the improvements in metabolic health occurred only in female mice and male mice demonstrated little to no changes with miRNA-155 deletion. Potentially, there may be sex-specific alterations in metabolism that can explain the resistance to improvements in male mice. The disparities in conclusions by the different studies may not be explained by the mouse line, however, as all three investigations included C57BL/6J mice.

The translational aspect of the Lin et al. (74) study, where miRNA-155 in the serum of 30 T2D patients was shown to be downregulated compared to 30 healthy subjects, provides convincing evidence in humans which is not provided by Chen et al. (107) or Gaudet et al. (112). These results are in accordance with the data provided by Corral-Fernandez et al. (113) who also showed lower expression of miRNA-155 in T2D patients, which was associated with hemoglobin A1c (HbA1c), fasting blood glucose and body mass index (BMI). Further, Fichtlscherer et al. (114) reported significantly lower serum miRNA-155 in patients with coronary artery disease. While the animal and cell culture models report contradicting conclusions, the evidence in humans thus far lends to the idea that more data from human participants including a combination of circulating and tissue-specific expression is needed to make conclusions regarding the function of miRNA-155. Nonetheless, there appears to be a role for miRNA-155 in regulating whole-body and cellular insulin resistance. Future studies to
examine the relationship between miRNA-155 and T2D progression warrant further investigation.

**miRNAs and Exercise**

Exercise is considered a powerful stimulus of several biological processes including acute responses and chronic adaptations. A myriad of cellular and systemic alterations induced by exercise, thus have significant influences on the metabolic health of various tissues (115). As with diseases such as T2D, it is important to examine the cross-talk between various tissues required to exhibit whole-body adaptations. The premise that skeletal muscle is a critical mediator of energy metabolism, particularly with exercise, has long been understood (116), however the idea of skeletal muscle as an endocrine organ did not arise until several decades later (117,118). In a study of trained endurance runners participating in a 20 km race, the cytokines interferon-γ (IF-γ), TNF-α, interleukin-1β (IL-1β) and IL-6 were upregulated immediately after the race in plasma or urine, suggesting a link between exercise and enhancements in the immune system (117). Evidence from B. K. Pederson and colleagues (118) identified that skeletal muscle secretes IL-6 during prolonged exercise, an inflammatory marker that has since received considerable attention in the context of exercise inflammatory responses (119). These data set the stage for subsequent work to investigate both the mechanisms and clinical significance of exercise-induced cross-talk to enable improvements in health. Given the central role of skeletal muscle with exercise to regulate endocrine actions and recent discoveries related to miRNA secretion, it is important to address how exercise and miRNA in the circulation contribute to changes in energy metabolism.

Endurance and resistance exercise are each considered potent stimuli for metabolic responses and long-term adaptations including improvements in insulin sensitivity (120–124). The mechanisms that drive the metabolic improvements with each exercise mode continue to be explored, however it is well-understood that exercise poses major energy demands of several tissues, and accordingly it is likely that a whole-body coordination to meet these energy demands
occurs. Evidence has shown that a single bout of exercise as well chronic exercise training have the capacity to induce a secretory response which alters circulating miRNAs (125). The emergence of miRNAs in the bloodstream, thus introduces a critical insight to establish molecular links between specific exercise treatments and disease prevention by understanding the cross-talk between tissues. The understanding of circulating miRNAs has the potential to progress efforts by the medical and scientific communities to provide individualized patient care.

miRNAs, in the context of exercise, were introduced by examining muscle-enriched miRNAs (myomiRs) in skeletal muscle of healthy, young males before and immediately after a bout of moderate intensity cycle exercise at baseline and after 12 weeks of training (61). Two of the four myomiRs tested (miRNA-1, miRNA-133a) were found to be upregulated acutely with exercise at baseline, but unchanged after a bout of exercise post-intervention. This suggested that in the untrained state, these myomiRs may respond to the greater degree of stress that is corrected by an ability to maintain homeostasis in the trained state. With evidence that miRNAs respond to exercise, this study initiated questions regarding the mechanisms and functional roles of specific miRNAs to mediate the responses to exercise.

Given the fundamental role of skeletal muscle in energy metabolism, the investigation of myomiRs during exercise, provided important grounds for future studies. A report by Baggish et al. (126), soon after, determined a potential value for studying miRNAs in the circulation following exercise. Collegiate rowers were recruited for analysis of circulating miRNAs immediately before and after a maximal exercise test. The miRNAs -21, -146a, -221 and -222 were found to be significantly upregulated acutely, while all (except miRNA-221) returned to baseline 1h post-exercise. Following 90 days of a controlled rowing program, changes in miRNA expression with acute exercise were re-examined. The increases in miRNA-146a and -222 with acute exercise were more profound post-intervention compared to baseline, while basal miRNA-21 and -221 were upregulated post-intervention and unaltered acutely with exercise. Interestingly, there was a strong relationship between the increase in miRNA-146a during a bout of cycling exercise and baseline cardiorespiratory fitness (VO₂max) (126). These data indicated a
role of circulating miRNAs in regulating the physiological responses to exercise, including whole-body adaptations such as cardiorespiratory fitness.

Despite limited research, the circulating miRNA response with exercise has implications for improving health outcomes at a group or individual level. High responders to a 16-week diet and exercise intervention displayed a different profile of circulating miRNAs compared with low responders (127). Thus, the 13 circulating miRNAs displayed in individuals with the greatest weight loss may reveal an individual’s potential to respond to a resistance training and diet program. In addition, the effects of resistance exercise may vary between age groups, as the expression of 10 miRNAs were upregulated in young men, but not healthy, older men after a bout of resistance exercise (128). In a mouse model of disuse atrophy, serum miRNA-129 is significantly downregulated compared with young, healthy controls (129). Since elderly adults lose muscle tissue more rapidly than young adults following physical inactivity (130), the ability to regain muscle mass and function may be represented by circulating miRNA expression. Thus, it is possible that circulating miRNAs may become biomarkers of age-related losses in muscle mass and may reflect adaptations with resistance exercise training.

It is important to consider the type of training when considering the circulating miRNA response. Four miRNAs (miRNA-21, -146a, -221, 222) were significantly upregulated in resting endurance athletes compared with strength athletes (131), suggesting that glycolytic compared with oxidative capacity rely on different miRNA actions. Moreover, these same four miRNAs were measured after a single bout of resistance exercise in recreationally active young men, but no significant alterations were displayed immediately after exercise (132). Although, miRNA-146a and miRNA-221 significantly declined compared with baseline three days after the resistance exercise session (132), in agreement with the notion that a strength-trained phenotype exhibits lower miRNA-146a and -221 (131). Interestingly, one study examined whether miRNAs in skeletal muscle compared with circulation exhibited a similar response after resistance exercise. D’Souza et al. (133) identified six miRNAs in muscle that were significantly affected by a single bout of high intensity resistance exercise, however only minimal alterations of the
same miRNAs occurred in circulation. Among the miRNAs significantly altered by this resistance exercise session, miRNA-133a and -146a were upregulated in muscle and circulating miRNA-133a was significantly elevated four hours post-exercise, but not immediately after the session (133). This suggests more research is needed to determine the time-course of miRNA secretion. It is conceivable that miRNA-133a exhibits a response in tissue before being released in circulation either as a by-product or to act on other tissues. In addition, resistance-trained individuals may regulate miRNAs differentially (in tissue and circulation) compared with endurance-trained (131) and elderly individuals (128).

While resistance exercise studies that examine circulating miRNAs have generally demonstrated the response related to functional strength outcomes (i.e., one-repetition maximum (1RM)), endurance exercise studies indicate that differential expression of several miRNAs may be related to improved health outcomes such as hypertension (134), bone disease (135) and aerobic capacity (136). The pool of circulating miRNAs associated with cardiac muscle damage grows with increased exercise duration concomitant with markers of inflammation (137). The elevations of miRNAs associated with cardiac dysfunction after a marathon quickly returned to baseline during recovery. This provides evidence that these miRNAs may reflect the extent of heart damage, important for treatment of individuals with cardiovascular disease. The insight into the role of circulating miRNAs in vascular function extends to other conditions including chronic kidney disease (CKD) (138). Patients in various stages of CKD demonstrate lower aerobic capacity and plasma miRNA levels of miRNA-146a and -150 are associated with estimated glomerular filtration rate, pulse wave velocity and VO2peak (138).

In healthy individuals, the endurance exercise protocols have varied; nonetheless demonstrating significant alterations in miRNA response. Cui et al. (139) compared the effects of high intensity interval exercise vs. vigorous intensity continuous exercise on the circulating profile of miRNAs. Immediately after exercise, there was an increase in the same 13 miRNAs regardless of the protocol (139). Conversely, uphill running induced upregulation of a different set of miRNAs in the circulation compared with downhill running (140), suggesting exercise
intensity may affect the release certain miRNAs into circulation. In contrast, miRNA-126 and miRNA-133 are upregulated regardless of exercise mode (141). However, basal expression of these two miRNAs were not affected by chronic exercise (126). Gomes et al (142) characterized miRNA profiles affected by varying endurance exercise durations and intensities (increasing treadmill running duration or speed). miRNA-1 responded in an intensity-dependent manner while miRNA-133a and -222 increased in a dose-dependent manner with duration (142). Interestingly, the upregulation in plasma miRNA-133a was concomitant with a depletion of muscular miRNA-133a, supporting the hypothesis that certain miRNAs may perform intracellular actions before secretion.

It is clear that circulating miRNAs have been a prominent topic for exercise physiologists in recent years, as distinct miRNAs continue to emerge (62,125,143). Furthermore, the list of biological pathways and health contexts related to exercise and miRNAs continues to grow (125). In turn, the advancements in exercise-related miRNA research appear to be expanding the range of targets, while the depth of understanding specific functions remains underdeveloped. This is a challenge for researchers to focus on certain miRNAs as potential therapeutic targets since > 100 circulating miRNAs are reported in response to exercise (62,125,143). While some studies evaluate miRNAs with high-throughput screening to assess large groups of miRNAs affected by exercise (132,144), many select a cluster of miRNAs to study (126,136,140,141,145,146). Therefore, researchers are challenged with selecting from a pool of dozens of miRNAs that may be relevant, but may have limited support. In this context, there are gaps in the literature to determine the roles of miRNAs related to specific health outcomes such as the degree of insulin resistance.

It is not understood to what degree exercise mode or intensity affect circulating miRNAs. Further, most exercise studies focus on single bouts of endurance or resistance exercise, therefore the effects of chronic exercise are limited. To our knowledge, there is only one study reporting the effects of exercise training on insulin sensitivity-related miRNAs in circulation (147). Among the miRNAs identified by exercise studies, miRNA-133 has been reported to be
altered by different protocols (61,133,139,141,146). In addition, miRNA-133 may be regulated by insulin resistance, as Gallagher et al (148) identified significantly lower expression of miRNA-133a in T2D patients.

**miRNA-133**

miRNA-133 and its subfamily, miRNA-133a and -133b, have received considerable attention related to changes in its expression with acute aerobic exercise (65,126,128,132,139–141,145,146,149–153). Results from these studies have been conflicting, however, indicating increased, decreased or no change in expression of circulating miRNA-133. As a result, there are challenges in drawing firm conclusions related to the roles and functions of this miRNA. However, this can be attested to the lack of consistency among exercise protocols/bouts. Nonetheless, the nature of circulating miRNA-133 deserves attention considering its expression level has been shown to be perturbed by exercise.

