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CIRCULATING MIRNAS AND HUMAN INSULIN RESISTANCE

by

ELIZABETH MA

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A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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2017

CIRCULATING MIRNAS AND HUMAN INSULIN RESISTANCE

ELIZABETH MA

NUTRITION SCIENCES

ABSTRACT

Insulin resistance (IR) is central to the pathophysiology of Type 2 diabetes (T2DM), and is a risk factor for cardiovascular disease. The mechanisms behind IR are not clear, but microRNA (miR; short, non-coding RNA strands that base-pair with mRNA to modify gene expression) have garnered interest as potential contributors. miRs have recently been shown to be detectable in the circulation, and can be taken up and regulate target genes within recipient cells.

Our primary objective was to identify circulating miRs (c-miRs) that play a role in regulating systemic metabolism and participate in the pathophysiology of IR and cardiometabolic disease. Three studies were performed to 1) ascertain the relationship between candidate c-miRs and IR in humans, 2) assess the association of these miRs with oxidative stress as a potential mechanism for the development of IR, and 3) evaluate the impact of an insulin-sensitizing intervention on c-miRs.

We found that miR-16, -107, -33, -150, -222, -34a, -126, -320, and let-7a are related to measures of insulin sensitivity in cross-sectional analyses. miRs were also associated with related metabolic risk factors (e.g., lipids). While we made the novel observation that F2-isoprostanes may reflect oxidative stress relating to age (decreases in bone and lean mass), there was no association between F2-isoPs and insulin sensitivity, nor between miRs and F2-isoPs. In patients with obesity placed on weight-loss diets, c-miRs were not affected by the amount of weight loss or macronutrient diet composition.

However, changes in miR-222 were correlated with enhanced insulin sensitivity and changes in miR-16 and miR-122a with decrements in HDL. The amount of weight loss affected the relationships between miR species and metabolic traits; after 15 weeks, miR-126, -34a, -320, and let-7a were correlated with insulin sensitivity in low responders and miR-16, and -223 with lipids in high responders. The data indicate that miRs could determine the metabolic response to dietary interventions in obesity.

Taken together, our results suggest that circulating miRs may be involved with insulin resistance pathophysiology, and thus have potential as biomarkers and/or therapeutic agents for diabetes and cardiometabolic disease. Future studies are warranted to better elucidate the mechanisms through which circulating miRs regulate metabolism.

Keywords: miRNA, circulating miR, insulin sensitivity, insulin resistance, metabolic risk factors, F2-isoprostanes

DEDICATION

This dissertation is dedicated to my family – particularly my parents, Miaoching Chu and Hsien-Ming Ma – for their love and support throughout my life and especially during these last few years.

This work is also dedicated to God, who has led me here, and without whom I could not have been accepted into this program, or have the strength to finish this degree.

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INTRODUCTION

Diabetes

Diabetes is currently ranked as the 7th leading cause of death in the United States, with 76,488 reported deaths (2.9% total) in 2014[1]. It affects 29.1 million people nationally (9.3% of the U.S. population), and is one of the leading causes of preventable blindness, kidney failure, and non-traumatic amputations. In addition, it is associated with other metabolic disturbances that put diabetics at a higher risk for developing concomitant diseases and complications, such as cardiovascular disease (CVD), dyslipidemia, and hypertension[2]. This clustering of metabolic disturbances was recognized in the 2000s as cardiometabolic syndrome (CMS)[3] after the observation that these metabolic abnormalities increased a patient's risk for cardiovascular disease. In fact, diabetes itself is an independent risk factor for strokes and CVD[4], which has generally remained the leading cause of death in the U.S. since at least the 1950s[5]. In 2014 alone, there were 614,348 deaths (23.4% of all deaths) from CVD, and an additional 133,103 deaths from cerebrovascular disease, which was listed as the 5th leading cause of death[1]. Thus, in addition to the poorer quality of life experienced by patients, diabetes is a costly disease, both on a personal level, and in regards to national healthcare expenditures. In 2014, the total estimated cost of diabetes was $245 billion, which includes both direct (medical) and indirect (disability, loss of work, premature death) costs[2].

The two major forms of diabetes are Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM). The former has autoimmune origins, wherein the body begins to attack its own pancreatic β cells, leading to cell injury and death, and develops a subsequent inability of the body to produce insulin – a key hormone required for decreasing blood glucose levels and storing excess energy. T2DM, which is the most common type of diabetes, is thought to be an interaction between genes, environment, and behavior (80% of diabetics are overweight or obese), and its pathophysiology involves the combination of insulin resistance and impaired insulin secretion. It is the insulin resistance in T2DM that will be the focus of this dissertation.

Diabetes is a disease of chronically elevated blood glucose, and it is diagnosed by one of the following: random blood sugar ≥ 200 mg/dL, fasting blood glucose levels ≥ 126 mg/dL on two separate tests, glycated hemoglobin (A1C) ≥ 6.5% on two separate tests, or blood glucose ≥ 200 mg/dL after an oral glucose tolerance test (OGTT), in which patients are given a bolus of glucose (75g or 100g) and their blood glucose levels are monitored for 2-3 hours to assess their body's response to the high glucose load. Prediabetes, a condition of elevated blood glucose that is not quite at diabetic levels, is diagnosed if fasting blood sugar is between 100-125 mg/dL, A1C is between 5.7 – 6.4%, or if the OGTT reading is between 140 and 199 mg/dL at 2 hours post glucose ingestion. An estimated 86 million people have prediabetes nationally[2], but it often goes undiagnosed, which is concerning because prediabetes is a significant risk factor for future diabetes and its accompanying metabolic sequelae. This may partly be due to the amount of time and energy it takes for these diagnostic tests (requiring either at least two test visits, or the uncomfortable, lengthy OGTT), which makes checking for prediabetes

cumbersome, and results in the majority of diabetes cases being diagnosed only after symptoms of hyperglycemia are present. Thus, if there were a simple way to test for insulin resistance, which precedes both of these conditions, early detection and treatment of patients who are at risk for developing prediabetes or T2DM would be more likely to occur.

Insulin Resistance

Insulin is a hormone produced in the β cells of the pancreas that plays a key role in promoting energy storage in the body. Its secretion is primarily stimulated by high levels of blood glucose following a meal, and its main target tissues are the liver, skeletal muscle, and fat, where it promotes uptake of glucose and storage of lipids while simultaneously suppressing gluconeogenesis and β-oxidation[6].

Insulin resistance (IR) occurs when these tissues no longer properly respond to stimulation by insulin. This puts pressure on the β cells to secrete more insulin to compensate. Over time, the β cells begin to fail with a decreased ability to sense insulin, and insulin secretory responses become impaired. This leads to impaired glucose tolerance (IGT), impaired fasting glucose (IFG), and eventually, T2DM. With progression to diabetes, hyperglycemia itself can worsen insulin resistance (i.e. "glucose toxicity") and add to the underlying degree of insulin resistance.

Currently, insulin resistance can be assessed through insulin sensitivity tests, which include both direct (the hyperinsulinemic-euglycemic clamp technique) and surrogate methods (indices calculated through either fasting insulin, glucose, and/or triglycerides, or through values obtained during the OGTT)[7]. The gold standard measure

for insulin sensitivity is the hyperinsulinemic-euglycemic clamp, first described by DeFronzo et al in 1979[8], where after a 12 hour fast, patients are given an insulin infusion to raise plasma insulin concentrations to a certain steady-state level that is maintained for 120 min. A glucose infusion is then given shortly after the initial priming dose to keep glucose levels "clamped" at the patient's baseline glucose levels. Blood samples are collected at regular intervals, and whole-body insulin sensitivity is determined through the glucose disposal rate (GDR) calculated during the steady-state period, adjusted for lean body mass.

While the gold standard clamp technique is currently the most direct way to measure insulin sensitivity, it is a time intensive, relatively invasive, and expensive method to use. Thus, surrogate measures for insulin sensitivity were developed for clinical use. One commonly used measure is the homeostasis model assessment-insulin resistance (HOMA-IR) which is based on fasting insulin and glucose values (calculated as (fasting insulin* fasting glucose)/constant) and has relatively good correlation with clamps[9]. The quantitative insulin-sensitivity check index (QUICKI) is another measure that uses these fasting values, and is given by 1/(log(fasting insulin) + log(fasting glucose)). It has also been shown to have good linear correlation with the gold standard clamp, possibly even better than that of HOMA-IR[10]. However, while HOMA-IR and QUICKI seem to be fairly useful from a population perspective, there are some concerns that they may not be any better than fasting insulin for diagnostic purposes on the individual level[11]. Thus, there is a need for better biomarkers for insulin sensitivity in humans.

The Matsuda index[12], on the other hand, takes both liver and peripheral tissue insulin sensitivity into account by utilizing values from the OGTT ($10000/\sqrt{fasting\ glucose * fasting\ insulin * \ mean\ glucose * \ mean\ insulin}$)[7], and is a better measure of insulin sensitivity than HOMA-IR[11]. Another approach for determining insulin sensitivity is the frequently sampled intravenous glucose tolerance test (fsIVGTT) and minimal model. However, logistics often preclude use of the Matsuda index and the fsIVGTT in clinical practice.

In addition to diabetes, insulin resistance also leads to dysmetabolic processes and worsens cardiovascular disease risk factors[13]. IR is central to the development of dyslipidemia (low HDL-c and high triglycerides), hypertension, fatty liver disease, cardiovascular disease, certain cancers, and can even impact the brain, such as in Alzheimer's disease. The combination of impaired glucose tolerance, dyslipidemia, elevated blood pressure, and abdominal obesity is called Metabolic Syndrome (MetS)[14]. Fortunately, studies have shown that improving insulin sensitivity in patients with prediabetes or MetS, such as through lifestyle interventions, weight loss, and/or pharmacotherapy interventions, can help prevent the progression to T2DM and other cardiometabolic outcomes. Thus, while the exact mechanism behind the development of IR is currently unclear, the elucidation of causal molecular processes uncovering some of the physiology behind insulin sensitivity would help identify more effective therapeutic targets with which to treat or prevent millions of people from being affected by these diseases.

MicroRNAs and Insulin Sensitivity

In 1993, Lee *et al.* described a short, non-coding RNA that seemed to modulate gene expression in *C. elegans*[15]. This would become the first known documentation of the existence of microRNAs (miRNA; miR) – short, non-coding strands of RNA, usually ~22 nt long, that can base pair with mRNA and modify gene expression post-transcriptionally. Since then, miRs have been found in both animals and humans, some of which are conserved across species, and others that are not. Additionally, some miRs seemed to be specifically expressed in certain tissues, while others seem to be present in multiple cell types.

The biogenesis and actions of miRNAs are summarized as follows[16]: in brief, miRNAs are first transcribed in the nucleus by RNA polymerase II as primary miRNA (pri-miRNA), which are cleaved by Drosha and Pasha into precursor miRNA (pre-miRNA) that have a hairpin loop. They are then exported to the cytoplasm by Exportin 5, where the hairpin loop is cleaved by Dicer and transactivation-responsive RNA-binding protein (TRBP), leaving a double-stranded miR duplex. Once the two strands are separated by RNA helicase, one strand becomes the "active" strand and is incorporated into the RNA-induced silencing complex (RISC), a group of proteins which include members of the Argonaute family and GW182, and help suppress protein translation or guide the degradation of target mRNA. The miR strand that is incorporated is often called the "guide strand" or "leading strand" of miR. The other strand (often denoted the "passenger strand" or "miR*") usually ends up being quickly degraded. Based on the level of complementarity between the miR and its target mRNA, translation of the mRNA is either repressed if the base-pairing is only partially complementary, or directly

cleaved and degraded by the RISC through proteins such as Argonaute2 (Ago2) if the base-pairing is completely complementary. The ability of miRs to modulate gene expression post-transcriptionally makes them an intriguing candidate to investigate as potential regulators in the molecular processes underlying insulin resistance and T2DM.

The majority of work done thus far relating miRs with diabetes and insulin sensitivity has been done *in vitro* or in animal models[17], and results suggest that alterations in tissue levels of miR may affect insulin sensitivity and/or whole-body metabolism. For example, studies have shown that miR-9, -34, and -375 suppress insulin secretion in rodent pancreatic β cells *in vitro*[18] and transfection of miR-135 into C2C12 muscle cells significantly decreased glucose uptake, similar to levels in insulin resistant cells[19]. *In vivo*, silencing of miR 103/107 in the liver and fat of obese mice increased their insulin sensitivity and improved whole body glucose tolerance[20]. Both genetically (*db/db*) and diet induced obese mice have increased miR-143 in their liver, and miR-143-145 deficient mice were protected against insulin resistance from diet-induced obesity[21]. miR-181b was found to be reduced in adipose tissue endothelial cells of obese mice, and systemic delivery of miR-181b improved glucose homeostasis and insulin sensitivity[22]. In diabetic rats, levels of miR-106b, -27a, and -30d were higher in skeletal muscle, and knocking them down in L6 rat cells *in vitro* significantly improved insulin-stimulated glucose uptake[23]. Our own lab has previously found that miR-150 knockout mice have lower body weight, higher glucose tolerance, and improved insulin sensitivity on a high fat diet compared with controls[24]. Thus, results from these rodent models indicate that there is some relationship between cellular/tissue miRs and insulin sensitivity.

In human tissues, however, the literature is sparser. Liver biopsies in obese women showed 14 miRs were associated with non-alcoholic fatty liver disease, and miR-146b was found to be associated with glucose metabolism and fatty acid mobilization[25]. Subcutaneous adipose tissue from 19 severely obese individuals showed alterations of a few miRs after 15 weeks on a weight loss intervention (hypocaloric diet and exercise)[26]. Muscle biopsies from healthy men showed that miR-148b levels increased in skeletal muscle during the early transition phase from an active lifestyle to an inactive one, and treatment with miR-148b in primary cultures of myocytes reduced insulin-stimulated glucose uptake[27]. However, studies like these are limited in population size and scope, due to the inherently invasive nature of tissue biopsies.

Recently, however, miRs have also been found to be present in the circulation, opening up a completely new avenue of investigation. Studies have shown that miR can be secreted and travel through the bloodstream[28], and that they can be protected from ribonucleases normally present in the blood by either being packaged into microvesicles[29] for export/transport, complexed to proteins such as Ago2 or nucleophosmin1 (NMP1), or even bound to lipoproteins such as HDL[30]. After arriving at the target cell, the miR can then act specifically on their target mRNA to alter gene expression within that cell[29]. Additionally, some circulating miRs (c-miRs) seem to be fairly stable over time and freeze-thaws[31], and there are many reports of differential expression of c-miRs in various disease states, including cancer[31, 32] and cardiovascular disease[33]. Thus, c-miRs have garnered attention as having the potential for becoming useful biomarkers of disease in humans, and may themselves be useful for therapeutic purposes.

Regarding the relationship between circulating miR and diabetes and/or insulin sensitivity specifically, several studies have shown that certain c-miRs are differentially expressed in patients with T2DM[34-36] compared with non-diabetic patients. This suggests that c-miRs may play a role in insulin resistance and might be useful as a potential biomarker for diabetes. However, studies are conflicting in regards to which miRs are relevant, which may partly be due to the fact that there is currently no consensus on a standard for normalizing samples[37], and other challenges of working with c-miRs[38]. Additionally, most of these studies look at disease compared to disease-free states, such as the presence or absence of diabetes[39] or metabolic syndrome[40], rather than specifically evaluating insulin sensitivity. This is an important distinction, since, as mentioned above, insulin resistance precedes the development of disease states like diabetes or metabolic syndrome. In addition, of the few studies that do address the connection between c-miRs and insulin sensitivity in humans, most use a surrogate measure for insulin sensitivity such as HOMA-IR[41-43] instead of the gold standard clamp method. Thus, we wanted to evaluate which c-miRs were specifically related to insulin sensitivity, as opposed to the diabetic disease state, and to use the gold-standard hyperinsulinemic-euglycemic clamp as the measure of insulin sensitivity. If successful, patients could be informed with a simple blood draw about their insulin status before they develop pre/diabetes, and this might additionally help to identify potential therapeutic targets for insulin resistance in humans.

Objectives

The underlying hypothesis of this dissertation is that circulating miRs may help regulate systemic metabolism and participate in the pathophysiology of insulin resistance and cardiometabolic disease. To this end, there were three main objectives: 1) ascertain the relationship between candidate c-miRs and insulin sensitivity in humans, 2) assess the association of these miRs with oxidative stress as a potential contributor to the pathophysiology of IR, and 3) evaluate the impact of a diet-induced, weight loss (insulin-sensitizing) intervention on c-miRs.

The first two studies utilized banked samples from participants that were sequentially recruited for metabolic characterization at the University of Alabama at Birmingham's (UAB) Clinical Research Unit. Participants were non-diabetic, sedentary, and weight-stable for at least 3 months prior to the study (± 3% body weight). Participants were excluded if they had BMI <21 kg/m^2 or >50 kg/m^2, had evidence of T2DM, cardiovascular, renal, thyroid, or hepatic disease, or if they were using any medications that could affect body composition, lipid, or carbohydrate metabolism. Demographics were given by self-report.

Participants were then equilibrated on a weight-maintenance, isocaloric diet (28-32 kcal/kg/d, 50% carbohydrates, 30% fat, and 20% protein) for 3 days while in the metabolic ward. Measurements taken included anthropometric measures, body composition as measured by dual-energy x-ray absorptiometry (DXA), blood pressure, insulin sensitivity as measured by the gold standard hyperinsulinemic-euglycemic clamp technique, fasting plasma/serum measures of glucose, insulin, circulating lipids, substrate

oxidation rates from indirect calorimetry, and oxidative stress markers through a spot morning urine collection. Plasma and urine samples were stored at -80°C until use.

The first study used plasma samples from a subset of these patients across a spectrum of insulin sensitivity to determine whether c-miRs are associated with insulin sensitivity, or other relevant metabolic parameters. A preliminary screening study was done using QIAGEN's Diabetes microarray, using pooled plasma samples of a subset of the most insulin resistant and most insulin sensitive of the sample population to identify candidate miRs of interest. All available patient samples were then individually assayed with each candidate miR of interest and subsequently assessed for their relationship with insulin sensitivity, as well as associated metabolic risk factors.

The second study aimed to evaluate whether miRs might influence insulin sensitivity through regulation of oxidative stress, based on the common assumption that oxidative stress is related to the pathophysiology of insulin resistance. Thus, urine samples were used from participants who underwent metabolic characterization to determine the relationship of urinary F2-isoprostanes (F2-isoPs; the gold standard biomarker of whole-body oxidative stress in humans) with insulin sensitivity as assessed by clamp. The urinary F2-isoPs were then also assessed for relationships with other metabolic factors and body composition. These findings are summarized in the published, second paper (3rd chapter) of this dissertation. Plasma miR data were also available for a subset of these patients from the first study, so urinary F2-isoprostanes were additionally analyzed with each candidate c-miR for potential associations. Results from these analyses are incorporated into the second paper (see *italics*) and Discussion section of this dissertation.

The last study attempted to determine how an insulin-sensitizing intervention would affect levels of c-miR, in conjunction with other metabolic risk factors. This was done through a pilot, prospective, diet-induced, weight-loss intervention study conducted at UAB in collaboration with Dr. Barbara Gower, as weight loss has been shown to improve insulin sensitivity and metabolic outcomes[44, 45]. The study aimed to evaluate the effects of three different diets (very low carbohydrate, low fat, and a very low calorie, meal-replacement diet) on weight loss and body composition, along with associated metabolic changes, such as with fasting glucose and lipids. Patients were overweight or obese, at least 19 years old, and not actively on a weight loss program prior to enrolling as a new patient to UAB Weight Loss Medicine. Exclusion criteria were BMI > 50 kg/m^2, pregnancy/lactation, steroid medication, thyroid disease, and smoking.