There is evidence from 11 studies reporting significant increases in one of the circulating miRNA-133 members, if not both, with acute exercise. The exercise modes in these studies ranged from high intensity interval cycling to a marathon and primarily included healthy, trained men. Seven studies assessed miRNA-133 expression before and after a marathon race and six of these identified acutely upregulated miRNA-133. Baggish et al. (149) found increased expression in 21 participants of the Boston Marathon with previous marathon experience. The authors compared the changes in miRNA expression with markers of skeletal muscle damage and inflammation including creatine phosphokinase (CPK), troponin 1 and high-sensitivity c-reactive protein (hsCRP). There was significant upregulation of CPK and troponin 1 immediately after the race along with increased miRNA-133a, suggesting that miRNA-133a may be involved in mediating muscle damage and/or inflammation during prolonged exercise. Interestingly, CPK remained elevated 24 hours after the race, while miRNA-133a returned to basal expression and the change in miRNA-133a was not correlated with any of the damage or inflammatory markers
Thus, it is possible that miRNA-133a induces muscle damage, but the sustained CPK levels are influenced by other molecules.

These data are in accordance with results shown by Clauss et al. (154) in both elite and non-elite marathon runners participating in the Munich Marathon. In both elite and non-elite runners, miRNA-133a was significantly increased immediately after the race and the creatine kinase-MB (CK-MB) isoform was increased only in non-elite runners. In this study, however, post-race miRNA-133a was positively correlated with creatine kinase (CK) in all runners, CK-MB in elite runners and troponin in non-elite runners. Similar to results from the Boston Marathon study, CK and CK-MB remained elevated 24h after the Munich race, while miRNA-133a returned to baseline. Clauss and colleagues speculated that damaged cells released from skeletal muscle or cardiac muscle tissue may secrete miRNA-133a (154), however mechanistic studies are needed to confirm this idea. This speculation is consistent with the upregulation of circulating miRNA-133 expression found in participants engaging in downhill backward walking, a highly muscle-damaging eccentric exercise but not with uphill walking, considered a more concentric movement with less muscle damage (140). Uhlemann et al. (141) conducted a cross-sectional analysis to investigate the effects of varying intensities of exercise on miRNA-133 expression including steady-state cycle exercise, a maximal exercise test and a marathon in healthy, trained men and women (no women were included in the steady-state cycle exercise or marathon). These results confirmed the premise that miRNA-133 may be involved in muscle damage, as the only exercise mode that induced upregulation of circulating miRNA-133 was the marathon (141).

In the marathon study that did not identify changes in miRNA-133 expression (no change in miRNA-133b; miRNA-133a was not detected), only 9 participants were included, although de Gonzalo-Calvo and colleagues provided an extensive summary of the methodological approaches to miRNA analysis (150). Similar to the other marathon studies, several inflammatory markers were upregulated post-race and de Gonzalo-Calvo identified a large miRNA profile that was upregulated immediately after the race that did not distinctly include
either of the miRNA-133 isoforms. The authors did not specifically address the discrepant findings in miRNA-133 expression, however they found conflicting results related to other miRNAs as well, and attributed these differences to environmental background and age (150).

The extent to which miRNA-133 is affected by exercise-induced inflammation and muscle damage after prolonged exercise remains unclear, although there appears to be a link between damaging exercise and release of miRNA-133 to the circulation. The findings of Mooren et al. (136) challenge the notion that miRNA-133a is solely related to inflammation, however. In trained endurance runners, there was an association between the change in miRNA-133a and VO$_2$max as well as running speed at lactate threshold (136). Interestingly, the populations in these studies are comparable (young-middle aged runners) and therefore contribute to the complexity of understanding the direct relationships between miRNA secretion and the observed outcome measures. It is possible that the source of miRNA secretion (i.e., lymphocytes, skeletal muscle, liver) plays a distinctive role in the target cell/tissue as well as the subsequent molecular pathway that is involved.

Only two studies have examined the effects of resistance exercise on circulating miRNA-133 expression. In the findings with resistance exercise compared with those of aerobic exercise, there is a clearer message related to muscle damage and increased miRNA-133. In the study comparing different forms of exercise, Uhlemann et al. (141) also implemented a resistance exercise protocol in healthy men and women that aimed to induce a high degree of muscle damage (141). Participants performed three different resistance training exercises using specialized weight training machines to increase the load by 25% during the eccentric movement. Along with increased CPK activity, the authors found a two-fold increase in serum miRNA-133 expression (141). To corroborate the miRNA-133 and skeletal muscle damage hypothesis, a separate resistance exercise study examined the effects of bench press and leg press exercises. The exercises consisted of a moderate load (70% of 1-repetition maximum [RM]) and assistance was provided when the load became too heavy. As expected with a less exhaustive
protocol, the authors did not find increased miRNA-133a expression nor any significant correlations with markers of inflammation or muscle damage (141).

Tissue-specific analysis of miRNA-133 expression was investigated in a mouse model of skeletal muscle hypertrophy. McCarthy & Esser (155) implemented a synergistic ablation technique to induce hypertrophy of the plantaris muscle. Here, two synergists of plantar flexion were completely ablated, thus subjecting the plantaris to functional overload. After 7 days of overload with resistance exercise, there was a 45% increase in muscle wet weight and a 50% reduction in miRNA-133a compared to controls with intact synergist muscles (155). These data suggest that miRNA-133a expression may inhibit skeletal muscle hypertrophy via suppression of mRNAs involved in muscle growth, although the direct targets and their direct link have yet to be investigated.

Together, the evidence regarding miRNA-133 in skeletal muscle function indicates a relationship between exercise and acute muscular damage as well as serving as a potential marker for maximal aerobic capacity. The data thus far supports the former, while further investigation is warranted to confirm the latter. Interestingly studies have included either aerobic or resistance exercise protocols, however there is no evidence that has determined whether combined exercise induces significant alterations in the circulating expression of miRNA-133a or -133b.

As it relates to chronic exercise, miRNA-133 has only been investigated by three studies. In the study including college rowers, Baggish et al. (149) did not find changes in circulating miRNA-133a basal expression or with acute exercise after 90 days of rowing exercise training. Nielson et al. (153) similarly did not find a significant difference in plasma miRNA-133a or -133b after 12 weeks of cycle exercise. In this study, however, stringent statistical analysis was conducted; the authors used a 0.00032 significance level with a Dunn-Bonferroni correction method to minimize false positives at the 0.05 significance level. An additional “borderline significance level” was set for samples with p-values less than 0.001 (153). The authors reported that both miRNA-133 isoforms were borderline significantly upregulated immediately following
a cycle exercise test after the training intervention. In addition, basal expression of both miRNA-133 isoforms was borderline significantly downregulated post-training. In a four-week study of cycle exercise, Aoi et al. (145) also did not find a difference in circulating miRNA-133 expression.

The relevance of miRNA-133 with exercise training remains inconclusive. Although only studies including acute muscle-damaging exercise have found increased miRNA-133 expression, this circulating molecule serves the potential to become a marker for hypertrophic response or resistance to the effects of combined exercise. Considering one study identified a relationship between maximal aerobic capacity and miRNA-133 (136), as well as one study found that repressed miRNA-133a may be involved in muscle hypertrophy (141), it is important to investigate the interaction between miRNA-133 and these two divergent exercise modes. Moreover, it is not known whether chronic exercise training, particularly combined training, alters the expression of circulating miRNA-133a or -133b. Therefore, our study will provide a comprehensive analysis of the relationship between acute as well as chronic exercise and both miRNA-133 isoforms. Additionally, the role of miRNA-133 with exercise and insulin sensitivity has yet to be reported.

**Specific Aims and Hypotheses**

Defining the role of circulating miRNAs and exercise has been an elusive topic thus far for several reasons including the challenges in identifying the source of release, standardization in measurement techniques, lack of consistency in exercise protocols and a lack of studies in general. Nonetheless, the potential use for circulating miRNAs as clinical biomarkers, underlie the need to progress the understanding of miRNAs and training adaptations. Reviews of circulating miRNAs related to acute and/or chronic exercise have been published recently, together outlining more than 100 distinct miRNAs (125,143,156). The challenge in drawing conclusions, in addition to the reasons listed above, lie in the poor overlap of miRNAs between studies. Similarly, some studies do not identify the use of specific isoforms when more than one exist. While it has been common to evaluate individual miRNAs selected based on a priori
hypotheses, the pool of potential miRNAs poses a serious challenge in making significant strides to understand specific roles of miRNAs. Ideally, future studies will include a combination of approaches using animals and/or cell culture as well as validation in humans. These studies will provide strong evidence for selection of candidate miRNAs related to exercise and establish a foundation for subsequent work.

Although comprehensive exercise studies are limited, there have been considerable efforts in the context of T2D including both human and pre-clinical data. Lin et al. (74) and Massart et al. (98), for example, reported the mechanisms of miRNA-155 and miRNA-29, respectively, in cell culture models and concurrently measured their expression in T2D patients. The roles of individual miRNAs and glucose metabolism are better understood compared with exercise, therefore an investigation to bridge the gap between mechanistic studies and human exercise studies is warranted. With the rates of obesity and insulin resistance reaching epidemic proportions, as well as levels of physical inactivity continuing to rise, it is imperative to identify biomarkers that can detect insulin resistance before the onset of T2D. Individuals with a greater predisposition to diabetes, such as those with a FH, are therefore an important population to target. Furthermore, to understand whether exercise-induced improvements in insulin sensitivity can be detected by circulating miRNA can lead to more effective prescription of exercise. Our study aims to define a relationship between seven circulating miRNAs, insulin sensitivity assessed by the gold-standard hyperinsulinemic euglycemic clamp and eight weeks of combined exercise training. Additionally, we will be the first to report the effects of combined exercise training on circulating miRNA expression in humans as well as the effects of a family history of type 2 diabetes on changes in circulating miRNA with exercise training.

Specific Aims

1. To determine whether a family history of type 2 diabetes alters the circulating profile of insulin sensitivity and exercise-related miRNAs in young, normoglycemic males (miRNA-29a, miRNA-29b, miRNA-29c, miRNA-133a, miRNA-133b, miRNA-143 and miRNA-155).
2. To determine the effects of 8 weeks of combined exercise training on circulating miRNAs (miRNA-29a, miRNA-29b, miRNA-29c, miRNA-133a, miRNA-133b, miRNA-143 and miRNA-155).

3. To determine whether exercise-induced improvements in insulin sensitivity, metabolic flexibility and aerobic capacity are associated with circulating miRNAs (miRNA-29a, miRNA-29b, miRNA-29c, miRNA-133a, miRNA-133b, miRNA-143 and miRNA-155).

**Hypotheses**

We hypothesize that 1) individuals with a FH will exhibit repression of miRNA previously shown to be positive regulators of insulin sensitivity (miRNA-143 and miRNA-155) and elevation of miRNA previously shown to be negative regulators of insulin sensitivity (miRNA-29 family members), 2) eight weeks of combined exercise training will normalize any aberrations in circulating miRNA expression despite a FH and 3) eight weeks of combined exercise training will increase the expression of miRNA-133a and -133b in serum of young, normoglycemic males due to the degree of energetic stress imposed by combined exercise.
Chapter 2: Methods

Clinical Exercise Trial

19 normoglycemic, healthy, sedentary Mexican-American males between the ages of 18 and 40 years were recruited to participate in this study and were assigned to one of two groups based on the absence of familial history of type 2 diabetes (FH-, n=9) or one/both parent(s) diagnosed with type 2 diabetes (FH+, n=10). The study protocol was approved by the UTEP Institutional Review Board (IRB). Each participant signed a written informed consent form.