Given that this was a pilot study and not a randomized, controlled trial, the variable number of enrollees across diets made it difficult to interpret/gain meaningful data through analysis by diet type. However, by pooling all the patients together and categorizing patients by amount of weight lost, we were able to examine the impact of weight loss on circulating miRs. Our criteria for categorizing patients was based on the fact that success in medication-assisted weight loss is defined by the Food and Drug Administration as ≥ 5% weight loss at 3 months. If weight loss is < 5% at 3 months, the recommendation is to discontinue the current drug and substitute it with another. The ability to achieve ≥ 5% weight loss at 3 months has also been associated with better weight loss at 2 years, along with improvements in insulin sensitivity and other metabolic parameters[46]. Thus, patients were categorized as high responders if they were able to successfully lose ≥ 5% of weight by 15 weeks, and low responders if they were not.

Individual miRs were then assayed in each patient sample at baseline and 15 weeks, and results were compared between the groups. Finally, additional analyses were conducted to correlate the relative expression of miRs with associated metabolic risk factors.

CIRCULATING MIRNAS, INSULIN SENSITIVITY, AND ASSOCIATED METABOLIC RISK FACTORS IN HUMANS

by

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Format adapted for dissertation

Abstract:

Objective: Insulin resistance results in disruption of metabolic processes and leads to various chronic disease states such as diabetes and metabolic syndrome. However, the mechanism linking insulin resistance with cardiometabolic disease pathophysiology is still unclear. One possibility may be through circulating microRNAs (miRs), which have garnered attention in recent years due to their ability to alter gene expression in target tissues. Our goal was to assess the relationship of circulating miRs with insulin sensitivity, as measured by the gold standard, hyperinsulinemic-euglycemic clamp technique.

Methods: 81 non-diabetic, sedentary and weight-stable patients with BMI >21 kg/m^2 across a wide range of insulin sensitivities were chosen for this study. Measurements were taken for blood pressure, anthropometric data, fasting glucose and lipids, and insulin sensitivity, as measured by the hyperinsulinemic-euglycemic clamp. After an initial screening array in plasma samples to identify candidate miRs, all patients were assessed for levels of these circulating miRs, which were then compared to insulin sensitivity and associated metabolic factors.

Results: A total of 13 miRs were chosen as candidates for this study for analysis in all patient samples: miR-133a, -200a, -34a, -150, -199a, -140, -16, -107, -222, -218, and -33, as well as SNORD61 and RNU6-2. miR-16 and miR-107 were positively associated with insulin sensitivity ($R^2 = 0.09$, $p = 0.0074$ and $R^2 = 0.08$, $p = 0.0417$), and remained so after adjustment with BMI. After adjusting for BMI, miR-33, -150, and -222 were also

found to be related to insulin sensitivity. Regarding metabolic risk factors, miR-16 was negatively associated with waist circumference (r = -0.25) and triglycerides (r = -0.28), and positively associated with HDL (r = 0.22), while miR-33 was inversely associated with systolic blood pressure (r = -0.29). No significant relationships were found between any candidate miRs and either BMI, diastolic blood pressure, or fasting glucose.

Conclusions: Our results show that relative levels of circulating miR-16, -107, -33, -150, and -222 are associated with insulin sensitivity and metabolic risk factors. These may help point towards potential mechanisms of metabolic dysfunction in insulin action, inflammatory response, lipid accumulation, or other metabolic changes, paving the way for novel therapeutic targets or identifying the miRs that may act as therapeutic agents themselves.

Highlights

- Specific miRs (mir-16, -107, -33, -150, and -222) are related to insulin sensitivity as measured by hyperinsulinemic-euglycemic clamps.
- Some of these miR are also associated with at least one other metabolic risk factor
- No significant relationship was found between any candidate miRs and either BMI, diastolic blood pressure, or fasting glucose.

Keywords: plasma, circulating, microRNA, insulin sensitivity, insulin resistance, metabolism

1. Introduction

Insulin resistance is one of the major contributors to the development of Type 2 diabetes mellitus (T2DM), as well as other metabolic disease states, such as metabolic syndrome (MetS) and cardiovascular disease (1). The development of insulin resistance precedes diagnosis of these diseases, and its mechanism is still unclear. In addition, interventions that increase insulin sensitivity in patients with prediabetes or MetS, such as exercise, weight loss, or thiazolidinedione medications, have been shown to prevent or delay progression to T2DM and improve metabolic risk factors. Thus, understanding the pathophysiology underlying insulin sensitivity could inform the development of better therapies for patients with cardiometabolic disease.

The role of microRNA (miRNA; miR) in the pathophysiology of insulin resistance has been an emerging interest in recent years, but has not been fully explored. miRNA are short, non-coding RNA strands (about 22 nucleotides long) that base-pair with mRNA to post-transcriptionally modify gene expression. They are first transcribed in the nucleus by RNA polymerase II (Pol II) in the form of primary miRNA (pri-miRNA), which then gets cleaved by into precursor miRNA (pre-miRNA) with a hairpin loop. Pre-miRNAs are exported into the cytoplasm, processed into single-stranded mature miRs, and incorporated into the RNA-induced silencing complex (RISC), which either suppresses protein translation or degrades the target mRNA, based on complementarity between the miR and corresponding mRNA.

miRs are found in all tissues, but recent studies have suggested that they can also be secreted and enter the bloodstream, where they can influence gene expression in remote recipient cells (2). Multiple mechanisms are operative to protect circulating miRs

against degradation, including packaging into microvesicles (MVs) (3), association with proteins like Argonaute2 (AGO2) or nucleophosmin 1 (NPM1), or bound to HDL (4). Zhang et al (3) showed that exogenous administration of miR through MVs could be specific and cause subsequent changes in cellular gene expression within the target cell: incubation of miR-150 in MVs secreted from THP-1 macrophages with human microvascular endothelial cells (HMEC-1) increased cellular miR-150 content by 12-fold, and also significantly decreased the intracellular levels of *c-myb*, an miR-150 target gene. Studies have also shown associations between circulating levels of miR species and various forms of cancer (5, 6), cardiovascular disease (7), and diabetes (8).

In animal models, manipulation of miRs in tissues has been shown to affect whole-body metabolism and insulin sensitivity. For example, silencing miR-103/107 in the liver and fat tissue of obese mice improved their glucose tolerance and insulin sensitivity (9). In dogs, changes in insulin and glucose metabolism pathway genes, such as PPARγ, GLUT4, and PGC1α, after a diet intervention for weight loss resulted in decreased miR-107 in muscle tissues and downregulation of both miR-103 and miR-107 in adipose tissue (10). Hepatic overexpression of miR-143, which occurs in obese mice, seems to inhibit insulin-stimulated glucose metabolism (11), and knockdown of a set of 3 miRs (miR-106b, -27a, and -30d), which were higher in the skeletal muscle of diabetic rats, was found to significantly improve glucose metabolism in L6 rat cells through regulation of GLUT4, MAPK 14, and the PI3K regulatory subunit beta (12). Whole-body and hepatic-specific knockout of miR-21/miR-21* was shown to help prevent glucose intolerance and steatosis in mice on a high fat diet (13), and we have previously

found that whole-body knockout miR-150 mice have lower body weights and improved glucose tolerance and insulin sensitivity on a high fat diet compared with controls (14).

However, much less is known about the relationship between levels of circulating miRs (c-miRs) and insulin sensitivity, especially in humans. Most studies use screening methodology to profile patterns of miR differences between disease and disease-free states (i.e. patients with diabetes, MetS, or PCOS vs. controls) (15-19), rather than specifically looking at insulin sensitivity, and studies that did assess insulin sensitivity often used the homeostatic model assessment for insulin resistance (HOMA-IR) (20-22) instead of the gold standard hyperinsulinemic-euglycemic clamp technique. Thus, to better elucidate the relationship between circulating miRs and insulin sensitivity, we identified candidate miRs in plasma samples and individually evaluated each miRs association with insulin sensitivity, as measured with the gold standard clamp technique. The goal of this study was to identify miRs that are related to human insulin sensitivity and its associated risk factors for metabolic disease.

2. Methods

2.1 Participants and sample collection/processing

Participants were sequentially recruited for metabolic characterization at the University of Alabama at Birmingham's (UAB) Clinical Research Unit from 2004-2011. Participants had BMI >21 kg/m^2, did not engage in regular exercise, and were weight-stable for at least 3 months prior (± 3% of body weight). Participants were excluded if they had BMI >50 kg/m^2, evidence of T2DM, cardiovascular, renal, thyroid, or hepatic disease, or if they were taking any medications that could affect body composition, lipid,

or carbohydrate metabolism. All protocols were approved by the UAB institutional review board and written consent was obtained from all participants.

2.2 Clinical measurements

Anthropometric measures of height, weight, and waist circumference were recorded and standardized blood pressure measurements were obtained. Serum and plasma (in EDTA tubes) were obtained after an overnight fast and stored at -80°C until use. Lipids, glucose, and insulin levels were determined using a conventional lipid panel colorimetric assay (Stanbio Laboratory, Boerne, TX), glucose analyzer (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH), and immunofluorescence (TOSOH A1A-II analyzer, TOSOH Corp., South San Francisco, CA), respectively. Insulin sensitivity, assessed as the glucose disposal rate (GDR), was measured using the hyperinsulinemic-euglycemic clamp technique as described previously (23, 24). Briefly, participants were given a primed-continuous infusion of insulin (Humulin; Eli Lilly, Indianapolis, IN) at 200 $mU/m^2/min$ to maximally stimulate glucose uptake and suppress hepatic glucose production, and serum glucose was clamped at 5.0 mmol/L for at least 3 hours. Maximal glucose uptake was determined as the mean glucose infusion rate over the last three 20-minute intervals and the GDR was calculated based on the glucose infusion rate after adjustments for glucose pool size and normalization per kilogram of lean body mass (LBM) as assessed by dual-energy x-ray absorptiometry.

2.3 miR microarray screening

For screening purposes, 15 participants with the highest GDR (Insulin Sensitive; IS) and 15 with the lowest GDR (Insulin Resistant; IR) were chosen to assess for differential miR expression based on insulin sensitivity. Total RNA (including miRs) was extracted from 200 µl of plasma samples of these individuals using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the manufacturer's protocol. Within each group, participants were divided into 3 pooled samples of 5 individuals each, which were then reverse transcribed with the miScript II RT Kit (QIAGEN) into cDNA using the miScript HiSpec Buffer for mature miR profiling. miRNA microarrays were performed with diluted pooled samples using a quantitative, real-time polymerase chain reaction (qrt-PCR; StepOnePlus Real-Time PCR System, Applied BioSystems, USA) on the Human Diabetes miScript miRNA PCR Array (QIAGEN; MIHS-115Z). Analysis of the miR microarray data was performed with miScript miRNA PCR Array Data Analysis software (SABiosciences/QIAGEN). Only one invariant miR (miR-490) emerged from this analysis, and was found to be stably expressed across subsequent individual assays, so it was used as the reference gene for the remainder of the study. For screening purposes, samples were determined to be candidate miRs if they had a greater than 4-fold difference between the two groups, p-value < 0.30. Additional miRs not included in the array were also added as candidate genes if they had the potential to be related to insulin sensitivity based on the literature.

2.4 Candidate miR evaluation

A subset of 81 participants were chosen for this study based on their GDR, with an effort to include an equal number of whites and blacks, males and females, and the availability/viability of the plasma sample (needed enough plasma for the miR extraction and those with overt hemolysis were excluded). Candidate miRs were individually evaluated in each of the 81 plasma samples. RNA extraction and reverse transcription to cDNA were the same as described above. cDNA samples were diluted 1:10 for determining relative expression using qrt-PCR with the miScript SYBR Green PCR kit (QIAGEN) in duplicate. Primers used for evaluation of individual mature miR expressions were as follows (All from QIAGEN, Cat. No. 218300, miScript Primer Assays): mi-33, mi-490, mi-150, RNU6, mi-16, mi-140, mi-133a, mi-199a, mi-218, mi-200a, SNORD61, mi-34a, mi-107, and mi-222. All data were normalized to miR-490 and subsequent analyses used –ΔCt values for relative expression.

2.5 Statistical analyses

Descriptive data are presented as means ± standard deviation, with comparisons made through Student's t-test or non-parametric equivalents, as appropriate. miR-107 and miR-133a were transformed by adding a constant and square-rooting the values, and triglycerides, HDL, and fasting glucose were log-transformed for normality for subsequent analyses. Simple linear regressions were conducted between insulin sensitivity and relative expression levels of circulating candidate miR (-ΔCt), along with an adjusted multiple linear regression model including BMI, with standardized β coefficients reported for the miR and BMI. Pearson's correlations were also determined

between circulating miRs and relevant metabolic risk factors (measures of obesity, blood pressure, lipids, and fasting glucose). All statistical analyses were performed with the SAS 9.4 statistical software package (SAS Institute Inc., Cary, NC).

3. Results

A total of 81 samples were used for this study, across a wide range of insulin sensitivity, as measured by the hyperinsulinemic-euglycemic clamp technique. The majority of patients were female (67.9%), and there was a relatively even representation of African-Americans and European Americans in our study (51.9% AA; 45.7% EA; 2.7% other). Patient characteristics are depicted in Table 1.

3.1 miRs and Insulin Sensitivity

Based on the screening array criteria above, five candidate miRs were chosen for further analysis from the microarray: miR-133a, -200a, -34a, SNORD61, and RNU6-2. miRs -150, -199a, -140, -16, -107, -222, -218, and -33 were additionally included for this study, as they have previously been shown to have differential expression in diabetic patients (9, 25, 26), and/or a potential relationship to insulin sensitivity (14, 27-30). Thus, a total of 13 candidate miRs were chosen for further analysis in each of the 81 samples. Four of these miRs were excluded from our final results (miR-218, -200a, SNORD61, and RNU6-2) due to having a majority of samples past the limits of detection (Ct values >35).

Simple linear regressions of candidate miRs with glucose disposal rate (Table 2) showed that miR-16 and miR-107 were positively associated with insulin sensitivity (R^2

= 0.09, p = 0.0074 and R^2 = 0.08, p = 0.0417, respectively) (Figure 1). After adjustment for BMI, miR-16 and -107 were still significantly associated with GDR, and miR-33, -150, and -222 were additionally found to be related to GDR. BMI was a significant covariate for all models (Table 2).

3.2 Candidate miRs and risk factors for metabolic disease

Candidate miRs were then tested for associations with common risk factors for cardiometabolic disease, including BMI, waist circumference (WC), systolic and diastolic blood pressure (SBP, DBP), circulating triglycerides (TG), HDL-cholesterol, and fasting glucose (Table 3). miR-16 was negatively associated with WC and triglycerides, and positively associated with HDL. miR-33 was negatively associated with SBP. No significant relationships were found between any candidate miRs with either BMI, DBP, or fasting glucose.

4. Discussion

Using miRNA microarrays, we screened pooled samples of human plasma from insulin sensitive and insulin resistant participants to identify miRNA species that were potentially affected by insulin sensitivity. Thirteen specific miRNAs were identified for validation in a larger number of individual plasma samples from patients who had been metabolically characterized for their degree of insulin sensitivity and Metabolic Syndrome (MetS) traits.

Circulating levels of miR-16 and miR-107 were found to be positively associated with insulin sensitivity, as measured by the gold standard hyperinsulinemic-euglycemic

clamp technique. Furthermore, after controlling for BMI, three other miRNAs, namely miR-150, -222, and -33, were significantly associated with insulin sensitivity in addition to miR-16 and miR-107. These miRNAs were also variably related to traits that comprise the MetS, including triglyceride levels (miR-16), HDL cholesterol (miR-16), waist circumference (miR-16), and systolic blood pressure (miR-33). Our findings demonstrate that insulin sensitivity in humans is associated with higher circulating levels of a small assembly of miRNA species, and that some of these miRNA species are also quantitatively associated with other MetS traits.

Our data showing that circulating miR-16 is associated with insulin sensitivity in humans corresponds with observations regarding tissue content of miR-16 in rodent models. Lee *et al.* (31) found that miR-16 levels in the skeletal muscle of genetically obese, insulin resistant Zucker rats were decreased by approximately 50% compared with insulin-sensitive lean rats, and miR-16 was also found to be significantly downregulated in muscle, adipose, and liver tissue of high sucrose diet mice (29). Circulating adipocyte-derived exosomal miR-16 has also been shown to decrease after gastric bypass surgery in humans, concomitant with improvements in insulin sensitivity (32). However, in contrast to these studies, a pilot study profiling women who acquired gestational diabetes showed a higher expression of miR-16 than those with healthy pregnancies (33). It is also difficult to say whether levels of miR-16 affect glucose metabolism and insulin sensitivity or vice versa, as levels of glucose in the microenvironment also seem to impact miR-16 both in cells and the circulation (34). We found no correlation of miR-16 with fasting glucose, however. Of note, miR-16 has at least 27 target genes *in silico* that participate in the insulin signaling pathway, including the insulin receptor itself (32).

miR-16 also appears to be involved with immune function, as it is decreased in M (LPS + IFNγ) polarized macrophages, and overexpression of miR-16 in these cells significantly decreases their secretion of pro-inflammatory cytokines. miR-16 overexpression also enhanced insulin-stimulated glucose uptake in muscle cells that had been rendered insulin resistant by exposure to conditioned media from cultured M (LPS + IFNγ) polarized macrophages (29). Interestingly, miR-16 has also been shown to be involved with skeletal muscle turnover (31), and has been implicated in the cross-talk between skeletal muscle and various organs, including the pancreas. Intramuscular injection of exosome-like vesicles (ELVs) containing miR-16 from insulin resistant skeletal muscle after a high palmitate diet (which induces insulin resistance) showed that the vesicles were able to travel to the pancreas *in vivo*, and could be taken up by adipocytes, and affect *Ptch1* in beta cells *in vitro* (35). These data from the literature, taken together with our findings, suggest that miR-16 may play a role in insulin resistance and the chronic inflammation underlying cardiometabolic disease.

It should also be noted that that some studies have used miR-16 as a constitutive miRNA for data normalization to examine alterations in other circulating miRs (36). However, this has been refuted by other studies (37), and our data show that expression of miR-16 in plasma is variable and quantitatively related to metabolic traits. In fact, we found that the miR that was the most stable across all samples was miR-490, even more so than the spike-in control (data not shown). Thus, future studies might consider using miR-490 as a potential endogenous control for qPCR studies on miR analyses conducted on human plasma samples.

Our data linking plasma miR-107 and insulin sensitivity in humans is consistent with the observation that circulating levels of miR-107 increased after 12 weeks of endurance training (38). However, a study measuring platelet and platelet-poor plasma miR levels in patients with either ischemic stroke, diabetes, both, or none, showed no difference in miR-107 among these groups (39). The current data are also somewhat at odds with previous reports in pre-clinical model systems. miR-107 was shown to be upregulated in the livers of obese mice, and silencing of miR-107 (and miR-103) in liver and adipose tissue improved glucose tolerance and insulin sensitivity in both *ob/ob* and diet-induced obese mice (9) through the actions of caveolin-1. In addition, miR-107 has been shown to promote lipid accumulation in liver cells through targeting of FASN (40) and inhibiting mitochondrial β-oxidation (41), and other potential targets within lipid metabolism pathways. The combined data point to an interesting possibility to explain the discrepancy between the current human data and preclinical studies. As described, circulating levels of miR-107 are increased in insulin-sensitive humans, and in rodent tissues and cultured cells, increased content of miR-107 mediates insulin resistance and lipid accumulation. Thus, perhaps under insulin resistant states, circulating miR-107 levels might decrease because they are being delivered to cells and tissues of need in order to compensate for impaired substrate metabolism and insulin action at those sites. This would explain a sort of counter-balance, featuring an inverse regulation of miRNA levels in plasma versus tissues.

It is interesting to note that miR-107 may promote angiogenesis in tissues under hypoxic conditions through downregulating Dicer-1, which normally helps process precursor miR (42). Circulating miR-107 is also downregulated after ischemic stroke in

rats (43), and both brain and plasma levels of miR-107 increase after patients have an ischemic stroke (44). This compels the speculation that hypoxic stressors may also promote tissue uptake of circulating miR-107. In any event, it is possible that circulating levels of miRNAs may not reflect expression levels or biological effects of the miRNAs in target tissues.

Our results regarding the positive association of miR-150 with insulin sensitivity corresponds well with a study that showed miR-150 KO mice exhibited significant increases in insulin resistance and obesity-induced inflammation, as well as poorer glucose tolerance, on a high fat diet despite similar gains in weight compared to controls (28). This was found to be primarily due to miR-150's effects on B cells present in the VAT. However, this is at odds with our previous data showing that whole-body knockout miR-150 mice had lower body weights, reduced expression of inflammatory cytokines, and improved glucose tolerance and insulin sensitivity on a high fat diet compared with controls (14). Circulating miR-150 has also been shown to be increased in one study of patients with T2DM (26), though it seemed to be somewhat decreased in another (8).

We also found that miR-222 was positively associated with insulin sensitivity after adjusting for BMI. However, these results are discrepant with a couple studies that indicate circulating miR-222 is associated with insulin resistance in post-menopausal women (45), is increased with T2DM (25) and decreases both after treatment with Metformin and after an insulin infusion during clamp (25). miR-222 was also higher in omental tissue of patients with gestational diabetes, and seems to regulate ERα expression related to estrogen-induced insulin resistance *in vitro* (46).

miR-33 has also previously been implicated in insulin sensitivity and glucose metabolism. Dávalos *et al.* showed that miR-33 targets both a key histone deacetylase SIRT6 in glucose homeostasis, as well as the insulin receptor IRS2 (30). The latter was shown to affect downstream PI3K/AKT pathways and reduce insulin-stimulated glucose uptake *in vitro.* Given these data, our findings that circulating miR-33 was positively associated with insulin sensitivity seem at odds with the current literature. On the other hand, anti-miR-33 treatment in high fat diet-fed mice showed increased total cholesterol and decreased serum triglycerides, but no impact on insulin resistance (47). Additionally, while the miR-33 family has been associated with regulation of cholesterol homeostasis, fatty acid metabolism, and circulating lipids (48-51), we did not observe any correlations between the relative expression of miR-33 with circulating lipids in our study. These discrepancies highlight the need for further study in this area.

One of the strong features of the current study is the use of hyperinsulinemic glucose clamps to quantify systemic insulin sensitivity in our volunteers and correlate miRs with GDR. To our knowledge, this is the first study that correlates circulating miRs with insulin sensitivity as measured by this gold standard.

However, there were a few limitations in our study inherent in its design that is relevant to discussion of circulating miRNAs. First, we used banked, frozen, plasma samples, some of which had been subjected to multiple freeze thaws before use, and miRNA species may vary in their stability under these conditions (52). Secondly, while our data show a remarkable constancy of miR-490 levels across all samples, miR-490 does not seem to have been used as a reference gene in previous reports relating to diabetes or insulin sensitivity. However, it was the only invariant miR that fit the criteria

in our screening arrays and was the most stable miR across our sample population, so we felt it was the best one to use as a reference for our study. Third, it is unclear whether different degrees of hemolysis in human blood samples could affect the data (53, 54). For example, miR-16 is present in red blood cells and miR-107 in basophils (55, 56), and these cells could have been disrupted during processing to release falsely elevate the levels of these miRs in the samples. In fact, one study showed that levels of both of these miR may be affected by hemolysis (56). However, efforts were made to exclude samples with overt hemolysis in our study based on visual inspection prior to beginning the miR extraction from samples. Finally, it is also important to note that the R^2 values in our study were relatively modest, indicating that each single miRNA can explain only a small degree of individual variation in insulin sensitivity and other traits. It may therefore be more useful to look at multiple miR signatures together to determine the utility of these miRs as biomarkers or therapeutics for insulin resistance.

In conclusion, our study implicates a cluster of miRNA species as having a role in human insulin resistance. Our study found that plasma levels of miR-16, -107, -150, -33, and -222 were significantly associated with insulin sensitivity, and that miR-16 and miR-33 were additionally associated with and Metabolic Syndrome traits. These miRNAs could serve as biomarkers in humans, or more importantly, act as pathophysiological mediators in diseases characterized by insulin resistance. Finally, it is known that miRNAs can be transported in blood and delivered to specific tissues where they can impact biological functions (3). This raises the prospect for the use of miRNAs we have identified, or others, as pharmacological agents that could be introduced into the bloodstream for targeted delivery to metabolically-relevant tissues, and could constitute a

novel therapeutic approach for the treatment and prevention of cardiometabolic disease. At the same time, it is important to point out that for individual miRs, the R^2s were modest, indicating that each miR can only explain a small portion of the variance in insulin sensitivity. Thus, subsets of miRs may be more efficacious when considering miRs for use as biomarkers and/or therapeutic agents.

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Disclosure

The authors state no conflicts of interest related to this work.

Author Contributions

EM designed the study with help from YF and WTG. EM and WTG worked together on data interpretation. EM conducted the miR assays, performed the statistical analyses, and wrote the manuscript, while WTG oversaw the project and edited the manuscript. YF helped with manuscript revision.

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Table 1. Patient clinical characteristics

Age (yrs)	39.0 ± 10.3
Sex	55 female, 26 male
Race	1 Asian, 42 Black, 37 White, 1 Hispanic
Body Mass Index (kg/m^2)	31.4 ± 5.2
Waist Circumference (cm)	97.4 ± 14.1
Systolic Blood Pressure (mmHg)	116.8 ± 14.1
Diastolic Blood Pressure (mmHg)	68.1 ± 9.5
Triglycerides (mg/dL)	110.0 ± 58.6
HDL-cholesterol (mg/dL)	50.0 ± 20.5
GDR (mg/min/kg LBM)	14.4 ± 4.7
Fasting glucose (mg/dL) (n=80)	92.9 ± 10.9
Fasting insulin (µU/mL) (n=48)	19.3 ± 12.6
Total body fat (%)	41.1 ± 10.0
Total fat mass (kg) (n=71)	34.9 ± 11.7

n = 81 unless otherwise noted. Data are presented as means ± standard deviation or *n* if categorical. Glucose disposal rate (GDR) is adjusted for lean body mass (LBM).

Table 2. Linear regressions of candidate miRs with insulin sensitivity

		Unadjusted Model		Adjusted Model			
miR	n	R^2	p-value	miR Partial β	BMI Partial β	Model R^2	Model p-value
miR-16	81	0.0873	0.0074**	0.25*	-0.39**	0.2183	<0.0001**
miR-33	52	0.0526	0.1018	0.26*	-0.36**	0.1814	0.0028**
miR-34a	44	0.0005	0.8851	0.05	-0.34*	0.0693	0.0865
miR-107	56	0.0746	0.0417*	0.32**	-0.43**	0.2310	0.0004**
miR-133a	73	0.0177	0.2623	0.13	-0.39**	0.1496	0.0013**
miR-140	66	0.0163	0.3072	0.12	-0.41**	0.1559	0.0018**
miR-150	81	0.0334	0.1025	0.21*	-0.43**	0.1994	<0.0001**
miR-199a	67	0.0436	0.0898	0.22	-0.40**	0.1807	0.0006**
miR-222	81	0.0321	0.1097	0.20*	-0.43**	0.1962	<0.0001**

Insulin sensitivity was measured with a hyperinsulinemic-euglycemic clamp technique for glucose disposal rate adjusted for lean body mass. Unadjusted model is a simple linear regression between candidate miRs and insulin sensitivity. Adjusted model includes BMI as a covariate, with standardized β estimates and adjusted R^2 reported. miR-107 and miR-133a were transformed with a constant and square-rooted for normality.

* = p <0.05

** = p <0.01

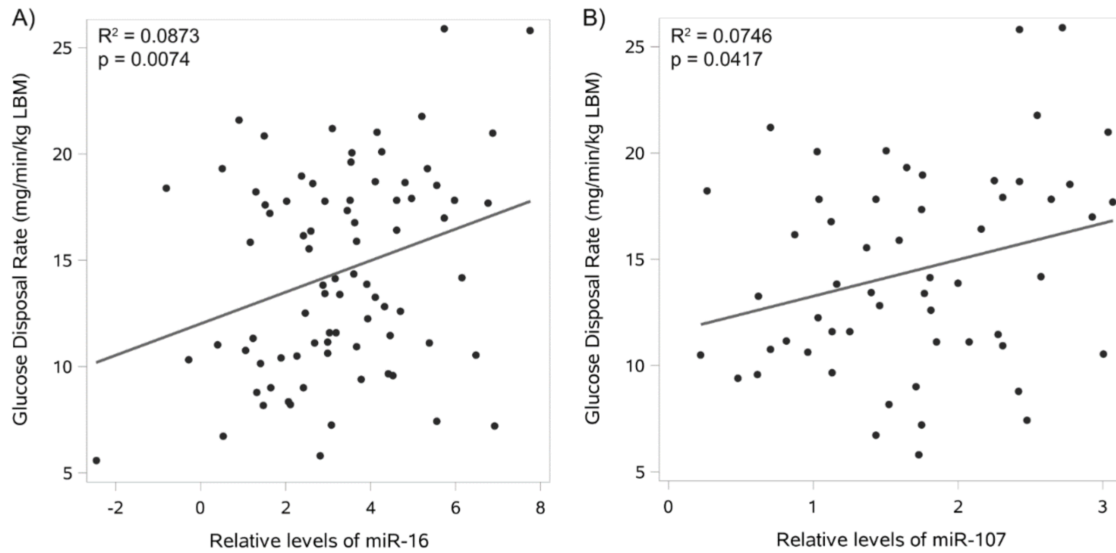

Figure 1. Relationship of miR-16 and miR-107 with insulin sensitivity. Both (A) miR-16 and (B) miR-107 were positively associated with insulin sensitivity, as measured by glucose disposal rate, adjusted for lean body mass. miR-107 was transformed with a constant and square-rooted for normality.

Table 3. Correlation of candidate miRs with metabolic parameters related to insulin sensitivity

miR	n	BMI	WC	SBP	DBP	TG	HDL	Fasting glucose
miR-16	81	-0.11	**-0.25***	-0.15	-0.11	**-0.28***	**0.22***	0.01 (n=80)
miR-33	52	0.08	-0.05	**-0.29***	-0.19	-0.20	0.21	0.02
miR-34a	44	0.09	0.19	0.16	0.18	-0.07	0.05	0.08
miR-107	56	0.11	-0.005	-0.13	-0.08	-0.23	0.09	-0.05
miR-133a	73	-0.01	-0.13	-0.20	-0.11	-0.14	0.10	0.18 (n=72)
miR-140	66	-0.01	-0.09	-0.02	0.04	-0.21	0.08	0.07 (n=65)
miR-150	81	0.07	-0.05	-0.01	0.01	-0.15	0.08	0.12 (n=80)
miR-199a	67	0.03	-0.05	-0.08	-0.07	-0.22	0.09	0.08 (n=66)
miR-222	81	0.06	-0.05	-0.09	-0.09	-0.19	0.13	0.11 (n=80)

Pearson's correlation coefficient is reported for each metabolic parameter as individually assessed with each miRNA. Circulating triglycerides, HDL, and fasting glucose were log-transformed for normality (though this did not affect results for the most part) and miR-107 and miR-133a were transformed with a constant and square-rooted for normality. BMI, Body Mass Index (kg/m^2); WC, waist circumference (cm); SBP, systolic blood pressure (mmHg); DBP, diastolic blood pressure (mmHg); TG, triglycerides (mg/dL); HDL, high-density lipoprotein cholesterol (mg/dL).
* = $p < 0.05$

F2-ISOPROSTANES REFLECT OXIDATIVE STRESS CORRELATED WITH LEAN MASS AND BONE DENSITY BUT NOT INSULIN RESISTANCE

by

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Format adapted and errata corrected for dissertation. In addition, data and discussion have been added that were not present in the publication regarding the relationship between microRNAs and F2-isoprostanes. This supplementary material is represented in *italics*.

Abstract

Context: F2-isoprostanes (F2-isoPs) are biomarkers for oxidative stress in humans and have been shown to be elevated in obesity, cardiovascular disease, and diabetes. Therefore, F2-isoPs are often implicated in oxidative stress contributing to insulin resistance, although this has not been rigorously examined.

Objective: To determine whether urinary F2-isoPs are predictive of insulin sensitivity and other clinical metabolic parameters.

Participants: Sedentary, weight-stable, nondiabetic adults equilibrated on a standard isocaloric diet.

Main Outcome Measures: Insulin sensitivity via hyperinsulinemic-euglycemic clamp, urinary F2-isoPs by gas chromatography-mass spectrometry, and body composition by dual-energy x-ray absorptiometry.

Results: No correlation was found between 15-F_{2t}-IsoP nor its major metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, with insulin sensitivity, even after adjusting for age, race, sex, and smoking status. 15-F_{2t}-IsoP was also not associated with body fat. However, there was a strong negative correlation between 15-F_{2t}-IsoP and lean body mass (LBM; r= - 0.46, P = 0.0001), bone mineral content (BMC; r = -0.58, P < 0.0001), bone mineral density (BMD; r = -0.65, P < 0.0001), and skeletal muscle 4-HNE (r = -0.54, P = 0.0239), another marker of oxidative stress. 15-F_{2t}-IsoP was also positively associated

with circulating triglycerides and total cholesterol, and increased as a function of age. *Circulating microRNA species that have been associated with insulin resistance were also not correlated with urinary F2-isoP levels.*

Conclusions: Urinary 15-F_{2t}-IsoP and its major metabolite are not associated with insulin sensitivity, suggesting the lipid peroxidation process that produces F2-isoPs does not reflect oxidative stress reactions operative in insulin resistance. However, urinary F2-isoPs were negatively correlated with LBM, BMC, BMD, and muscle 4-HNE. Because lean and bone mass decline as a function of biological aging, F2-isoPs may reflect the oxidative stress operative in the aging process.

Keywords: F2-isoprostanes, oxidative stress, insulin resistance, lean body mass, bone density, aging

Oxidative stress has been implicated in the development of insulin resistance (1) and related disease states, such as type 2 diabetes (T2DM), obesity, and metabolic syndrome (2). An important consequence of oxidative stress and overproduction of reactive oxygen species is lipid peroxidation, which, in particular, has been linked with insulin resistance and diabetes (3, 4). F2-isoprostanes (F2-isoPs) are prostaglandin-like compounds formed by nonenzymatic, free radical-induced peroxidation of arachidonic acid (5) and thus represent predominant lipid biomarkers for oxidative stress in humans *in vivo*.

F2-isoPs have been associated with many disease states relating to insulin resistance, including T2DM and cardiometabolic disease (3, 6-9), and are associated with increased mortality from coronary heart disease or stroke in postmenopausal women (10). Laight *et al.* showed that obese mice had elevated plasma F2-isoPs in addition to hyperglycemia and hyperinsulinemia, all of which decreased after animals were given supplementary dietary vitamin E as an antioxidant (11). In humans, obese, insulin-resistant patients had higher levels of urinary F2-isoPs and circulating oxidized low-density lipoprotein (LDL) after a high-fat meal compared with normal weight controls (12). Other studies have shown that elevated F2-isoPs in T2DM (13) are reduced as a result of antioxidant supplementation (14). Among nondiabetic individuals, F2-isoPs have been observed to be elevated as a function of high body mass index (BMI), waist-to-hip ratio, and fasting insulin (15). Based on these findings, oxidative stress contributing to the production of F2-isoPs is widely interpreted to be involved in the pathogenesis of insulin resistance in cardiometabolic disease. However, most studies have examined this relationship using surrogate measures of insulin sensitivity, rather than the gold standard,

hyperinsulinemic-euglycemic clamp technique. Thus, we evaluated levels of urinary F2-isoPs in nondiabetic patients across a wide range of BMI and insulin sensitivity, as quantified through use of this clamp technique.

Both urinary and plasma F2-isoPs can be utilized as biomarkers for *in vivo* oxidative stress; however, urinary isoprostanes are stable both at room temperature and over multiple freeze-thaw cycles, do not undergo auto-oxidation (6), and are indicative of systemic "whole-body" oxidant stress over time. Urinary F2-isoprostanes were chosen for use in this study, with a focus on the most abundant and bioactive form, 15-F_{2t}-IsoP (8-iso $PGF_{2\alpha}$), along with its major urinary metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP. Additionally, we further evaluated the association of urinary F2-isoPs with other clinical variables related to insulin resistance and cardiometabolic risk factors, including body composition, substrate oxidation rates, and circulating lipids. *Since we have previously established that certain circulating microRNAs are associated with measures of systemic insulin resistance (unpublished), we further assessed whether these miRs were also related to F2-isoPs, which would suggest that the miRs could mediate insulin resistance by virtue of their influence on oxidative stress.*

1. Materials and Methods:

A. Patient Characteristics

Sixty five subjects were sequentially recruited for metabolic characterization at the University of Alabama at Birmingham Clinical Research Unit through advertisements and word of mouth. Patients with a BMI >21 kg/m^2 were included, with an effort toward equal recruitment of white and black participants. Patients were required to have been

sedentary (no regular exercise) and weight-stable for at least 3 months prior to the study (± 3% of body weight). Exclusion criteria included BMI >50 kg/m^2, a diagnosis of plasma glucose data indicative of T2DM, the presence of cardiovascular, renal, thyroid, or hepatic disease, and use of pharmacological agents that could affect body composition, lipid, or carbohydrate metabolism. Demographics, such as age, race and sex were self-reported.

Subjects were equilibrated on a weight maintenance diet (28-32 kcal/kg/day; 50% carbohydrates, 30% fat, and 20% protein) for a 3-night stay in the metabolic ward. The study was approved by the institutional review board and written informed consent was obtained from every subject.

B. Body Composition

Anthropometric data (weight, height, waist circumference) were collected and BMI was calculated from weight (kg)/height (m^2). Dual-energy x-ray absorptiometry (Lunar Radiation, Madison, WI) was used to assess lean body mass (LBM), body fat, bone mineral content (BMC), and bone mineral density (BMD).

C. Insulin Sensitivity

Insulin sensitivity was assessed as glucose disposal rate (GDR) *in vivo* using the hyperinsulinemic-euglycemic clamp technique, as previously described (16, 17). Briefly, after a 12-hour fast, subjects were administered a primed-continuous infusion of regular insulin (Humulin; Eli Lilly, Indianapolis, IN) at a rate of 200 $mU \cdot m^{-2} \cdot min^{-1}$, producing steady-state insulin concentrations >3,000 pmol/L, which are maximally

effective for stimulating glucose uptake into skeletal muscle (17). Serum glucose was clamped at 5.0 mmol/L for at least 3 hours, and maximal glucose uptake for each individual was determined as the mean glucose infusion rate over the final three 20-minute intervals. Whole-body glucose uptake was calculated based on the glucose infusion rate corrected for changes in the glucose pool size, assuming a distribution volume of 19% body weight and pool fraction of 0.65. The GDR was then normalized per kilogram of LBM assessed by dual-energy x-ray absorptiometry.