Figure 3. Study Design

Upon subject recruitment, an over-the-phone questionnaire was administered to determine eligibility, including Mexican-American status and familial history of Type 2 Diabetes. After informed consent was obtained during the subject’s visit to the clinic, it was determined whether subjects met any exclusion criterion (Table 1). If no exclusion criteria were met, subjects were issued an accelerometer to assess physical activity levels. Less than 60 minutes spent in moderate to vigorous physical activity per week was considered sedentary (1). If the participant met the sedentary physical activity criteria, the participant was then instructed...
to return for assessments of maximal aerobic capacity (VO₂max), upper and lower body muscular strength (1-repetition maximum [1RM]) and body composition.

Table 1. Inclusion & Exclusion Criteria.

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Exclusion Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 18 ≤ Age ≤ 40 years</td>
<td>1. Evidence of significant cardiovascular disease or diabetes</td>
</tr>
<tr>
<td>2. 18 ≤ BMI ≤ 30 kg/m²</td>
<td>2. A fasting blood glucose ≥100 mg/dl</td>
</tr>
<tr>
<td>3. Both parents are</td>
<td>3. Screening blood pressure ≥140/90</td>
</tr>
<tr>
<td>4. Mexican/Mexican-American)</td>
<td>4. Hyperlipidemia- Total cholesterol ≥240mg/dl</td>
</tr>
<tr>
<td>5. Sedentary lifestyle</td>
<td>5. Use of drugs affecting energy metabolism or body weight</td>
</tr>
<tr>
<td>Less than 60 minutes/wk. of moderate to vigorous intensity physical activity (MVPA)</td>
<td>6. Excess alcohol, drug abuse, and smoking</td>
</tr>
<tr>
<td></td>
<td>7. Eating disorder or eating attitudes interfering with study</td>
</tr>
<tr>
<td></td>
<td>8. Unwillingness to be abide by randomization</td>
</tr>
</tbody>
</table>

Table 2. Screening Measurements

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary Physical Activity Level</td>
<td>Physical activity questionnaire &amp; Time spent in Moderate to Vigorous Physical Activity (MVPA) &lt;60 minutes per week.</td>
</tr>
<tr>
<td>Fasting Blood Glucose</td>
<td>Blood sample via lancet stick/Analysis via True Result Blood Glucose Meter</td>
</tr>
<tr>
<td>Lipid Profile (screening) (HDL, LDL, Total Cholesterol)</td>
<td>Blood sample via lancet stick/Analysis through Point of Care Testing (Alere, Cholestech LDX)</td>
</tr>
<tr>
<td>Blood Pressure</td>
<td>Automated Blood Pressure Device</td>
</tr>
<tr>
<td>Body Mass Index (BMI)</td>
<td>Height &amp; Weight Measurements</td>
</tr>
<tr>
<td>Medical History</td>
<td>Questionnaire</td>
</tr>
</tbody>
</table>

Table 3. Clinical Study Outcome Measures

<table>
<thead>
<tr>
<th>Variable</th>
<th>Method of Measuring</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Composition</td>
<td>Air displacement plethysmography (BodPod) &amp; Dual Energy X-ray Absorptiometry (DXA)</td>
</tr>
<tr>
<td>Maximal Aerobic Capacity</td>
<td>Standard Graded Treadmill Exercise Test</td>
</tr>
<tr>
<td>Insulin Sensitivity</td>
<td>Hyperinsulinemic-euglycemic clamp</td>
</tr>
<tr>
<td>Glucose Tolerance</td>
<td>Oral Glucose Tolerance Test (OGTT)</td>
</tr>
</tbody>
</table>
Table 4. Data Collection Schedule

<table>
<thead>
<tr>
<th>Screening</th>
<th>Baseline</th>
<th>Exercise Training</th>
<th>Post Training</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S-1 S1-S7 B1 B2 B3 B4 B5 B6</td>
<td>D1 &amp; D2</td>
<td>D-2 D1 D2 D3 D4 D5 PT1 PT2</td>
</tr>
<tr>
<td>Accelerometer applied</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accelerometer removed- MVPA</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Consent Form</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasting glucose screening</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipids- Screening</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO2 max</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Bod Pod</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>DXA</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>OGTT</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Clamp</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Insulin</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Glucose</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Myokines</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>RMR</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>1 RM</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Diet (55%Cho/15%Pro/30&amp;Fat)</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Exercise training</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Subjects were provided a five-day standard diet (55% carbohydrate, 15% protein, 30% fat) to control for dietary effects on insulin sensitivity. Subjects reported any food allergies prior to the study. Participants were instructed to arrive at the UTEP Health Sciences building the day of the clamp after an overnight fast for assessment of insulin sensitivity and metabolic flexibility using the hyperinsulinemic euglycemic clamp. Indirect calorimetry was used to assess resting metabolic rate and metabolic flexibility before and after insulin stimulated conditions during the clamp. Fasting blood samples were collected to determine miRNA expression, a complete blood metabolic panel, thyroid function, lipid profile, insulin, and myokine concentrations.

Subjects were instructed to report the Monday after the baseline hyperinsulinemic euglycemic clamp for the first day of training. Exercise training consisted of 3 combined exercise training sessions (aerobic & resistance exercise) per week for a total of eight weeks. During the first week of training, blood was collected before and immediately after the first bouts of aerobic exercise (Table 2; day 1 of week 1) and resistance exercise (Table 2; training day 2 of week 1) Similarly, blood was drawn before and immediately after the last bouts of aerobic
(Table 2; day 1 of week 8) and resistance (Table 2; day 3 of week 8) exercise after the intervention to assess the effects of acute aerobic/resistance exercise in the sedentary vs. the trained state. 1RM was assessed at baseline, mid-intervention and post-intervention (Table 2). All outcome variables performed at baseline were repeated after eight weeks of combined exercise training.

**Physical Activity Measurement**

After eligibility was confirmed, sedentary level of physical activity was determined by calculating the time spent in moderate to vigorous physical activity (<60 minutes per week) (1). An ActiGraph activity Monitor (GT3XP-BTLE 2GB) was clipped to the belt or pants of the subjects closest to the anterior superior iliac crest and was worn for 7 days (Monday-Sunday) including sleep time. After the 7-day wear time (>90% wear time), the subject reported to the research facility for determination of physical activity level.

**Air Displacement Plethysmography (BodPod)**

Participants were asked to sit in an air displacement plethysmography chamber (BodPod) (Life Measurement, INC. Concord, CA) for approximately 45 seconds, maintaining the sitting position and normal breathing. Compression/tight fitting clothing such as tights/spandex, along with a swimming cap were required. The subject’s body mass, body volume, and amount of air displaced were determined to calculate the percentage of fat and fat free mass.

**Dual Energy X-ray absorptiometry (DXA)**

Following an overnight fast, subjects were asked to lie supine on the scanner table of a GE Dual Energy X-ray Absorptiometry (GE Medical Systems. Madison, WI) system. Arms were kept close to the body while knees and ankles were lightly strapped to prevent movement in the lower extremities (157). The scanner bar traveled from head to toe. The time spent for the measurement was about 15 to 20 minutes, depending on the body the size of the subjects. Lean mass, fat mass, bone mineral density (BMD), and bone mineral content (BMC) measurements were obtained.
Maximal Aerobic Capacity

Maximal aerobic capacity (VO$_{2\text{max}}$) was measured using a standardized graded treadmill laboratory protocol. Subjects began the test on a treadmill at 4.8 km/hr and a 3% incline for the first 3 minutes to determine the oxygen consumption at an absolute workload followed by a 2-minute warm up at 8 km/hr at 0% incline. The remainder of the test was performed at a comfortable speed (8 - 9.6 km/hr) pre-selected by the participant while increasing 1% incline each minute. Subjects’ rate of perceived exertion (RPE) and heart rate (HR) were monitored throughout the test. Once the subject reached volitional exhaustion the test was terminated. Standard measurements of VO$_2$ and VCO$_2$ were used to determine respiratory exchange ratio (RER) throughout the exercise test using a Parvo Medics TrueOne 2400 metabolic measurement system (Salt Lake City, UT). Maximal aerobic capacity was determined once the subject met 2 out of the 4 following criteria: 1) respiratory exchange ratio (RER) >1.1, 2) ±10 beats per minute from age predicted maximal heart rate, 3) RPE >17, 4) plateau in oxygen consumption with increased intensity for 1 minute, as previously described (158).

Hyperinsulinemic Euglycemic Clamp

The hyperinsulinemic euglycemic clamp is considered the gold-standard measurement for insulin sensitivity (159). Participants were asked to arrive at the UTEP Health Sciences building following an overnight (12-hour) fast. A catheter was inserted into a forearm vein for infusion of insulin and glucose; another catheter was inserted into a contralateral dorsal hand vein warmed by a heating blanket for arterialized-venous blood sampling. Prior to insulin and glucose infusion, fasting insulin and blood glucose samples (6 ml for each) were obtained. A primed continuous infusion of regular insulin (2.5 ml) (Humulin, Eli Lilly and Co., Indianapolis, IN) was added to a 250 ml saline bag and was administered at an initial rate of 313 mU/min/m$^2$. This large bolus of insulin was used to suppress endogenous β-cell insulin secretion. The initial insulin loading dose of 80 mU/min/m$^2$ was calculated by multiplying the body surface area (BSA) by the desired insulin dose (80 mU/min) then dividing this product by 1000 (conversion...
to milliliters) and multiplying the remainder by 60 (minutes in an hour). This product yields the insulin infusion rate (ml/h). Insulin was infused along with a 20% dextrose solution to maintain blood glucose at a concentration of 90 mg/dl throughout the clamp period. A 0.5 mL blood sample from the forearm vein was drawn every 5 minutes throughout the duration of the clamp. A waste sample of 1.5 times the dead space of the micro-bore extension set (approximately 1 mL) was withdrawn prior to each collection of the 0.5 mL blood sample. The samples were analyzed immediately for glucose concentrations (YSI 2300 STAT Plus Glucose/Lactate Analyzer). Insulin concentrations were measured from blood drawn pre- and post-clamp. Insulin sensitivity was determined from the glucose disposal rate (mg/kg estimated metabolic size (EMBS)/min) during the last 15 minutes of the clamp (160).

Following cessation of insulin administration, the final glucose infusion rate (ml/hr) was increased by 30% for 10 min to prevent hypoglycemia, as endogenous insulin secretion is ensued. Once euglycemia was maintained, the glucose infusion rate was decreased to 10% of the final glucose infusion rate for 5 minutes. At the end of this 15-minute period, if euglycemia was maintained (i.e. 3 consecutive concentrations within 10mg/dl), there was cessation of glucose infusion. The participant continued to be monitored for 30 minutes. Blood samples were collected at 10 minute intervals. Following the 2-hour procedure, the participant was provided a snack and beverage and allowed to leave the laboratory.

**Oral Glucose Tolerance Test (OGTT)**

Participants were instructed to avoid eating, drinking, smoking or participating in strenuous exercise during the 10-12 hours prior to testing. Participants were asked to arrive at the UTEP Health Sciences building following an overnight (12-h) fast. Participants remained seated while a fasting blood sample was collected via finger stick. The participant then orally ingested 75 grams of glucose dissolved in water as quickly as possible. Blood samples were collected at timed intervals of 15, 30, 60, 90, 120, 150, and 180 minutes following ingestion of the glucose. Blood glucose was analyzed via true result handheld blood glucose meter. Glucose tolerance was
assessed from the area under the curve determined from blood glucose concentrations during the 3 hours of the test.