D. Substrate Oxidation Rates

Basal lipid and carbohydrate oxidation rates were determined through indirect calorimetry after an overnight fast as previously described (18). Resting energy expenditure (REE) was measured using a Deltratrac metabolic monitor (Deltatrac II; SensorMedics, Yorba Linda, CA) after subjects rested supine on a bed for 30 minutes. The instrument was calibrated with ethanol combustion tests every month, and against standard gases prior to each test. Expired air was collected with an adult-size, ventilated canopy system for 20 minutes after a 10-minute equilibration. Whole body oxygen consumption and carbon dioxide production were calculated by measuring gradients across the face with known flow rates of air using the Haldane transformation. Substrate oxidation rates were then determined from the respiratory quotient value and the tables of Lusk (19), normalized per kilogram of metabolically active body mass, according to Ravussin *et al.* (20).

E. Assays

Blood samples were collected after patients fasted overnight for at least 12 hours. Plasma glucose was measured by the glucose oxidase method using a glucose analyzer (YSI 2300; Yellow Springs Instruments, Yellow Springs, OH). Serum insulin levels were measured using immunofluorescence on a TOSOH A1A-II analyzer (TOSOH Corp., South San Francisco, CA); 50µl serum, sensitivity, 1.0 uU/ml; interassay CV, 4.0%; and intra-assay CV, 1.5%. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were determined by the colorimetric method on a SIRRUS analyzer (Stanbio Laboratory, Boerne, TX); 3µl for each; sensitivity, 2 to 5 mg/dL; all assay CVs < 6.6%. LDL cholesterol was calculated using the Friedewald equation. *Candidate miR that might be related to insulin sensitivity (identified in a previous, yet-unpublished study) were extracted from 200µl of a subset of available plasma samples using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the manufacturer's protocol, reverse transcribed with the miScript II RT Kit (QIAGEN) and analyzed via a quantitative, real-time polymerase chain reaction (qrt-PCR) (StepOnePlus; Applied Biosystems, USA) with the miScript SYBR Green PCR kit (QIAGEN) in duplicate. Samples were normalized to miR-490, with –ΔCt values used as relative expression of these miRs.*

F. Urinary F2-IsoP/Metabolite

Spot urine samples were collected in the morning from each subject, stored at -70°C, and submitted to Dr. G. Milne at Vanderbilt University for analysis. A total of 250 µl of urine was used to analyze the principal urinary F2-isoP, 15-F_{2t}-IsoP (aka 8-iso

$PGF_{2\alpha}$), along with its major urinary metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, via gas chromatography/negative-ion chemical ionization mass spectrometry (21, 22). Both the F2-isoP and its metabolite were normalized to urine creatinine, as determined through a modified Jaffe reaction using a commercial chemistry analyzer (Roche COBAS Integra 800; F. Hoffmann-La Roche AG. Basel, Switzerland). The lower limit of detection for the assay was 0.0001 ng/mL, with a CV of 8%. Normal concentrations of F2-isoPs in human urine are 1.6 + 0.6 ng/ml (23).

G. Statistical Analyses

Descriptive data are presented as means ± standard deviation and range. In a few instances, statistical outliers were identified and removed; however, the conclusions were not affected by the inclusion or exclusion of these data points. Pearson and Spearman correlation coefficients were used to assess the linear relationship between 15-F_{2t}-IsoP and its major metabolite with insulin sensitivity, age, body composition measurements, and metabolic parameters, as appropriate. Multiple linear regression analyses were conducted to ascertain the relationship between the F2-isoP values and body composition measures, with additional adjustments made for age, sex, race, and smoking status, because these have all previously been associated with difference in F2-isoP levels in other studies. Body composition measures were checked for collinearity prior to entering them into the model. Marginal means were calculated for F2-isoP levels for race, obesity, and smoking status, adjusted for sex. All statistical analyses were conducted using the SAS 9.4 statistical software package (SAS Institute Inc., Cary, NC).

2. Results

A. General Subject Characteristics

The sample population had more females (70%) than males, but was racially balanced with 33 blacks (73% female) and 32 whites (69% female). The average age of participants was 38.6 years. Study subjects were recruited over a wide range of BMI (21.2 to 46.9 kg/m^2) and insulin sensitivity as reflected by GDR values (5.46 to 21.82 mg/min/kg LBM). Clinical characteristics of subjects are presented in Table 1.

B. Insulin Sensitivity/Substrate Metabolism

No correlation was found between insulin sensitivity as measured by GDR and either 15-F_{2t}-IsoP or its major metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (Fig. 1). This lack of association persisted even after adjusting for age, race, sex, and smoking status. Similarly, there were no significant correlations between 15-F_{2t}-IsoP or its major metabolite with either basal carbohydrate oxidation rate or basal fat oxidation rate, although there was a trend for a positive correlation between fasting glucose and 15-F_{2t}-IsoP (P = 0.071) (Table 2).

C. Body Composition

Urinary 15-F_{2t}-IsoP was negatively correlated with body weight and LBM (Table 2, Fig. 1), but not with BMI. 15-F_{2t}-IsoP was not associated with measures of adiposity, including total fat mass, body fat percentage, trunk fat mass, waist circumference, or waist-to-hip ratio (all P = NS).

The F2-isoP metabolite was strongly and negatively correlated with LBM and positively correlated with body fat percentage and trunk fat (Table 2). It was not correlated with body weight.

D. BMC/BMD

Strong inverse correlations were observed between both 15-F_{2t}-IsoP and its major metabolite with both BMC and BMD (Table 2, Fig. 1).

E. Age

Because decrements in LBM, BMC, and BMD (Fig. 1) all occur as a function of progressive biological aging, we also examined whether 15-F_{2t}-IsoP and its metabolite were correlated with age. Levels of urinary 15-F_{2t}-IsoP were found to increase with advancing chronological age (Fig. 2).

F. Circulating Lipids and Lipoproteins

As shown in Table 2, 15-F_{2t}-IsoP was found to have a significant positive correlation with circulating triglycerides and total cholesterol, whereas the trend for an association with LDL-cholesterol did not achieve statistical significance. There were no significant correlations found between these circulating lipids and the metabolite 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP. No correlation was found between either the F2-isoprostane or its metabolite with HDL-cholesterol (Table 2). In addition, neither systolic nor diastolic blood pressures were correlated with either of them.

G. Effects of Gender, Race, and Smoking Status and Multiple Linear Regression Analyses

Urinary levels of 15-F_{2t}-IsoP and its metabolite were higher in females than males (2.53 ± 0.22 vs. 0.88 ± 0.07, $P = 0.0074$; 1.67 ± 0.22 vs. 0.50 ± 0.06, $P = 0.0003$ respectively). In addition, after adjusting for sex, urinary 15-F_{2t}-IsoP values were higher in whites than blacks (0.68 ± 0.11 and 0.44 ± 0.11, respectively; $P = 0.0169$), and higher among former and current cigarette smokers compared with those who have never smoked (0.72 ± 0.18, 0.66 ± 0.22, and 0.49 ± 0.09, respectively; $P = 0.0375$). Additionally, although F2-isoPs were not significantly correlated with BMI, those who were overweight had the highest mean levels of urinary 15-F_{2t}-IsoP (0.77 ± 0.13) compared with either normal weight (0.51 ± 0.15) or obese patients (0.40 ± 0.12) ($P = 0.0151$) after adjusting for sex.

After ensuring no collinearity between body composition variables (variance inflation factor for LBM = 1.64, BMC = 3.18, BMD = 2.52), a regression model for urinary 15-F_{2t}-IsoP and LBM with BMC and BMD showed that BMD was an independent predictor whereas BMC was not (Table 3). Subsequent multiple regression analyses revealed that the relationship between 15-F_{2t}-IsoP and either LBM or BMD was still significant after adjusting for age, race, sex, and smoking status (Table 4), although age was a significant covariate in the regression model for lean mass.

H. Additional Markers of Oxidative Stress

In a subgroup of subjects (58.8% black and 70.6% female, which is very similar to our total sample population), we had available measures of protein 4-hydroxynonenal (protein-HNE; 4-HNE) and carbonyl content in skeletal muscle, which indicate local

levels of oxidative stress in the tissue (4). In these patients (n = 17), urinary 15-F_{2t}-IsoP was correlated with protein-HNE (r = -0.54, *P* = 0.0239), but not with protein carbonyl content (r = 0.12, *P* = 0.6643) (Fig. 3).

I. Circulating microRNAs

In a subset of patients, we also measured levels of circulating miRs that we found were potentially associated with measures of systemic insulin resistance (not published). Simple linear regressions showed no significant associations between 15-F_{2t}-IsoP and any of these candidate miRs (Table 5).

3. Discussion

Oxidative stress participates in the pathophysiology of cardiometabolic disease, and markers of oxidative stress are elevated in those with diabetes, obesity, atherosclerosis, and increased cardiovascular disease risk (23-25). F2-isoPs have been used widely as biomarkers of oxidative stress *in vivo*. Plasma and urinary F2-isoP levels have been shown to be elevated in both obesity (12) and diabetes (13), and are reduced following improved glycemic control (14) and weight loss (26). Furthermore, plasma F2-isoprostane levels are acutely increased with induced hyperglycemia (27).

Given the central role of insulin resistance in diabetes, obesity, and CVD, one might expect that F2-isoprostane levels would directly reflect oxidative stress that is contributing to the pathogenesis of insulin resistance. However, in the current study, we found no association of either urinary 15-F_{2t}-IsoP or its major metabolite 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP with insulin sensitivity, as measured by a hyperinsulinemic-

euglycemic clamp. Neither did we observe any association of either 15-F_{2t}-IsoP or its metabolite with basal carbohydrate or lipid oxidation rates*, and no relationship was observed between 15-F_{2t}-IsoP and any of the miRs thought to be associated with insulin resistance*.

Our observation that F2-isoPs are not quantitatively related to insulin sensitivity in humans suggests that the lipid peroxidation process that produces urinary F2-isoPs may not reflect the oxidative stress processes operative in insulin resistance. F2-isoPs are prostaglandin-like compounds formed by peroxidation of arachidonic acid and constitute a minor component of lipid peroxidation products (24). F2-isoPs can mediate biological effects such as vaso-reactivity and platelet aggregation; however, these effects are believed to be mediated by receptor binding and not via inherent chemical reactivity and protein modification (6). This is in contradistinction to 4-HNE, another marker of lipid peroxidation, which is elevated in the skeletal muscle of patients with T2DM and is negatively correlated with GDR in nondiabetic subjects (4).

4-HNE protein adducts are formed in response to mitochondrial generation of reactive oxygen species that interact with *n*-6 polyunsaturated fatty acids, including arachidonic acid, and other fatty acids such as linoleic acid (28). It is a highly reactive lipid electrophile, with a prolonged half-life that can diffuse from sites of formation because of its amphiphilic properties (29). The 4-HNE protein can bind to glutathione and can form covalent bonds with functional proteins in a manner that can alter their function. The 4-HNE adducts form preferentially with specific proteins and can therefore alter function in a cell-specific manner depending on the pattern of protein expression (28, 29). These different properties may partially explain the observations that 4-HNE

adducts in skeletal muscle are correlated with insulin sensitivity (4) whereas F2-isoPs are not. Additionally, the negative correlation between urinary F2-isoPs and tissue 4-HNE noted in our subgroup of patients with skeletal muscle biopsies suggests that local oxidative stress intrinsic to muscle itself can be related to insulin sensitivity in ways that are not reflected by whole-body F2-isoPs. This is likely the result of biochemical differences in the formation of these markers, and further demonstrates that the oxidative process that produces F2-isoPs may not be relevant to insulin sensitivity. Collectively, these findings underscore the complex relationship between various oxy-lipid species and insulin sensitivity.

Our results regarding the lack of relationship between F2-isoPs and insulin sensitivity as measured by GDR are consistent with a recent study in healthy youth across the weight spectrum that found no relationship between F2-isoPs and insulin sensitivity as measured by the Homeostatic Model Assessment of Insulin Resistance (30). Moreover, recent analyses of the Insulin Resistance Atherosclerosis Study discovered an inverse relationship between urinary F2-isoPs and the risk of developing T2DM (31, 32). These findings, however, are discrepant with those of a smaller study (n = 31) reported by Urakawa *et al.,* who observed a negative correlation between plasma 8-iso-prostaglandin-$F_{2\alpha}$ and clamp measures of insulin sensitivity in lean and obese white men (33).

In terms of body composition, no linear association was found between F2-isoPs and either BMI or measures of body fat (total fat mass, body fat percentage, trunk fat, waist and hip circumference). This finding seems contrary to studies demonstrating that F2-isoprostane levels are correlated with BMI (15, 34), and with studies that show

oxidative stress tends to decrease with weight loss (35). One possibility for this incongruence is that we provided all participants with a standardized, isocaloric diet for three days before study measurements. Because meal intake can affect levels of oxidative stress (36), normalizing the diet for our subjects may have blunted the differences that might otherwise have been observed in our measure of whole-body oxidative stress. Another consideration is that approximately half of our subjects were African-American, as Il'yasova *et al.* reported in the Insulin Resistance Atherosclerosis Study that F2-isoPs rose with BMI in whites but not blacks. Even so, we failed to find a relationship between F2-isoPs with BMI and measures of fat mass even after controlling for race and in a stratified analysis of whites only (data not shown). However, we observed a higher level of F2-isoPs in overweight subjects compared with lean or obese subjects, while the F2-isoPs were similar in obese and lean subjects.

An alternative explanation for this discrepancy in our findings and the literature may be the relationship of F2-isoPs with LBM. We observed that F2-isoPs values have a strong negative relationship with LBM, which was independent of BMI or measures of fat mass. Because BMI is a poor predictor of obesity in those with higher LBM, it is possible that the lack of association between F2-isoPs and BMI in our study could be explained by the influence of LBM.

The findings of a relationship between urinary F2-isoPs and LBM may be an important consideration for future studies utilizing F2-isoPs. We also observed that both urinary 15-F_{2t}-IsoP and its major metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP, exhibited strong, significant, negative correlations with BMC and BMD. The strength of these correlations was not diminished by adjusting for additional covariates like age, race, sex,

and smoking status. Because decrements in LBM, BMC, and BMD occur as a function of biological aging, it is possible that levels of F2-isoPs reflect oxidative stress contributing to aging. Our data support this hypothesis, as we found a positive correlation between urinary 15-F_{2t}-IsoP levels and age, at least over the delimited age range of our study subjects (*i.e.*, 20-60 years).

Regarding sex differences, we found that F2-isoPs were higher in females than males, which is consistent with other studies. However, sex was not a significant factor in the multiple regression equations relating F2-isoP and body composition measures, suggesting that the differences in F2-isoP levels by sex in this study are explained by the effects of LBM and BMD. This may also explain the differences in F2-isoP levels by sex observed in other studies. After controlling for sex, we also observed that whites had higher levels of F2-isoPs than blacks, which is similar to findings by Il'yasova et al (37), and that smokers and former smokers both had higher F2-isoPs levels than in nonsmokers, as expected (38).

No significant correlation was found between F2-isoP with total body fat (%), even after adjusting for age, race, sex, and smoking status (Table 4). There was, however, a significant correlation between both total body fat (%) and trunk fat with the F2-isoP metabolite. It has been suggested that this metabolite might be a more accurate measure of whole-body oxidative stress over time (because it reduces the likelihood of potential local contribution of free F2-isoP from the kidney) (22). Thus, future studies are warranted to investigate this compound and its relationship with measures of fat mass and metabolism in more detail.

The strength of this study lies in the use of gold standard measures of *in vivo* insulin sensitivity in humans, as well as state-of-the-art methodology for quantification of F2-isoPs. Our subjects represented a wide range of BMI and insulin sensitivity and excluded the potentially confounding disease state of diabetes. Subjects were weight-stable upon entering the study and were fed an isocaloric diet with a standard macronutrient composition (14) to account for potential dietary effects on study measurements (39, 40).

The main limitation of this study was the use of a single spot morning urine sample instead of a 24-hour collection for F2-isoprostane analysis, which may have affected outcomes due to the potential of intra-subject daily variability (41). Even so, the equilibration of research volunteers on a standardized, isocaloric diet with studies conducted in a metabolic ward would minimize daily variation. Another limitation is that our study sample was characterized by an uneven distribution of sex, although we used marginal means to adjust for this unbalanced design in regression analyses. Most importantly, our correlation studies in a cross-section sample population cannot be used to indicate causality; therefore, mechanistically, the results should be regarded as hypothesis-generating. However, the focus of our study was to assess the utility of F2-isoPs as a biomarker of oxidative stress in insulin resistance. Despite wide use of F2-isoPs in this context, we found that there is no quantitative relationship with individual differences in insulin sensitivity in a nondiabetic population.

4. Conclusion

In conclusion, oxidative stress, as measured by urinary F2-isoPs, was not associated with insulin sensitivity, substrate oxidation, BMI, or fat mass. However, a significant negative correlation was found between both 15-F_{2t}-IsoP and its major metabolite with LBM, as well as BMC and BMD. Since LBM, BMC, and BMD decline as a function of biological aging, F2-isoPs may reflect the oxidative stress operative in the aging process independent of changes in insulin sensitivity. Future studies are needed to identify how aspects of oxidative stress differentially affect pathways involved in the pathogenesis of insulin resistance, bone loss, and aging.

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Table 1. Descriptive Characteristics

Characteristic	Value
Age (y)	38.6 ± 11.1 (20 – 60)
Race	33 black, 32 white
Sex	19 male, 46 female
Weight (kg)	87.9 ± 19.0 (61.2 – 139.8)
BMI (kg/m^2)	31.0 ± 6.3 (21.2 – 46.9)
Body composition	
LBM (kg)	49.2 ± 10.2 (30.7 – 73.3)
LBM (%)	56.9 ± 10.6 (40.9 – 87.9)
Total fat mass (kg) (n = 52)	33.9 ± 14.6 (4.1 – 71.5)
Total body fat (%)	40.6 ± 11.2 (7.2 – 58.0)
Trunk fat mass (kg) (n = 63)	18.58 ± 7.73 (4.28 – 40.73)
Waist circumference (cm)	98.0 ± 14.9 (74.0 – 141.5)
Hip circumference (cm) (n = 64)	111.5 ± 13.0 (87.5 – 148.5)
Waist-to-hip ratio (n = 64)	0.88 ± 0.08 (0.72 – 1.04)
BMC (g) (n = 50)	2928.2 ± 525.5 (1892 – 4192)
BMD (g/cm^2) (n = 51)	1.26 ± 0.12 (1.09 – 1.62)
Fasting glucose (mg/dL) (n = 64)	95.1 ± 11.54 (72.4 – 133)
GDR (mg/min/kg LBM) (n = 63)	13.51 ± 4.16 (5.46 – 21.82)
Carbohydrate oxidation rate (kcal/day/kg LBM) (n = 63)	133.8 ± 58.5 (0 – 245.68)
Fat oxidation rate (kcal/day/kg LBM) (n = 64)	75.1 ± 35.4 (10.4 – 197.1)
Lipid markers	
Triglycerides (mg/dL) (n = 64)	109.6 ± 55.0 (36 – 247)
Total cholesterol (mg/dL) (n = 63)	181.2 ± 43.3 (111 – 340)
LDL-cholesterol (mg/dL) (n = 64)	112.7 ± 38.0 (45.4 – 211.8)
HDL-cholesterol (mg/dL)	47.6 ± 14.0 (26 – 81)
Systolic blood pressure (mm Hg)	117.1 ± 15.3 (87 – 158)
Diastolic blood pressure (mm Hg)	67.5 ± 10.0 (48 – 91)
Urinary F2-isoP (ng/mg creatinine)	2.28 ± 1.42 (0.5 – 7.0)
Urinary F2-isoP metabolite (ng/mg creatinine) (n = 64)	0.77 ± 0.48 (0.16 – 2.36)
Smoker	7 Yes, 10 Former, 42 No

Data are presented as mean ± standard deviation (n = 65 unless otherwise noted), unless variables are categorical, in which case the *n* for each category is reported.

Table 2. Correlation of Metabolic Risk Factors With Urinary F2-isoP and its Major Metabolite

Metabolic Risk Factor	**Urinary F2-Isoprostane** (ng/mg Cr) (N=65)	***P* value**	**Urinary F2-Isoprostane Metabolite** (ng/mg Cr) (N=64)	***P* value**
Age (y)	0.28	0.0241*	0.10	0.4220
Weight (kg)	-0.28	0.0261*	0.02	0.9009
BMI⁺ (kg/m²)	-0.09	0.4787	0.21	0.1035
Body composition				
LBM (g)	-0.46	0.0001**	-0.33	0.0076**
LBM (%)	-0.20	0.1126	-0.41	0.0008**
Total fat mass (g) (n = 52; 51)	-0.04	0.7844	0.23	0.1013
Total body fat⁺ (%)	0.18	0.1492	0.40	0.0011**
Trunk fat mass⁺ (g) (n = 63; 62)	0.01	0.9219	0.25	0.0471*
Waist circumference (cm)	-0.06	0.6319	0.11	0.4054
Hip circumference (cm) (n = 64; 63)	-0.07	0.5637	0.23	0.0712
Waist-to-hip ratio (n = 64; 63)	-0.01	0.9234	-0.13	0.3014
BMC (g) (n=50; 49)	-0.58	<0.0001**	-0.40	0.0043**
BMD (g/cm²) (n = 51; 50)	-0.65	<0.0001**	-0.30	0.0358*
Fasting glucose⁺ (mg/dL)	0.23	0.0706	0.03	0.8180
GDR (mg/min/kg LBM) (n = 63; 62)	-0.01	0.9423	0.003	0.9798
Carbohydrate oxidation rate⁺ (kcal/day/kgLBM)	-0.001	0.9928	-0.03	0.8379
Fat oxidation rate (kcal/day/kg LBM) (n=64)	-0.07	0.5922	-0.01	0.9193
Lipid markers				
Triglycerides (mg/dL)	0.36	0.0031*	0.23	0.0711
Total cholesterol⁺ (md/dL)	0.32	0.0096**	0.11	0.4079
LDL-cholesterol (mg/dL) (n = 64; 63)	0.23	0.0670	0.08	0.5478
HDL-cholesterol⁺ (mg/dL)	0.04	0.7331	-0.18	0.1580
Systolic blood pressure (mm Hg)	0.13	0.2924	0.07	0.5868
Diastolic blood pressure (mm Hg)	0.13	0.3228	-0.13	0.3027

All correlations are Pearson's correlation coefficients, unless indicated as Spearman's correlation coefficients. Metabolic parameters were otherwise normalized with natural log transformations as appropriate. All values for the urinary isoprostane and its metabolite are natural log-transformed, unless otherwise indicated for Spearman's correlation.

⁺ = Spearman's correlation coefficient
* = $p < 0.05$
** = $p < 0.01$

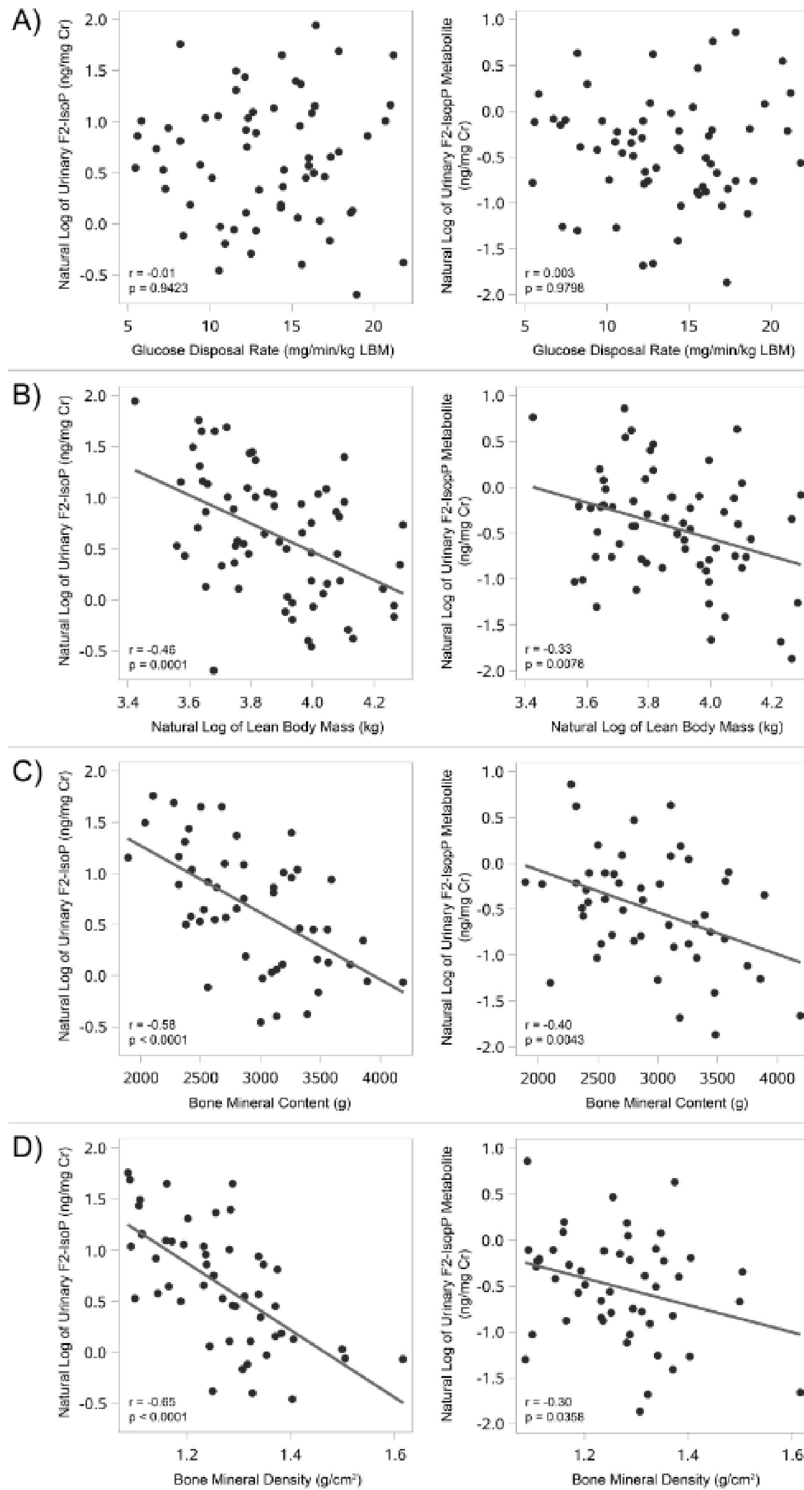

Figure 1. Relationships of urinary F2-isoprostane and its metabolite with clinical parameters. (A) No correlation was observed between either the urinary F2-isoprostane or its metabolite with insulin sensitivity, as measured by glucose disposal rate via a hyperinsulinemic-euglycemic clamp. Both the urinary F2-isoprostane and its metabolite are negatively correlated with (B) LBM, (C) BMC, (D) and BMD. Cr, creatinine.

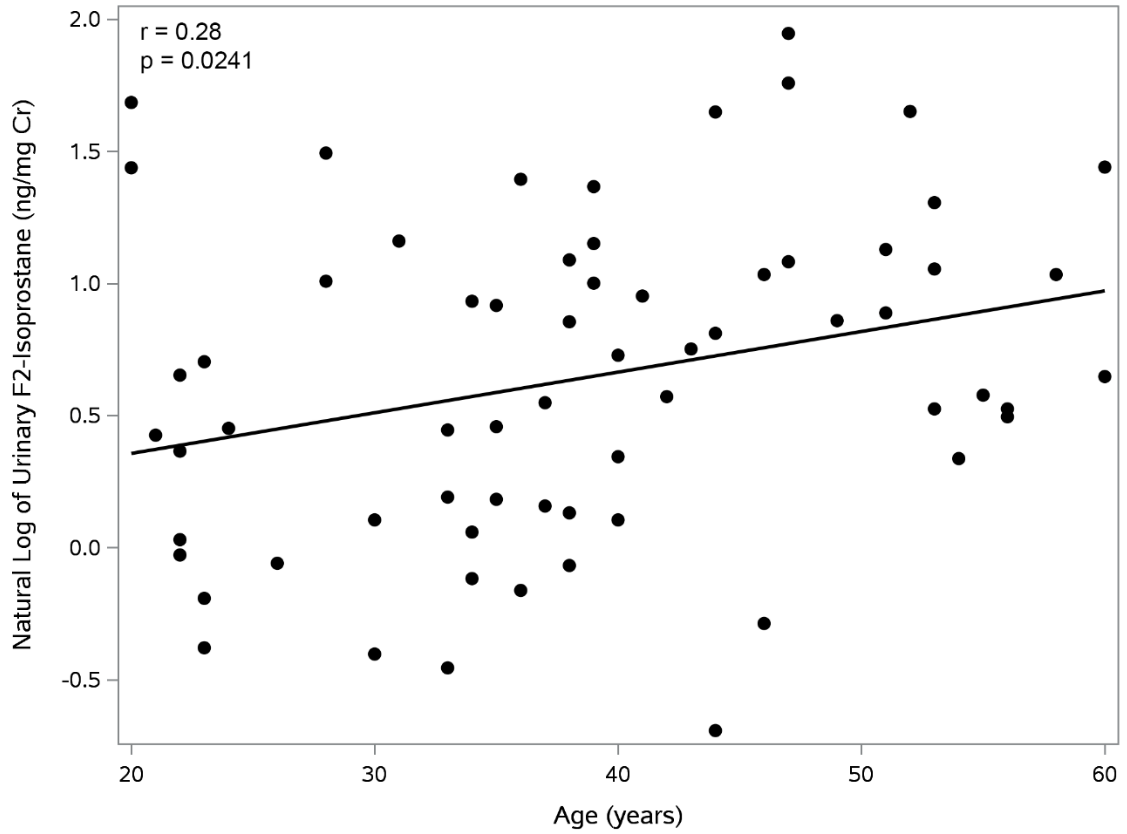

Figure 2. Correlation of urinary F2-isoprostane with age. 15-F_{2t}-IsoP is positively correlated with age.

Table 3. Multiple Linear Regression Model of 15-F_{2t}-IsoP with Lean and Bone Mass (n = 46)

	Standardized β	P-value
Lean Body Mass	-0.27	0.0646
Bone Mineral Content	-0.06	0.7853
Bone Mineral Density	-0.45	0.0166

Full model: adjusted R^2 = 0.42, P <.0001

Table 4. Multiple Linear Regression Analysis of 15-F_{2t}-IsoP With Body Composition and

	Lean Body Mass (n = 59)		Bone Mineral Density (n = 48)		Total Body Fat (%) (n = 59)	
	Standardized β	**P-value**	**Standardized β**	**P-value**	**Standardized β**	**P-value**
Variable of interest	-0.62	0.0004	-0.62	<.0001	0.045	0.7997
Age	0.29	0.0182	0.11	0.4251	0.29	0.0360
Race						
Black	-0.10	0.3984	0.11	0.4231	-0.14	0.3040
White	0	.	0	.	0	.
Gender						
Female	-0.15	0.3759	0.14	0.2955	0.28	0.1195
Male	0	.	0	.	0	.
Smoking Status						
Former	-0.18	0.2722	-0.16	0.3652	-0.10	0.5868
No	-0.27	0.0912	-0.16	0.3261	-0.14	0.4413
Yes	0	.	0	.	0	.
Full model	**R^2 = 0.33**	**0.0001**	**R^2 = 0.38**	**0.0002**	**R^2 = 0.15**	**0.0222**

Relationships between 15-F_{2t}-IsoP and each body mass composition variable of interest (LBM, BMD, or body fat, respectively). Adjusted R^2 values are reported.

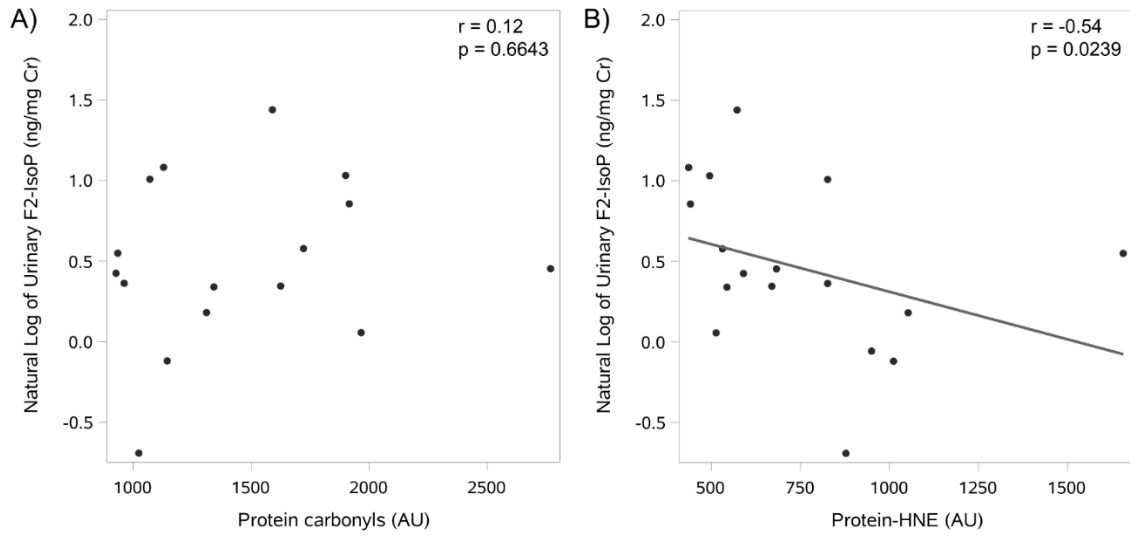

Figure 3. Skeletal muscle markers of oxidative stress. The relationship between 15-F_{2t}-IsoP and (A) skeletal muscle protein carbonyl content (n = 16) and (B) skeletal muscle protein-HNE content (n = 17). AU, arbitrary units.

Table 5. Simple Linear Regression of 15-F_{2t}-IsoP with Candidate Circulating miRs

miR	n	R^2	p-value
miR-16	32	0.0002	0.9319
miR-33	19	0.0614	0.3064
miR-34a	17	0.0217	0.5726
miR-107	22	0.0567	0.2860
miR-133a	28	0.0400	0.3078
miR-140	26	0.0870	0.1436
miR-150	32	0.0005	0.9046
miR-199a	25	0.0874	0.1514
miR-222	32	0.0019	0.8124

No significant association was found between 15-F_{2t}-IsoP (natural log-transformed) and relative expression of any candidate circulating miRs.

EFFECT OF WEIGHT LOSS ON CIRCULATING MIRNAS AND THEIR RELATIONSHIP TO CHANGES IN INSULIN SENSITIVITY AND METABOLIC TRAITS

by

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Abstract:

Background: Insulin resistance, a key risk factor for the development of cardiometabolic disease, can be improved by weight loss. Our goal was to determine whether circulating miRNAs (miRs) potentially related to insulin sensitivity would change after dietary weight loss interventions as a function of improvements in insulin resistance and associated metabolic traits.

Methods: Non-diabetic, overweight or obese individuals were newly enrolled for a weight-loss program at the UAB Weight Loss Medicine clinic and given the choice of 3 diets: Low Fat, Very Low Carbohydrate, or Very Low Calorie Diet (VLCD). Patients were classified by the amount of weight lost by 15 weeks (high responders ≥ 5% weight loss, low responders <5%), and assessed for anthropometric measures, body composition, fasting glucose and lipids, and insulin resistance (HOMA-IR) at baseline and 15 weeks. Patients were also assessed for levels of 15 candidate circulating miRs, which were then analyzed for their relationship to weight loss and corresponding improvements in metabolic risk factors.

Results: 21 low responders and 32 high responders were assessed at baseline and 15 weeks. No differences were found in levels of circulating miR between high and low responders, or between the low fat and low carbohydrate diets. Changes in plasma levels of miR-222 were associated with changes in insulin sensitivity, and changes in miR-16 and miR-122a were associated with changes in HDL after 15 weeks. The amount of weight loss affected the relationships between miRs and metabolic traits. After 15 weeks

on reduced calorie diets, a cross sectional analysis identified multiple miRs associated with insulin sensitivity in the low responder group (miR-34a, -126, -320, and let-7a) while a different pattern was observed in the high responder group with miRs associated with triglycerides (miR-16, -223) and HDL cholesterol (miR-16).

Conclusions: In patients with obesity placed on dietary weight-loss interventions, circulating levels of miRs were not affected by the amount of weight loss or diet macronutrient composition. However, the amount of weight loss did affect relationships between miR species and metabolic traits. The data indicate that miRs may be involved with the metabolic response to reduced calorie diets in obesity regarding improvements in insulin sensitivity and lipids.

Keywords: diet, weight loss, miRNA, metabolism

Introduction

Insulin resistance is a key risk factor for the development of Type 2 Diabetes Mellitus (T2DM) and cardiometabolic disease. Insulin resistance is also commonly noted in obesity, along with a host of associated signs of metabolic dysfunction, such as dyslipidemia and hypertension, which in aggregate represent the Metabolic Syndrome (MetS) and places patients at higher risk for T2DM and cardiovascular disease (CVD) (1). A 5-10% weight loss is generally recommended by clinicians for those who are overweight or obese to prevent the development of these diseases, as studies suggest this amount of weight loss can significantly improve various metabolic risk factors and clinical sequelae. A 5% weight loss has been shown to increase whole body and multi-organ insulin sensitivity, and progressive weight loss above 5% seems to have dose-dependent effects (2). An intensive lifestyle intervention with a goal of at least 7% weight loss via diet and exercise was more effective than either placebo or Metformin treatment on standard lifestyle recommendations to prevent or delay onset of diabetes in high-risk patients (3), and lifestyle interventions have been shown to effectively decrease incidence of diabetes in multiple countries (4, 5) and improve insulin sensitivity in patients with non-alcoholic steatohepatitis (which is often associated with diabetes) (6). In addition, pharmacological treatment for weight loss has also been shown to prevent diabetes in high risk patients (7).

In recent years, microRNAs (miRs; short, non-coding RNA that can modulate gene expression post-transcriptionally by base-pairing with target mRNA) have been implicated in obesity and associated insulin resistance (8, 9), and have been shown to be protective against high fat diets and associated metabolic dysfunction in mice (10). Some

miRs are also differentially expressed after weight loss and show associated improvements in metabolic parameters. For example, in dogs, a dietary intervention for weight loss down-regulated miR-107 in muscle and adipose tissue, along with changes in certain insulin and glucose metabolism pathway genes, such as PPARγ, GLUT4, and PGC1α (11). Studies have also shown that levels of various circulating miR (c-miR) can change after weight loss concomitant with improvements in metabolic function. In humans, patients with T2DM who underwent Roux-enY gastric bypass surgery showed a significant decrease of miR-29a-3p, 122-5pm, 124-3p, and -320a in serum samples 12 months after surgery in one study, along with significant decreases in BMI and improvements in glucose metabolism (12), while another study showed differences in let-7, miR-16, -107 -320, and others after 3 months (13). Patients with MetS treated with an 8-week, hypocaloric, Mediterranean diet for weight loss showed changes in let-7b and various miRs in their white blood cells, along with improvements in MetS risk factors (14). However, there is currently only a limited number of studies assessing relationships between circulating (plasma) miRs and changes in body weight, insulin resistance, and associated metabolic factors, with little agreement across studies. In addition, while there are a couple studies that show that the type of diet may affect circulating miRs (15-17), to our knowledge, there are no studies that compare a low carbohydrate diet and a low fat diet regarding the differential impact on levels of circulating miRNAs. Thus, we aimed to assess the impact of weight loss on miRs relevant to insulin sensitivity, as well as the potential impact of diet on this relationship.