**Resting Metabolic Rate**

After an overnight fast, resting metabolic rate (RMR) was measured via indirect calorimetry using a Parvo Medics TrueOne 2400 metabolic measurement cart (Salt Lake City, UT). On clamp days, RMR and respiratory quotient (RQ) were determined via indirect calorimetry. Participants were placed into a semi-recumbent position and a hood was placed over the head for measurement of oxygen utilization and carbon dioxide production. Participants were instructed to breathe normally during the 30-min collection period. The procedure was completed in fasting conditions and repeated during the last 30-min of the clamp (insulin stimulated conditions).

**Metabolic Flexibility**

The ratio of CO$_2$ expired to volume of O$_2$ inspired (i.e., RQ) acquired during indirect calorimetry was used to calculate metabolic flexibility, as previously described (22). Delta RQ was derived by subtracting the fasting RQ from the insulin-stimulated RQ. This value was used to determine to what degree subjects were able to shift from fat to carbohydrate oxidation during upon insulin stimulation.

**Lipid Profile**

On the same day of the clamp, an intravenous blood sample was obtained from the IV line for analysis of low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides (TG) and total cholesterol. Blood samples were evaluated by Laboratory Corporation of America (Burlington, NC.).

**Fasting Glucose and Fasting Insulin**

On the same day of the clamp, an intravenous blood sample was obtained from the IV line for analysis of fasting insulin and fasting glucose. Fasting blood glucose was analyzed via
the YSI 2300 STAT PLUS glucose and L-Lactate analyzer. Fasting blood insulin was analyzed by Laboratory Corporation of America (Burlington, NC).

**Exercise Intervention**

All participants exercised at the UTEP Research Fitness Facility located in the Ross Moore building. All exercise sessions were supervised by trained graduate students to ensure safety and compliance to exercise prescriptions. Exercise training consisted of 8 weeks of combined aerobic and resistance exercise performed 3 days of the week (30 min aerobic and 30 min resistance per training day). During aerobic training, subjects began exercise at 55% of maximum aerobic capacity (VO$_2$max), increasing 5% every two weeks up to 75% of VO$_2$max. During resistance training, subjects performed 3 upper-body and 3 lower-body resistance exercises, including 3 sets of 8 repetitions for each exercise with one minute of rest between sets. Exercises were rotated from a pool of 20 exercises (Lower body: barbell squat, dumbbell squat, dumbbell goblet squats, dumbbell sumo squats, dumbbell lunges, barbell deadlift, dumbbell deadlift, weighted glute bridges; Upper body: flat barbell bench press, incline barbell bench press, flat dumbbell bench press, incline dumbbell bench press, latissimus pull-down, cable row, dumbbell row, barbell row, dumbbell shoulder press, dumbbell lateral raises, dumbbell front raises, triceps extensions). Assessments of maximal strength were performed before the first week (baseline), on the first day of training of the fifth week (mid-intervention), and the last week (post-intervention) of the exercise training period (1RM bench press & back leg strength dynamometer).

**Dietary Control**

To control for the effects of diet on insulin sensitivity, breakfast, lunch, dinner and snacks were provided five days prior to clamp days. Nutrition expertise, including meal planning and food choices, was provided by Dr. Andrew McAinch. Meals were organized and labeled in containers, by student researchers, to provide instruction for study participants. Meals were designed to comply with the USDA 2010 Dietary Guidelines for Americans and individualized
to participant preferences. The macronutrient composition of the diets comprised of: ~55% energy from Carbohydrate, ~15% energy from protein and ~30% energy from fat (with no more than 10% from saturated fat). The Mifflin St Jeor equation was utilized to match the participants estimated energy requirements with the diet provided. During the exercise training intervention, participants were encouraged to follow the USDA 2010 Dietary Guidelines for Americans (as detailed above) and to consume an energy balanced diet.

**miRNA METHODS**

**Blood Sampling**

Blood samples were obtained during ten time points for each participant throughout the intervention (Figure 3). Venous blood samples were collected using 22-gauge needles and Becton Dickinson (BD) serum, clot-activating tubes. Whole blood was obtained from the subject’s antecubital vein at baseline (1: pre-clamp, 2: immediately before and 3: after an aerobic exercise bout, 4: immediately before and 5: after a resistance exercise bout) and repeated post-intervention to assess the effects of acute exercise in the sedentary and the trained state as well as the effects of chronic exercise training. Blood was allowed to clot by leaving it undisturbed at room temperature, then centrifuged at 2800 rpm for 15 minutes at 4°C. The resulting supernatant – the participant's serum – was transferred and aliquoted to polypropylene tubes using extended, sterile, filtered pipette tips that were free of RNase, DNase, ATP, pyrogen, PCR inhibitors, endotoxin and DNA. The derived sera was then transferred and stored immediately in 0.5 mL aliquots at -80°C. To prevent detrimental effects of RNA and PCR applications, freeze-thaw cycles were minimized and samples containing visual hemolysis (red in color) were not used for analysis. Standard operating procedures were consistent with recommendations for serum processing and subsequent miRNA analysis (161,162).

**Circulating miRNA Extraction**

Prior to conducting RNA and qRT-PCR experiments, a designated bench space as well all supplies and materials were thoroughly cleaned with RNase decontamination solution
Serum samples were allowed to thaw on ice and then total RNA was extracted from 100 μl of serum using bead-based MagMAX™ mirVana™ Total RNA Isolation Kits (A27828; Applied Biosystem Inc, Foster City, CA) which allow for extraction and purification of total RNA, including miRNA from serum samples. The RNA extraction protocol was conducted using the KingFisher™ Duo Prime Purification System (Thermo Fisher Scientific, Foster City, CA) which allows for partially automated isolation of RNA from several types of specimens, including serum. The internal environment of the purification system was sterilized via ultraviolet (UV) light for 20 minutes before each run. The RNA isolation protocol was conducted using 96-well plates that were custom-made for the KingFisher™ Duo Prime Purification System and allowed for extraction of RNA from 12 samples at a time. Considering each participant provided ten serum samples and 12 were allowed per run, six time points were selected from one participant in the FH- group and six time points from one participant the FH+ group to account for potential variability between runs.

Upon thawing of serum samples, 50 μl of a denaturing agent in solution (Proteinase K Digestion Mix) were added and incubated for 30 minutes at 65° C. Due to the small amount of RNA in serum and the need for estimation of extraction efficiency, an exogenous miRNA, without homologous sequence in humans (Caenorhabditis elegans, cel-miR-39) (see Table 5 for details regarding miRNA primers), was spiked-in. According to manufacturer’s recommendations, 10 pmoles of the exogenous miRNA was spiked-in to serum samples after addition of denaturing agents to avoid degradation by endogenous RNase activity. The addition of cel-miR-39 was used to determine the yield of miRNA extraction and sample-sample variability. Next, 100 μl of lysis buffer containing 1% concentration of a reducing agent to degrade RNases (2-mercaptoethanol), was added. In addition, 20 μl of RNA Binding Beads were added followed by incubation for 15 minutes at 67° C. Next, 270 μl of 100% molecular-grade isopropanol was added to samples for purification of nucleic acids. In rows C, D, F and G of the plate, 150 μl of wash buffers containing 200-proof (absolute) molecular-grade ethanol (row C) or isopropanol (rows D, F, G) were added. Using magnetic tips, the purification system picks up
RNA that is bound to magnetic beads inside each well (i.e., sample) and transfers the beads from row to row to perform washing steps (see link for video demonstration: https://www.youtube.com/watch?v=h6NvCd94eng). In addition, a DNase treatment step is performed in row E of the plate. This step allowed for a sequence of washing, DNase removal (TURBO DNase Solution™) and washing again with additional loading of 100 μl of isopropanol. During the second addition of alcohol for purification, a RNA Rebinding Beads Mix was added to prevent loss of RNA during washing steps. In a separate 12-well strip loaded in the instrument, 75 μl of an RNase-, DNase-free Elution Buffer was added, where the RNA lysate was transferred by the magnetic tip. The final eluted product was immediately transferred to 0.6 mL tubes free of RNase and DNase in 20-25 μl aliquots to prevent evaporation and multiple free-thaw cycles. To confirm the yield of RNA following the RNA isolation protocol, sample concentration and purity were assessed via spectrophotometry (DeNovix DS-11; DeNovix, Inc, Wilmington, DE) and then stored in -80°C. The RNA isolation protocol using the KingFisher™ Duo Prime Purification System was created with the help of an instrument specialist.

Reverse Transcription and miRNA Pre-Amplification

Reverse transcription (RT) of miRNA templates was conducted using TaqMan™ Advanced miRNA cDNA Synthesis Kits (A28007; Applied Biosystems) and a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems) for thermal cycling. RT experiments were conducted using MicroAmp™ Fast Optical 96-Well Reaction Plates (4346907; Applied Biosystem). Using the manufacturer’s instructions, 2 μl of sample eluent containing total RNA was used for input. First, polyadenylation was conducted to prevent enzymatic degradation via addition of 3 μl of a Poly(A) Enzyme diluted in 10X Poly(A) Buffer, ATP and RNase-free water to samples. Plates were then covered and sealed, briefly vortexed and centrifuged using the “pulse” function of a bench-top centrifuge (Allegra X-12R; Beckman Coulter, Indianapolis, IN). The polyadenylation step was completed by incubation in a thermal cycler (45 minutes at 37°C,
10 minutes at 65° C, 4° C hold). Next, adaptor ligation of the 5’ end of the miRNA transcript was added to extend the template. Here, 10 µl of an Adaptor Ligation Mix was added to each sample followed by a brief vortex and centrifugation of the plate. Adaptor ligation was completed by incubation in a thermal cycler (60 minutes at 16°C, 4° C hold; no cycles). The RT step comprised an addition of 15 µl of a RT Reaction Mix (5X RT Buffer, dNTP Mix, 20X Universal Primer, 10X RT Enzyme Mix, RNase-free water) to each sample. Thermal cycling was performed with 15 minutes at 42° C, 5 minutes at 85° C and a 4° C hold. To assess DNA contamination during RNA preparation, 3 no-RT controls (NRT) were randomly selected. These samples included everything in the reaction mix except the RT enzyme and instead replaced the volume with RNase-free water.

Pre-amplification of cDNA prior to quantitative real-time polymerase chain reaction (qRT-PCR) has been considered an effective means of exponentially increasing the amount of template before conducting gene expression analysis without being affected by the quality of RNA or introducing bias to miRNA detection (161,163,164). For low yield specimens such as isolated RNA from serum and miRNA expression analysis, pre-amplification is therefore advantageous for downstream qRT-PCR applications. miRNA pre-amplification was completed by addition of 45 µl of miR-Amp Reaction Mix (included with cDNA synthesis kit) with 5 µl of the RT reaction product. Upon brief mixing via vortex and pulse centrifugation, thermal cycling was performed for: enzyme activation (5 min at 95° C; 1 cycle), denature (3 seconds at 95° C) and anneal/extend (30 seconds at 60° C) (14 cycles), stop reaction (10 minutes at 99° C) and hold (4° C).

The RT reaction product yields 30 µl, while only 5 µl are used for pre-amplification, therefore the remaining cDNA template was transferred from a plate to 0.6 mL tubes and stored at -20° C.
Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Samples were allowed to thaw on ice before the PCR experiments. According to the manufacturer’s instructions, cDNA templates were prepared in a 1:10 dilution, 5 µl of diluted cDNA template was combined with 15 µl of the PCR Reaction Mix containing 10 µl 2X TaqMan™ Fast Advanced Master Mix (4444557; Applied Biosystems), 1 µl 2X TaqMan™ Advanced miRNA Assay (A25576; Applied Biosystems) and 4 µl RNase-free water. PCR reactions for each miRNA were performed in triplicate in 384-well plates. The plates were sealed, vortexed and pulse centrifuged then loaded for thermal cycling. The thermal cycling conditions included: enzyme activation (20 seconds at 95°C; 1 cycle), denature (1 second at 95°C) and anneal/extend (20 seconds at 60°C) (40 cycles) and hold (4°C). Negative controls included the three NRT controls as well as three no-template controls (NTC) that comprised solely of 15 µl of RNase-free water to determine contamination and primer-dimer formation (non-specificity).