The miRs assessed in this study were chosen based on previous preliminary work (unpublished), and/or if they have been either differentially expressed in diabetes/obesity

or related to glucose homeostasis in multiple previous studies. To illustrate, miR-126 seems to be the circulating miR that is most consistently associated with diabetes, as shown in three separate studies (18-20). One of these studies also notes miR-320 as being within the top 5 most significant miRs related to diabetes (19), and circulating miR-320 was also found to be associated with insulin resistance based on samples from the Framingham study, along with miR-122 and miR-16 (21), the latter two of which were also found to be increased after gastric bypass and associated improvements in insulin sensitivity (22). Other references for each miR are noted within the methods section after their respective primers. Our purpose was to identify circulating miRs that change in response to 15 weeks of dietary weight loss interventions as a function of improvements in insulin resistance and associated metabolic traits.

Methods

Participants

Participants were recruited to a pilot research study through UAB Weight Loss Medicine, local communities via flyers, online advertising on the UAB website, and word of mouth from February 2014 – February 2017. Patients were initially screened on the phone for age ≥ 19 years, BMI ≥ 25 kg/m^2, and not actively being on a weight loss program prior to enrolling as a new patient at the UAB Weight Loss Medicine clinics for the study. Exclusion criteria included BMI >50 kg/m^2, pregnancy/lactation, steroid medication, thyroid disease, and smoking. All protocols were approved by the UAB institutional review board and written consent was obtained from all subjects.

Study design

The study was a pilot program designed with the primary aim of determining long-term changes to resting metabolic rate and body composition among participants in 3 different diet interventions for weight loss. Secondary outcomes were changes in metabolic risk factors and miRNA on these diets. The study was not intended to be a randomized control trial. The three diets included were: 1) **Low Fat** (EatRight® – low calorie (500 calories less than current energy intake based on Resting Energy Expenditure (REE)) and low fat: 63% carbohydrate: 13-23% protein: 10-25% fat), 2) **Very Low Calorie Diet** (VLCD; Optifast® – low fat meal replacement/supplement plan. Starting calorie allotment was 800 Cal; 50%:35%:16%), and 3) **Very Low Carbohydrate** (low carb; starting with a total of 20g carbohydrate intake daily, then increasing carbohydrates to 30g/day after reaching a weight loss goal, and finally increasing it to 45g ("low carb for life" phase). No caloric prescription was given for this diet).

After the initial phone screen, patients arrived for a baseline study visit, where they gave informed consent and baseline clinical measurements were taken (see section below for more details). Patients then came back for a clinic visit with a 3-day food diary to discuss and choose their diet plan with either a clinician or a dietitian under supervision of a clinician. During this visit, they were given nutrition counseling for their diet plan, including meal plans to take home with them (for the low fat and low carb diets). They were then counseled by either a clinician or dietitian at regular intervals during clinic visits (2 weeks, 4 weeks, 8 weeks, 12 weeks, 15 weeks, and every month thereafter until completion of the study) during which they were told to keep food diaries. Patients also had the option to attend weekly support groups throughout the study. Study

visits for clinical measurements were taken at baseline, 4 weeks (added as an amendment later in the study), 15 weeks, 24 weeks, and 1 year, for the patients who remained in the study.

Due to the uneven distribution and relatively small number of enrollees across the diets and time points, for this study, we chose to combine patients from all three diets for our sub-analyses and grouped them according to whether they were able to achieve ≥ 5% weight loss (high responder) or not (low responder) by 15 weeks. This was chosen as a cutoff value as the Food and Drug Administration uses 5% weight loss as a standard goal for pharmacotherapy for weight loss, and ≥ 5% weight loss by 3 months is predictive of better weight loss at 2 years, along with improvements in insulin sensitivity and other metabolic parameters (23). For completeness' sake, however, we did include some sub-analyses of diet type with circulating miR.

Clinical measurements

Prior to starting on their chosen diet intervention, participants were required to bring in a 3-day food journal to estimate daily intake patterns for a baseline visit. At each study visit, patients arrived after an overnight fast of at least 8 hours, and anthropometric measurements were taken, along with resting metabolic rate by indirect calorimetry, body composition by dual-energy x-ray absorptiometry (iDXA, GE Lunar, Madison, WI, including use of the core scan from the iDXA software for visceral adipose tissue (VAT)) and blood samples for serum and plasma (in EDTA tubes), which were stored at -70°C until use. Only measurements from the baseline and 15 week study visits were used for these sub-analyses.

Resting energy expenditure (REE; kcal/day) was measured in the NORC Metabolism Core using an open-circuit metabolic monitor (Vmax ENCORE 29N) according to standard protocol. Participants rested supine for 10 minutes and a canopy hood was used to collect expired air for oxygen consumption (VO_2) and carbon dioxide production (VCO_2) every minute for 30 minutes. The first 10 minutes of testing were excluded in final analyses, and the VO_2 and VCO_2 values recorded over the last 20 minutes were averaged and used to calculate REE by the equation of Weir (24). Lipids and glucose were determined using a colorimetric assay (SIRRUS analyzer, Stanbio Laboratory, Boerne, TX), and serum insulin levels were determined through immunofluorescence (TOSOH A1A-II analyzer, TOSOH Corp., South San Francisco, CA). LDL was calculated using the Friedewald equation, and HOMA-IR was calculated from the fasting insulin and glucose values.

Plasma miRNA extraction and analysis via quantitative, real-time PCR

As studies have shown that contaminants from biobanked plasma samples may impact levels of circulating miR (25, 26) plasma samples were centrifuged twice after thawing at speeds higher than the amount needed for platelet separation (27) to remove potential contamination from platelets (28) and other cell debris in our samples. We first centrifuged the plasma samples at 2,500 x g for 20 minutes at 4°C, transferred the supernatant to a new tube, centrifuged them for 15 minutes at the same speed and temperature, and finally transferred the supernatant to a new tube for RNA extraction.

Total RNA (including miRs) was extracted from 200µl of the doubly-spun plasma samples using the miRNeasy Serum/Plasma Kit (QIAGEN) according to the

manufacturer's protocol. The samples were then reverse transcribed with the miScript II RT Kit (QIAGEN) into cDNA using the miScript HiSpec Buffer for mature miRs and diluted 1:10 for subsequent use in a quantitative, real-time polymerase chain reaction (qrt-PCR) (QuantStudio Flex 6; Applied Biosystems, USA) with the miScript SYBR Green PCR kit (QIAGEN) in duplicate. Based on previous studies in our lab that showed miR-490 had relatively stable expression in all plasma samples, after verifying its stability in this population, it was used as the reference gene for this study. Primers used for evaluation of individual mature miR expressions were as follows (All from QIAGEN, Cat. No. 218300, miScript Primer Assays): mi-33 (29), mi-490, mi-150 (30, 31), mi-16 (32), mi-122a (33-35), mi-103 (11, 32, 36), mi-29a (37-41), mi-146a (37, 42, 43), mi-126 (19, 44), let-7a (32, 45), mi-34a (37, 46), mi-107 (11, 32), mi-222 (47, 48), mi-223 (15, 19, 49), mi-320 (42, 50), mi-143 (8, 9), and ce-mi-39. Fold-change of miR expression levels from baseline was determined through the -ΔΔCt method (51).

Statistical analyses

Descriptive data are presented as means ± standard deviation, and after checking for normality, comparisons from baseline were made through paired t-tests, and comparisons between groups with 2 sample t-tests. Differences in miR fold-change from baseline were determined through a signed-rank test ($\mu_0 = 1$) and differences between groups were determined through the Mann-Whitney U test (the VLCD was excluded for dietary sub-analyses due to low *n*). Correlations between relative expression of circulating miRs (-ΔCt) and relevant metabolic risk factors (measures of obesity, fasting insulin, glucose, HOMA-IR and lipids) were compared at both baseline and 15 weeks

with Pearson's correlation coefficients after checking for normality. At 15 weeks, additional correlation analyses were conducted based on whether ≥ 5% weight loss was achieved. Lastly, the correlation between changes in relative c-miR expression (-ΔΔCt, aka $\log_2$-transformed fold-change expression) and change in metabolic factors over 15 weeks were also evaluated. $P < 0.05$ was considered significant. All statistical analyses were performed with the SAS 9.4 statistical software package (SAS Institute Inc., Cary, NC).

Results

A total of 103 overweight or obese patients were enrolled in the study, with 55 participants placed on the low carb diet, 14 on the VLCD, and 44 on the low fat diet. For the purposes of these sub-analyses, patients were only included if they had both a baseline and a 15-week weight measurement. Patients were then categorized as low responders (< 5% weight loss) or high responders (≥ 5% weight loss) based on the degree of weight loss at 15 weeks from baseline. When grouped this way, there was a total of 21 low responders and 32 high responders, as shown in Table 1. Most of the patients were female (85.7% and 75% respectively) and white (71.4% and 71.9% respectively) .

Metabolic changes after 15 weeks of dietary interventions

After 15 weeks on the dietary interventions, both high and low responders lost a significant amount of total body fat percentage and visceral adipose tissue (VAT), though high responders lost significantly more of both. High responders also had decreased lean body mass (LBM), fasting insulin, and triglycerides, as well as improved insulin

sensitivity (as measured by HOMA-IR) compared to baseline, while low responders exhibited no significant differences in these parameters compared to baseline. Between groups, high responders had significantly lower levels of triglycerides and LDL and higher levels of HDL compared with low-responders after 15 weeks (Table 1).

Circulating miR levels after 15 weeks of dietary intervention

A total of 15 miRs were assessed in plasma samples from each participant at both baseline and 15 weeks. 6 miRs had a majority of samples with levels below the limits of detection (Ct > 35), so these were excluded from further analysis: miR-29a, -33, -103, -107, -143, and -146a. Of the remaining 9 miRs, high responders had significantly higher levels of miR-222 at 15 weeks compared to baseline, while low responders had significantly higher levels of miR-223. Relative fold-change of miRs at 15 weeks were not significantly different between groups (Fig. 1).

To assess whether diet type might play a factor in the relationship between weight change and changes in circulating miRs, the fold-change of miRs was compared between the low carb and low fat diets. The low fat diet showed a significant increase in fold change of miR-126 and miR-223 from baseline, while no significant changes were observed in the low carb diet. There were no differences between the diets for any of the miRs (Fig. 2).

Correlation of circulating miRs with metabolic factors

At baseline, a cross-sectional analysis demonstrated that the relative expression of 4 miRs were found to be significantly correlated to metabolic risk factors: miR-16, -122a,

-150, and let-7a. Age was negatively correlated with miR-16, let-7a, and miR-150, while lean body mass (LBM) was positively correlated with let-7a. In terms of circulating lipids, triglycerides were positively associated with miR-122a, while HDL-cholesterol was negatively associated for all 4 miRs (Table 2). No significant correlations were found between any circulating miRs tested with either baseline weight, total body fat percentage, visceral adipose tissue (VAT), fasting glucose, fasting insulin, or HOMA-IR.

The cross-sectional analysis at 15 weeks showed that miR-34a was positively correlated with both fasting insulin and HOMA-IR, and that let-7a and miR-150 were correlated with HOMA-IR. Triglycerides were associated with miR-222, and HDL was negatively correlated with miR-16. When grouped by weight loss, low responders had significant associations of c-miRs with weight (miR-16), fasting insulin (miR-34a), and HOMA-IR (miR-34a, -126, -320, and let-7a), while high responders showed significant associations with triglycerides (miR-16, -223) and HDL (miR-16) (Table 2). Again, no significant correlations were found between any of the c-miRs tested with either total body fat percentage, VAT, or fasting glucose.

When the relative changes in miR expression were compared with changes in metabolic factors over 15 weeks on the dietary interventions, miR-126 was negatively associated with changes in LBM, miR-222 was negatively associated with changes in both fasting insulin and insulin sensitivity (Fig. 3), and miR-16 and miR-122a were negatively associated with a change in HDL (Table 4). No significant correlations were detected between changes in any of the c-miRs tested with either changes in weight, total body fat percentage, VAT, fasting glucose, or triglycerides.

Discussion

In patients with obesity, a 5-10% weight loss is recommended, as this degree of weight loss is associated with improvements in metabolism and reduced risk of progression to type 2 diabetes (52). Our current study is in accord with this, as those who lost ≥5% body weight by 15 weeks (high responders) had significant decreases in total body fat and VAT, as well as improvements in insulin sensitivity (HOMA-IR), fasting insulin, and triglycerides compared to baseline, while those who lost <5% body weight (low responders) experienced lesser decrements in total body fat (%) and VAT without significant improvements in insulin sensitivity and lipids.

We examined the association of circulating miRs on weight loss and the metabolic response to weight loss in patients with obesity placed on one of three reduced calorie diets: low fat, low carbohydrate, and VLCD. The candidate miRs examined were selected based on a previous miRNA array study (unpublished) that identified circulating miRs associated with clamp measures of insulin sensitivity, as well as miRs found to be altered in diabetes, obesity, and/or insulin resistance by other authors (8, 9, 11, 12, 14, 15, 18-22, 29-35, 37-40, 45, 47, 49, 50). After 15 weeks of a dietary intervention, high responders had a significant increase in miR-222 over baseline, while low responders had an increase in miR-223 regardless of the assigned diet. The latter is in agreement with a study by Parr *et al*, who also observed that miR-233 increased in both low and high (≥ 10%) responders to a weight loss intervention at 16 weeks involving a diet and exercise regimen (49), and the fact that circulating miR-223 is decreased in obese and morbidly obese patients. Importantly, however, despite these few changes from baseline within groups, we found no significant differences in levels of miRs in comparing low and high

weight loss responders. In addition, there were no differences in circulating miR levels among patients assigned to the low fat and low carb diets. Thus, the degree of weight loss and dietary macronutrient composition did not affect levels of the candidate circulating miRs.

Interestingly, however, miRs seemed to be involved with the metabolic response to dietary intervention regarding improvements in insulin sensitivity and lipids. Since neither the amount of weight loss nor the assigned diet affected miR levels, we assessed the relationship between changes in miRs over 15 weeks and changes in metabolic parameters in the study group as a whole. Changes in miR-222 were associated with improvements in insulin sensitivity as measured by HOMA-IR and fasting insulin. In addition, changes in miR-16 and miR-122a were related to changes in HDL cholesterol, and miR-126 to reductions in lean body mass. These observations indicate that miRs may determine the metabolic response to the diets with respect to insulin sensitivity and Metabolic Syndrome traits rather than on the amount of weight loss, and independent of the macronutrient composition of the diet.

Circulating miRs were also associated with metabolic traits in cross sectional analyses at both baseline and after 15 weeks of dietary intervention. At baseline, let-7a was associated with LBM, miR-122a was associated with triglycerides, and miR-16, -122a, -150, and let-7a were associated with HDL. After 15 weeks on the dietary interventions, different patterns of relationships between miRs and metabolic parameters emerged. A number of miRs were related to measures of insulin sensitivity including miR-34a, -150, -and let-7a, while miR-16 was correlated with HDL and miR-222 with triglycerides. Interestingly, the amount of weight lost affected the relationships between

miRs and metabolic measures at 15 weeks. Low responders exhibited a cluster of miRs that were associated with fasting insulin (miR-34a) and insulin sensitivity (miR-34a, -126, -320, let-7a), while the high responders had miRs correlated with circulating lipids, including triglycerides (miR-16 and miR-223) and HDL (miR-16).

Our identification of miRs linked to insulin sensitivity and metabolic traits corroborates previous reports in the literature. Regarding insulin sensitivity, our findings that changes in miR-222 were associated with changes in both fasting insulin and HOMA-IR is in agreement with the fact that circulating miR-222 has been associated with insulin resistance in post-menopausal women (47), patients with PCOS (53) and T2DM (20), and is upregulated in the omental adipose tissue of women with gestational diabetes (54). In the Ortega *et al.* study, circulating miR-222 also decreased with insulin infusion during clamp, as well as after treatment with metformin, and increased after an insulin plus intralipid/heparin infusion (which induces transient insulin resistance) (20). Thus, our findings help strengthen the idea that miR-222 may be involved in regulating metabolism and insulin sensitivity. In addition, miR-222 has been shown to decrease after hyperglycemia and hyperlipidemia induced injury in vascular smooth muscle cells, so miR-222 may also play a role in the vascular complications in diabetes (55).

Our finding that miR-34a was positively associated with fasting insulin and HOMA-IR at 15 weeks both overall and in low responders is consistent with observations of Kong *et al.*, who noticed that of the 7 miR they tested, miR-34a showed the most significant differences between patients with and without diabetes (37). Additionally, fasting serum miR-34a was higher in insulin resistant preschoolers compared with matched controls (56), which is in agreement with our findings (though they additionally

noticed an increase in miR-122 and miR-320a in the insulin resistant pre-schoolers, while we did not).

The correlation between miR-150 and insulin resistance after 15 weeks in the current study corresponds well with our previous study in miR-150 knockout mice, which were found to be lean, insulin sensitive, and diabetes-resistant when compared with wild type controls (30), as well as a study showing that miR-150 was higher in T2DM patients (31). However, it is at odds with a study that shows miR-150 KO mice have high insulin resistance and poor glucose tolerance on a high fat diet (57). Another study, however, showed that miR-150 was not differentially expressed between either normal, pre-diabetic, or diabetic patients, but was decreased in obesity (58). Given that certain miRs can distinguish between obese people with and without T2DM (59, 60), perhaps miR-150 plays different roles in these two disease processes that may be additionally confounded by weight loss.

Other miRs we found associated with insulin resistance included let-7a, miR-320, and miR-126, which correspond well to the literature for the most part. The let-7 family seems to play a role in regulating the insulin signaling (insulin-PI3K-mTOR) pathway in skeletal muscle (61), and knockdown of let-7 prevented the glucose intolerance in diet-induced obesity and restored insulin signaling in the muscle and liver of mice (45). miR-320 was found to be 50-fold higher in insulin-resistant 3T3-L1 adipocytes compared to insulin sensitive adipocytes, and treatment with an anti-miR-320 oligonucleotide improved insulin stimulated glucose uptake in insulin-resistant adipocytes, along with observed increases in p85, p-Akt, and protein expression of GLUT-4 (50). *In vivo*, there is also evidence that circulating miR-320 is higher in insulin resistant adults, and

decreases after thiazolidinedione therapy (62). miR-126 has been one of the few circulating miRs that has consistently shown up in multiple studies to be differentially expressed in patients with diabetes, and was also found to increase in serum after a dietary and exercise intervention (63).

While several studies have shown an association of circulating miR-122a with insulin resistance (33-35, 56) and progression to diabetes is marked by increasing levels of circulating miR-122a in rats (64), we did not find any significant association of miR-122a with insulin resistance in our study. A possible explanation is that all of the human studies looked at miRs in serum, while our samples were with plasma. However, we did find that miR-122a was associated with circulating lipids. Specifically, miR-122a was positively associated with triglycerides and negatively associated with HDL at baseline, and changes in miR-122a were associated with changes in HDL at 15 weeks. This corroborates well with a study that showed serum miR-122 was elevated in obese patients compared to normal weight controls, and was also significantly positively correlated with triglycerides and negatively associated with HDL (33). miR-122 is also higher in, and predictive of, fatty liver, and has been associated with small VLDL, IDL, and large LDL particle concentrations (65). Interestingly, statins have been shown to decrease serum miR-122 levels along with the concomitant decreases in total and LDL cholesterol (35).