Normalization

Accurate normalization is an important prerequisite to appropriate interpretation of gene expression data, however there remains to be a standardized approach to normalization of miRNAs in tissue or circulation (161). Therefore, in addition to an exogenous spike-in control, it has been recommended to include more than one endogenous control. Chen et al. (165) recently reported that let-7d, let-7g and let-7i can concomitantly serve as stable references for serum miRNA analysis after comparing their invariability versus other commonly used reference genes. All miRNA were normalized to geometric mean of the cel-miR-39 and the three internal controls (let-7d, let-7g and let-7i). The use of both external and internal controls presents normalization for technical and sample-sample variability. Moreover, calculation of the geometric mean has been considered a valid approach to normalization of gene expression data when more than one internal control is included (166).

The normalization steps include:
1. Determination of Geometric Mean. The cycle threshold (Ct) values of let-7d, let-7g and let-7i were used to calculate the geometric mean, would serve as a reference Ct for subsequent normalization of sample-sample variability. The Ct values of each internal control were entered in the following equation:

$$\left(\left[let-7d \text{ Ct}\right] \times \left[let-7g \text{ Ct}\right] \times \left[let-7i \text{ Ct}\right]\right)^{1/3}$$

The geometric mean of each sample replicate was averaged.

2. Determination of Spike-In Normalization Factors for each sample to control for variation in RNA input is required due to the inability to measure miRNA-specific concentrations via spectrophotometry. The spike-in factors are obtained by subtracting the sample cel-miR-39 Ct value (average of three replicates) from the median of all cel-miR-39 Ct’s. Each sample therefore had a corresponding spike-in factor that was consistent for normalization across all target miRNAs.

Equation: (Median cel-miR-39 Ct – average sample cel-miR-39 Ct)

3. Determination of a Median-Normalized Ct. The Ct value of each target miRNA were added to the sample’s corresponding Spike-in Normalization Factor to determine a “new” Ct value based on the input of total RNA. The subsequent median-normalized Ct of each replicate was averaged to represent the sample’s miRNA expression level.

4. Determination of Delta Ct. The relative expression levels of each miRNA were determined by subtracting the sample Geometric Mean from the sample Median-Normalized Ct. This value was expressed as delta Ct ($\Delta$Ct) and is representative of the miRNA’s relative expression level.

**Comparative Gene Expression Quantitation**

To determine differences in expression levels of target miRNAs with exercise and/or between groups, the comparative cycle threshold (Ct) method was used, otherwise known as the delta delta Ct ($\Delta\Delta$Ct) method. To assess exercise training-induced changes in miRNA expression,
the relative expression levels of a miRNA at baseline was subtracted from relative expression at the end of the eight week intervention (Equation: post-intervention ΔCt – baseline ΔCt).

**Statistical Analysis**

Two-way ANOVA with repeated measures was used to compare groups (FH-, FH+), time (before vs. after intervention) and group by time effects. To compare miRNA baseline expression levels between groups, unpaired student t-tests were used. Relationships between miRNA expression and clinical outcome measures were determined via Pearson correlation analysis. Results are reported as mean ± SEM and significant differences were to be assumed for p<0.05. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc, La Jolla, California).

**Table 5. Target miRNAs**

<table>
<thead>
<tr>
<th>Mature miRNA (Assay ID)</th>
<th>miRNA Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cel-miR-39-3p (478293_mir)</td>
<td>UCACCGGGUGUAAAUCAGCUUG</td>
</tr>
<tr>
<td>hsa-miR-29a-3p (478587_mir)</td>
<td>UAGCACCAUCUGAAAAUCGGUUA</td>
</tr>
<tr>
<td>hsa-miR-29b-3p (478369_mir)</td>
<td>UAGCACCAUUGAAAAUCAGGUUU</td>
</tr>
<tr>
<td>hsa-miR-29c-3p (479229_mir)</td>
<td>UAGCACCAUUGAAAAUCGGUUA</td>
</tr>
<tr>
<td>hsa-miR-133a-5p (478706_mir)</td>
<td>AGCUGGUAAAAUUGGAACAAAU</td>
</tr>
<tr>
<td>hsa-miR-133b (480871_mir)</td>
<td>UUGGUCCCCUCUAACCAACGCUA</td>
</tr>
<tr>
<td>hsa-miR-143-5p (478713_mir)</td>
<td>GGUGCAGUGCUCAUCUCUGGU</td>
</tr>
<tr>
<td>hsa-miR-155-3p (477926_mir)</td>
<td>CUCUCAAUUUAUGCAUAACAA</td>
</tr>
</tbody>
</table>

cel: *Caenorhabditis elegans*; hsa: *Homo sapiens*
Chapter 3: Results

The expression of three out of the seven or the expression of miRNAs after acute exercise were not detected by qRT-PCR, therefore the results presented in the following sections will consider baseline and post-intervention miRNA expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155.

Baseline miRNA Expression

There were no significant differences between FH- and FH+ in expression levels of miRNA-29a (FH- vs. FH+, mean ± SEM: -3.82 ± 4.92 vs. 3.56 ± 1.48; p = 0.17), miRNA-133a (0.89 ± 3.97 vs. 1.76 ± 3.85; p = 0.88), miRNA-133b (-1.73 ± 4.4 vs. -3.52 ± 5.44; p = 0.80) and miRNA-155 (2.01 ± 5.06 vs. 3.31 ± 9.47; p = 0.89) (Figure 4).

**Figure 4.** Relative expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D) in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes (FH+). Significance is indicated with *, representing p < 0.05. Graphs are presented as mean ± SEM.
Associations Between miRNA Expression and Insulin Sensitivity & Metabolic Flexibility

Insulin sensitivity at baseline was not associated with the expression of miRNAs when analyzed by family history status or when groups were combined (Figure 5). However in the FH+ group, exercise training-induced improvements in insulin sensitivity displayed a significant positive correlation with miRNA-133a ($r^2 = 0.50$, $p = 0.03$). In FH+, the correlations between training-induced improvements in insulin sensitivity approached significance with miRNA-133b ($r^2 = 0.37$, $p = 0.10$) and miRNA-155 ($r^2 = 0.86$, $p = 0.07$) (Figure 6). The correlation with miRNA-155 should be considered with caution, as the sample size of miRNA-155 detected in the FH+ group was four. When all participants regardless of family history of T2D were included in the correlation between the change in insulin sensitivity and expression of miRNA=133a, there was a trend toward a positive association ($r^2 = 0.19$, $p = 0.08$) (Figure 7).

**Figure 5.** Correlations for baseline insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$. 
Figure 6. Correlations for combined exercise training-induced improvements in insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes vs. baseline expression levels of miRNA-29a (A,E), miRNA-133a (B,F), miRNA-133b (C,G) and miRNA-155 (D,H). Significance is represented as $p < 0.05$.

Figure 7. Correlations for combined exercise training-induced improvements in insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$. 
Notably, there was a significant negative association in the FH+ group between miRNA-29a expression and the improvements in metabolic flexibility observed with exercise training ($r^2 = 0.55$, $p = 0.03$) (Figure 8). Whereas no other significant correlations between metabolic flexibility and miRNAs were displayed (Figures 8 & 9).

**Figure 8.** Correlations for baseline metabolic flexibility in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes (FH+) vs. baseline expression levels of miRNA-29a (A,E), miRNA-133a (B,F), miRNA-133b (C,G) and miRNA-155 (D,H). Significance is represented as $p < 0.05$. 
Figure 9. Correlations for baseline metabolic flexibility in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.

In addition to insulin sensitivity assessed via the clamp technique, glucose tolerance was assessed. It was observed that participants with lower glucose tolerance, indicated by a greater glucose area under the curve (AUC), exhibited lower expression of miRNA-133b at baseline ($r^2 = 0.35, p = 0.01$) (Figure 10). Similarly, there were significant negative associations between fasting blood glucose and expression levels of miRNA-29a ($r^2 = 0.63, p = 0.01$), miRNA-133a ($r^2 = 0.52; p = 0.001$), miRNA-133b ($r^2 = 0.30, p = 0.02$) and miRNA-155 ($r^2 = 0.80, p = 0.0002$) (Figure 12). Interestingly, miRNA-29a ($r^2 = 0.46, p = 0.003$), miRNA-133a ($r^2 = 0.43; p = 0.004$), miRNA-133b ($r^2 = 0.39, p = 0.008$) and miRNA-155 ($r^2 = 0.50, p = 0.01$) expressions were positively associated with the reductions in fasting blood glucose with exercise training (Figure 13). There were no significant correlations between miRNA expression and fasting insulin or the change in insulin with training (data not shown).
Figure 10. Correlations for baseline glucose tolerance in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.

Figure 11. Correlations for baseline fasting blood glucose in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.
Figure 12. Correlations for baseline fasting blood glucose in subjects without a family history of Type 2 Diabetes (FH−; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.
Figure 13. Correlations for combined exercise training-induced improvements in fasting blood glucose in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.

Associations Between miRNA Expression and Substrate Utilization

There were no significant associations observed between miRNAs and substrate oxidation under fasting and insulin-stimulated conditions.

Associations Between miRNA Expression and Blood Lipids

When blood lipid levels were correlated with miRNA expression, negative associations between very low-density lipoprotein (VLDL) levels and expressions of miRNA-133a ($r^2 = 0.33$, $p = 0.01$) and miRNA-155 ($r^2 = 0.40$, $p = 0.3$) were observed, while the negative association with miRNA-133b did not reach significance ($r^2 = 0.22$, $p = 0.06$) (Figure 14). Moreover, expressions of miRNA-133a and miRNA-133b were negatively associated with triglyceride levels ($r^2 = 0.33$, $p = 0.01$ and $r^2 = 0.21$, $p = 0.06$, respectively) (Figure 15), and miRNA-133a was positively associated with high-density lipoprotein (HDL) levels in FH+ ($r^2 = 0.66$, $p = 0.008$) (Figure 16).
Figure 14. Correlations for baseline very low-density lipoprotein (VLDL) levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline miRNA expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.
Figure 15. Correlations for baseline triglyceride levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline miRNA expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.

Figure 16. Correlations for baseline high-density lipoprotein (HDL) levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline miRNA expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.
Figure 17. Correlations for baseline high-density lipoprotein (HDL) levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline miRNA expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as \( p < 0.05 \).

Associations Between miRNA Expression and Exercise Outcomes

There were no relationships between miRNA expression levels and baseline cardiorespiratory fitness (VO\(_{2\text{max}}\)) (Figure 18), however the training-induced improvements in VO\(_{2\text{max}}\) were negatively associated with the four miRNAs in FH+. miRNA-29a (\( r^2 = 0.73, p = 0.007 \)), miRNA-133a (\( r^2 = 0.66, p = 0.01 \)), miRNA-133b (\( r^2 = 0.63, p = 0.01 \)) and miRNA-155 (\( r^2 = 0.94, p = 0.03 \)), exclusively in the FH+ group, displayed significant negative relationships with the changes in VO\(_{2\text{max}}\) (Figure 19).
Figure 18. Correlations for baseline aerobic capacity (VO$_{2\text{max}}$ ml/kg/min) in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline miRNA expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.
Figure 19. Correlations for combined exercise training–induced improvements in aerobic capacity ($\Delta VO_{2\text{max}}$ ml/kg/min) in subjects with a family history of Type 2 Diabetes (FH+) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$.