Regarding other miRs related to circulating lipids, miR-150 (66), let-7b, miR-16, -126, -222, -223, and -320 have all been shown to be travel in the bloodstream in association with HDL particles, and some of these are also increased in dyslipidemic states, such as in Familial Hypercholesterolemia (67). In our study, however, only let-7a (of the same family as let-7b), miR-150, and miR-16 showed any significant correlations

with HDL cholesterol, the last of which is in agreement with a study showing that miR-16 is decreased in lipid excess both *in vivo* and *in vitro* (68). While miR-222 was not associated with HDL in our study, we did find a positive correlation of miR-222 with triglycerides at 15 weeks, which is in agreement with Li *et al.* (47) and a study showing that miR-222 was upregulated in patients with hypertriglyceridemia induced acute pancreatitis (69).

In several instances, multiple miRNAs are simultaneously related to the same trait, suggesting that subsets of miRNAs act in concert to regulate metabolism, perhaps by altering expression of genes in multiple pathways. Together, the coordinated regulation of a matrix of biochemical pathways might produce changes in systemic metabolism. In the current study, the coordinated action of multiple miRs appears to be operative in determining the metabolic responses to the dietary, weight loss interventions. In addition, multiple miRNAs may be required to produce the various associated components of the Metabolic Syndrome trait complex.

Our study has several limitations. First, this was not a randomized control trial. Patients with obesity were clinically assigned to the different diets, so selection bias could confound our ability to identify diet-specific effects. The current study was, in effect, ancillary to an intervention cohort study in which we studied only the completers. In the cohort study, volunteers were not compensated for their time, and the study experienced high drop-out rates perhaps in part for this reason. Additionally, the Optifast program had an associated fee that patients had to pay out-of-pocket, which may explain the low number of enrollees in that particular diet. Patient adherence was also likely to be variable, which could have impacted study outcomes differentially in each diet group.

Finally, as the group support classes were optional, the amount of diet education and support differed across patients even within the same group. For these reasons, a randomized trial specifically powered to detect effects on c-miRs should be conducted to rigorously determine whether dietary macronutrient composition could impact c-miR levels. In addition, insulin sensitivity was measured through HOMA-IR, which is a surrogate index derived from fasting insulin and glucose measures, and is a less optimal measure than a direct assessment of insulin sensitivity, such as the hyperinsulinemic euglycemic clamp (70). It should also be noted that 3 samples showing evidence of hemolysis from visual inspection were included in this study, as they did not seem to dramatically change our overall results; however, it is possible that they may have affected the relative expression levels of certain miRs within those samples.

In summary, in patients with obesity placed on dietary, weight loss interventions, circulating levels of miRs were not affected by the amount of weight loss or diet macronutrient composition. However, changes in miR-222 were associated with improvements in insulin sensitivity and changes in miR-16 and miR-122a were negatively associated with HDL cholesterol. The amount of weight loss affected the relationships between miR species and metabolic traits; after 15 weeks, levels of miR-34a, -126, -320, and let-7a were correlated with insulin sensitivity in the low responder group, while miR-16 and -223 were correlated with lipids in the high responder group. The data indicate that these miRs may play a role in the metabolic response to dietary, weight loss interventions in obesity regarding improvements in insulin sensitivity and lipids, rather than in the amount of weight lost per se. We also found that there seemed to be no difference in the miRs between a very low carb and low fat diet, although the

ability of different diets to affect c-miRs requires further study due to limitations in our study design. Our study points to the potential importance of c-miRs in regulating metabolism, and their potential as therapeutic agents that could be applied to diseases characterized by insulin resistance, such as obesity, T2DM, and cardiometabolic disease.

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Disclosure

The authors state no conflicts of interest pertinent to this work.

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Table 1. Clinical characteristics

	Low-responders (n=21)			High-responders (n=32)			Difference between groups
	Baseline	Change at 15 weeks	p-value	Baseline	Change at 15 weeks	p-value	p-value
Age	52.7 ± 10.3			50.6 ± 11.6			0.5143
Sex	18 F, 3 M			24 F, 7 M			-
Race	6 B, 15 W			7 B, 24 W			-
Weight (lbs)	218.8 ± 36.12	-2.51 ± 6.49	0.0993	241.1 ± 62.6	-24.55 ± 11.76	**<.0001**	**<.0001**
Total Body Fat (%)	46.21 ± 6.66	-1.77 ± 1.83	**0.0003**	47.22 ± 6.43	-3.88 ± 2.56	**<.0001**	**0.0019**
VAT (g)	1748.8 ± 998.1	-149.8 ± 295.0	**0.0350**	1655.5 ± 1158.1+	-353.44 ± 368.17	**<.0001**	**0.0477**
Lean Body Mass (kg)	50.45 ±9.15	.407 ± 1.72	0.2910	53.54 ± 11.14	-1.43 ± 2.22	**0.001**	**0.0024**
Fasting glucose (mg/dL)	108.95 ± 30.99	-1.16 ± 31.1	0.7012	110.59 ± 40.98	-8.16 ± 33.96	0.1840	0.1606
Fasting insulin (μU/mL)	18.31 ± 11.80	4.22 ± 17.04	0.2825	14.23 ± 7.51	-3.83 ± 7.15	**0.0049**	0.0567
HOMA-IR	5.47 ± 5.61	0.98 ± 5.03	0.3925	4.09 ± 3.16	-1.37 ± 3.00	**0.015**	0.0693
Triglycerides (mg/dL)	123.90 ± 64.96	7.75 ± 60.5	0.5732	133.38 ± 72.03	-29.91 ± 54.90	**0.0043**	**0.0469**
HDL (mg/dL)	58.85 ± 16.35	2.65 ± 6.62	0.0894	64.72 ± 16.51	-3.09 ± 12.04	0.1561	**0.0248**
LDL (mg/dL)	106.37 ± 26.70	-0.3 ± 17.96	0.9412	108.45 ± 31.98	-2.88 ± 30.14	0.599	**0.0313**

Baseline values are reported as mean ± standard deviation, except for categorical variables, in which the *n* is reported. Sex: F = female, M = male; Race: B = black, W = white (in high responders, 1 hispanic patient was grouped into W). In low responders at baseline, n = 20 for all lab values (fasting glucose, insulin, HOMA, and lipids) and weight.

+n = 28

Table 2. Correlations between relative expression of miR and metabolic risk factors at baseline

	n	r	p-value
Age (yrs)			
miR-16	50	-0.57	<0.0001
let-7a	47	-0.37	0.0110
miR-150	51	-0.36	0.0098
Lean Body Mass (g)			
let-7a	47	0.30	0.0383
Triglycerides (mg/dL)			
miR-122a	44	0.41	0.0052
HDL cholesterol (mg/dL)			
miR-16	50	-0.35	0.0142
miR-122a	44	-0.39	0.0097
let-7a	47	-0.46	0.0011
miR-150	51	-0.38	0.0068

Pearson's correlation coefficients are reported for significant correlations ($p < 0.05$) between relative expression of miRs ($-\Delta Ct$) and metabolic traits at baseline. No significant correlations were observed between any miRs tested and weight, total body fat percentage, visceral adipose tissue, fasting insulin, fasting glucose, or HOMA-IR.

Table 3. Correlations between relative expression of miRs and metabolic risk factors at 15 weeks

	Total			Low-responders			High-responders		
	n	**r**	**p-value**	**n**	**r**	**p-value**	**n**	**r**	**p-value**
Weight (lbs)									
miR-16	–	–	–	20	0.44	0.0497	–	–	–
Fasting insulin (µU/mL)									
miR-34a	41	0.60	<0.0001	15	0.78	0.0007	–	–	–
HOMA-IR									
miR-34a	41	0.55	0.0002	15	0.73	0.0019	–	–	–
miR-126	–	–	–	20	0.45	0.0443	–	–	–
let-7a	47	0.29	0.0496	18	0.56	0.0150	–	–	–
miR-150	50	0.29	0.0383	–	–	–	–	–	–
miR-320	–	–	–	20	0.46	0.0423	–	–	–
Triglycerides (mg/dL)									
miR-222	25	0.39	0.0545	–	–	–	–	–	–
miR-16	–	–	–	–	–	–	30	0.42	0.0199
miR-223	–	–	–	–	–	–	30	0.36	0.0543
HDL cholesterol (mg/dL)									
miR-16	50	-0.34	0.0151				30	-0.38	0.0411

Pearson's correlation coefficients are reported for significant correlations ($p < 0.05$) between relative expression of miRs ($-\Delta Ct$) with metabolic traits at 15 weeks. No significant correlations were observed between any miRs tested and total body fat percentage, visceral adipose tissue, or fasting glucose.

Table 4. Correlations between change in relative miR expression and change in metabolic factors over 15 weeks

	n	r	p-value
Lean Body Mass (g)			
miR-126	50	-0.30	0.0317
Fasting insulin (µU/mL)			
miR-222	15	-0.51	0.0545
HOMA-IR			
miR-222	15	-0.54	0.0368
HDL cholesterol (mg/dL)			
miR-16	49	-0.40	0.0045
miR-122a	38	-0.40	0.0122

Pearson's correlation coefficients are reported for significant correlations ($p < 0.05$) between change in relative expression of miRs (-ΔΔCt) with change in metabolic traits over 15 weeks. No significant correlations were observed between any miRs tested and weight, total body fat percentage, visceral adipose tissue, fasting glucose, or triglycerides.

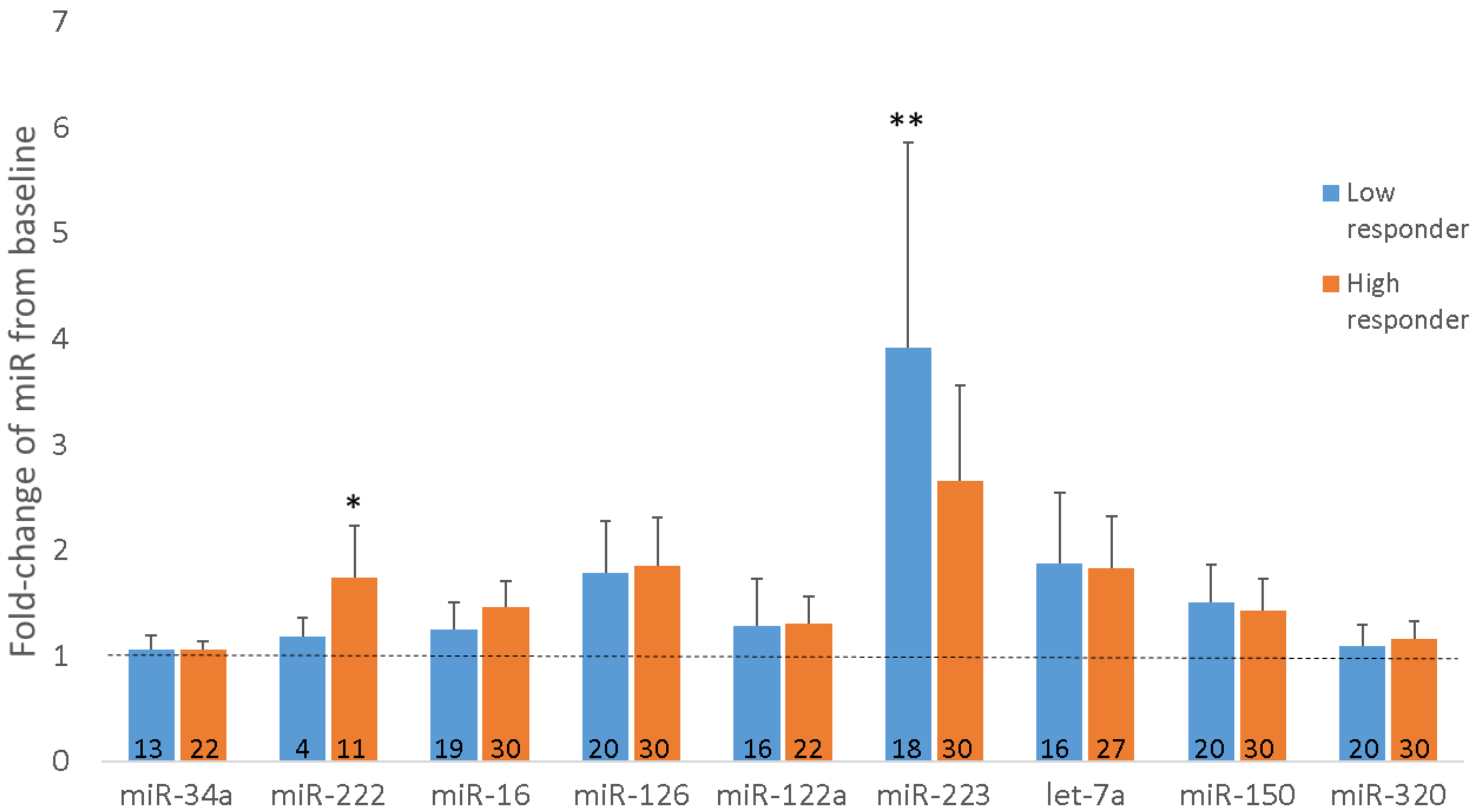

Figure 1. Fold-change of miRs at 15 weeks based on weight loss response. P-values are based on the signed-rank test for fold-change difference from baseline. No significant differences were observed between groups. Number of detectable c-miRs are listed within each bar.

* = $p < 0.05$; ** = $p < 0.01$

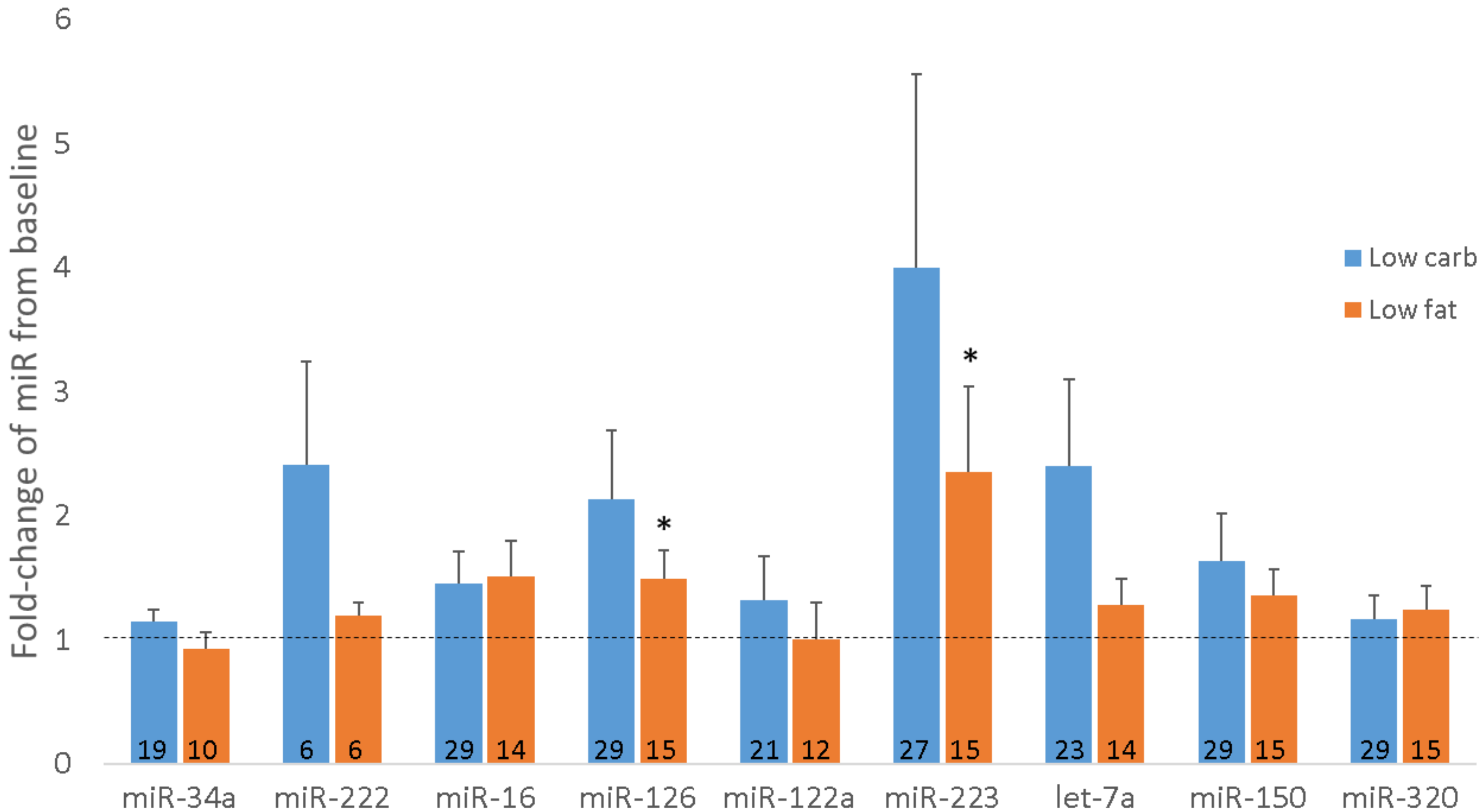

Figure 2. Fold-change of miRs at 15 weeks by diet type. P-values are based on the signed-rank test for fold-change difference from baseline. No significant differences were observed between groups. Number of detectable c-miRs are listed within each bar. The VLCD group was excluded for these analyses due to low *n*.

* = $p < 0.05$

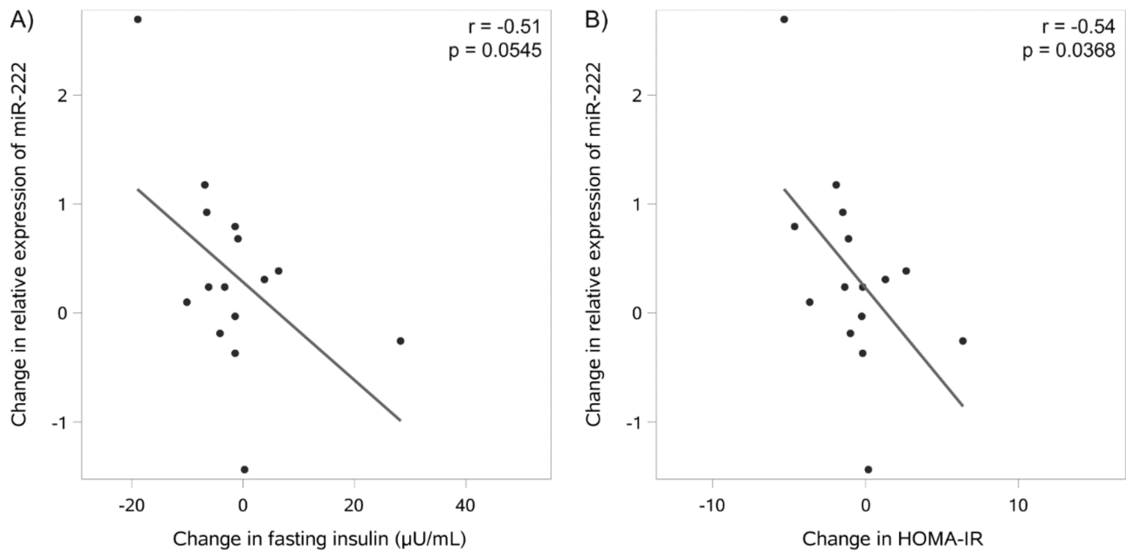

Figure 3. Changes in miR-222 with changes in insulin sensitivity over 15 weeks. Changes in miR-222 are negatively correlated with changes in (A) fasting insulin and (B) insulin resistance, as measured by HOMA-IR.