Neither upper- or lower-body strength at baseline was associated with miRNA expression (Figures 20 & 21), but the increases in lower body strength in the FH+ group were positively associated with expressions of miRNA-133a ($r^2 = 0.56, p = 0.02$) and miRNA-155 ($r^2 = 0.97, p = 0.01$) (Figure 22).
Figure 20. Correlations for baseline upper body strength (1RM) in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$.

Figure 21. Correlations for baseline lower body strength (1RM) in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$. 
Figure 22. Correlations for combined exercise training-induced improvements in lower body strength (1RM) in subjects with a family history of Type 2 Diabetes (FH+) vs. baseline expression levels of miRNA-133a (A) and miRNA-133b (B). Significance is represented as p < 0.05.
Associations Between miRNA Expression and Body Composition

In the FH- group, the negative correlation between miRNA-155 expression and body fat percentage trended toward significance ($r^2 = 0.44$, $p = 0.10$) (Figure 23). While in the FH+ group, there was a significant negative correlation between miRNA-133a expression and WHR at baseline ($r^2 = 0.48$, $p = 0.03$) (Figure 23). Similarly, when the two groups were analyzed together, WHR trended toward a negative association with miRNA-155 expression ($r^2 = 0.32$, $p = 0.07$) (Figure 23).

**Figure 23.** Correlations for baseline percent body fat (A) and waist-to-hip ratio (B-C) in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. baseline expression levels of miRNA-133a and miRNA-155. Significance is represented as $p < 0.05$. 

![Figure 23](image-url)
EXERCISE TRAINING-INDUCED CHANGES IN miRNA EXPRESSION

The expression levels of three miRNAs approached significant reductions with exercise training including miRNA-29a (Pre vs Post: -3.82 ± 4.92 vs. 6.07 ± 3.64; p = 0.11), miRNA-133a (0.89 ± 3.97 vs. 8.34 ± 3.89; p = 0.07) and miRNA-133b (-1.73 ± 4.39 vs. 5.71 ± 5.78; p = 0.08). There were no significant changes in the expressions of miRNAs in participants with a FH+ (Figure 24). Independent of FH, eight weeks of combined exercise training did not significantly alter miRNA expression (Figure 25).

Figure 24. Relative expression levels before and after eight weeks of combined exercise training in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes (FH+). miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D) Significance is indicated with *, representing p < 0.05. Graphs are presented as mean ± SEM.
Figure 25. Individual responses to eight weeks of combined exercise training in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes (FH+). miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is indicated with *, representing p < 0.05. Graphs are presented as mean ± SEM.

Associations Between Exercise Training-Induced Changes in miRNA Expression and Clinical Outcome Measures

The increase in miRNA-133b displayed a positive trend toward significance with insulin sensitivity at baseline in FH- ($r^2 = 0.52$, $p = 0.06$) (Figure 26). Interestingly, the changes in expression of the four miRNAs appeared to change concomitantly with improvements in insulin sensitivity in FH-, but not FH+ (Figure 28). The negative correlation between the change in miRNA-133a expression and change in insulin sensitivity was significant in the FH- group ($r^2 = 0.62$, $p = 0.03$) (Figure 28). There was a significant negative correlation between the change in miRNA-155 expression and the change in insulin sensitivity in the FH- group ($r^2 = 0.79$, $p = 0.04$), however, the change in insulin sensitivity was positively related to changes in miRNA-155 expression in the FH+ group ($r^2 = 0.88$, $p = 0.06$) (Figure 28). In addition, the change in miRNA-29a trended toward a negative association with the change in insulin sensitivity in the FH- group ($r^2 = 0.56$, $p = 0.08$) (Figure 28).
Figure 26. Correlations for baseline insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-; blue circles) vs. the training-induced change in expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$.

Figure 27. Correlations for baseline insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as $p < 0.05$. 
Figure 28. Correlations for combined exercise training-induced improvements in insulin sensitivity in subjects without a family history of Type 2 Diabetes (FH-) and with a family history of Type 2 Diabetes (FH+) vs. the training-induced change in expression levels of miRNA-29a (A), miRNA-133a (B), miRNA-133b (C) and miRNA-155 (D). Significance is represented as p < 0.05.

Related to metabolic flexibility, the changes in miRNA-133a (did not reach significance) and miRNA-133b were positively related to baseline metabolic flexibility in the FH- group ($r^2 = 0.42$, $p = 0.11$ and $r^2 = 0.57$, $p = 0.04$, respectively) (Figure 29). In the FH- group, the improvements in metabolic flexibility trended toward an inverse relationship with the change in miRNA-29a expression ($r^2 = 0.50$, $p = 0.11$) (Figure 29).
**Figure 29.** Correlations for baseline metabolic flexibility (A-D) combined exercise training-induced changes in metabolic flexibility (E-H) in subjects without a family history of Type 2 Diabetes (FH-) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.

Lower glucose tolerance (greater AUC) at baseline was inversely related with miRNA-29a in FH- ($r^2 = 0.69$, $p = 0.03$) (Figure 30). The change in miRNA-133a expression was negatively related with baseline AUC in the FH- group ($r^2 = 0.74$, $p = 0.01$), while the relationship in the FH+ group was inversely associated (trended toward significance) ($r^2 = 0.52$, $p = 0.10$) (Figure 30). Similar results were displayed when the change in AUC was correlated with the change in miRNA-133a. Where, the FH- group displayed a significant positive association ($r^2 = 0.62$, $p = 0.03$) and the FH+ displayed a trend toward a negative association ($r^2 = 0.61$, $p = 0.06$) (Figure 32). Moreover, there were positive trends toward associations between the change in AUC and changes in miRNA-29a ($r^2 = 0.52$, $p = 0.10$) and miRNA-155 ($r^2 = 0.72$, $p = 0.07$) in the FH- group (Figure 32).
Figure 30. Correlations for baseline glucose tolerance in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.

Figure 31. Correlations for baseline glucose tolerance in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.
Figure 32. Correlations for combined exercise training-induced changes in glucose tolerance in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.

Two miRNAs displayed significant negative associations between the change in expression level and baseline fasting blood glucose in the FH+ group (miRNA-29a: $r^2 = 0.48$, p = 0.05 and miRNA-133b: $r^2 = 0.72$, p = 0.003) (Figure 33).

Figure 33. Correlations for baseline fasting glucose levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.
Figure 34. Correlations for baseline fasting glucose levels in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.
The change in miRNA-133b expression induced by exercise training was positively associated with lower-body strength at baseline in the FH+ group ($r^2 = 0.55$, $p = 0.02$) (Figure 35).

![Figure 35](image)

**Figure 35.** Correlations for combined exercise training-induced changes in lower body strength in subjects with a family history of Type 2 Diabetes (FH+) vs. baseline expression levels of miRNA-133a and miRNA-155. Significance is represented as $p < 0.05$.

In the FH+ group, there was a trend toward a negative association between the change in miRNA-155 expression and RQ at baseline ($r^2 = 0.84$, $p = 0.08$) (Figure 36). In the insulin-stimulated state, the changes in miRNA-29a and miRNA-155 levels in FH- trended toward inverse associations with RQ ($r^2 = 0.60$, $p = 0.07$ and $r^2 = 0.64$, $p = 0.10$, respectively) (Figure 35). Whereas the change in miRNA-133b displayed a significant negative association with insulin–stimulated RQ ($r^2 = 0.69$, $p = 0.02$) (Figure 36). In FH-, changes in resting energy expenditure (REE) were negatively associated with changes in expression levels of miRNA-29a ($r^2 = 0.62$, $p = 0.06$), miRNA-133a ($r^2 = 0.61$, $p = 0.04$) and miRNA-133b ($r^2 = 0.87$, $p = 0.002$) (Figure 37).
**Figure 36.** Correlations for baseline substrate utilization (fasting respiratory quotient (RQ) and insulin-stimulated RQ) in subjects without a family history of Type 2 Diabetes (FH-; blue circles) and with a family history of Type 2 Diabetes (FH+; red squares) vs. the training-induced change in expression levels of miRNA-29a, miRNA-133b and miRNA-155. Significance is represented as p < 0.05.

**Figure 37.** Correlations for combined exercise training-induced changes in resting energy expenditure (REE) in subjects without a family history of Type 2 Diabetes (FH-) vs. the training-induced change in expression levels of miRNA-29a (A), miRNA-133a (B) and miRNA-133b (C). Significance is represented as p < 0.05.
Chapter 4: Discussion

T2D is a complex, multi-fac torial disease characterized by insulin resistance of peripheral tissues and compromised β-cell function. The role of miRNAs in glucose metabolism and the risk for T2D associated with a FH is not known. In this study, we determined whether a FH altered the expression levels of circulating miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155 and determined the effects of eight weeks of combined exercise training on circulating miRNAs associated with insulin sensitivity (miRNA29a, miRNA-133a, miRNA-133b, and miRNA-155) in healthy, sedentary, normoglycemic men with a FH. These four miRNA were selected since they have been previously associated with regulation of glucose metabolism.

The main outcomes of this study were that a FH did not alter circulating miRNA expression levels and that combined exercise training did not have a significant effect on expressions of miRNA-29a, -133a, -133b and miRNA-155 regardless of a FH. Although we recruited healthy, normoglycemic men, there appears to be an inverse relationship between circulating miRNAs and fasting blood glucose levels before and after exercise training. Additionally, we observed associations between circulating miRNA-133 isoforms and miRNA-155 with plasma triglycerides and very low-density lipoprotein (VLDL) levels. These findings suggest that a FH may not exhibit aberrant circulating miRNA expression in young, normoglycemic men. Independent of FH, these miRNAs (miRNA-29a, miRNA-133a, miRNA-133b, and miRNA-155) may be involved in the regulation of blood glucose and lipids.

Our study is novel in understanding the effects of combined exercise training on circulating miRNA expression in individuals with a FH. There are several studies that have examined the effects of exercise training on circulating miRNAs (126,127,138,145,147,153,167) as well as the role of certain miRNAs in glucose metabolism (168). However only one study (167), to our knowledge, has implemented combined exercise training to investigate changes in circulating miRNAs. The present study is the first to determine the effects of combined exercise
training on circulating expression levels of the miRNAs -29a, -133a, -133b and -155. Further, we are the first to determine whether circulating miRNAs are altered in healthy individuals with a FH.

We have preliminary findings indicating that normoglycemic, sedentary individuals with a FH exhibit a similar metabolic profile compared with individuals without a FH, including similar insulin sensitivity (assessed via hyperglycemic euglycemic clamp), metabolic flexibility, fasting fat oxidation and comparable aerobic capacity and strength. These data reflect our findings that expression levels of circulating miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155 were not significantly altered between subjects with/without FH. Previous studies using the clamp technique have reported significant impairments in insulin sensitivity with a FH in young, normoglycemic populations (29,31,169) including Mexican Americans with normal glucose tolerance (40). However, the characterization of a FH has been inconsistent, varying from second degree relatives (e.g., grandparents) (169) to a “strong family history of type 2 diabetes” characterized by at least two known first-degree relatives (40). Our study included subjects who self-reported at least one first-degree relative with T2D, which supports the findings from the European Prospective Investigation into Cancer and Nutrition (EPIC)-InterAct study, a cohort study of > 27,000 individuals (170). The authors found that a biparental family history was associated with a greater risk for development of T2D compared with only paternal or maternal FH (170). Thus, it is likely that, despite normoglycemia, the presence of T2D in both parents contributes to impaired insulin sensitivity and circulating miRNAs that are not observed with a FH from at least one self-reported first-degree relative.

Moreover, the subjects in our study were younger (23.3 ± 0.6 years) compared with other studies that have examined the effects of a FH on insulin sensitivity in adults (26,31,171–174). While insulin resistance has been observed in adolescents with a FH, the impact of a FH on normoglycemic college-age males has not been well-characterized (175). Our preliminary evidence shows no significant metabolic abnormalities in healthy, normoglycemic individuals with a FH. These findings have important clinical implications to offset predispositions for
abnormal metabolic health at an early age. Results from the Framingham Heart Study indicate that the age of obesity onset is a strong predictor of T2D (176). Likewise, the negative effects of T2D on morbidity are greatest in individuals diagnosed at a young age (177). Hence, we show that if normoglycemia can be sustained through early adulthood and obesity is prevented, metabolic health can be retained despite a predisposition for T2D. Further, the response to eight weeks of combined exercise training was similar regardless of FH in the present study. Therefore, our study suggests that healthy, normoglycemic males with a FH may not display aberrations in insulin sensitivity-related miRNAs, so long as normoglycemia exists. Notably, there was no apparent impediment to exercise training-induced improvements in metabolic health in normoglycemic, college-age males with a FH.

While it is known that lifestyle modifications counteract insulin resistance, the links between a FH and impaired metabolic phenotypes (29,32,37) complicate the pathophysiology of T2D disease. In turn, the scientific community has directed considerable efforts to understand the mechanisms underlying T2D. An integrative approach to studying the physiology of T2D, which considers the metabolic function of several tissues, may improve current assessments of T2D risk as well as improve the understanding of diabetes progression. Present examinations of T2D rely on fasting glucose levels, which may not represent the risk of insulin resistance, more than a mere diagnosis of diabetes when metabolic aberrations already exist. In this respect, circulating miRNAs represent a promising strategy to examine whole-body metabolic status due to their differential expression with disease (178) and their stability in circulation (71). The evidence showing distinct circulating miRNA profiles of healthy adults compared with prediabetic and T2D patients (105,167) suggests circulating miRNAs may be novel biomarkers to recognize individuals across different stages of insulin resistance.

Exercise is considered a major stimulus to improve insulin sensitivity, therefore examination of circulating miRNAs following exercise training warrants further attention. The exercise training-induced adaptations in insulin sensitivity require coordination by skeletal muscle, β-cells and the liver to control metabolic functions such as insulin action and hepatic
glucose production. If circulating miRNA are to be used as biomarkers for assessment of T2D risk and treatment efficacy, it is important to understand how exercise regulates miRNAs in tissue and circulation. It is not understood whether miRNAs are released into circulation passively or actively to target other cells. There is a significant gap in our understanding of the mechanisms that regulate export of miRNAs from tissues as well as the mechanisms that may signal specific delivery to other tissues. It has been suggested that miRNAs are merely waste products of metabolism (179), while many believe miRNAs possess paracrine signaling properties (180). That is, miRNAs may be actively secreted by a signal to “communicate” with other tissues. In this respect, the signature of circulating miRNAs may represent adaptations occurring in tissues with exercise training (181). Therefore, identification of biomarkers for T2D risk can improve preventative therapies, but understanding the modulation of circulating miRNAs with exercise adds necessary information to further personalize treatment and prevention.

**miRNA-29a**

The present study is the first to determine whether exercise training alters circulating miRNA-29a expression. We found that miRNA-29a expression is not different between normoglycemic males with/without a FH and is not significantly altered following eight weeks of combined exercise training. Dahlmans et al. (104) showed elevated expression levels of the three miRNA-29 isoforms in skeletal muscle of T2D patients compared with normoglycemic obese individuals and Karolina et al. (105) identified significantly elevated serum miRNA-29a in T2D patients. Interestingly, athletes may not display different expressions of miRNA-29a, miRNA-29b or miRNA-29c compared with lean sedentary individuals (104). Since enhanced oxidative capacity did not confer lower expression of the miRNA-29 family in athlete skeletal muscle, it is possible that hyperglycemia causes the dysregulation of miRNA-29. This notion supports our findings of similar miRNA-29a expression between normoglycemic healthy males with and without a FH. The differential expression of miRNA-29a in T2D has been documented
(98,104,106), but it is not clear whether miRNA-29a expression follows a linear relationship with elevations in blood glucose. Therefore miRNA-29a may be indicative of T2D and its role in development of T2D is yet to be determined. Our findings show that miRNA-29a expression may not be altered by a FH in a healthy, normoglycemic population.

To further understand the potential for miRNA-29a as a biomarker for T2D risk, the actions of circulating miRNA-29 need to be determined. Namely, the actions of miRNA-29a in target tissues may explain the elevated expression levels with hyperglycemia, but not in individuals with normal fasting glucose. In addition, understanding the mechanisms that regulate miRNA-29a secretion may elucidate the differential expression in T2D. Karolina et al. (105) observed elevated circulatory miRNA-29a in patients with T2D, which was concomitant with significantly downregulated Akt2 mRNA in skeletal muscle. Akt is a predicted target of miRNA-29a according to target prediction databases (TargetScan, MirBase) and the direct association of miRNA-29 isoforms on the 3’ UTR of the Akt3 mRNA was elegantly confirmed in C2C12 myoblasts via a fluorescent luciferase reporters (182). In addition, C2C12 cells transfected with pooled miRNA-29a, -29b and -29c led to decreased Akt3 protein and the p85 regulatory subunit of PI3-K despite normal Akt3 mRNA levels (182). This suggests that in conditions of hyperglycemia, miRNA-29 may suppress translation of the Akt3 mRNA to its functional protein, creating a compromised state for insulin signaling. The sustained levels of Akt3 mRNA indicate upregulated transcription of insulin signaling genes, but the decline in Akt3 protein is consistent with the observations of greater miRNA-29 expression in humans with T2D, who are known to exhibit impaired insulin signaling (31).

Similar defects in PI3-K-Akt phosphorylation were mediated by miRNA-29 overexpression in rodent skeletal muscle (98). Notably, the diminished proximal insulin signaling led to significant reductions in GLUT-4 mRNA and protein in vivo that manifested in lower insulin-stimulated glucose uptake (98). The evidence in vitro and in animal studies indicate that impairments in insulin signaling mediated by miRNA-29, are sufficient to reduce insulin sensitivity (98,182), which supports the two human studies showing elevated miRNA-29
in T2D skeletal muscle (104) and serum (106). These data elucidate the mechanisms underlying miRNA-29a-mediated impairments in skeletal muscle insulin signaling and glucose uptake in T2D. Whether these defects in skeletal muscle glucose metabolism related to miRNA-29a exist prior to T2D development, are not known. Whereas we show that regardless of a FH, insulin sensitivity and miRNA-29a expression are maintained during normoglycemia. Future studies should assess peripheral insulin sensitivity along with skeletal muscle insulin signaling to confirm this relationship with circulating miRNA-29 in humans. Additionally, it should be studied whether prediabetes and a FH contribute to upregulated miRNA-29 expression compared with normoglycemic adults with a FH.

The role of miRNA-29a with exercise training may further elucidate its ability to regulate glucose metabolism. Notably, we are the first to determine the effects of combined exercise training on miRNA-29 expression. Our preliminary evidence shows that eight weeks of combined exercise training significantly improved insulin sensitivity independent of FH. Moreover, combined exercise training did not decrease fasting blood glucose or circulating miRNA-29a levels regardless of FH. Thus, our results align with the findings by Dahlmans et al. (104), who did no observed any differences miRNA-29a expression in skeletal muscle of lean sedentary individuals compared with endurance athletes. However, we found a significant association between the exercise training-induced reductions in fasting blood glucose and baseline miRNA-29a expression. Given the recruitment of a normoglycemic population in the present study, significant reductions in fasting blood glucose were not anticipated; nonetheless, we found that individuals with lower miRNA-29a expression at baseline were more likely to reduce fasting blood glucose after combined exercise training. This was an unexpected finding, but provides more support for the link between miRNA-29a and control of fasting blood glucose. Although it is known that combined exercise training can improve fasting blood glucose and insulin signaling in human skeletal muscle (183), the ability of exercise training to diminish the repression of Akt by miRNA-29a warrants further investigation.
There are two studies to assess muscle miRNA-29a expression with endurance exercise training in rats (98,184) and only one study in humans (98). In rats, Massart et al. (98) found significantly lower skeletal muscle miRNA-29a after five days of swim training, but the authors did not report the effects of exercise on insulin signaling. In contrast, Soci et al. (184) found significantly elevated miRNA-29a expression in the left ventricle of female rats after 10 weeks of swim training. The effects of 14 consecutive days of endurance training in T2D patients resulted in unaltered skeletal muscle miRNA-29a, -29b and -29c (98). These data suggest an interesting divergence between the effects of exercise training on skeletal muscle miRNA-29a in rats vs. humans. Thus we provide the first evidence of circulating miRNA-29 expression after combined exercise training, supporting the notion that miRNA-29a expression may not be altered by exercise training in humans. Future studies should investigate whether longer exercise interventions may significantly reduce skeletal muscle or circulating miRNA-29a expression. Additionally, it is possible that our study did not observe significant reductions in serum miRNA-29 due to the normoglycemic status of our participants.

In conclusion, our findings show that eight weeks of combined exercise training may not significantly reduce miRNA-29a in normoglycemic males, but lower circulatory miRNA-29a at baseline remains favorable to improve glycaemia after exercise training. Additionally, young, normoglycemic men with a FH may not display altered circulating miRNA-29a. There is a need for more human studies to determine whether expression of miRNA-29a can be used as a biomarker for T2D risk. The evidence of elevated miRNA-29a in T2D (98,104) supports the hypothesis that its expression is only altered by hyperglycemia, but not in individuals with normal fasting glucose. Nonetheless, studies in cells (98), animals (98) and humans (98,104) are in agreement of miRNA-29 isoforms as negative regulators of insulin sensitivity in T2D.

**miRNA-133A & miRNA-133B**

The present study shows that serum miRNA-133a isoforms are similar between normoglycemic males with/without a FH. We observed significant negative associations between
baseline miRNA-133a and -133b expression and fasting blood glucose and baseline miRNA-133b with glucose area under the curve (AUC). Further, we found a significant relationship between miRNA-133a isoforms and reductions in fasting glucose following eight weeks of combined exercise training.

Increased miRNA-133a has been suggested as a marker of myocardial damage in patients with cardiovascular disease (185). In the context of T2D, miRNA-133 has largely been studied as a regulator of cardiac hypertrophy (186,187). One human study has identified lower mirNA-133a in skeletal muscle with T2D (148) and one reported greater miRNA-133b in T2D muscle (104). The direct binding of miRNA-133 with the 3’ UTR of serum response factor (SRF) – a mediator of skeletal muscle proliferation – was elucidated in C2C12 myoblasts (188).

Additionally, miRNA-133a downregulates the insulin-like growth factor-1 receptor (IGF-1R), a mechanism that significantly repressed insulin signaling in neonatal rat cardiomyocytes (189). These actions of miRNA-133a or -133b have not been identified. However the difference in miRNA-133a expression between T2D and adults with normal glucose tolerance was five-fold in the Gallagher et al. (148) study, and the authors found a negative association between miRNA-133a and HbA1c. These data suggest a link between miRNA-133a and insulin resistance, but the connection between reduced skeletal muscle miRNA-133a in T2D muscle and the mechanisms identified in the cell culture studies do not provide enough support for this miRNA as a candidate for a T2D biomarker.