GENERAL DISCUSSION

The development of insulin resistance plays a key role in the pathogenesis of cardiometabolic disease, increasing risk for diseases such as T2DM, MetS, and CVD. While the exact mechanism behind the development of insulin resistance is unclear, in recent years, miRNAs have come to attention as potential regulators of pathways participating in systemic glucose tolerance. Additionally, circulating miRs (c-miRs) have been shown to deliver miRs to target cells, opening up the possibility of miRs to not only be regulators of this pathway, but also to be potential biomarkers and/or therapeutic agents for insulin resistance and its metabolic sequelae. The overall objective of this dissertation was to assess whether c-miRs are related to insulin sensitivity, as a first step towards understanding the complex interplay between miRs, insulin resistance, and metabolic dysfunction in humans. This was accomplished through three specific aims: 1) ascertaining the relationship between candidate c-miRs and insulin sensitivity in humans, 2) assessing the association of these miRs with oxidative stress as a potential contributor to the pathophysiology of insulin resistance, and 3) evaluating the impact of an insulin-sensitizing intervention on c-miRs.

The first study utilized banked samples from metabolically characterized participants to screen for candidate miRs that might be related to insulin sensitivity, as assessed by the gold standard, hyperinsulinemic-euglycemic clamp technique. This was accomplished by pooling samples that had the most extreme values for glucose disposal rate (i.e., the highest and lowest GDR values) and using a microarray containing miRs

pertinent to diabetes (commercially sourced, based on levels of miRs in tissues in the literature). We identified 5 candidate miRs from this screening, and added an additional 8 miRs from literature searches for a total of 13 to evaluate: miR-133a, -200a, -34a, -150, -199a, -140, -16, -107, -222, -218, and -33, SNORD61, and RNU6-2. Four of these were excluded from analyses due to having too many samples in the undetectable range (Ct > 35), and the remaining 9 were analyzed for relationships with insulin sensitivity.

We found that miR-16 and miR-107 were associated with GDR ($R^2 = 0.09$, $p = 0.0074$ and $R^2 = 0.08$, $p = 0.0417$, respectively), the clamp measure of insulin sensitivity. After adjusting for BMI, however, in addition to these two miR, we found positive associations of miR-33, -150, and -222 with GDR, showing that the effect of these miR on insulin sensitivity is independent of BMI. miR-16 was additionally correlated to multiple traits in the Metabolic Syndrome, such as waist circumference, triglycerides, and HDL, while miR-33 was related to systolic blood pressure. Thus, it seems certain miRs might affect multiple parts of metabolic pathways that may be involved in the development of insulin resistance and its sequelae.

One of the proposed mechanisms underlying the pathophysiology of insulin resistance is oxidative stress. For this reason, we examined whether urinary F2-isoprostanes (F2-isoPs) were quantitatively associated with GDR and whether miRs influenced insulin sensitivity by modulating oxidative stress as reflected by isoPs. Thus, we first endeavored to confirm whether isoPs were associated with insulin sensitivity, which has been commonly assumed in the literature even though definitive confirmation has been lacking. However, contrary to assumptions expressed in the literature, we found that there was no relationship between either the most abundant F2-isoP, 15-F_{2t}-IsoP, or

its major metabolite, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP with insulin sensitivity, as measured by the hyperinsulinemic-euglycemic clamp. Additionally, since there was an overlap in the patients from study 1 and study 2, we were able to assess the relationship between the circulating miRs associated with insulin resistance and urinary F2-isoPs in a subset of the participants. The fact that no significant relationships were found between any of the candidate miRs with urinary F2-isoPs is consistent with the idea that the oxidative stress pathways that produce urinary F2-isoPs are likely not related to insulin resistance.

On the other hand, we made the novel observation that 15-F_{2t}-IsoP was related to processes associated with aging, such as decrements in lean body mass and bone mass. After adjusting for height to account for its potential contribution to total LBM or bone mass (data not shown), we found that these relationships were still significant, which strengthens our conclusions. Thus, urinary F2-isoPs may be more closely related to the oxidative stress processes operative in biological aging, rather than those underlying the development of insulin resistance.

In the third study, we wanted to assess whether circulating miRs would change in response to an insulin-sensitizing intervention. Since it is well known that weight loss $\geq$ 5% can improve insulin sensitivity and associated metabolic traits[44], we collaborated with Barbara Gower, PhD, on a study that aimed to evaluate the differences in metabolic outcomes when patients with obesity were assigned one of three different dietary, weight-loss interventions: low fat, very low carbohydrate, or very low calorie. We categorized patients based on weight loss (high responder: $\geq$ 5% weight loss, low responder: $<$ 5%

weight loss) after 15 weeks and evaluated levels of c-miRs and metabolic outcomes based on these groupings.

We found that there were no differences in circulating miR based on amount of weight loss or diet type. However, some c-miRs were associated with metabolic responses to the dietary weight loss interventions. For example, we observed that changes in miR-222 over the course of the intervention were associated with improvements in insulin sensitivity, as measured by HOMA-IR and fasting insulin. We also assessed relationships between c-miRs and metabolic traits in cross-sectional analyses after 15 weeks on the diets, and found that miR-34a was associated with both fasting insulin and HOMA-IR, and that miR-150 and let-7a were also associated with HOMA-IR. Additionally, the amount of weight loss seemed to affect relationships between miRs and metabolic parameters at 15 weeks. In low-responders, correlations with measures of insulin sensitivity seemed to predominate, with miR-34a, -126, -320, and let-7a showing associations with fasting insulin and/or HOMA-IR. In contrast, relationships with lipids were prominent in the high responders as evidenced by significant correlations between triglycerides and both miR-16 and miR-223, as well as between HDL cholesterol and miR-16. This may suggest differential regulation of metabolic processes that may help predict how well a patient may respond to a dietary intervention.

In summary, the analyses of patients with obesity being treated with dietary, weight-loss interventions demonstrated that miRs were not affected by the degree of weight loss or macronutrient composition of the diet, but instead may be part of the metabolic response to weight loss with respect to improvements in insulin sensitivity and

dyslipidemia. Since we know that miRs can travel through the blood and be taken up with some specificity into target tissues[29, 47], it is theoretically possible that some miRs present in the circulation are relevant to the communication between tissues that regulate insulin sensitivity and systemic metabolism. In fact, one study showed that miR-16 might play a role in the cross-talk between skeletal muscle and various organs, including the pancreas. Exosome-like vesicles (ELVs) from insulin resistant skeletal muscle were able to travel to the pancreas *in vivo* after being injected intramuscularly, and they were also able to be taken up by adipocytes and to affect *Ptch1* in β cells *in vitro*[48].

Given the results of our studies, we have determined that certain miRs (miR-16, -107, -33, -150, -222, -34a, -126, -320, and let-7a) are related to insulin sensitivity measures in cross-sectional analyses, and changes in miR-222 were observed to be significantly (positively) associated with improvements in insulin sensitivity in patients with obesity after dietary intervention for weight loss. The relationship between miRs and insulin sensitivity can be complex however, as we observed that miR-150 was positively associated with insulin sensitivity in the cross-sectional analysis in our first study, but then found it was negatively associated after 15 weeks on a diet intervention, suggesting a potential difference in how miRs may act when responding to an insulin-sensitizing intervention.

We also found that there was overlap between c-miRs associated with insulin sensitivity and those that were correlated with other metabolic risk factors. For example, miR-16 was correlated with circulating lipids in both studies, miR-33 with systolic blood pressure in the first study, and let-7a was associated with LBM and HDL at baseline in the third study. Our finding of this overlap between miRs with both insulin sensitivity

and metabolic factors corroborates with other studies, which have also noted that relative expression of miRs linked to insulin sensitivity seem to be related to other metabolic risk factors[41]. This suggests that these miRs may help determine which metabolic traits become associated with insulin resistance in the development of the Metabolic Syndrome and the cardiometabolic disease process. Indeed, there is some evidence that certain miR involved in diabetes may also play a role in the development of cardiovascular disease and other diabetic complications[49, 50].

It is important to note, however, that in some studies, the R^2 values linking miRs with insulin resistance and metabolic traits are quite modest and explain only a small component of overall variance. In addition, multiple miRs are often associated with multiple metabolic parameters, suggesting that metabolic traits may be regulated by subsets of multiple miRs. Along these lines, it is interesting that many of the relative expressions of c-miRs were very highly correlated with each other in both study 1 and 3 (data not shown). Taken together, the data provide some support for our idea that multiple miRs may work together to act upon insulin and metabolic pathways, engaging networks of molecular pathways that together mediate changes in systemic metabolism. From this perspective, subsets of miRs may be more useful as biomarkers of disease than single miR species, and combinations of miRs might be more effective in developing miRs for therapeutic purposes[23]. Future studies may thus wish to examine the mechanisms behind which these miR may interact/work together, and what the effector and target cells are for these circulating miR.

On the other hand, given that we noticed some miRs were mutually correlated even in the absence of being associated with metabolic traits, it's possible that there is

another underlying factor that is regulating these miRs in tandem, such as immune or inflammatory processes. For example, miR-16, -126, -146a, and -223, which were evaluated in study 3, were all shown to be upregulated after patients with rheumatoid arthritis were treated with an anti-TNFα drug, suggesting they may play a role in immune function and anti-inflammatory processes[51]. Additionally, many of these c-miR have been shown to be differentially expressed in various other disease states, such as cancer[31, 32] and tissue injury[47, 52], so it would be difficult to interpret single, or even combinations of, miRs as being solely a biomarker for insulin sensitivity.

Our research points to some challenges in assessing c-miRs and the need for further technological development. For example, despite the fact that we used a single, stably-expressed miR as the reference miR across studies, as well as the same primers from the same commercial source for both studies 1 and 3, it was surprising that there were so many discrepancies in the miRs linked to metabolic traits in cross-sectional analyses involving different cohorts. For example, while miR-107 was found to be significantly correlated with GDR in the first study, and miR-33 was associated after adjusting for BMI, in the diet study, both of these miRs were almost completely undetectable in most samples at both baseline and 15 weeks. miR-16, which was also found to be significantly correlated with GDR in the first study, did not show any correlation with change in either fasting insulin or HOMA-IR in the diet study. Additionally, while miR-16 was associated with circulating lipids in both studies, it seemed to have the exact opposite relationship between the first study and the third. In the first study, miR-16 was found to be positively correlated with HDL and negatively associated with triglycerides, but in the third study, it was consistently negatively

associated with HDL and positively associated with triglycerides. Some potential reasons for these differences may be attributed to challenges associated with circulating miRs, such as inherently low levels of miRs in the blood, and/or the fact that the associations in study 1 were characterized by modest R^2 values. The populations were also different, as the first (cross-sectional) study was fairly equal in terms of blacks and whites, whereas the diet study was predominantly white. The diet study also did not exclude patients with T2DM, which tends to be associated with multiple areas of metabolic dysregulation and increased cardiovascular risk.

There are other limitations in our studies that do bear noting. For example, the first study utilized banked plasma samples to assess c-miR, some of which have been through multiple freeze-thaws prior to the study. While some studies show that miRs tend to be fairly stable over time and are unaffected by freeze-thaws[32], there can be variation in the stability of the miR depending on the miR species, which may have affected our results. Indeed, in our own preliminary assessments (data not shown), we noticed that there was some fluctuation in miR expression after multiple freeze thaws for a limited number of miRs.

There are also innate challenges when working with circulating miRs[53], including the relatively low abundance of miR in serum/plasma as mentioned above, the lack of standardization of reference genes for normalization[37], issues with sample processing[54], and variable methods of analysis used across studies. Sample handling is also an important potential source of variation, as many of the miRs we observed also have been shown to be present on platelets[55] and other hematopoietic cells, so disruption of any of these blood components may falsely elevate miRs contained in these cells. All of these

challenges with c-miR assays probably contribute to the large discrepancies within the literature itself, as well as differences in our study findings. It is thus also prudent to note the reference gene(s) used and statistical analyses used when perusing the literature on circulating miRs and associations with disease for the sake of reproducibility and interpretation. Along these lines, we found that miR-490 was the most stably expressed across all samples tested across studies, even more so than the spike-in control. To our knowledge, this has not been used as a reference gene before, so future studies may be needed to verify the generalizability of its use as a potential endogenous control for qrt-PCR studies on miR analyses conducted on human plasma samples.

Additionally, while some studies suggest the amounts of miRs in serum and plasma tend to be fairly similar[31, 32], there is also evidence that there are differences in patterns of circulating miR between serum and plasma[56] for various disease states. Thus, even though both are derived from blood samples, due to the differences in collection and processing, certain miRs may be preferentially lost (such as lipid-bound miR that may become trapped within the serum tubes). Variation can also be attributed to the use of reagents, such as the Taqman probe system or Locked Nucleic Acid based platforms, compared with the SYBR green reagents that were used in our study, which some have claimed may be less specific. Future studies may thus wish to compare our results with these systems for confirmation. Other technologies are also being developed in this area that may help speed the process of biomarker discovery, such as Abcam's Fireplex platform that allows for multiplexing via flow cytometry. Finally, we only evaluated a handful of circulating miRs that may have potential relevance to insulin resistance in our studies. Future studies may be interested in assessing others of the ~119 miR that have

been shown to be consistently expressed in serum and plasma samples[53] for their relationship with insulin sensitivity and associated metabolic traits.

CONCLUSIONS

In conclusion, circulating miRs are intriguing potential candidates to better elucidate the mechanisms behind the development of insulin resistance. We have found certain miRs to be associated with both insulin sensitivity (as measured by clamp, HOMA-IR, and fasting insulin) and metabolic risk factors, and that specific miRs can be related to metabolic responses in patients with obesity placed on dietary, weight loss interventions. We also noted a shift in the patterns of miR associated with metabolic risk factors before and after dietary intervention: low responders tended to have more significant correlations of miR with insulin sensitivity, while high responders tended to have more relationships between miR and circulating lipids. Taken together, these results suggest that c-miRs may work in concert to affect insulin sensitivity and metabolism, and may thus hold potential as biomarkers for metabolic status, and/or as therapeutic targets or agents for treatment and prevention of diabetes and cardiometabolic syndrome. We additionally found that there was no relationship between F2-isoPs, the gold-standard, whole-body measure of oxidative stress in humans, and insulin sensitivity, suggesting that the mechanism behind insulin resistance is not related to the oxidative stress processes that produce F2-isoPs. However, F2-isoPs were negatively correlated with lean and bone mass, which suggest they may be more relevant to the oxidative stress processes behind aging. Further studies are warranted to better elucidate the mechanisms through with circulating miRs may act in relation to insulin sensitivity and its associated metabolic sequelae.

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APPENDIX

INSTITUTIONAL REVIEW BOARD APPROVAL

OMB No. 0990-0263
Approved for use through March 31, 2018

Protection of Human Subjects
Assurance Identification/IRB Certification/Declaration of Exemption
(Common Rule)

Policy: Research activities involving human subjects may not be conducted or supported by the Departments and Agencies adopting the Common Rule (56FR28003, June 18, 1991) unless the activities are exempt from or approved in accordance with the Common Rule. See section 101(b) of the Common Rule for exemptions. Institutions submitting applications or proposals for support must submit certification of appropriate Institutional Review Board (IRB) review and approval to the Department or Agency in accordance with the Common Rule.

Institutions must have an assurance of compliance that applies to the research to be conducted and should submit certification of IRB review and approval with each application or proposal unless otherwise advised by the Department or Agency.

1. Request Type	2. Type of Mechanism	3. Name of Federal Department or Agency and, if known, Application or Proposal Identification No.
[ ] ORIGINAL [X] CONTINUATION [ ] EXEMPTION	[X] GRANT [ ] CONTRACT [ ] FELLOWSHIP [ ] COOPERATIVE AGREEMENT [ ] OTHER:	

4. Title of Application or Activity	5. Name of Principal Investigator, Program Director, Fellow, or Other
Mechanisms of Human Insulin Resistance	GARVEY, W TIMOTHY

6. Assurance Status of this Project *(Respond to one of the following)*

[X] This Assurance, on file with Department of Health and Human Services, covers this activity:
Assurance Identification No. FWA00005960, the expiration date 01/24/2017 IRB Registration No. IRB00000726

[ ] This Assurance, on file with *(agency/dept)*______, covers this activity.
Assurance No.______, the expiration date______ IRB Registration/Identification No.______ *(if applicable)*

[ ] No assurance has been filed for this institution. This institution declares that it will provide an Assurance and Certification of IRB review and approval upon request.

[ ] Exemption Status: Human subjects are involved, but this activity qualifies for exemption under Section 101(b), paragraph______.

7. Certification of IRB Review (Respond to one of the following IF you have an Assurance on file)

[X] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.
by: [ ] Full IRB Review on (date of IRB meeting)______ or [X] Expedited Review on (date) 9/19/16
[ ] If less than one year approval, provide expiration date______

[ ] This activity contains multiple projects, some of which have not been reviewed. The IRB has granted approval on condition that all projects covered by the Common Rule will be reviewed and approved before they are initiated and that appropriate further certification will be submitted.

8. Comments
Protocol subject to Annual continuing review.
HIPAA Waiver Approved?: No

Title X030603009
Mechanisms of Human Insulin Resistance

IRB Approval Issued: 9-19-16

IRB Approval No Longer Valid On: 9/19/17

9. The official signing below certifies that the information provided above is correct and that, as required, future reviews will be performed until study closure and certification will be provided.	10. Name and Address of Institution
11. Phone No. *(with area code)* (205) 934-3789 12. Fax No. *(with area code)* (205) 934-1301 13. Email: irb@uab.edu	University of Alabama at Birmingham 701 20th Street South Birmingham, AL 35294
14. Name of Official Expedited Reviewer	15. Title IRB Member
16. Signature Leslie Cooper, CIP	17. Date 9/19/16

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According to the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0990-0263. The time required to complete this information collection is estimated to average 30 minutes per response. If you have comments concerning the accuracy of the time estimate(s) or suggestions for improving this form, please write to: U.S. Department of Health & Human Services, OS/OCIO/PRA, 200 Independence Ave., S.W., Suite 336-E, Washington D.C. 20201, Attention: PRA Reports Clearance Officer.

Institutional Review Board for Human Use

Form 4: IRB Approval Form
Identification and Certification of Research
Projects Involving Human Subjects

UAB's Institutional Review Boards for Human Use (IRBs) have an approved Federalwide Assurance with the Office for Human Research Protections (OHRP). The Assurance number is FWA00005960 and it expires on November 8, 2021. The UAB IRBs are also in compliance with 21 CFR Parts 50 and 56.

Principal Investigator:	SOLEYMANI, TARANEH
Co-Investigator(s):	MA, ELIZABETH
Protocol Number:	**F140115008**
Protocol Title:	*Resting Metabolic Rate and Body Composition Changes from Three Different Weight Management Programs*

The IRB reviewed and approved the above named project on 2/15/2017. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services. This Project will be subject to Annual continuing review as provided in that Assurance.

This project received FULL COMMITTEE review.

IRB Approval Date: 2/15/2017

Date IRB Approval Issued: 4/3/17

IRB Approval No Longer Valid On: 2/15/18

Identification Number: IRB00000196

Ferdinand Urthaler, M.D.
Chairman of the Institutional Review
Board for Human Use (IRB)

Partial HIPAA Waiver Approved?: Yes

Investigators please note:

The IRB approved consent form used in the study must contain the IRB approval date and expiration date.

IRB approval is given for one year unless otherwise noted. For projects subject to annual review research activities may not continue past the one year anniversary of the IRB approval date.

Any modifications in the study methodology, protocol and/or consent form must be submitted for review and approval to the IRB prior to implementation.

Adverse Events and/or unanticipated risks to subjects or others at UAB or other participating institutions must be reported promptly to the IRB.

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