Our findings indicate that circulating miRNA-133a and miRNA-133b expression are similar in healthy, normoglycemic individuals regardless of a FH. Even with normoglycemia, individuals with lower fasting glucose and better glucose tolerance exhibited greater expression of miRNA-133b at baseline, while greater miRNA-133a was negatively associated with fasting glucose. The relationship between miRNA-133a and fasting glucose is in agreement with greater miRNA-133a expression in individuals with normal fasting glucose compared with T2D (148). However two other animal studies have reported greater miRNA-133a expression in islet cells of obese mice (190) and blood of rats with T2D (105). To our knowledge, the Gallagher et al. (148)
study is the only study to report human miRNA-133 expression, which agrees with our observations of greater miRNA-133a in those with lower fasting glucose. Moreover, when the primary miRNA-133a (pri-miRNA-133a) transcript was compared between metabolic groups (148), the similar abundance suggested that downregulation of skeletal muscle miRNA-133a in T2D was likely due to altered target mRNA degradation and not impaired transcription of miRNA-133 precursors. Thus, miRNA-133a may be upregulated in healthy conditions to degrade mRNAs that negatively influence insulin signaling. Whereas the relationship between circulating miRNA-133b and glucose control in our study is a novel finding.

We show that greater miRNA-133 in circulation may indicate favorable fasting glucose levels and in the only other human studies to evaluate miRNA-133, Gallagher et al. (148) reported greater miRNA-133a in muscle. On the other hand, elevated circulatory miRNA-133a has been identified after myocardial infarction in cardiovascular disease patients (185). Considering miRNA-133a inhibits cardiomyocyte IGF-1 (189) and may contribute to cardiac hypertrophy (186,187), it is not clear why upregulated miRNA-133a may confer better glucose metabolism. An investigation of the various types of vesicles (exosomes, apoptotic bodies, microvesicles) that may transport miRNA-133 in the circulation can provide insight to determine whether cell-to-cell communication is altered by properties of the vesicle.

The role of miRNA-133 with exercise may demonstrate mechanisms underlying its relationships with cardiovascular health or glucose metabolism. We showed that eight weeks of combined exercise training did not significantly alter circulating miRNA-133a or -133b. The mixed responses of circulating miRNA-133 (126,139,145) with acute exercise suggest that muscle contraction alone does not secrete miRNA-133 isoforms. Rather, circulating miRNA-133 expression may be regulated by muscle damage, supporting the notion that miRNA-133a may be a biomarker for patients with heart failure (185). miRNA-133a has been repeatedly shown to be upregulated immediately after a marathon, a competition known to induce great degrees of muscle damage (126,136,154). Baggish et al. (149) determined the circulatory miRNA response was specific to skeletal muscle damage, as brain- and endothelial-specific miRNAs were
minimally affected by a marathon, but miRNA-133a was robustly upregulated. Furthermore, a comparison of different exercise modes determined that eccentric exercise (i.e., highly muscle damaging) resulted significantly upregulated circulatory miRNA-133a (141). Such insight regarding the role of skeletal muscle damage with miRNA-133a and -133b may explain the reasons for discrepant findings between miRNA-133 with insulin sensitivity and cardiac damage. For instance, miRNA-133 may only be acutely upregulated by muscle-damaging exercise, but chronic downregulation could be indicative of muscular adaptation. In female rats, 10 weeks of swim training resulted in significantly lower miRNA-133a and miRNA-133b expression in muscle (184). This improvement may be due to enhanced oxidative metabolism, as Mooren et al. (136) reported a relationship between the marathon-induced increase in miRNA-133a and runners’ cardiorespiratory fitness ($\text{VO}_2\text{max}$). This is in agreement with our findings of lower baseline miRNA-133a and miRNA-133b associated with the greatest improvements in aerobic capacity.

Findings from the present study indicate that in sedentary, normoglycemic males, eight weeks of combined exercise training do not significantly alter miRNA-133a and miRNA-133b expression. However, the ability to improve aerobic capacity after combined exercise training may be related to the regulation of miRNA-133 isoforms, as displayed by the FH+ group. Given that obesity is characterized by chronic low-grade inflammation (191) further investigation of miRNA-133 may provide a link between insulin resistance and the ability of miRNA-133 to regulate inflammation.

miRNA-155

The role of miRNA-155 in regulating glucose metabolism remains unclear after divergent mechanisms were recently published by two animal studies (74,112). To our knowledge, this is the first study to examine circulating miRNA-155 expression following an exercise intervention. Our findings indicate that circulating miRNA-155 expression are not be altered between individuals with/without a FH who are healthy and normoglycemic. In addition, we found that
eight weeks of combined exercise training did not significantly alter circulating miRNA-155 expression in healthy, normoglycemic males, regardless of FH status. The expression of serum miRNA-155 was inversely related with fasting blood glucose at baseline and significantly associated with the exercise training-induced improvements in fasting blood glucose.

The effects of miRNA-155 deletion in female mice indicate protection against a number of metabolic abnormalities induced by a high fat diet (HFD) (112). Compared with female wild types (WT), female miRNA-155 KO mice gained significantly less body weight (including 74% less white adipose tissue), upregulated insulin signaling genes (GLUT4 mRNA and IRS-1 mRNA) and demonstrated greater energy expenditure due to uncoupling in brown adipose tissue (112). Lower blood glucose levels at the 60 min mark of a glucose tolerance test (GTT), compared with WT fed a HFD, suggest improved 2nd phase insulin secretion, which is important to sustain inhibition of hepatic glucose production in the post-prandial state (192). These data suggest that elevated miRNA-155 may negatively regulate hepatic insulin signaling and impair suppression of hepatic glucose production.

In contrast, Lin and colleagues (74,193) reported that miRNA-155 has a positive role in regulating glucose metabolism. Liver-specific overexpression of miRNA-155 alleviated nonalcoholic fatty liver induced by a HFD and improved the serum lipid profile (193). Global miRNA-155 overexpression in vivo significantly improved glucose tolerance and reduced fasting blood glucose. In C2C12 cells, miRNA-155 overexpression improved glucose uptake and phosphorylation of several insulin signaling proteins (74). On the other hand, miRNA-155 deficiency induced glucose intolerance and increased fasting blood glucose (74).

There may be multiple roles of miRNA-155 in regulating glucose metabolism, as determined by opposing results in the preclinical studies. Our findings indicate that circulating miRNA-155 is similar in a normoglycemic population, regardless of FH. However, we observed a significant relationship, where individuals with greater miRNA-155 expression exhibited lower fasting glucose levels at baseline. This finding supports the animal studies by Lin and colleagues (74,193), who also showed that serum miRNA-155 was significantly lower in T2D patients.
compared with healthy adults (74). This downregulation of miRNA-155 in T2D was negatively associated with insulin resistance (HOMA-IR). To further understand the mechanisms underlying miRNA-155-mediated improvements in insulin sensitivity, a predicted target of miRNA-155 was validated (74). Phosphatase and tensin homolog (PTEN), an inhibitor of insulin signaling in skeletal muscle upstream of Akt (194), was significantly reduced by miRNA-155 overexpression (74). Thus, our study aligns with the hypothesis that elevated miRNA-155 may inhibit negative regulators of insulin sensitivity. We provide evidence that greater circulating miRNA-155 is associated with better control of fasting glucose levels and its expression is not aberrantly expressed in normoglycemic individuals.

In the first study to assess miRNA-155 expression with exercise training, we showed that eight weeks of combined exercise training did not affect serum miRNA-155 expression regardless of a FH. However, there was a negative association between miRNA-155 expression at baseline and very low-density lipoprotein (VLDL) levels, independent of FH. Only in those with a FH, exercise training-induced improvements in aerobic capacity were inversely related to baseline miRNA-155 expression. To determine why greater circulating miRNA-155 may enhance insulin sensitivity, but improvements in aerobic capacity are related to lower miRNA-155 expression, the mechanisms that control miRNA-155 secretion need to be examined. The role of miRNA-155 related to mitochondrial content/function warrants further investigation.

**SUMMARY**

In conclusion, our study shows that sedentary, normoglycemic, healthy Mexican American males with a family history of type 2 diabetes do not display altered expression of circulating miRNAs associated with insulin sensitivity (miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155). In the first study to determine the effects of combined exercise training in healthy individuals on circulating miRNAs related to insulin sensitivity, we showed that basal expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155 are not significantly affected. Our findings show that even when normoglycemia exists in sedentary
populations, individuals who exhibited lower expression levels of miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155 displayed lower fasting blood glucose at baseline and the greatest reductions in fasting glucose following exercise training. Thus, we provide a link between circulating miRNA-29a, miRNA-133a, miRNA-133b and miRNA-155 and an important marker of T2D, fasting glucose levels, in normoglycemic individuals. The coordination of blood glucose requires efforts from multiple tissues and the extent which circulating miRNAs regulate glucose metabolism needs further investigation.

Understanding the role of miRNAs in the pathophysiology of obesity and type 2 diabetes is a challenging, yet imperative task with a potential to enhance diabetes screening and treatment. To establish a blood-based biomarker for individuals at risk for type 2 diabetes, will address the metabolic disorders of millions across the globe and prevent undue healthcare expenses, but more importantly preserve healthy lives. Efforts to elucidate mechanisms of individual miRNAs in vitro are valuable, but without the proper validation in human studies, there remains a significant gap between the physiological knowledge and implementation in medical practice. Future research should combine methods that detect miRNA in circulation and in target tissues, while validating mRNA targets. Advancements in technology such as next generation sequencing (NGS) can clarify the secretory profile of miRNAs and their related cargo in circulation to determine tissue-tissue cross-talk in diabetes. Several circulating miRNAs have emerged as candidate biomarkers in only a decade of research, hence the potential for clinical application holds a promising prospective to manage, screen and potentially prevent a disease that affects individuals across different ages and ethnic backgrounds.
Figure 1. The Central Dogma of Molecular Biology.
Figure 2. miRNA synthesis and biogenesis (78). miRNA transcription occurs in the nucleus via polymerase II, generating a primary-miRNA (pri-miRNA). The enzyme, Drosha, cleaves the structure resulting in a precursor-miRNA (pre-miRNA), which is then exported to the cytoplasm by exportin-5 (EXPO5). Dicer then cleaves the precursor further, removing the stem loop, to generate a mature double-stranded miRNA. The mature miRNA is recognized by a RNA-induced silencing complex (RISC) facilitating the binding of miRNAs to messenger RNAs (miRNA) to repress translation or induce degradation.
Figure 3. Study Design
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Curriculum Vita

Cesar Meza is a graduate student researcher pursuing a career in academia. Cesar earned a Bachelor of Science in Kinesiology from The University of Texas at El Paso (UTEP) and is earning his Master of Science in Kinesiology. After completion of his master’s degree, Cesar will enter his doctoral studies at Florida State University to earn a PhD in Exercise Physiology. During his time as a graduate student under the supervision of Dr. Sudip Bajpeyi, Cesar worked as a Teaching Assistant for the Departments of Kinesiology and Biological Sciences.

As a research assistant in the Metabolic, Nutrition & Exercise Research (MiNER) Laboratory, Cesar earned several awards including a 2017-2018 Dodson Research Grant, a Master’s Scholar Award from the American Kinesiology Association, the Outstanding Graduate Student Award from the Department of Kinesiology, the 2017-2018 Allien and Paul C. Davidson Scholarship, 1st and 2nd place for research poster presentations at regional conferences and several travel awards.

Cesar plans to pursue a research career to focus on the pathophysiology of type 2 diabetes and to understand how exercise mitigates impairments in cardiometabolic health.

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